Hepatic interferon-stimulated genes are differentially regulated in the liver of chronic hepatitis C patients with different interleukin-28B genotypes

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Supplemental material and methods

RNA preparation and GeneChip analysis

The liver biopsy samples were divided into 3 parts: the first part was immersed in formalin for histological assessment, the second part was immersed immediately in RNAlater (QIAGEN, Valencia, CA, USA) for RNA isolation, and the final part was frozen in OCT compound for LCM analysis and stored at -80 °C until use as described previously.(1, 2)

Liver tissue RNA was isolated using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Isolated RNA was stored at -70 °C until use. The quality of the isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer (Agilent, Santa Clara, CA, USA). Aliquots of total RNA (50 ng) isolated from the liver biopsy specimens were subjected to amplification using the WT-Ovation^{TM} Pico RNA Amplification System (NuGen, San Carlos, CA, USA) as recommended by the manufacturer. Briefly, double-stranded cDNA was synthesized from template RNA by using a unique SPIATM DNA/RNA chimeric primer. The resultant cDNA with a unique DNA/RNA heteroduplex at one end was subjected to linear isothermal DNA amplification in the presence of RNase H and DNA polymerase. RNase H degraded RNA in the DNA/RNA heteroduplex, generating a single-stranded DNA site at the 3'-end for the attachment of a new chimeric primer and synthesis of single-stranded cDNA. This amplification cycle generates approximately 10 μg cDNA from 50 ng total RNA. For the hybridization, 5 μg of the resultant cDNA were used for fragmentation and biotin labeling using an FL-Ovation^{TM} cDNA Biotin Module V2 (NuGen) as recommended by the manufacturer. The biotin-labeled cDNA was suspended in 220 μL hybridization cocktail (NuGen), and 200 μL were used for the hybridization.(2, 3)

Blood samples collected in PAXgene Blood RNA tubes (BD, Franklin Lakes, NJ, USA) were incubated and stored according to the manufacturer's instructions. Total RNA was isolated after thawing the samples at room temperature using a PAXgene Blood RNA System Kit (QIAGEN) following the manufacturer's instructions. We amplified 5 μg total RNA using a GeneChip® One-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA, USA). The resultant cDNA was used as a template for an *in vitro* transcription (IVT) reaction for complementary RNA (cRNA) amplification and biotin labeling using GeneChip® IVT Labeling Kit (Affymetrix). Following fragmentation, 10 μg of cRNA were used for hybridization. An Affymetrix Human 133 Plus 2.0 Microarray Chip (Affymetrix) containing 54,675 gene transcripts was used for the analysis. Hybridization (45 °C for 16 h at 60 rpm), washing, and staining (using a Fluidics Station) were performed according to the standard protocol (Affymetrix). The probe array was scanned using a GeneChip® Scanner 3000 (Affymetrix). Hybridized data files (CEL) were obtained with the GeneChip® Operating Software 1.4 (GCOS) (Affymetrix).

Cells in liver lobules (CLL) and cells in portal areas (CPA) were isolated by laser capture microdissection (LCM) using a PALM MicroBeam (Carl Zeiss, Oberkochen, Germany) from the liver biopsy specimens frozen in OCT compound. For LCM, the frozen liver biopsy specimens in OCT compound were sliced into 8-μm thick sections, immediately fixed in methanol for 5 min, and kept on dry ice. Tissue samples were quickly stained with toluidine blue and dissected. Approximately 1000 lymphoid cells and hepatocytes were excised on 6 slides and immersed in a denaturing solution. Dissection was completed within 5 min for each slide. Total RNA in LCM was isolated with a carrier nucleic acid (20 ng polyC) using RNAqueous^{TM}-Micro Kit (Ambion, Austin, TX, USA). The quality of the isolated RNA was estimated after electrophoresis using an

Agilent 2001 Bioanalyzer. Usually, from 1000 cells, 40–80 ng total RNA were isolated. Half of the total RNA isolated from the LCM specimens was amplified twice using a TargetAmp™ 2-Round Aminoallyl-aRNA Amplification Kit 1.0 (EPICENTRE, Madison, WI, USA). The amplification protocol was based on the standard antisense RNA amplification procedure using T7 RNA polymerase and an Eberwine oligo-dT/T7 primer. Two rounds of amplification yielded 30–60 μg aRNA, and 20 μg aRNA were used for further biotin labeling using Biotin-X-X-NHS (provided by EPICENTRE) according to the manufacturer's protocol. The biotin-labeled aRNA was suspended in 300 μL hybridization cocktail (Affymetrix), and 200 μL were used for hybridization with a Affymetrix Human 133 Plus 2.0 Microarray Chip. Hybridization, washing, staining, and scanning procedures were performed as described earlier. Hybridized data files (CEL) were obtained with GCOS (Affymetrix).

Processing the GeneChip data

The intensity of each GeneChip was normalized by one patient using GCOS and further processed by BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Absent detection calls were excluded and the filtered data were log-transferred and normalized using median over the array (BRB-ArrayTools). A class comparison was performed to find the differentially expressed genes among the different groups of patients. Hierarchical clustering was performed using average linkage with centered correlation.

We performed pathway analysis by using MetaCore™ (Thomson Reuters, New York, NY, USA). Differentially expressed genes were applied to MetaCore™ to find which categories of genes were included. In MetaCore™, functional ontology

enrichment was performed to compare the Gene Ontology process distribution of the differentially expressed genes ($p < 0.05$).

Quantitative real-time detection PCR

We performed quantitative real-time detection PCR (RTD-PCR) using TaqMan Universal Master Mix (PE Applied Biosystems, Foster City, CA, USA) as described previously.(3) Primer pairs and probes for myxovirus (influenza virus) resistance 1 (Mx1), 2'-5'-oligoadenylate Synthetase 2 (OAS2), interferon-induced protein 44 (IFI44), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), wingless-related MMTV integration site 5A (WNT5A), GTPase activating protein (SH3 domain) binding protein 1 (G3BP1), IFN-α, IFN-β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the TaqMan assay reagents library. The primer pairs and probes for IL28B and IFN-λ4 were designed as described previously.(3, 4) For the primer and probe sequences for IL28B that are shared with IL28A, we use IL28A/B henceforth. A standard curve was obtained in each assay using the RNA obtained from a normal liver. The expression values were normalized by GAPDH and normalized values indicate the relative fold expression to a normal liver. For the measurement of IFN-λ4, DNase I-treated RNA was used and represented as relative fold of GAPDH using the delta Ct method.

Immunohistochemical (IHC) staining

Immunohistochemical (IHC) staining was performed by immunoperoxidase technique with an Envision kit (DAKO Japan). Primary antibodies used were: rabbit polyclonal IFI44 (1:100 dilution) (Sigma-Aldrich, St. Louis, MO, USA), rabbit polyclonal FZD5 (1:100 dilution) (abcam, Cambridge, MA, USA), mouse monoclonal CD163 (1:100

dilution) (Leica Biosystems, Minato-ku, Tokyo, Japan) and mouse monoclonal CD8 (1:100 dilution). CD163 and CD8 positive cells were observed in lower power field (LPF) (x100) and cell numbers were counted in ten different filed in each samples.

Cell lines

THLE-5b,(5) TTNT,(6) HEK293T, and Huh-7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Huh-7 cells continuously infected with JFH-1 (Huh7-JFH1) were described previously.(7) The cells were maintained in normal medium by passaging every 3–4 days for approximately 6 months. Approximately 20–30% of the cells were consistently positive for HCV core protein.

Recombinant lentivirus preparation

VSV-G-pseudotyped lentiviral vectors were provided by Prof. Arthur Nienhuis (St. Jude Children's Research Hospital, Memphis, TN, USA).(8) The WNT5A expression vector, pCMV6-WNT5A, was purchased from ORIGENE (Rockville, MD, USA) (Cat. No. RC209206). The EGFP (control) and WNT5A genes were cloned separately into the pCL20c lentivirus vector to make pCL20c-EGFP and pCL20c-WNT5A, respectively. Recombinant lentiviruses were produced by co-transfecting HEK293T cells with a mixture of 4 plasmids: pCAGkGP1R, pCAG4RTR2, pCAG-VSV-G, and pCL20c-EGFP or pCL20c-WNT5A using the FuGENE®6 Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA). The medium, containing the viral particles, was harvested at 72 h after transfection. The medium samples were filtered through 0.45-μm membranes, centrifuged at 25,000 rpm

for 90 min, resuspended in phosphate-buffered saline (pH 7.4), and stored as frozen aliquots at -80 °C until use. Huh-7 cells were infected with viral particles at a multiplicity of infection (MOI) of 1. At 24 h after infection, the medium was replaced with fresh medium, and culturing was continued for another 7 days.

HCV replication analysis

pH77S3 is an improved version of pH77S, a plasmid containing the full-length sequence of the genotype 1a H77 strain of HCV with 5 cell culture-adaptive mutations that promote its replication in Huh-7 hepatoma cells.(9, 10) pH77S.3/*Gaussia* luciferase (Gluc) 2A is a related construct in which the Gluc sequence, fused to the 2A autocatalytic protease of foot-and-mouth virus RNA, was inserted in-frame between p7 and NS2.(7, 11) A 10 μg aliquot of synthetic RNA transcribed from pH77S.3/Gluc2A was used for electroporation. Control lentivirus-transduced Huh-7 cells (Huh7-control) and WNT5A-transduced cells (Huh7-WNT5A) were subjected to an electroporation pulse at 260 V and 950 μF using Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and plated in fresh normal medium. After 72 h, the cells and culture medium were collected and used for Gluc assays and quantitative RTD-PCR.

IFN treatment

Huh7-control cells and Huh7-WNT5A cells were infected with HCV at an MOI of 1. At 72 h after infection, the cells were treated with the indicated units of IFN-α for 24 h.

siRNA and plasmid transfection

An siRNA specific to WNT5A and a control siRNA were obtained from Thermo

Fisher Scientific (Waltham, MA, USA). Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Huh7-JFH1 cells were cultured for 24 h in 12-well plates, and were then transfected with pCMV6-WNT5A in a dose-dependent manner using the FuGENE®6 Transfection Reagent (Roche Applied Science). After 48 h, quantitative RTD-PCR was carried out as described earlier.

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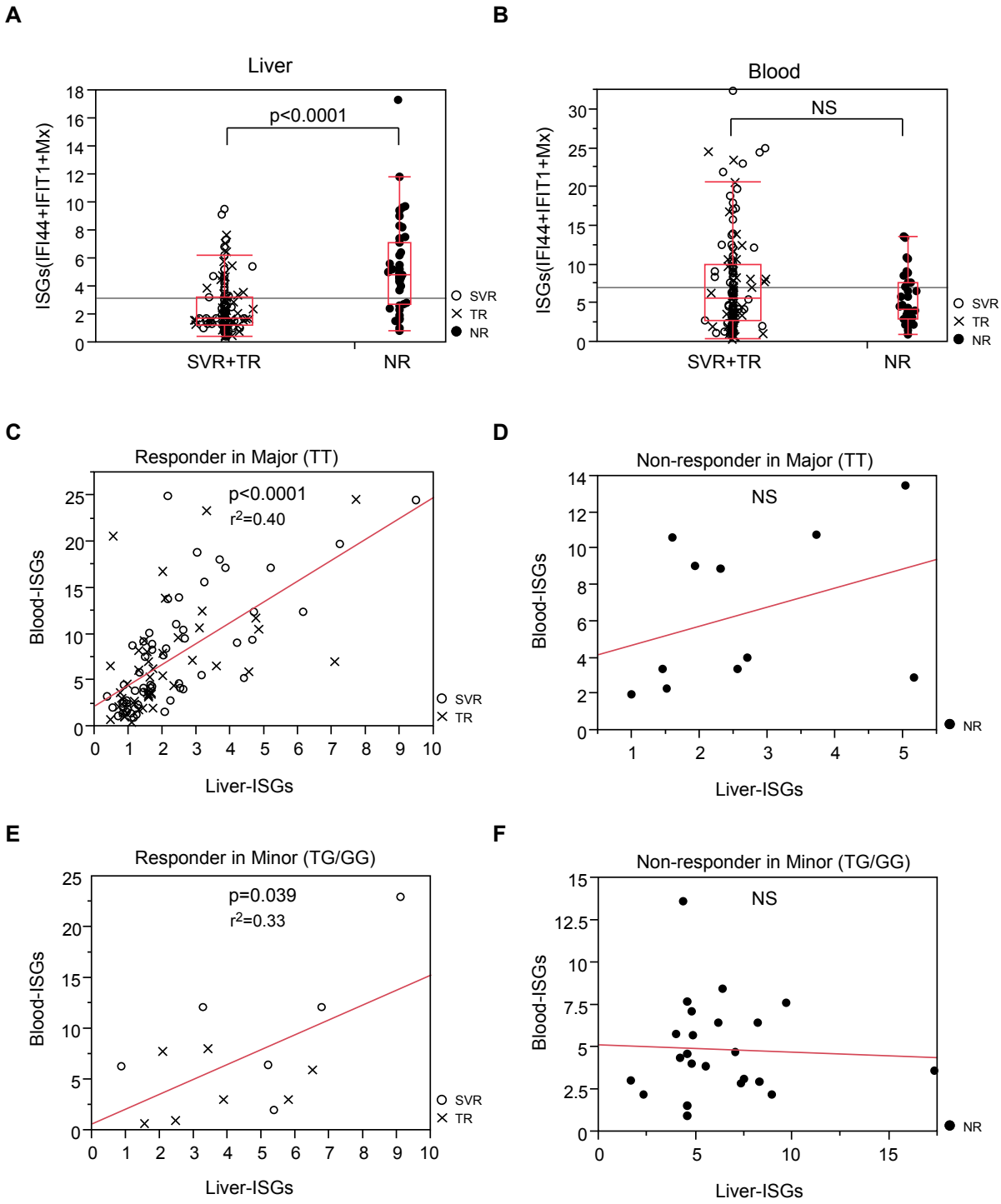
Supple Fig.1



Supplemental Figure 1. Comparison of ISG expression in the liver and blood of patients with different IL28B genotypes

A: RTD-PCR results of mean ISG expression (IFI44+IFIT1+Mx1) in the liver of responders (SVR+TR) and non-responders (NR) (n = 168).

B: RTD-PCR results of mean ISG expression (IFI44+IFIT1+Mx1) in the blood of responders (SVR+TR) and non-responders (NR) (n = 146).

C: Correlation of mean ISG expression (IFI44+IFIT1+Mx1) in the liver and blood of responders (SVR+TR) with the IL28B major genotype.

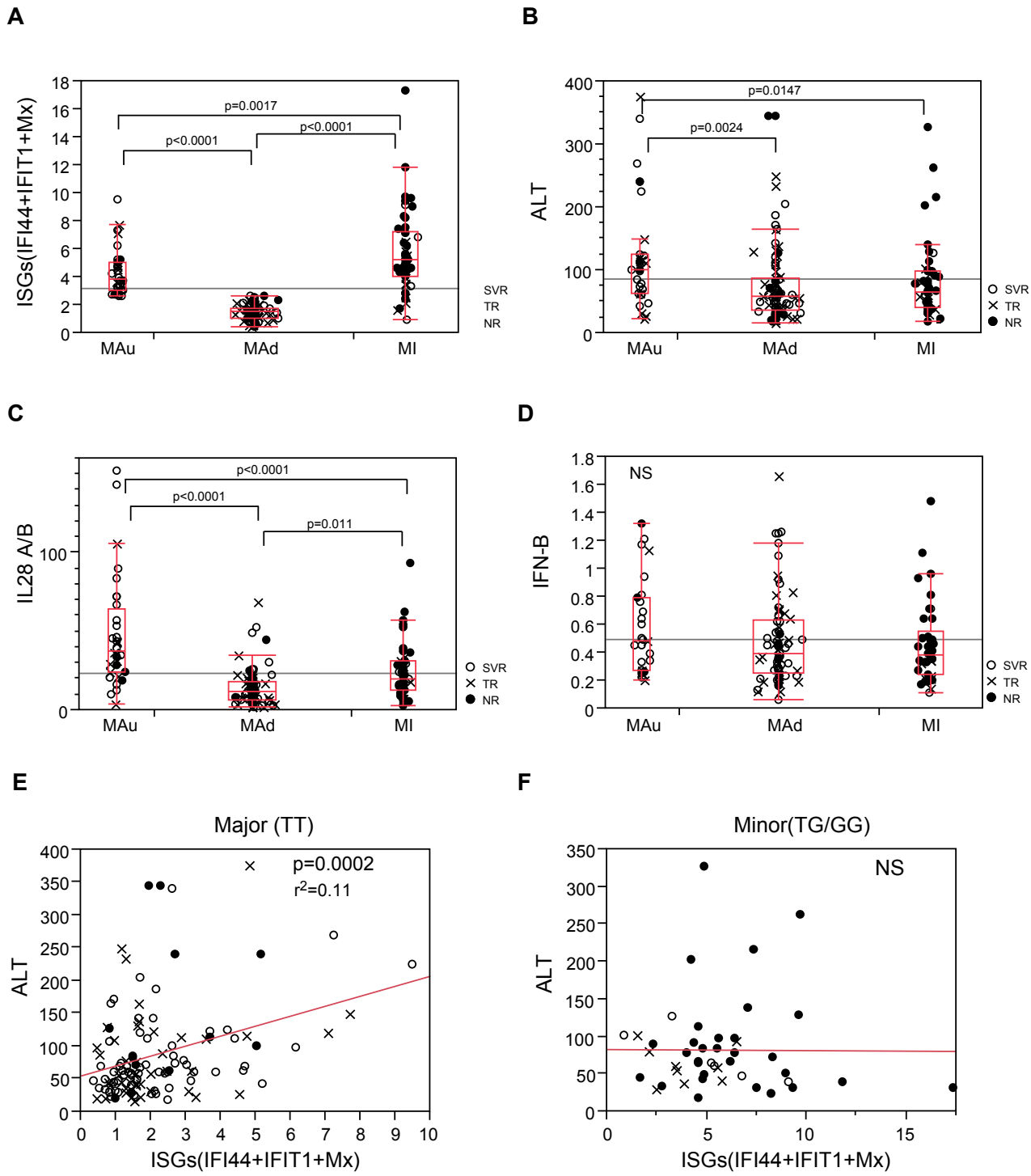
D: Correlation of mean ISG expression (IFI44+IFIT1+Mx1) in the liver and blood of non-responders (NR) with the IL28B major genotype.

E: Correlation of mean ISG expression (IFI44+IFIT1+Mx1) in the liver and blood of responders (SVR+TR) with the IL28B minor genotype.

D: Correlation of mean ISG expression (IFI44+IFIT1+Mx1) in the liver and blood of non-responders (NR) with the IL28B minor genotype.

IL28B major genotype: TT at rs8099917; IL28B minor genotype: TG/GG at rs8099917.

Supple Fig.2



Supplemental Figure 2. Comparison of hepatic ISG, IL28A/B, IFN-β, and ALT levels in MAu, MAd, and MI patients

A: Mean ISG expression (IFI44+IFIT1+Mx1) in the liver of MAu, MAd, and MI patients (n = 168).

B: Serum ALT levels in MAu, MAd, and MI patients (n = 168).

C: IL28A/B expression in the liver of MAu, MAd, and MI patients (n = 168).

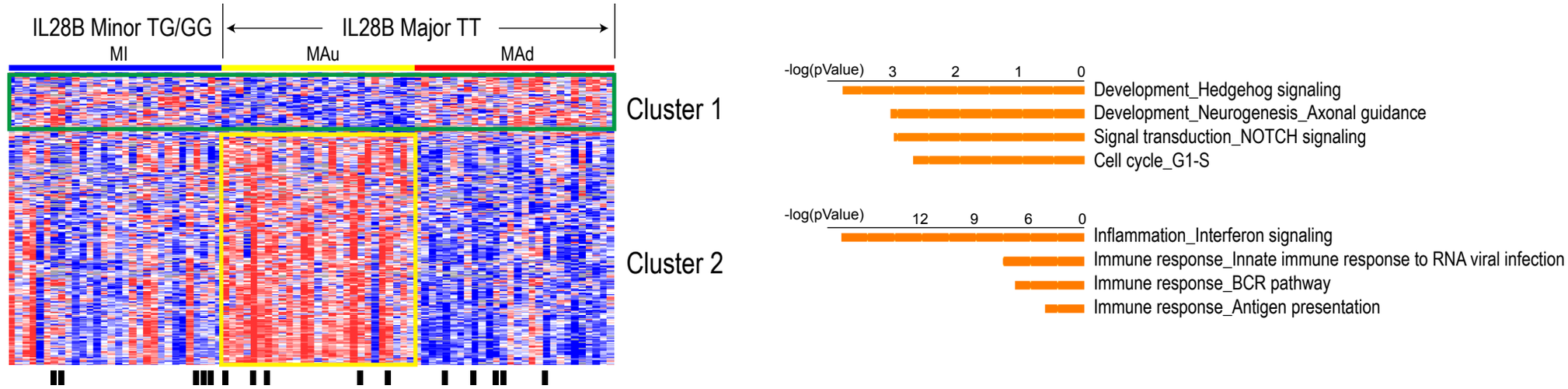
D: IFN-β expression in the liver of MAu, MAd, and MI patients (n = 140).

E, F: Correlation of serum ALT levels and hepatic ISG expression in IL28B major (E) and IL28B minor (F) genotype patients.

MAu: patients with the IL28B major genotype and up-regulated ISGs; MAd: patients with the IL28B minor genotype and down-regulated ISGs; MI: patients with the IL28B minor genotype.

Supple Fig.3

Blood



Supplemental Figure 3. One-way hierarchical clustering of 334 differentially expressed genes (p < 0.005) in the blood of MAu, MAd, and MI patients

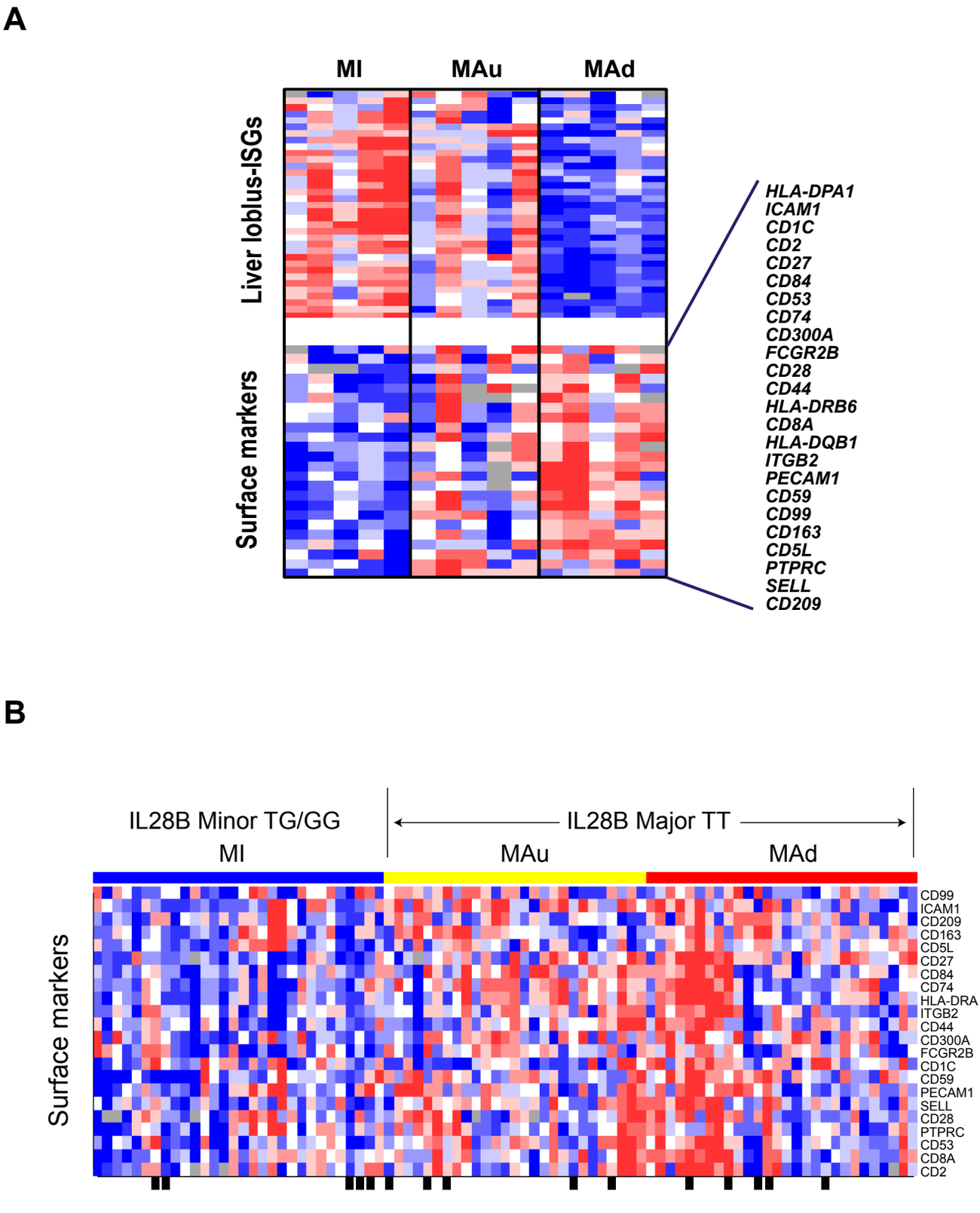
Two major clusters are shown on the left, one (cluster 1) is up-regulated in the MAd patients, while the other (cluster 2) is up-regulated in the MAu patients. Gene categories of each cluster deduced using MetaCore™ are shown on the right. Cluster 1 includes development process of immune cells and cluster 2 includes active immune signaling, including IFN signaling.

The small black bars under the heat map indicate the patients who were analyzed using LCM.

MAu: patients with the IL28B major genotype and up-regulated ISGs; MAd: patients with the IL28B minor genotype and down-regulated ISGs;

MI: patients with the IL28B minor genotype.

Supple Fig.4



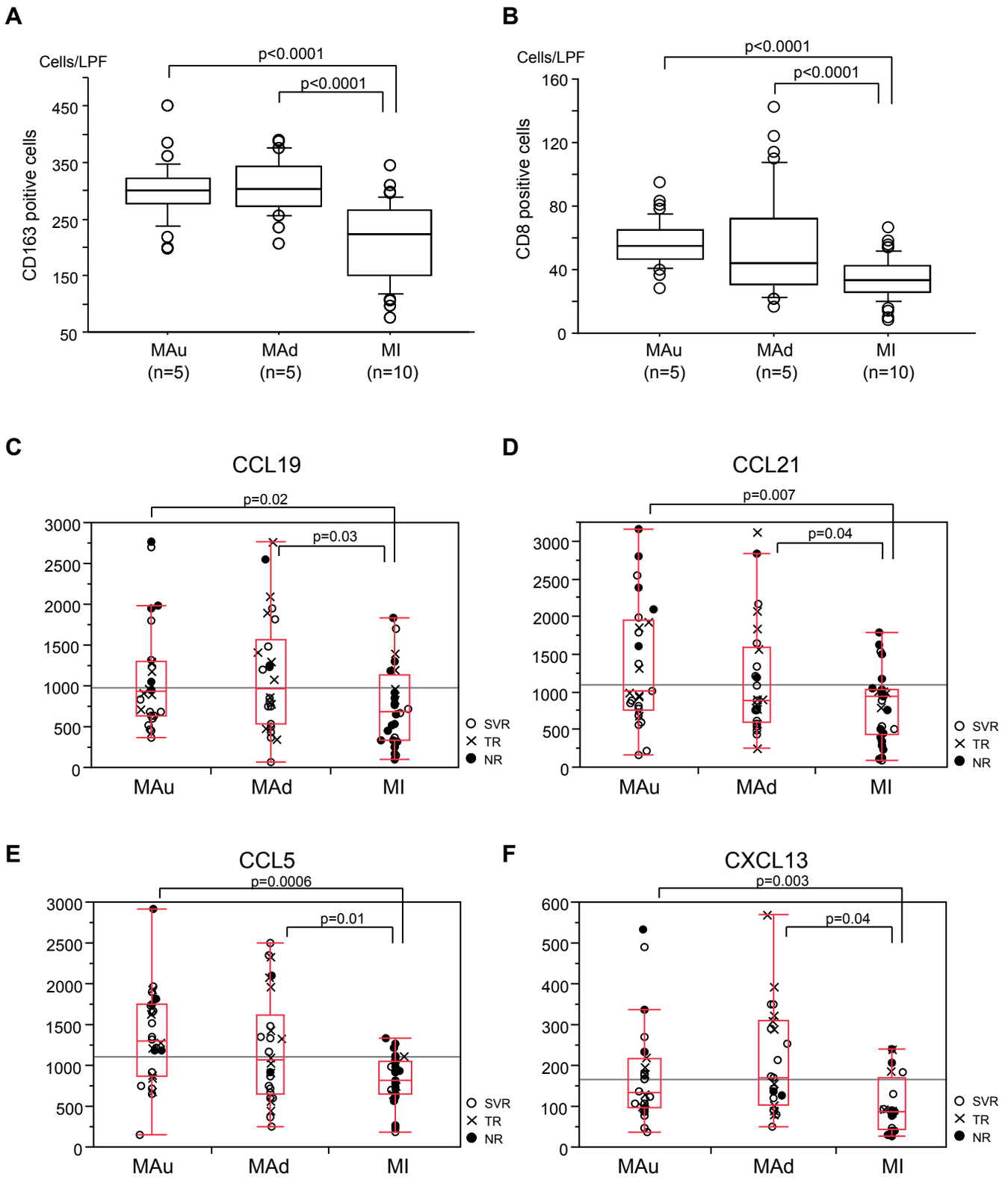
Supplemental Figure 4. Expression of immune cell surface markers in CLL (A) and whole liver (B)

A: The expression of ISGs in CLL is shown in the upper panel and the corresponding expression of immune cell surface marker in CLL is shown in the lower panel.

B: The expression of immune cell surface markers in the whole liver of 85 patients.

MAu: patients with the IL28B major genotype and up-regulated ISGs; MAd: patients with the IL28B minor genotype and down-regulated ISGs; MI: patients with the IL28B minor genotype.

Supple Fig.5



Supplemental Figure 5.

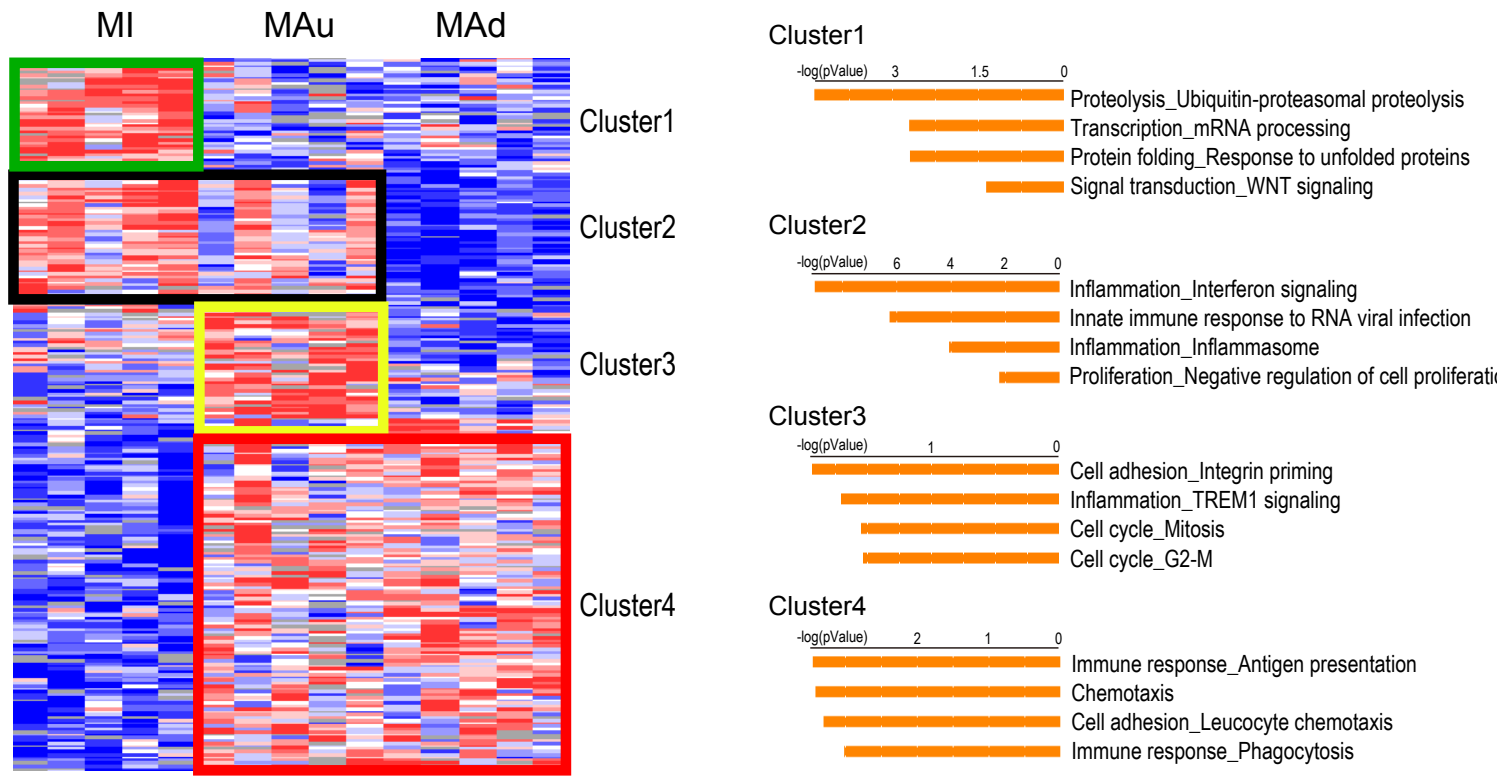
Semi-quantitative cell count of lymphocytes and chemokine expression in CH-C patients with different IL28B genotypes.

A, B: Semi-quantitative cell counts of CD168 positive (A) and CD8 (B) positive cells infiltrated in liver lobules.

Cell number was counted in ten different filed of low power magnification (LPF) (X100) in each samples (MAu=5, MAd=5, MI=10). Average numbers of cells in one LPF was shown.

C, D, E, F: Probe intensities of CCL19 (C), CCL21 (D), CCL5 (E), and CXCL13 (F) derived from Affymetrix GeneChip data using liver samples from 91 patients with CH-C (Supplemental Table 3).

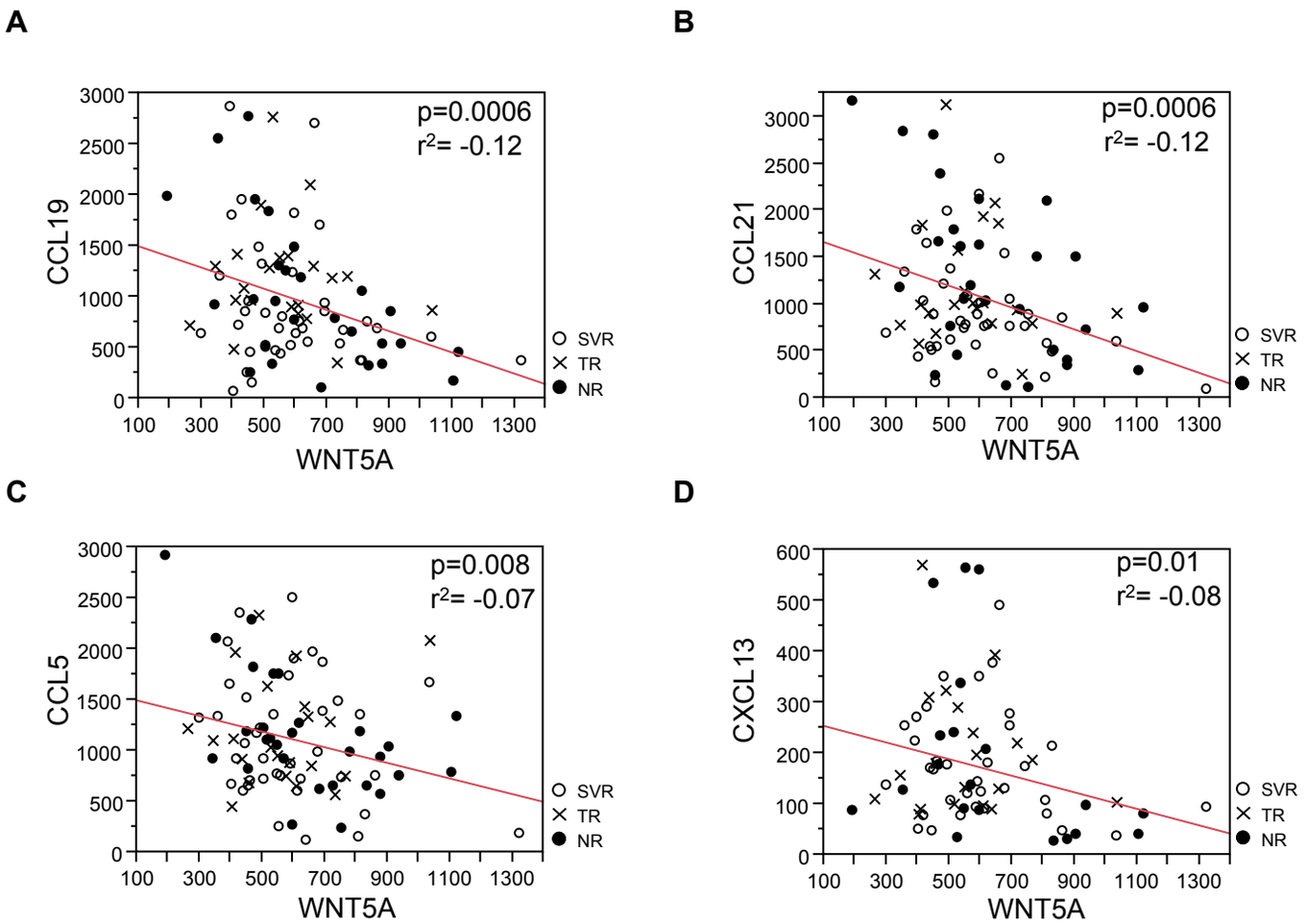
Supple Fig.6



Supplemental Figure 6. One-way hierarchical clustering of 197 differentially expressed genes (p < 0.01) in CLL among the MAu, MAd, and MI patients

Four major clusters are shown on the left, one (cluster 1) is up-regulated in the MI patients, the second (cluster 2) is up-regulated in the MI and MAu patients, the third (cluster 3) is up-regulated in the MAu patients, and the fourth (cluster 4) is up-regulated in the MAu and MAd patients. Cluster 1 includes protein folding and WNT signaling, cluster 2 includes interferon signaling, cluster 3 includes inflammation and cell damage, and cluster 4 includes chemokine signaling.
MAu: patients with the IL28B major genotype and up-regulated ISGs; MAd: patients with the IL28B minor genotype and down-regulated ISGs;
MI: patients with the IL28B minor genotype.

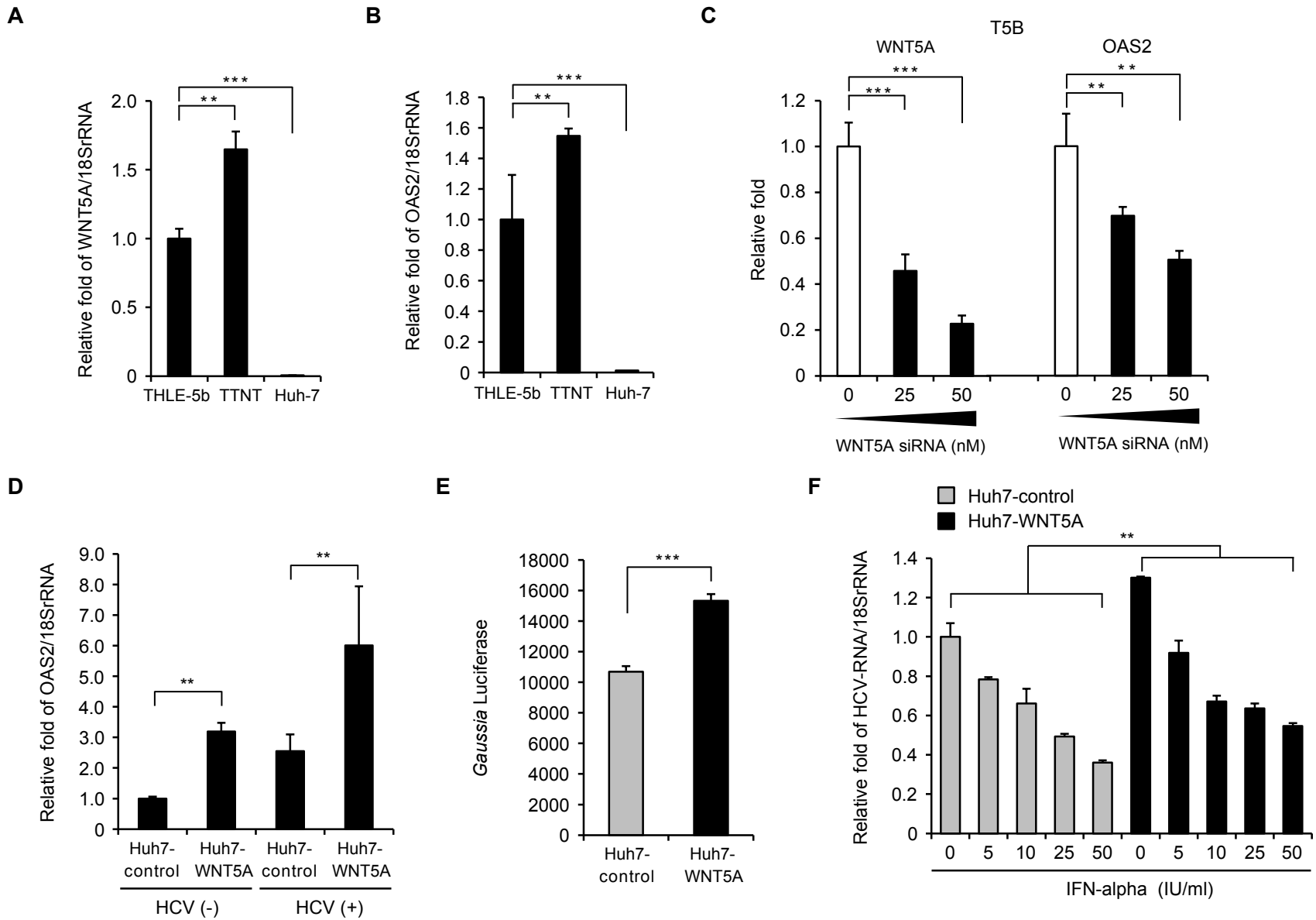
Supple Fig.7



Supplemental Figure 7. Correlation analysis of chemokine and WNT5A expression in the liver of CH-C patients

The correlation of probe intensities of chemokines (CCL19 (A), CCL21 (B), CCL5 (C) and CXCL13 (D)) and WNT5A derived from Affymetrix GeneChip data from liver samples of 91 patients with CH-C (Supplemental Table 3).

Supple Fig.8



Supplemental Figure 6. WNT5A induces OAS2 expression and increases HCV replication in Huh-7 cells.

A: WNT5A expression in THLE-5b, TTNT and Huh-7 cells.

B: OAS2 expression in THLE-5b, TTNT and Huh-7 cells.

C: Knocking down of WNT5A by siRNA and the change of OAS2 expression in THLE-5b cells

D: OAS2 expression in control lentivirus transduced Huh-7 cells (Huh7-control) and recombinant WNT5A lentivirus transduced Huh-7 cells (Huh7-WNT5A).

E: HCV replication deduced by Gaussia Luciferase activity in Huh7-control and Huh7-WNT5A.

F: Suppression of HCV infection by IFN-α in Huh7-control and Huh7-WNT5A.

All experiments were performed in duplicate and repeated 3 times (n=6). Values are means ± SE. **p <0.01 ***p<0.005.

Supplemental Table 1

Clinical characteristics of 168 patients whose liver biopsy samples were analyzed by RT-PCR

Clinical category	Major (MA)				Minor (MI)		p value
	Major ISG up (MAu)		Major ISG down (MAd)				
No. of patients	n = 44		n = 78		n = 46		NA
Age and sex							
Age (years)	55	(30–72)	56	(31–72)	55	(30–73)	NS
Sex (M vs. F)	28 vs. 16		40 vs. 38		24 vs. 22		NS
Treatment responses							
SVR/TR/NR	26/12/6		38/34/6		6/9/31*		MAu vs. MI <0.0001, MAd vs. MI <0.0001
IL28B genotype (TT vs. TG+GG)	TT		TT		TG/GG (40/6)		NA
Liver factors							
F stage (1/2/3/4)	14/14/11/5		36/22/13/7		16/13/13/4		NS
A grade (A0–1 vs. A2–3)	17 vs. 27		44 vs. 34		26 vs. 20		NS
ISGs (Mx1, IFI44, IFIT1)	3.79*	(2.14–9.48)	1.29*	(0.36–2.89)	5.72*	(0.82–17.3)	MAu vs. MAd <0.0001, MAu vs. MI <0.0001, MAd vs. MI <0.0001
IL28A/B	41.5*	(4-151)	12.3*	(1-53)	24.1*	(3-93)	MAu vs. MAd <0.0001, MAu vs. MI =0.0003, MAd vs. MI =0.008
Laboratory parameters							
HCV-RNA (KIU/mL)	2407	(160–5000)	2567	(140–5000)	2017	(126–5000)	NS
BMI (kg/m^2)	23.9	(18.1–31.9)	24.3	(16.3–40.6)	22.9	(19.1–30.5)	NS
AST (IU/L)	86*	(22–258)	52	(18–192)	66	(21–283)	MAu vs. MAd =0.0004
ALT (IU/L)	111*	(17–376)	74	(16–345)	82	(18–326)	MAu vs. MAd =0.017
γ-GTP (IU/L)	88*	(19–392)	31	(4–367)	53	(20–298)	MAu vs. MAd =0.0001
WBC (/mm^3)	4804	(2100–8100)	5068	(2800–11100)	4837	(2500–8200)	NS
Hb (g/dL)	14.1	(9.3–16.5)	13.9	(9.3–16.7)	14.3	(11.2–17.2)	NS
PLT (×10^4/mm^3)	15.1	(9.2–27.8)	17.2	(7–39.4)	16.6	(9–27.8)	NS
TG (mg/dL)	112	(42–248)	103	(42–260)	136*	(30–323)	MAd vs. MI =0.008
T-Chol (mg/dL)	162	(90–221)	169	(107–229)	169	(81–237)	NS
LDL-Chol (mg/dL)	77	(36–123)	83*	(43–134)	72	(29–107)	MAd vs. MI =0.014
HDL-Chol (mg/dL)	40	(18–67)	43	(27–71)	48*	(27–82)	MAd vs. MI =0.011
Viral factors							
ISDR mutations ≦1 vs. ≧2	23 vs. 21*		57 vs. 20		34 vs. 12		MAu vs. MAd =0.015, MAu vs. MI =0.032
Core aa 70 (wild-type vs. mutant)	25 vs. 19		54 vs. 23		20 vs. 26*		MAu vs. MI =0.0035

BMI, body mass index; AST, aspartate aminotransferase
ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase
WBC, leukocytes; Hb, hemoglobin; PLT, platelets; TG, triglycerides
T-chol, total cholesterol; LDL-chol, low density lipoprotein cholesterol
HDL-chol, high density lipoprotein cholesterol
NA, not applicable
NS, not significant
*p<0.05

Supplemental Table 2

Clinical characteristics of 85 patients whose liver and blood samples were analyzed by GeneChip.

Clinical category	Data	Range
No. of patients	n = 85	
Age and gender		
Age (years)	56	(30–71)
Sex (M vs. F)	52 vs. 33	
Treatment responses		
SVR/TR/NR	37/21/27	
IL28B genotype (TT vs. TG/GG)	55 vs. 26/4	
Liver factors		
F stage (1/2/3/4)	29/22/23/11	
A grade (A0–1 vs. A2–3)	42 vs. 43	
ISGs (Mx1, IFI44, IFIT1)	3.2	(0.7–17.3)
Laboratory parameters		
HCV-RNA (KIU/mL)	2005	(126–5000)
BMI (kg/m^2)	23.8	(16.2–31.9)
AST (IU/L)	56	(21–258)
ALT (IU/L)	71	(18–376)
γ-GTP (IU/L)	58	(4–392)
WBC (/mm^3)	4400	(2100–11100)
Hb (g/dL)	14.3	(11.2–16.7)
PLT (×10^4/mm^3)	15.4	(7–39.4)
TG (mg/dL)	99	(42–323)
T-Chol (mg/dL)	160	(81–229)
LDL-Chol (mg/dL)	80	(29–134)
HDL-Chol (mg/dL)	41	(18–82)
Viral factors		
ISDR mutations ≦1 vs. ≧2	56 vs. 28	
Core aa 70 (wild-type vs. mutant)	53 vs. 31	

BMI, body mass index; AST, aspartate aminotransferase
ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidas
WBC; leukocytes, Hb; hemoglobin, PLT; platelets

Supplemental Table 3
Clinical characteristics of 91 patients whose liver biposy samples were analyzed by GeneChip.

Clinical category	Data	Range
No. of patients	n = 91	
Age and gender		
Age (years)	56	(21–73)
Sex (M vs. F)	57 vs. 34	
Treatment responses		
SVR/TR/NR	41/21/29	
IL28B genotype (TT vs. TG/GG)	60 vs. 27/4	
Liver factors		
F stage (1/2/3/4)	32/23/25/11	
A grade (A0–1 vs. A2–3)	47 vs. 44	
ISGs (Mx1, IFI44, IFIT1)	3.3	(0.6–17.3)
Laboratory parameters		
HCV-RNA (KIU/mL)	2000	(126–5000)
BMI (kg/m^2)	23.8	(16.2–40.6)
AST (IU/L)	56	(20–283)
ALT (IU/L)	71	(16–376)
γ-GTP (IU/L)	58	(4–392)
WBC (/mm^3)	4500	(2100–11100)
Hb (g/dL)	14.3	(11.2–17.2)
PLT (×10^4/mm^3)	15.6	(6.6–39.4)
TG (mg/dL)	109	(30–03)
T-Chol (mg/dL)	171	(114–237)
LDL-Chol (mg/dL)	79	(42–123)
HDL-Chol (mg/dL)	44	(18–77)
Viral factors		
ISDR mutations ≦1 vs. ≧2	61 vs. 29	
Core aa 70 (wild-type vs. mutant)	57 vs. 33	

BMI, body mass index; AST, aspartate aminotransferase
ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase
WBC, leukocytes; Hb, hemoglobin; PLT, platelets; TG, triglycerides
T-chol, total cholesterol; LDL-chol, low density lipoprotein cholesterol
HDL-chol, high density lipoprotein cholesterol