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ORIGINAL ARTICLE

Characteristics of Changes in Inflammatory Cytokines as a Function of Hepatic Ischemia-Reperfusion Injury Stage in Mice

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Abstract— Liver ischemia-reperfusion injury (IRI) can severely compromise the prognosis of patients receiving liver surgery. While inflammation contributes to the damage resulting from IRI, only a limited number of inflammation biomarkers have been identified as being associated with the different stages of hepatic IRI. As an approach to identify some of these inflammatory cytokines and the molecular mechanisms involved within different stages of hepatic IRI, we used an advanced antibody array assay to detect multiple proteins. With this technology, we observed specific differences in the content of inflammatory cytokines between ischemic and sham controls, as well as a function of the different reperfusion stages in a hepatic IRI mouse model. For example, while liver tissue content of IL-12p40/p70 was significantly increased in the ischemic stage, it was significantly decreased in the reperfusion stage as compared with that of the sham group. For other inflammatory cytokines, no changes were obtained between the ischemic and reperfusion stages with levels of IL-17, Eotaxin-2, Eotaxin, and sTNF RII all being consistently increased, while those of TIMP-1, TIMP-2, BLC, and MCSF consistently decreased as compared with that of the sham group at all reperfusion stages examined. Results from protein function annotation Gene Ontology and the KEGG pathway revealed that inflammatory cytokines are enriched in a network associated with activation of inflammatory response signaling pathways such as TLR, TNF, and IL-17 when comparing responses of the IR *versus* sham groups. The identification of cytokines along with their roles at specific stages of IRI may reveal important new biological markers for the diagnosis and prognosis of hepatic IRI.

KEY WORDS: hepatic ischemia-reperfusion injury; inflammatory cytokines; diagnosis and prognosis; biomarker.

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INTRODUCTION

Hepatic ischemia-reperfusion injury (IRI) can severely compromise liver metabolic function, thus affecting the clinical prognosis and survival of patients undergoing liver surgery [1–3]. It appears that inflammation associated with the occurrence and development of various liver diseases represents an important contributing factor with regard to liver diseases [4] and functions as a critical link involved with hepatic IRI [5]. The damage resulting from IRI progresses through a series of stages. In the initial stages of hepatic ischemia, hepatocellular edema and cell death are observed. The inflammatory reactions associated with these effects disrupt the homeostasis that exists between pro- and anti-inflammatory responses and involves multiple cytokines. Pro-inflammatory mediators, such as TNF-α, IL-6, and CXC, are rapidly released from injured tissue during the acute phase of hepatic IRI, which then induces the recruitment and activation of inflammatory cells [6–8]. Following liver injury, the release of inflammatory cytokines, such as TNF-a and IL-6, and subsequent inflammatory effector cells are involved in this inflammatory process [9, 10].

However, only a limited number of these inflammatory cytokines have been identified. Nor is it known whether the same marker was expressed differently at different stages of hepatic IRI. These factors reveal the complexity involved with regard to the screening of hepatic IRI biomarkers. Accordingly, screening for non-invasive biomarkers with high sensitivity and specificity for early diagnosis and clinical prognosis of liver IRI would play an important role in the clinical treatment of liver diseases.

Antibody microarray technology, which represents a novel technology that can be applied for the simultaneous detection of multiple proteins, has the advantage of a system capable of high throughput applicability [11]. In this way, it can serve as an effective means of identifying promising biomarkers. Therefore, in this report, we used an advanced antibody array detection chip technology for the rapid detection of multiple proteins with the goal of identifying protein markers associated with liver IRI. The ability to characterize the types and molecular mechanisms of inflammation biomarkers within specific stages of hepatic IRI has the potential to improve the diagnosis and clinical management of this condition.

MATERIALS AND METHODS

Experimental Animals

Male C57BL/6 mice (about 5–6 weeks, 20 ± 1 g) were purchased from the Experimental Animal Center of the Chinese Academy of Military Medical Sciences. Mice were housed under specific pathogen-free conditions. All mice used in the experiment were housed in an animal breeding room controlled at 25 °C. All animal protocols were in accordance with the National Institutes of Health (NIH) guidelines, and all experimental mice were housed in accordance with laboratory animal care principles.

Animal Liver IRI Model

The IRI mouse model used in this experiment has been described previously [12]. In this study, we used the segmental (70%) liver ischemia model. The 12 mice were randomly divided into four groups ($N = 3$/group): (1) Sham, (2) IR at 0 h, (3) IR at 6 h, and (4) IR at 12 h.

Serology Detection

Blood collected from all mice was centrifuged to separate the serum. Serum AST and ALT levels were measured using a transaminase detection kit (Nanjing Jiancheng Biotechnology, China).

Histopathological Detection of Liver in Mice

Fresh liver specimens of each group of mice were fixed in 4% neutral isotonic formaldehyde solution for 24 h, then dehydrated and embedded in paraffin. Sections (4 μm) were cut from each paraffin embedded tissue and subjected to H&E staining to evaluate the extent of liver damage.

Antibody Array Assay

A separate portion of fresh liver tissue was minced to obtain protein extracts for measurements using the Mouse Inflammation Array G1 (RayBio Mouse Cytokine Antibody Array, RayBiotech, Norcross, GA, United States). With this assay, it was possible to simultaneously detect 40 separate cytokines. According to the internal positive control provided by RayBiotech analysis tool, the signal values were read and normalized.

Detection of Serum Indicators by ELISA

In order to validate results from the antibody array, ELISA assay kits (Nanjing Jiancheng Biological

Technology, China) were used to measure specific cytokines considered of particular significance (TNF-α and IL-6). Using ELX800NB microplate reader (Biotek, Winooski, CT, USA), the optical density was measured immediately at 450 nm wavelength.

Statistical Analysis

The data are presented as mean ± standard deviation (SD). One-way ANOVA and the Newman Keuls post hoc test were used to analyze differences among the groups. The SPSS software 22.0 program was used in these analyses, and a $P < 0.05$ was required for results to be considered as statistically significant.

RESULTS

Changes in Serum AST and ALT Levels and Liver Histopathology

As shown in Fig. 1a, histopathological analysis of liver showed that a large amount of hepatocyte edema, congestion, apoptosis, or necrosis were observed in the IR group at 0, 6, and 12 h after reperfusion. Moreover, we quantified IR-induced liver injury by measuring Suzuki's score. Suzuki's score gradually increased (Fig. 1b, $P < 0.05$). As shown in Fig. 1c, d levels of liver AST and ALT changed with the time of reperfusion, with levels in the IR group showing a gradual increase ($P < 0.05$). TNF-a levels in the IR group showing a gradual increase ($P < 0.05$) and peaked at 6 h after reperfusion ($P < 0.01$). Moreover, IL-6 levels in the IR group showing a gradual increase ($P < 0.05$) *versus* the sham group.

Expression Characteristics of IRI-Induced Inflammatory Cytokines

Figure 2 a contains a map of the mouse inflammation antibody array G series I including the 40 cytokines detected. In addition, the array distribution of positive correlation between fluorescent intensities and expression levels is shown in Fig. 2b. This presentation further reveals the variations of these proteins observed in the IR groups over the three reperfusion times. Moreover, with use of an unsupervised-hierarchical cluster, it was possible to distinguish among the three reperfusion times of the IR groups (Fig. 2c).

Altered Inflammatory Cytokines Levels in the 0 H Group

The parameter used for evaluating statistical significance was that of the fold change. For this analysis, results included (log2) fold changes for each protein and for each individual contrast. Differentially expressed proteins (DEPs) were defined as those demonstrating a fold change over 1.2 or less than 0.83 (absolute logFC > 0.263). Based on this analysis, we found that BLC, MCP-1, IL3, IFN-gamma, TIMP-2, MIG, IL4, IL-17, Lymphotactin, Fas, ligand, CD 30L, I-TAC, IL-12p40/p70, TCA-3, sTNF RI, sTNF RII, RANTES, Eotaxin, and Eotaxin-2 were all significantly upregulated in the 0 h group as compared with that of the sham group (Fig. 3a, b, $P < 0.05$). In contrast, Leptin, TECK, TIMP-1, MCSF, and BLC were all significantly downregulated in the IR 0 h group ($P < 0.05$).

In order to better understand the role of inflammatory cytokines in mouse hepatic IR injury, protein function annotation gene ontology (GO) and the KEGG pathway were analyzed with use of the R package "clusterProfiler." GO analysis includes three subtypes: BP (biological process, Fig. 3c), MF (molecular function, Fig. 3e), and CC (cellular component, Fig. 3d). KEGG involves a systematic analysis of protein functions, linking genomic information with higher-order functional information (Fig. 3f). When comparing inflammatory cytokines between the IR and sham groups at 0 h, we found that those of the IR group were enriched in a network associated with the activation of inflammatory response pathways such as cytokine-cytokine receptor interaction, chemokine signaling pathway, TNF signaling pathway, rheumatoid arthritis, and IL-17 signaling pathway (Fig. 3f).

Altered Inflammatory Cytokines Levels in the 6 H Group

As shown in Fig. 4a, Leptin, TIMP-2, BLC, IL-1 beta, TECK, IL-12p40/p70, and I-TAC were all significantly downregulated in the IR *versus* sham group at the 6-h reperfusion period (Fig. 4b, $P < 0.05$). In contrast, TCA-3, MIP-1 gamma, lymphotactin, IL-1 alpha, SDF-1, LIX, MIG, Eotaxin-2, Fractalkine, sTNF RII, KC, and Eotaxin were all upregulated in the 6-h group ($P < 0.05$). Protein function annotation GO (Fig. 4c, d) and KEGG pathway (Fig. 4e) results showed that inflammatory cytokines of the IR 6 h and the sham samples were enriched in a network associated with activation of inflammatory response pathways, for example, Toll-like receptor signaling pathway, Pertussis, Salmonella infection, rheumatoid arthritis, and IL-17 signaling pathway.

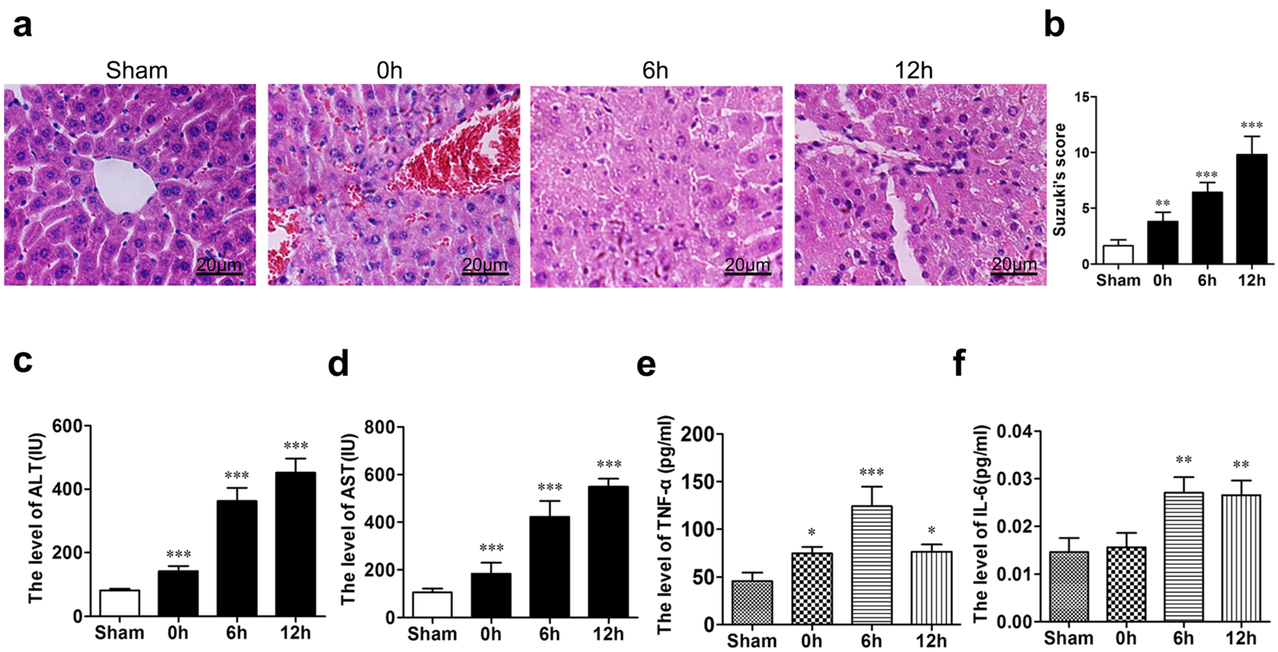

Fig. 1. IR induces liver injury in mice. **a** Hepatic IRI produced varying degrees of liver ischemia, necrosis, and edema as a function of reperfusion times (0, 6, and 12 h) in mouse livers (magnification, × 200, scale bars = 20 μm). **b** Suzuki's score in IR-induced liver injury. **c**, **d** IRI mice showed emporally dependent increases in serum levels of ALT and ASTas a function of reperfusion times. **e**, **f** IR treatment significantly increased serum TNF-α and IL-6 levels as compared with that of the sham group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the sham group.

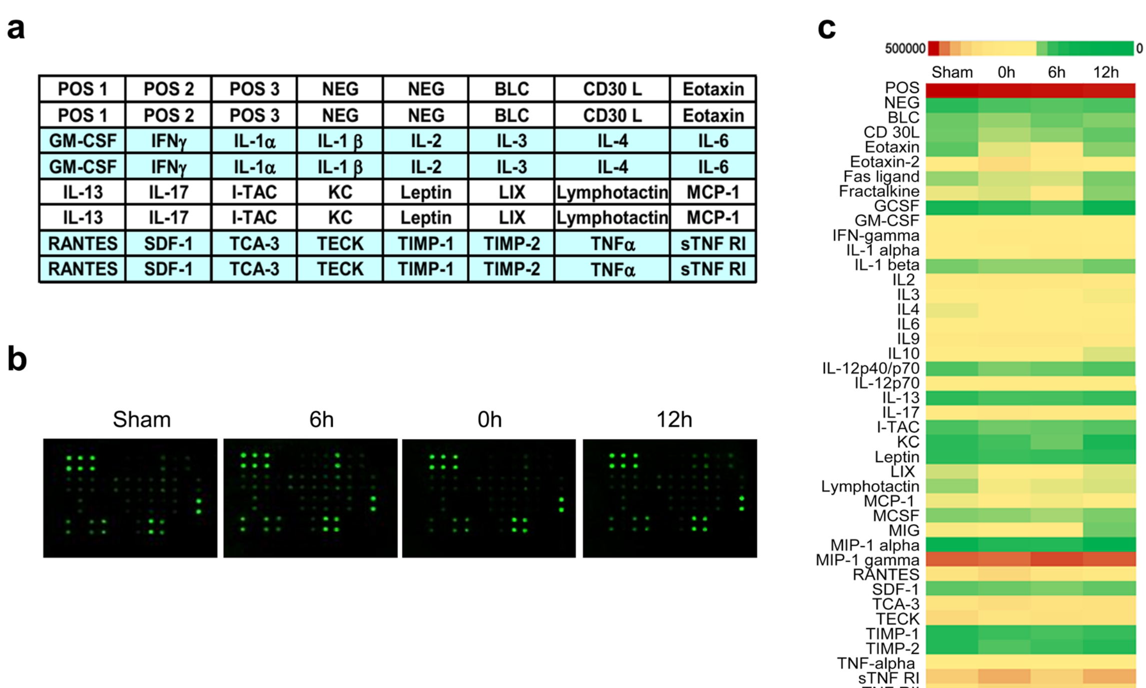

Fig. 2. Antibody array profiles and expression levels of inflammatory cytokines. **a** A map of mouse inflammation antibody array G series I including 40 cytokines. **b** The levels of cytokines are proportional to their fluorescent intensities. **c** Hierarchical cluster analysis of inflammatory cytokines among the three groups.

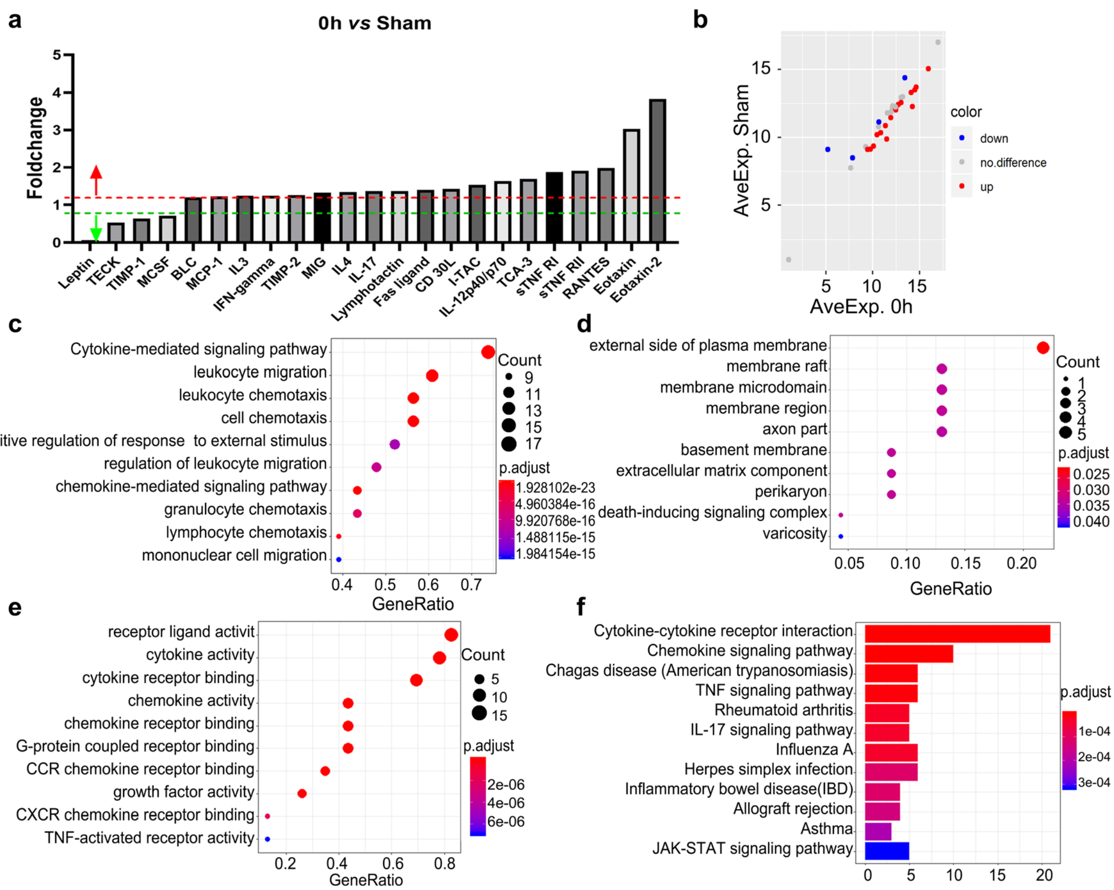

Fig. 3. Protein function annotation GO and KEGG pathway analysis (0 h *vs* sham). **a** DEPs as defined in IR 0 h and sham groups. **b** Scatter plot of inflammatory cytokines in 0 h and sham groups. Red indicates upregulation, blue downregulation, and gray no difference. Biological process (**c**), molecular function (**e**), and cellular component (**d**). **f** KEGG analysis of protein functions, linking genomic information with higher-order functional information.

Altered Inflammatory Cytokines Levels in the 12 H Group

A comparison of inflammatory cytokine levels obtained between the sham and IR groups as determined at the 12-h reperfusion period revealed that Leptin, KC, TIMP-2,TIMP-1, MIG, CD 30L, Fractalkine, IL-1 beta, MCSF, RANTES, TECK, Fas ligand, SDF-1, IL10, and IL-12p40/p70 were all significantly downregulated in the IR group (Fig. 5a, $P < 0.05$). But BLC, IL-17, MIP-1 gamma, IL-1 alpha, Eotaxin-2, Lymphotactin, TCA-3, Eotaxin, sTNF RII, and sTNF RI were all significantly upregulated in this IR 12-h group (Fig. 5a, b, $P < 0.05$). Protein function annotation GO (Fig. 5c–e) and KEGG pathway (Fig. 5f) results showed that inflammatory cytokines of the IR 12-h group were enriched in a network associated with activation of inflammatory response pathways.

DISCUSSION

The exact mechanisms that underlay the injury associated with hepatic ischemia-reperfusion injury are quite complex. However, it is clear that inflammation represents a key factor inf ischemia-reperfusion injury [2], and, in specific, in hepatic IRI [13–15], which has been shown to include the participation of a number of inflammatory cells and inflammatory cytokines [6, 16]. Complementing these processes is activation of the innate and adaptive immune systems, which plays an immunomodulatory and chemotactic role [17]. Therefore, any procedure involving a reduction in these inflammatory responses would serve as an important method for use in the treatment of liver IRI.

While it has been well established that the inflammatory response plays an important role in the pathogenesis and

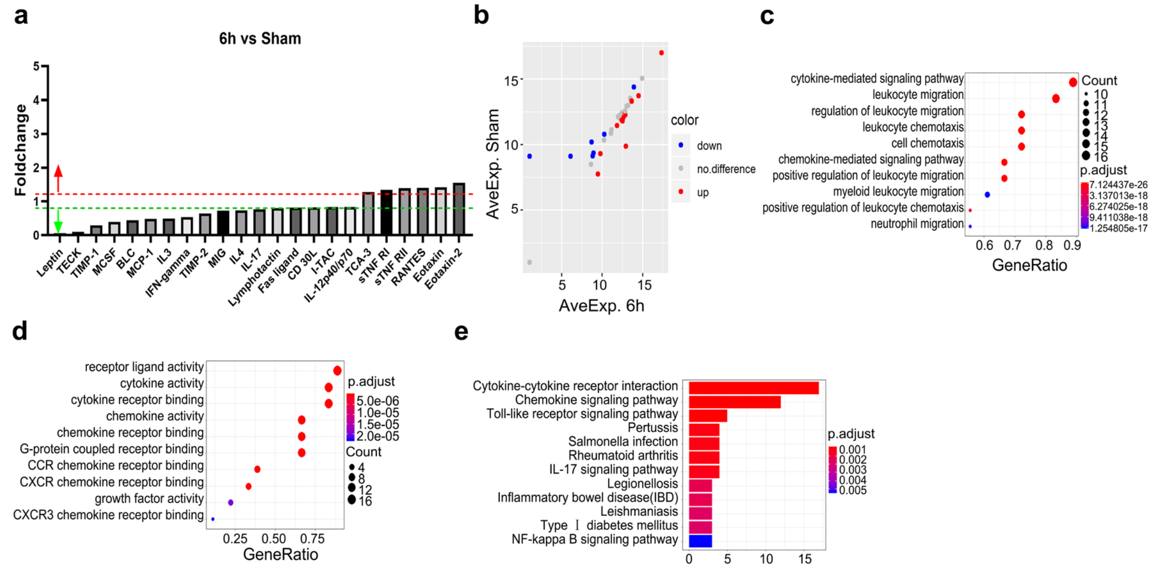

Fig. 4. Protein function annotation GO and KEGG pathway analysis (6 h *vs* sham). **a** DEPs are defined in IR 6 h and sham groups. **b** Scatter plot of inflammatory cytokines in 6 h and sham groups. Red indicates upregulation, blue downregulation, and gray presents no difference. Biological process (**c**) and molecular function (**d**). **e** KEGG analysis of protein functions, linking genomic information with higher-order functional information.

pathological processes of IRI [18], the identification and molecular mechanism of inflammatory cytokines in hepatic IRI is sorely absent. In this report, an advanced antibody array detection chip technology was employed to identify protein markers associated with liver IRI. Compared to the sham group, MCP-1, IFN-gamma, TIMP-2, IL-17, CD 30L, I-TAC, IL-12p40/p70, TCA-3, sTNF RI, sTNF RII, RANTES, Eotaxin, and Eotaxin-2 were all upregulated at the 0-h reperfusion period. However, Leptin, TECK, TIMP-1, MCSF, and BLC were all significantly downregulated in this group. At the 6-h reperfusion period, Leptin, TIMP-2, BLC, IL-1 beta, TECK, IL-12p40/p70, and I-TAC were all significantly downregulated in the IR *versus* sham group, while TCA-3, MIP-1 gamma, Lymphotactin, IL-1 alpha, SDF-1, LIX, MIG, Eotaxin-2, Fractalkine, sTNF RII, KC, and Eotaxin were all significantly upregulated in this IR 6-h group. Finally, when examining these inflammatory cytokine levels at 12 h after reperfusion between the sham and IR groups, we found that Leptin, TIMP-2,TIMP-1, CD 30L, IL-1 beta, MCSF, RANTES, TECK, Fas ligand, SDF-1, IL10, and IL-12p40/p70 were all significantly downregulated in the IR groups. In contrast, BLC, IL-17, MIP-1 gamma, IL-1 alpha, Eotaxin-2, Lymphotactin, TCA-3, Eotaxin, sTNF RII, and sTNF RI were all significantly upregulated in the IR group at this 12-h reperfusion period. These results not only demonstrate some of the inflammatory cytokines that are activated in response to hepatic IRI, but of greater significance, the specificity in this cytokine activation that exists as a function of different reperfusion stages. For example, in the ischemic stage, liver tissue content of IL-12p40/p70 was increased, while in the reperfusion stage, this IL-12p40/p70 content was significantly decreased. For other inflammatory cytokines, here were no differences between responses observed in the ischemic *versus* reperfusion stages of the liver. Specifically, IL-17, Eotaxin-2, Eotaxin, and sTNF RII all significantly increased *versus* that obtained in the sham group at all IR stages, while TIMP-1, TIMP-2, BLC, and MCSF were all significantly decreased at all IR stages.

To better understand the role of these inflammatory cytokines in hepatic injury in mice, protein function annotation GO and the KEGG pathway were analyzed with use of the R package. Protein function annotation GO and KEGG pathway revealed that inflammatory cytokines of the IR group as determined at the three reperfusion periods (0 h, 6 h, and 12 h) were enriched in a network associated with activation of inflammatory response signaling pathways such as TLR, TNF, and IL-17.

Toll-like receptor (TLR) is a transmembrane receptor that plays a sentinel role in innate and adaptive immune responses. In this way, it can function as a component through which the innate immune system perceives the invasion or tissue damage of pathogenic microorganisms by identifying specific molecular patterns in microbial products or endogenous molecules released by damaged tissues [19–21]. In livers subjected to a partial warm ischemia, the ischemia activates innate immune cells in the liver through ER stress

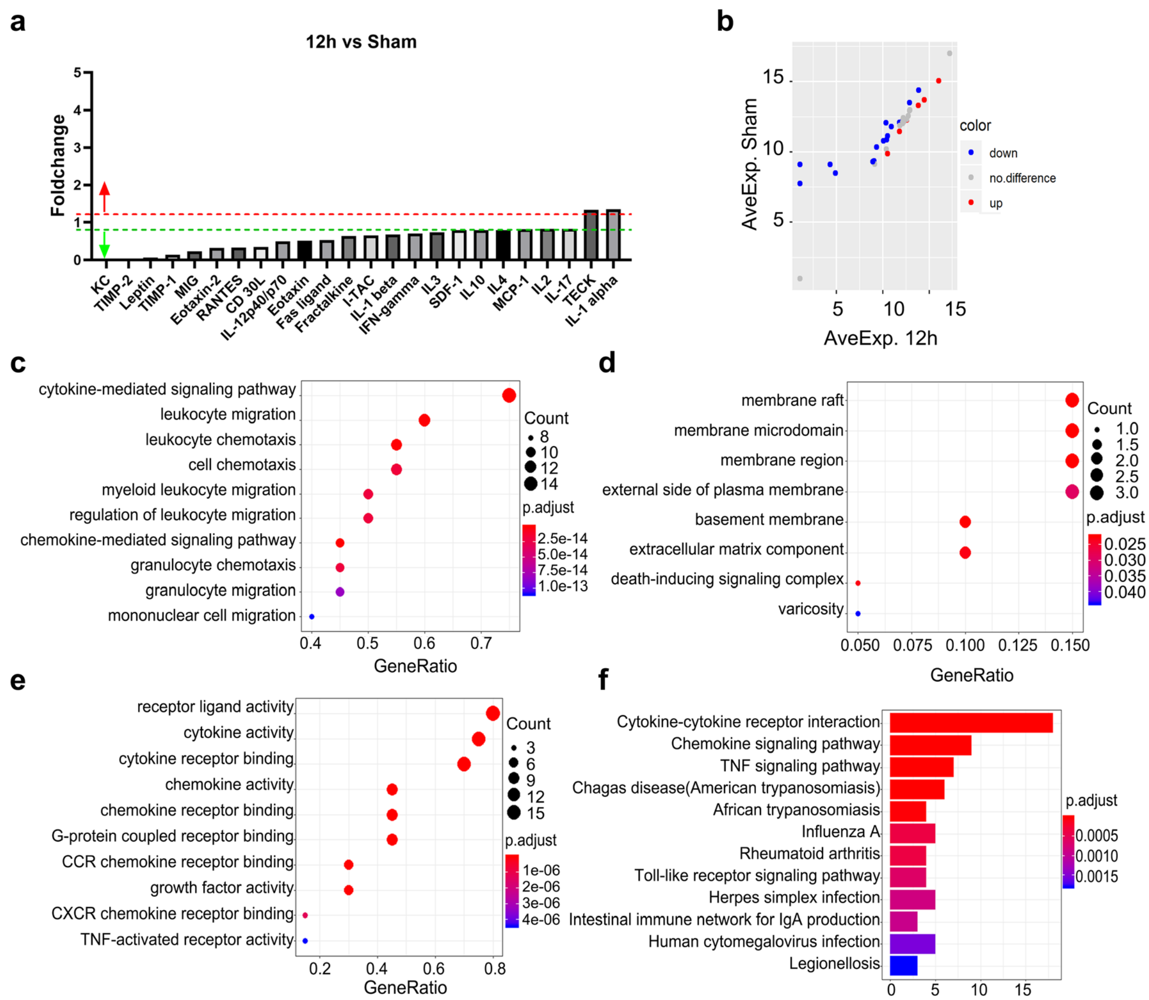

Fig. 5. Protein function annotation GO and KEGG pathway analysis (12 h *vs* sham). **a** DEPs are defined in IR 12 h and sham groups. **b** Scatter plot of inflammatory cytokines in 12 h and sham groups. Red indicates upregulation, blue downregulation, and gray no difference. Biological process (**c**), molecular function (**e**), and cellular component (**d**). **f** KEGG analysis of protein functions, linking genomic information with higher-order functional information.

mediated by ATF6. Ischemia-reperfusion-induced metabolic stress and TLR activation co-activate tissue inflammatory immune response [22]. Ischemia-induced liver injury and inflammation require the binding of TLR4 to phagocytic non-substantive cells such as Kupffer cells [23]. Interestingly, in myocardial IRI, the expression of NF-κB in TLR-4 null mice was found to be significantly decreased as compared with that observed in homozygous mice. These findings suggest that TLR-4/NF-κB pathway is closely related to myocardial injury, which plays a role by releasing inflammatory cytokines [24].

Tumor necrosis factor (TNF), a mediator involved with IR, plays a critical role in hepatocyte injury. In particular, TNF-α exerts a number of effects in hepatic IRI due to the variety of interactions associated with this protein [25]. In part, the hepatocellular apoptosis that commonly occurs in IRI requires TNF to bind to TNF receptor 1 (TNFR1) and form a death-inducing signal complex. The cytoprotective effect of HO-1 is mediated by inhibiting TNF/TNFR1-mediated apoptotic signals, such as by regulating apoptotic disc formation and mitochondrial TNFR1 translocation in hepatic IRI [26]. Liver secretion of TNF-a following IR can also be controlled by NOX2 and p47phox, with inhibition of Nox1-dependent TNF-a production resulting from Kupffer cytokines and Nox2 in liver [27]. This TNF-a and IL-17 synergistically mediate ATII cells to produce CXCL1 after IR through a NADPH oxidase-dependent mechanism, thereby inducing neutrophil infiltration and lung IRI [28].

Ischemia and reperfusion involve a complex cascade of inflammatory mediators involved in the pathogenesis of liver injury. One notable factor is that of IL-17, which was significantly upregulated in the IR group. The significance of increased IL-17 production has been demonstrated in IRF3-deficient mice where IL-17 neutralization has been shown to enhance hepatocellular damages and liver inflammation in these animals. These IRF3-dependent events downstream of TLR4 control the IL-23/IL-17 axis in the liver. Therefore, this regulatory function of IRF3 plays an important role in hepatic IRI [29]. Further evidence implicating IL-17 has been provided from results obtained in the partial warm ischemia model, where it was reported that IL-17 was involved in the inflammation of hepatic IRI during different reperfusion periods. Tritolide inhibited the production of IL-17 by downregulating Stat3 transcription and inhibiting the migration of neutrophils after hepatic IRI [30].

Taken together, our findings indicate that IL-17, Eotaxin-2, Eotaxin, and sTNF RII may all serve as significant biomarkers in response to ischemia and at specific stages of reperfusion in hepatic IRI. In addition, the signaling pathways of TLR, TNF, and IL-17 are all activated and likely play key roles during hepatic ischemia-reperfusion injury.

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AUTHOR CONTRIBUTIONS

Li SP, Wang FF, and Zhang WK performed the majority of experiments and analyzed the data; Bian MZ and Zhang SY performed the molecular investigations; Li SP, Fang Y, Yan H, and Zhang WK participated equally in treatment of animals; Li SP and Zhang HM designed and coordinated the research; Li SP wrote the paper.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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