Expression of Inhibitory Receptors on T and NK Cells Defines Immunological Phenotypes of HCV Patients with Advanced Liver Fibrosis

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HIGHLIGHTS
We compared T/NK cell phenotypes of HCV patients in different fibrosis stages
t-SNE showed co-expression clusters of high frequency in patients with advanced fibrosis T/NK cluster co-expressing GAL-9, LAG-3, and PD-1 of high frequency in these patients NK cluster co-expressing TIM-3 and TIGIT is also of high frequency in these patients

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SUMMARY
Chronic HCV can result in advanced liver disease, including cirrhosis. Patients with advanced fibrosis experience poor clinical outcomes and increased risk for hepatocellular carcinoma (HCC). These outcomes are, in part, a consequence of immune dysfunction. Increased inhibitory receptor and Galectin-9 (GAL-9) expression is a possible mechanism promoting lymphocyte dysfunction. In this study, we measured the expression of inhibitory receptors and GAL-9 on T/NK cells of patients with chronic HCV with no to moderate fibrosis (F0-F2) and advanced fibrosis (F3-F4). To analyze their co-expression, we employed t-SNE analysis. Notably, we found that F3-F4 patients had higher frequencies of >3 inhibitory receptor co-expression on NK cells. Moreover, F3-F4 patients manifest a higher frequency of NK cells co-expressing TIGIT and TIM-3, and CD4/NK cells co-expressing LAG-3 and GAL-9. In conclusion, we identified phenotypes of immune dysregulation that could explain the increased susceptibility to infection and HCC in patients with chronic HCV with advanced fibrosis.

INTRODUCTION
Untreated, acute hepatitis C virus (HCV) progresses to chronic infection in 50%–80% of infected individuals (Blackard et al., 2008). Chronic HCV affects ~70 million people worldwide and is the leading infectious cause of cirrhosis, hepatocellular carcinoma (HCC), liver transplantation, and liver-related deaths (Chhatwal et al., 2016; Gane et al., 2015). Chronic HCV infection results in liver fibrosis through hepatocellular damage and inflammation. TGF-β production activates hepatic stellate cells that produce and deposit collagen, and continual scar tissue accumulation causes patients with chronic HCV to develop cirrhosis, portal hypertension, and HCC (Friedman, 2010; Kawada, 2011). Interestingly, patients with advanced liver fibrosis have a poor response to vaccination, recurrent infections, and an increased risk of developing HCC (Aggeletopoulou et al., 2017; Bonnel et al., 2011; Gurtsevitch, 2008).

Lymphocytes play a crucial role in anti-viral responses and cancer surveillance at both the innate and adaptive immune levels. Natural killer (NK) cells are innate immune cells vital to defense against tumors and virus-infected cells. Through the recognition of infected cells and cancerous cells by activating receptors, NK cells are able to perform their effector function by releasing cytokines and cytotoxic granules (Lanier, 2005; Lee et al., 2007; Ljunggren and Karre, 1990). CD8⁺ T cells or cytotoxic T lymphocytes (CTLs) play a role in the adaptive immune response to tumors and viral infections, as they recognize viral and tumor antigens presented on MHC class I molecules and release perforin and granzymes to induce apoptosis of the target cell (Farhood et al., 2019). CD4⁺ T cells also act to maintain and boost immune cell functions, including those of CTLs, in order to orchestrate an effective response to infections. During optimal immune responses, expression of both activating and inhibitory receptors is homeostatically maintained on T and NK cells to ensure that they are adequately activated to perform their effector or helper function.

Inhibitory receptors have a critical role in regulating immune responses to infections, thereby limiting autoimmunity and/or immunopathology (Bi and Tian, 2019; Joller and Kuchroo, 2017). However, increased and sustained expression of inhibitory receptors, often found in patients with chronic viral infections and malignancies, is a principle mechanismpriming lymphocytes to be dysfunctional. The suppressive role of some inhibitory receptors, programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated
protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), and, more recently, T cell immunoglobulin and ITIM domain (TIGIT), is relatively well understood (Bi and Tian, 2019; Golden-Mason and Rosen, 2013; Joller and Kuchroo, 2017; Lee et al., 2010).

With chronic antigen availability, the surface expression of the inhibitory receptor is significantly upregulated and maintained on T and NK cells (Bi and Tian, 2019; Joller and Kuchroo, 2017). It leads to T and NK cell dysfunction, which manifests as an inability to effectively perform their effector function. Moreover, the sustained expression of these inhibitory receptors creates an environment permitting the development of advanced stages of cancer as dysfunctional immune cells are unable to conduct tumor immunosurveillance. Notably, T cells highly expressing inhibitory receptors showed impaired effector functions (Singer et al., 2016). For this reason, the targeting of inhibitory receptors is actively being explored in cancer immunotherapies (Pauken and Wherry, 2015).

Inhibitory receptor expression has also been implicated in chronic virus infections. Studies have shown that the progression of acute to chronic HCV infection is associated with high PD-1 expression on HCV-specific CTLs (Rutebemberwa et al., 2008). During chronic HCV infections, HCV-specific CTLs in the liver have also been shown to co-express PD-1 and CTLA-4, but this phenotype was not observed in the peripheral blood lymphocytes (Nakamoto et al., 2009). Additionally, prior to the revolutionary impact of direct-acting antiviral (DAA) therapy on the treatment of chronic HCV infection, anti-PD-1 therapy showed positive potential as a means to persistently suppress HCV replication in chronic patients (Gardiner et al., 2013). Importantly, the upregulation of PD-1 and LAG-3 expression is observed on not only antigen-specific CTLs but also the bulk CD8+ T cell population in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) (Blackburn et al., 2009).

More recently, the role of Galectin-9 (GAL-9) surface expression in immune cell dysfunction has been identified. GAL-9 is a TIM-3 ligand and is widely expressed in tissues but is abundant in the liver (Wada and Kanwar, 1997). During chronic viral infections, specifically HCV, the circulating levels of GAL-9 and its expression in the liver are increased, primarily by GAL-9 producing macrophages and monocytes (Golden-Mason and Rosen, 2013; Mengshol et al., 2010). Studies have shown that the presence of soluble GAL-9 during NK cell stimulation impairs their cytokine production and cytotoxic ability (Golden-Mason and Rosen, 2013). Most recently, NK and T cells with surface expression of GAL-9 (GAL-9+ cells) in patients with HIV were shown to have reduced cytotoxicity with reduced expression of granzyme B and perforin (Motamedi et al., 2019; Shahbaz et al., 2020). Studies have also shown that HCC tumors express GAL-9, and currently, the potential use of GAL-9 expression as a biomarker of HCC is being explored (Sideras et al., 2019).

As previously mentioned, the introduction of DAAs transformed the HCV treatment landscape as most therapies show an over 95% cure rate in all HCV genotypes (Martinello et al., 2018). However, patients with chronic HCV with advanced liver fibrosis face immune dysregulation that persists after viral clearance. We have previously shown that bulk CD8+ T cells of HCV-infected individuals with advanced liver disease were dysregulated and showed sustained activated status compared with those with minimal liver fibrosis, and this persisted post DAA treatment (Vrankovíc et al., 2019). Importantly, this differential nature of immune suppression is present not only in patients with chronic HCV relative to healthy individuals but specifically in patients with advanced fibrosis compared with those with minimal or no fibrosis. Therefore, it is imperative to characterize the immune dysregulation associated with advanced liver fibrosis to develop targeted strategies for restoring the effective immune function of T and NK cells in order to treat or reduce the risk of adverse clinical outcomes of cirrhosis in HCV+ individuals such as increased HCC risk. To do this, we examined the patterns of GAL-9 expression alongside inhibitory receptors on bulk T cells and NK cells and identified immune phenotypes associated with immune dysregulation in patients with chronic HCV with advanced liver fibrosis.

**RESULTS**

**Proportions of T and NK Cell Subsets in Patients with HCV with Liver Fibrosis**

To characterize the nature of immune dysregulation associated with advanced liver fibrosis during chronic viral infection, we recruited patients with chronic HCV and placed them into two groups based on their stage of fibrosis. The characteristics of study participants are summarized in Table 1 with F0-F2 patients having no to moderate fibrosis (n = 15) and F3-F4 patients having advanced fibrosis, including cirrhosis (n = 15). F3-F4 patients are older than F0-F2 patients, presumably owing to the years required to develop
As expected, higher levels of AST and ALT are observed in F3-F4 patients, indicating advanced liver damage. Given the pivotal role that T and NK cells play in immunosurveillance, we examined T cells and NK cells in PBMC samples to detect any changes in subset frequencies associated with liver fibrosis severity. Advanced liver fibrosis was not associated with altered frequencies of CD8⁺CD4⁻ (CD8⁺), CD8⁻CD4⁺ (CD4⁺), CD8⁺CD4⁺ (double-positive), and CD8⁻CD4⁻ (double-negative) T cell or CD56Bright and CD56Dim NK cell frequencies compared with frequencies in minimal fibrosis (Figures S1 and 1A). T cell subsets were identified using CD45RO and CCR7 markers with CD45RO⁻ CCR7⁺ denoting naive cells (TNaive), CD45RO⁻ CCR7⁻, terminal effector cells (TEMRA), CD45RO⁺ CCR7⁺, effector memory cells (TEM), and CD45RO⁺ CCR7⁻, central memory cells (TCM). Although CD8 and CD4 subsets were not significantly different between the HCV-infected groups, there was a trend showing a lower TNaive frequency and a higher TCM frequency in CD4 subsets of F3-F4 patients when compared with F0-F2 patients (Figure 1B). Lastly, the expression of CCR7 on CD56Dim NK cells was higher in the F3-F4 group compared with the F0-F2 group (Figures 1C and 1D).

The regulatory T cells (Treg) play an important role in downregulating immune responses and are defined in part by the expression of IL-2 receptor-α chain, CD25High, and the transcriptional factor FoxP3. We found no significant difference in Treg frequency between F0-F2 patients and F3-F4 patients (Figures 1E and 1F).

| Characteristics | F0-F2 | F3-F4 |
|-----------------|------|------|
| Age (years)     | 46.8 ± 2.0 | 55.3 ± 2.4 |
| Male/Female (N) | 11/3 | 13/2 |
| Race (N-Caucasian) | 15 | 15 |
| LSM (kPa)       | 6.05 ± 0.5 | 27.8 ± 4.9 |
| AST (U/L)       | 48 ± 8 | 97 ± 19 |
| ALT (U/L)       | 75 ± 10 | 104 ± 19 |
| HCV genotype (N) |       |        |
| -1a             | 10   | 6    |
| -2              | 0    | 3    |
| -3              | 2    | 5    |
| -4              | 1    | 1    |
| Metavir fibrosis (N) |   |      |
| -F0             | 4    | −    |
| -F1             | 6    | −    |
| -F2             | 3    | −    |
| -F3             | −    | 3    |
| -F4             | −    | 11   |

Table 1. Characteristics of Patients with Chronic HCV
*All data are presented as mean ± SEM.
**Two patients within this group could not be identified in the clinic post analysis but at the time of sample collection were placed in this group by the clinician. Thus, we are lacking AST, ALT, HCV genotype and Metavir stage for the two patients and the gender of one patient.
*One patient within this group did not have a definitive Metavir classification owing to lack of liver stiffness measurement or liver biopsy but was placed in this group after evaluation by the clinician.
*Five patients were excluded owing to lack of LSM data.
*AST, aspartate transaminase.
*ALT, alanine transaminase.

Liver fibrosis. As expected, higher levels of AST and ALT are observed in F3-F4 patients, indicating advanced liver damage.
Figure 1. Proportions of T and NK Cell Subsets in Patients with HCV with Liver Fibrosis

Freshly thawed PBMCs of F0-F2 (n = 15) and F3-F4 (n = 15) chronically infected patients with HCV were stained for surface and intracellular expression of T and NK cell population markers.

(A) Cumulative data showing the percentage of T cell subpopulations (CD4+CD8- , CD4+CD8- , CD4+CD8- , and CD4-CD8- ) and NK cell subpopulations (CD56DimCD16+ and CD56BrightCD16- ) of patients with chronic HCV in fibrosis stages F0-F2 and F3-F4.

(B) Cumulative data showing the percentage of CD8 and CD4 T cell subsets of patients with chronic HCV in fibrosis stages F0-F2 and F3-F4.

(C) Cumulative data comparing the percentage of CD56BrightCD16- and CD56DimCD16- NK cells expressing CCR7 of patients with chronic HCV in fibrosis stages F0-F2 and F3-F4.

(D) Representative plot showing the expression of CCR7 on CD56Dim NK cells of F0-F2 and F3-F4 patients.

(E) Representative plot showing the Treg (CD25+FoxP3+) population.

(F) Data comparing the frequency of Tregs between CD4 T cells of patients with chronic HCV in fibrosis stages F0-F2 and F3-F4.

(G) Cumulative data comparing CD25 expression on NK cells of patients with chronic HCV in fibrosis stages F0-F2 and F3-F4. Terminal effector (EMRA), Effector memory (EM), Central memory (CM) and T Regulatory cells (Treg). Data are represented as median with interquartile range (IQR) and each point represents an individual. *p < 0.05, ****p < 0.0001.
Notably, the F3-F4 patients showed a significantly higher proportion of CD25+ CD56Dim NK cells compared with F0-F2 patients (Figure 1G).

Higher Frequencies of T and NK Cells Expressing Multiple Inhibitory Receptors in Patients with Advanced Liver Fibrosis

Given the critical role of inhibitory receptors and their ligands in modulating T and NK cell function, we measured the surface expression of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 on CD8+/CD4+ T cells and CD56Bright/CD56Dim NK cells. In the CD8+ T cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of PD-1, LAG-3, TIGIT, and TIM-3 cells (Figures 2A and 2B). F0-F2 patients had higher frequencies of CTLA-4 cells than F3-F4 patients. F3-F4 patients had a higher frequency of GAL-9 cells than F0-F2 patients. In the CD4+ T cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of PD-1, CTLA-4, LAG-3, and TIM-3 cells (Figure 2C). Notably, F3-F4 patients had a higher frequency of TIGIT and GAL-9 cells than F0-F2 patients.

On the CD56Bright NK cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of PD-1 and TIGIT cells (Figure 2D). Interestingly, F3-F4 patients had a higher frequency of TIM-3, CTLA-4, and GAL-9 cells on CD56Bright NK cells than F0-F2 patients (Figure 2D). We also observed a trend toward a higher frequency of LAG-3 cells, although this did not reach the level of statistical significance (p = 0.0514). Lastly, on the CD56Dim NK cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of CTLA-4, TIM-3, and TIGIT cells (Figure 2E). F3-F4 patients had a higher frequency of PD-1, LAG-3, and GAL-9 cells on CD56Dim NK cells than F0-F2 patients. Notably, the higher expression of GAL-9 was consistent on all tested T and NK cells of F3-F4 patients. The CTLA-4 surface expression was found minimal on all tested subsets, presumably owing to its rapid internalization in the absence of ligand binding (Qureshi et al., 2012).

Extensive lymphocyte exhaustion is characterized by the simultaneous expression of multiple inhibitory receptors, and likewise, functionally superior lymphocytes express limited inhibitory receptors (Blackburn et al., 2009; Wherry and Kurachi, 2015). Thus, using Boolean analysis, we compared the frequency of T and NK cells simultaneously expressing more than three inhibitory receptors, PD-1, CTLA-4, LAG-3, TIGIT, and TIM-3 (Figure 2F), and the frequency of T and NK cells negative for all five receptors (Figure 2G). We chose to measure the expression of >3 receptors as the surface expression of CTLA-4 on both T and NK cells was minimal.

Notably, we observed a lower frequency of both CD56Bright and CD56Dim NK cells positive for >3 (i.e., 4 or 5) inhibitory receptors in F0-F2 patients compared with F3-F4 patients (Figure 2F). No difference was observed in the CD4+ and CD8+ T cell populations. Conversely, there was a clear trend showing a lower frequency of cells negative for all inhibitory receptors in F3-F4 patients, even though it only reached a significance on the CD4+ T cell and CD56Bright NK cell populations (Figure 2G). Altogether, the results suggest that a higher frequency of extensively exhausted NK cells and a lower frequency of functionally superior T and NK cells are characteristics of patients with advanced liver fibrosis.

Higher Frequencies of CD25⁺TIGITmed-hi CD4⁺ T Cells and PD-1med CD4⁺ T Cells Co-expressing LAG-3 and GAL-9 in Patients with Advanced Liver Fibrosis

We have shown that the expression of multiple inhibitory receptors is a characteristic of patients with advanced liver fibrosis. Thus, we decided to investigate the co-expression of inhibitory receptors on the surface of T cells. In addition, we included GAL-9 expression for the co-expression analysis. The t-SNE analysis was employed to dimensionally reduce the expression data of cell surface markers, inhibitory receptors, and GAL-9 from all 30 patients with HCV and to visualize expression patterns in two dimensions (Belkina et al., 2019). t-SNE analysis of all 30 patients (F0-F4) revealed several clusters, and separating the plots based on the fibrosis scores showed the distribution of cell densities (Figure 3A). We analyzed the expression of T cell subset markers (CD8, CD4, CD45RO, CCR7, and CD25) to characterize T cell clusters (Figure 3B). To visualize the co-expression patterns of the inhibitory receptors and GAL-9, we classified their expressions as low, medium (med), and high (hi) (Figure S3). Through this classification, T cells were found to be PD-1low or PD-1med, CTLA-4low or CTLA-4med, LAG-3low, LAG-3med, or LAG-3hi, TIGITlow, TIGITmed, or TIGIThi, TIM-3low, TIM-3med, or TIM-3hi, and GAL-9low, GAL-9med, or GAL-9hi (Figure 3C).
Figure 2. The Expression of Inhibitory Receptors and GAL-9 on Lymphocytes of Patients with HCV with Liver Fibrosis

Freshly thawed PBMCs of F0-F2 (n = 15) and F3-F4 (n = 15) chronically infected patients with HCV were stained for surface expression of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9.

(A) Representative plot of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on CD8+ T cells of patients with chronic HCV in fibrosis stages F0-F2 and F3-F4.

(B–E) Cumulative data comparing PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on CD8+ T cells (B), CD4 T cells (C), CD56Bright NK cells (D) and CD56Dim NK cells (E) of patients with chronic HCV in fibrosis stages F0-F2 and F3-F4.

(F) Cumulative data comparing Boolean analysis of the frequency of T and NK cells expressing >3 inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3).

(G) Cumulative data comparing Boolean analysis of the frequency of T and NK cells expressing none of the five inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3). Data are represented as median with IQR and each point represents an individual. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Prior to cluster analysis, t-SNE plots revealed a high co-expression of GAL-9hi and LAG-3hi on both CD8+ and CD4+ T cells with PD-1med and TIM-3low expression (Figure 3C). The t-SNE cluster analysis further generated eight clusters based on the expression of cell surface markers and inhibitory receptors as follows: (1) naive CD8 T cells, (2) TEM and TCM CD8 T cells, (3) TEMRA TIGITmed-hi CD8 T cells, (4) TEM and

Figure 3. t-SNE Analysis of T Cells of Patients with Chronic HCV
CD3+ cells excluding CD4-CD8- cells from F0-F2 (n = 15) and F3-F4 (n = 15) chronically infected patients with HCV were combined to conduct t-SNE analysis.

(A) Merged T cell t-SNE analysis of 30 patients with chronic HCV followed by the analysis of F0-F2 and F3-F4 patients separately.

(B) Expression of T cell subset markers (CD8, CD4, CD45RO, CCR7, and CD25) in t-SNE analysis.

(C) Expression of inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3) and GAL-9 in t-SNE analysis.

(D) Clustering of t-SNE analysis based on subset markers, inhibitory receptor, and GAL-9 expression.

(E) Cumulative data comparing the proportion of F0-F2 and F3-F4 patients T cells found in each cluster.

(F) Linear regression analysis of cluster 1, 6, and 7 frequencies against LSM of patients with chronic HCV (n = 25). Liver Stiffness Measurement (LSM). R² is the coefficient of determination. Cluster analysis data are represented as median with IQR and each point represents an individual. *p < 0.05, **p < 0.01. Statistically significantly regression p values are identified in red.
TCM CD4 T cells, (5) TNaive and TCM CD4 T cells, (6) CD25+ TIGITmed-hi CD4 T cells, (7) LAG-3med-hi GAL-g9med-hi PD-1med CD4 T cells, and (8) LAG-3med-hi GAL-9med-hi PD-1med CD8 T cells (Figure 3D). We next compared the T cell frequency of F0-F2 patients and F3-F4 patients in each cluster (Figure 3E). Notably, we found that F3-F4 patients had less of their T cells found in cluster 1 (naive CD8 T cells), but more found in clusters 6 (CD25+ TIGITmed-hi CD4 T cells) and cluster 7 (Lag-3med-hi GAL-9med-hi PD-1med CD4 T cells) when compared with F0-F2 patients. These data indicate that more advanced stages of fibrosis are associated with a higher frequency of CD25+ CD4 T cells with high TIGIT expression. Interestingly, TIGIT is highly expressed on Tregs compared with Teffs, CD25+ FoxP3- CD4 T (Figure S2). In addition, it indicates that GAL-9 and LAG-3 are highly co-expressed on PD-1med cells, and this co-expression is found in both CD8+ T and CD4+ T cells. Lastly, F3-F4 patients have an increased frequency of these LAG-3med-hi GAL-g9med-hi CD4+ T cells.

To correlate the identified cell clusters with liver damage, we conducted linear regression analyses between patients’ Liver Stiffness Measurements (LSM) and the frequency of cells found in significantly different clusters (clusters 1, 6, and 7). We found a significant correlation of cluster 6 and 7 frequencies with LSM, but not cluster 1 (Figure 3F). Taken together, using the t-SNE analysis, we identified high TIGIT expression on CD25+ CD4+ T cells and high LAG-3 and GAL-9 co-expression on PD-1med CD4+ T cells and these expression patterns are associated with advanced liver fibrosis.

Higher Frequencies of CD56Dim NK Cells Co-expressing LAG-3 and GAL-9 as well as TIGIT and TIM-3 in Patients with Advanced Liver Fibrosis

To determine co-expression patterns of inhibitory receptors on NK cells, we employed t-SNE analysis on CD56+ NK cells, first combining the plots derived from all 30 patients irrespective of fibrosis stage and then separating plots based on the stage of fibrosis, F0-F2 and F3-F4 (Figure 4A). The expression of subset markers on NK cells was classified as CD56Dim or CD56Bright; CD16-, CD16Dim, or CD16Bright; CCR7- or CCR7+; and CD25+ or CD25− (Figure 4B). The expression of inhibitory receptors was classified as PD-1low or PD-1med, CTLA-4low or CTLA-4med, LAG-3low, LAG-3med, or LAG-3hi, TIGITlow, TIGITmed, or TIGIThi, TIM-3low, TIM-3med, or TIM-3hi, and GAL-glow, GAL-g9low, GAL-g9med, or GAL-g9hi (Figure 4C). Using this classification, NK cells were divided into six clusters (Figure 4D) as follows: (1) CD56BrightCD16 TIM-3med-hi cells, (2) CD56DimCD16+ TIM-3med-hi cells, (3) CD56DimCD16+ TIM-3med-hi cells, (4) CD56Dim CD25+ TIGITmed-hi TIM-3med-hi cells, (5) CD56Dim CCR7 PD-1med LAG-3med-hi GAL-g9med-hi cells, and (6) CD56BrightDim CD16Dim cells.

We compared the frequency of patients’ NK cells in each cluster (Figure 4E) and found that F3-F4 patients had a significantly higher frequency of their NK cells found in cluster 4 (CD56Dim CD25+ TIGITmed-hi TIM-3med-hi cells) and cluster 5 (CD56Dim CCR7 PD-1med LAG-3med-hi GAL-g9med-hi cells). Interestingly, the higher expression on CCR7+ NK cells in F3-F4 patients was observed in Figure 1C, and the t-SNE analysis identified the co-expression of LAG-3, GAL-9, and PD-1 on the CCR7+ NK cells. Linear regression analysis was employed correlating patients’ LSM with the frequency of cells found in clusters 4 and 5. We found a significant correlation of cluster 4 frequencies with LSM and a correlation in cluster 5, although this did not rise to the level of statistical significance, p = 0.0863 (Figure 4F). Taken together, using t-SNE analysis, we identified CD25+ TIGITmed-hi TIM-3med-hi cells and CCR7 PD-1med LAG-3med-hi GAL-g9med-hi cells, and these co-expression patterns on CD56Dim NK cells are associated with advanced liver fibrosis.

DISCUSSION

NK and T cells play a vital role in the anti-viral and anti-tumor responses. Patients with chronic HCV with advanced stages of fibrosis have an increased susceptibility to infection and an increased risk of HCC post-viral clearance with DAAs (Aggeletopoulou et al., 2017; Bonnel et al., 2011; Gurstevitch, 2008; Vranjkovic et al., 2019). Thus, it is imperative to understand the immune dysfunction in these patients to improve their immune function after they are cured by DAAs. Previously, it has been shown that the expression of inhibitory receptors is a mechanism contributing to immune cell suppression of the NK cell and bulk T cell population (Blackburn et al., 2009; da Silva et al., 2014; Motamedi et al., 2019; Woo et al., 2012; Xu et al., 2015). Therefore, there is a need to understand the differences in immune dysregulation between patients with minimal liver fibrosis and those in the advanced stages to create targeted strategies to improve immune function.

Although the expression of only one inhibitory receptor does not suggest lymphocyte exhaustion, the expression of multiple inhibitory receptors is a clear indicator of extensively exhausted immune cells
On the other hand, immune cells not expressing any inhibitory receptors are known to exhibit superior effector function (Blackburn et al., 2009). We observed that F3-F4 patients had a higher frequency of NK cells, which expressed more than 3 (i.e., 4 or 5) inhibitory receptors, suggesting the extensive exhaustion of these cells. Conversely, these patients showed a lower

Figure 4. t-SNE Analysis of NK Cells of Patients with Chronic HCV
CD56+ NK cells from F0-F2 (n = 15) and F3-F4 (n = 15) chronically infected patients with HCV were combined to conduct t-SNE analysis.
(A) Merged NK cell t-SNE analysis of 30 patients with chronic HCV followed by the analysis of F0-F2 and F3-F4 patients separately.
(B) Expression of NK cell subset markers (CD56, CD16, CCR7, and CD25) in the t-SNE analysis.
(C) Expression of inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3) and GAL-9 in the t-SNE analysis.
(D) Clustering of t-SNE analysis based on subset markers, inhibitory receptor, and GAL-9 expression.
(E) Cumulative data comparing the proportion of F0-F2 and F3-F4 patients NK cells found in each cluster.
(F) Linear regression analysis of cluster 4 and 5 frequencies against LSM of patients with chronic HCV (n = 25). Liver Stiffness Measurement (LSM). R² is the coefficient of determination. Cluster analysis data are represented as median with IQR and each point represents an individual. **p < 0.01, ***p < 0.001. Statistically significantly regression p values are identified in red.

(Blackburn et al., 2009; Wherry and Kurachi, 2015). On the other hand, immune cells not expressing any inhibitory receptors are known to exhibit superior effector function (Blackburn et al., 2009). We observed that F3-F4 patients had a higher frequency of NK cells, which expressed more than 3 (i.e., 4 or 5) inhibitory receptors, suggesting the extensive exhaustion of these cells. Conversely, these patients showed a lower
frequency of T and NK cells that did not express any of the measured inhibitory receptors. Therefore, our data demonstrate that patients with advanced liver fibrosis contain higher proportions of NK cells expressing multiple inhibitory receptors and lower proportions of T and NK cells free from inhibitory receptors expression.

As CD25 is the IL-2 receptor α-chain, its expression plays a crucial role in the response of NK cells in patients with chronic HCV. Co-culturing of NK cells with HCV-infected hepatocytes has been shown to increase their CD25 surface expression (Pollmann et al., 2018). CD56\textsuperscript{Dim} NK cells with increased expression of CD25 have enhanced responsiveness to low doses of IL-2, thus improving their production of IFN-γ and cytotoxicity in these conditions (Leong et al., 2014). Here we showed that patients with advanced stages of fibrosis have a higher frequency of CD25\textsuperscript{+} CD56\textsuperscript{Dim} NK cells than patients with minimal fibrosis. The t-SNE analysis revealed that these CD25\textsuperscript{+} cells also highly co-express TIM-3 and TIGIT. TIM-3 expression denotes functionally exhausted CD56\textsuperscript{Dim} NK cells with impeded cytokine production and cytotoxicity (da Silva et al., 2014). Similarly, TIGIT engagement on NK cells directly inhibits their cytotoxicity and cytokine secretion by prematurely terminating the activity of NF-κB (nuclear factor-κB), PI3K (phosphoinositide 3-kinase), and MAPK (mitogen-activated protein kinase) (Li et al., 2014; Liu et al., 2013). TIM-3 expression has also been observed on HCV-specific CD8 T cells, but this increased expression was restricted to the cells in the intrahepatic compartment and not the peripheral blood (Golden-Mason et al., 2009). Knowing TIM-3 and TIGIT are co-expressed on NK cells, which are already primed for enhanced function (CD25\textsuperscript{+}), a combined immune blockade therapy can be employed to unleash these exhausted cells, thus conferring protection to the host against other infections and HCC. Tumor infiltrating lymphocytes (TILs) of B16F10 melanoma in mice highly co-express TIM-3 and TIGIT, and their effector function is only restored in TIGIT\textsuperscript{−/−} mice treated with anti-TIM-3 antibodies (Kurtulus et al., 2015). Blockade of TIM-3 or TIGIT has separately been shown to restore NK cell function (Xu et al., 2015; Zhang et al., 2018). Thus, our data suggest that high co-expression of TIM-3 and TIGIT on CD25\textsuperscript{+} NK cells hinders their function, and a combined blockade therapy using anti-TIGIT and anti-TIM-3 antibodies could provide a potential therapeutic benefit by improving NK cell function in patients with chronic HCV with advanced liver fibrosis.

Our t-SNE analysis also revealed a higher frequency of T\textsuperscript{reg}med-hi CD25\textsuperscript{+} CD4\textsuperscript{+} T cells (T\textsubscript{reg} and T\textsubscript{effs}) in F3-F4 patients when compared with F0-F2 patients. T\textsubscript{reg} begin to express IL-10 and FgI2 after TIGIT engagement, and FgI2 expression allows T\textsubscript{reg} to selectively suppress Th1 and Th17 pro-inflammatory responses (Joller et al., 2014). TIGIT expression on T\textsubscript{reg} is also implicated in their lineage stability (Fuhrman et al., 2015). Altogether, our data reveal that patients with advanced liver fibrosis have an increase in the frequency of T\textsubscript{reg}med-hi CD25\textsuperscript{+} CD4\textsuperscript{+} T cells, which we found to be approximately 50% T\textsubscript{IGIT}\textsuperscript{+} T\textsubscript{reg}, and evidence suggests TIGIT\textsuperscript{+} T\textsubscript{reg} could possess increased immunosuppressive capabilities and increased stability.

GAL-9 is one of the ligands of TIM-3, and the role of TIM-3/GAL-9 interactions on modulating immune cell functions has been well studied (Elsegood et al., 2017; Mengshol et al., 2010). We found that CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells as well as CD56\textsuperscript{Dim} and CD56\textsuperscript{Bright} NK cells in patients with advanced liver fibrosis had higher frequencies of GAL-9\textsuperscript{+} cells when compared with F0-F2 patients. GAL-9\textsuperscript{+} NK cells in patients with HIV had reduced cytotoxicity with lower expression of perforin and granzyme B (Motamedi et al., 2019). Furthermore, GAL-9 binding to TIM-3 has been shown to trigger cell death in Th1 and Tc1 cells (Zhu et al., 2005). t-SNE analysis of patients with chronic HCV showed that the T cells co-expressing LAG-3 and GAL-9 were PD-1\textsuperscript{+} cells. It also showed that F3-F4 patients had a higher frequency of CD4\textsuperscript{+} T cells with this expression pattern. Given the vital role of the TIM-3/GAL-9 interactions in modulating T/NK cell functions, the higher frequency of T and NK cells expressing GAL-9 in patients with advanced liver fibrosis when compared with F0-F2 patients is a phenotype potentially denoting immune dysregulation.

Lastly, we observed a higher frequency of CCR7\textsuperscript{+} expressing CD56\textsuperscript{Dim}CD16\textsuperscript{+} NK cells in patients with advanced fibrosis than in those with less fibrosis (Figure 1D). CCR7 is a receptor vital for the migration of immune cells into secondary lymphoid organs such as the lymph nodes, and CD56\textsuperscript{Dim} NK cells in the peripheral blood lacking CCR7 have been shown to be cytotoxic effector cells (Berahovich et al., 2006; Campbell et al., 2001). The increase in CD56\textsuperscript{Dim} CCR7\textsuperscript{+} NK cells in F3-F4 patients suggests that the frequency of cytotoxic effector NK cells is reduced. The phenotype was accompanied by the close co-expression of LAG-3, GAL-9, as well as PD-1. Studies have previously shown LAG-3 and PD-1 are co-expressed on TILs in various human tumors as well as LCMV chronically infected mice, and LAG-3 blockade was not sufficient to reverse the CTL exhausted phenotype (Blackburn et al., 2009; Matsuzaki et al., 2010; Woo et al., 2012).
Notably, combined blockade of LAG-3 and PD-1 vastly improves CTL function to control tumor growth (Woo et al., 2012). The combined blockade of LAG-3 and PD-L1 was also shown to improve viral control in LCMV chronically infected mice (Blackburn et al., 2009). Thus, our findings reason that combined blockade of LAG-3, GAL-9, and PD-1 could potentially improve the function of T/NK cells in patients with advanced liver fibrosis.

In conclusion, we investigated PBMCs from patients with chronic HCV with minimal to no fibrosis and chronic HCV with advanced fibrosis by analyzing the co-expression of five inhibitory receptors and GAL-9 on T/NK cells. A recent meta-analysis found that PD-1 inhibitor monotherapy and anti-CTLA-4 monotherapy had 18.6% efficacy when used for treating liver cancer in patients with chronic HBV and HCV (Pu et al., 2020). Ultimately, they concluded that immune checkpoint inhibitors are safe for these patients, but virus reactivation remains a possibility. Our findings revealed multiparametric phenotypes that characterize immune suppression in patients with chronic HCV with advanced liver fibrosis. Mechanisms underlying these phenotypes would provide a better understanding of immune dysregulation associated with advanced liver damage and increased susceptibility to infections and HCC and will lead to the development of targeted therapies to improve their immune function.

**Limitations of the Study**

Although we utilize an extensive number of inhibitory receptors, there are other inhibitory receptors at play, which were not included in this study, but could be critical in inducing and maintaining immune dysregulation. Owing to the limited sample size, we acknowledge that statistically significant differences do not necessarily translate into biological significance; thus, we are looking forward to our results being reproduced in other studies with larger sample sizes. In addition, our data represent the status of circulating immune cells and may not reflect the phenotypic and functional picture of their counterparts in the liver. Nonetheless, information regarding the immunological status of T and NK cells in the peripheral blood should be vital to understand the susceptibility to other infections and HCC in patients. Lastly, we did not include healthy controls in the study. Thus, our data itself might not be sufficient to distinguish phenotypes of immune dysregulation associated with advanced liver damage from those found in healthy controls. Nonetheless, there is ample literature that has compared the expression of inhibitory receptors in healthy controls with that in patients with chronic HCV.

**Resource Availability**

**Lead Contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Seung-Hwan Lee (seunglee@uottawa.ca).

**Materials Availability**

This study did not generate new unique reagents.

**Data and Code Availability**

This study did not generate/analyze datasets or codes.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101513.

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AUTHOR CONTRIBUTIONS
Conceptualization, C.I.A.O. and S.-H.L.; Methodology, C.I.A.O., J.S.O., and S.-H.L.; Investigation, C.I.A.O.; Formal Analysis, C.I.A.O.; Visualization, C.I.A.O.; Resources, C.L.C. and J.S.O.; Writing – Original Draft, C.I.A.O. and S.-H.L.; Writing – Review & Editing, C.I.A.O., S.-H.L., C.L.C., and A.M.C.; Funding Acquisition, S.-H.L.; Supervision, S.-H.L.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Expression of Inhibitory Receptors on T and NK Cells Defines Immunological Phenotypes of HCV Patients with Advanced Liver Fibrosis

Chisom Ifeoma Adaeze Okwor, Jun Seok Oh, Angela Marie Crawley, Curtis Lindsey Cooper, and Seung-Hwan Lee
Figure S1. [Gating strategy to identify and analyze T and NK cell subsets], Related to Figure 1. We gated on lymphocytes based on SSC-A and FSC-A and subsequently performed doublet discrimination. (A) We gated T cells following gating on live cells. CD4 and CD8 was used to identify T cell subsets: CD4-CD8+, CD4+CD8+, CD4+CD8- and CD4-CD8- T cells. (B) We gated on Live CD3-CD19-CD14- cells and using CD56 and CD16 identified CD56+ NK cells.

Figure S2. [TIGIT expression on Tregs and Teffs], Related to Figure 3. Freshly thawed PBMCs of F0-F2 (n=15), and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of CD25 and TIGIT, and intracellular expression of FoxP3. Cumulative data comparing TIGIT expression on Tregs and Teffs of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. Regulatory T cell (Tregs) and T effector (Teffs). Data are represented as median with IQR and each point represents an individual. ****p<0.0001.
Figure S3. [Representative histograms used to identify and analyze inhibitory receptor and GAL-9 expressions in T and NK cell t-SNE plots], Related to Figure 3 and 4. The expression of receptors in each t-SNE cluster was classified as either low, med or hi based on the histogram expression profile of the cluster. Only low and med PD-1 and CTLA-4 expression were identified. Multiple histograms were overlayed to define the differential expression.
Transparent Methods

Study subjects

Blood samples from chronically infected HCV+ individuals (i.e. > 6 months HCV RNA-positive) who were direct acting antiviral (DAA) treatment-naive were collected. The study cohort was composed of 2 groups with 30 patients: 15 chronic HCV patients in fibrosis stage F0-F2 and 15 chronic HCV patients in stage F3-F4. 27 of these patients were interferon treatment naïve while 3 patients had previously received interferon treatment. Written informed consent was obtained from all participants, and the study was approved by The University of Ottawa Health Sciences and Sciences Research Ethics Board.

Fibrosis scoring

Fibrosis stages of the liver were determined by transient elastography (FibroScan, Echosens) or liver biopsies and were grouped in accordance with the METAVIR stages (F0: no fibrosis, F1: minimal fibrosis, F2: spreading of fibrosis to other areas of the liver including blood vessels, F3: spreading and presence of fibrosis network in the liver, F4: cirrhosis). Fibrosis evaluation was performed at The Ottawa Hospital Viral Hepatitis Clinic.

Extraction and storage of Peripheral Blood Mononuclear Cells from blood samples

PBMCs were isolated by Ficoll gradient density centrifugation (Ficoll-Paque PLUS, GE Healthcare, Mississauga, ON, Canada), aliquoted in freezing media consisting of 10% DMSO (Fisher BioReagents, Fisher Scientific) and 90% FBS (Gibco, Life Technologies, Burlington, Ontario, Canada), and stored at
-80°C for future use. The cells were thawed in complete RP-10 media, consisting of RPMI 1640 (HyClone, GE Healthcare) supplemented with 10% FBS, 2mM L-glutamine (HyClone), 10mM HEPES buffer (HyClone), 1% penicillin/streptomycin (HyClone), and 55μM 2-Mercaptoethanol (Gibco), before assessing cell surface and intracellular protein expression.

**Flow cytometry analysis**

Anti-human fluorophore-conjugated antibodies were purchased from BD Bioscience, Biolegend and Invitrogen. The following antibodies were used: anti-PD-1 (MIH4), anti-CTLA-4 (14D3), anti-Lag-3 (T47-530), anti-TIGIT (MBSA43), anti-Tim-3 (7D3), anti-Gal-9 (9M1-3), anti-CD3 (HIT3a), anti-CD4 (SK3), anti-CD8 (RPA-T8), anti-CD56 (NCAM 16.2), anti-CD16 (3G8), anti-CD45RO (UCHL1), anti-CCR7 (2-L1-A), anti-CD25 (M-A251), anti-FoxP3 (236A/E7), anti-CD19 (SJ25C1) to exclude B cells, anti-CD14 (MΦP9) to exclude monocytes and near IR fluorescent reactive dye (Life Technologies, California, USA) was used to exclude dead cells. Cells were fixed using paraformaldehyde (2%) and acquired using BD LSRFortessa. For T_reg analysis, cells were fixed using eBioscience™ Foxp3/Transcription factor staining buffer set and acquired using Attune™ NxT Flow cytometer. All flow cytometry data were analyzed using FlowJo v.10 software.

**t-SNE analysis**

t-SNE analysis was performed using FlowJo v.10 t-SNE plugin. Each subject’s T cells were down-sampled to randomly select 50 000 cells for t-SNE analysis. Each 50 000 cell down-sample was then concatenated into a single file. Prior to analysis, we excluded double negative (CD4+CD8-) T cells due
to over-representation from one study subject and ultimately performed t-SNE analysis on 1 402 236 cells. Analysis of T cells was done using surface expression of CD8, CD4, CD45RO, CCR7, CD25, PD-1, CTLA-4, LAG-3, TIGIT, TIM-3 and GAL-9. T cells were clustered made by qualitatively accessing expression of subset markers, inhibitory receptors and GAL-9. The analysis was done using: 1 000 iterations, 100 perplexity, 98 156 learning rate (eta), Exact (vantage point tree) KNN algorithm and Barnes-Hut gradient algorithm. For t-SNE analysis on NK cell, each subject’s CD3⁺CD14⁻CD19⁻ lymphocytes were down-sampled to randomly select 15 000 cells for t-SNE analysis. Each 15 000 cell down-sample was then concatenated into a single file. Prior to analysis, we selected CD56⁺cells and ultimately performed t-SNE analysis on 317 727 cells. Analysis of NK cells was done using the surface expression of CD56, CD16, CCR7, CD25, PD-1, CTLA-4, LAG-3, TIGIT, TIM-3 and GAL-9. NK cells were clustered by qualitatively accessing expression of subset markers, inhibitory receptors and GAL-9. The analysis was done using: 2 000 iterations, 100 perplexity, 22 240 learning rate (eta), Exact (vantage point tree) KNN algorithm and Barnes-Hut gradient algorithm.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 8. Unpaired nonparametric Mann-Whitney U test was used for all analysis between the F0-F2 and F3-F4 groups. Results are presented as median with IQR with a p value< 0.05 being considered statistically significant. Regression analysis was performed following a linear regression model and using liver stiffness measurement (LSM) of chronic HCV patients. R² and p-values are reported for all regression analyses. LSM regression analysis was done using 25 chronic HCV patients due to the lack of LSM of 5 patients.
Graphical Abstract

The graphical abstract was created with BioRender.com