High-Programmed Death-1 Levels on Hepatitis C Virus-Specific T Cells during Acute Infection Are Associated with Viral Persistence and Require Preservation of Cognate Antigen during Chronic Infection


*J Immunol* 2008; 181:8215-8225; doi: 10.4049/jimmunol.181.12.8215
http://www.jimmunol.org/content/181/12/8215

---

**References**

This article cites 42 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/181/12/8215.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
High-Programmed Death-1 Levels on Hepatitis C Virus-Specific T Cells during Acute Infection Are Associated with Viral Persistence and Require Preservation of Cognate Antigen during Chronic Infection

Alleluiah Rutebemberwa,2* Stuart C. Ray,2*† Jacquie Astemborski,*† Jordana Levine,* Lin Liu,*§ Kimberly A. Dowd,§ Shalyn Clute,§ Changyu Wang,§ Alan Korman,¶ Alessandro Sette,¶ John Sidney,¶ Drew M. Pardoll,¶ and Andrea L. Cox3*†

Hepatitis C virus (HCV) is an important human pathogen that represents a model for chronic infection given that the majority of infected individuals fail to clear the infection despite generation of virus-specific T cell responses during the period of acute infection. Although viral sequence evolution at targeted MHC class I-restricted epitopes represents one mechanism for immune escape in HCV, many targeted epitopes remain intact under circumstances of viral persistence. To explore alternative mechanisms of HCV immune evasion, we analyzed patterns of expression of a major inhibitory receptor on T cells, programmed death-1 (PD-1), from the time of initial infection and correlated these with HCV RNA levels, outcome of infection, and sequence escape within the targeted epitope. We show that the level of PD-1 expression in early HCV infection is significantly higher on HCV-specific T cells from subjects who progress to chronic HCV infection than from those who clear infection. This correlation is independent of HCV RNA levels, compatible with the notion that high PD-1 expression on HCV-specific CD8 T cells during acute infection inhibits viral clearance. Viral escape during persistent infection is associated with reduction in PD-1 levels on the surface of HCV-specific T cells, supporting the necessity of ongoing antigenic stimulation of T cells for maintenance of PD-1 expression. These results support the idea that PD-1 expression on T cells specific for nonescaped epitopes contributes to viral persistence and suggest that PD-1 blockade may alter the outcome of HCV infection. The Journal of Immunology, 2008, 181: 8215–8225.

Chronic viral infections, such as those caused by HIV, hepatitis C virus (HCV),4 and hepatitis B virus (HBV) are among the leading causes of death in the world (1). Whereas most viral infections induce successful T cell responses that eliminate the infections, HCV, HBV, and HIV have developed mechanisms to evade immune elimination, allowing them to persist in many if not all infected individuals. HCV is found in virtually every region of the world with an estimated 170 million people and 1–2% of the general population of most countries infected (2). Chronic HCV may cause cirrhosis or liver cancer and, in the United States, is the most common indication for liver transplantation as well as the most common cause of hepatocellular carcinoma (3–7). In the United States, there are ~4 million people with persistent hepatitis C infection and over 10,000 HCV-related deaths each year, with mortality from HCV expected to double in this decade and possibly surpass that caused by HIV (4, 8, 9).

HCV is not only an important cause of disease but also an ideal infection in which to study viral evasion mechanisms because infection persists in the majority of infected individuals but not all, given that ~25% of those infected successfully clear the virus. This permits comparison of immune responses between those individuals who successfully control the infection and those who fail. The immune correlates that determine whether a patient resolves infection or proceeds to chronic infection are not completely defined, but viral escape is likely a contributing factor in HCV as well as in HBV and HIV infection. Because immune responses take weeks to develop, and pathogens replicate rapidly, it is well recognized that immune escape mutations may blunt the effectiveness of the immune response (10). Indeed, evasion of the immune response via substitution within T cell epitopes has been demonstrated during HCV, HBV, SIV, and HIV infections (11–22). However, detectable levels of CD8 T cell recognition do not always result in escape substitutions, and many CD8 T cell epitopes remain intact while others mutate within the same host (11, 23). Reversion of immune escape mutations after transmission to a new host suggests that immune escape can be associated with a significant cost to the virus in terms of fitness (17). Therefore, it is

4 Abbreviations used in this paper: HCV, hepatitis C virus; HBV, hepatitis B virus; PD-1, programmed death-1; Flu, influenza; MFI, mean fluorescence intensity.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

www.jimmunol.org
Table I. Demographic characteristics of the 20 adult study subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (yr) at seroconversion</td>
<td>24 [20–46]a</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>100</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
</tr>
<tr>
<td>% male</td>
<td>60</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>16</td>
</tr>
<tr>
<td>1b</td>
<td>1</td>
</tr>
<tr>
<td>2b</td>
<td>1</td>
</tr>
<tr>
<td>3a</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>Outcome of infection (%)</td>
<td></td>
</tr>
<tr>
<td>Persistent</td>
<td>14 (70)c</td>
</tr>
<tr>
<td>Control</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Mode of infection drug usec</td>
<td>19 (95)</td>
</tr>
<tr>
<td>Mean length of follow-up</td>
<td>673 days [106–1521]</td>
</tr>
<tr>
<td>Mean no. of time points studied</td>
<td>3.5 [1–6]</td>
</tr>
<tr>
<td>HCV RNA levels during the first 180 days after infection</td>
<td>Median 95,700 IU/ml [0–6.66 × 10^6 IU/ml]</td>
</tr>
<tr>
<td>HCV RNA levels after &gt;180 days of infection</td>
<td>Median 47,300 IU/ml [0–7.56 × 10^6 IU/ml]</td>
</tr>
<tr>
<td>Mean no. of epitopes tested per patient</td>
<td>2.5 [1–5]</td>
</tr>
</tbody>
</table>

a Numbers in brackets, range.
b For one subject who cleared HCV viremia rapidly and missed a monthly visit, HCV Ab seroconversion but no period of viremia was observed. Therefore, the genotype of that subject’s infection is unknown.
c Numbers in parentheses, percent.
d Two subjects were lost to follow-up before 300 days of infection, the minimum time of follow-up required to meet the definition of any outcome.
e The only study subject not infected via injection drug use was infected in a common source outbreak.

possible that some substitutions result in virus with such significantly reduced fitness that substitution at that position is not observed. Reversion of immune escape mutations can even occur in the original host without viral elimination. This finding not only highlights the presumed fitness cost of certain immune escape mutations but also emphasizes the importance of additional immune evasion mechanisms that inhibit the immune system from eliminating HCV despite an expanded T cell repertoire capable of recognizing viral epitopes. Among others, cell surface receptors associated with T cell regulation have been shown to play important roles in regulating T cell function and responsiveness in chronic murine viral infections and may play a role in control of human viral infections.

One of the most important categories of candidate cell membrane receptors that could be participating in the hyporesponsiveness of human virus-specific T cells is the growing family of inhibitory (or regulatory) receptors. A number of inhibitory receptors that down-regulate T cell function have been recently characterized and their engagement appears to inhibit CD8 effector function. Programmed death-1 (PD-1) is an ITIM-containing inhibitory receptor expressed on activated T cells that binds two known ligands: B7-H1/PD-L1, which is expressed at high levels in the liver; and B7-DC/PD-L2, which is predominantly expressed on dendritic cells (24–27). Although B7-DC/PD-L2 binds PD-1 with higher affinity than B7-H1/PD-L1, B7-H1/PD-L1 appears to be the major PD-1 ligand in vivo responsible for inhibition of T cell function. Ab blockade of B7-H1/PD-L1 and PD-1 partially reversed the inactivity of exhausted lymphocytic choriomeningitis virus-specific T cells. PD-1 has recently been shown to be up-regulated on HIV- and HCV-specific T cells, suggesting that PD-1 up-regulation may be an important mechanism for viral immune evasion in these chronic human viral diseases (28–32). However, the absence of analysis from the time of documented infection of PD-1 expression and of viral sequence evolution precluded determination of the role of Ag persistence in maintenance of PD-1 expression. A recent study examining PD-1 expression on CD8 T cells from SIV-infected macaques demonstrated that PD-1 expression gradually declined on CD8 T cells specific for an SIV-derived epitope that had undergone mutational escape (33). The authors suggested that sustained Ag-specific TCR stimulation is the primary determinant of PD-1 expression; but the study examined just one mutated epitope, so the decline could have been characteristic of some other feature of the host or the epitope rather than the mutation. In addition, the mutation studied completely abrogates recognition; therefore, it is not clear whether a decline in PD-1 expression would be observed with mutations that decrease or decrease but do not completely abrogate recognition.

To more clearly define variables associated with PD-1 expression and outcome of HCV infection, we performed a detailed analysis of PD-1 expression on HCV-specific T cells monitored longitudinally in a cohort of patients studied from the time of infection. PD-1 levels were ascertained at multiple time points postinfection and correlated with HCV RNA levels, sequence variation within the cognate epitopes, and outcome of infection. We demonstrate that PD-1 up-regulation precedes epitope mutation and that viral escape is associated with reduction in PD-1 expression on the surface of HCV-specific T cells, supporting the necessity of ongoing antigenic stimulation of T cells for up-regulation of PD-1. We observed an inverse correlation between PD-1 expression during acute infection and subsequent viral clearance, independent of HCV RNA levels, indicating that PD-1 expression on T cells specific for nonescaped epitopes contributes to viral persistence.
Materials and Methods

Study subjects

Blood samples were obtained from consenting HCV-infected adults participating in a prospective study of young intravenous drug users in Baltimore, MD, as previously described (34). Table I shows the demographics of subjects who were studied. At each visit, participants were provided counseling to reduce the risks of drug use. Blood was drawn for the isolation of serum, plasma, and PBMC in a protocol designed for monthly follow-up. Serum and plasma were stored at −80°C, PBMC were isolated by Ficoll-Hypaque (GE Healthcare Bio-Sciences) gradient centrifugation and cryopreserved in liquid nitrogen.

HCV RNA levels

HCV RNA levels were determined using the quantitative RT-PCR assay (COBAS AMPLICOR HCV Monitor version 2.0 or COBAS TaqMan HCV Test (Roche Molecular Systems) according to the manufacturer’s instructions. The COBAS AMPLICOR HCV and COBAS TaqMan assays have a lower limit of quantitation of 2.8 log_{10} and 1.7 log_{10} IU/ml, respectively. HCV clearance was defined as the presence of anti-HCV Ab with HCV RNA undetectable by the COBAS TaqMan HCV Test in serum or plasma specimens from ≥2 consecutive visits obtained at least 300 days after initial detection of viremia. Persistence was defined by detection of HCV RNA by either COBAS HCV assay in serum or plasma specimens obtained at least 300 days after initial viremia.

Multimers

Allophycocyanin-labeled multimers were obtained through ProImmune and Beckman Coulter Immunomics. They comprised a panel of nine HCV multimers: A2 (KLVAGINAY_N3:1406–1415); A2 (ALYDVVTKL_N3:2643–2652); RLWHYPCTV_N2:614–622; CINGVCWTV_N3:1073–1081; VLSDFKTWL_N4:1987–1995); A01 (ATDALMTGY_N3:1435–1443); B7 (GPRLGVRAT_Core:41–49); B35 (HPALVFDIT_N3:1359–1357); A1-Flu (CTELKLSDYNP 44–52); A2-Flu (GILGFVFTLMP 58–66); A2-EBV (GLCTLVAML_BMLF1 44–52); A2-Flu (GILGFVFTL_HMP 56–62); A2-EBV (GLCTLVAML_BMLF1 259–267; CLGGLLTVMV_LMP-2 426–434), and B8-EBV (RAFKQKLL_RZLZ-1 190–197).

FIGURE 1. PD-1 staining patterns for two representative subjects. PD-1 expression on HCV-specific T cells was essentially uniformly positive and generally unimodal. Therefore, the mean levels of PD-1 expression (MFI) on the entire multimer+ population of T cells rather than percentage positivity was used as an unbiased estimate of PD-1 expression. The numbers in the top boxes indicate the percentage of isotype control (top row) or PD-1+ (bottom row) CD8+ HCV-specific T cells. HlgG4, Human IgG4. PE-A, PE area; APC-A, APC area.
Phenotypic analysis

PBMC were thawed and rested at 37°C, 5% CO2 for at least 5 h; 1–2 × 10⁶ cells were resuspended in 50 μl/well of 10% human serum (Gemini Bio-Products), 10% FCS (HyClone). 50 μg of human IgG (Jackson Immuno-Research Laboratories) was added; the cells were incubated at room temperature for 10 min. 5 μl of Ag-specific multimer were added, and the incubation was continued for a further 20 min. Either biotinylated PD-1 (anti-human PD-1–5) or IgG4 isotype control (Medarex) was added at a final concentration of 5 μg/ml, and the cells were incubated for an additional 20 min at room temperature (35). PBMC were washed twice with FACS buffer and resuspended in 200 μl of 10% human serum (Gemini Bio-Products), 10% FCS. To this, streptavidin-PE (10 μg/ml; eBioscience) and C56-PerCP Cy5.5 (BD Pharmingen) were added, and PBMCs were incubated at room temperature for 20 min. PBMCs were washed twice with FACS buffer and resuspended in 200 μl of 2% paraformaldehyde (J. T. Baker) and analyzed on a FACSCanto flow cytometer within 24 h. At least 50,000 –100,000 cells in 200 μl of 2% paraformaldehyde were selected, and PD-1+ cells were isolated. Subsequently, CD8+ multimer+ cells were selected, and PD-1 expression was measured in the gated total CD8+ multimer+ T cells.

Viral sequencing

From 140 to 280 μl of serum or plasma, the 5.2-kb region from the 5’- untranslated region to the NS3-NS4A junction was amplified as previously described (36). For each specimen, 40 clones were isolated and stored, and the first 20 were selected for preliminary clonal analysis. Preliminary clonal analysis was performed by sequencing and phylogenetic analysis of a 450-nt region spanning the E1-E2 junction including hypervariable region 1. Sequence contigs were assembled and aligned using Aliigner version 2 (CodonCode) and trimmed to equal length using BioEdit (37). The general time-reversible (GTR) + I + G analytical model (parameters available on request from the authors) was selected using the Akaike criterion as implemented in ModelTest version 3.7 (38) and PAUP* version 4b10 (Sinauer Associates). Phylogenetic trees were estimated using the neighbor-joining algorithm implemented in PAUP*. For each specimen, the sequence closest to the phylogenetic center of the tree was identified as previously described (39) and sequenced. These 5.2-kb contigs were assembled, aligned, and edited as described in this paragraph. Because longitudinal sequencing provided validation of conserved clonal sequences, only novel epitope substitutions required validation by sequencing additional stored cDNA clones.

Assessment of potential escape mutations

Peptides originating from the HCV H77 genome (genotype 1a) were used to assess T cell function by the ELISPOT assay. Amino acid replacements in epitopes were tested as potential immune escape substitutions by synthesizing each of the variant peptides and testing them in serial dilution as Ags for bulk PBMC and T cell lines raised to original and mutated versions of the epitope in an IFN-γ ELISPOT assay. These methods have been described in detail previously (11). The observed amino acid substitutions were classified as escape substitutions if they reduced autologous T cell recognition of the epitope in which it was located, as measured by at least a 2-fold reduction in SFC at two or more peptide dilutions.

Statistical analysis

Linear regression was used to examine the relationship between the co-variates and the level of PD-1. Generalized estimating equations methods were used to account for the multiple observations per participant (40). Analysis was performed using SAS version 9. Differences were considered significant if p values were <0.05.

Results

Characteristics of study populations

The demographic characteristics of the 20 subjects studied are listed in Table I. Subjects were selected on the basis of expression of HLA alleles for which multimers were available as well as on the basis of having previously demonstrated peptide-specific responses by IFN-γ ELISPOT. Subjects with HIV infection were excluded. Using peptides representing T cell epitopes identified previously, HLA-matched MHC multimers with peptides recognized by the subject were used to assess PD-1 expression on HCV-specific CD8 T cells obtained from the subjects at time points from initial detection of CD8 T cell responses through the first 4 years after infection. As controls, we performed multimer-Ab costaining for EBV-, CMV-, and Flu-specific T cells. Statistical analysis of the relationship between expression of PD-1 with gender, age, HCV RNA levels, status of Ag (intact or mutated), and virological outcome of infection was then performed. PD-1 expression on HCV-specific T cells was essentially uniformly positive and generally unimodal (Fig. 1). Therefore, we used mean levels of PD-1 expression (mean fluorescence intensity; MFI) on the entire multimer+ population of T cells rather than percentage positivity, as an unbiased quantitative estimate of PD-1 expression. Other studies of PD-1 have also demonstrated that the relative expression level of PD-1 is probably the more useful marker than is the presence or absence of PD-1 as measured by percentage (41).

Statistical analysis for correlations with PD-1 level

Given that we had different numbers of subjects in each outcome group and they were sampled at varying numbers of time points after infection, we chose the generalized estimating equations as our statistical method. Using this method, which accounts for different numbers of subjects sampled at different numbers of time points, we determined that PD-1 levels, as measured by the MFI on HCV-specific T cells, correlate positively with HCV RNA levels but not with subject gender or age (Table II). Race could not be considered given that all study subjects were Caucasian. When the levels of PD-1 were compared among subjects based on outcome of infection, they were significantly higher on HCV-specific T
cells in subjects with persistent infection than on T cells from subjects who cleared infection, both in the first 180 days of infection and using all time points assessed. In a multivariate model, both log_{10} HCV RNA and clearance were significantly associated with the PD1 level. When the model was changed so that clearance was outcome, PD-1 levels were again associated with clearance.

FIGURE 2. PD-1 expression on HCV-specific T cells is higher than on Flu-specific T cells and the general CD8 T cell population regardless of outcome of infection or the phase of infection. The MFI of PD-1 on the T cell surface was compared among HCV-specific T cells from subjects with either outcome of infection, control-specific T cells, and the general CD8 population in the first 180 days of HCV infection (HCV⁺ acute to chronic or cleared (a) and at time points after 180 days of HCV infection (b, HCV⁺ chronic or cleared). The level of PD-1 expression is also higher in acute infection on HCV-specific T cells from subjects who remain persistently infected than those who clear HCV infection. Each data point represents the MFI of the PD-1 level on a specific tetramer⁺ population within an individual.

FIGURE 3. PD-1 expression on HCV-specific T cells stratified by HCV RNA level during early and late infection. HCV RNA levels at the time the T cells were acquired are indicated by colored symbols: red, >100,000 copies/ml; yellow, 600–100,000 copies/ml; green, <600 copies/ml. Acute infection was defined as time points <180 days from initial viremia. PD-1 levels are highly variable in chronic infection. Some HCV-specific T cells from subjects with chronic infection expressed low PD-1 levels in the setting of high circulating HCV RNA levels (red dots at low PD-1 MFI values).
Due to the limited variability of HCV RNA in the clearance group, a model which included HCV RNA could not be tested. Earlier studies on PD-1 expression in chronic viral infections have not assessed whether the difference in T cell PD-1 expression between subjects who control HIV or HCV and those with progressive HIV or chronic HCV infection is based on the level of virus even though our study and others have shown that levels of virus (HIV or HCV) and PD-1 expression on virus-specific T cells are positively correlated. Given that HCV RNA levels are higher in those with persistent infection than in those who have cleared infection, we analyzed this association controlling for HCV RNA levels and found that PD-1 levels are independently associated with outcome of infection. In fact, multivariate analysis revealed that the first 180 days of infection, PD-1 levels were correlated only with outcome of infection, not with HCV RNA levels. These data are most compatible with the notion that PD-1 expression or a factor that regulates it has causal effects on outcome of infection rather than that PD-1 expression simply represents a marker for presence and levels of Ag.

PD-1 levels on HCV-specific T cells vs the total CD8 population T cell population

PD-1 is significantly up-regulated on HCV-specific T cells vs the total CD8 T cell population at all stages of infection regardless of the outcome of HCV infection (Fig. 2). Given that the level of PD-1 remains high on the cell surface of T cells specific for recognized HCV Ags relative to the level on the general CD8 T cell population long after control of HCV infection, continued recognition of Ag does not seem to be required to maintain PD-1 levels above that of the general CD8 population (Fig. 2b).
PD-1 levels on HCV-specific T cells in subjects who progress to chronic infection vs those who clear virus

PD-1 is significantly up-regulated on HCV-specific T cells vs T cells specific for influenza from the acute phase through chronic infection in individuals who progress to chronic HCV infection (Fig. 2). PD-1 levels are also significantly higher on HCV-specific T cells than on T cells specific for EBV and CMV in the first 6 mo of HCV infection but not in later stages of HCV infection in subjects who progress to chronic infection (Fig. 2). This suggests a change in PD-1 modulation on HCV-specific T cells in the later stages of infection relative to EBV- or CMV-specific T cells and that regulation of PD-1 might differ in the acute and chronic phases of infection.

There were not enough data points in the first 6 mo of infection for control (EBV, CMV or Flu) Ag-specific T cells from subjects who cleared HCV infection to compare them with HCV-specific responses in the early phase of infection. However, the level of PD-1 on HCV-specific T cells from subjects who cleared infection was lower than on HCV-specific T cells from subjects who failed to clear HCV infection (Fig. 2a; $p < 0.0001$). Smaller differences were observed between these two groups when PD-1 levels were assessed beyond 6 mo after initial infection (Fig. 2b; $p = 0.06$). The range of PD-1 expression on HCV-specific T cells in the chronic phase of HCV infection is broad with some levels exceeding 2000. However, the bulk of the data points for the HCV+ chronic population in Fig. 2b falls in the lower half of PD-1 MFIs measured, resulting in an insignificant difference between the two groups 6 mo after infection. This further suggests that regulation of PD-1 may differ between the acute and chronic phases of infection. We therefore further analyzed the association between PD-1 levels, infection outcome, and HCV RNA levels in early and later infection.

**PD-1 levels on HCV-specific T cells stratified by HCV RNA levels and outcome of infection**

As shown in Table II and Fig. 2, the level of PD-1 is significantly higher in early infection on HCV-specific T cells from those who
fail to control infection vs T cells from those who clear HCV infection. Despite high HCV RNA levels being generally associated with high PD-1 levels, the levels of PD-1 on HCV-specific T cells ranged widely at all HCV RNA levels both early and late in infection (Fig. 3). We observed that some HCV-specific T cells from subjects with chronic infection expressed low PD-1 levels in the setting of high circulating HCV RNA levels (Fig. 3). Low levels of PD-1 have also been observed on some HIV-specific T cells with high HIV RNA levels (28). We hypothesized that T cells with low levels of PD-1 expression in the setting of high viral RNA levels recognize epitopes that have mutated to escape the immune response. Viral amino acid substitutions in an epitope that abrogate the generation of peptide-MHC complexes on the cell surface result in functional Ag loss even in the setting of high HCV RNA levels. If intact Ag is required for a T cell to receive the signals that induce PD-1 up-regulation, T cells specific for HCV epitopes that have escaped via impaired presentation on APCs would be expected to have low levels of PD-1 expression.

Correlations between viral sequence evolution and PD-1 levels on HCV-specific T cells

To determine whether the low PD-1 levels seen on T cells in patients with high circulating HCV RNA levels were due to escape mutations, the viral sequence was assessed at initial infection and surrounding the time at which the PD-1 levels were found to be low relative to initial PD-1 levels. Low levels of PD-1 have also been observed on some HIV-specific T cells with high HIV RNA levels (28). We hypothesized that T cells with low levels of PD-1 expression in the setting of high viral RNA levels recognize epitopes that have mutated to escape the immune response. Viral amino acid substitutions in an epitope that abrogate the generation of peptide-MHC complexes on the cell surface result in functional Ag loss even in the setting of high HCV RNA levels. If intact Ag is required for a T cell to receive the signals that induce PD-1 up-regulation, T cells specific for HCV epitopes that have escaped via impaired presentation on APCs would be expected to have low levels of PD-1 expression.

FIGURE 5. Amino acid substitutions in T cell epitopes reduce recognition by cognate T cells, validating them as escape mutations. PBMC were cultivated in the presence of the peptide present at initial infection (ocz) or at the point where PD-1 levels decreased (ocz) in subject 17 (a) or subject 30 (b). After 20 days of stimulation with the peptide present in initial infection (left) or the variant peptide (right), cells were tested for recognition of both peptides in serial dilutions in an IFN-γ ELISPOT assay. The number of spot-forming colonies (SFC) per 30,000 cells is shown at each peptide dilution. In both cases, the variant peptide was less well recognized than the initial peptide by T cells expanded with the initial peptide (left). Stimulation with either of the variant peptides also resulted in reduced recognition of either the initial or variant peptides (right) and in reduced expansion of T cells as measured by T cell numbers (data not shown) compared with stimulation with the initial peptide. These substitutions were thus deemed escape mutations.
reemergence (Fig. 4c). The kinetics and magnitude of PD-1 re-expression are quite different for the two distinct epitopes, suggesting that additional factors may contribute to regulation of PD-1 expression besides simply Ag dosage. In subject 30, sequence escape at epitope C63B is observed at day 1144 after infection and is associated with a 5-fold drop in PD-1 levels (Figs. 4d and 5b). On Day 1521 after initial infection, the epitope reverted back to the original sequence. This reversion is associated with a 6-fold increase in PD-1 expression on the cognate T cells. The A2-61 epitope recognized by subject 30 does not undergo substitution, and the level of PD-1 expression over the same time frame varies <2-fold. In contrast to levels on T cells specific for epitopes that underwent substitution, PD-1 levels remained stable (declined <2-fold) or increased over time for epitopes in four additional subjects with no viral escape (Fig. 4, e–h). Seven of the eight subjects studied had detectable T cell responses to at least one control (EBV, CMV, or Flu) Ag, and PD-1 levels differed by <2-fold over multiple time points on control epitope-specific T cells in the same time periods. (data not shown) In summary, epitope escape (or as in subject 175, decrease in viral titer to nearly undetectable levels) consistently resulted in decreases in PD-1 levels of 3- to 10-fold, whereas decreases in PD-1 levels were never >2-fold and usually increased over time when the cognate epitope sequence was maintained.

Discussion
We present the first detailed longitudinal analysis of PD-1 expression on HCV-specific T cells from the time of acute infection, correlating levels of surface expression with viral load, clearance vs persistence, and viral sequence evolution. Our data from early infection demonstrate that PD-1 up-regulation precedes epitope mutation and correlates inversely with viral clearance independent of viral load. Analysis of patients with persistent infection indicates that maintenance of high PD-1 levels as a mechanism of immune evasion may be necessary only when the virus fails to escape via epitope mutation. Taken together, these results demonstrate that although intact Ag is not the sole factor determining levels of PD-1 expression, maintenance of PD-1 levels on an HCV-specific T cell requires persistence of intact Ag and that restoration of intact Ag (either by reemergence of virus or reversion back to the cognate epitope) following escape is associated with an increase in PD-1 levels. Although changes in PD-1 levels on CD8 T cells specific for a given epitope are closely correlated with presence vs absence of mutation or major changes in viral load (i.e., subject 175), the initial set points for PD-1 level on cognate T cells for each epitope vary widely even within the same patient. This could be due to any of a number of variables, including epitope density or mean affinity within the cognate TCR repertoire for each epitope.

There are conflicting data on whether the level of PD-1 on HCV-specific T cells differs significantly between those who control HCV infection and those with persistent infection (31, 32). All studies to date agree that PD-1 levels are high on HCV-specific T cells vs levels on the general CD8 T cell population or on T cells specific for at least some control Ags in the acute phase of infection regardless of the outcome of infection. Our finding of higher PD-1 levels in the acute phase of infection on T cells from those who fail to control infection vs levels seen in those who clear matches the results of Urbani et al., but we did not see differences in the later stages of infection. A study by Kasprwicz et al. found that PD-1 levels do not differ significantly between those who control infection and those who do not in the acute or chronic phase of infection. It is not clear why their results differ from ours, but sexual transmission is a more common route of infection in the cohort studied by Kasprwicz et al., and the majority of subjects in both of the other studies presented with symptomatic infection. Our subjects almost all acquired the infection via injection drug use, and all but one were asymptomatic, which is more common for acute HCV infection. There are known differences between patients who present with symptoms and those who do not, including the rate of clearance, but the reasons for those differences are not understood. It is therefore possible that some of the biological differences that account for the development of symptoms also affect PD-1 expression. In addition, the duration of infection of those defined as acutely infected may be different between studies. The time from infection to manifestation of symptoms is highly variable, making it harder to pinpoint the duration of infection in studies that identify subjects on the basis of symptoms. Each of the three groups also used a different Ab against PD-1, which could also be responsible for the differences observed.

HCV and HIV RNA levels are known to be associated with outcome of infection, with persistence of HCV RNA with chronic HCV infection and higher HIV RNA levels associated with more rapid disease progression. Although previous studies have shown PD-1 levels to be positively correlated with viral RNA levels, those studies have not controlled for viral RNA levels in the assessment of the correlation between PD-1 levels and outcome of infection (28–31). We find that the level of HCV RNA is positively correlated with expression of PD-1 on HCV-specific T cells, but also that the level of PD-1 expression is significantly higher in early infection on HCV-specific T cells from those who progress to chronic HCV infection compared with those who clear infection independent of HCV RNA levels. It is not possible from the current data to conclude that PD-1 levels during acute infection determine ultimate outcome, but the correlation between PD-1 levels on HCV-specific T cells during acute infection and outcome, independent of HCV RNA levels, certainly supports a contributory role. These data also suggest that another as yet undefined factor regulating PD-1 expression is associated with clearance of HCV infection. It is possible that initial innate responses to acute HCV infection differ between the two outcomes of infection and may affect PD-1 expression independently of viral RNA levels.

While there has been much focus on persistent Ag in the induction of chronically high PD-1 expression, it has recently been shown that PD-1 levels can also be substantially affected by the presence vs absence of proinflammatory signals at the time of initial TCR engagement (42, 43). A recent chimpanzee study also suggests that outcome of infection and loss of PD-1 expression may not be absolute and that PD-1 levels may not always be a marker of exhaustion (41). Consistent with data in HIV indicating that PD-1 may be differentially expressed on epitope-specific CD8 T cells within a single individual at the same time, we saw differential expression of PD-1 on contemporaneous HCV epitope-specific CD8 T cells from single individuals (28). HCV RNA levels and the cytokine milieu are constant in a patient at a given time point. Therefore, although distinct innate responses could create different cytokine milieus that affect PD-1 expression, HCV RNA levels and the cytokine milieu cannot be the sole determinants of PD-1 expression based on our data, and there must be epitope-specific differences.

Generation of escape mutations within T cell epitopes is a well-described mechanism for viral escape from CD8 T cell responses. We sought to determine whether escape affected the level of PD-1 expression and therefore represented an epitope-specific determinant of cell membrane levels of PD-1. Our data demonstrate that viral escape is associated with a reduction in PD-1 expression on the surface of HCV-specific T cells and that reversion is associated with an increase in the level of PD-1, supporting the necessity of
ongoing antigenic stimulation of T cells for up-regulation of PD-1. However, maintenance of PD-1 on levels on the surface of HCV-specific T cells following control of HCV infection in those who cleared HCV above the levels seen on the general CD8 T cell population suggests that Ag need not be present to maintain some PD-1 on the surface of T cells after Ag exposure. Our data also suggest that maintenance of high levels of PD-1 as a mechanism of immune evasion is necessary only when the virus fails to escape via epitope mutation, given that PD-1 does not persist at high levels on the cell surface in the setting of escape.

Given that we observed lower levels of PD-1 on the cell surface of T cells specific for mutated epitopes than those specific for epitopes in the same individual who did not undergo substitution, variable PD-1 levels on T cells specific for different epitopes in the same subject at the same time point may in part be explained by the presence or absence of escape mutations in specific epitopes. However, the levels of PD-1 between T cell epitopes that did not undergo substitution also varied as much as 5-fold; thus, there must be alternative explanations for the variability of epitope-specific PD-1 expression that are not due to escape, HCV RNA levels, and the cytokine milieu. Varying strength of TCR engagement, host TCR repertoire, Ag presentation, and other factors should be investigated as additional mechanisms of PD-1 expression modulation.

In summary, we demonstrated that high HCV RNA levels and maintenance of intact HCV epitopes are associated with high levels of PD-1 on the surface of HCV-specific T cells. However, these factors did not fully explain the variability of PD-1 on the surface of HCV-specific T cells or the association of high PD-1 levels on HCV-specific T cells in the acute phase with persistence of HCV infection. Although additional research is needed to identify other factors affecting PD-1 expression and the outcome of HCV infection, these results suggest that PD-1 expression on T cells specific for nonescaped viral epitopes contributes to viral persistence and that PD-1 blockade may alter the outcome of HCV infection.

Acknowledgments
We gratefully acknowledge Minhua Han of Medarex Corporation and The Johns Hopkins University Immunology Core Laboratory for technical assistance.

Disclosures
Shalyn Clute, Changyu Wang, and Alan Korman work for Medarex Corp. The authors otherwise have no conflicting financial agreements.

References