HCC immune-surveillance and antiviral therapy of hepatitis C virus infection

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Supplementary Information

MATERIALS AND METHODS

Table 1: Characteristics of patients that developed on- or post-treatment HCC

Patient	Sex	Age	HCV Gt.	BL Child Pugh score	BL MELD-Score	Time between prior HCC diagnosis and DAA TS (months)	HCC Treat-ment prior DAA therapy	DAA regimen	Treatment outcome	Time to HCC after DAA therapy (months)	HCC status	Characteristics of current HCC	HCC Treatment after DAA
Pt #1	M	80	1b	A6	9	23	RFA, PEI	SOF/ LDV	SVR	1	Recurrence	1 nodule, 30mm, BCLC A2	Atypical liver resection
Pt #2	M	65	1a	A6	9	n/a	n/a	SOF/ LDV/ RBV	SVR	1.5	De novo	1 nodule, 23mm, BCLC A2	RFA
Pt #3	F	47	1b	B7	7	n/a	n/a	SOF/SMV/RBV	SVR	2	De novo	1 nodule, 24.6mm, BCLC A4	Liver segment resection
Pt #4	M	63	1a	A5	10	n/a	n/a	SOF/RBV	SVR	3	De novo	1 nodule, 26.3mm, BCLC A3	Microwave ablation
Pt #5	M	61	3a	B7	14	n/a	n/a	SOF/RBV	Relapse	4	De novo	1 nodule, 24mm, BCLC A3	TACE, RFA
Pt #6	M	77	1b	B7	10	23	RFA	3D regimen/ RBV	SVR	10	Recurrence	1 nodule, 6.7mm, BCLC A2	RFA
Pt #7	F	51	1a	A5	8	4	RFA	SOF/RBV	Relapse	12	Recurrence	4+ nodules, >30mm, BCLC B	TACE, RFA 4 months later
Pt #8	M	50	3a	C10	20	n/a	n/a	SOF/RBV	Relapse	22	De novo	2 nodules, 12.2mm, BCLC A4	RFA
Pt #9	F	54	1a	B8	11	n/a	n/a	SOF/ LDV/RBV	SVR	9	De novo	1 nodule, 42mm, BCLC A2	TACE
Pt #10	M	62	1b	A6	7	n/a	n/a	SOF/ LDV	SVR	0.5	De novo	1 nodule, 28mm, BCLC A1	Atypical liver resection
Pt #11	M	51	1a	A6	8	n/a	n/a	SOF/RBV	SVR	23	De novo	1 nodule, 87mm, BCLC C	None

Abbreviations: Pt #- patient number; RBV- ribavirin; SOF-sofosbuvir; SMV-simaprevir; LDV-ledipasvir; SVR-sustained virologic response; BCLC-Barcelona clinic liver cancer; RFA- radiofrequency ablation; TACE-transarterial chemoembolization; PEI-percutaneous ethanol injection; Gt-genotype; DAA-direct-acting antiviral; HCC-hepatocellular carcinoma; MELD-model for end-stage liver disease; BL-baseline; n/a-not applicable.

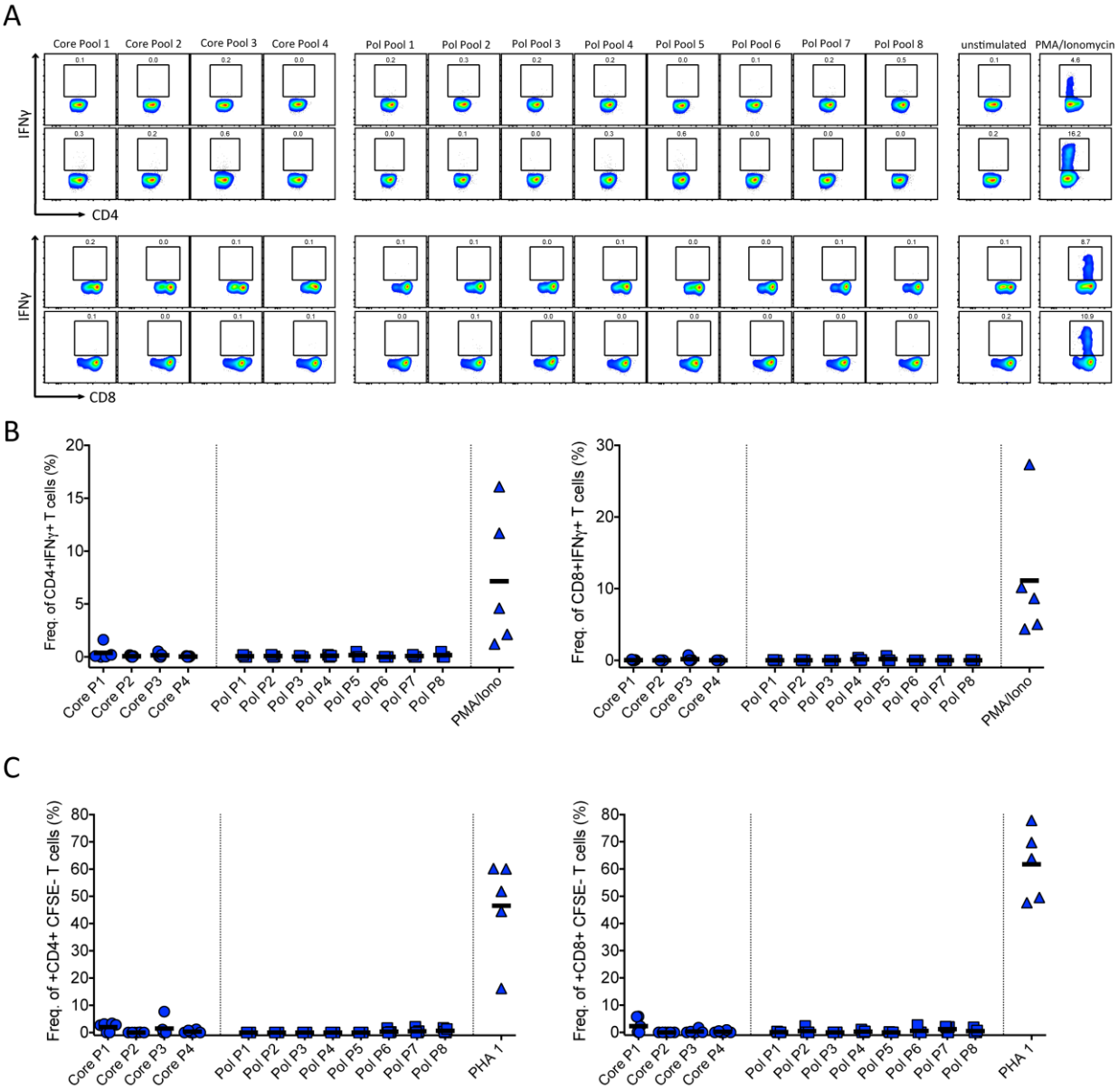
Supplementary Table 2: GPC-3-derived overlapping peptides and pool arrangement

ID	Position	Sequence	Pool
1	1	MAGTVRTACLVVAMLLSL	1
2	9	CLVVAMLLSLDFPGQAQP	1
3	17	SLDFPGQAQPPPPPPDAT	1
4	25	QPPPPPPDATCHQVRSFF	1
5	33	ATCHQVRSFFQRLQPGLK	1
6	41	FFQRLQPGLKWVPETPVP	1
7	49	LKWVPETPVPGSDLQVCL	1
8	57	VPGSDLQVCLPKGPTCCS	1
9	65	CLPKGPTCCSRKMEEKYQ	1
10	73	CSRKMEEKYQLTARLNME	1
11	81	YQLTARLNMEQLLQSASM	1
12	89	MEQLLQSASMELKFLIIQ	1
13	97	SMELKFLIIQNAAVFQEA	2
14	105	IQNAAVFQEAFEIVVRHA	2
15	113	EAFEIVVRHAKNYTNAMF	2
16	121	HAKNYTNAMFKNNYPSLT	2
17	129	MFKNNYPSLTPQAFEFVG	2
18	137	LTPQAFEFVGEFFTDVSL	2
19	145	VGEFFTDVSLYILGSDIN	2
20	153	SLYILGSDINVDDMVNEL	2
21	161	INVDDMVNELFDSLFPVI	2
22	169	ELFDSLFPVIYTQLMNPG	2
23	177	VIYTQLMNPGLPDSALDI	2
24	185	PGLPDSALDINECLRGAR	2
25	193	DINECLRGARRDLKVFGN	3
26	201	ARRDLKVFGNFPKLIMTQ	3
27	209	GNFPKLIMTQVSKSLQVT	3
28	217	TQVSKSLQVTRIFLQALN	3
29	225	VTRIFLQALNLGIEVINT	3
30	233	LNLGIEVINTTDHLKFSK	3
31	241	NTTDHLKFSKDCGRMLTR	3
32	249	SKDCGRMLTRMWYCSYCQ	3
33	257	TRMWYCSYCQGLMMVKPC	3
34	265	CQGLMMVKPCGGYCNVVM	3
35	273	PCGGYCNVVMQGCMAGVV	3
36	281	VMQGCMAGVVEIDKYWRE	3
37	289	VVEIDKYWREYILSLEEL	4
38	297	REYILSLEELVNGMYRIY	4
39	305	ELVNGMYRIYDMENVLLG	4
40	313	IYDMENVLLGLFSTIHDS	4
41	321	LGLFSTIHDSIQYVQKNA	4
42	329	DSIQYVQKNAGKLTTTIG	4
43	337	NAGKLTTTIGKLCAHSQQ	4
44	345	IGKLCAHSQQRQYRSAYY	4
45	353	QQRQYRSAYYPEDLFIDK	4
46	361	YYPEDLFIDKKVLKVAHV	4
47	369	DKKVLKVAHVEHEETLSS	4
48	377	HVEHEETLSSRRRELIQK	4
49	385	SSRRRELIQKLKSFISFY	5
50	393	QKLKSFISFYSALPGYIC	5
51	401	FYSALPGYICSHSPVAEN	5
52	409	ICSHSPVAENDTLCWNGQ	5
53	417	ENDTLCWNGQELVERYSQ	5
54	425	GQELVERYSQKAARNGMK	5
55	433	SQKAARNGMKNQFNLHEL	5
56	441	MKNQFNLHELKMKGPEPV	5
57	449	ELKMKGPEPVVSQIIDKL	5
58	457	PVVSQIIDKLKHINQLLR	5
59	465	KLKHINQLLRTMSMPKGR	5
60	473	LRTMSMPKGRVLDKNLDE	5
61	481	GRVLDKNLDEEGFESGDC	6
62	489	DEEGFESGDCGDDEDECI	6
63	497	DCGDDEDECIGGSGDGMI	6
64	505	CIGGSGDGMIKVKNQLRF	6
65	513	MIKVKNQLRFLAELAYDL	6
66	521	RFLAELAYDLDVDDAPGN	6
67	529	DLDVDDAPGNSQQATPKD	6
68	537	GNSQQATPKDNEISTFHN	6
69	545	KDNEISTFHNLGNVHSPL	6
70	553	HNLGNVHSPLKLLTSMAI	6
71	561	PLKLLTSMAISVVCFFFL	6
72	569	AISVVCFFFLVHMAGTVR	6
73	577	FLVHMAGTVRTACLVVAM	6

The highlighted peptides are those that could not be synthesized.

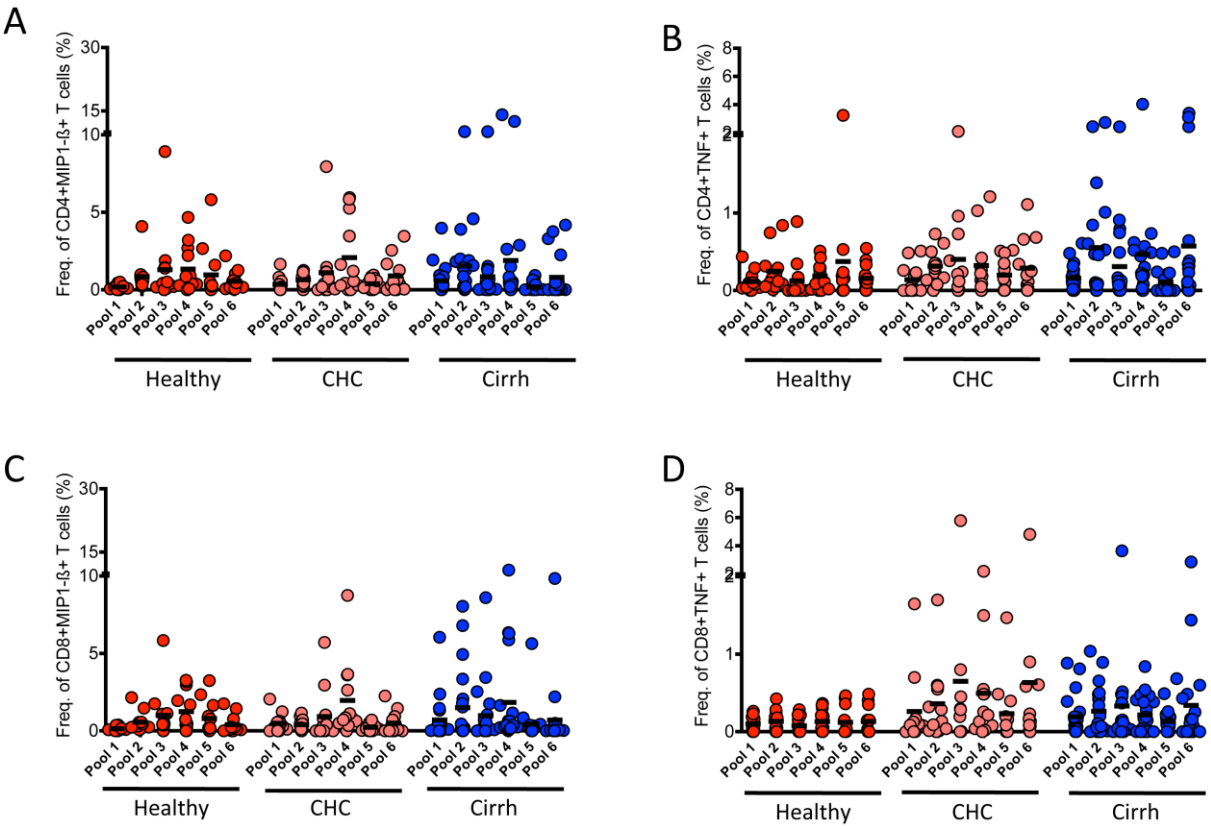
RESULTS

Supplementary Figure 1: Effect of control peptide stimulation on Tumor-Associated Antigen-specific T cell responses.



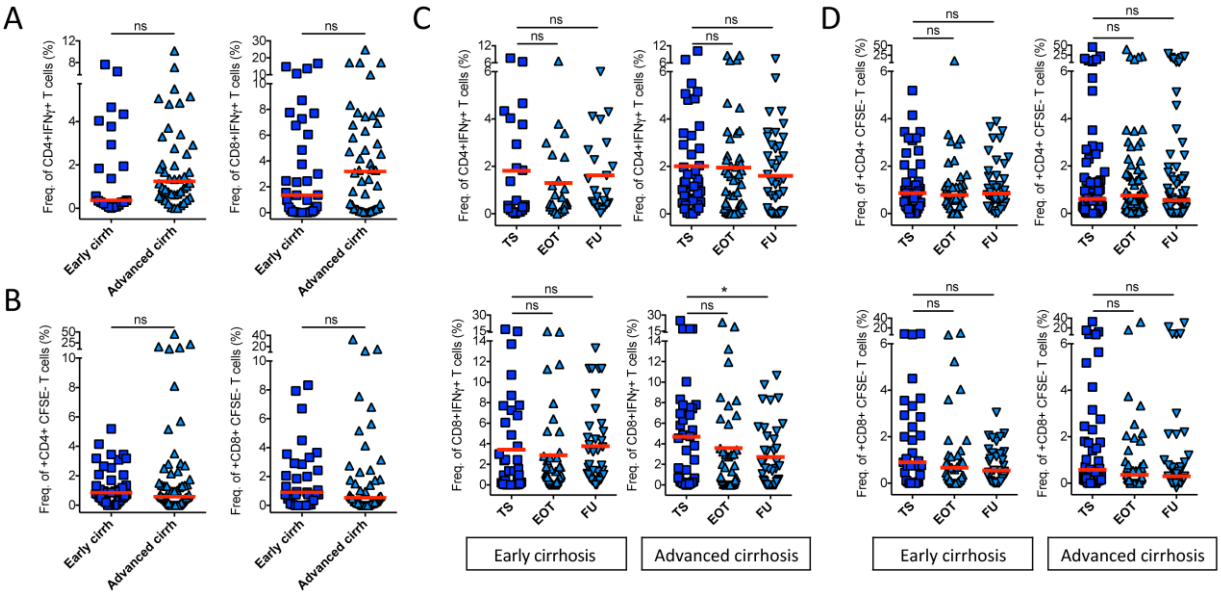
PBMC from selected HBsAg negative, anti-HBsAg/anti-HBcAg cirrhosis patients were stimulated with HBV-specific overlapping peptides covering the entire length of the HBV core and polymerase regions as negative control antigens. (A) Representative FACS plots from two patients each showing IFNγ secretion by $CD4^+$ and $CD8^+$ T cells. Summary plots of IFNγ secretion (B) and proliferation (C) by $CD4^+$ and $CD8^+$ T cells upon stimulation with negative control peptides are also shown. Black bars represent the respective mean values. Abbreviations: Pol - Polymerase, P - Peptide Pool,

Supplementary Figure 2: Magnitude and breadth of GPC-3-specific T cell responses in cirrhosis patients before antiviral therapy for hepatitis C.



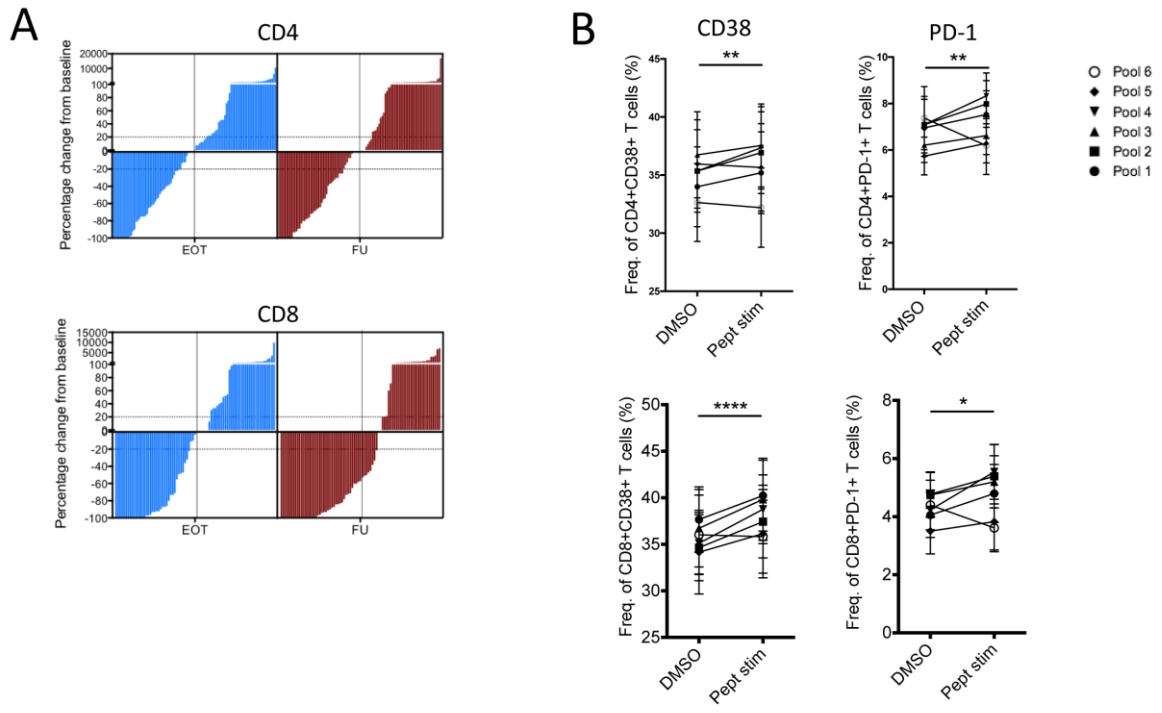
Cytokine production by circulating TAA-specific T cells to overlapping peptides covering the entire length of GPC-3 were tested in cirrhosis patients and non-cirrhotic controls at baseline *in vitro*. GPC-3-specific $CD4^+$ T cells producing (A) MIP1-ß (B) TNF are shown. Also shown are GPC-3-specific $CD8^+$ T cells responses of (A) MIP1-ß and (B) TNF secretion between cirrhosis patients and the non-cirrhotic controls. Mean values of all subjects in each cohort are indicated by black horizontal bars. Abbreviations: Cirrh - Cirrhosis, CHC - Chronic hepatitis C.

Supplementary Figure 3: Differential TAA-specific T cell response profiles in different sub-groups of cirrhosis patients and their fates following IFN-free antiviral therapy.



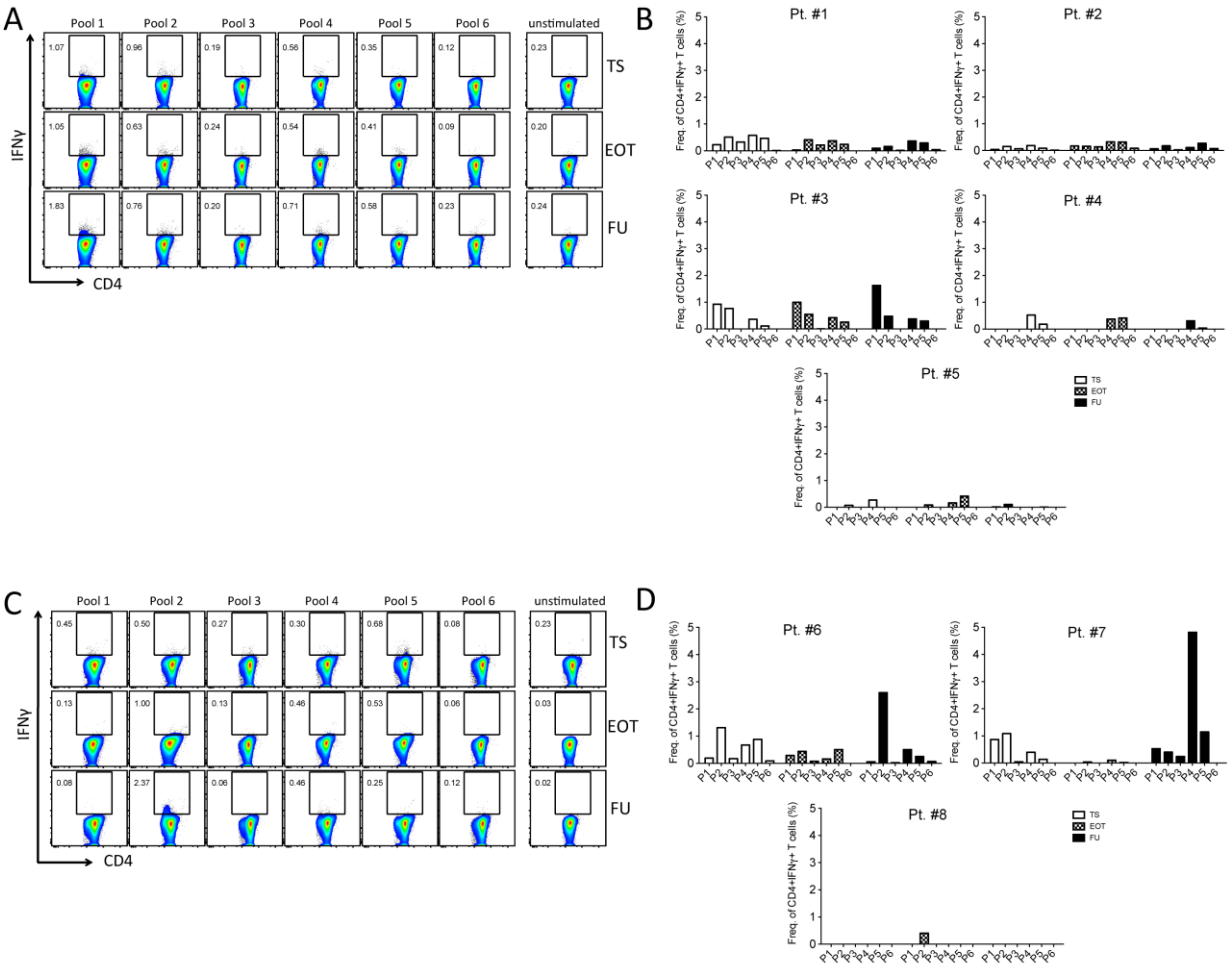
Baseline IFNγ production (A) and proliferative responses (B) of $CD4^+$ and $CD8^+$ T cells to GPC-3-specific peptides compared between early and advanced cirrhosis patients within the cirrh-to-No HCC cohort. Longitudinal changes in IFNγ production (C) and proliferation (D) of $CD4^+$ and $CD8^+$ T cells to GPC-3-specific peptides compared between early and advanced cirrhosis patients during the course of DAA therapy and beyond.

Supplementary Figure 4: Fate of TAA-specific T cell responses in cirrhosis patients following IFN-free antiviral therapy for hepatitis C.



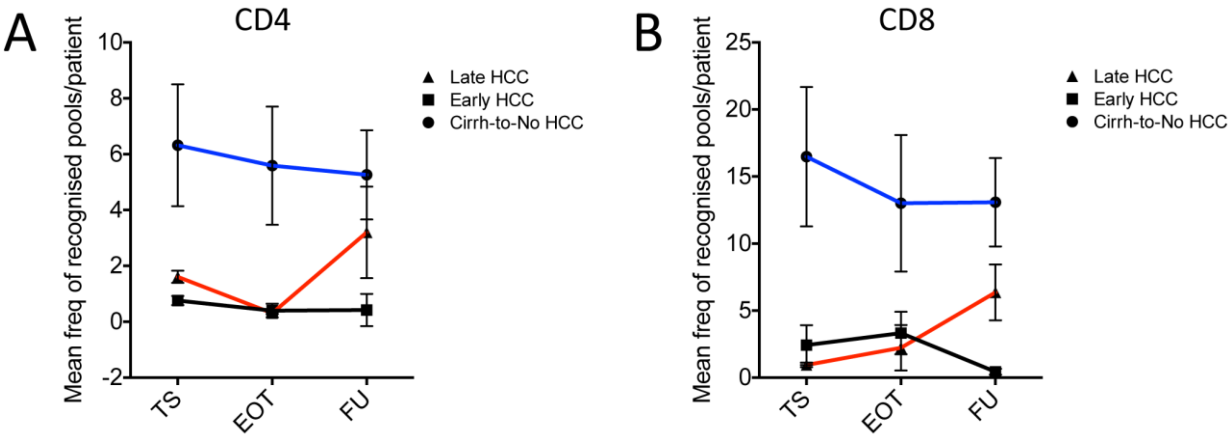
TAA-specific T cell responses to GPC-3 overlapping peptides were longitudinally assessed in cirrhosis patients at therapy start (TS), end of therapy (EOT) and at 12 or 24 weeks follow-up (FU) *in vitro*. (A) Percentage change of proliferative responses to individual pools by GPC-3-specific $CD4^+$ (top) and $CD8^+$ (down) at EOT and FU from respective baseline responses. (B) Expression of CD38 and PD-1 by $CD4^+$ and $CD8^+$ T cells between GPC-3 peptide stimulated conditions and unstimulated controls. Abbreviations: EOT – End-of-therapy, FU – Follow-up, PD-1 – Programmed cell death protein 1, Pept stim – Peptide stimulated.

Supplementary Figure 5: Longitudinal dynamics of CD4$^+$ T cell responses to GPC-3-specific peptides in cirrhosis patients who developed HCC following IFN-free therapy.



GPC-3-specific T cell responses were assessed at TS, EOT and FU in the sub-group of cirrhosis patients who developed on/post-treatment HCC upon receiving IFN-free DAA therapy for hepatitis C. Representative FACS plots of 1 Early (A) and 1 Late HCC patient (C) indicating the changes in frequency of IFNγ-secreting CD4$^+$ T cells during the course of DAA therapy and beyond. Bar graphs showing longitudinal IFNγ secretion by GPC-3-specific CD4$^+$ T cells for individual patients within the Early (B) and Late (D) HCC cohorts are displayed. Abbreviations: TS – Therapy start, EOT – End-of-therapy, FU – Follow-up, Pt. # – patient number.

Supplementary Figure 6: Magnitude and breadth of GPC-3-specific T cell responses at different time point during DAA therapy in cirrhosis patients that developed on/post treatment HCC.



Strength of IFNγ-secreting responses by GPC-3-specific $CD4^+$ (A) and $CD8^+$ (B) T cells at different time points during IFN-free DAA therapy compared between cirrh-to-No HCC and cirrh-to-HCC patients. Abbreviations: TS – Therapy start, EOT – End-of-therapy, FU – Follow-up

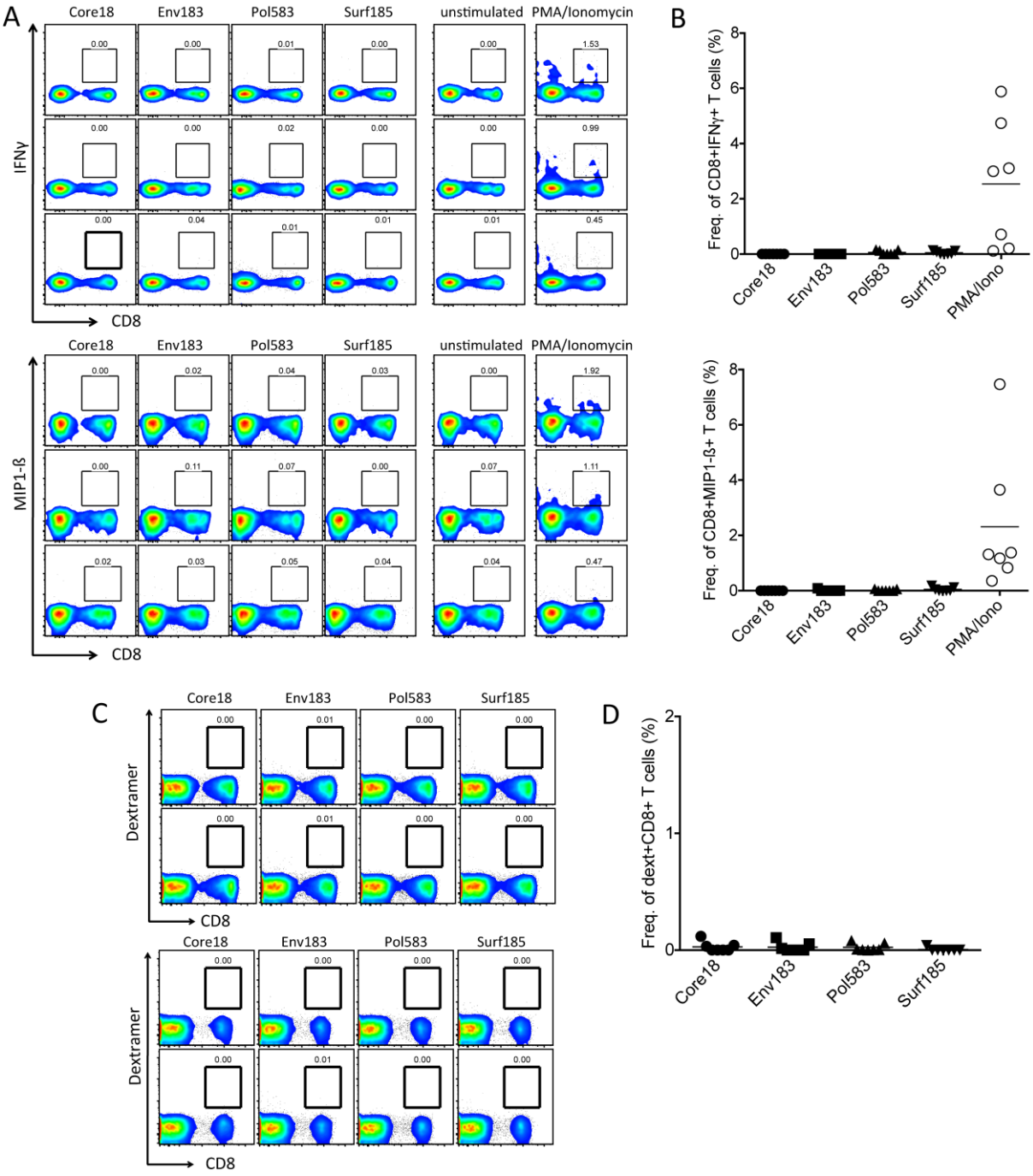
Supplementary Table 3: Summary of IFNγ-secreting T cells responses in cirrh-to-No HCC and cirrh-to-HCC patients at different time points during antiviral therapy with DAAs.

		TS			EOT			FU		
		Cirrh-to-No HCC	Early HCC	Late HCC	Cirrh-to-No HCC	Early HCC	Late HCC	Cirrh-to-No HCC	Early HCC	Late HCC
Mean number of recognised pool per patient	CD4	2,35	1,2	1,67	2,15	0,6	0,67	2,15	0,4	1,67
	CD8	2,75	1,6	1,33	2,74	1,6	1,33	3,10	0,6	2,00
Mean frequency (%) of recognised pool per patient	CD4	6,32*†	0,76	1,61	5,59*†	0,40	0,31	5,26*†	0,42	3,20
	CD8	16,48*†	2,43	0,95	13,01*†	3,33	2,23	13,08*†	0,44	6,36
Overall mean frequency (%) per patient	CD4	6,59*†	1,18	1,95	5,89*†	1,23	0,68	5,47*†	0,99	3,54
	CD8	16,68*†	2,78	1,35	13,22*†	3,79	2,47	13,24*†	0,98	6,51

Abbreviations: Cirrh – Cirrhosis, TS – Therapy start, EOT – End-of-therapy, FU – Follow-up.

*$p < 0.05$ versus Early HCC; †$p < 0.05$ versus Late HCC.

Supplementary Figure 7: HCC epitope-specific CD8$^+$ T cell responses to stimulations by negative control peptides.



PBMC from selected cirrhosis patients free from HBV co-infection were stimulated with HLA-A2-restricted HBV-specific peptides ($Core_{18}$, Env_{183}, Pol_{583}, and Surf185) as negative controls *in vitro.* (A) Representative FACS plots from three patients each depicting IFNγ and MIP1-ß secretions by CD8$^+$ T cells. (B) Respective summary plots of IFNγ and MIP1-ß secretions by CD8$^+$ T cells. (C) Representative FACS plots of HBV epitope-specific CD8$^+$ T cell expansion in two patients. (D) Overall data displaying expansion of dextramer$^+$CD8$^+$ T cells following *in vitro* stimulations with negative control peptides.