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 have upregulated 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.

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 chronic 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 postinfection.
Infection, 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/H11032 cells and Huh7 cells producing JFH1 generated increased amounts of IDO mRNA in response to IFN-α/H9253 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-α, a proinflammatory cytokine that is upregulated in the livers of patients with CHC (1). IDO upregulation in response to IFN-α does not affect the replicative activity of HCV in the infected cells, since treatment of the cells with IFN-α 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-α 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

![Graph A](image)

**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 18S-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.
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 Dynabeads CD3/CD28 T-cell expander (Dynal biotech, Oslo, Norway) to activate T cells. After 1 day, the coculture was terminated by quantitative real-time PCR (IFN-γ sense primer, CTTCTGCATCGTTTTGGGTTC; antisense, GCGTTGGACCTCTGCATCGTTTTGGGTTC; antisense, GCCTTGAGATCTTGGTGGTGC; antisense, GGCTTGGACATTACAGTCAG). 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 IFN-γ 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, TCATGTACCCACCGCATTAC; antisense, TAGACCCCCGTGTTGTAAAGAGTGT), 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 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
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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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.