主 論 文

Spred2 Deficiency Exacerbates D-Galactosamine/Lipopolysaccharide-induced Acute Liver Injury in Mice via Increased Production of TNFα
(Spred2 欠損は、TNFα産生増加を介してD-ガラクトサミン / LPS 誘発肝傷害を悪化させる)

Introduction

ALF is associated with sepsis, which is frequently caused by gram-negative bacteria, lipopolysaccharide (*LPS*) is the outer cell membrane of gram-negative bacteria and plays a role in the progression of ALF under specific conditions. D-Galactosamine (D-GalN) /LPS-induced ALI in mice is a well-established experimental hepatitis model. D-GalN is an amino sugar that blocks RNA synthesis and greatly increases the sensitivity of LPS-induced hepatotoxicity and LPS can stimulate inflammation cells to produced cytokines, particularly tumor necrosis factor α (TNFα). Recent studies showed that MAPKs are involved in the inflammatory response during ALI and ERK-MAPK is involved in liver pathology, it is reasonable to speculate that ERK-MAPK plays a role in ALI. Dysregulation of ERK-MAPK may exacerbate D-GalN/LPS-induced ALI. Sprouty-related EVH1-domain-containing proteins (Spreds) are a family of proteins that inhibit Ras-dependent ERK signaling and is ubiquitously expressed in various tissues, including the liver. In this study, we demonstrated that Spred2 controls the development of D-GalN/LPS-induced ALI by negatively regulating the ERK-MAPK pathway.

Materials and Methods

Animal model

Spred-2 knockout (Spred-2$^{-/-}$) and wild type(WT) female mice (8–12 weeks old) were used in this study. Mice were given intraperitoneal (i.p.) injection of LPS (20 μg/kg) and D-GalN (400 mg/kg) dissolved in phosphate-buffered saline (PBS). In some experiments, mice were pre-treated by i.p. injection of D-GalN (700 mg/kg) 30 min before intravenous (i.v.) injection of mouse recombinant TNFα (10 μg/kg). In a different set of experiments, mice were treated i.p. with 0.5 μM U0126 or vehicle control (DMSO) 2 h before D-GalN/LPS administration. To neutralize endogenous TNFα, neutralizing rabbit anti-murine TNFα IgG (750 μg/mouse) was administered i.p. 14 h before D-GalN/LPS administration.

Detection of apoptosis and caspase activity

Apoptotic cells in the liver were detected by TUNEL assay using an in-situ apoptosis detection kit. Caspase-3/-8/-9 activities were measured by a colorimetric assay kit.

Isolation of hepatic leukocytes and Kupffer cells

After digestion of livers with HBSS containing collagenase and DNase I, hepatic leukocytes were isolated by Percoll centrifugation. For Kupffer cell isolation, $CD11b^+$ cells were isolated from the cells using anti-CD11b microbeads over MS^+ MiniMACS separation columns.

Isolation and culture of primary hepatocytes

Hepatocytes were isolated using a modified two-step collagenase perfusion technique. Hepatocytes were suspended in hepatocyte maintenance medium and planted on collagen I-coated plates, and stimulated with either D-GalN/TNFα or LPS.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from the cultured cells and whole lungs using a High Pure RNA Isolation Kit or High Pure RNA Tissue Kit. cDNA was constructed from total RNA using oligo $(dT)_{12–18}$ primers, and the cDNAs were used as templates for PCR. RT-qPCR analysis was performed using StepOne with Taqman PCR master mix.

ELISA

Murine cytokines were measured using a standard sandwich ELISA.

Immunoblot analysis

Samples were fractionated by SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. After incubation with a primary antibody, the membrane was counter-stained with secondary antibody and visualized with an enhanced chemiluminescence system.

Immunofluorescence assessment

Frozen livers were sectioned at 4 μm-thick, and incubated with a primary antibody (anti-F4/80 antibody, anti-TNFα antibody) and then Alexa Flour conjugated second antibody. DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining. The tissue sections were analysed using an Olympus confocal microscope system.

Statistics

All data were expressed as the mean ± SEM. Differences of $p < 0.05$ were considered significant. All statistical calculations were performed using GraphPad Prism 6

Results

Spred2 deficiency exacerbates D-GalN/LPS-induced ALI

Compared to WT mice, Spred2$^{-/-}$-mice showed exacerbated ALI after D-GalN/LPS challenge, as determined by serum levels of alanine aminotransferase (ALT), hepatic inflammation and tissue damage. Apoptotic hepatocytes and caspase activities were increased in Spred2$^{-/-}$-livers relative to WT mice.

ERK-MAPK pathway is augmented in Spred2$^{-/-}$ mice

ERK activation, represented by phosphorylated-ERK, was significantly augmented in Spred2$^{-/-}$-livers after D-GalN/LPS challenge compared to WT mice. Treatment with U0126, an inhibitor of the ERK-MAPK pathway, decreased ALT levels and tissue damage in Spred2$^{-/-}$ mice. Thus, the hepatotoxicity was largely dependent on the ERK-MAPK pathway, and the augmented liver injury in Spred2$^{-/-}$ mice was due to upregulated ERK activation.

Enhanced TNFα is responsible for increased ALI in Spred-2$^{-/-}$ mice

The expressions of TNFα and IL-1β in the livers were increased after D-GalN/LPS challenge, both of which were significantly enhanced in Spred2$^{-/-}$-livers. Neutralization of TNFα with anti-TNFα antibody ameliorated ALI, as demonstrated by the histology results and serum levels of ALT in both WT and Spred2$^{-/-}$ mice. Under the situations, the expression of TNFα and IL-1β mRNA were decreased. When mice were challenged with D-GalN/TNFα, compared to WT mice, Spred2$^{-/-}$ mice showed drastically enhanced ALI with increased expressions of TNFα and IL-1β. These results indicate that augmented expressions of endogenous TNF α in Spred2$^{-/-}$-livers were responsible for the enhanced ALI after D-GalN/LPS challenge.

Kupffer cells play a causative role in D-GalN/LPS-induced ALI

Immunofluorescence double staining demonstrated that F4/80^{+} cells, likely Kupffer cells, were positive for TNFα after D-GalN/LPS challenge. TNFα production was significantly higher in Spred2$^{-/-}$-Kupffer cells than in WT controls after LPS stimulation in vitro. U0126 pretreatment decreased TNFα production from Spred2$^{-/-}$-Kupffer cells. Thus, Spred2$^{-/-}$-Kupffer cells produce higher levels of TNFα upon stimulation with LPS, possibly through an enhanced activation of ERK-MAPK pathway.

Discussion

Despite advances in medicine and health care, the management of ALI is currently a significant challenge. In the model of D-GalN/LPS-induced ALI, we demonstrate that over-action of ERK-MAPK is deleterious during ALI and endogenous Spred2 down-regulates the ERK/MAPK pathway.

D-GalN/LPS-induced ALI depends on endogenous TNFα. We also examined whether the production of TNFα might cause the endogenous expression of TNFα and IL-1β that could also induce liver injury. Neutralization of TNFα reduced the expression of TNFα and IL-1β mRNA after D-GalN/LPS challenge by 40% and 53%, respectively. Thus, the expression of TNFα and IL-1β appeared to be partly regulated by the production of endogenous TNFα in response to D-GalN/LPS.

We demonstrated that Kupffer cells produced TNFα in D-GalN/LPS-induced hepatotoxicity. We showed that Spred2$^{-/-}$-Kupffer cells produced more TNFα compared to WT-Kupffer cells after LPS stimulation. Sperd2$^{-/-}$-hepatocytes may also contribute to the augmented TNFα in the Spred2$^{-/-}$-livers; however, this is unlikely because TNFα expression in hepatocytes was extremely lower than in Kupffer cells. We showed that the MEK inhibitor U0126 decreased the level of TNFα in Spred2$^{-/-}$-Kupffer cells. Thus, augmented TNFα production in Spred-2$^{-/-}$-livers appeared to result from enhanced ERK activation in Kupffer cells.

Conclusion

We showed that Spred2 deficiency exacerbates hepatotoxicity via increased production of TNFα in a murine model of D-GalN/LPS-induced ALI. A better understanding of the signalling pathway involved in this animal model may provide insight and lead to identification of potential therapeutic targets