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Upregulation of Indoleamine 2,3-Dioxygenase in Hepatitis C Virus Infection▽

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Indoleamine 2,3-dioxygenase (IDO) is induced by proinflammatory cytokines and by CTLA-4-expressing T cells and constitutes an important mediator of peripheral immune tolerance. In chronic hepatitis C, we found upregulation of IDO expression in the liver and an increased serum kynurenine/tryptophan ratio (a reflection of IDO activity). Huh7 cells supporting hepatitis C virus (HCV) replication expressed higher levels of IDO mRNA than noninfected cells when stimulated with gamma interferon or when cocultured with activated T cells. In infected chimpanzees, hepatic IDO expression decreased in animals that cured the infection, while it remained high in those that progressed to chronicity. For both patients and chimpanzees, hepatic expression of IDO and CTLA-4 correlated directly. Induction of IDO may dampen T-cell reactivity to viral antigens in chronic HCV infection.

Indoleamine 2,3-dioxygenase (IDO) mediates conversion of tryptophan to catabolites collectively known as kynurenines (22). This enzyme is expressed by both epithelial and dendritic cells induced by proinflammatory cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor alpha (20, 25). Also, engagement of CTLA-4 with CD80/CD86 on the membrane of dendritic cells stimulates IDO transcriptional expression and activity (4, 9, 19). Increased IDO activity provokes tolerogenicity of antigen-presenting cells and deprives T cells of tryptophan, leading to proliferation arrest and T-cell apoptosis (15). Kynurenine, on the other hand, has been shown to act as an immunoregulatory molecule that mediates immunosuppressive effects in the tissue microenvironment (7, 22, 26). IDO activity contributes to maternal tolerance in pregnancy (21), control of allograft rejection (9), and protection against autoimmunity (8).

Chronic infection caused by hepatitis C virus (HCV) is characterized by weak T-cell responses, recognizing very few epitopes. In contrast, viral clearance after acute infection or after interferon therapy is associated with the presence of a robust and polyclonal T-cell reaction (2, 3, 6, 10, 14, 18, 23, 24). Thus, HCV has developed efficient means to escape T-cell immunity, thus causing a high rate of chronic infections. The molecular mechanisms that are responsible for immune tolerance to HCV antigens remains ill understood. Since IDO activity may dampen T-cell reactivity and can contribute to tolerogenicity of dendritic cells (17), we have analyzed IDO expression by quantitative real-time PCR using β-actin gene expression as an endogenous control (12, 13) (IDO sense primer, TGGCACACGCTATGGAAAAC; antisense, ATGC ATCCCAGAACTAGACG; β-actin sense primer, AGCCTC GCCTTTGCCGA; antisense, CTGGTGCCTGGGGCG) in liver samples from patients with chronic hepatitis C (CHC), subjects with sustained virological response (SVR) after interferon therapy, and patients with other forms of chronic liver inflammation (chronic hepatitis B and steatohepatitis) and in normal liver samples (Table 1, cohort 1). IDO mRNA levels were significantly higher in the CHC group than in the other groups. Patients with other forms of liver disease had values higher than those for normal livers but lower than the CHC values (Fig. 1A). Subjects with SVR showed values similar to those for controls.

As an index of IDO activity, we measured the serum kynurenine/tryptophan ratio (KTR) for equivalent groups of patients and for healthy controls (Table 1, cohort 2). KTR was determined by high-performance liquid chromatography (27). We found that KTR was significantly higher for the CHC group than for the other groups, which did not show significant differences among them (Fig. 1B). Since both IDO mRNA levels and serum KTRs are significantly higher for CHC than in other forms of liver disease (see Fig. 1A and B), it seems possible that HCV might be especially efficient at facilitating IDO overexpression in an inflamed milieu.

To determine whether HCV replication may enhance IDO expression in response to proinflammatory cytokines, we stimulated with IFN-γ (100 U/ml; R&D Systems, Minneapolis, MN), for 16, 24, and 40 h, Huh7 cells containing the full-length HCV replicon (Huh7-Core-3′) (12, 16), Huh7 cells producing JFH1-HCV viral particles (28), and control cells. JFH1-Huh7 cells were used at 30 to 35 days postin-

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TABLE 1. Characteristics of patient cohorts

Variable[a]	Value for patient group			
	Normal liver	Chronic hepatitis C	Sustained virological response	Miscellaneous liver diseases
Amt of aspartate aminotransferase (IU/liter)				
Cohort 1	14.7 ± 5	42.1 ± 28	10.8 ± 2	35.4 ± 24
Cohort 2		46.2 ± 28.3	13.8 ± 5	67.0 ± 55
Amt of alanine aminotransferase (IU/liter)				
Cohort 1	19.3 ± 9	78.8 ± 74	10.7 ± 2	53.1 ± 31
Cohort 2		36.5 ± 17.9	15.4 ± 5	50.3 ± 44.5
Viral load (mean, IU/ml)				
Cohort 1		6.5×10^7	0	
Cohort 2		1.1×10^8	0	
No. of samples with viral genotype (1/non-1/not determined)				
Cohort 1		15/5/4		
Cohort 2		12/4/3		
Liver biopsy (Knodel's score) inflammatory activity				
Cohort 1		4.8 ± 1.8	2.3 ± 1.0	5.6 ± 2.5
Cohort 2		5.4 ± 2.2	0.25 ± 0.5	5.4 ± 2.5
Fibrosis score				
Cohort 1		0.5 ± 0.8	0.5 ± 0.5	1.5 ± 1.5
Cohort 2		1.4 ± 1.5	0.25 ± 0.5	1.1 ± 1.2

[a] Cohort 1, liver tissues from normal liver, $n = 13$ (samples obtained at surgery of liver metastasis or cholelithiasis); miscellaneous liver diseases, $n = 23$ (of whom 11 were chronic hepatitis B patients and 12 were steatohepatitis patients); chronic hepatitis C, $n = 24$ (of whom 11 were naive and 13 were nonresponders to pegylated IFN-α2 plus ribavirin); sustained virological response, $n = 11$. Cohort 2, serum samples from healthy subjects, $n = 14$; miscellaneous liver diseases, $n = 17$ (of whom 6 were chronic hepatitis B patients and 11 steatohepatitis patients); chronic hepatitis C, $n = 19$ (of whom 7 were naive and 12 nonresponders to pegylated IFN-α2 plus ribavirin); sustained virological response, $n = 19$. Miscellaneous liver diseases and chronic hepatitis C patients did not differ in terms of aspartate aminotransferase/alanine aminotransferase levels and histological grading. The study was approved by the local ethical committee.

fection, when about 50 to 60% of cells were positive for the HCV core protein, as determined by immunofluorescence. As shown in Fig. 2A and B, both Huh7-Core-3′ cells and Huh7 cells producing JFH1 generated increased amounts of IDO mRNA in response to IFN-γ at all time points compared to control Huh7 cells. These findings indicate that HCV replication sensitizes the cells to produce IDO at high levels in response to IFN-γ, a proinflammatory cytokine that is upregulated in the livers of patients with CHC (1). IDO upregulation in response to IFN-γ does not affect the replicative activity of HCV in the infected cells, since treatment of the cells with IFN-γ plus an IDO inhibitor (1-methyl tryptophan) or plus kynurenine did not provoke changes in HCV-RNA levels in the infected cells with respect to those observed with treatment of the cells with IFN-γ alone (data not shown). It seems, therefore, that IDO upregulation may represent a strategy of HCV to escape T-cell immunity rather than a mechanism directly influencing HCV replication.

Our data suggest that one of the strategies used by HCV to

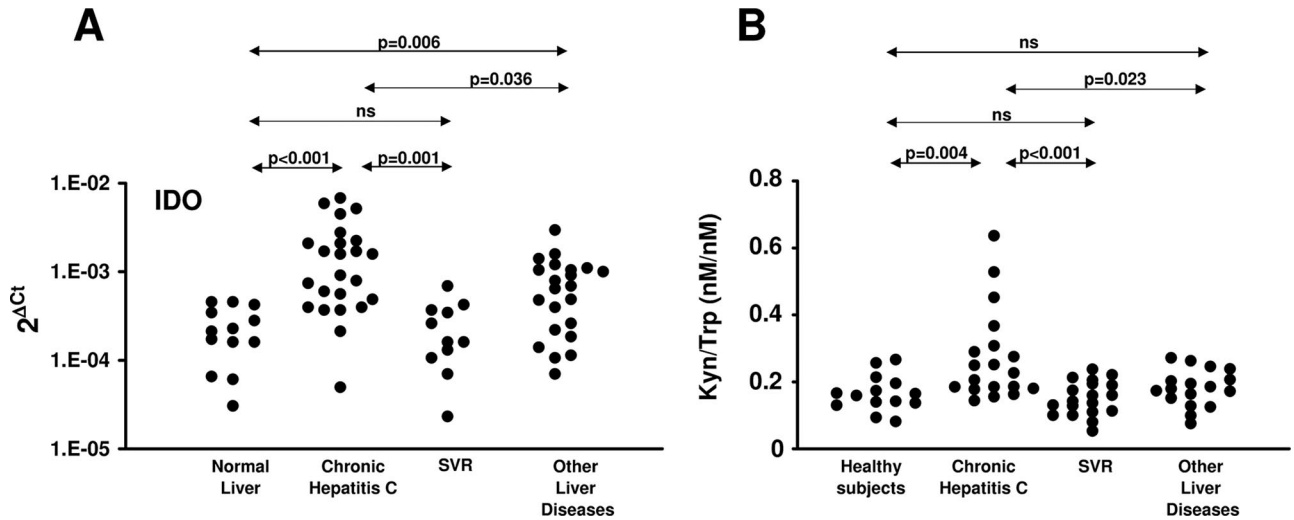

FIG. 1. IDO and HCV infection. (A) Real-time PCR quantitation of IDO mRNA in liver samples from normal livers, from patients with chronic hepatitis C (CHC), from patients with CHC who cleared the virus after interferon therapy (SVR), or from a miscellaneous group of patients with liver disorders unrelated to HCV. Results are normalized with β-actin. (B) Kynurenine/tryptophan ratio in serum samples from individuals belonging to groups equivalent to those shown in panel A. Statistical analyses were performed using nonparametric Kruskal-Wallis and Mann-Whitney U tests. ns, not significant.

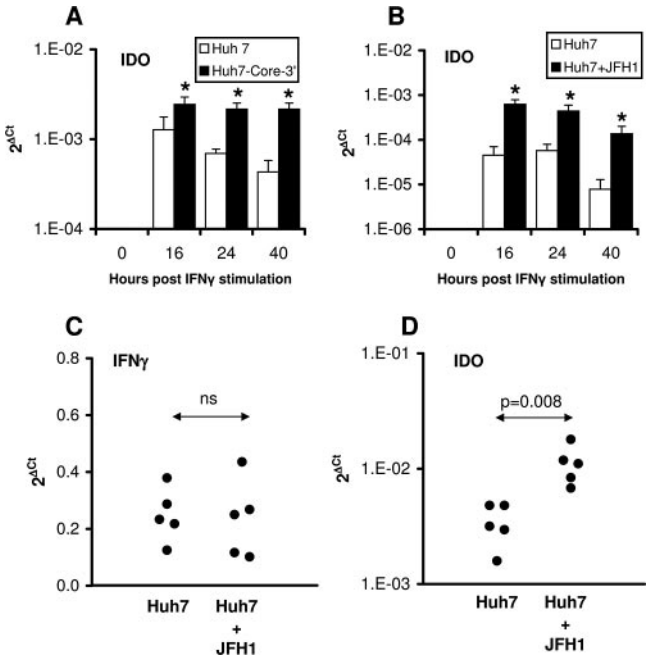

FIG. 2. Induction of IDO mRNA expression by HCV replication. Quantitation of IDO mRNA levels by real-time PCR in Huh7 cells with or without HCV replicon (Huh7-Core-3′) (A) or JFH1 virus (B), treated with 100 U/ml of IFN-γ for 0, 16, 24, or 40 h. Results are expressed as the mean ± standard deviations of one representative experiment out of three experiments performed in sextuplicate. (C and D) IDO and IFN-γ mRNA levels measured by real-time PCR in $CD4^+$ $CD25^-$ cells cocultured for 1 day with Huh7 cells with or without HCV-JFH1 in the presence of Dynabeads CD3/CD28 T-cell expander. All results are normalized with β-actin. Statistical analyses were performed using the nonparametric Mann-Whitney U test. (*, $P < 0.01$; Huh7-core 3′ versus Huh7 or Huh7 plus JFH1 versus Huh7). ns, not significant.

resist immune attack is by promoting IDO expression when infected hepatocytes interact with effector T cells producing IFN-γ. To test this hypothesis, Huh7 cells infected with JFH1 and control Huh7 cells were cocultured with 1.2×10^5 $CD4^+$ $CD25^-$ cells from a healthy subject (using the negative fraction of the CD4+CD25+ Regulatory T Cell Isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence of the Dynabeads CD3/CD28 T-cell expander (Dynal biotech, Oslo, Norway) to activate T cells. After 1 day, the coculture was collected and both IFN-γ mRNA and IDO mRNA were determined by quantitative real-time PCR (IFN-γ sense primer, CTCTGCATCGTTTTGGGTTC; antisense, GCGTTGGAC ATTCAAGTCAG). As shown in Fig. 2C and D, the induction of IFN-γ was similar in cocultures containing control and infected Huh7 cells, but the expression of IDO was significantly higher in HCV-infected cultures. Since IDO levels were about 100-fold higher in coculture experiments than in experiments using exogenous IFN-γ, whether other factors apart from IFN-γ, such as cell contact, might be involved in this high IDO upregulation was studied. Thus, when supernatant from activated $CD4^+$ $CD25^-$ T cells was added to infected or noninfected Huh7 cells, differences in IDO upregulation were not observed (data not shown). It appears, therefore, that IDO induction is mainly facilitated by cell contact between infected cells and activated T lymphocytes. Whether IDO induction in livers with CHC takes place in infected hepatocytes and/or in inflammatory mononuclear cells has not been analyzed in the present work. However, our data for HCV-infected hepatoma cells suggest that hepatocytes are at least partially responsible for the elevated hepatic levels of IDO found in CHC.

There is an intricate cross talk between IDO and CTLA-4 (17). It has been shown that tryptophan depletion together with the presence of kynurenines promotes the expression of inhibitory molecules, such as CTLA-4 and Foxp3, in T cells (5). On the other hand, CTLA-4 stimulates IDO expression and IDO activity in antigen-presenting cells, inducing tolerogenic dendritic cells (17). Thus, we investigated whether IDO expression in the liver might correlate with the abundance of CTLA-4 mRNA in this organ. By using quantitative real-time PCR (CTLA-4 sense primer, TCATGTACCCACCGCCATAC; antisense, TAGACCCCTGTTGTAAGAGG), we found that CTLA-4 mRNA levels were increased in liver biopsy samples from HCV-infected patients over those in normal hepatic tissue or in samples from patients with SVR or other forms of liver disease (Fig. 3A). A significant direct correlation was found between IDO mRNA levels and CTLA-4 mRNA levels in liver tissue from HCV-infected patients ($r = 0.52$; $P < 0.01$) (Fig. 3B).

Liver biopsies are not routinely performed for patients with acute hepatitis C. Thus, in order to investigate the role of hepatic IDO expression in the evolution of HCV infection, we analyzed serial liver biopsy samples obtained from six chimpanzees after they were infected with 25 50% chimpanzee infectious doses of the HCV 1b J4 virus stock (Robert H. Purcell, NIAID, NIH, Bethesda, MD). This study was approved by independent ethical committees in accordance with international regulations (International Animal Care and Use Committee). As shown in Fig. 3C, hepatic IDO mRNA declined after an initial peak and remained low during evolution in animals that cleared the virus, while in the chimpanzees that evolved to chronicity, the initial peak of IDO expression was lower but the levels remained elevated during evolution. Thus, both in chimpanzees and in humans, chronic HCV infection is associated with persistently high IDO expression in the liver. An initial short-lived upregulation of IDO in the animals that cleared the virus might be secondary to the induction of a potent and efficient immune response. In fact, an early and transient upsurge of IDO might take place in association with activation of dendritic cells and T-cell immunity (11), while persistent IDO overexpression may favor tolerance (17). Our findings for acute infection in chimpanzees lend support to this contention.

In parallel to IDO results, for chimps that cured the infection, CTLA-4 expression in the liver showed an initial peak and then remained stable at very low levels during the evolution of the disease (Fig. 3D). In contrast, for chimps that became chronic carriers, expression of CTLA-4 showed little change during the early phase of infection but tended to persist above basal values along the course of the infection (Fig. 3D). As with humans, we found a significant direct correlation between IDO and CTLA-4 mRNA values in the liver (Fig. 3E).

In summary, we show upregulation of IDO in the livers of patients and chimpanzees with chronic hepatitis C. This finding is associated, and correlates, with overexpression of CTLA-4 in liver tissue. Our data indicate that HCV infection facilitates

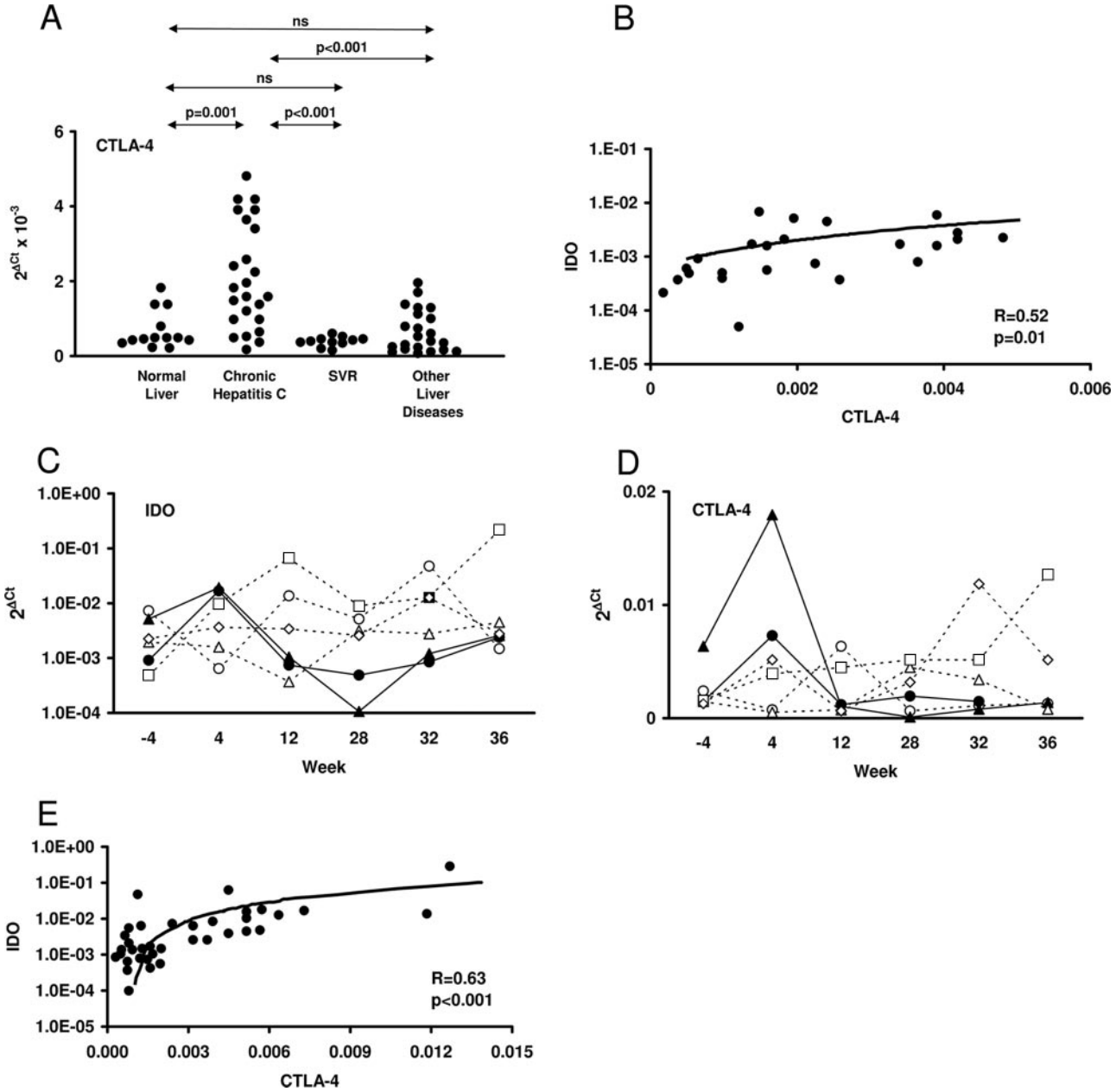

FIG. 3. CTLA-4 and HCV infection. (A) Real-time PCR quantitation of CTLA-4 mRNA in samples from normal livers or from livers from patients with CHC, from patients with CHC who cleared the virus after interferon therapy (SVR), or from a miscellaneous group of patients with liver disorders unrelated to HCV. Statistical analyses were performed using nonparametric Kruskal-Wallis and Mann-Whitney U tests. ns, not significant. (B) Correlation between mRNA levels of IDO and CTLA-4 in liver samples from CHC patients. (C and D) Real-time PCR quantitation of IDO and CTLA-4 mRNA levels in liver samples from chimpanzees obtained at different time points before and after infection with infective HCV inocula, with 0 being the week of infection. Solid lines, chimpanzees who cleared HCV infection; dotted lines, chimpanzees who did not clear HCV infection. (E) Correlation between mRNA levels of IDO and CTLA-4 in liver samples from the chimpanzees described above. Results in panels A, C, and D are normalized with β-actin.

the induction of IDO in response to proinflammatory cytokines and activated T cells. This may constitute an efficient strategy of the virus to escape T-cell immunity. Our findings point to novel targets for therapeutic intervention.

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