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TLR2/4 ligand-amplified liver inflammation promotes initiation of autoimmune hepatitis due to sustained IL-6/IL-12/IL-4/IL-25 expression

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ABSTRACT

Autoimmune hepatitis (AIH), a serious autoimmune liver disease, can be a lifelong illness, leading to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). So far the mechanisms for disease initiation are largely unknown. Here we report that the amplified non-AIH liver inflammation could promote the initiation of AIH due to the sustained increase of IL-6, IL-12, IL-4, and IL-25 in the liver. The liver injury resulting from virus (adenovirus) or chemicals (CCl_4) could induce an amplified (stronger/long-lasting) hepatic inflammation by releasing the ligands for TLR2/TLR4. The amplified inflammation resulted in the increase of multiple cytokines and chemokines in the liver. Among them, the sustained increase of IL-6/IL-12 resulted in the activation of STAT3 and STAT4 in hepatic $CD4^+CD25^+$ Treg cells, thus suppressing *Foxp3* gene expression to reduce the suppressive function of Treg cells in the liver, but not those in the spleen. The increase of IL-12 and the impairment of Treg function promoted Th1 response in presence of self-mimicking antigen (human CYP2D6). Intriguingly, the amplified inflammation resulted in the increase of IL-4 and IL-25 in the liver. The moderate increase of IL-4 was sufficient for cooperating with IL-25 to initiate Th2 response, but inefficient in suppressing Th1 response, favoring the initiation of autoimmune response. Consequently, either adenovirus/CYP2D6 or CCl_4/CYP2D6 could induce the autoimmune response and AIH in the mice, leading to hepatic fibrosis. The findings in this study suggest that the amplified non-AIH inflammation in the liver could be a driving force for the initiation of autoimmune response and AIH.

1. Introduction

Autoimmune hepatitis (AIH) is a serious autoimmune liver disease that is characterized by a progressive destruction of the liver parenchyma and the development of chronic fibrosis (Christen and Hintermann, 2016). It is defined as a chronic immune-mediated liver injury, characterized in its classic form by lympho-plasmacytic hepatitis, autoantibodies, and the elevated immunoglobulins (Corrigan et al., 2015). AIH can be a lifelong illness, which can lead to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Lapierre and Lamarre, 2015). The mechanisms for disease initiation are largely unknown, although genetic susceptibility, molecular mimicry and impaired immunoregulatory networks are likely to be involved in the pathogenesis of AIH (Liberal et al., 2015; Manns et al., 2015; DeFilippis and Kumar, 2015).

Molecular mimicry is involved in the induction and acceleration of autoimmune processes (Holdener et al., 2008). Self/non-self molecular mimicry is a prerequisite for breaking T-cell tolerance in the liver (Ehser et al., 2013). On the other hand, however, it has also been proposed that molecular mimicry results in autoimmune disease only when there is appropriate secondary stimulation (von Herrath et al., 2003). Several lines of experimental evidence indicate that molecular mimicry alone might be incapable of causing autoimmune disease, and can do so only in conjunction with other factors (von Herrath et al., 2003). These factors could be provided by virally induced inflammation or a nonspecific inflammatory process (von Herrath et al., 2003). Both viruses and drugs could induce non-AIH inflammation, the hepatic inflammation that is not related to the autoimmune response. AIH can be distinguished from virus-induced or drug-induced hepatitis by seropositive autoantibodies and the histological feature such as significant interface hepatitis (Wang et al., 2016). It has been clinically observed that de novo AIH followed the infection of several types of viruses (Guido and Burra, 2011), and that AIH could be induced by drugs (Björnsson et al., 2010). These findings suggest that virus- and

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chemical-induced non-AIH inflammation could promote the initiation of AIH.

Both viruses and drugs could induce the liver injury (Holdener et al., 2008, Wang et al., 2016), resulting in the release of intracellular molecules. Many of them have been identified as TLR2/4 ligands, including HMGB1, HSP60, HSP70, gp96, S100 A8, S100 A9, SAA3 and so on (Tsan and Gao, 2004; Lotze et al., 2007; Ehrchen et al., 2009). These TLR2/4 ligands are not only involved in promoting inflammatory response in infections but also identified as the potent amplifier of inflammation in autoimmunity (Tsan and Gao, 2004; Lotze et al., 2007; Ehrchen et al., 2009). During the process of virus- and chemical-induced non-AIH inflammation, TLR2/4 ligands might be able to induce the production of the factors that promote the initiation of autoimmune response, favoring the development of autoimmune hepatitis. But the underlying mechanisms are still not fully understood. In this study, we tried to ascertain whether virus- and chemical-induced non-AIH inflammation could provide the factors for breaking immune tolerance and promoting the initiation of AIH, and gain a better understanding of the underlying mechanisms. For this purpose, we induced non-AIH inflammation in the liver of mice by using adenovirus and CCl_4, a hepatotoxin that causes direct hepatocyte injury (Kovalovich et al., 2000). Human CYP2D6, a protein with 75% sequence homology to mouse Cyp2D isoenzymes, could be used as self-mimicking antigen to trigger autoimmune hepatitis in mice (Holdener et al., 2008). We therefore used human CYP2D6 as a triggering antigen to induce autoimmune response. The results showed that TLR2/4 ligand-amplified inflammation, but not just a weak inflammation or an inflammation within short time-frame, is crucial for breaking immune tolerance and initiating autoimmune hepatitis in the presence of self-mimicking antigen, due to the requirement for the sustained increase of IL-6/IL-12/IL-4/IL-25 in the liver.

2. Materials and methods

2.1. Reagents

Recombinant human CYP2D6 was purchased from Abcam (Cambridge, UK). Adenovirus (Ad) was purchased from Viraltherapy Technology Co., Ltd (Wuhan, China). CCl_4 was purchased from aladdin Biochemical Technology Co., Ltd (Shanghai, China).

2.2. Plasmids and in vivo gene transfection

Plasmids pCYP2D6, pIL6, pIL12, psTLR2 and psTLR4 are expression vectors (pcDNA3.1, Invitrogen, Carlsbad, CA) carrying the cDNA encoding human CYP2D6, murine IL-6, murine IL-12, the extracellular domain of murine TLR2 and TLR4, respectively, which were constructed in our laboratory (Li et al., 2017; Yan et al., 2013; Liu et al., 2010; Wu et al., 2012).

For *in vivo* gene transfection, plasmids were prepared and analyzed as described previously (Geng et al., 2006). Mice received the injection of plasmid DNA via the tail vein (i.v. injection) using the hydrodynamics-based gene delivery technique (Geng et al., 2006).

2.3. Animal model and treatment protocols

Female C57BL/6 mice, 7–8 weeks old, were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies. All animal experiments were approved by the Animal Care and Use Committee of Tongji Medical College. To induce hepatic inflammation, the mice received the i.v. injection of adenovirus or the i.p. injection of CCl_4. To express human CYP2D6, the mice received the i.v. injection of pCYP2D6 plasmid. To induce autoimmune response and autoimmune hepatitis, pCYP2D6 plasmid was injected to mice together with adenovirus or CCl_4. To block the ligands for TLR2 and TLR4 in the liver, mice received the i.v. injection of plasmids psTLR2/psTLR4. The protocols for the injection of different agents are shown in Supplementary Figures, and indicated in the corresponding figure legends.

2.4. Histology

Liver tissues from median and left lobes were collected, and embedded in paraffin according to standard histological procedures. Tissue sections were prepared and subjected to H&E staining for observation under a light microscope. To evaluate AIH inflammation, an inflammation score was performed as previously described (Bulau et al., 2011; Siegmund et al., 2002).

2.5. Analysis of gene expression by real-time RT-PCR

The quantification of the expression of genes was performed using real-time RT-PCR. The sequences of the primers were as follows: *CYP2D6*, sense 5′-ACCAGGCTCACATGCCCTA-3′, antisense 5′-TTCGAT GTCACGGGATGTCAT-3′; *Il1b* (IL-1β), sense 5′-TGGACCTTCCAGGATG AGGACA-3′, antisense 5′-GTT CATCTCGGAGCCTGTAGTG-3′; *Il6* (IL-6), sense 5′-CTGCAAGAGACTTCCATCCAG-3′, antisense 5′-AGTGGTATAG ACAGGTCTGTTGG-3′; *Tnfa* (TNF-α), sense 5′-CAGGCGGTGCCTATGT CTC-3′, antisense 5′-CGATCACCCCGAAGTTCAGTAG-3′; *Col1a1*, sense 5′-ATGGATTCCCGTTCGAGTACG-3′, antisense 5′-TCAGCTGGATAGCG ACATCG-3′; *Col1a2*, sense 5′-CACCCCAGCGAAGAACTCAT A-3′, antisense 5′-GCCACCATTGATAGTCTCTCCTAAC-3′; *Foxp3*, sense 5′-TCCC AGAG TTCTTCCACA-3′, antisense 5′-AACATGCGAGTAAACCAAT-3′; *Il12* (IL-12), sense 5′-CC AGGTGTCTTAGCCAGTCC-3′, antisense 5′-GCAGTGCAGGAATAATGTTTCA-3′; *Il2* (IL-2), sense 5′-GCGGCATG TTCTGGATTTGACTC-3′, antisense 5′-CCACCACAGTTGCTGACTC ATC-3′; *Ifng* (IFN-γ), sense 5′-ATGAACGCTACACACTGCATC-3′, antisense 5′-CCATCCTTTTGCCAGTTCCTC-3′; *Il4* (IL-4), sense 5′-GGTCTC AACCCCCAGCTAG T-3′, antisense 5′-GCCGATGATCTCTCTCAAGT GAT-3′; *Il13* (IL-13), sense 5′-CCTGGCTCTTGCTTGCCTT-3′, antisense 5′-GGTCTTGTGTGATGTTGCTCA-3′; *Actb* (β-actin), sense 5′-AGGGAA ATCGTGCGTGAC-3′, antisense 5′-CGCTCGTTGCCAATAGTG-3′. The resulting data were analyzed with the comparative C_T method for relative gene expression quantification against house keeping gene *Actb* (Gong et al., 2008).

2.6. Western blot assay

The cells were lysed, and the tissue samples were homogenized for Western blot as described previously (Gong et al., 2008; Liu et al., 2007). Antibodies were purchased from Cell Signaling (MA), Abcam (UK), and Santa Cruz (Santa Cruz, CA). When the soluble interstitial molecules from liver tissues were analyzed by Western blot, the concentration of protein samples was quantitated. Equal amount of protein sample was loaded for SDS-PAGE.

2.7. Preparation of soluble interstitial molecules from liver tissues

The liver tissues were dissected. The mixture of soluble interstitial molecules from liver tissues was prepared by digesting the tissues with collagenase and removing debris by centrifugation. The concentration of the mixture was defined by the concentration of protein, which was determined by using Coomassie Bradford reagent (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions.

2.8. ELISA analysis

Recombinant human CYP2D6 (0.25 μg/ml) or normal hepatic proteins (500 μg/ml) were used to coated 96-well microtiter plates. Normal hepatic proteins were prepared by homogenizing the liver tissues from normal mice, followed by centrifugation. Sera were added in PBS containing 2% FCS. The dilution series for each serum started at 1:100,