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Transient CD86 Expression on Hepatitis C Virus-Specific CD8+ T Cells in Acute Infection Is Linked to Sufficient IL-2 Signaling

Henry Radziewicz,⁎,†,‡ Chris C. Ibegbu,⁎,† Huiming Hon,† Nathalie Bédard,§ Julie Bruneau,§,¶ Kimberly A. Workowski,‡ Stuart J. Knechtle,‖ Allan D. Kirk,‖ Christian P. Larsen,‖ Naglao H. Shoukry,§,¶,#, and Arash Grakoui⁎,†,‡

Costimulatory signals via B7/CD28 family molecules (signal 2) are critical for effective adaptive CD8+ T cell immune responses. In addition to costimulatory signals, B7/CD28 family coinhibitory receptor/ligands that modulate immune responses have been identified. In acute hepatitis C virus (HCV) infection, programmed death receptor 1, an inhibitory receptor in the CD28 family, is highly expressed on virus-specific CD8+ T cells, yet vigorous immune responses often develop. We hypothesized that other costimulatory signals present during the acute phase of HCV infection would be important to counter this negative signaling. In this study, we found that CD86 was highly expressed on HCV-specific CD8+ T cells early in acute HCV infection and was lost on transition to chronic HCV infection; the expression of CD86 was different from other activation markers, because expression was delayed after in vitro TCR stimulation and required sufficient IL-2 signaling; and HCV-specific CD8+ T cells in the liver of patients with chronic HCV infection were highly activated (CD69, CD38, and HLA-DR expression), but only a minority expressed CD86 or showed evidence of recent IL-2 signaling (low basal phosphorylated STAT5), despite persistent viremia. Our study identified B7 ligand expression on HCV-specific CD8+ T cells as a distinct marker of effective T cell stimulation with IL-2 signaling in acute HCV infection. Expression of costimulatory molecules, such as CD86, early in HCV infection may be essential in overcoming inhibitory signals from the high level of programmed death receptor 1 expression also seen at this phase of infection. The Journal of Immunology, 2010, 184: 2410–2422.

A majority of patients infected with hepatitis C virus (HCV) do not spontaneously clear the virus and become chronically infected. It is hypothesized that in those individuals developing persistent infection, an effective adaptive T cell response fails to develop during the acute phase of HCV infection or is lost on progression to the chronic phase of infection (see review in Ref. 1). In the acute phase of HCV infection, a clinical hepatitis is often observed that is marked by a significant elevation in liver transaminase levels. In contrast, during chronic infection, a clinical hepatitis is often absent; only a mild elevation in liver transaminases is noted. Over many years, this mild inflammation can lead to liver fibrosis and the eventual development of cirrhosis. Evaluation of HCV-specific CD8+ T cells from the peripheral blood of patients with acute infection showed that a majority are highly activated, expressing the markers CD69, CD38, and HLA-DR (2–7). Likewise, during the chronic phase of infection, HCV-specific CD8+ T cells in the liver are highly activated and prevalent (7–9); however, they induce only mild liver injury, as measured by serum liver transaminase levels. The loss of functionality of HCV-specific CD8+ T cells, despite the continued high level of activation that is seen in chronic infection (at the site of infection), is not completely understood.

Recently, it was reported that the expression of programmed death receptor 1 (PD-1), an inhibitory receptor in the B7/CD28 family, during chronic viral infection was associated with a loss of T cell function and a state of exhaustion that could be reversed by PD-1 blockade (10). In chronic HCV infection, a high level of PD-1 was expressed on HCV-specific CD8+ T cells infiltrating the liver of patients with chronic HCV infection (11–13), and expression of PD-1 on these cells contributed to their decreased function, despite the high level of activation (12, 13). Somewhat surprisingly, high levels of PD-1 expression have also been noted on HCV-specific CD8+ T cells in the acute phase of infection, yet these cells still induce significant liver injury (7, 13–15). We hypothesized that other stimulatory signals are important to counteract this high PD-1 expression to enable vigorous T cell responses in acute infection.

B7 ligands, such as CD80 and CD86, are typically described as being expressed on APCs. However, these molecules can also be expressed on in vitro activated T cells (16–23), and the expression of these ligands on T cells is hypothesized to be important in T–T cell interactions (17). For example, after 10 d of stimulation with
anti-CD3 and IL-2, >80% of human CD4+ and CD8+ T cells expressed CD80 (16), and stimulation of human PBMCs with anti-CD3 and IL-2 led to maximal CD86 expression (60% of T cells) 3 wk after stimulation (18). Expression of the B7 ligands CD80, CD86, and PD-L1 was also identified directly ex vivo on CD3+ T cells from patients with HIV infection (24, 25) and autoimmune diseases (26, 27), and the expression of these ligands was hypothesized to be a marker of disease progression (24–27).

Despite these studies, the expression of B7 family ligands on HCV-specific CD8+ T cells isolated from the peripheral blood or liver of patients with HCV infection has not been investigated. Because CD80 and CD86 expression on T cells can provide a costimulatory signal to other T cells (16, 18), the study of the expression of these ligands on HCV-specific CD8+ T cells in acute and chronic infection is important for a more complete understanding of the signals driving a functional adaptive T cell response to this virus. In the current study, we evaluated B7 ligand expression in four patients with acute HCV infection and detectable HCV-specific tetramer responses. We report that HCV-specific CD8+ T cells expressed high levels of CD86 in all of the patients in the acute phase of HCV infection at a time when significant liver injury was clinically apparent. We found that CD86 expression on CD8+ T cells was linked to effective TCR stimulation with sufficient IL-2 signaling. This differed from the expression of the activation markers CD69, CD38, HLA-DR, and CD25 that occurred rapidly after TCR stimulation alone, without the addition of IL-2 to in vitro cultures of PBMCs. Unlike in acute HCV infection, in chronic infection, despite the high-level expression of activation markers, the majority of HCV-specific CD8+ T cells did not express CD80 or CD86, even at the site of infection, and there was no evidence of recent common γ-chain cytokine signaling. This study improves our understanding of the deficits in T cell stimulation at the site of HCV infection: HCV-specific CD8+ T cells are activated partially (expression of CD69, CD38, and HLA-DR) but not effectively [low IL-2 signaling, low CD86 expression, and low proliferation (7)]. Furthermore, this study highlights the early loss of supportive cytokine stimulation of HCV-specific CD8+ T cells during the waning response to HCV infection and identities B7 ligand expression (CD80 and CD86) on T cells as an indicator of effective TCR stimulation with supportive IL-2 signaling.

Materials and Methods

Subjects

Four patients with acute HCV infection, as evidenced by HCV Ab seroconversion in the presence of a clinical syndrome of acute hepatitis, and 28 patients with chronic HCV infection (HCV Ab and HCV PCR positive) were enrolled in the study from the clinic or hospital of Emory University, Atlanta Veterans Affairs Medical Center, Grady Memorial Hospital, Atlanta, or the Montreal Acute Hep C Cohort at Centre de Recherche du Centre Hospitalier de l’Universite de Montreal (CRCHUM), Hôpital St-Luc, as previously described (28, 29). The patient characteristics are summarized in Table II. Patient identifiers are represented as “a” followed by a number, and chronically infected patients are denoted by a “c” preceding the patient number. Nine of the chronically infected patients were enrolled in the Emory Liver Transplant Program, and liver specimens were procured at the time of hepatectomy for liver transplantation (explant liver). An “E” following the patient number denotes these patients. Patients a802, a240, a808, a4915, c113E, and c671 were HLA-A2 positive and chronically infected patients were denoted by a “c” preceding the patient number. The following mAbs were purchased from BD Pharmingen (San Diego, CA): anti-CD25 FITC, CD28 FITC, CD32 FITC, K607 FITC, IgG1k FITC, HLA-DR PE, CD86 PE, PD-L1 PE, PD-L2 PE, IgG1k PE, IgG2Ak PE, Granzyme B PE, and PD-L1 PE. The following mAbs or tetramers according to the manufacturers’ instructions, and flow cytometry was performed using FACSFlowJo software. FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR). The following mAbs were purchased from BioLegend (San Diego, CA): anti-CD25 PE, CD45RA PE, CD62L PE, CD127 PE, and PD-L1 PE. The following mAbs were purchased from eBioscience (San Diego, CA): anti-CD80 PE, anti-CD86 PE, and PD-L1 PE. The following mAbs were purchased from Abcam (Cambridge, MA): anti-CD8 PE, anti-CD95 PE, and PD-L1 PE. The following mAbs were purchased from R&D Systems (Minneapolis, MN): anti-CD4 PE, anti-CD16 PE, and anti-CD64 PE. The following mAbs were purchased from Invitrogen: anti-CD3 PE, anti-CD28 PE, and PD-L1 PE. The following mAbs were purchased from BioLegend (San Diego, CA): anti-CD25 PE, anti-CD45RA PE, CD62L PE, CD127 PE, and PD-L1 PE. The following mAbs were purchased from eBioscience (San Diego, CA): anti-CD80 PE, anti-CD86 PE, and PD-L1 PE. The following mAbs were purchased from Abcam (Cambridge, MA): anti-CD8 PE, anti-CD95 PE, and PD-L1 PE. The following mAbs were purchased from BioLegend (San Diego, CA): anti-CD25 PE, anti-CD45RA PE, CD62L PE, CD127 PE, and PD-L1 PE. The following mAbs were purchased from eBioscience (San Diego, CA): anti-CD80 PE, anti-CD86 PE, and PD-L1 PE. The following mAbs were purchased from Abcam (Cambridge, MA): anti-CD8 PE, anti-CD95 PE, and PD-L1 PE. The following mAbs were purchased from BioLegend (San Diego, CA): anti-CD25 PE, anti-CD45RA PE, CD62L PE, CD127 PE, and PD-L1 PE. The following mAbs were purchased from eBioscience (San Diego, CA): anti-CD80 PE, anti-CD86 PE, and PD-L1 PE. The following mAbs were purchased from Abcam (Cambridge, MA): anti-CD8 PE, anti-CD95 PE, and PD-L1 PE. The following mAbs were purchased from BioLegend (San Diego, CA): anti-CD25 PE, anti-CD45RA PE, CD62L PE, CD127 PE, and PD-L1 PE. The following mAbs were purchased from eBioscience (San Diego, CA): anti-CD80 PE, anti-CD86 PE, and PD-L1 PE. The following mAbs were purchased from Abcam (Cambridge, MA): anti-CD8 PE, anti-CD95 PE, and PD-L1 PE.
PBMCs or isolated CD8+ T cells were washed in FACS buffer (PBS containing 0.1% NaN3) and then negatively selected using a CD8 negative-selection kit (Invitrogen) and then stained for 30 min with anti-CD3 and other Abs. For pSTaT analysis on cultured cells, cells were first washed in FACS buffer and then fixed with Fix Buffer I (BD Biosciences) prior to permeabilization. Flow cytometry was performed using FACS Calibur or LSRII cytometers. FACS data were analyzed with FlowJo software.

### Results

**Early and transient expression of CD86 on HCV-specific CD8+ T cells during acute HCV infection**

The characteristics of four patients with acute HCV infection (a802, a240, a808, and a4915) are shown in Table I. Acute HCV infection was identified by HCV Ab seroconversion in the presence of a clinical syndrome of acute hepatitis. At the time of blood sampling, all of the patients had significantly elevated liver transaminases. None of the patients spontaneously cleared HCV. Patient a802 was also HIV positive and had undetectable HIV viremia with HAART for ~9 mo prior to acquiring HCV. The other three patients were HIV negative. These patients had detectable HCV-specific CD8+ T cell responses by tetramer analysis. Blood sampling from each of these patients was obtained at the earliest time points after acquisition of acute HCV infection. Day 0 corresponds to the first time point sampled for this study, rather than day 0 of infection, because the exact time point of HCV acquisition was not known.

B7 ligand expression on HCV-specific CD8+ T cells from the peripheral blood of each patient was measured at the earliest time points available for evaluation (day 0 of sampling for patients a802 and a240, day 25 for patient a808, and day 33 for patient a4915; Fig. 1A). Approximately 30–60% of HCV-specific CD8+ T cells expressed CD86 at these early time points in HCV infection for all patients (Fig. 1A). For a240, two HCV-specific class I tetramer responses could be identified for two epitopes (HCV/1107 and HCV/1406), and HCV-specific CD8+ T cells directed at both epitopes highly expressed CD86 (Fig. 1A). CD80 expression was not detected on HCV-specific CD8+ T cells from a802, a240, or a4915 at the earliest time points (Fig. 1A), nor at any time point post-infection (data not shown). In contrast, HCV-specific CD8+ T cells from a808 expressed CD80 and CD86 at the earliest time point studied (Fig. 1A). Approximately 30% of HCV-specific CD8+ T cells expressed CD86 and 40% expressed CD80 at the earliest time point sampled for a808 (Fig. 1A). Neither CMV- nor Flu-specific CD8+ T cells expressed CD80 or CD86 during acute HCV infection (Fig. 1A). In addition, HCV-specific CD8+ T cells did not express the other B7 ligands, PD-L1, or PD-L2 at the earliest time points sampled or at any time point of infection (data not shown).

Frequent blood sampling of a802 and a240 enabled a precise longitudinal assessment of CD86 expression on HCV-specific CD8+ T cells. For both patients, the high-level expression of CD86 on HCV-specific CD8+ T cells was transient (Fig. 1B). After the first month of evaluation, ~10% of HCV-specific CD8+ T cells continued to express CD86 (Fig. 1B). For a802 and a240, an early and transient increase in CD86 expression on bulk CD8+ T cells was also noted (Fig. 1C). For a240, who was HIV negative, a peak ~5% of bulk CD8+ T cells expressed CD86 at the earliest time points of HCV infection, which decreased to <1% of CD8+ T cells within the first month of follow-up (Fig. 1C). For a802, who was HIV positive, ~14% of CD8+ T cells expressed CD86 at the earliest time point, which decreased to ~11% after the first month (Fig. 1C). Because elevation of CD86 on CD3+ T cells was reported in patients with HIV infection (24, 25), we hypothesize that continued elevation of CD86 on bulk CD8+ T cells from a802 was related to HIV infection. In contrast to these findings in acute HCV infection, <1% of bulk CD8+ T cells typically expressed CD86 in healthy donors (Fig. 1D).

Lack of frequent blood sampling for a808 precluded a complete prospective evaluation of CD80 and CD86 expression for this patient; however, peak CD80 expression was noted on day 25 (40% of HCV-specific CD8+ T cells), which decreased to <5% by day 49 (Fig. 1E). CD86 expression was noted on ~30% of HCV-specific CD8+ T cells on day 25 and 25% of HCV-specific CD8+ T cells at day 49 (Fig. 1E); at the next available blood sampling...
Specific CD8+ T cells preceded the loss of detectable HCV-specific CD38 and HLA-DR, during the acute phase of infection (Fig. 1). The initiation on day 80 of follow-up. The frequency of HCV-specific CD8+ T cells expressing CD38 remained >10% after the first month (Fig. 1). For a802 (Fig. 1G), by day 25 only ~5% of HCV-specific CD8+ T cells expressed CD86, whereas ~30% continued to express CD38, and 70% expressed HLA-DR. As with a802, the level of CD69 expression correlated most closely with the level of CD86 expression. For a802 and a240, the costimulatory molecule CD28 remained highly expressed (>75%) on HCV-specific CD8+ T cells throughout infection (Fig. 1F, 1G).

Addition of IL-2 to culture media induces high-level expression of CD80 and CD86 on CD8+ T cells after TCR stimulation but is not required for CD69, CD38, HLA-DR, or CD25 expression after brief in vitro culture of PBMCs

We next investigated the signals driving CD80 and CD86 expression on CD8+ T cells and compared this with the expression of other activation markers in response to the same stimulation (Fig. 2A). Although TCR stimulation alone led to very high levels of CD69 expression (>50% of CD8+ T cells) within 1–2 d (Fig. 2A). Significant expression of CD86 on bulk CD8+ T cells after brief in vitro culture (7 d) with anti-CD3 could be achieved by the addition of IL-2 to the culture media (Fig. 2B). Furthermore, increasing amounts of IL-2 led to

### Table I. Patient characteristics

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Patients with acute HCV infection are denoted by an “a” preceding the HCV number; patients with chronic infection are denoted by a “c” preceding the HCV number. An “E” after the patient number denotes an Emory Transplant patient and explant liver sample. For acute infection, viral load and ALT are shown for the first time point sampled.

<sup>a</sup>Liver biopsy inflammation score (based on Scheuer scoring system where available, otherwise as reported).

<sup>b</sup>Liver biopsy fibrosis score (based on Scheuer scoring system or as reported).

<sup>c</sup>For a802, HIV viral load was undetectable (<50 copies/ml) on HAART, and CD4 count was 220 cells/µl.

<sup>d</sup>A qualitative positive test with the COBAS Amplicor test.

<sup>e</sup>Scheuer scoring not available.

+, positive; –, negative; NP, not performed.
increasing CD86 expression (Fig. 2B). We further assessed the importance of IL-2 for CD86 expression after brief in vitro culture on sorted CD8+ T cells from 11 patients with chronic HCV infection (c128, c144, c148, c152, c157, c161, c163, c167, c176, c177, and c181) using a negative bead-selection protocol (Invitrogen). After TCR stimulation without IL-2 added to the culture media, minimal CD86 expression was detected on the sorted CD8+ T cells (Fig. 2C). Addition of IL-2 led to consistent and significant CD86 expression on CD8+ T cells after TCR stimulation with anti-CD3 (Fig. 2C). Although the expression of CD69 could be enhanced by coculture with IL-2 among the sorted CD8+ T cells, TCR stimulation alone with anti-CD3 led to the significant expression of CD69, in distinct contrast with B7 ligand upregulation (data not shown). Hence, although CD86 expression during acute HCV infection (Fig. 1) most resembled CD69 expression in terms of the timing of expression (both seen transiently at the earliest phase of acute infection only), the signal driving the expression of CD86 was unique in its dependence on additional cytokine signaling and in its delayed expression in vitro.  

We also investigated the signals driving CD80 and CD86 expression on virus-specific CD8+ T cells from the peripheral blood (Fig. 2D). Fresh PBMCs from a808 (day 0) were used in 5-d culture to assess the ability of TCR stimulation plus IL-2 to increase CD86 expression on HCV-specific CD8+ T cells (Fig. 2D). Few detectible HCV-specific CD8+ T cells could be obtained after 5-d culture for the no-stimulation condition (Fig. 2D). This was in concordance with our previous work, indicating that a large fraction of these HCV-specific CD8+ T cells at this early time point were highly susceptible to cytokine-withdrawal apoptosis (7). Five-day stimulation with HCV peptide plus IL-2 led to high expression of CD86 (Fig. 2D) and CD80 (data not shown) to nearly 100% of HCV-specific CD8+ T cells.  

Expression of CD86 on virus-specific CD8+ T cells after in vitro culture with IL-2 was not unique to HCV infection. After 5-d
culture of fresh PBMCs from a healthy donor (HD209), culture with CMV peptide alone led to only low-level CD86 expression on CMV-specific CD8+ T cells (Fig. 2E). Culture with CMV peptide plus IL-2 (50 U/ml) led to high expression of CD86 (Fig. 2E). Culture with CMV peptide plus the common γ-chain cytokines, IL7 (5 ng/ml) and IL-15 (5 ng/ml), also led to high-level expression of CD86 on CMV-specific CD8+ T cells (Fig. 2E).

De novo production of CD86 on CD8+ T cells

Two mechanisms exist by which CD86 (and other B7 ligands) could be expressed on CD8+ T cells in general. The first is via de novo generation in highly stimulated cells (16, 30); the second is by a relatively recently described mechanism termed trogocytosis, whereby T cells capture surface molecules through the immunological synapse from APCs (31–33). Both mechanisms were reported to occur in T cells: trogocytosis occurring after stimulation for ≤24 h and endogenous production occurring after 3 d of stimulation (30). In support of de novo production of CD86 on CD8+ T cells, we demonstrated that sorted CD8+ T cells, without the presence of CD86-expressing APCs from which to steal CD86, expressed CD86 after TCR stimulation with IL-2 supplementation in 7-d culture (Fig. 2C). In addition, sorted CD8+ T cells from these patients with chronic HCV infection (c128, c134, c144, c148, c152, c157, c161, c163, c167, c176, c177, and c181) increased the mRNA expression of CD86 after in vitro culture with anti-CD3 and IL-2 compared with no stimulation (Fig. 3A) or stimulation with anti-CD3 alone (Fig. 3B). For the sorting experiments for c134, the quantity of sorted CD8+ T cells only allowed mRNA analysis and not flow cytometry analysis. In further support of the ability of HCV-specific CD8+ T cells to synthesize B7 ligands de novo after stimulation, we generated an HCV-specific CD8+ T cell line that has been propagated via anti-CD3+ IL-2 stimulation without APCs. These cells highly expressed CD86 (Fig. 3C).
Early loss of pSTAT5 of HCV-specific CD8+ T cells in a patient with acute HCV infection

pSTAT5 in T cells is critical for signal transduction after common γ-chain cytokine binding (34). No study has evaluated pSTAT5 directly ex vivo or in vitro on Ag-specific CD8+ T cells using tetramer analysis. Because we noted a dependence on common γ-chain cytokine signaling for maximal CD80 and CD86 expression of HCV-specific CD8+ T cells in vitro, we assessed the level of pSTAT5 in T cells directly ex vivo from fresh blood of a808 during acute infection (Fig. 4). Only frozen PBMCs were available for the other three acutely infected patients, precluding an ex vivo, basal pSTAT analysis for these patients. We found an increase in the basal level of pSTAT5 in HCV-specific CD8+ T cells compared with pSTAT1 or pSTAT6 for a808 (Fig. 4A). This increase in pSTAT5 was also detected in CD3+ T cells compared with CD3+ T cells from a patient with chronic HCV infection (Fig. 4B). Prospective evaluation of a808 showed that the increased level of detectable pSTAT5 in HCV-specific CD8+ T cells, as well as in CD3+ T cells, was lost early in the acute phase of infection, because by day 49 of blood sampling we were unable to detect increased pSTAT5 (Fig. 4C). These findings in HCV infection contrast with the recent report in HIV infection, in which increased basal pSTAT5 was noted in bulk CD4+ and CD8+ T cells, even in chronic HIV infection (35), which likely indicated the persistent high level of immune activation that often characterizes HIV infection.

We also assessed the ability of HCV-specific CD8+ T cells during early HCV infection to further signal through common γ cytokines and found that brief culture with IL-15, in particular, increased pSTAT5 (Fig. 4D, red box). Although the concentrations of IL-2 (100 U/ml), IL-7 (5 ng/ml), and IL-15 (5 ng/ml) were adequate to cause phosphorylation of STAT5 on bulk CD3+ T cells after very brief culture (10 min) (note the shift in the nontetramer CD3+ T cells in Fig. 4D), there was a lack of increase in pSTAT5 via IL-2 and -7 stimulation of HCV-specific CD8+ T cells. This deficit in pSTAT5 in response to IL-7 and -2 stimulation in HCV-specific CD8+ T cells may be related to the low level of IL-7R (CD127) and IL-2R (CD25) characterizing the HCV-specific CD8+ T cells from a808 at that time point (day 25) (Fig. 4E). CD127 expression, in particular, was also reported to be low on HCV-specific CD8+ T cells during acute infection (7, 28, 36–38). Our finding of relatively low pSTAT5 in HCV-specific CD8+ T cells after IL-2 stimulation in vitro in acute HCV infection corresponds with deficits in signaling of CD4+ and CD8+ T cells seen in chronic HIV infection, in which increased basal levels of pSTAT5 correlated with poor pSTAT5 signaling in response to further IL-2 stimulation (35).

Low-level CD80, CD86, and basal pSTAT5 expression in HCV-specific CD8+ T cells in the liver of patients with chronic HCV infection despite high-level expression of activation markers CD69, CD38, and HLA-DR

To fully understand the deficits in the adaptive T cell response to HCV infection, it is important to study immune cells at the site of infection. Studying bulk CD8+ T cells from the liver of five patients with chronic HCV infection (c159, c280, c147E, c671, and c684), we found that the majority of CD8+ T cells expressed the activation markers CD69, CD38, and HLA-DR (Fig. 5A). Despite high-level expression of these activation markers in the liver of patients with chronic HCV infection, only a minority of CD8+ T cells expressed the B7 ligands CD80 and CD86. We identified two patients with chronic HCV infection and a detectable HCV-specific CD8+ T cell response by tetramer analysis in the liver (c671 and c113e). In previous work, we (7) and other investigators (8, 9) found high-level expression of the activation markers CD69, CD38, and HLA-DR on HCV-specific CD8+ T cells from the liver of patients with chronic HCV. Nearly 100% of HCV-specific CD8+ cells in the liver of patients with chronic HCV infection expressed these activation markers (7). Despite expressing high levels of these activation markers (7), the frequency of liver-infiltrating HCV-specific CD8+ T cells from c671 and c113e that expressed CD86 (~14%) or CD80 (<1%) was low, despite persistent HCV viremia (Fig. 5B). Similarly, in blood, no detectible CD80 or CD86 expression was found on HCV-specific CD8+ T cells from c671 (Fig. 5B). C113e did not have a detectible HCV-specific CD8+ T cell response in the peripheral blood.

When evaluating the basal level of pSTAT5 in bulk CD3+ T cells and in HCV-specific CD8+ T cells for c113e, no basal elevation in recent common γ-chain cytokine signaling could be detected (Fig. 5C, upper plots), nor could we detect a difference in pSTAT5 in the liver versus blood (Fig. 5C, lower plot). We analyzed ex vivo, bulk pSTAT5 in the blood and liver of five additional patients with chronic HCV infection (c128E, c135E, c140E, c142E, and c270) and did not detect an increase in the basal level of pSTAT5 in CD8+ T cells in blood or liver of these patients (data not shown).

We further evaluated for the presence of any underlying impairment in IL-2, -7, or -15 signaling of liver-infiltrating CD3+ T cells that could explain the low expression of CD80 and CD86, despite persistent activation (Fig. 5D). We found no deficit in
pSTAT5 in response to IL-2 or -15 (Fig. 5D). In comparison, we found a relatively lower level of pSTAT5 for the IL-7 condition for the liver CD3$^+$ T cells (Fig. 5D). We also investigated the ability of HCV-specific CD8$^+$ T cells in the liver to signal after exposure to IL-2, -7, and -15 (Fig. 5E). Similar to c166 (Fig. 5D), for patient c113E, liver-derived CD3$^+$CD8$^+$ T cells increased pSTAT5 after exposure to IL-2 and -15 efficiently (Fig. 5E, upper row). However, again, there was lower pSTAT5 for the IL-7 condition (Fig. 5E, upper row). Mirroring these liver CD3$^+$CD8$^+$ T cells, liver HCV-specific CD8$^+$ T cells also efficiently increased pSTAT5 after brief exposure to IL-2 and -15; however, we noted lower pSTAT5 after IL-7 exposure (Fig. 5E, lower row). We hypothesize that this deficit in IL-7 signaling in the liver that is seen in these patients may be related to the decreased frequency of CD127 expression seen on bulk T cells and HCV-specific CD8$^+$ T cells in the liver of patients with chronic HCV infection in general (11). This contrasts
with the deficit in pSTAT5 expression in acute HCV, in which HCV-specific CD8+ T cells showed deficiency in pSTAT5 in response to IL-2 and -7 exposure (Fig. 4D). Future studies with larger cohorts of acute and chronically infected patients will be important to characterize the noted deficits in pSTAT5 signaling. The defect in pSTAT5 phosphorylation of HCV-specific CD8+ T cells in the liver in response to IL-7 that we detected could contribute to the lack of CD80 and CD86 expression seen in the majority of CD8+ T cells in the liver of patients with chronic HCV infection. However, overall, we conclude that there is not an overriding, underlying deficit in the ability of HCV-specific CD8+ T cells from the liver to respond to cytokine (particularly IL-2), which might explain the lack of B7 ligand expression on HCV-specific CD8+ T cells in the liver. Rather, based on these findings, we hypothesize that a dearth of common γ-chain cytokines at the site of chronic HCV infection explains the low frequency of CD80 or CD86 expression on HCV-specific CD8+ T cells in the liver.

In further support of a lack of an underlying deficit in the ability of liver-infiltrating T cells to signal after exposure to cytokines, 5-d in vitro culture of liver CD8+ T cells with anti-CD3 and IL-2 led to high expression of CD86 (Fig. 6A). High-level CD80 expression was also seen on CD8+ T cells after culture with anti-CD3 and IL-2 (Fig. 6B). Comparing blood and liver for c671, 5-d culture with IL-2 alone led to greater expression of CD86 on CD8+ T cells in blood compared with liver. We hypothesize that these findings indicate that liver-infiltrating T cells of patients with chronic HCV infection recently received stimulation via their TCR without
supporting cytokine. As such, they respond poorly to further TCR stimulation alone (via anti-CD3) but do respond rapidly to IL-2 alone (or the combination of anti-CD3 + IL-2) by increased expression of CD80 and CD86.

Expression of CD86 is linked to STAT5 signaling

Our studies identified the importance of IL-2 signaling for the expression of CD86 on T cells. In fact, without IL-2 in the culture media, minimal CD86 expression was observed on sorted CD8+ T cells (Fig. 2C). However, we noted low-level CD86 expression on CD8+ T cells after in vitro stimulation of whole PBMCs from some patients with anti-CD3 alone (Figs. 2A, 6B) in contrast to a lack of CD86 expression after anti-CD3 stimulation alone of sorted CD8+ T cells. Thus, we investigated the level of pSTAT5 expression in relation to CD86 expression in cell culture after TCR stimulation with anti-CD3 alone (Fig. 7A) and the level of pSTAT5 expression after anti-CD3 stimulation alone in PBMCs versus sorted CD8+ T cells (Fig. 7B, 7C). The anti-CD3 and IL-2 conditions are shown as positive controls. Importantly, we found that anti-CD3 stimulation alone of PBMCs also led to transient levels of STAT5 phosphorylation in CD8+ T cells (25% noted at day 1) and to transient, low-level expression of CD86 (Fig. 7A, upper row). Addition of IL-2 to anti-CD3 stimulation led to a greater frequency of CD8+ cells expressing pSTAT5 and to prolonged pSTAT5 expression, as expected (Fig. 7, lower row). Evaluating CD8+ T cells in culture over time showed that all CD8+ T cells eventually expressing CD86 also expressed pSTAT5, whether stimulated with anti-CD3 alone or with anti-CD3 and IL-2 (Fig. 7A). Because we did not observe the expression of CD86 on sorted CD8+ T cells in culture after anti-CD3 stimulation alone (Fig. 2C), we repeated the pSTAT5 experiment on sorted CD8+ T cells (Fig. 7B, 7C). In concordance with the importance of IL-2 signaling for CD86 expression, there was no pSTAT5 expression on sorted CD8+ T cells after anti-CD3 stimulation alone, in contrast to the findings in PBMCs (Fig. 7B). With the anti-CD3 stimulation alone of PBMCs, ∼20–30% of CD8+ T cells expressed pSTAT5 at day 1 (Fig. 7A, 7C). Consistently, we found a lack of pSTAT5 expression after anti-CD3 stimulation alone of sorted CD8+ T cells, in contrast to anti-CD3 stimulation of PBMCs (data not shown). We hypothesize that IL-2 released by CD4+ T cells in cultured PBMCs after anti-CD3 stimulation alone contributed to the expression of CD86 on CD8+ T cells in this condition and that the lack of CD4+ T cells in sorted CD8+ T cells led to a lack of pSTAT5 expression (Fig. 7) and a lack of CD86 expression (Fig. 2A) after anti-CD3 stimulation alone. Based on these studies, we conclude that pSTAT5 expression in CD8+ T cells is critical for CD86 expression after TCR stimulation of bulk PBMCs and sorted CD8+ T cells.

Discussion

B7/CD28 family molecules play a central role in the generation and modulation of the adaptive T cell immune response. A balance of costimulatory and coinhibitory signaling governs the activation and function of the responding T cells (see review in Ref. 21). Classically, it is the B7 ligand expressed on the APC signaling to the CD28 family receptor on the T cell that directs the T cell response. However, a number of studies also identified B7 ligand expression on T cells (16–23) and linked its expression to an enhancement of the T cell response (16). Expression of CD86 on a human CD4+ T cell clone enhanced an MLR response in resting peripheral blood responder T cells (16), and expression of CD86 on anti-CD3 stimulated and paraformaldehyde-fixed human T cells enhanced IFN-γ production and proliferation of naive CD4+ T cells responding to suboptimal concentrations of anti-CD3 (18). Furthermore, fixed CD86+, but not CD86−, T cells induced an MLR response that was partly decreased by neutralizing anti-CD86 mAbs (18). Despite these studies, the importance of B7 ligand expression on virus-specific CD8+ T cells is not known.

In the acute phase of infection, the inhibitory receptor PD-1 is often highly expressed on HCV-specific CD8+ T cells (7, 15), so we hypothesized that other costimulatory signals are important at this early phase of infection to enable an effective immune response. In the current study, we demonstrated that the B7 ligand CD86 is highly expressed on HCV-specific CD8+ T cells during the early acute phase of infection and not during the later phase of acute infection or during chronic infection, even at the site of acute infection or during chronic infection.
infection in the liver. Significant CD86 expression on HCV-specific CD8+ T cells was not detected at any later time points for any of the acutely infected patients developing chronic infection nor for any other chronically infected patients that we evaluated. For some patients in the acute phase of HCV infection, HCV-specific CD8+ T cells also expressed CD80, although expression was not seen in other patients with acute infection. It is not well understood why CD80 is expressed on HCV-specific CD8+ T cells from some patients with acute infection but not others. We hypothesize that this may be related to differing kinetics of CD86 and CD80 expression or to different levels of signaling required for the expression of CD86 and CD80.

In this study, we investigated the significance of B7 ligand expression on T cells and found that high-level expression was delayed after brief in vitro culture (5–7 d) and was linked with recent common \( \gamma \)-chain cytokine signaling. This was in contrast with other activation markers, such as CD69, CD38, HLA-DR, or CD25, whose expression could rapidly (1–3 d) be induced by TCR stimulation alone after brief in vitro culture of PBMCs. Hence, our findings support the hypothesis that B7 ligand expression on T cells is a unique marker that identifies recent stimulation via TCRs in the presence of sufficient supportive cytokine. The lack of B7 ligand expression on liver-infiltrating HCV-specific CD8+ T cells in chronic infection, despite the high-level expression of activation markers, highlights a critical deficit in sufficient supportive cytokine signaling that contributes to the waning immune response to HCV. Recent studies in mice demonstrated that IL-7 can be produced by hepatocytes themselves and that IL-7 is important in regulating the expansion of T cells in response to LPS (39). Although hepatocyte IL-7 was not found to be important in pathogen-specific CD8+ T cell proliferation in this study (39), improved understanding of the cytokine milieu in the liver of patients with hepatotropic viral infection is clearly important.

IL-2, in particular, was shown to be important in the generation of effective immune responses to HCV infection, and secretion of IL-2 by CD4+ T cells during the acute phase of infection is critical for sustained and effective adaptive CD8+ T cell responses (38, 40, 41). CD4+ T cells from patients with self-limited evolution of infection produced considerably more IL-2 in response to HCV recombinant proteins compared with patients with chronically evolving disease (38, 40, 41). In the chimpanzee model of HCV infection, depletion of CD4+ T cells prior to infection led to an inability to clear viremia (42). A preferential loss of IL-2–secreting CD4+ T cells has been noted on progression to chronic HCV infection (43), and HCV-specific CD8+ T cells from the peripheral blood of patients with chronic infection have an impaired ability to proliferate that can be rescued in vitro by exposure to IL-2 (44). Our study further supports the critical loss of IL-2 during progression to chronic infection and identifies ex vivo pSTAT5 signaling and expression of B7 ligands (CD86 and CD80) as important markers of recent effective signaling. Although our study highlights the role of common \( \gamma \)-chain cytokines, such as IL-2, in the expression of CD86 on T cells, future studies will need to investigate the role of other inflammatory cytokines in the expression of CD86 or CD80 on T cells during HCV infection. Furthermore, determining whether the level of CD86 expression or the timing of CD86 expression is a determinant of viral clearance versus persistence will require longitudinal studies with larger numbers of patients with acute disease.

Studies of liver-infiltrating HCV-specific CD8+ T cells are critical to understand the failure of the immune response seen in most patients with HCV infection. Previous studies demonstrated...
the high activation state of these cells in the liver but poor functionality in the chronic phase of infection (7–9). A number of factors likely contribute to the waning immune response and include high-level expression of PD-1 (11–13), infiltration by regulatory T cells (Tregs) [45] and reviewed in Ref. 46], and a loss of CD4+ T cell help [43]. We hypothesize that a central feature of each of these mechanisms is the loss of IL-2 signaling on HCV-specific CD8+ T cells. Recent studies on the mechanism of action of PD-1 signaling indicate the possibility that PD-1 signaling might directly prevent STAT5 phosphorylation via activation of the Src homology 2-containing tyrosine phosphatase (45, 47). In addition, one of the proposed mechanisms of action of Tregs is to act as an IL-2 sink and deplete the immunological milieu of the supportive cytokine (48). Our study is the first to characterize pSTAT5 on virus-specific CD8+ T cells using tetramers. Our findings indicate an early loss of pSTAT5 that occurs in the acute phase of infection and a lack of high-level pSTAT5 in liver-infiltrating HCV-specific CD8+ T cells, despite persistent infection and persistent activation. Future studies will need to determine the relative contribution of PD-1 signaling, Treg infiltration, and CD4+ Th cell loss in the reduction of pSTAT5 in HCV-specific CD8+ T cells.

Clearly, differences in the acute versus chronic immune response are evident in HCV infection, and this can be seen in the differing clinical responses and degree of liver injury as measured by alanine aminotransferase (ALT) in the patients infected with HCV. Based on our study, we hypothesize that other B7 molecules, and in particular, CD86, which are expressed at this early phase of infection, provide costimulatory signals via T–T interactions that enhance the immune response at this early stage of infection. In this study, we assessed the ability of HCV-specific CD8+ T cells expressing CD86 to function as APCs in a T–T-dependent manner by sorting on CD3+CD8+ T cells from fresh PBMCs from a808 expressing CD86 to function as APCs in a T–T-dependent manner by sorting on CD3+CD8+ T cells from fresh PBMCs from a808 (day 0) and culturing in the presence of HCV peptide, HCV peptide plus IL-2, and HCV peptide plus anti-CD86 with/without IL-2 (data not shown). We were unable to demonstrate an ability of HCV-specific CD8+ T cells to present Ag in this manner, indicating that these T cells functioned poorly in presenting Ag, despite the expression of B7 ligands (data not shown). Although we did not see evidence of direct Ag presentation by HCV-specific CD8+ T cells to other T cells, we hypothesize that the expression of CD86 on HCV-specific CD8+ T cells is important for other T–T interactions, by providing costimulation to neighboring T cells interacting with an APC or via an cell-autonomous costimulatory signal. Unfortunately, given the difficulty in separating the effect of CD86 expression on APCs from CD86 expression on T cells during in vitro assays, we were unable to demonstrate this effect directly.

In this study, we found that CD86 expression was lost early during HCV infection (Fig. 1), despite persistent high-level PD-1 expression on HCV-specific CD8+ T cells in acute infection (7, 15). This loss of CD86 expression also coincided with decreased liver inflammation, as measured by ALT levels. Thus, we hypothesize that as HCV progresses to chronic infection, the persistent negative signals via receptors, such as PD-1, and the loss of positive signals via CD86 on T cells tip the balance in favor of a waning response. Net negative costimulatory/coinhibitory signals to T cells at this phase of infection may be adaptive for a host that is unable to clear a virus, and waning CD86 expression may be a mechanism to prevent further high-level liver damage. If CD86 expression on HCV-specific CD8+ T cells is shown to provide direct costimulation to other HCV-specific CD8+ T cells in acute infection, prolonging or modulating CD86 expression on these T cells may also be a mechanism that can be used to enhance future therapies for patients with chronic HCV infection.

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Disclosures

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References


