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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

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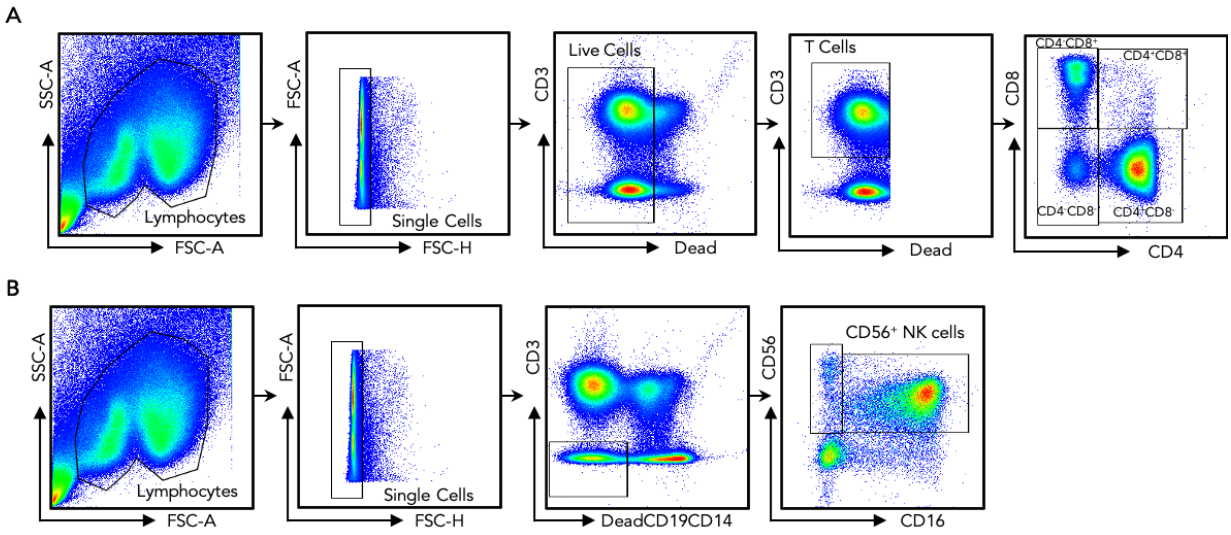

Figure S1. [Gating strategy to identify and analyze T and NK cell subsets], Related to Figure 1. We gated on lymphocytes based on SSC-A and FSC-A and subsequently performed doublet discrimination. (A) We gated T cells following gating on live cells. CD4 and CD8 was used to identify T cell subsets: $CD4^{-}CD8^{+}$, $CD4^{+}CD8^{+}$, $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{-}$ T cells. (B) We gated on Live $CD3^{-}CD19^{-}CD14^{-}$ cells and using CD56 and CD16 identified $CD56^{+}$ NK cells.

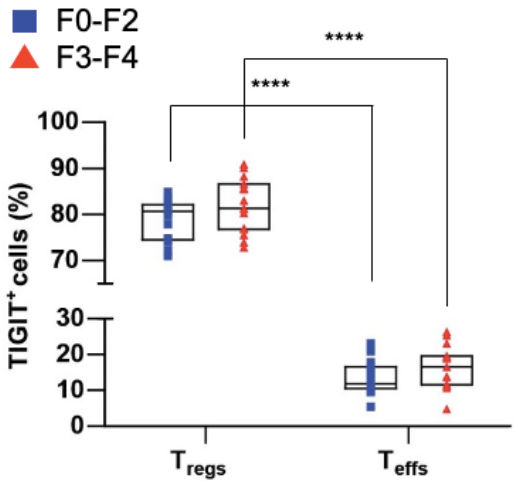

Figure S2. [TIGIT expression on T_{regs} and T_{effs}], Related to Figure 3. Freshly thawed PBMCs of F0-F2 (n=15), and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of CD25 and TIGIT, and intracellular expression of FoxP3. Cumulative data comparing TIGIT expression on T_{regs} and T_{effs} of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. Regulatory T cell (T_{reg}) and T effector (T_{effs}). Data are represented as median with IQR and each point represents an individual. ****$p<0.0001$.

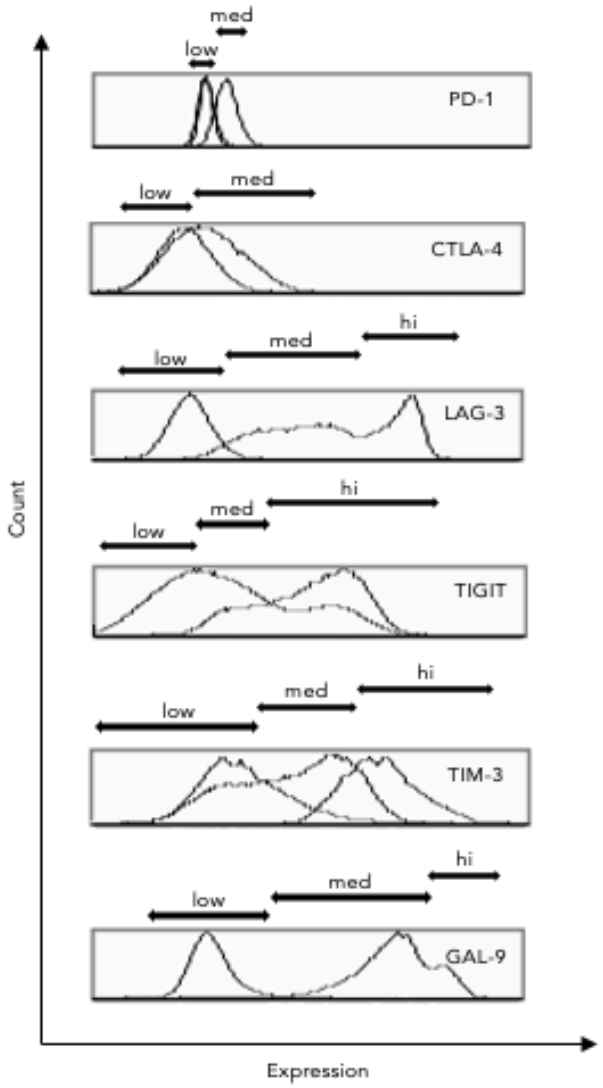

Figure S3. [Representative histograms used to identify and analyze inhibitory receptor and GAL-9 expressions in T and NK cell t-SNE plots], Related to Figure 3 and 4. The expression of receptors in each t-SNE cluster was classified as either low, med or hi based on the histogram expression profile of 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. R^2 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