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_J Immunol_ 2008; 180:3637-3641; doi: 10.4049/jimmunol.180.6.3637

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Cutting Edge: Programmed Death-1 Expression Is Increased on Immuneocytes in Chronic Hepatitis C Virus and Predicts Failure of Response to Antiviral Therapy: Race-Dependent Differences

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Up-regulation of programmed death-1 (PD-1) identifies exhausted T cells in various mouse and human viral models including chronic hepatitis C virus (HCV) infection, which is characterized by impaired CTL function. A large proportion of patients fail to eradicate HCV with current IFN-based antiviral therapy; in particular, African Americans are less likely to respond, but the mechanisms for these differences are not fully elucidated. In this study, in 72 treatment-naive patients with persistent HCV, we found that PD-1 was significantly up-regulated on CD4+ and CD8+ T cells, HCV-specific CTLs, and NK cells. Increased PD-1 on HCV-specific CTLs was significantly associated with failed early and sustained virologic response to therapy in African American but not Caucasian American patients. Patients with sustained virologic response showed decreases in PD-1 on total CD4+ and CD8+ T cells, HCV-specific CTLs, and the CD56bright NK subset after therapy completion. Collectively, these data indicate that PD-1 is critical in persistent HCV and successful therapy results in global down-regulation of its expression. The Journal of Immunology, 2008, 180: 3637–3641.

Most individuals exposed to hepatitis C virus (HCV) develop viral persistence. HCV subverts the host immune response at multiple levels (1, 2), including impairment of the proliferative, cytokine-secreting, and cytotoxic effector functions of HCV-specific T cells. Recent evidence (3–6) from other viral infections in humans and mice indicates a critical role for programmed death-1 (PD-1), a CD28 homologue and costimulatory molecule (7, 8) that inhibits T cell functions by recruiting intracellular Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2, deactivating downstream signal transducers (9). In these models, PD-1 demarcates functionally exhausted CTLs. Accordingly, chronic HCV infection is characterized by the marked up-regulation of PD-1 on HCV-specific CTLs in the peripheral and intrahepatic compartment of patients relative to subjects with spontaneous recovery (10–13). Moreover, in vitro disruption of the interaction of PD-1 with its ligand(s) significantly enhances the effector function of HCV-specific CTLs, even in those individuals lacking CD4+ T cell help (10).

Based on early experimental evidence (14) and mathematical models (15) suggesting a central role for cytotoxic lymphocytes in mediating viral clearance, we hypothesized that the pretreatment PD-1 expression might be associated with the virologic response to combination therapy. Moreover, based on our findings in patients with spontaneous HCV recovery (10), we hypothesized that successful therapy would down-regulate the expression of PD-1. A subset of 72 treatment-naive patients was selected from the Viral Resistance to Antiviral Therapy in Hepatitis C (Virahep-C) study cohort of 401 patients with chronic HCV genotype 1 infection based on their expression of relevant HLA alleles. We used multiparametric flow cytometry to characterize PD-1 on T and NK cells before and after therapy with combination pegylated interferon (peginterferon) and ribavirin.

Materials and Methods

Subjects

The average age of the 72 patients was 46.8 years, 63.9% were male, 30 were African Americans (AA), 42 were Caucasian Americans (CA), and 43.1% African American; 42 were Caucasian American; CI, confidence interval; NT, natural T cell; PD-1, programmed death-1; RR, risk ratio; SHP, Src homology region 2 domain-containing phosphatase; SVR, sustained virologic response (defined by lack of detectable serum HCV RNA at least 6 mo after cessation of antiviral therapy); Virahep-C, viral resistance to antiviral therapy in hepatitis C.
experienced sustained virologic response (SVR). The Virahep-C study design, including treatment course and response definitions, has been detailed previously (16).

**Pentamers**

Patients expressing the appropriate HLA class I alleles were assessed for Ag-specific responses to HCV by a pentamer incorporating frequently targeted HCV epitopes (10), including an additional B35-restricted epitope (HPNIEEVAL).

**Statistical analyses**

Statistical analyses were performed using SAS 8.02 (SAS Institute) and the R language and environment. Descriptive statistics, including measures of central tendency and estimates of spread, were used for continuous variables such as PD-1 expressions. Wilcoxon’s rank sum/Kruskal-Wallis test was used to compare continuous variables (such as PD-1 expression) between groups. When sample size was small, p values from permutation tests were used. The paired signed rank test was used to compare PD-1 levels pretreatment and posttreatment. To model the association between repeated PD-1 expressions (multiple pentamer) and covariates such as race, we have used generalized estimating equations with unstructured correlation between the repeated observations (17). Because SVR was a prospective outcome, the association between PD-1 expression and SVR after adjusting for potential confounders was assessed through relative risks from Poisson regression models with robust SE. When repeated PD1 expressions were considered as a covariate, mean expressions over multiple pentamers were used. Results are reported as relative risks with 95% confidence intervals.

**Results and Discussion**

Comparison of total CD4+ and CD8+ T cell subsets revealed that PD-1 expression was >2-fold higher on CD8+ than on CD4+ T cells. Moreover, HCV-infected subjects had significantly higher PD-1 expression on both T cell subsets compared with normal subjects (Fig. 1). Because only a minority of T cells are HCV specific, these data indicate that chronic HCV infection has a global effect on PD-1 expression. In contrast to the restricted expression of other CD28 family members to T cells, PD-1 is more broadly expressed, being induced upon activation, for example, on peripheral B cells and monocytes (18). Its expression on NK and natural T (NT) cells has not been previously characterized, although the recruitment and activation of SHP-1 and SHP-2 are essential for initiation of the inhibitory signaling pathways in these cells (19); however, direct evidence to implicate the PD-1 pathway in the regulation of NK/NT cell responses is lacking. In light of emerging data that HCV infection leads to functional impairment of NK and NT cells (20, 21), we determined the expression of PD-1 on these cell types. By convention, NK cells have been divided functionally according to their expression of CD56 (22). CD56bright NK cells are considered immature and demonstrate low natural cytotoxicity, whereas CD56dim NKs are considered mature effectors. Among healthy subjects, PD-1 expression was similar between CD56bright and CD56dim NKs. However, in patients with chronic HCV, CD56bright NKs expressed higher levels of PD-1, consistent with their greater functional incompetence and less mature differentiation state. NT cells, which coexpress CD56 and CD3, expressed PD-1 at levels comparable to CD56bright NKs and total CD8+ T cells. Among the lymphocytes examined, the highest PD-1 expression was noted for HCV-specific CTLs (p < 0.0001 compared with total CD8+ T cells). It should be noted that pentamer binding may identify CTLs of varying affinity or even cross-reactive T cells, and performing it without knowledge of autologous sequence data has limitations. The effect of epitope mutations on the capability of the pentamer-positive cells to control virus and on PD-1 expression was not accounted for in the present study. Collectively, these data demonstrate that PD-1 is typically expressed at only low levels in most immune cell types in normal individuals but is up-regulated in the presence of hepatitis C viremia. These results are congruent with the concept that HCV may induce global immune suppression (23) as supported by the recent demonstration that patients with HCV had a significantly higher prevalence of other blood-borne virus infections, including HIV, hepatitis B, and CMV, as well as cryptococcus, tuberculosis, and sexually transmitted diseases (24).

There are major racial differences in the natural history of chronic hepatitis C and treatment responses to anti-HCV therapy (16). We compared the relative PD-1 expression in 42 CA and 30 AA patients with chronic hepatitis C, who were enrolled in the Virahep-C trial in preparation for antiviral therapy with peginterferon and ribavirin. There were no statistically significant differences in PD-1 expression on total CD4, CD8, and NK cells between AA and CA patients. However, PD-1 expression was significantly higher on HCV-pentamer+ CTLs in CA compared with AA patients (p = 0.0058). Pretreatment viral level, previously shown to be associated with the likelihood of an individual patient experiencing SVR (25), did not correlate with the levels of PD-1 on immunocytes. However, pretreatment PD-1 on HCV-specific CTLs among AAs was statistically higher among those who ultimately failed to develop an SVR to combination therapy compared with those who did (Fig. 2B). PD-1 expression as a predictor of SVR was further examined for each cell type by using relative risks in a Poisson regression analysis with adjustment for race. Among AAs with chronic HCV, PD-1 expression on HCV-specific CTLs before treatment was...
negatively associated with SVR ($p < 0.0001$). This relationship did not hold among CA patients. The plots of the proportion of SVR vs PD-1 expression on HCV-specific CTLs in the two racial groups (Fig. 2C) indicated that the higher the mean pretreatment PD-1 on HCV-specific CTLs in AAs, the lower the likelihood of developing SVR.

The association between pretreatment PD-1 expression and early viral kinetics was assessed by racial group. Previous studies (15) have postulated that the host immune response (specifically viral-specific CTLs) plays a major role in early kinetics and clearance of virus-infected cells; however, support for this concept is limited (26). In the Virahep-C study, all patients underwent careful study of viral response during the first 28 days of therapy and were categorized as having a poor, intermediate, or marked viral kinetics response. The poor response was defined by a $1.4 \log_{10}$ drop in HCV RNA levels between baseline and day 28, the intermediate response as a $1.4 – 3.5 \log_{10}$ drop, and the marked response as a $3.5 \log_{10}$ drop (or decrease to undetectable). AA patients with poor early viral kinetics demonstrated significantly higher pretreatment PD-1 on HCV-specific CTLs than AAs with an intermediate or marked viral decline ($p = 0.0005$). Remarkably, PD-1 expression on HCV-specific CTLs was, on average, 15% higher among AA poor responders compared with the other kinetics groups. As displayed graphically in Fig. 2D, the PD-1 expression on HCV-specific CTLs was not significantly associated with early viral response in CA patients. Taken together, these findings suggest that negative regulation by the PD-1 pathway attenuates antiviral CTL responses in AAs and is associated with nonresponse to antiviral therapy; in contrast, in CAs the outcome of antiviral therapy appears to be independent of PD-1 expression.

Because it has recently been shown that subjects with spontaneously resolved HCV infection demonstrate relatively lower levels of PD-1 expression on viral-specific CTLs than patients with persistent viremia (10), we explored the effect of therapy-induced viral clearance on PD-1 expression. Levels of PD-1 were examined before treatment and 6 mo following cessation of therapy. Patients experiencing SVR had a statistically significant decrease in PD-1 expression on total CD4$^+$ T cells (Fig. 3A). In patients experiencing SVR, there was a statistically significant decrease in PD-1 expression on total CD4$^+$ T cells. B. In total, NKs PD-1 expression increases in those who failed to respond to therapy. C. Successful antiviral therapy also led to sustained down-regulation of PD-1 on the CD56$^{bright}$ NK cell subset. D. The largest decrease in PD-1 expression occurred in the HCV-specific CTLs of responders to therapy.
In summary, our data demonstrate that PD-1 is differentially up-regulated on immunocytes in patients with chronic HCV infection naive to antiviral therapy, being highest in HCV-specific CTLs. Although it has been known for some time that AAs as a group demonstrate lower rates of response to antiviral therapy, potential mechanisms have remained enigmatic; we found that the pretreatment level of PD-1 expression on HCV-specific CTLs was highly predictive of both early and sustained virologic responses. Thus, our data are consistent with the notion that PD-1 demarcates functionally exhausted cells and that baseline immunity is important in determining a response to antiviral therapy. Antiviral therapy that led to sustained elimination of circulating HCV RNA was associated with down-regulation of PD-1 on a wide range of cells, including CD4+ T cells, HCV-specific CTLs, and NK cells. Knowledge of the basic immune mechanisms governing the outcome of antiviral treatment will hopefully lead to improved therapeutic strategies.

Acknowledgments
We thank the members of Viral Hep-C group for contributing to the study, the patients for their willingness to participate, Nicole Castelblanco for technical assistance, and Jia Li for statistical analysis. We thank Jay Hoofnagle, MD for insightful suggestions and recommendations into drafts of the manuscript.

Disclosures
The authors have no financial conflict of interest.

References


