Carlsbad, CA). Based on the recent studies [17, 19–21] as well as our analysis using an oligonucleotide DNA chip, Genopal (Mitsubishi Rayon CO., LTD. Tokyo, Japan) which can detect 208 genes related to innate immune responses (data not shown), we selected the following ISGs and IFN-λs: *ISG15, A20, zc3h12a*, ring finger protein 125 (*RNF125*), myxovirus resistance protein A (*MxA*), *IL1β, IL10*, interferon regulatory transcription factor 1 (*IRF1*), *SOCS1, SOCS2, SOCS3*, 2'-5'-oligoadenylate synthetase 1 (*OAS1*), double stranded RNA-dependent protein kinase (*PKR*), *IL28A, IL28B*, and *IL29*. We then quantified their mRNA levels by real-time detection polymerase chain reaction (PCR). The primers and probes for *IL28A* and *IL28B* were designed according to the previous report [22], and those of other genes were obtained from Applied Biosystems (Carlsbad, CA) as TaqMan Gene Expression Assays (Table A in S1 File). Amplification and detection were carried out using an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Levels of mRNAs for ISGs were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control, and those for IFN-λs were measured using the calibration curves for each cDNA clone.

Statistical Analysis

Categorical variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorical variables by the Mann-Whitney U test. Correlations between continuous variables were analyzed using Pearson's correlation coefficient test. $P < 0.05$ was considered significant in all tests.

Results

Patient characteristics and distribution of *IL28B* genetic variants

The baseline clinical characteristics of the study population are described in Table 1. The unfavorable *IL28B* genotype, TG or GG (TG/GG) at rs8099917 was possessed by 38% (19/50) of the

Table 1. Baseline clinical characteristics of the 50 chronic hepatitis C patients treated with PEG-IFN, RBV and protease inhibitor.

Characteristic	(n = 50)
Male gender	30 (60%)
Age, years	55 (29–70)
Hemoglobin, g/dL	14.8 (12.0–17.1)
Platelet count, $\times 10^4$ /μL	16.2 (9.8–27.9)
ALT, IU/L	34 (13–212)
γ-GTP, IU/L	28 (12–258)
HCV RNA, log IU/ml	6.7 (4.8–7.5)
rs8099917, TT / TG / GG	31 / 16 / 3
Fibrosis stage, F0 / 1 / 2 / 3 / 4 / N.D.	5 / 20 / 6 / 3 / 1 / 15
Prior treatment	
naïve / IFN mono / IFN +RBV / PEG-IFN+RBV	14 / 2 / 2 / 32
Treatment efficacy of PEG-IFN+RBV, TVR / NVR	19 / 13

Abbreviations: ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; N.D., not determined; IFN, interferon; RBV, ribavirin; PEG-IFN, pegylated interferon; TVR, transient virological response; NVR, non-virological response.

rs8099917: TT is favorable for treatment efficacy.

Data are expressed as numbers for categorical data or the median (range) for continuous data.

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patients. Fourteen patients were treatment-naive. Of the 32 patients previously treated with PEG-IFN/RBV, 19 and 13 had TVR and NVR, respectively. Of the 13 NVR patients, 4 were null responders, defined as having an HCV RNA decrease of < 2 log IU/mL at week 12 after the start of therapy, relative to baseline. In addition, the clinical characteristics of the subsets of patients receiving telaprevir or faldaprevir are described in Table B in S1 File. The proportions of patients with an unfavorable *IL28B* genotype and NVR on prior PEG-IFN/RBV therapy were higher in patients who received faldaprevir.

All 31 patients with a favorable *IL28B* genotype and 13 of 19 with an unfavorable genotype achieved SVR on PEG-IFN/RBV/PI treatment. Hence, the total SVR rate was 88% (44/50). The detailed information of the six non-SVR cases are described as follows: one patient did not response to PEG-IFN/RBV/telaprevir up to week 12 (quantity of HCV RNA at week 12 was 3.7 log IU/mL) and the therapy was discontinued; three had virological breakthrough at week 17, 38, 40 during PEG-IFN/RBV/faldaprevir and the therapies were discontinued; two were relapsed after the completion of PEG-IFN/RBV/faldaprevir. Thus these six patients resulted in non-SVR, though they were given enough doses of drugs. In four SVR cases, the therapies were discontinued at week 9, 11, 18, 20 during PEG-IFN/RBV/telaprevir due to adverse events. Other clinical characteristics of the patients according to *IL28B* genotype and treatment efficacy are described in Table C in S1 File.

Gene expression of ISGs and IFN-λs induced by PEG-IFN/RBV/PI in patients stratified according to *IL28B* genotype

Eight hours after the initial administration of PEG-IFN/RBV/PI, levels of mRNAs for *A20, SOCS1*, and *SOCS3* known to be genes suppressing antiviral activity via the IFN signaling pathway, as well as *IRF1* were found to be significantly higher in patients with TG/GG at rs8099917, an unfavorable *IL28B* genotype ($P = 0.007$, 0.026, 0.0004, and 0.0006, respectively). In contrast, the levels of mRNAs for *IL28A, IL28B*, and *IL29* were not different regardless of the *IL28B* genotype, although the expression of *IL28B* itself tended to be higher in patients with a favorable *IL28B* type (Fig. 1). There were also no significant differences in the levels of other mRNAs for *ISG15, IL1β, RNF125* (Fig. 1), *zc3h12a, MxA, IL10, SOCS2, OAS1* or *PKR* at 8 h (data not shown). We analyzed changes in expression of the genes for *A20, SOCS1, SOCS3* and *IRF1* between baseline and 8 h and found that the fold-changes of *SOCS3* and *IRF1* were significantly higher in patients with an unfavorable *IL28B* genotype ($P = 0.005$ and 0.030, respectively) (Fig. 2).

Correlations of gene expression in PEG-IFN/RBV/PI treatment

We evaluated the correlations of the levels of mRNAs for genes implicated in suppressing the antiviral state each other and with those promoting it, (*ISG15* and *IL28B*), in all 50 cases. The expression levels of most of the mRNAs for suppressive genes such as *A20, SOCS1* and *SOCS3*, as well as *IRF1* were significantly correlated with each other at 8 h (Fig. 3) as well as at baseline (Figure A in S1 File) and 24h (Figure B in S1 File). However, they did not correlate with those of *ISG15* and *IL28B* at 8 h (Figure C in S1 File) as well as at baseline and 24h (data not shown).

Associations between ISGs including suppressive genes against the antiviral state and prediction of treatment efficacy

To examine the association between the expression of genes suppressing antiviral activity and treatment efficacy, we divided the patients into three groups according to *IL28B* genotype and treatment outcome, as follows: TT: SVR (n = 31); TG/GG: SVR (n = 13); TG/GG: non-SVR

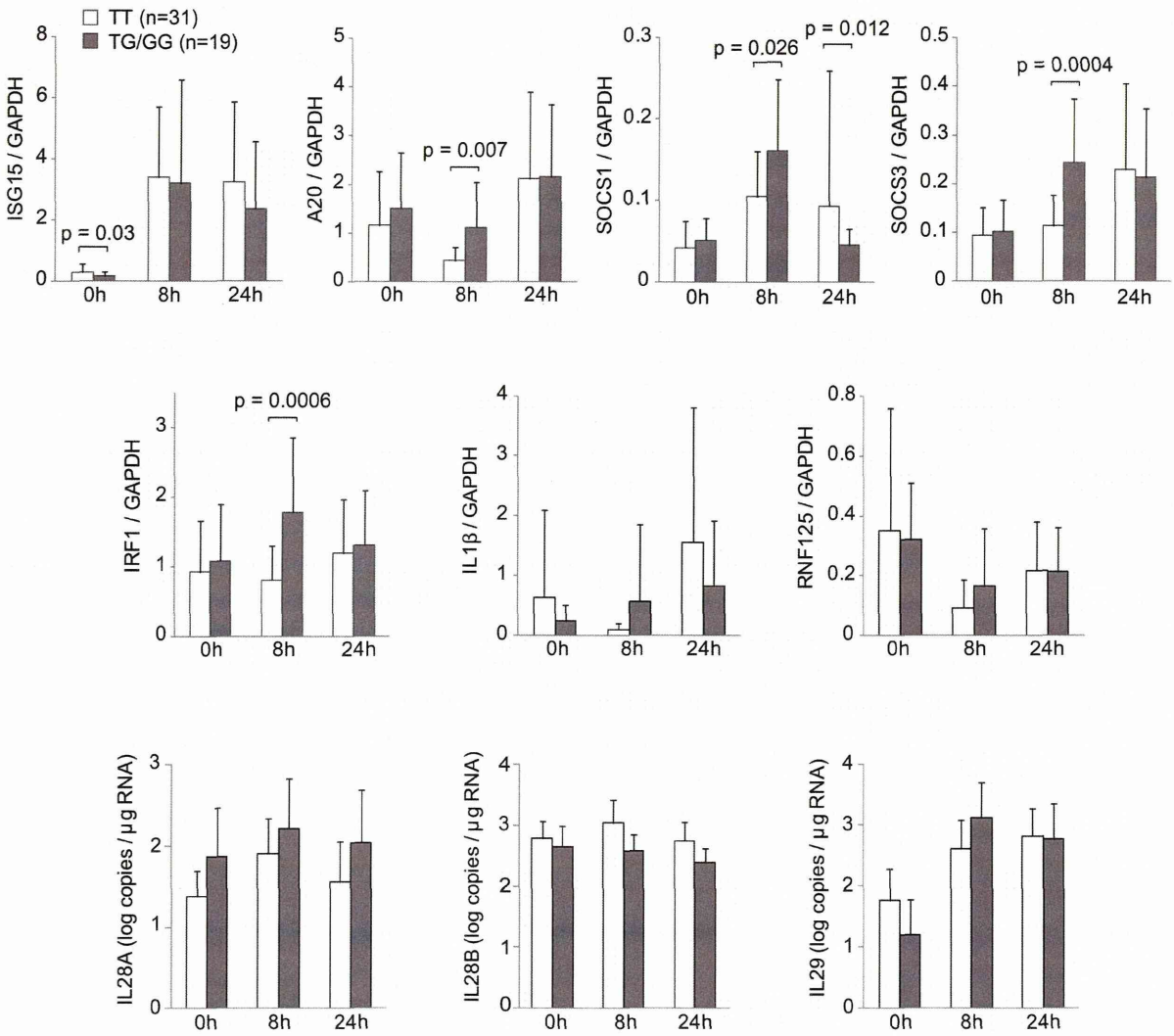

Fig 1. Expression of interferon-stimulated genes (ISGs) and interferon-lambdas (IFN-λs) in peripheral blood mononuclear cells at baseline, 8, and 24 hours after the initial administration of pegylated interferon, ribavirin, plus NS3/4A protease inhibitor, in patients stratified according to *IL28B* genotype. Levels of mRNAs for ISGs were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and those for IFN-λs were measured using the calibration curves for each cDNA clone. Bars and error bars represent means and standard deviations, respectively. TT and TG/GG at rs8099917 is a favorable and an unfavorable *IL28B* genotype for treatment responses, respectively.

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(n = 6) (Table C in S1 File). We then compared the levels of mRNAs for *A20, SOCS1, SOCS3, IRF1, ISG15*, and *IL28B* among the groups. We found that the levels of mRNAs for *A20, SOCS3* and *IRF1* at 8 h were significantly higher in TG/GG: non-SVR than in TT: SVR ($P = 0.002$, 0.001, and 0.002, respectively). Moreover, the levels of mRNAs for *SOCS3* and *IRF1* were also higher in TG/GG: SVR than in TT: SVR ($P = 0.012$ and 0.015, respectively) (Fig. 4A). Whereas the level of mRNA for *IL28B* tended to be higher in the order TT: SVR, TG/GG: SVR, TG/GG: non-SVR, there were no significant differences among the three groups. Although we also compared the expression levels of these genes at baseline and 24h among the same three groups, we could not find the definite tendency (data not shown). Next, we analyzed the changes in expression of *A20, SOCS1, SOCS3*, and *IRF1* from baseline to 8 h and found that the fold-change of *IRF1* was significantly higher in TG/GG: non-SVR than in TG/GG: SVR as well as in

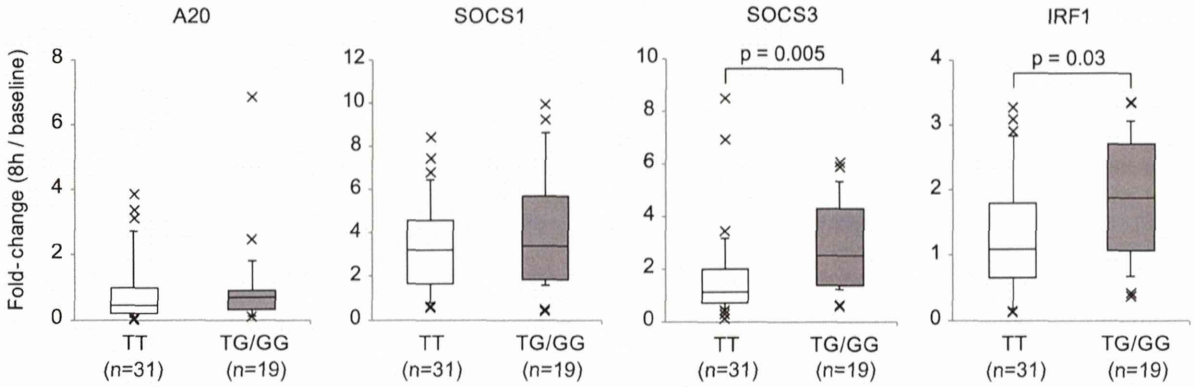

Fig 2. Fold-changes of mRNAs including suppressive genes in PBMCs at 8 hours relative to baseline in patients stratified according to *IL28B* genotype. TT and TG/GG at rs8099917 is a favorable and an unfavorable *IL28B* genotype for treatment responses, respectively. Boxes represent the interquartile range of the data. The lines across the boxes and the numbers indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

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TT: SVR ($P = 0.035$ and 0.003, respectively). Similarly, the fold-change of *SOCS3* was higher in TG/GG: non-SVR and SVR than in TT: SVR ($P = 0.021$ and 0.032, respectively) (Fig. 4B). Collectively, one can conclude that levels of expression of mRNAs including these suppressive genes early after the initial administration of PEG-IFN/RBV/PI were different in patients with different *IL28B* genotypes and different treatment efficacies.

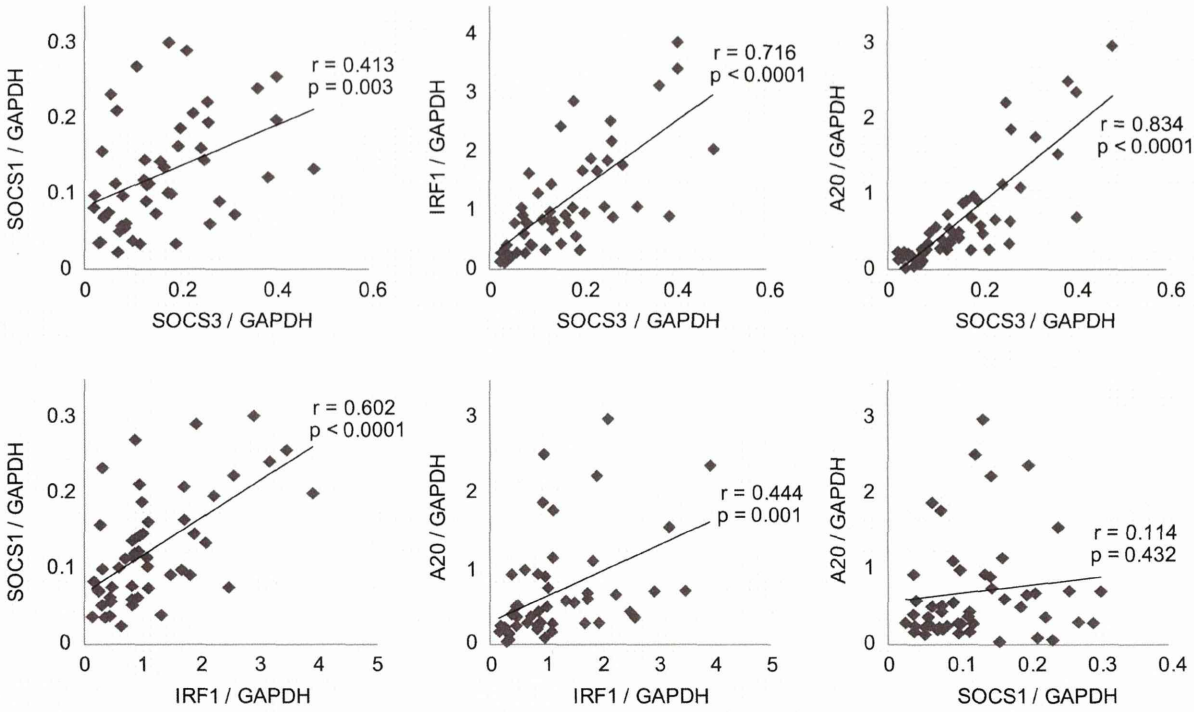

Fig 3. Relationships between levels of mRNAs including suppressive genes in PBMCs 8 hours after the initial administration of pegylated interferon, ribavirin, plus NS3/4A protease inhibitor in all patients. Levels of mRNAs including suppressive genes were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

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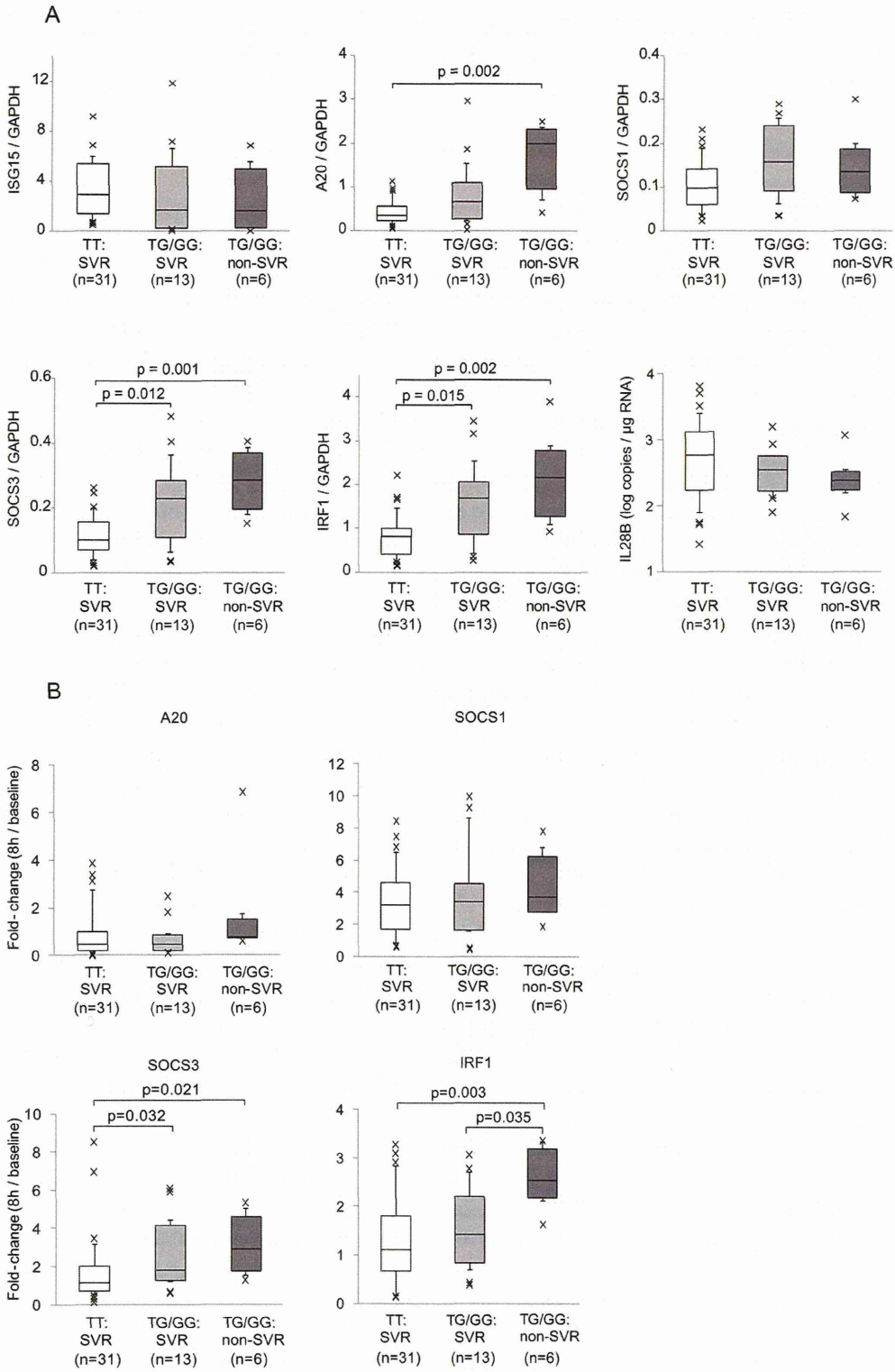

Fig 4. Associations between the expression of ISGs or IFN-λ3 and treatment efficacy. Patients were divided into three groups according to *IL28B* genotype at rs8099917 and treatment outcome: TT; SVR (n = 31), TG/GG; SVR (n = 13), and TG/GG; non-SVR (n = 6). (A) Expression of *ISG15, IL28B* and suppressive genes in PBMCs at 8 hours after the initial administration of pegylated interferon, ribavirin, plus NS3/4A protease inhibitor in each group. (B) Fold-changes of mRNAs including suppressive genes at 8 hours relative to baseline in each group. Levels of mRNAs including suppressive genes and

ISG15 were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and those for *IL28B* were measured using the calibration curves of cDNA clone. TT and TG/GG at rs8099917 is a favorable and an unfavorable *IL28B* genotype for treatment responses, respectively. Boxes represent the interquartile range of the data. The lines across the boxes and the numbers indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

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Discussion

In the present study, we determined that mRNAs for *A20, SOCS1* and *SOCS3*, known to be genes suppressing antiviral activity via the IFN signaling pathway, as well as *IRF1* were highly expressed in PBMCs early after the initial administration of PEG-IFN/RBV/PI in patients with an unfavorable *IL28B* genotype, especially the non-SVR group. The correlations of mRNA expression levels of these genes, *ISG15*, and *IL28B* suggest that the expression levels of these suppressive genes show similar dynamics independently with the genes promoting the antiviral state in the interferon signaling pathway. Asahina *et al.* showed that the induction of several ISGs in PBMCs after the initial administration of PEG-IFN/RBV tended to be stronger in SVR than in NVR, but in their study the difference was not statistically significant [20]. The HCV NS3/4A protease cleaves and inactivates two important signaling molecules in the innate immune system, the mitochondrial antiviral signaling protein (MAVS), an essential component of the RIG-I pathway [23], and the Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), an adaptor in the TLR3 pathway [24]. Because PI inhibits the function of NS3/4A protease, it is expected to affect these pathways and the expression of ISGs. Indeed, Kalkeri *et al.* showed that PIs including telaprevir, boceprevir, and simeprevir can restore innate immunity by directly inhibiting NS3/4A protease-mediated cleavage of MAVS at clinically achievement concentrations *in vitro* using HCV replicon cells [25]. Therefore, in PEG-IFN/RBV/PI therapy, the expression of ISGs, IFN-λs, and molecules related to the innate immune system may be more markedly altered early after the start of this therapy than PEG-IFN/RBV therapy without PI. This may be the reason why we were able to determine the differences of expression of these suppressive gene mRNAs. We preliminarily compared the mRNA levels of the suppressive genes, *ISG15, MAVS* and *TRIF* in PBMCs between in patients of this study (data of two patients were unavailable) and in those with PEG-IFN/RBV therapy, whose characteristics are described in Table D in S1 File. There were no differences for these genes at 8h/baseline, however, the inductions of mRNA for several genes such as *A20, IRF1, SOCS3*, and *MAVS* at 24h/baseline were greater in PEG-IFN/RBV/PI (Figure D in S1 File). In general, previous studies have shown that HCV mainly could replicate in liver and lympho-trophic HCV would be minor, therefore it is not main event that HCV NS3/4A cleaves MAVS or TRIF in PBMCs. We speculate that inhibiting cleavages of MAVS and TRIF by PI in liver more strongly induces IRF3 activation and subsequent IFN-α/β and ISGs production, resulting in the activation of RIG-I, TLR3, and IFN signaling pathway in livers and PBMCs. For these reasons, we guess that the several genes related with these pathways were more strongly induced at 24 h in patients treated with PEG-IFN/RBV/PI. Further studies will be required to evaluate the effect of PI itself on the IFN signaling pathway in PBMCs or liver. In the present study, levels of mRNAs for IFN-λs as well as common ISGs promoting the antiviral state at baseline and during therapy were not found to be significantly associated with the *IL28B* genotype or treatment efficacy. Recently, Honda *et al.* showed that there was no difference of pretreatment mRNA expression of ISGs as well as *IL28A/B* in blood between *IL28B* genotypes or responses to PEG-IFN/RBV [26]. These results support our data at baseline. Interestingly, they also indicated that the expression of ISGs at baseline correlated significantly between liver and blood in patients with a favorable *IL28B* genotype, not in those with an unfavorable genotype [26].

As previously reported, *SOCS1* suppresses the Jak/STAT pathway, specifically STAT1 [27]. *SOCS3* inhibits expression of ISGs such as *OAS1* and *PKR* through inactivation of the Jak-STAT pathway [28]. *A20* is a suppressive factor of the nuclear factor-kappa B pathway [29] and a candidate negative regulator of the signaling cascade leading to *IRF3* activation in the innate antiviral response [30]. *IRF1* is well known as a transcription factor that activates the expression of *IFN-β*, leading to enhancement of IFN signaling [31, 32]. However, Moore *et al.* showed that *IRF1* enhances the expression of *SOCS1* using rat pancreatic β-cells, and suggested that *IRF1* provides a negative feedback on STAT1 and downstream signaling via STAT1 dephosphorylation by SOCS1 up-regulation [33]. Furthermore, in our preliminary *silico* analysis, *IRF1* is expected to bind the promoter region of *A20* (data not shown), and thus may influence the functional expression of *A20* through transactivation of *A20* promoter, resulting in negative regulation of IFN signaling cascade. Collectively, these suppressive factors may negatively affect the IFN signaling pathway and the production of ISGs or IFN in HCV infection. Abe *et al.* showed that pretreatment intrahepatic levels of two ISGs suppressing the antiviral state, *A20* and *Zc3h12a*, were significantly higher in patients with a favorable *IL28B* genotype, and that a high level of *SOCS1* was a predictive factor for NVR. In contrast, they found that levels of most of the ISGs promoting the antiviral state via the IFN signaling pathway and *IL28* were significantly lower in patients with a favorable *IL28B* genotype [34]. Thus, the expression of these suppressive genes in the liver might influence treatment efficacy. Taking this previous report together with our results using PBMCs presented here, we may conclude that the levels of mRNAs for suppressive genes in liver and PBMCs are associated with *IL28B* polymorphisms.

The mechanism of interaction between IFN-λ and ISG expression in liver or PBMC resulting in the elimination of HCV has not yet been elucidated. Using primary hepatocytes from humans and chimpanzees, Thomas *et al.* found that type III but not type I IFNs are primarily induced after HCV infection, and that their degree of induction is closely correlated with the levels of ISGs [35]. These results strongly suggest that hepatic IFN-λ production may have important roles and could be a principal driver of ISG induction in response to HCV infection. On the other hand, in a chronically HCV-infected chimeric mouse model, larger amounts of IFN-λs were produced by HCV-infected human hepatocytes with a favorable *IL28B* genotype on treatment with IFN-α [36]. Recently, it has been shown in *ex vivo* experiments that a certain subset of dendritic cells (DCs) within human PBMCs recognized HCV and produced large amounts of IFN-λs [37, 38], and that the capacity for producing IFN-λ3 was superior in subjects with a favorable *IL28B* genotype [38]. Furthermore, IFN-α directly affected DC function and significantly increased IFN-λ production [37]. These findings suggest that in addition to HCV-infected hepatocytes, DCs within PBMCs may play a crucial role in the response to IFN treatment via production of IFN-λs and ISGs. We speculate that the levels of several suppressive ISGs in liver and DCs might be different according to the *IL28B* genotype, implying a difference of response to treatment. In addition, it has not been fully elucidated how IFN-λs or ISGs influence effector cells such as natural killer (NK) cells or cytotoxic T lymphocytes in HCV infection. Although we also investigated several cytokines such as IL-2, 4, 5, 6, 10, 12, IFN-γ, and tumor necrosis factor (TNF)-α in patients′ serum during PEG-IFN/RBV/PI, we did not find any differences attributable to *IL28B* genotype or any associating with treatment efficacy (data not shown). Intriguingly, recent study showed that infiltration of various immune cells including DCs, NK cells, and T cells, and expression of various chemokines in liver were repressed in patients with an unfavorable *IL28B* genotype, and their up-regulation of intrahepatic ISGs was mediated by multiple factors, including *IL28A/B*, IFN-λ4, and wingless-related MMTV integration site 5A [26]. Further studies will be required to identify the role of ISGs suppressing the antiviral state in hepatocytes or DCs, and how IFN and ISGs effect the elimination of HCV.

This study has several limitations. The treatment regimens were different for different patients, including the type of PI, its dose, and duration of therapy, especially in the patients receiving faldaprevir, even though faldaprevir dose and treatment duration reportedly had little influence on SVR rates in some clinical trials [39]. Furthermore, there was bias in that the proportion of intractable cases was higher in the patients receiving faldaprevir. Second, the number of analyzed cases was small, especially the non-SVR cases. Third, we analyzed the expression of the selected genes in PBMCs at baseline and the only early periods after the initial administration of PEG-IFN/RBV/PI. Further comprehensive gene expression analysis including more prolonged kinetics of genes are necessary in a large number of patients treated with the same regimen to verify the results of the present study.

The findings in this study contribute to our understanding of immune response to HCV during PEG-IFN/RBV/PI therapy. IFN-free therapy is expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. Future study should evaluate immune responses under IFN-free therapy as well as IFN-based therapy to clarify the mechanism of HCV elimination.

In conclusion, the expression of several genes, which suppress antiviral activity by interfering IFN signaling pathway, in PBMCs during PEG-IFN/RBV/PI was found to be different according to the patient's *IL28B* genotype and treatment response.

Supporting Information

S1 File. Table A, Primers and probes for quantitative real-time PCR of ISGs and IFN-λs. Table B, Clinical characteristics of chronic hepatitis C patients treated with PEG-IFN/RBV plus telaprevir or faldaprevir. **Table C,** Clinical characteristics of chronic hepatitis C patients according to *IL28B* genotype and treatment efficacy. **Table D,** Clinical characteristics of chronic hepatitis C patients treated with PEG-IFN/RBV. **Figure A,** Correlations between levels of mRNAs including suppressive genes at baseline. **Figure B,** Correlations between levels of mRNAs including suppressive genes at 24 hours after the initial administration PEG-IFN, RBV, plus NS3/4A protease inhibitor. **Figure C,** Correlation between levels of mRNA including suppressive genes and those for *IL28B* or *ISG15* at 8 hours after the initial administration PEG-IFN, RBV, plus NS3/4A protease inhibitor. **Figure D,** Fold-changes of mRNAs for ISGs, *TRIF* and *MAVS* in PBMCs at 8, 24 hours relative to baseline in PEG-IFN/RBV and PEG-IFN/RBV/PI therapy.
(PDF)

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Author Contributions

Conceived and designed the experiments: SI KM YT. Performed the experiments: SI KO TF KI. Analyzed the data: SI KM. Contributed reagents/materials/analysis tools: KM EI TM KF NS AK ME SN TJ YT. Wrote the paper: SI KM TW YT.

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