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BRIEF REPORT

Hepatitis C Virus Relapse After Ultrashort Direct-Acting Antiviral Therapy Associates With Expression of Genes Involved With Natural Killer-Cell and CD8$^+$ T-Cell Function

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To identify immunologic correlates of hepatitis C virus (HCV) relapse after direct-acting antiviral (DAA) therapy, we quantified select immune transcripts in whole blood from noncirrhotic HCV subjects treated with 4–6 weeks of DAAs. We identified specific markers of natural killer-cell and CD8$^+$ T-cell function (*GZMB*, *PRF1*, *NKp46*) with higher expression in subjects who relapsed. These findings suggest a role for host immunity in HCV eradication with ultrashort DAA therapy.

Keywords. direct acting antiviral; gene expression analysis; hepatitis C virus; relapse; sustained virologic response.

Most, but not all, chronic hepatitis C virus (HCV) patients treated for 8–12 weeks with direct-acting antivirals (DAAs) achieve a sustained virologic response (SVR), synonymous with cure. Virologic relapse occurs in some patients, but not others, for reasons that are poorly understood and do not always relate to viral resistance or medication adherence [1–3]. The majority of patients treated with DAAs experience rapid downregulation of endogenous interferon (IFN) activity and changes in the distribution and function of innate and adaptive immune cells in liver and peripheral blood [4–8]. Although functional markers on total and antigen-specific CD4$^+$ T lymphocytes, CD8$^+$ T lymphocytes, and natural killer (NK) cells change during DAA therapy, it has not been delineated which changes might affect or reflect the odds of achieving SVR [9–12].

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Our prior work identified changes in whole blood that correlated with virologic relapse after HCV treatment with sofosbuvir and ribavirin [13]. In peripheral blood, pretreatment NK-cell concentration, higher pretreatment expression of genes associated with T-cell dysfunction and tolerance, and differential expression of select genes associated with type-I IFN signaling correlated with the odds of virologic relapse [13]. In this current report, we analyzed whole blood from subjects who relapsed after ultrashort treatment with 4–6 weeks of sofosbuvir-based DAA therapy to further explore immune correlates of treatment outcome [2, 14]. Our overarching hypothesis is that peripheral markers of immune function may reflect intrahepatic events that influence relapse risk irrespective of the specific DAA regimen used for treatment. Understanding the correlates of treatment outcome could have significant practical implications for predicting relapse and identifying patients who can benefit from shorter treatment. Given high rates of SVR achieved with currently approved regimens, analysis of samples from ultrashort DAA therapy is an ideal way to assess the contribution of host immunity to HCV treatment outcome.

MATERIALS AND METHODS

Clinical Samples

We used whole blood collected during clinical trials from subjects receiving combination DAA therapy for 4 or 6 weeks and relapsed (n = 33) or achieved SVR (n = 55). In the 4-week clinical trial, subjects had F0-F2 liver fibrosis and received therapy with sofosbuvir and ledipasvir combined with vedroprevir (n = 25) or vedroprevir + radalbuvir (n = 25) [14]. Sofosbuvir (an NS5B inhibitor) and ledipasvir (an NS5A inhibitor) are US Food and Drug Administration-approved medications, whereas vedroprevir (GS-9451) and radalbuvir (GS-9669) are investigational NS3/4A and NS5B inhibitors, respectively. Sustained virologic response was achieved in 10 of 25 subjects treated with 3-drug therapy and 5 of 25 subjects receiving 4-drug therapy [14]. Baseline viral load, genotype 1b infection, age, and presence of resistance variants in the NS3 and NS5 loci associated with odds of virologic relapse, as previously reported [14]. In the 6-week clinical trial, subjects had F0-F3 liver fibrosis and received therapy with sofosbuvir and ledipasvir combined with radalbuvir (n = 20) or vedroprevir (n = 20) [2]. Sustained virologic response was achieved by 19 of 20 subjects in each arm, with 1 relapse occurring in the radalbuvir arm and 1 patient lost to follow up in the vedroprevir arm after having undetectable viral load 4 weeks after treatment (SVR4) [2]. For the purpose of this molecular analysis, the subject lost to follow up was presumed to have achieved SVR.

Paired pre- and posttreatment whole blood collected in PaxGene tubes and stored at −80°C was available from 88 subjects in these trials (Supplemental Table 1). Posttreatment samples were collected immediately after completing treatment and before virologic relapse. Ribonucleic acid (RNA) extraction was performed using the QIAGEN PAXgene Blood miRNA Kit following the manufacturer instructions, as previously described [13]. RNA integrity was determined using an Agilent Bioanalyzer (Genomics Shared Resource, MUSC Hollings Comprehensive Cancer Center) yielding a median RNA integrity number of 8.3 for all samples.

Patient Consent Statement

As previously reported with the initial publication of the clinical trial results [2, 14], all subjects provided written informed consent, which included permission to use samples and data collected in the initial trial for future studies. The current study was approved by the Institutional Review Board at the Medical University of South Carolina.

Gene Quantitation

Direct quantitation of selected transcripts was performed using 100 ng of total RNA on a Nanostring nCounter system using 30 preselected immune markers and 5 housekeeping genes (gene list provided in Supplemental File 1). RCC files were imported into nSolver 4.0 software and analyzed using the default analysis pipeline. Normalization was performed using 6 internal control probes to account for differences between sample cartridges. Five preselected housekeeping genes (*TBP*, *PPIA*, *TUBB*, *GUSB*, and *GADPH*), identified as high performing in our prior analysis [13], were quantitated to generate a normalization factor to adjust and remove variance across samples within nSolver.

Statistical Analysis

Statistical analysis was performed on $\log_2$-transformed normalized counts using GraphPad Prism software (version 9.0.0). Paired samples were analyzed by *t* test using parametric assumptions. Unpaired pretreatment and posttreatment expression values comparing SVR versus relapse subjects were analyzed by unpaired *t* test using parametric assumptions.

RESULTS

We used whole blood collected before and after 4–6 weeks of DAA treatment to identify transcriptional correlates of HCV treatment outcome. We quantitated expression of 30 preselected genes of interest (Supplemental File 1) based on prior work suggesting their potential to correlate with the risk of HCV relapse [13].

Multiple IFN-stimulated genes (ISGs) decreased during treatment, consistent with previous findings (Figure 1) [4–6, 13], whereas type-I and type-III IFN receptor expression did not change. Expression of ISGs and IFN receptors did not differ pre- or posttreatment based on treatment outcome (data not shown). We identified 4 genes that correlated with higher odds of relapse. Subjects who relapsed had higher pretreatment

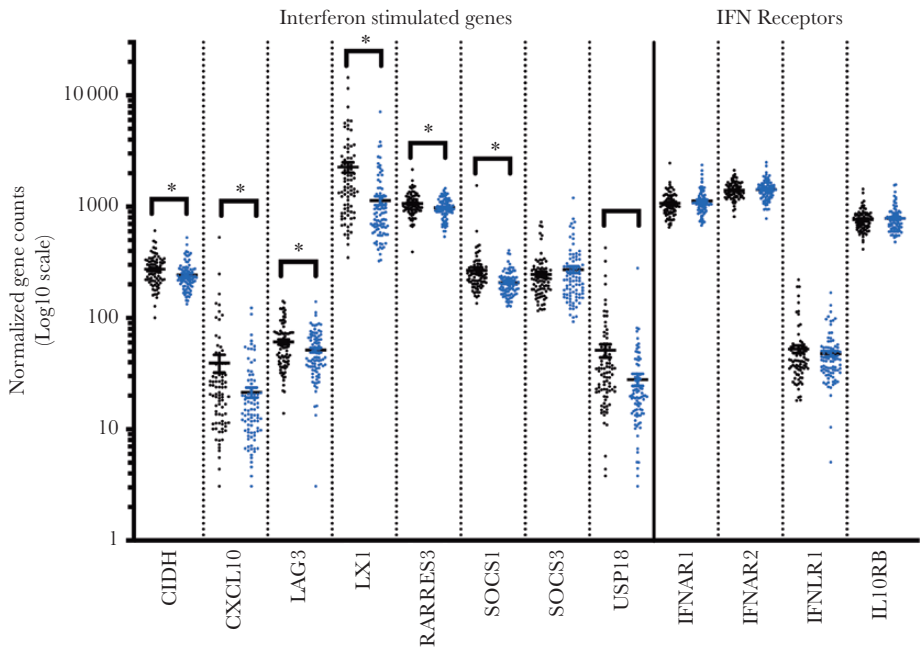

Figure 1. Interferon (IFN)-stimulated gene expression decreases in whole blood during short course direct-acting antiviral therapy, but IFN receptor expression does not. Shown are paired pretreatment (in black) and posttreatment (in blue) expression values for the indicated genes (n = 88 subjects). The * represents genes whose expression changes significantly ($P < .05$ by paired *t* test). Individual data points, mean, and standard error are shown. None of these genes had differential expression between sustained virologic response and relapse either pre- or posttreatment.

gene expression of *GZMB* (granzyme B), *PRF1* (perforin), and *NKp46* (*NCR1*), and lower expression of *CTLA4* relative to subjects who achieved SVR (Figure 2). In posttreatment whole blood obtained before virologic relapse, relapsers continued to have higher expression of *GZMB* and *NKp46*, whereas *PRF1* and *CTLA4* expression were no longer significantly different (Figure 2). Multiple genes that differed in our prior analysis of subjects treated with sofosbuvir and ribavirin did not change over the course of treatment or differ by treatment outcome in this study. These included genes associated with adaptive immune function (*CD274/PDL1*, *HAVCR2/TIM3*, *IDO1*), innate immune signaling (*IRAK4*, *IFNAR1*, *IFNAR2*, *SOCS3*, *TLR8*, *TOLLIP*), and NK-cell function (*CD244*, *KLRC3*, *KLRC4*, *KLRD1*, *KLRG1*, *KLRK1*) (data not shown).

DISCUSSION

Our primary finding is that higher pretreatment expression of *GZMB*, *PRF1*, and an activating NK-cell receptor (*NCR1/NKp46*) associate with higher odds of HCV relapse in subjects treated with ultrashort courses of DAAs for 4–6 weeks. *GZMB* and *NCR1* expression remained higher at the end of treatment in relapsers relative to subjects achieving SVR. In a prior study by our group in subjects treated with sofosbuvir and ribavirin for 24 weeks, each of these 3 markers had higher expression posttreatment in relapsers [13]. These genes are associated with NK-cell and cytotoxic $CD8^+$ T-cell function, and their consistent association with relapse across multiple DAA studies suggests that viral relapse may have an immunologic basis irrespective of the DAA regimen that is used for treatment. Although ISG expression decreased during treatment in this study (Figure 1), we did not identify a correlation with relapse, which suggests that ISG expression alone is inadequate to explain differential treatment outcome for short-course therapy.

Our findings are consistent with prior work showing restoration of NK-cell subsets and a reduction in exhaustion markers on $CD8^+$ T cells in subjects achieving SVR with different DAA regimens [9, 10, 12]. Our observation of differential *NKp46* expression both pre- and posttreatment in relapsers is also consistent with prior reports that associated hepatic *NKp46* expression and NK-cell activation with treatment outcome after IFN-based therapy [15, 16]. More importantly, cytolysis of HCV-infected hepatocytes is executed by perforin and granzyme B that are secreted by cytotoxic $CD8^+$ T cells and NK cells [17]. The higher expression of *GZMB* and *PRF1* we observed in relapsers suggests that a heightened dysfunctional immune response to HCV may mechanistically relate to treatment relapse.

In the initial clinical trial report, higher HCV viral load, younger age, genotype 1b infection, and baseline resistance variants in the NS3 and NS5 region of HCV were associated with relapse [2, 14]. Taken together, these studies suggest immune dysfunction may prevent the hosts' ability to overcome high viral loads and baseline-resistant variants when therapy is shortened, helping to explain why ultrashort DAA therapy is only effective for a subset of patients. Because longer treatment with DAAs for patients with unfavorable baseline characteristics typically results in SVR, it is thus plausible that DAAs are capable of achieving SVR without significant help from the host immune system when given for longer periods of time. The immune correlates identified in this study may be relevant for understanding the rare instances of relapse that occur with currently approved DAA therapies and may also relate to the risk of reinfection.

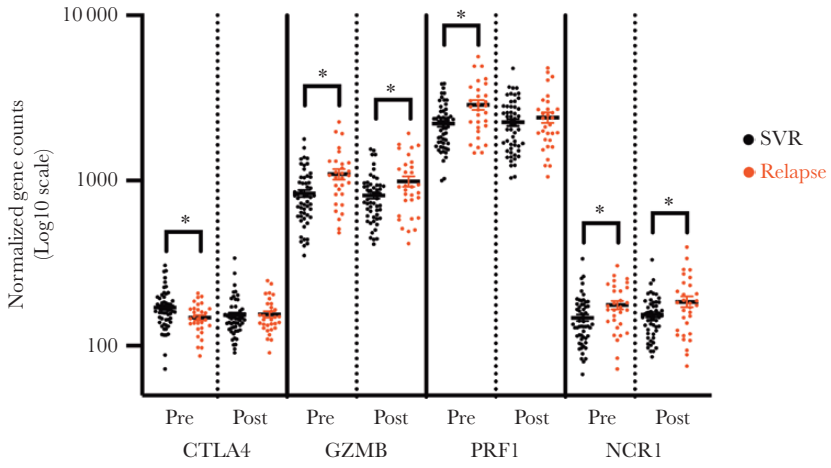

Figure 2. Genes with differential expression between sustained virologic response (SVR) and relapse patients either pre- or posttreatment. Two genes had differential expression both pre- and posttreatment (*GZMB, NCR1*) and 2 others differed pretreatment (*CTLA4, PRF1*) between SVR (n = 55) and relapse (n = 33) patients. The * represents genes with significant differential expression ($P < .05$ by unpaired *t* test). Individual data points, mean, and standard error are shown. None of these genes had a significant change in expression over the course of treatment in the overall cohort.

Limitations of this study include the use of whole blood for gene expression analysis. Although this approach was taken based on the ease of collection and analysis, attribution of gene expression findings to specific cell-types is not possible. Moreover, no individual gene or group of genes examined had enough differential expression by outcome to suggest potential for clinical utility to guide treatment decisions for individual patients. In addition, we did not enumerate specific cellular populations in this study to assess whether differences in NK-cell or $CD8^+$ T-cell abundance either before or after treatment correlated with the observed gene expression patterns, nor did we have the ability to examine functional correlates of the gene expression patterns on immune cells. Most markers we measured that were related to NK-cell function did not change over the course of treatment (see results above), and our prior analysis of samples from a different trial identified no change in NK-cell or $CD8^+$ T-cell frequencies 4–6 weeks after starting DAA therapy [7]. This makes it less likely, but still possible, that differences in the abundance of specific cellular populations might explain the differential transcriptional profiles observed. Finally, because only 8 of 88 subjects analyzed in these trials had IFNL4 TT/TT genotype (rs368234815), we could not examine any association of IFNL4 genotype with the reported findings.

CONCLUSIONS

In conclusion, after ultrashort HCV treatment with DAA therapy for 4–6 weeks, relapse of HCV is associated with expression of genes associated with NK-cell and $CD8^+$ T-cell function in whole blood. This suggests that immune function may be an important determinant of response to short-course DAA therapy, and that pharmacologic augmentation of immune function might enhance the efficacy of less costly, more efficient short courses of HCV therapy.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. E. G. M. has served on an Advisory Board for Theratechnologies, Inc and receives research support from Viiv Healthcare. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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