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Supplemental Methods

Subject enrollment and inclusion

Pregnant women with hepatitis C viremia were recruited from The Ohio State University Substance Treatment Education and Prevention Program (STEPP), other OSU clinics, and a satellite site in Portsmouth, Ohio. All subjects provided informed consent before participation in the study. Peripheral blood was collected during the third trimester of pregnancy, (gestational week 27 until delivery hospitalization), 3 months after delivery, and, in a subset of women, at 6 and 12 months after delivery. Thirty-three HCV-infected women negative for HIV antibody and hepatitis B surface antigen with follow-up visits through at least 3 months postpartum were included in this analysis. Thirty-two were included in functional studies, and one additional subject was added for tetramer-based analyses. All protocols were approved by the institutional review boards of both The Ohio State University and Nationwide Children's Hospital and were conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Many of the women analyzed in this paper were included in our previous publication (1). Patient information can be found in **Supplemental Table 1**. Briefly, of the 34 mothers in the prior study, 29 are included in the current analyses. The remaining 5 were not included due to availability of samples. Four newly enrolled women were added to the current cohort. We refer to the 11 women with ≥1 $\log_{10}$ reduction in viremia at 3 months postpartum (3PP) as compared to the third trimester (T3) as controllers; all other women are classified as non-controllers.

Blood processing and virus testing

EDTA anticoagulated whole blood was separated by Ficoll gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC). 1 mL aliquots were then frozen in fetal bovine serum with 10% DMSO at a concentration of 5 x 10^6 cells/mL. HCV RNA levels in EDTA plasma were quantified by the Roche Taqman COBAS RT-PCR. Viral genotypes were determined by the line probe assay (LiPA 2.0 HCV Genotype, Siemens) or via RT-PCR followed by direct sequencing of the 5′UTR.

HCV-specific intracellular cytokine staining assay

PBMC were thawed rapidly in a 37°C water bath and slowly diluted 1:20 in pre-warmed R10 medium (RPMI + pen/strep + 10% FBS). Cells were then pelleted, washed, and re-suspended in R10. 7.5 x10^5 cells were plated in a 96 well plate and allowed to rest overnight before stimulation. For our initial assay, cells were stimulated with genotype-matched peptide pools (2 ug/mL) corresponding to HCV proteins NS3 (pools 4 and 5) and NS4A-B (pool 6), PMA + ionomycin, or peptide dilution medium as negative control. In the functional studies of tetramer epitope-specific CD4+ T-cells in M071, M025, and M026, cells were stimulated with peptides (2 ug/mL per peptide) matching the sequence of the epitopes presented by the class II tetramers listed in **Supplemental Table 2**. In both experiments, after one hour, brefeldin A (Golgi Plug, BD Biosciences) and monesin (Golgi Stop, BD Biosciences) were added at 1 ug/mL to inhibit protein transport. Stimulated cells were incubated overnight (<16 hours).

Stimulated cells were washed extensively before blocking with 20% human serum in FACS buffer (PBS + 5% FBS + 0.5% sodium azide). The following antibodies were used to stain for extracellular surface molecules: CD4 V500 (BD Biosciences, clone RPA-T4), CD8 Qdot605 (Life Technologies, clone 3B5), CD14 PE-Cy7 (Biolegend, clone HCD14), CD16 PE-Cy7 (Biolegend, clone 3G8), and CD19 PE-Cy7 (Biolegend, clone HIB19). Extracellularly stained samples were washed and stained with Live Dead Blue viability dye (Life Technologies). Cells were washed,

and then permeabilized with Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instructions. Cells were washed with Perm Wash (BD Biosciences) and then stained intracellularly. The following antibodies were used for intracellular staining: CD3 PerCP (Biolegend, clone UCHT1), IL2 FITC (Biolegend, clone MQ1-17H12), IFNγ Pacific blue (Biolegend, clone 4S.B3), IL10 APC (Biolegend, clone JES3-19F1) or TNFα APC (BD Biosciences, clone Mab11), IL17a Alexafluor 700 (Biolegend, clone BL168), and IL21 PE (Biolegend, clone 3A3-N2). Cells were washed, fixed in 1% paraformaldehyde, and run on the BD LSRII within 24 hours.

Flow cytometry analysis was applied to CD4+ CD8- CD3+ cells that were negative for CD14, CD16, and CD19 as well as Live Dead Blue. Negative controls were used to set gates for cytokine analysis for each subject.

Class II tetramer staining

PBMC from 11 women with sufficient T-cells at 3 month postpartum time points were evaluated by direct ex vivo HCV-specific class II tetramer staining. All available validated class II tetramers matching a subject's HLA-DR type were pooled in order to facilitate detection and to preserve samples. The tetramers used for each subject can be found in **Supplemental Table 2**. Briefly, 10 x10^6 PBMC per time point per subject were thawed in R10 medium and pelleted by centrifugation. Class II tetramer staining was performed in the dark at 37°C for 60 minutes. Cells were then washed and stained for extracellular and intracellular markers, as described in (2). Antibodies used for extracellular and intracellular marker staining include: CD14 APC-Cy7 (Biolegend, clone H1B19), CD19 APC-Cy7 (Biolegend, clone M5E2), CD56 APC-Cy7 (Biolegend, clone HCD56), CD3 PE-Dazzle (Biolegend, clone UCHT1), CD4 BUV395 (BD Biosciences, clone SK3), CD8 PE-Cy7 (Biolegend, clone SK1), PD-1 BUV737 (BD Biosciences, clone EH12.1), CTLA-4 BV421 (BD Biosciences, clone BN13), CD127 BV410 (BD Biosciences,

clone AO19D5), CCR7 PerCP-Cy5.5 (Biolegend, clone G043H7), CD45RA BV605 (Biolegend, clone HI100), Tbet BV711 (Biolegend, clone 4B10), and Eomes FITC (eBioscience, clone W01928). Exclusion of dead cells was achieved via staining with Live Dead Near IR (Life Technologies). Analysis was performed on a BD LSRII.

Statistics

Antigen-specific tetramer+ or cytokine-producing CD4+ T-cells were measured as a frequency (%) of total Live, CD14-, CD19-, CD56-, CD3+, CD4+ CD8- T-cells. Frequencies used to perform statistical analyses of cytokine production were determined by subtracting background cytokine production (by unstimulated cells) from raw frequencies of individual responses to the 3 separate peptide pools spanning NS3-4 and then summing the positive responses. Differences in the frequencies of tetramer+ or cytokine producing T-cells between various time points were analyzed by repeated measures analyses for each subject (Wilcoxon signed-rank test, $\alpha=0.05$). Differences in frequencies of tetramer+ and cytokine-producing T-cells between groups at each time point were analyzed by the Mann-Whitney U test ($\alpha=0.05$). Bivariate associations were determined by Pearson or Spearman correlation. Univariable and multivariable linear regression analyses were used to assess independent effects genetic polymorphisms and other factors on postpartum HCV-specific CD4+ Th1 cytokine responses and viral control. All statistical analyses of viral load were performed using $\log_{10}$-transformed values.

References

1. Honegger JR, et al. Influence of IFNL3 and HLA-DPB1 genotype on postpartum control of hepatitis C virus replication and T-cell recovery. *Proceedings of the National Academy of Sciences of the United States of America.* 2016;113(38):10684-9.
2. Schulze Zur Wiesch J, et al. Broadly directed virus-specific CD4+ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J Exp Med.* 2012;209(1):61-75.

Supplemental Table 1: Primary cohort characteristics

	Overall (n=32)	Controllers* (n=10**)	Non-controllers* (n=22)
Age at delivery, years [median (IQR)]	25.8 (24.7-29.2)	25.6 (25-27.3)	26.7 (24.5-29.8)
Estimated duration of infection at delivery, years [median (IQR)]	2.7 (1.5-5.7)	1.5 (1.1-4.8)	3.2 (1.8-7.2)
Gestational age at delivery, weeks [median (IQR)]	39.1 (38.5-39.6)	39.1 (38.9-39.5)	39 (38-39.7)
HCV RNA level at third trimester, log_{10} IU/ml [median (IQR)]	5.9 (5.4-6.7)	6 (5.3-6.8)	5.8 (5.5-6.7)
Route of HCV infection [n (%)]			
Injection drug use	28 (88%)	10 (100%)	18 (82%)
Vertical transmission	2 (6%)	0 (0%)	2 (9%)
Unknown	2 (6%)	0 (0%)	2 (9%)
HCV genotype [n (%)]			
1a/1b	21 (66%)	6 (60%)	15 (68%)
2a/2b	7 (22%)	3 (30%)	4 (18%)
3a	4 (13%)	1 (10%)	3 (14%)
Host genotype [n (%)]			
IFNL3 rs12979860 genotype CC***	13 (41%)	7 (70%)	6 (27%)
IFNL4 rs368234815 genotype TT/TT***	13 (41%)	7 (70%)	6 (27%)
≥1 high-expression *HLA-PBB1* allele	18 (56%)	9 (90%)	9 (41%)

*The term "controllers" indicates women with HCV viral load reductions of at least 1 log_{10} between the third trimester and 3 months postpartum. **The primary cohort for functional T-cell studies shown in Figure 1 includes 10 women with postpartum viral control. An additional woman with postpartum viral control (M003) was included for tetramer studies shown in Figures 2 and 3. ***The favorable *IFNL3* rs12979860 C allele was in 100% linkage disequilibrium with *IFNL4* rs368234815 TT allele in this cohort. IQR: interquartile range.

Supplemental Table 2: HCV-specific HLA class II tetramers used for individual subjects

HLA-class II allele	Epitope	M071	M025	M026	M003-2	M033	M048	M051	M024-2	M034	M031	M072
DRB1*0101	NS4 1806 TLLFNILGGWVAA			X				X				
DRB1*0301	NS4 1690 GKPAIIPDREVLYRE				X	X					X	
	NS5 2801 VYYLTRDPTTPLARAA				X	X					X	
DRB1*0401	NS3 1248 GYKVLVLNPSVAATL				X				X			
	NS4 1770 SGIQYLAGLSTLPGNPAIASL				X				X			
DRB1*0404	NS4 1773 QYLAGLSTLPGNPAIASL						X					
DRB1*0701	NS4 1540 RAYMNTPGLPV		X						X		X	X
DRB1*1101	NS4 1773 QYLAGLSTLPGNPAIASL		X	X				X				
DRB1*1104	NS4 1773 QYLAGLSTLPGNPAIASL					X						
	NS4 1911 GAVQWMNRLIAFAS					X						
DRB1*1501	NS3 1411 GINAVAYYRGLDVSV	X					X			X		
	NS4 1582 NFPYLVAYQATVCARA	X					X			X		

Supplemental Table 3: Univariable linear regression of predictors of HCV-specific IL2+IFNγ+ CD4+ T-cell frequency at 3 months postpartum in 32 women

Independent Variables	Coefficient* (95% CI)	P
IFNL3 rs12979860 genotype: CC	0.05 (-0.32, 0.42)	0.793
high expression *HLA-DPB1* alleles: ≥1	0.17 (-0.19, 0.54)	0.341
Age at delivery, years	-0.19 (-0.56, 0.18)	0.295
Estimated duration of infection at delivery, years	-0.13 (-0.56, 0.28)	0.501
Gestational age at delivery, weeks	-0.11 (-0.48, 0.26)	0.550
HCV genotype: 1	-0.34 (-0.69, 0.01)	0.059
HCV RNA level at T3, log_{10} IU/ml	-0.38 (-0.72, -0.03)	**0.034**

*standardized coefficients. CI: confidence interval. T3: third trimester. 3PP: 3 months postpartum.

Supplemental Table 4: Multivariable linear regression models predicting HCV-specific IL2+IFNγ+ CD4+ T-cell frequency at 3 months postpartum in 32 women

Model	Coefficient* (95% CI)	P
Model 1 (P=0.002, Adjusted R^2 = 0.313)		
IFNL3 rs12979860 genotype: CC	0.56 (0.19, 0.93)	**0.004**
HCV-RNA level at T3 (log_{10} IU/ml)	-0.69 (-1.06, -0.32)	**0.001**
Model 2 (P=0.11, Adjusted R^2 = 0.08)		
high expression *HLA-DPB1* alleles: ≥1	-0.04 (-0.40, 0.32)	0.812
HCV-RNA level at T3 (log_{10} IU/ml)	-0.39 (-0.75, -0.02)	**0.038**
Model 3 (P=0.004, Adjusted R^2 = 0.309)		
IFNL3 rs12979860 genotype: CC	0.58 (0.2, 0.95)	**0.004**
high expression *HLA-DPB1* alleles: ≥1	-0.1 (-0.42, 0.22)	0.532
HCV-RNA level at T3 (log_{10} IU/ml)	-0.72 (-1.11, -0.34)	**0.001**

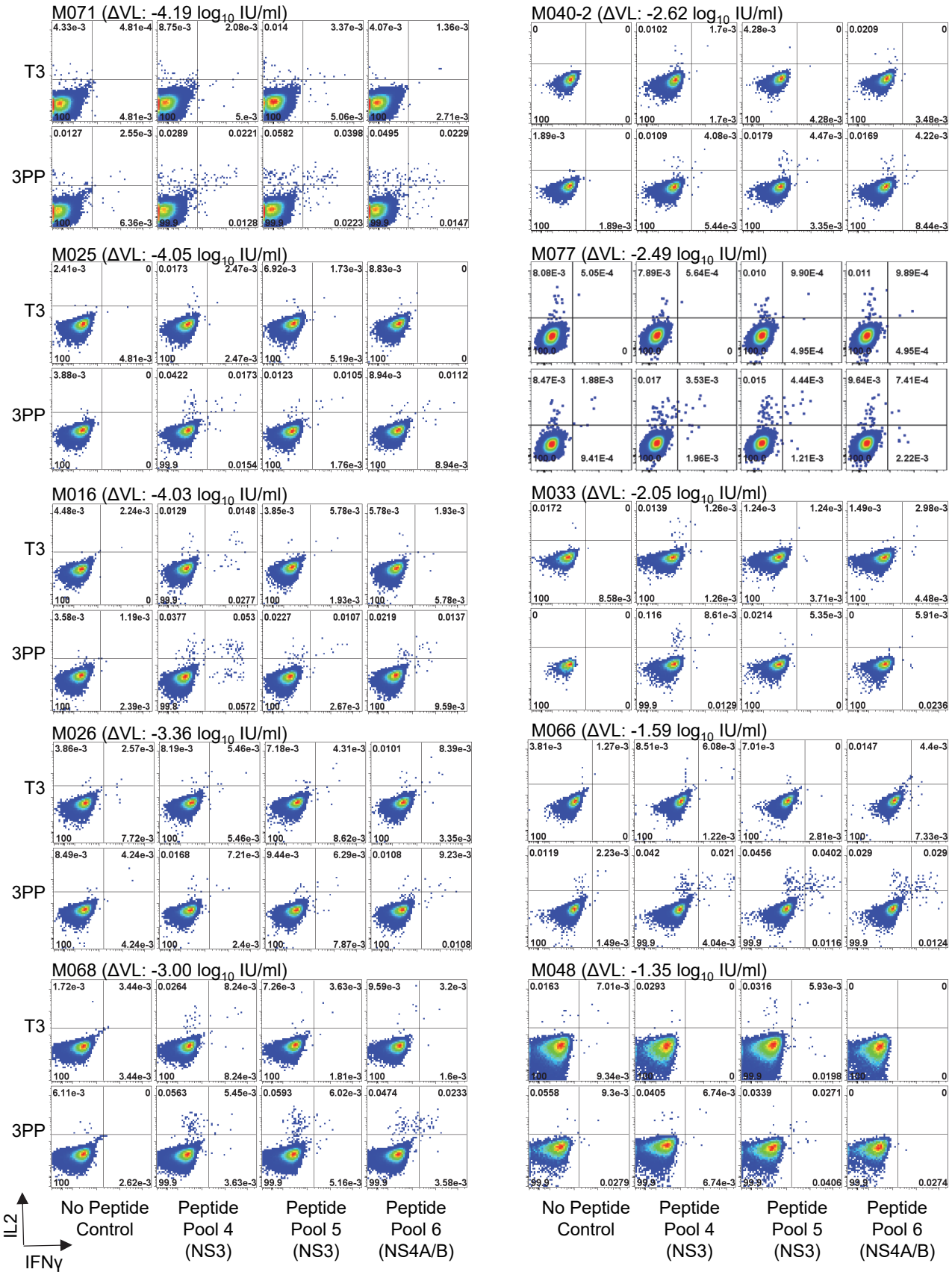
*standardized coefficients. CI: confidence interval. T3: third trimester. 3PP: 3 months postpartum.

Supplemental Table 5: Multivariable linear regression models predicting change in viral load from third trimester to 3 months postpartum in 32 women.

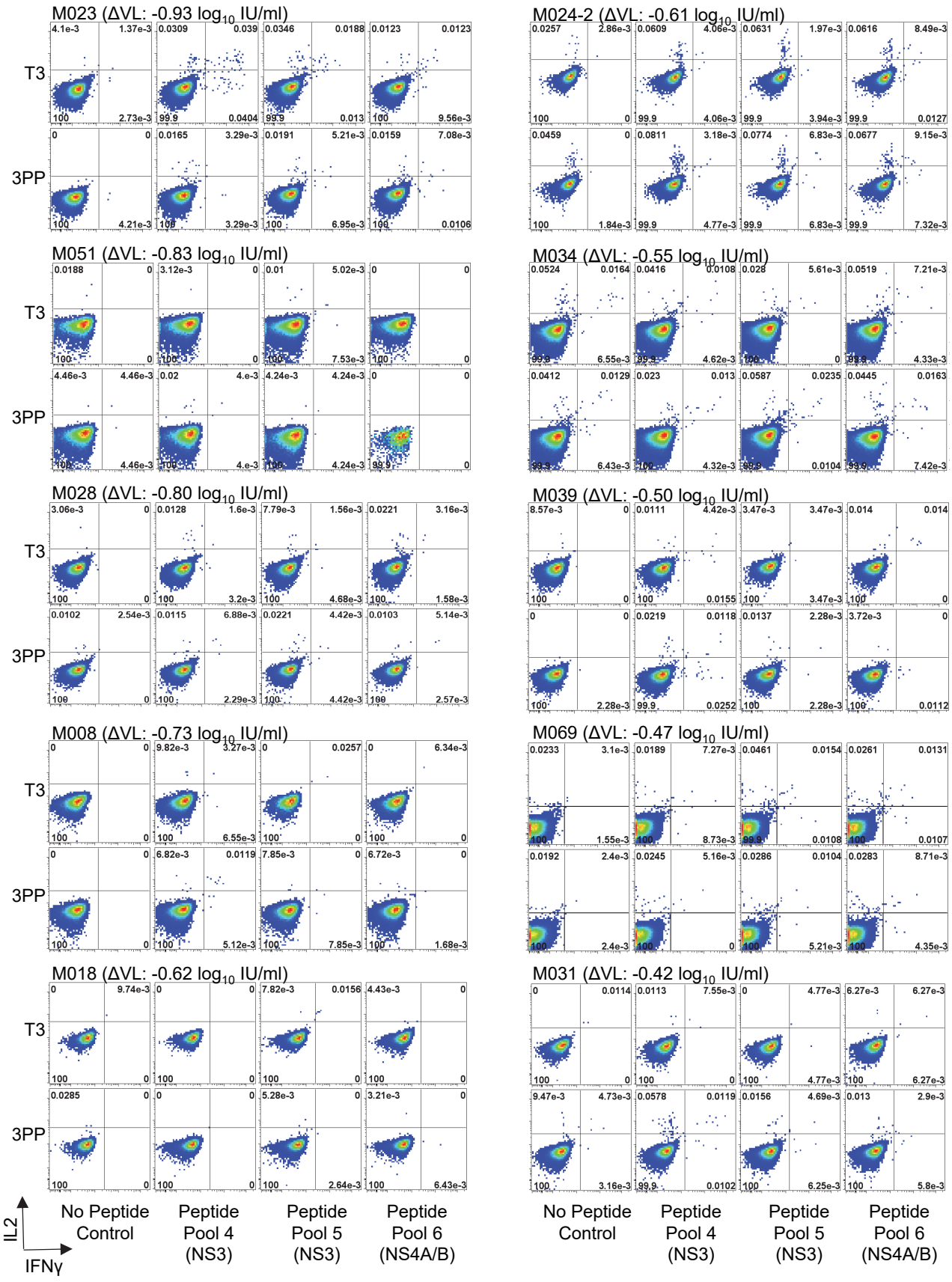
Model	Coefficient* (95% CI)	P
Model 1 (P=0.013, Adjusted R^2 = 0.209)		
IFNL3 rs12979860 genotype: CC	-0.38 (-0.71, -0.05)	**0.024**
high expression *HLA-DPB1* alleles: ≥1	-0.36 (-0.68, -0.03)	**0.034**
Model 2 (P=0.001, Adjusted R^2 = 0.360)		
IFNL3 rs12979860 genotype: CC	-0.31 (-0.61, -0.01)	**0.043**
high expression *HLA-DPB1* alleles: ≥1	-0.33 (-0.63, -0.04)	**0.029**
HCV-specific CD4+ T-cell response: % IL2+IFNγ+ at 3PP	-0.41 (-0.71, -0.11)	**0.009**

*standardized coefficients. CI: confidence interval. T3: third trimester. 3PP: 3 months postpartum.

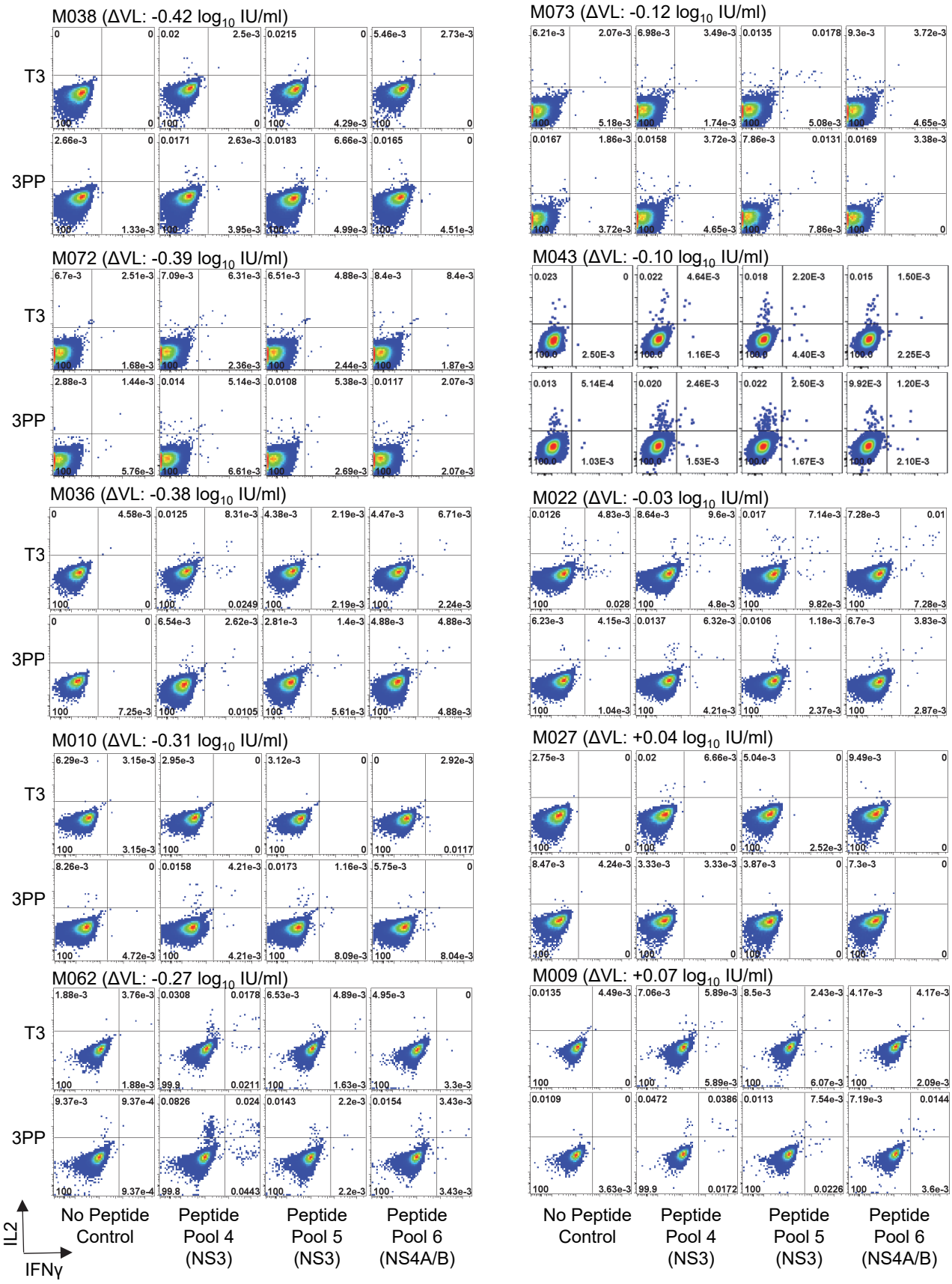
Supplemental Figure 1



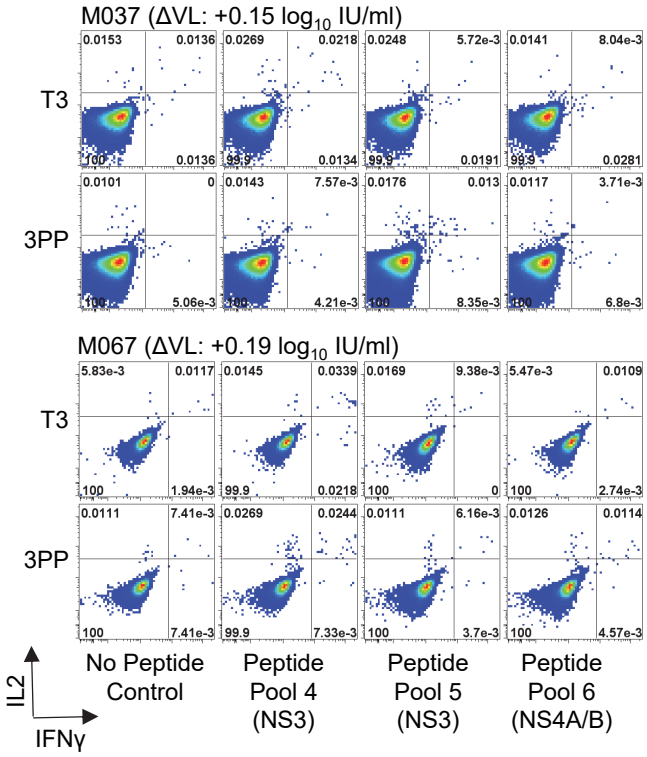
Supplemental Figure 1 continued



Supplemental Figure 1 continued

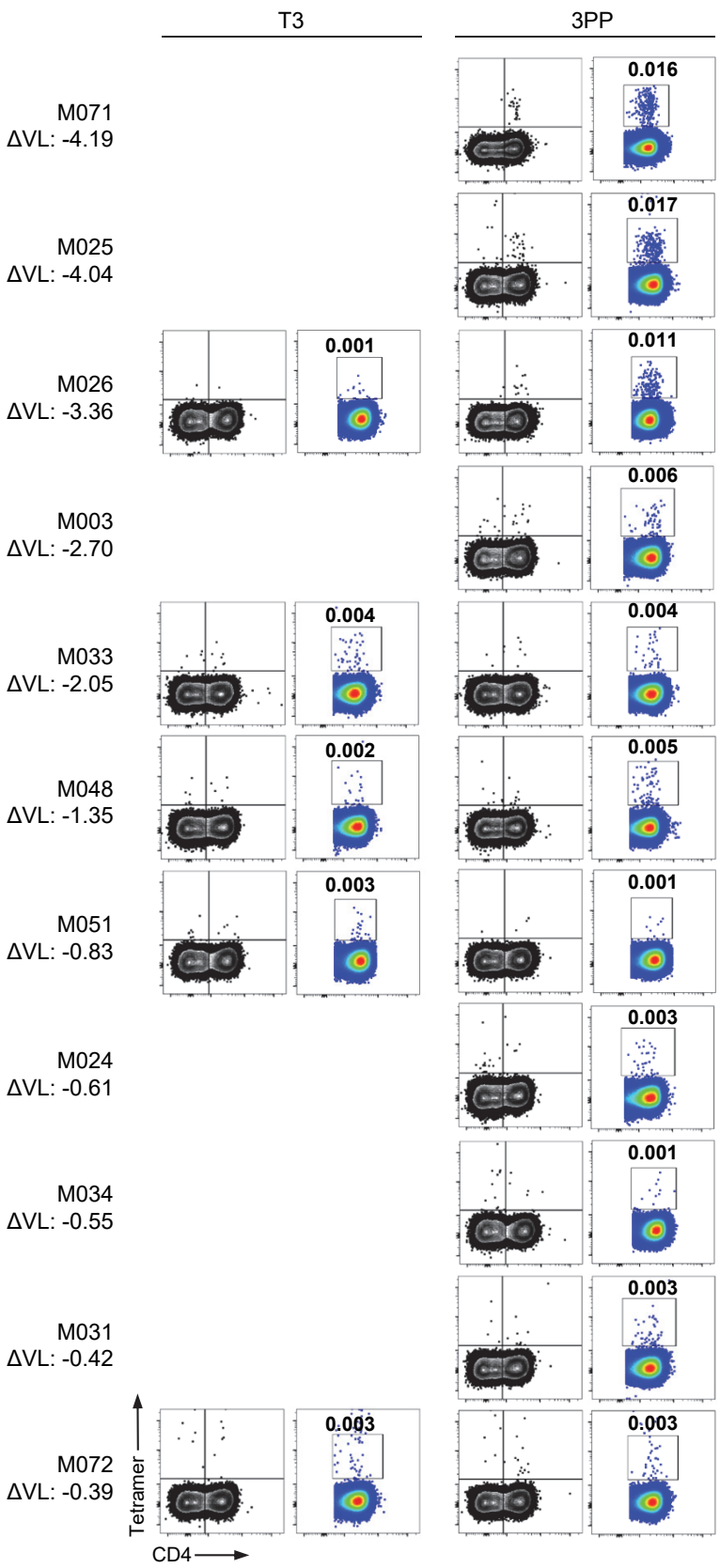


Supplemental Figure 1 continued



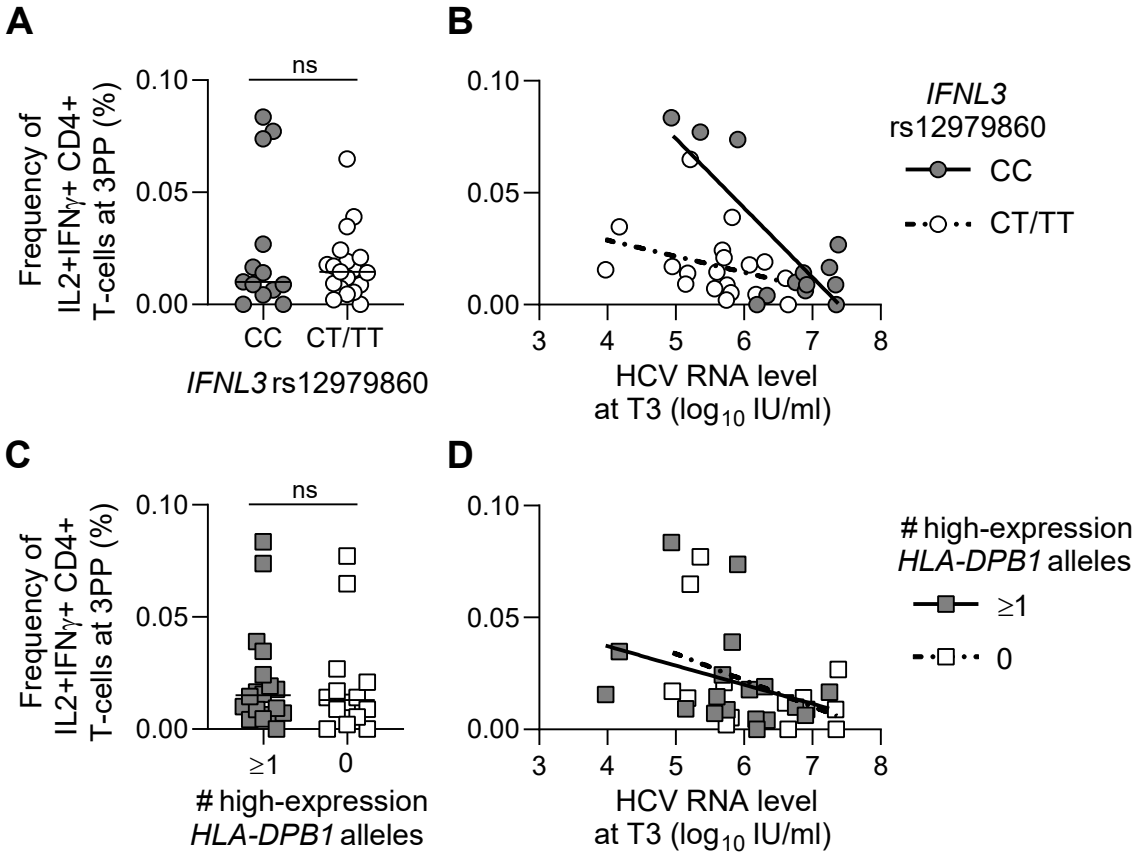
Supplemental Figure 1. Individual flow cytograms of HCV-specific CD4+ T-cell IL2 and IFNγ responses of 32 women during the third trimester of pregnancy (T3) and 3 months postpartum (3PP). Virus-specific IL2 and IFNγ production by CD4+CD8-CD3+ T-cells was assessed by intracellular cytokine stain of PBMC that were stimulated with 3 separate genotype-matched peptide pools spanning HCV NS3-NS4 or a negative control. The subjects are ordered by degree of viral control. ΔVL: change in viral load from T3 to 3PP.

Supplemental Figure 2



Supplemental Figure 2. HCV-specific CD4+ T-cell frequencies measured by class II tetramer staining of PBMC collected in the third trimester (T3) and 3 months postpartum (3PP). For each sample, tetramer stains are shown for the total CD3+ T-cell population (left) using 2% contour plots to depict relative frequencies and for CD4+CD8-CD3+ T-cells (right) using pseudocolor plots to show all tetramer+ events. Listed frequencies represent the % of tetramer+ events per CD4+CD8-CD3+ T-cell. Subjects are ordered by degree of viral control. ΔVL: change in viral load from T3 to 3PP.

Supplemental Figure 3



Supplemental Figure 3. Effect of *IFNL3* and *HLA-DPB1* polymorphisms on HCV-specific CD4+ T-cell responses. **A**. Comparison of frequencies of HCV-specific IL2+IFNγ+ CD4+ T-cells at 3PP of 13 women with the favorable *INFL3* 12979860 CC genotype (grey circles) and 19 women with CT or TT genotypes (white circles, Mann Whitney U Test). **B**. HCV-specific CD4+ T-cell responses of *INFL3* 12979860 CC (grey circles, solid line) and CT/TT (white circles, dashed line) subjects plotted versus baseline HCV RNA levels at T3. **C,D**. Comparison of HCV-specific IL2+IFNγ+ CD4+ T-cell frequencies of 18 women carrying at least one high-expression *HLA-DPB1* allele (grey squares) and 14 women with no high-expression alleles (white squares) directly (**C**) and versus T3 HCV RNA levels (**D**). ns, $P \geq 0.05$