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Liver Environment and HCV Replication Affect Human T-Cell Phenotype and Expression of Inhibitory Receptors

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Abstract

Background & Aims—There is an unclear relationship between T-cell expression of inhibitory receptors and their ability to control viral infections. Studies of human immune cells have been mostly limited to T cells from blood, which is not always the site of infection. We investigated the relationship between T-cell location, expression of inhibitory receptors, maturation, and viral control using blood and liver cells from patients with hepatitis C virus (HCV) and other viral infections.

Methods—We analyzed 36 liver samples from HCV antibody-positive patients (30 from patients with chronic HCV infection, 5 from patients with sustained virologic responses [SVRs] to treatment, and 1 from a patient with spontaneous clearance), with 19 paired blood samples, and 51 liver samples from HCV-negative patients, with 17 paired blood samples. Intrahepatic and circulating lymphocytes were extracted; T-cell markers and inhibitory receptors were quantified, for total and virus-specific T cells, by flow cytometry.

Results—Levels of the markers PD-1 and 2B4 (but not CD160, TIM3, or LAG3) were increased on intrahepatic T cells from healthy and diseased liver tissues, compared to T cells from blood. HCV-specific intrahepatic CD8⁺ T-cells from patients with chronic HCV infection were distinct in that they expressed TIM3 along with PD1 and 2B4. In comparison, HCV-specific CD8⁺ T cells from patients with SVRs and T cells that recognized cytomegalovirus (CMV) lacked TIM3, but expressed higher levels of LAG3; these cells also had different memory phenotypes and proliferative capacity.

Conclusions—T cells from liver express different inhibitory receptors than T cells from blood, independent of liver disease. HCV-specific and CMV-specific CD8⁺ T cells can be differentiated based on their expression of inhibitory receptors; these correlate with their memory phenotype and levels of proliferation and viral control.

Keywords

immune regulation; co-stimulation; T-cell exhaustion; inflammation

Introduction

Inhibitory and co-stimulatory T-cell receptors regulate T-cell responses in order to allow for effective control of pathogens and tumor cells while limiting immunopathology and autoimmunity^{1, 2}. A disturbed balance between stimulatory and inhibitory signals can lead to ineffective T-cell responses that are unable to clear infected or malignant hepatocytes³. Increased expression of a variety of T-cell inhibitory receptors such as PD-1, 2B4, LAG-3, TIM-3 and CD160 has been described for chronic infections caused by LCMV, SIV, HIV-1, HBV and HCV⁴⁻⁷ among others, demonstrating that this pathway enabling viral persistence is widely operative in chronic infections in animals and humans. Dissecting the mechanisms leading to T-cell exhaustion generates opportunities for immunotherapeutic interventions by reversing T-cell inhibition, as demonstrated by the recent success of PD-1 blockade in human malignancies⁸. In order to optimize the impact of these therapies while minimizing the risk for immunopathologies and autoimmunity, it is paramount to develop our understanding of the co-regulation patterns of inhibitory receptors, since T-cell regulation is likely adapted to the infecting pathogen, but also the immunological features of the site of infection.

Hepatitis C Virus (HCV) infection offers the rare opportunity to study the immune correlates required for viral control mediated by virus-specific T-cells, since an estimated 20-30% of infected individuals are able to clear infection spontaneously or on therapy⁹. Furthermore,

since HCV infection is limited to hepatocytes, access to liver tissue allows comparison of T-cells from the site of infection with those circulating in the blood. Previous studies in HCV infection have demonstrated the up-regulation of T-cell inhibitory receptors, especially PD-1^{5, 10}, on HCV-specific T-cells and the association of continuous antigenic stimulation with higher expression levels of PD-1^{11, 12}. Emerging data further suggest that co-expression of multiple T-cell inhibitory receptors on HCV-specific CD8 T-cells in the blood leads to a more severely exhausted phenotype than expression of PD-1 alone, with a variety of additional inhibitory receptors suggested as important¹³⁻¹⁵. While these data suggest that T-cell exhaustion through expression and engagement of T-cell inhibitory receptors may be a causative factor leading to persistent HCV infection, these studies were predominantly performed on circulating T-cells in blood. Recent studies in chronically HCV infected livers have shown that intrahepatic T-cells, in comparison to T-cells in the blood, express higher levels of PD-1 and lower levels of the T-cell maturation marker CD127^{5, 16, 17}. In addition, co-expression of PD-1 with TIM-3 has been suggested to identify HCV-specific T-cells with advanced exhaustion^{13, 18}. These studies provide rationale to fully define the expression of a larger set of inhibitory receptors on intrahepatic T-cells in chronic HCV infection. They also suggest that the liver might represent a special microenvironment in the context of T-cell regulation that needs better definition in order to interpret profiles of intrahepatic T-cells adequately.

Based on the previous findings in HCV infection and the remaining gaps in our understanding of how HCV-specific T-cells are regulated we tested the following hypotheses: 1) that intrahepatic T-cells are in general characterized by T-cell inhibitory expression patterns that are distinct from T-cells in the blood, 2) that co-expression patterns of T-cell inhibitory receptors on intrahepatic T-cells are distinct in different disease types and 3) that in the case of HCV infection, distinct levels of viral control are directly associated with differential expression of T-cell inhibitory receptors and with different T-cell phenotypes. To this end we studied bulk CD4 and CD8 T-cell responses in blood and liver from subjects with a variety of liver conditions also in conjunction with virus-specific CD8 T-cell responses targeting HCV and CMV. Importantly we were able to study intrahepatic T-cells in not only persistent, but also resolved HCV infection, allowing us to define differences in T-cell properties that can be directly attributed to persistent viremia. The results suggest an overarching influence of the liver environment on all intrahepatic T-cells, but also identify unique T-cell phenotypes that correspond to different levels of viral control.

Materials and Methods

Subjects

87 liver specimens were obtained from explantation or resection liver tissue of subjects recruited at Massachusetts General Hospital. The study was approved by the local IRB (Title: 'Cell mediated immunity in Hepatitis C virus infection'; Protocol # 1999-P-004983/54; MGH #: 90-7246).

Liver tissue was obtained from liver explants in end stage liver disease (ESLD) and from liver resection for malignant or benign lesions. When intrahepatic lesions were present, the tissue for this study was collected from the most distant section of the specimen. One HCV Ab+ RNA- subject was undergoing a second liver transplant and was on Tacrolimus.

Overall we analyzed 36 HCV Ab+ liver tissues (30 chronically infected, 5 with sustained virological response (SVR), 1 after spontaneous clearance) with 19 paired blood samples and 51 HCV negative liver tissues with 17 paired blood samples. Patient characteristics are described in Table 1.

Intrahepatic lymphocytes were extracted by mechanical disruption of liver tissue. The cell suspension was washed twice in R10 (RPMI 1640 with 10% FBS), followed by centrifugation at 300 rpm in order to remove cell debris and hepatocytes. The final washing step was followed by centrifugation at 1500 rpm in order to pellet the IHL. PBMC were used cryopreserved as previously described ⁵.

HLA typing

High resolution HLA typing was performed at NCI Frederick. Samples were genotyped for HLA-A/B/C by the PCR-SSOP (sequence-specific oligonucleotide probing) typing protocol and PCR-SBT (sequence based typing). All HLA class I loci were defined to 4-digit resolution with the exceptions of A*74:01/2, B*81, Cw*17 and Cw*18, which were determined to 2-digits.

Flow cytometry

For expression analysis of T-cell inhibitory receptors on bulk CD4+ and CD8+ PBMC and IHL, 0.3-0.5 Million cells were stained with Invitrogen viability dye (L23105) for 30 minutes at room temperature followed by a washing step with PBS and incubation with surface antibodies 30 min on ice (CD3-Pacific Blue, BD Biosciences 558117; CD4 APC-Cy7 BD Biosciences 557871; CD8 AF700 BD Biosciences 557945; PD-1 PerCp/Cy5.5 BIOLEGEND 329914 or PE-Cy7 BIOLEGEND 109110; CD160 PE eBioscience 12-1601-81 or AF488 eBioscience 53-1609-42; CD244 PC5, Beckman Coulter IM2658 or PerCpCy5.5 BIOLEGEND 329514; LAG-3 FITC, Lifespan Biosciences LSB2237 or PerCp R&D FAB2319C; TIM-3 APC (clone 11G8, Rosemary de Kruffy); CCR7 APC-Cy7 BIOLEGEND 353212; CD45RA Qdot605 eBioscience 93-0458-42). The cells were washed twice with PBS/2% FCS and then fixed in 1% formalin/PBS. Flow cytometric analysis was performed on an LSR-II flow cytometer (Becton Dickinson) using FlowJo® software (Tree Star Inc., Ashland, OR). Gating for the different antibodies was determined by FMO (fluorescence minus one) with sample flow plots shown in Supplementary Figure 1. Due to application of different flow cytometry panels, CD160 or TIM-3 staining is missing in some patients (Figure 4C and 4D). For staining with HCV-peptide tetramers or pentamers, the samples were incubated with tetrameric or pentameric complexes for an additional 30 min at room temperature after addition of the viability dye, followed by a washing step, as described previously¹⁹. A list of the used multimers can be found in Supplementary Table 1.

Statistical analysis

All data were normally distributed. When comparing continuous variables within 2 groups, students T-test (paired and unpaired) as applicable were used. P-values were corrected using Bonferroni's method whenever applicable. All the statistical tests were performed using Graph-Pad Prism® software version 5 and SAS® version 9.3.

For Figure 4 we acknowledge that the n=15 or n=8 includes 2 patients in both of these experiments (11-13 and 10-96 for chronic HCV; 10N and 10V for resolved HCV) that contributed more than one observation. To account for this discrepancy we re-calculated our sample size. Assuming a moderate intra-class correlation coefficient of 0.5, which in our opinion is a conservative estimate, we determined our effective sample size to be 12 and 6.6 respectively (calculation not shown). This technique is justifiable considering the statistical significance remains unchanged even if said observations are removed from the analysis.

SPICE analysis

In order to analyze co-expression profiles of different T-cell inhibitory receptors on CD4+ and CD8+ T-cells as well as on HLA multimer+ CD8+ T-cells, we used SPICE, which is a

freeware analysis program provided by the NIH (<http://exon.niaid.nih.gov/spice/>). Data analysis is based on the “Boolean gates” function in Flow Jo (TreeStar).

Results

Expression of PD-1 and 2B4, but not CD160, LAG-3 or TIM-3 is higher on intrahepatic CD4 and CD8 T-cell populations compared to circulating T-cells

In order to compare the expression levels of different T-cell inhibitory receptors between intrahepatic and peripheral lymphocytes we analyzed bulk CD4+ and CD8+ T-cells using liver tissue derived lymphocytes and matched PBMC from 19 individuals with chronic HCV infection and 14 HCV antibody negative patients. (Clinical characteristics in Table 1, flow cytometry sample in Supplementary Figure 1). We detected higher expression levels for PD-1, as previously described^{5, 16, 17}, but could also detect much higher expression of 2B4 on bulk CD4+ and CD8+ T-cells in the liver compared to matched PBMC in HCV infected individuals (Figure 1A, 1B, first and third graph). Interestingly, the same differential expression levels of PD-1 and 2B4 between liver and periphery could be observed in HCV negative individuals (Figure 1A, 1B, second and fourth graph). Because the liver contains virtually no naïve T-cells, we also compared the two compartments while limiting the analysis to the non-naïve cell pool (CD45RA– CCR7–) in blood and liver. Removing naïve T-cells, which typically do not express inhibitory receptors, from the analysis did not alter the general pattern of differential expression of inhibitory receptors in liver versus blood, though the differences were less pronounced (Supplementary Figure 2).

In contrast to PD-1 and 2B4, the global expression levels of CD160, TIM-3 and LAG-3 did not differ significantly between intrahepatic and peripheral CD4+ and CD8+ T-cells in either HCV+ and HCV– patients, and overall their expression levels were generally much lower compared to those observed for PD-1 and 2B4 (Figure 1C, 1D, 1E). One exception was that CD160 expression was higher on intrahepatic CD8 T-cells from HCV negative liver tissues (Figure 1C, forth graph). All together our data show that, irrespective of HCV status, intrahepatic CD4 and CD8 T-cells are characterized by the global upregulation of PD-1 and 2B4, while expression levels of TIM-3, LAG-3 and CD160 are comparable to what is observed in blood.

Expression patterns of inhibitory T-cell receptors on IHL are similar in all liver disease types and even in healthy liver

Given the surprising finding that T-cell inhibitory receptor expression patterns were similar in HCV positive and negative liver tissues we extended our analysis to a total of 81 liver tissues, allowing us to compare a variety of different infectious and non-infectious liver pathologies. 30 liver tissues were from HCV Ab-positive subjects and 51 from HCV Ab negative subjects with different liver diseases, including 8 tissues without any signs of infection, inflammation or malignancy (obtained from subjects with resections of liver adenomas or cysts). We did not detect any major differences in the expression levels of T-cell inhibitory receptors between different liver pathologies, including IHL from control liver tissue from patients with benign lesions (Figure 2). We did not find any correlation between expression of inhibitory receptors and ALT levels (Supplementary Figure 3 left graphs) or the presence or absence of cirrhosis (Supplementary Figure 3, right graphs) and patients younger or older than 60 years (data not shown) also failed to reveal significant differences. When we analyzed the correlation between inhibitory receptor expression and viral load, we could not detect any expression pattern relationship (data not shown). These data identify an expression pattern of T-cell inhibitory receptors on intrahepatic bulk CD4+ and CD8+ T-cells that is characteristic for the liver environment and not a function of liver disease etiology or liver pathology.

HCV specific IHL in chronic hepatitis C are characterized by co-expression of multiple T-cell inhibitory receptors that correlates with the degree of viral control

We next assessed to what degree virus-specific CD8 T-cells co-express the different T-cell inhibitory receptors using class I multimers specific for HCV and CMV epitopes. Sixteen HCV Ab positive subjects were assessed with 22 CD8 T cell responses to 14 HCV antigens. Furthermore, 11 CMV antigens of 4 HCV Ab positive and 7 HCV Ab negative patients could be identified directly ex vivo. Representative example stainings on specific cells are presented in Figure 3. Using the Boolean gate function in FlowJo® together with SPICE® analysis (a software analysis tool provided by the NIH), we integrated the expression data for the different T-cell regulatory receptors. Interestingly, a significantly higher proportion of HCV-specific CD8+ T-cells express 3 or more inhibitory receptors, compared with the total CD8+ population (Figure 3D). This was observed for both the 15 CD8 responses in 10 subjects with persistent viremia and the 8 responses in 6 individuals who were HCV Ab+, HCV RNA-. Of these persons with resolved HCV infection for a minimum of 5 years, 5 had achieved SVR after therapy and one had resolved HCV spontaneously.

Looking at individual T-cell inhibitory receptors we found that PD-1 expression, although already generally high on all IHL (Figure 1), was further increased on HCV-specific T-cells compared to bulk IHL by paired analysis (Figure 4A, left graphs), which is in line with previous studies⁵. There was no significant difference in the expression of 2B4 between specific and bulk CD8+ IHL (Figure 4B, left graphs), whereas CD160 expression was increased on some HCV-specific cells (Figure 4C, left graphs). Expression levels for PD-1, 2B4 and CD160 in CMV-specific responses were even more closely aligned with those of the bulk CD8 T-cell population,

In contrast to the results for PD-1, 2B4 and CD160, expression levels for TIM-3 and LAG-3 were distinct between specific and bulk CD8 T-cells and, more importantly, between T-cells associated with different levels of viral control. While TIM-3 was detected in only a minority of intrahepatic bulk CD8+ T-cells, a majority of HCV-specific cells in chronic HCV infection were TIM-3 positive (Figure 4D, left graphs). HCV-specific CD8 T-cells in resolved HCV infection rarely displayed elevated levels of TIM-3. These cells, however, almost universally expressed LAG-3 (Figure 4E), distinctively from all other T-cell populations we studied. CMV responses were on the lower side of both TIM-3 and LAG-3 expression, though individual responses expressing these molecules were observed (Figure 4A-E).

The co-expression of 3 or more inhibitory receptor molecules differentiated HCV-specific CD8 T-cell responses from bulk CD8 populations in the liver (Figure 3D, Figure 5). Expression of TIM-3 in addition to PD-1 and 2B4 was characteristic for HCV-specific T-cells in chronic HCV infection (Figure 5B) whereas co-expression of LAG-3 with PD-1 and 2B4 was predominantly observed in subjects who controlled HCV successfully (Figure 5C). CMV-specific responses contained few cells co-expressing either TIM-3 or LAG-3, but instead had a significant proportion of CD160 co-expressing cells (Figure 5D). Together the data show that virus-specific CD8+ T-cells have a distinct inhibitory receptor expression profile compared to other intrahepatic T-cells, and that distinct inhibitory receptor profiles are associated with different levels of viral control.

The expression pattern of inhibitory receptors in chronic versus resolved infection defines HCV-specific T-cells with differences in memory phenotype and proliferation

In order to determine whether the different patterns of inhibitory receptor expression associated with different levels of viral control translate into distinct T-cell memory phenotypes and T-cell functions, we analyzed the virus-specific CD8+ T-cell populations in

the liver for the memory molecules CD45RA and CCR7 as well as for the proliferation marker Ki-67.

No differences in the distribution of different memory phenotypes were observed on bulk CD8⁺ IHL from the different subgroups. Bulk CD8⁺ IHL are almost universally effector memory cells (CCR7⁻, CD45RA⁻), regardless of the disease status (Figure 6A; chronic HCV, resolved HCV, HCV negative diseased tissue, healthy controls). HCV-specific CD8⁺ T-cells in chronic infection were of the same effector memory phenotype, while those from resolved infection were mostly TEMRA cells (terminally differentiated RA⁺ effector cells; CCR7⁻, CD45RA⁺) (Figure 6B) that expressed Ki67 as a marker of recent proliferation (Figure 6C-E). CMV responses displayed a phenotype that was a mix of those detected in chronic HCV and resolved HCV infection, with both CD45RA⁻ and CD45RA⁺ cells and a minority of cells expressing Ki-67 (Figure 6B, E). These data indicate that different degrees of viral control are associated with different inhibitory receptor patterns, with distinct memory development and with different functional properties of those T-cells.

Discussion

Our first aim in this study was to establish the general differences in inhibitory receptor expression profiles between T-cells circulating in the blood and those residing in the liver. Previous reports had described increased expression of PD-1 on intrahepatic T-cells and associated this observation with T-cell exhaustion in chronic viral Hepatitis C^{16, 17}. Studying PD-1 and 4 additional inhibitory receptors we found that some, but not all, are expressed by more T-cells in the liver compared to blood. This differential expression was only observed for PD-1 and 2B4, whereas CD160, TIM-3 and LAG-3 were expressed at similar levels in blood and liver. These expression differences between liver and blood were similar for both CD4 and CD8 T-cells, though it was more pronounced in the CD8 T-cell population. Importantly, differences in inhibitory receptor expression levels between T-cells from the liver versus those in the blood were not just a consequence of intrahepatic T-cell populations consisting entirely of antigen experienced T-cells and PBMC consisting of a significant proportion of naïve, inhibitory receptor negative, T-cells. Exclusion of naïve T-cells from analysis in PBMC did not change the overall pattern.

Having hypothesized that elevated expression of inhibitory receptors on IHL was a consequence of T-cell exhaustion in viral infection and also malignancy, we were surprised to see that the IHL phenotype characterized by increased expression of 2B4 and PD-1 was actually not limited to diseases known for exhausted T-cells expressing T-cell inhibitory receptors. Expression patterns of the 5 receptors on intrahepatic T-cells were identical to those observed in HCV infection for all liver tissues, whether it was hepatic tissue surrounding cancer or from sterile inflammation as seen in nonalcoholic steatohepatitis (NASH). Most importantly, IHL from liver tissue without infection, inflammation or malignancy displayed the same patterns, indicating that higher expression levels of PD-1 and 2B4 are not driven by pathological processes (Figure 2 and Supplementary Figure 4). Our interpretation of these data is that higher expression levels of PD-1 and 2B4 are a physiological response to the liver environment and not a reflection of a failing immune response in disease. As an example, the expression of 2B4 and PD-1 on intrahepatic T cells could be important for the prevention of tissue damage by liver infiltrating lymphocytes, both in scenarios with large numbers of such cells (e.g. viral hepatitis) or with few IHL in normal liver tissue. In this context it will be important to determine whether T-cells acquire this phenotype upon entering the liver or whether T-cells with this phenotype are specifically recruited to this site. In either case, our data suggest that expression of inhibitory receptors on T-cells cannot be automatically equated with a pathological immune response, rather we need to define the physiological roles of T-cell inhibitory receptors for adaptation

to tissue microenvironments. We describe the characteristic inhibitory receptor expression patterns for intrahepatic T-cells as a starting point for future investigations into the relationship between tissue environment and physiological T-cell regulation and an important standard for the interpretation of studies on liver-resident T-cells in disease.

Defining the baseline pattern of inhibitory receptor expression of T-cells in liver tissues allowed us to determine what further differentiates virus-specific T-cells in this specific microenvironment. The analysis of HCV-specific T-cells from persistent and resolved HCV infection and T-cells specific for CMV allowed us to directly assess the impact of viral replication on T-cell phenotype. The first important finding from studying both HCV- and CMV-specific T-cells was that they are more susceptible to regulation by co-inhibitory pathways, since a majority co-expressed 3 or more inhibitory receptors, in contrast to most other CD8 T-cells co-expressing two or less receptors. Since this pattern was found in T-cells from both chronic and resolved HCV infection and those targeting CMV, a chronic infection with controlled viremia, expression of multiple different inhibitory receptors may not be per se pathological, but rather enable the critical regulation of T-cells in order to balance the need for sufficient antiviral activity with the threat of excessive immunologic response.

While the co-expression of PD-1 and 2B4 with additional inhibitory receptors was a unifying feature of virus-specific T-cells, certain co-expression patterns of the inhibitory receptors, memory phenotypes and functional properties, were distinct between T-cells in different settings of viral control. It has been previously suggested that co-expression of TIM-3 and PD-1 defines more severely exhausted T-cells than those expressing PD-1 alone^{13, 18}. Our data strongly support an even more critical role for TIM-3, since the differential expression of TIM-3, but not that of PD-1, was the defining feature of T-cells in persistent HCV infection, compared to resolved HCV and CMV infection. Our interpretation of these findings is not that the PD-1 pathway is irrelevant for T-cell exhaustion in HCV infection, but that PD-1 expression alone is not a sufficient marker for exhausted T-cells and needs to be understood within a complex network of T-cell regulation. The fact that HCV-specific T-cells co-expressing TIM-3 were functionally deficient was supported by the finding that they did not express Ki-67, a marker of proliferation, despite high levels of antigen expression. This data conforms to our recent observation that most intrahepatic HCV-specific T-cells in chronic infection express the T-box transcription factor Eomes, but not T-bet, characteristic of terminally exhausted T-cells that have undergone extensive division and have lost further proliferative potential²⁰. Accordingly, these intrahepatic T-cells display an effector memory phenotype with low expression of both CCR7 and CD45RA. These findings differ from what has been described for circulating TIM-3 expressing T-cells in chronic viral infection, which are central memory T-cells¹³ with high T-bet expression²¹. Together, our data on intrahepatic HCV-specific T-cells in chronic HCV infection suggest that TIM-3 expression is the hallmark of a distinct intrahepatic T-cell population that has differentiated into a phenotype unable to sustain an effective antiviral response.

While the critical role of TIM-3 for T-cells in persistent viral infection was not surprising based on previous studies, the up-regulation of LAG-3 on HCV-specific T-cells in the liver after eradication of HCV was unexpected. Indeed we and others have seen extremely few LAG-3 expressing T-cells in human samples in general²², in contrast to mouse lymphocytes that almost universally express LAG-3 upon activation²³. The PD-1, 2B4 and LAG-3 co-expressing populations were almost completely compartmentalized to the liver, and within the liver they represent a unique and rare phenotype of CD45RA positive memory T-cells, in contrast to the rest of the intrahepatic CD8+ T-cell population that consists almost exclusively of CD45RA negative effector memory cells. This memory phenotype had

originally been described as terminally differentiated with short-term survival ²⁴, but recent evidence suggests a critical role for cells with this phenotype in long-term antiviral memory ²⁵. It is striking that these cells were maintained in the liver many years after presumed complete eradication of HCV and also continued to display markers of recent proliferation. It is also noteworthy that intrahepatic CMV-specific T-cells displayed phenotypes on a continuum between what we observe for HCV with and without viremia, with most CMV-specific populations expressing LAG-3 and TIM-3 at levels between the extremes of controlled and viremic HCV. This fits the clinical picture of CMV as a controlled chronic infection with latency and intermittent episodes of viral replication. To what degree LAG-3 is driving the T-cell phenotype and thus is not an inhibitory receptor, or whether the expression of LAG-3 is critical to maintain small memory T-cell populations after viral control needs to be determined.

In summary, we find that elevated expression of T cell inhibitory receptors is not necessarily a sign for an exhausted T cell response. We actually have identified a T-cell inhibitory receptor signature for T-cells in both healthy and diseased liver, which suggests a physiological role for the expression of PD-1 and 2B4 on IHL and which serves as a point of reference for interpreting studies on T-cells in this important microenvironment. We further show that virus-specific T-cells in the liver are characterized by inhibitory receptor co-expression patterns that correlate with the status of viral control and that are associated with differences in memory phenotype and functional properties in addition to the differences in transcriptional regulation that we reported recently ²⁰. Further development of safe and effective immunological interventions through modulation of T-cell co-inhibitory pathways will require our understanding of the physiological role of these pathways and the downstream effects of these distinct inhibitory receptor co-expression patterns on T-cell function and T-cell differentiation in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression analysis of PD-1, 2B4, CD160, TIM-3 and LAG-3 on CD4+ (two left graphs) and CD8+ T-cells (two right graphs) for patient-matched PBMC and IHL. The analysis includes 19 chronically HCV infected and 14 HCV negative patients (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, paired t-test). A) PD-1, B) 2B4, C) CD160, D) TIM-3, E) LAG-3 expression on CD4+ and CD8+ T-cells on PBMC versus IHL in [%].

Figure 2.

Inhibitory receptor expression on CD4+ and CD8+ IHL in 4 different subgroups: HCV positive (30), HBV positive (7), virus-negative with other liver disease (36) and benign liver lesions (8). PD1 (A), 2B4 (B), CD160 (C), TIM-3 (D) and LAG-3 (E) on CD4+ (left graphs) and CD8+ IHL (right graphs). P values <0.008 were considered positive using unpaired T-tests with Bonferroni's correction.

A chronic HCV

B resolved HCV

C CMV

D

■ ≤ 2 receptors
■ ≥ 3 receptors

Bulk CD8+ IHL

HCV-specific CD8+ IHL

HCV experienced

HCV RNA+

HCV RNA-

CMV specific
CD8+ IHL

Figure 3.

Representative flow plots for analysis of virus-specific IHL (red dots) and PD1, 2B4, CD160, TIM-3 and LAG-3 in comparison to bulk CD8+ IHL for a chronically infected HCV patient (A), a patient with resolved HCV (B) as well as for an intrahepatic CMV-specific response (C). D) Co-expression patterns of T-cell inhibitory receptors in different T-cell populations by SPICE analysis (each Spice diagram represents all experiments performed in this subgroup): Total intrahepatic CD8 T-cell population (left panel), intrahepatic HCV-specific CD8 T-cell responses (middle panels, separated into all responses combined, responses in resolved HCV infection and responses in chronic HCV infection) and intrahepatic CMV

responses. Green pie sections represent T-cells with co-expression of 2 or less inhibitory receptors, red pie sections co-expression of 3 or more inhibitory receptors.

Figure 4. Expression of T-cell inhibitory receptors on bulk CD8+ IHL (blue bars) compared to HCV specific CD8+ IHL (red bars). Patients with chronic HCV infection are shown in the left graphs **A-E**, patients with resolved HCV are depicted in the middle graphs **A-E** and the right graphs **A-E** display results for CMV specific IHL. **F)** summarizes the IHL results from the left for **A)** PD-1, **B)** 2B4, **C)** CD160, **D)** TIM-3 and **E)** LAG-3. (*p<0.01, ***p<0.0001 in paired and unpaired T-tests with Bonferroni's correction).

Figure 5.

SPICE co-expression analysis of T cell inhibitory receptors (PD1, 2B4, CD160, TIM-3 and LAG-3) on HCV specific IHL of 4 chronically infected individuals, 3 patients with resolved infection and 3 patients with CMV infection (IgG positive). **A)** Co-expression of inhibitory receptors on HCV specific CD8⁺ IHL compared to patients with resolved infection. **B)** Predominant receptor combination on HCV specific CD8⁺ IHL in chronically infected patients is the combination of PD1, 2B4 and TIM-3. **C)** Combination of PD1 with 2B4 and LAG-3 is the dominating receptor combination on HCV specific CD8⁺ IHL in patients with resolved HCV infection. **D)** CMV specific IHL display co-expression of PD-1 with 2B4 and CD160.

Figure 6.

Memory phenotype and proliferation marker for IHL in chronic and resolved infection. We defined central memory (CM, CD45RA-/CCR7+), effector memory (EM, CD45RA-/CCR7-), effector memory RA (TEMRA, CD45RA+/CCR7-) and naïve (CD45RA+/CCR7+) CD8+ T-cell subsets for **A**) total intrahepatic CD8 lymphocytes in patients with chronic HCV infection (red dots, n=5), resolved HCV infection (green dots, n=3), HCV negative liver disease (blue dots, n=5) and in healthy liver tissue (light blue dots, n=4) and **B**) intrahepatic virus-specific CD8 T-cells in patients with chronic HCV infection (red dots, n=4), resolved HCV infection (green dots, n=5) and on intrahepatic CMV-specific populations (blue dots, n=7). *= $p < 0.01$ in unpaired T-test with Bonferroni's correction.

Ki-67 expression. Examples of Ki-67 analysis for intrahepatic HCV-specific CD8 responses in **C)** a patient with chronic and **D)** a patient with resolved HCV infection. **E)** summarizes Ki-67 expression for virus-specific intrahepatic CD8 responses in chronic and resolved HCV infection and for CMV-specific populations. $\ast=p<0.01$ in unpaired T-test with Bonferroni's correction

Table 1

Cohort	N	Avg age	Male (%)	Cirrhosis (%)	Jaundice (%)	Avg. ALT	Avg. AST
	Total N=87						
<u>Viral Hepatitis</u>	43 (50%)	59.4	90	73	42	58	82
HCV Cohort	36 (83%)	59.8	89	60	43	64	90
<i>Chronic HCV</i>	30	60.3	87	89	52	70	99
Malignancy (19), ESLD (11)							
<i>Resolved HCV</i>	6	57.2	100	60	40	31	43
Malignancy (5), Rejection (1)							
HBV Cohort	7 (17%)	57.9	100	57	43	36	47
Malignancy (6), ESLD (1)							
<u>Non viral Cohort</u>	36 (43%)	55.8	50	22	25	60	65
Malignancy (20), other liver diseases (16)							
<u>Control Cohort</u>	8 (7%)	48.7	17	0	0	28	23
benign liver lesions (8)							