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CD4 T cells promote liver inflammation/injury by reverse signaling via CD40 without *de novo* activation¹

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Abstract

Although the role of CD4 T cells in tissue inflammation and organ injury resulting from ischemia and reperfusion (IRI) has been well documented, it remains unclear how CD4 T cells are activated and function in the absence of specific Ag. We used a murine liver warm IRI model to dissect first, whether *de novo* Ag-specific CD4 T cell activation was required and then what was its functional mechanism. The critical role of CD4 T cells in liver immune activation against IR was confirmed in CD4 KO mice and CD4 depleted WT mice. Interestingly, the inhibition of CD4 T cell activation without target cell depletion failed to protect livers against IRI, suggesting that T cells function in liver IRI without Ag-specific *de novo* activation. To dissect T cell functional mechanism, we found that only CD154 blockade, but not IFN- γ neutralization, inhibited local immune activation and protected livers from IRI. Furthermore, agonist anti-CD40 Abs restored liver IRI in otherwise protected CD4-deficient hosts. Finally, FACS analysis of liver CD4 T cells revealed the selective infiltration of effector cells, which constitutively expressed higher level of CD154, as compared with their peripheral counterparts. IR triggered a significant liver increase of CD40, but not CD154 expression, and macrophages responded to TLR4 and type I IFN stimulation to upregulate CD40 expression. These novel findings provide evidence that CD4 T cells function in liver IRI via CD154 without *de novo* Ag-specific activation, and innate immune induced CD40 upregulation may trigger the engagement of CD154-CD40 to facilitate tissue inflammation and injury.

Introduction

Although tissue damage resulting from ischemia and reperfusion injury (IRI) can develop in the absence of exogenous Ag, studies in liver and kidney murine models have documented the key role of T cells in the activation of local immune responses (1,2). The observation that systemic immunosuppression (CsA, FK506) attenuated hepatocellular damage, provided initial indirect evidence for T cell involvement in the mechanism of IRI (3). In addition, the inhibition of lymphocyte adherence to endothelium, previously thought to affect primarily neutrophils, was shown to target T cells. Finally, results from T cell-, and CD4-deficient mice have provided direct proof that T cell, particularly of CD4 phenotype, is the key player in the early IRI phase (4–7). Indeed, adoptive transfer of T cells or CD4+ T subset, readily restored IRI in T cell-deficient mice (4,6). Thus, the question arises, as to

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how T cells function in this innate immunity-dominated response, and in the absence of exogenous Ag stimulation?

T cells may function in Ag-independent manner by secreting cytokines and upregulating costimulatory molecules. The role of T cell derived -CD28, -CD154 and -IFN- γ in IRI has been demonstrated in both mouse and rat models (8–10). We have shown the importance of CD28 and CD154 expression for the activation of liver pro-inflammatory response leading to IR-triggered hepatocellular damage. Indeed, livers in CD154 KO, CD28 KO mice or in WT mice treated with anti-CD154 Ab or CTLA-4-Ig were protected from IRI (6). Our recent study on type I vs. type II IFN receptors indicated that IFN- γ might be dispensable in liver IRI (11). Although the role of CD154 in IRI has been attributed to its T cell activation, the nature of IR-triggered T cell activation remains elusive. Since no specific Ags are required, we hypothesize that T cells become activated during IR by Ag-non-specific pro-inflammatory milieu and independent of Ag-specific first signaling; and that CD154 triggers CD40 to facilitate innate immune activation. In this study, we first confirmed the role of CD4 T cells in liver immune activation, and then determined the nature of T cell activation in liver IRI. CD4 blocking Ab, YTS177.9 (12,13), which inhibits Ag-specific CD4 T cell activation without target cell depletion (14–18), was utilized and contrasted with CD4 depleting, GK1.5 Ab. To determine the functional mechanisms of CD4 T cells, we first analyzed the effects of CD154 blockade and IFN- γ neutralization in liver IRI, followed by CD40 triggering in CD4 KO mice. Our novel findings indicate that CD4 T cells function in liver IRI without the requirement of *de novo* Ag-specific activation, and are dependent on CD154-CD40, but not IFN- γ signaling.

Materials and Methods

Animals

Male wide-type (WT), nude (B6.Cg-Foxn1^{nu}/J), CD4-deficient (B6.129S2-Cd4^{tm1Mak}/J) and CD8-deficient (B6.129S2-Cd8a^{tm1Mak}/J) mice (C57BL/6 strain, 8–12 weeks old) were used (Jackson Laboratory, Bar Harbor, ME). Animals were housed in 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 (NIH publication 86–23 revised 1985).

Mouse warm hepatic IRI model

We have used a warm partial hepatic IRI model in mice (6,19,20). After 90 min of local ischemia, animals were sacrificed serially at reperfusion. Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured by auto analyzer (ANTECH Diagnostics, Los Angeles, CA). Liver, spleen and peripheral blood samples were collected. Liver specimens, fixed in 10% buffered formalin and embedded in paraffin, were stained with hematoxylin and eosin, and then analyzed blindly. For molecular biology, liver specimens were rinsed in PBS prior to freeze in liquid nitrogen. Sham WT controls underwent the same procedure, but without vascular occlusion.

CD4 blocking (YTS177) or depleting (GK1.5) Abs were administered (1 mg/mouse i.v.) 24 h prior to the experiment. Anti-CD154 and anti-IFN- γ Abs were given (500 μ g/mouse i.v.) prior to the onset of liver ischemia. Anti-CD40 Ab was infused (250 μ g/mouse) at the onset of reperfusion via portal vein.

CD4 T cell activation *in vitro*

Splenocytes from naive B6 mice cells were labeled with carboxy-fluorescein diacetate succinimidyl ester (4mM CFSE; Molecular Probe, Eugene, OR) (21), and incubated with

irradiated B6 (syngeneic) or B/c (allogeneic) stimulator cells (2×10^6 /ml), or ConA (2U/ml, Sigma) in the absence or presence of anti-CD4 Abs, GK1.5 or YTS177 (10 μ g/ml). At day 4, cells were harvested, and stained with anti-mouse CD4-PE (eBiosciences). Topro 3 (1nM) was added as viable dye. Flow cytometry was performed on a FACSCalibur dual-laser cytometer (Becton Dickinson). Cells in lymphocyte gate, topro 3 negative (viable cells), CD4 positive were analyzed for CFSE intensities. To measure CD154 expression, fresh naive splenocytes were stimulated with ConA (5U/ml) in the presence of anti-CD154-PE (5 μ g/ml) at 2.5×10^6 /ml for 4 h in the presence of GolgiStop (eBiosciences). The stimulated cells were stained with rat anti-mouse CD8-FITC, CD4-PE-Cy5. To measure cytokine production, cells were stimulated with PMA (10 ng/ml)/ionomycin (400 ng/ml) at 2.5×10^6 /ml for 4 h in the presence of GolgiStop. Cells were first stained with anti-CD4/CD8, then fixed/permeabilized using the CytoStain Kit (eBiosciences), followed by PE conjugated rat anti-mouse IFN- γ (XMG1.2) (eBiosciences). The stained cells were analyzed by three-color FACS.

Macrophage activation in vitro and CD40 staining

Murine bone marrow macrophages (BMM) were differentiated from marrow of 6–10-week old C57B/6 mice by culturing in 1xDMEM, 10% FBS, 1% penicillin/streptomycin, and 30% L929 conditioned medium for 6 days (22). The cell purity was assayed to be 94–99% CD11b+. In addition, we used mouse leukemic macrophage line RAW264.7 (TIB-71; ATCC, Manassas, VA), which was maintained in DMEM medium supplemented with 10% FBS. Cells were treated with LPS (1ng–10 μ g/ml, Sigma, St. Louis, MO), or IFN- β (10–1000U/ml, R&D Systems, Minneapolis, MN) for 1–24h. Treatment did not affect macrophage viability (>95%). Cells were collected and stained with FITC-anti-CD11b and PE-anti-CD40 (Ebiosciences, San Diego, CA).

Liver lymphocyte isolation and FACS analysis

We used mechanical method to separate intrahepatic lymphocytes from liver PCs. Livers, perfused *in situ* with 10 ml of cold PBS to remove circulating PBLs, were pressed through a sterile stainless steel screen in 30 ml RPMI media with 5% FBS. The hepatocytes were removed by low-speed centrifugation. The supernatant was collected, centrifuged, and the pellet was resuspended. The cell suspension was then layered on top of a density cushion of 25%/50% discontinuous Percoll (Pharmacia) and centrifuged to obtain lymphocyte fraction at the interface. Lymphocytes were collected, washed, and subjected to FACS analysis. Approximately half to one million of liver resident lymphocytes were obtained from one mouse liver by this method. Spleen and liver lymphocytes were first stained with CD4-FITC, CD8-APC and CD62L-Cy5. After wash, cells were fixed with 1% paraformaldehyde-PBS, followed by permeabilization with 0.5% Saponin. CD154 (both cell surface and intracellular) were detected by anti-CD154-PE.

Quantitative RT-PCR

Two and a half μ g of RNA was reverse-transcribed into cDNA using SuperScriptTM III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative-PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25 μ l, the following were added: 1xSuperMix (Platinum SYBR Green qPCR Kit, Invitrogen, Carlsbad, CA), cDNA and 0.5 mM of each primer. Amplification conditions were: 50 °C (2 min), 95 °C (5 min) followed by 50 cycles of 95 °C (15 s), 60 °C (30 s). Primers to amplify specific gene fragments, TNF- α , IL-1 β , CXCL10, were described (20). Target gene expressions were calculated by their ratios to the house-keeping gene HPRT.

Statistical analysis

All values are expressed as mean $\pm$ SD. Data were analyzed with an unpaired two-tailed Student's *t* test. *P* < 0.05 was considered to be statistically significant.

Results

CD4 T cells are critical for the pro-inflammatory immune response in liver IRI

To document the role of CD4 T cells in liver pro-inflammatory immune response against IR, CD4 KO and WT mice pre-treated with CD4 depleting Ab were subjected to 90 min of warm ischemia; the hepatocellular damage and gene induction were measured at 6 h of reperfusion. Indeed, both groups of mice were protected from IRI, as their sALT levels were lower (Fig. 1a: WT = 3775 $\pm$ 621, *n*=10, vs. CD4 KO = 445.8 $\pm$ 111.9, *n*=6 and anti-CD4 = 161.8 $\pm$ 27, *n*=6; *p*<0.04), and liver architecture was better preserved (Fig. 1b), as compared with WT controls, and similar to that in nude mice. Intrahepatic pro-inflammatory gene induction by IR was suppressed in the absence of CD4 T cells, as shown by TNF- α and CXCL10 transcript levels (Fig. 1c). Unlike CD4 T cells, CD8 T cells or NK cells were not essential, as their deficiency in respective KO mice or WT mice pre-treated with depleting Abs failed to protect livers from IRI. Both sALT levels (Fig. 1a: CD8 KO = 1960 $\pm$ 976, anti-CD8 Ab = 2784 $\pm$ 1355, anti-NK Ab = 2634 $\pm$ 978, *p*>0.1 vs. WT, *n*=4–8/group) and histology (H/E staining) revealed comparable tissue damage in CD8 KO/depleted, NK depleted, and WT mice (Fig. 1b). In parallel, intrahepatic levels of TNF- α and CXCL10 were upregulated in CD8 or NK depleted mice, comparable with WT mice (Fig. 1c). Thus, CD4 T cells are critical for IR-triggered pro-inflammatory response leading to the hepatocellular damage.

De novo Ag-specific CD4 activation is not required for liver IRI

To determine the nature of CD4 T cell activation by IR, we used a blocking CD4 Ab, capable to inhibit Ag-specific CD4 activation without target cell depletion. We have shown that treatment of cardiac allograft recipients with YTS177 Ab inhibits CD4-dependent alloreactive CD8 activation (23). To directly demonstrate that this blocking Ab did inhibit Ag-specific CD4 T cell activation, MLRs were set up in the absence or presence of the CD4 Ab. As shown in Fig. 2a, addition of YTS177 inhibited CD4 T cell proliferation against allo-Ag stimulation in the culture. CD4 depleting GK1.5 Ab showed similar capability to suppress T cell proliferation in MLRs. Interestingly, neither of CD4 Abs inhibited Con A-stimulated CD4 proliferation (Fig. 2a). *In vivo*, administration of GK1.5 depleted CD4 T cells by GK1.5, whereas YTS177 preserved CD4 T cells both in the spleens and livers (Fig. 2b).

Having confirmed its effects on CD4 T cells *in vitro*, we then analyzed the impact of non-deletional CD4 inhibition *in vivo*. In contrast to CD4 depletion, CD4 blockade failed to prevent IRI. Indeed, sALT levels (Fig. 3a) and liver pathology (Fig. 3b) showed similar degree of tissue damage in YTS177-treated mice and controls (Control = 10150 $\pm$ 2193 vs. YTS177 = 6989 $\pm$ 2168, *n*=4–6, *p*>0.3), which was in sharp contrast to the effect of CD4 depletion (GK1.5 = 445.8 $\pm$ 111.9, *n*=6, *p*<0.01 vs. YTS177 group). Correlated with hepatocellular injury, local TNF- α /CXCL10 levels remained upregulated following CD4 blockade, comparable with controls, yet higher than in CD4-depleted mice (Fig. 3c). Thus, inhibition of *de novo* Ag-specific CD4 T cell activation did not interfere with their function in liver IRI.

CD154, but not IFN- γ , is critical for liver IRI

Although both IFN- γ and costimulatory CD154 have been implicated in the mechanism by which CD4 T cells promote IRI, controversial data exist (10,11) and function of the two molecules has never been tested in parallel in the same model system. Both CD4 and CD8 T

cells can produce IFN- γ upon stimulation, but only CD4 T cells upregulate CD154 expression (data not shown). This suggests that CD154, but not IFN- γ , may represent the key mediator in the disease process, with CD8 T cells dispensable in liver IRI. To directly compare the function of the two molecules, groups of WT mice were infused with IFN- γ neutralizing Ab or CD154 blocking Ab. Figure 4 shows that untreated mice as well as those treated with anti-IFN- γ Ab suffered similar degree of liver injury, as evidenced by sALT levels (5993 ± 943.2 and 6972 ± 1549 , respectively; $n=6-8/\text{group}$; $p=\text{ns}$), and histology. Moreover, intrahepatic TNF- α and IL-1 β transcript levels remained upregulated in both recipient groups. In contrast, mice treated with anti-CD154 Ab were protected from liver IRI, as evidenced by lower sALT levels (1407 ± 336.2 , $n=4$, $p<0.02$ vs. anti-IFN group), preservation of liver architecture, and reduced local pro-inflammatory gene levels (TNF- α , IL-1 β and CXCL10). These results are supportive of our hypothesis that CD154, but not IFN- γ , mediates CD4 T cell function in liver IRI.

Ab-mediated CD40 ligation restores liver IRI in CD4 KO mice

As *de novo* Ag-specific activation is not required for CD4 T cell function in liver IRI, we then re-assessed the role of CD154. We tested the hypothesis that, instead of activating CD4 T cells, CD154 may trigger CD40 on liver innate immune cells to facilitate the pro-inflammatory immune response against IR. An agonist anti-CD40 Ab was infused into CD4 KO mice at the onset of liver reperfusion. This Ab by itself did not trigger liver pro-inflammatory response or hepatocellular injury if infused into naive animals (data not shown). However, treatment with anti-CD40 Ab readily recreated the hepatocellular damage following IR in CD4 KO mice, as evidenced by increased sALT levels (Fig. 5a; control = 4050 ± 1189 , $n=9$, vs. anti-CD40 = 10350 ± 1035 , $n=10$, $p<0.001$) and liver histology (Fig. 5b), compared with untreated CD4 KOs. Moreover, liver immune activation by IR was also restored, as both TNF- α and CXCL10 gene levels markedly increased after CD40 Ab infusion, compared with controls (Fig. 5b; $p<0.05$). Thus, cross-linking of CD40 recreated liver inflammation/damage in otherwise protected CD4 KO mice, which implies that CD4 T cells may function by activating CD40 in liver IRI.

Liver CD4 T cells are enriched with effector cells and constitutively express CD154

Having confirmed the critical roles of CD4 T cells and the CD154-CD40 signaling in immune activation against IR, we next addressed the question how liver CD4 T cells engage CD154-CD40 pathway without *de novo* activation. Liver lymphocytes were isolated and subjected to multi-parameter FACS analysis of their functional status, in parallel with splenocytes of the same animal. As shown in Fig. 6a, liver T cells, both CD4 and CD8 subsets, were highly enriched with pro-inflammatory effector type cells (CXCR3⁺CD62L^{low}): percentages of CD4+CXCR3+CD62L^{low} subset in total CD4 or CD8 were 44% and 51% vs. 16.9% and 17.1% in spleens. Interestingly, liver CD4 memory, represented by CD4+CD44^{high}, or regulatory, represented by CD4+CD25⁺, populations were not different from spleen CD4, indicating that effector rather than memory/regulatory T cells are selectively sequestered in the liver. To show that liver CD4 T cells are capable of engaging CD154-CD40, we directly measured CD154 expression in these T cells without further in vitro stimulation. As cell surface CD154 has a very fast turnover rate, with its majority stored intracellularly (24–26), we did intracellular staining of CD154 in combination with cell surface CD4/CD62L staining. In spleens, CD4 T cells, but not CD8 T cells; and only CD62L^{low} effector subset of CD4, but not CD62L^{high} subset, expressed CD154 (Fig. 6b). As liver CD4 T cells are enriched with the effector type, they expressed significantly higher levels of CD154 than their spleen counterparts. In fact, their CD154 levels were comparable with those of the spleen CD4⁺CD62L^{low} subset. These results indicate that liver CD4 T cells are highly enriched with effector type cells that constitutively express CD154.

Liver IR triggers CD40 upregulation

The question arises as to the mechanism that triggers CD154-CD40 activation in liver IRI. First, we determined gene expression profiles of CD40 and CD154 in livers during IR by qRT-PCR. Indeed, CD154 levels were low and with no significant changes observed throughout the 6h reperfusion period. In contrast, CD40 levels increased in livers undergoing IR, and peaked at 2–4 h post reperfusion (Fig. 7a). As macrophage CD40 represents the major receptor of CD154 on CD4 T cells for immune activation, we next determined as to whether CD40 macrophage expression was responsive to TLR4 stimulation. In addition, as type I IFNs represent the major functional pathway downstream of TLR4 activation in liver IRI (11), we also attempted to determine the effect of IFN- β on macrophage CD40 expression. As shown in Fig. 7b, both LPS and IFN- β upregulated CD40 expression in macrophages. Thus, CD40 upregulation in response to TLR4-type I IFN activation may represent the triggering event in the activation of the CD154-CD40 pathway during liver IR.

Discussion

Although the role of CD4 T cells in the pathogenesis of organ IRI has been well documented (4,7), the actual mechanisms remain elusive. The pro-inflammatory role of T cells in IRI has been recently questioned by showing that CD4 deficient mice suffered more severe hepatic IRI, compared with WT controls, despite decreased local neutrophil accumulation (27). Furthermore, RAG-1 KO mice, lacking both T and B cells, were susceptible to renal IRI, whereas T cell reconstitution in these KO mice exerted cytoprotection (28). In this study, we first confirmed the pathogenic role of CD4 T cells in the murine warm hepatic IRI model. Our results document the selective impact of CD4, but not CD8 or NK1.1, cells in promoting tissue pro-inflammatory immune response and injury. As no specific Ags are associated with IRI, we then tested whether *de novo* Ag-specific CD4 T cell activation was required for their function. The CD4 blockage interrupts CD4 T cell signaling, which effectively inhibits CD4 T cell activation in transplant recipients (23), and CD4 T cell proliferation in MLRs. However, in contrast to the effects of CD4 depletion, CD4 blockade failed to suppress liver pro-inflammatory response against IR in our study. This data provides direct evidence that CD4 T cells function in liver IRI without the requirement of *de novo* Ag-specific activation. This conclusion is supported by a recent study in which anti-MHC class II Ab failed to affect liver IRI (29). To dissect the functional mechanism of CD4 T cells in liver IRI, we then differentiated the role of CD154 and IFN- γ . Consistent with our previous study (11), our present data shows that IFN- γ was dispensable for both liver inflammation and injury against IR. To further analyze the role of CD154, we used agonist Ab against CD40 in CD4 KO mice to test the hypothesis that CD4 T cells function by CD154-triggered CD40 signaling in innate immune cells to promote tissue inflammation. Our results confirm that agonist Ab did indeed restore liver pro-inflammatory response and tissue injury in otherwise “protected” CD4 KO mice. These data is consistent with our previous findings in CD154 KO mice or after blockade of CD154 in WT mice (6,8,30). However, the mechanism of CD154 in liver IRI suggested by our current data is not the inhibition of T cell activation *per se* but rather the interruption of T cell help for innate immune activation. Indeed, the ligation of CD40 by soluble or cellular CD154 in macrophages/DCs leads to cell activation with increased TNF- α production *in vitro* (31–33). Thus, our results indicate that CD4 T cells function in liver IRI via CD154-mediated CD40 signaling without the requirement of *de novo* Ag-specific activation.

As activated CD4 T cells express CD154 (34–36), the absence of *de novo* CD4 T cell activation during IR suggests that only previously activated CD4 T cells may provide the key help for liver innate immune activation. Indeed, FACS analysis of liver T cells revealed the presence of a higher percentage of pro-inflammatory effector (CXCR3⁺CD62L^{low}) CD4

subset in liver resident lymphocytes, as compared with splenocytes. Importantly, these effector CD4 T cells in livers constitutively express higher levels of CD154. Thus, these cells have the capability of triggering CD40 signaling. The question is what triggers the activation of CD154-CD40 pathway during IR? As liver CD40 expression is upregulated during IR, we hypothesize that CD40 upregulation may represent the triggering event of CD154-CD40 activation. Indeed, the agonist anti-CD40 by itself triggered only mild liver injury at later time points, i.e., >12 h post injection (37); and it synergized with IR to activate liver pro-inflammatory response within 6h in our model. Additionally, we presented in vitro evidence that both TLR4 ligands and type I IFN can upregulate CD40 expression in macrophages.

As TLR4 activation, particularly its downstream IRF3-mediated type I IFN pathway, has been recently shown to represent the major pathway initiating liver pro-inflammatory response during IR (20,38,39), the obvious question arises on the relationship between CD4 T cell function and TLR4 activation, particularly whether CD154-CD40 signaling may synergize with TLR4 in macrophage (or Kupffer cell) activation. The demonstration that agonist anti-CD40 Ab upregulated TLR4-MD2 complex in murine DCs without increasing TLR4 transcript levels (40), provides a potential synergistic mechanism of CD40 and TLR4 signaling in macrophage activation. This may be particularly relevant to the liver innate immune system, as liver DCs, possibly KCs, express lower TLR4 levels (41). As TLR4 activation upregulates macrophage CD40, it may in turn enhance their response to CD4-derived CD154 signaling, which constitutes another putative synergistic mechanism. In vivo, Ag immunization with a combined TLR/CD40 stimulation elicited a potent cellular immunity, exponentially greater than those with any single stimulation (42,43). In addition, CD40 may synergize with TLR4 in different aspects of liver IRI. As hepatocyte CD40 activation leads to their death, CD40 signaling may also enhance TNF- α or reactive oxygen species-induced liver injury resulting from TLR4 activation.

In summary, this study provides evidence that effector CD4 T cells reside in livers and facilitate liver inflammation/tissue damage during IR by CD154 without *de novo* Ag-specific activation. These novel findings highlight the unique function of previously activated CD4 T cells in regulating tissue innate immune response.

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Figure 1.

CD4 T cells are critical for the liver pro-inflammatory immune response in IRI. (a). WT, T cell-deficient (nude) or CD4-, CD8-deficient mice, or WT mice treated with CD4, or CD8, or NK1.1 depleting Abs were subjected to 90 min of liver warm ischemia. Serum ALT levels were measured at 6 h post reperfusion. Each dot represents an individual animal. (b). Livers from WT, nude, WT w/CD4-, or CD8-, or NK-cell depleting Ab-treated groups were harvested after 6 h of reperfusion and analyzed by histology (Representative of 4–10/group; H/E staining x40). (c) Livers from WT or WT treated with CD4, CD8, or NK1.1 depleting Abs were harvested after 6 h of reperfusion. Liver expression of TNF- α and CXCL10 (IP-10) was measured by qRT-PCR.

Figure 2.

(a) Both depleting and blocking anti-CD4 Abs inhibit CD4 T cell proliferation against allo-Ags. Splenocytes were isolated from naive B6 mice, labeled with CFSE, and then stimulated with either x-irradiated syngeneic or allogeneic (B/c) splenocytes or ConA (4 U/ml) for 4 days in vitro. Stimulated cells were stained with fluorochrome-labeled anti-CD4 and subjected to FACS analysis. CD4 cells were gated and analyzed for CFSE histograms. Both CD4 Abs (YTS177 or GK1.5 treated, grey lines; Controls, black line; Filled, syngeneic stimulation) effectively inhibited alloreactive, but not ConA-induced, CD4 T cell proliferation; (b) GK1.5, but not YTS177, Ab depleted CD4 T cells in spleen and livers. Splenocytes and liver lymphocytes were isolated from B6 mice that were pretreated with control Ig, GK1.5 or YTS177 (day -1 at 1mg/mouse), and stained with fluorochrome-labeled anti-CD4, -CD8. Cells were then subjected to FACS analysis to quantitate CD4 and CD8 cells. Representative (n=3–4) dot plots are shown.

Figure 3. CD4 T blocking Ab fails to protect livers from IRI. (a). WT B6 mice treated with control Ig, GK1.5 or YTS177 Ab were subjected to liver IR. Serum ALT levels were measured at 6 h post reperfusion. Each dot represents an individual animal. (b). Livers were harvested after 6 h of reperfusion and analyzed by histology (Representative of 3–4/group; H/E staining x40). (c) Liver expression of TNF-α and CXCL10 (IP-10) was measured by qRT-PCR (n=3–4/group).

Figure 4.

IFN- γ is not critical in the mechanism of liver IRI. WT mice were treated with control Ig, anti-CD154 (MR1) or anti-IFN- γ Ab prior to being subjected to liver IR experiment. (a) Serum ALT levels were measured at 6 h post-reperfusion. Each dot represents one individual animal. (b) Liver histology (at 6h of reperfusion) was evaluated by H/E staining (40x). (c) Liver expression of TNF- α , CXCL10 (IP-10) and IL-1 β was measured by qRT-PCR (n=3–4/group).

Figure 5.

Agonist anti-CD40 Abs restores liver IRI in CD4 KO mice. (a) CD4 KO mice treated with control Ig, or anti-CD40 Ab were subjected to liver IR experiments. Serum ALT levels were measured at 6 h post-reperfusion. Each dot represents one individual animal. (b) Liver histology (at 6h of reperfusion) was evaluated by H/E staining (40x). (c). Liver expression of TNF- α and CXCL10 (IP-10) was measured by qRT-PCR (n=3–4/group).

Fig.6b

Figure 6.

Liver CD4 T cells are enriched with the pro-inflammatory effector type and constitutively express CD154. Splenocytes and liver lymphocytes were isolated from the same WT mice. (a). CD4+ or CD8+ cells were gated and analyzed for CD62L, CXCR3, CD25 or CD44 expression. (b). CD154 expression was evaluated by intracellular staining. CD4 or CD8 T cells were gated and analyzed for CD62L and CD154 expression by dot plots. CD154 expression in CD4 T cells or its subsets in spleen or livers were also plotted in histograms. Representative (n=4) dot plots and histograms were shown.

Figure 7.

CD40 expression patterns in livers and macrophages. (a) Ischemic livers were harvested serially after reperfusion and subjected to gene analysis by qRT-PCR. CD154 and CD40 expression kinetics was plotted as their average ratios to house-keeping gene HPRT ($n=2-4$ /time points). (b). RAW264.7 cells were either unstimulated (Ctl) or stimulated with LPS (10ng/ml) or IFN- β (50U/ml) overnight and harvested for FACS analysis of CD40 expression.