Macrophage Heterogeneity in Liver Ischemia Reperfusion Injury

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ABSTRACT

The liver has the largest population of tissue resident macrophages, i.e., Kupffer cells. How they react and function in liver ischemia and reperfusion injury (IRI) vs. bone marrow derived infiltrating macrophages, remains poorly defined. Taking advantage of newly developed Clec4F-DTR mice, we studied KC responses and determined the impact of their specific depletion via diphtheria toxin (DT) on the pathogenesis of liver IRI. Our results showed that liver IR resulted in a significant loss of embryonic derived KCs (TIM-4$^+$), in parallel with the infiltration of monocytes/macrophages and neutrophils in IR livers. The DT-mediated elimination of Clec4F$^+$KCs prior to the onset of liver ischemia changed liver susceptibilities to IRI in a time-dependent manner. Liver IRI was reduced at 24h, but increased at 14 days, post DT injection. The cytoprotective phenotype in the KC-depleted mice was due to the infiltration of immunoregulator macrophages (Gr-1$^+$CD11b$^+$ Ly6G$^-$), which exhibited immunosuppressive signature in RNA-seq analysis, as compared with KCs. Anti-Gr-1 depleting Abs restored liver IRI in these DT-treated mice. In comparison, the DT treatment in CD11b-DTR mice or CCR2 deficiency, which reduced monocyte infiltration, minimally impacted the severity of liver IRI. Neutrophils and monocytes but not macrophages dominated the liver myeloid cell population in the activation phase of the disease. Thus, liver resident, rather than infiltrating, macrophages play key roles in the inflammatory activation of liver IRI. New Gr-1$^+$ infiltrating macrophage after KC depletion are immune regulatory and protects livers from IRI.

METHODS

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 60 min of ischemia, the clip was removed to initiate hepatic reperfusion. Sham controls underwent the same procedure, but without vascular occlusion. Mice were sacrificed after 6h to 5 days of reperfusion, and liver and serum samples were collected. Serum alanine aminotransferase (sALT) levels were measured. Parts of liver specimens were fixed in 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. Anti-Gr-1 Abs (0.5mg/mouse, BioXCell, West Lebanon, NH) were administered, i.v., 24h prior to liver ischemia.

Macrophage Depletion. To deplete KCs or CD11b+ infiltrating macrophages, we injected diphtheria toxin at 10μg/g mouse body weight, i.v. 24h prior to the onset of liver ischemia. The selective depletion of KCs and CD11b macrophages in these mouse models as confirmed by FACS analysis of liver non-parenchymal cells and peritoneal exudes 24h post DT injection. KCs (F4/80+Clec4F+TIM4+) were depleted in Clec4F-DTR, but not CD11b-DTR mice, while peritoneal macrophages (F4/80+CD11b+) were depleted in CD11b-DTR, but not Clect4F-DTR mice.

Liver NPC/Kupffer cell isolation. Liver NPC/KCs were isolated from normal or IR livers of B6 mice by in situ collagenase perfusion. In brief, livers were perfused via the portal vein with calcium- and magnesium-free HBSS supplemented with 2% heat-inactivated FBS, followed by 0.27% collagenase IV (Sigma, St Louis, MO). Perfused livers were dissected and teased through 70 μm nylon mesh cell strainers (BD Biosciences, San Diego, CA). Non-parenchymal cells (NPCs) were separated from hepatocytes by centrifuging at 50g for 2 min three times. NPCs were stained with fluorescence-labeled Abs and analyzed by FACS.

KC and 24h iMΦs Comparison. Measurements for KCs and iMΦs 24 hours post-depletion were collected from NCBI's Gene Expression Omnibus under accession number GSE128657. Genes with a maximum read count below 10 across samples were removed; ribosomal genes (prefixed with "RPL" or "RPS") and spliceosome genes (prefixed with "SNP" or "SNR") were removed. Average gene expression was compared between KCs and 24h iMΦs via two-sample t-test; resulting p-values were corrected for multiple-hypothesis testing via Benjamini-Hochberg procedure. Concurrently, log2-fold change in average gene expression between KCs and 24h iMΦs was derived. Genes with (1) a log2-fold change greater than 1 and (2) a corrected t-test p-value less than 0.05 were considered differentially expressed (DEGs). Gene set enrichment analyses were performed via Enrichr through the GSEApy package to identify concerted biological trends in DEGs (1,2). DEGs were compared against the Gene Ontology: Biological Process 2025 terms (3,4).

RESULTS and CONCLUSIONS

1. Liver IR results in significant depletion of KCs.

In Clec4F-driven tdTomato reporter mice, we isolated non-parenchymal cells (NPCs) from sham or IR (60m or 90m) livers (both ischemia and non-ischemic lobes) at 24h post reperfusion. Resident KCs were identified as F4/80$^+$CD11b^{low}tdTomato$^+$TIM-4$^+$ cells. Compared with those in the sham livers (calculated based on the total# of cells isolated from IR livers and percentages of KCs) , KCs were severely depleted by liver IR: 90m ischemia resulted in more than 95% reduction, while 60m ischemia resulted in ~60% reduction, with simultaneous increases of infiltrating inflammatory myeloid cells (CD11b^{high}Gr-1$^+$) at 1500% and 500%, respectively (Fig.1B). KCs in non-ischemic lobes were also affected with significant but less degree of reduction: ~55% reduction after 90m ischemia. Systemic inflammation (induced by LPS injection i.v.) also reduced KCs in livers (data not shown). We confirmed KC depletion in IR-livers by Clec4F immunohistochemical staining. Indeed, average numbers of Clec4F$^+$ cells/area were significantly lower in IR livers vs. sham control (Fig.1C).

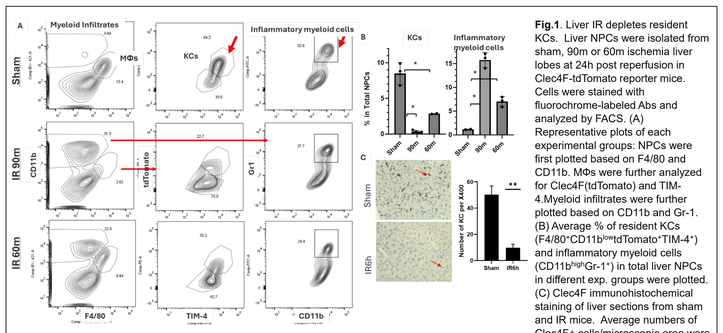

Fig.1. Liver IR depletes resident KCs. Liver NPCs were isolated from sham, 90m or 60m ischemia liver lobes at 24h post reperfusion in Clec4F-tdTomato reporter mice. Cells were stained with fluorochrome-labeled Abs and analyzed by FACS. (A) Representative plots of each experimental groups: NPCs were first plotted based on F4/80 and CD11b. MΦs were further analyzed for Clec4F(tdTomato) and TIM-4.Myeloid infiltrates were further plotted based on CD11b and Gr-1. (B) Average % of resident KCs (F4/80$^+$CD11b^{low}tdTomato$^+$TIM-4$^+$) and inflammatory myeloid cells (CD11b^{high}Gr-1$^+$) in total liver NPCs in different exp. groups were plotted. (C) Clec4F immunohistochemical staining of liver sections from sham and IR mice. Average numbers of Clec4F+ cells/microscopic area were plotted.

Figure 1

2. KC depletion results in a transient protection of livers from IRI.

To define roles of KCs in liver IRI, we depleted KCs in Clec4F-DTR mice with a single dose of DT and compared liver IRI in control vs KC-depleted cohorts at 24h post DT injection (Fig.2A). The KC-specific depletion by DT in this model was confirmed by FACS analysis of MΦs in livers and peritoneal cavity. Interestingly, these KC-depleted mice developed significantly reduced liver IRI, as evidenced by lower sALT levels (measured at 6h post reperfusion), better preserved liver histological architectures and lower inflammatory gene expressions in IR livers, vs. controls (Fig.2B, C). The overall downregulation of inflammatory genes, including both pro- and anti-inflammatory, such as TNF-a, IL-1b, IL-10, TGF-b, iNOS, Arg-1, as well as chemokines, such as CCL2 and CXCL1, indicates an immune suppressive status in KC-depleted livers at 24h post DT injection.

We analyzed liver non-parenchymal cells (NPCs) by FACS in KC-depleted vs. controls at 24h post DT injection. The DT treatment resulted in a complete elimination of Clec4F$^+$F4/80$^+$CD11b^{low} KCs and infiltration of F4/80^{low}CD11b$^+$Gr-1$^+$ infiltrates which were mostly Ly6C$^+$ cells (Fig.2E). To determine whether these Gr-1$^+$ infiltrates were cytoprotective, we administered anti-Gr-1 Abs following DT injection (at -20h) in Clec4F-DTR mice (Fig.2A). Liver IRI was increased by the Ab treatment in the KC-depleted mice (Fig.2B). The pre-treatment with anti-Gr-1 Ab by itself did not affect liver IRI in control mice. Thus, the Gr-1$^+$ infiltrates were responsible for the liver protection from IRI in KC-depleted mice. The depletion of Gr-1$^+$ cells in KC-depleted livers (prior to ischemia) was confirmed by FACS analysis (Fig.2E). The anti-Gr-1 Ab resulted in the upregulation of TNF-a and IL-10 (much less degree), but further downregulation of TGF-b and Arg-1, gene expressions in KC-depleted IR livers. Meanwhile IL-1β, CCL2 and CXCL1 levels remained mostly unchanged. These data indicate that the Gr-1+CD11b+Ly6C+ infiltrating cells in KC-depleted livers played immune regulatory roles in liver IRI.

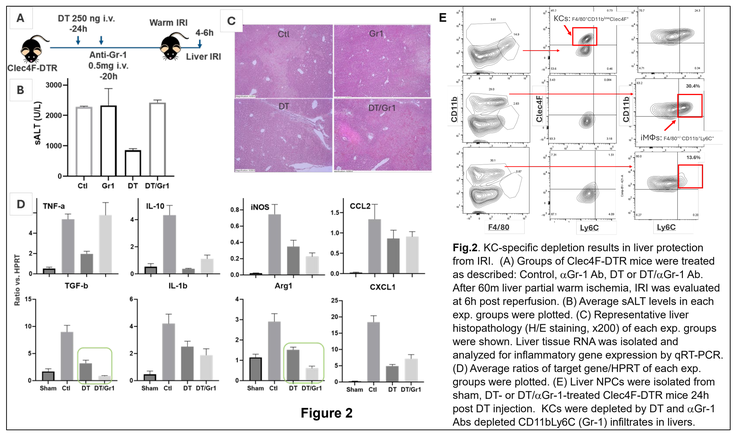

Fig.2. KC-specific depletion results in liver protection from IRI. (A) Groups of Clec4F-DTR mice were treated as described: Control, αGr-1 Ab, DT or DT/αGr-1 Ab. After 60m liver partial warm ischemia, IRI was evaluated at 6h post reperfusion. (B) Average sALT levels in each exp. groups were plotted. (C) Representative liver histopathology (H/E staining, x200) of each exp. groups were shown. Liver tissue RNA was isolated and analyzed for inflammatory gene expression by qRT-PCR. (D) Average ratios of target gene/HPRT of each exp. groups were plotted. (E) Liver NPCs were isolated from sham, DT- or DT/αGr-1-treated Clec4F-DTR mice 24h post DT injection. KCs were depleted by DT and αGr-1 Abs depleted CD11bLy6C (Gr-1) infiltrates in livers.

Figure 2

3. The iMΦs at 24h post DT injection exhibit immunosuppressive signatures.

To analyze the protective mechanisms of these Ly6C$^+$CD11b$^+$ cells in 24h DT-treated livers, we analyzed the RNA-seq data set (GSE128657) deposited by Sakai M et al. of FACS-sorted liver MΦs from Clec4F-DTR mice at different time points following DT injection. We compared average gene expression in KCs and iMΦs 24h post-depletion by two-sample t-test. Differentially expressed genes (DEGs) were evaluated for concerted transcriptomic patterns via gene set enrichment analyses. Compared to KCs, 24h iMΦs exhibited elevated expression of genes associated with negative regulation of immune responses (Fig.3A). Likewise, KCs exhibited elevated expression of genes associated with inflammatory responses relative to the 24h iMΦs. Further examination of differentials in gene expression found that immunoregulatory genes demonstrated near-universally greater expression in 24h iMΦs relative to KCs (Fig.3B). Inflammatory responses in KCs were driven primarily by genes associated with cytokine (IL1A, IL1B, IL18) and chemokine (CCL24, CCR5, CXCL13) responses (Fig.3C). Comparatively, immunoregulatory responses in iMΦs included elevated expression of Trem2, TIM-3 (HAVCR2), and Galectin-3, which have been shown to potently inhibit macrophage pro-inflammatory activation.

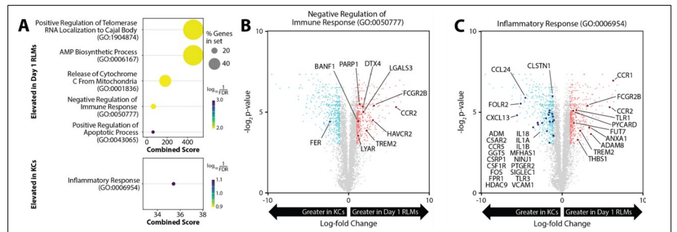

Fig.3. 24h iMΦs exhibit immunosuppressive signatures. A) Dot plots depicting enrichment analyses for genes with elevated expression in 24h iMΦs post-KC depletion (top) or Kupffer cells (KCs, bottom). Only genes with (1) log2-fold change (LFC) greater than 1 and (2) corrected t-test p-values below 0.05 between cell populations were considered for each enrichment analysis. B-C) Volcano plots comparing differences in average gene expression between 24h iMΦs and KCs. Colored points correspond to genes with (1) log2-fold change greater than 1 and (2) corrected t-test p-values below 0.05 between cell types. Red corresponds to genes with greater expression in 24h iMΦs; blue corresponds to genes with greater expression in KCs. Bolded points with gene labels correspond to genes with significant differential expression that are part of the GO Biological Process terms "Negative Regulation of Immune Response" (B) and "Inflammatory Response" (C).

Figure 3

4. CD11b depletion and CCR2 deficiency minimally impacted acute liver IRI

As a comparison, we also studied CD11b-DTR and CCR2 deficient mice to determine roles of BM-derived iMΦs in our model. CD11b depletion by a single dose of DT 24h prior to the onset of liver ischemia did not affect the severity of liver IRI (data not shown). Similarly, CCR2 deficient (CCR2-GFP homozygous) mice developed the same levels of liver IRI as their controls (CCR2-GFP heterozygous), as documented by sALT levels, histopathological pictures and inflammatory gene expressions (Fig.4A, B, C). These results indicate that CD11b$^+$ or CCR2$^+$ infiltration does not impact the acute phase of liver IRI.

FACS analysis of liver myeloid cells revealed that neutrophils (CCR2$^-$Ly6G$^+$) and monocytes (CCR2$^+$Ly6C$^+$) (all F4/80-) dominated IR livers at 6h post reperfusion (Fig.4D). CCR2 deficiency reduced selectively monocytes, but not neutrophil, infiltration in IR livers.

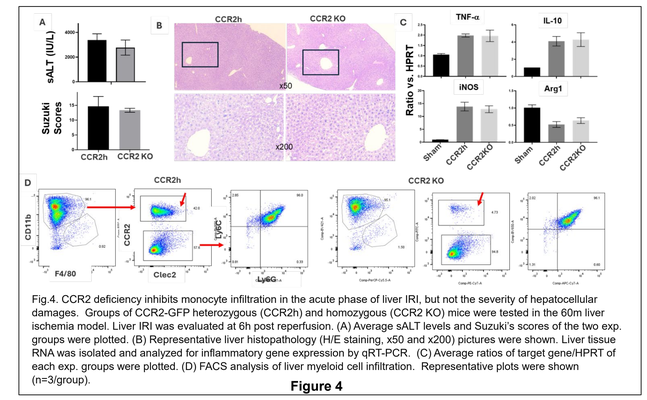

Fig.4. CCR2 deficiency inhibits monocyte infiltration in the acute phase of liver IRI, but not the severity of hepatocellular damages. Groups of CCR2-GFP heterozygous (CCR2h) and homozygous (CCR2 KO) mice were tested in the 60m liver ischemia model. Liver IRI was evaluated at 6h post reperfusion. (A) Average sALT levels and Suzuki's scores of the two exp. groups were plotted. (B) Representative liver histopathology (H/E staining, x50 and x200) pictures were shown. Liver tissue RNA was isolated and analyzed for inflammatory gene expression by qRT-PCR. (C) Average ratios of target gene/HPRT of each exp. groups were plotted. (D) FACS analysis of liver myeloid cell infiltration. Representative plots were shown (n=3/group).

Figure 4

ACKNOWLEDGMENT

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