Characterization of the liver immune microenvironment in liver biopsies from patients with chronic HBV infection

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Table of contents

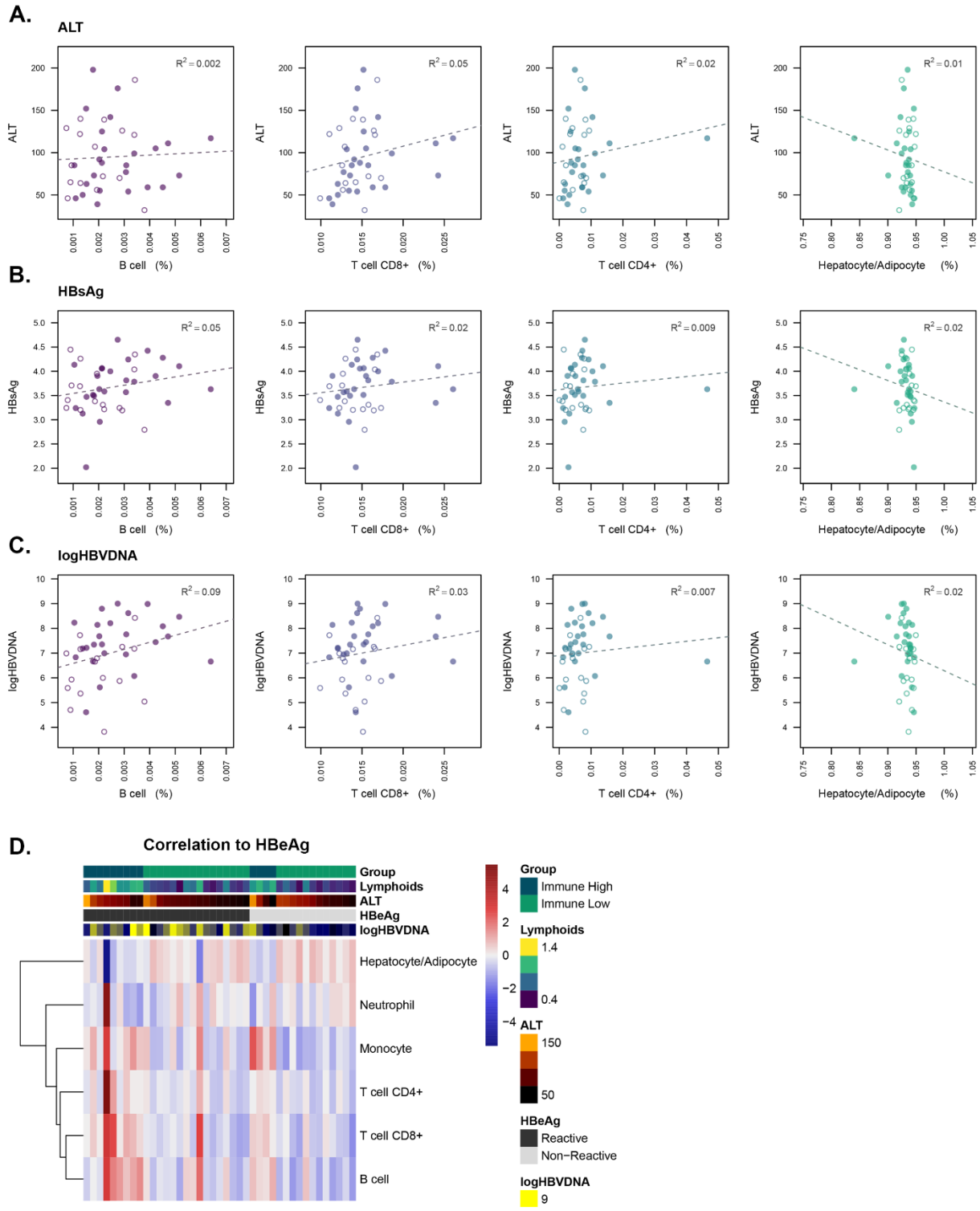

A.
ALT
R² = 0.002
R² = 0.05
R² = 0.02
R² = 0.01
ALT
B cell (%)
T cell CD8+ (%)
T cell CD4+ (%)
Hepatocyte/Adipocyte (%)
B.
HBsAg
R² = 0.05
R² = 0.02
R² = 0.009
R² = 0.02
HBsAg
C.
logHBVDNA
R² = 0.09
R² = 0.03
R² = 0.007
R² = 0.02
logHBVDNA
D.
Correlation to HBeAg
Group
Lymphoids
ALT
HBeAg
logHBVDNA
Hepatocyte/Adipocyte
Neutrophil
Monocyte
T cell CD4+
T cell CD8+
B cell
Group
Immune High
Immune Low
Lymphoids
1.4
0.4
ALT
150
50
HBeAg
Reactive
Non−Reactive
logHBVDNA
9

Fig. S1: Liver immune signatures independent of serum HBV DNA, ALT or HBeAg status. Individual cell type scores from EPIC cell deconvolution were correlated to peripheral biomarkers **(A)** ALT, **(B)** HBsAg, or **(C)** HBV DNA. **(D)** HBeAg status was used to cluster EPIC cell scores in a heatmap. No significant correlations were observed between immune cell signatures and these peripheral biomarkers.

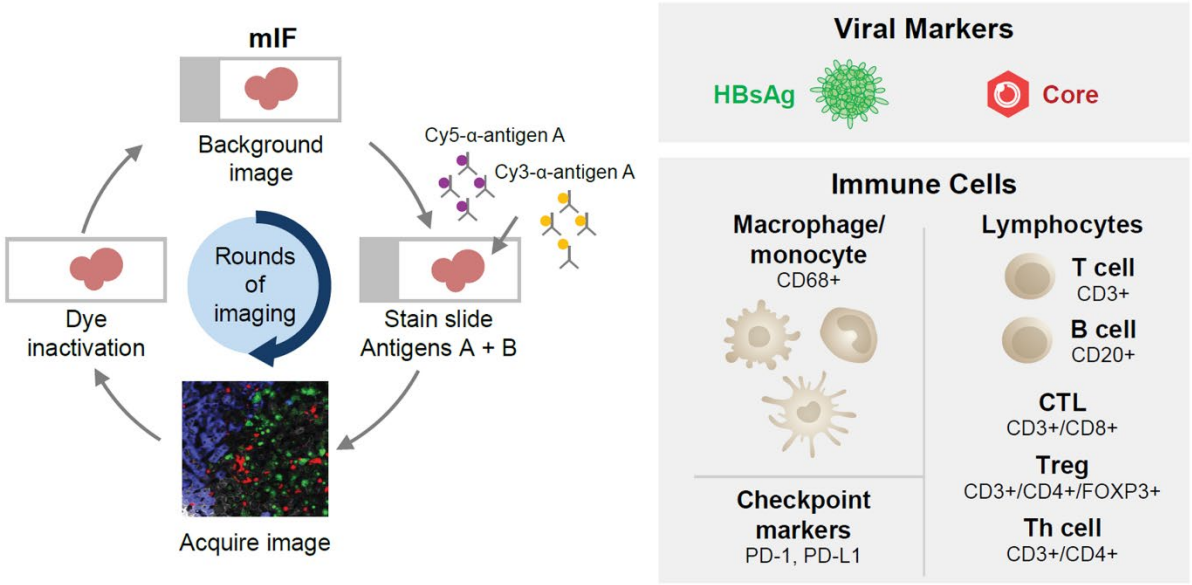


Fig. S2: Neogenomics mIF platform. All antibodies were conjugated to Cy5 and Cy3 dyes in pairs and liver biopsies were subjected to 7 rounds of staining, imaging and dye-inactivation. DAPI was used to align subsequent rounds of staining. The markers used for this analysis include HBcAg, HBsAg, CD3, CD4, CD8, CD68, FoxP3, Ki67, PD-1, PD-L1, CD299, and NaKATPase.

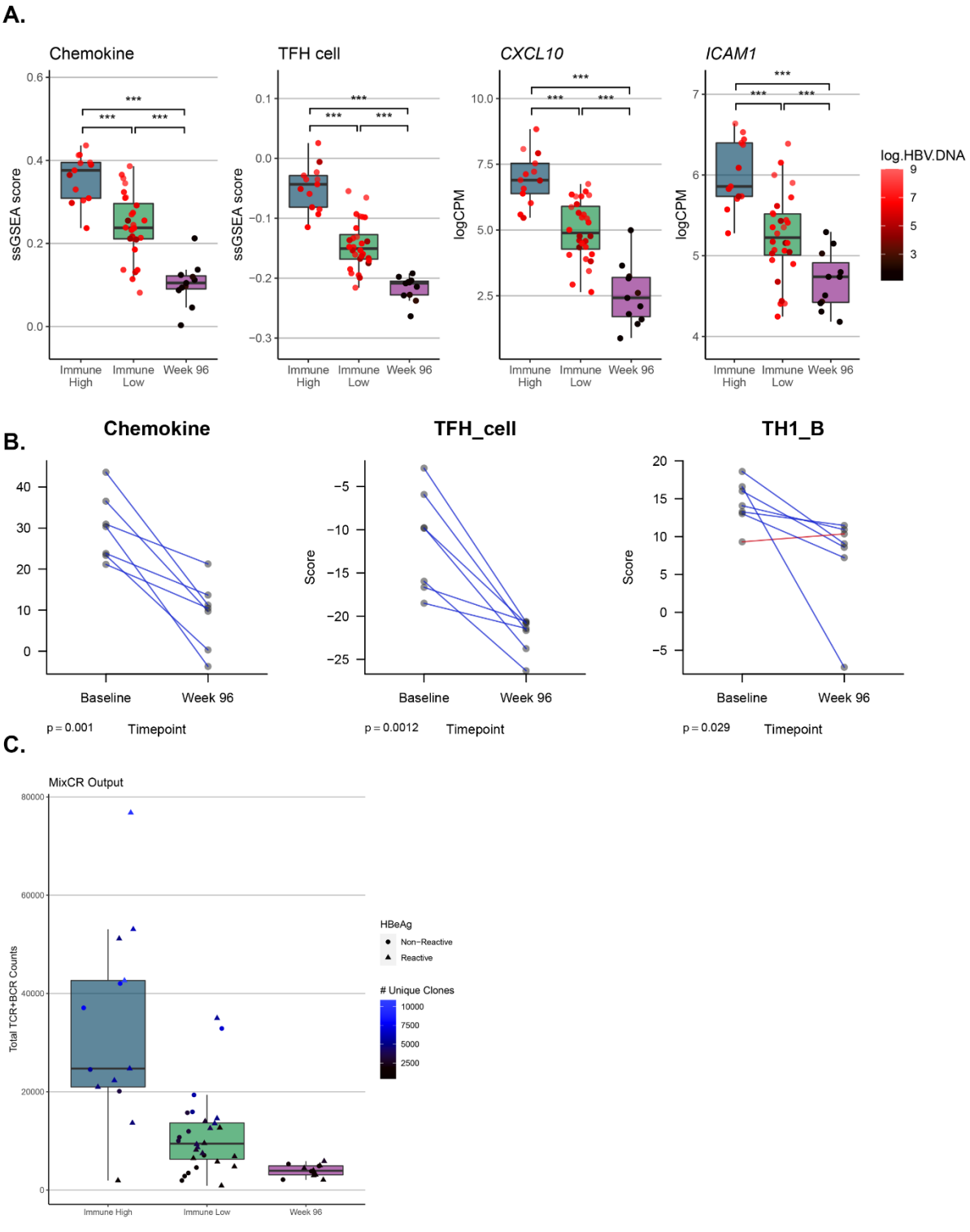
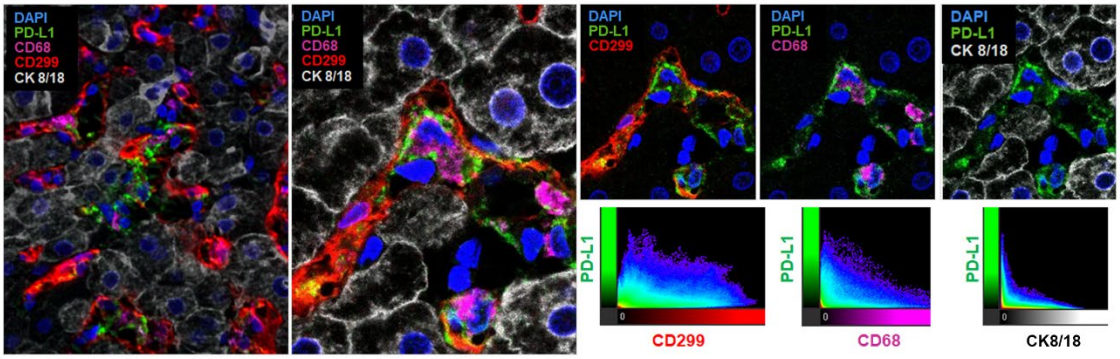

A.
Chemokine
TFH cell
CXCL10
ICAM1
ssGSEA score
logCPM
log.HBV.DNA
Immune High
Immune Low
Week 96
B.
Chemokine
TFH_cell
TH1_B
Score
Baseline
Week 96
p = 0.001
p = 0.0012
p = 0.029
Timepoint
C.
MixCR Output
Total TCR+BCR Counts
HBeAg
Non−Reactive
Reactive
Unique Clones
10000
7500
5000
2500

Fig. S3: TLS signatures reduced by treatment. A. Previously published TLS gene signatures were assessed in all liver biopsies [1-4]. **B.** TLS signatures were also quantified in seven longitudinal pairs. **C.** BCR and TCR clonality was quantified from all RNA-Seq data.

A.



B.

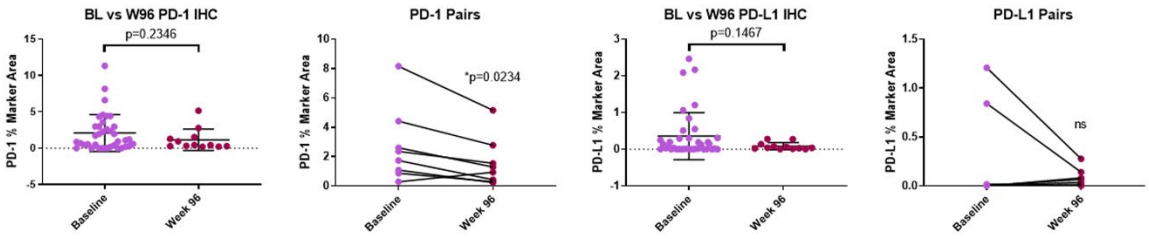


Fig. S4: PD-L1 is localized to liver sinudoidal endothelial cells and Kupffer cells and not hepatocytes. A. 4-plex mIF was developed to determine which liver cells express PD-L1. Biopsies with elevated PD-L1 by IHC were subjected to staining for CD68, CD299, PanCK, PD-L1 and DAPI. Image overlays represent a combination of all markers or individual PD-L1 overlays with CD68, CD299 and PanCK. Pearson's correlation analysis was performed to demonstrated colocalization with PD-L1. B. PD-L1 and PD-1 quantification by single-plex chromogenic staining. Only those with corresponding liver transcriptomes were presented in Figure 4B; this figure represents the comprehensive data set.

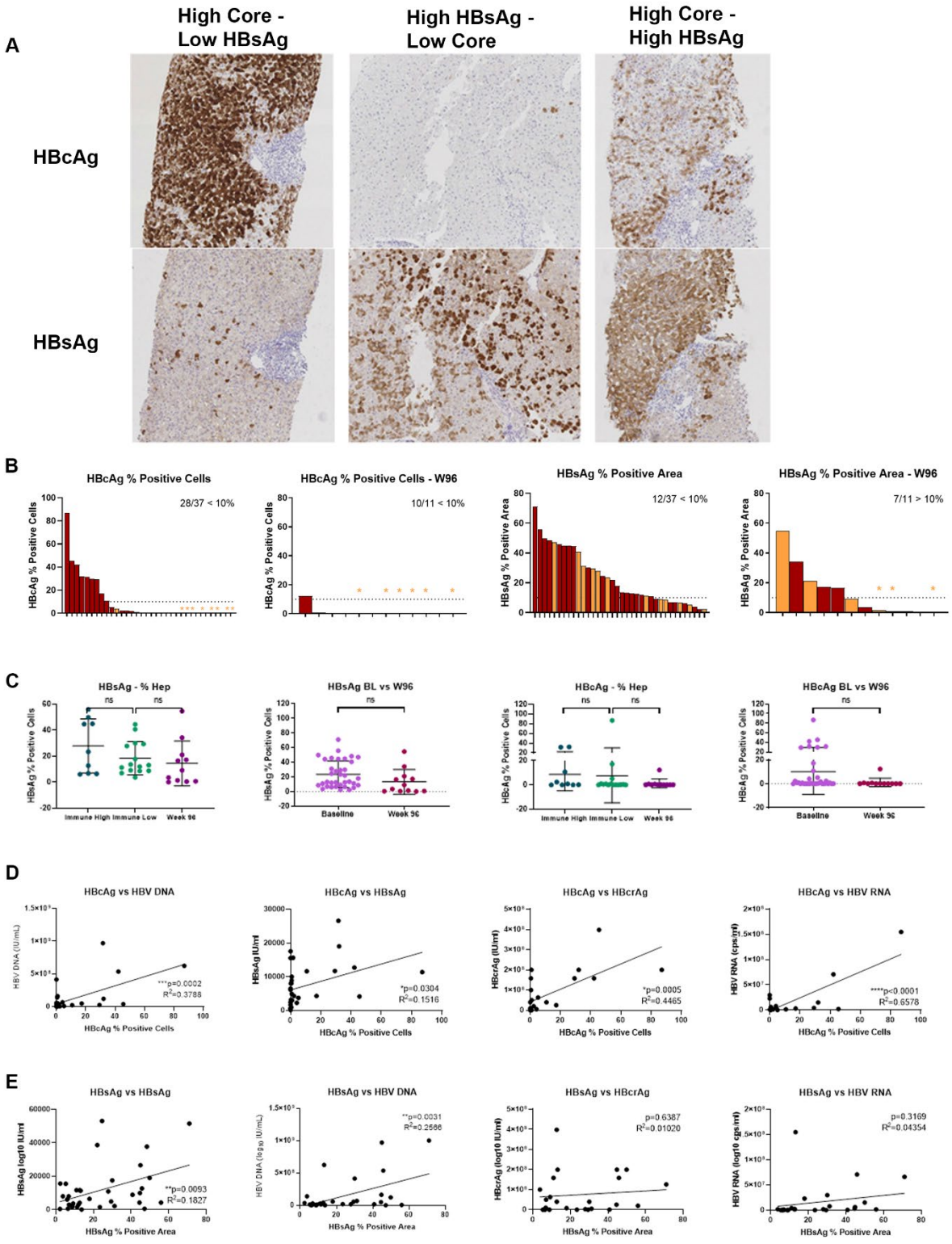
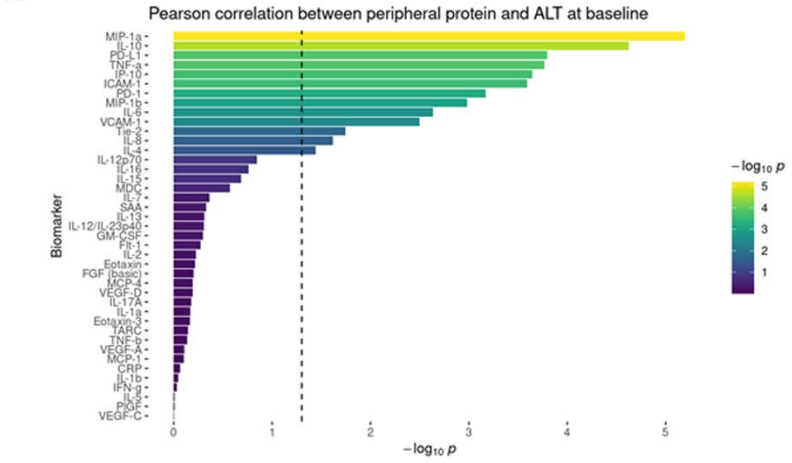
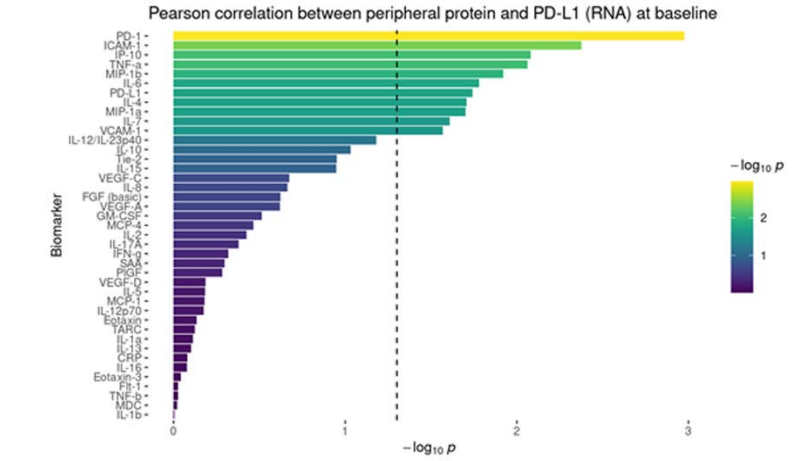

A
High Core - Low HBsAg
High HBsAg - Low Core
High Core - High HBsAg
HBcAg
HBsAg
B
HBcAg % Positive Cells
28/37 < 10%
HBcAg % Positive Cells - W96
10/11 < 10%
HBsAg % Positive Area
12/37 < 10%
HBsAg % Positive Area - W96
7/11 > 10%
C
HBsAg - % Hep
ns
HBsAg % Positive Cells
Immune High
Immune Low
Week 96
HBsAg BL vs W96
Baseline
HBcAg - % Hep
HBcAg BL vs W96
D
HBcAg vs HBV DNA
HBV DNA (IU/mL)
***p=0.0002
R²=0.3788
HBcAg % Positive Cells
HBcAg vs HBsAg
HBsAg IU/ml
*p=0.0304
R²=0.1516
HBcAg vs HBcrAg
HBcrAg (IU/ml)
*p=0.0005
R²=0.4465
HBcAg vs HBV RNA
HBV RNA (cps)/ml
****p<0.0001
R²=0.6578
E
HBsAg vs HBsAg
HBsAg log10 IU/ml
**p=0.0093
R²=0.1827
HBsAg % Positive Area
HBsAg vs HBV DNA
HBV DNA (log10 IU/mL)
**p=0.0031
R²=0.2566
HBsAg vs HBcrAg
HBcrAg (log10 IU/ml)
p=0.6387
R²=0.01020
HBsAg vs HBV RNA
HBV RNA (log10 cps/ml)
p=0.3169
R²=0.04354

Fig. S5: Analysis of HBcAg and HBsAg burden in liver biopsies using IHC. A. Heterogeneity was observed for HBcAg and HBsAg burden. Antigen burden was scored as % hepatocytes positive for HBcAg or HBsAg and representative images are shown. Each HBcAg image is a matched serial section to the HBsAg image shown below. **B.** HBcAg and HBsAg were quantified for each patient sample imaged by singe-plex IHC. Each bar represents an individual patient sample. HBeAg-positive samples are red and HBeAg-negative samples are orange. **C.** All data was plotted based on sampling time point. A subset of the entire collection had complementary intrahepatic transcriptome analyses that were used to classify samples by immune group. Statistical analysis was performed using unpaired t-tests. IHC scores for HBcAg **(D)** and HBsAg **(E)** were correlated to peripheral viral antigen levels including HBV DNA, HBV RNA, HBsAg and HBcrAg. Linear regression was used to quantify the correlation between liver biopsy staining scores and peripheral viral biomarkers.

A


Pearson correlation between peripheral protein and ALT at baseline
Biomarker
MIP-1a
IL-10
PD-L1
TNF-a
IP-10
ICAM-1
PD-1
MIP-1b
IL-6
VCAM-1
Tie-2
IL-8
IL-4
IL-12p70
IL-16
IL-15
MDC
IL-7
SAA
IL-13
IL-12/IL-23p40
GM-CSF
Flt-1
IL-2
Eotaxin
FGF (basic)
MCP-4
VEGF-D
IL-17A
IL-1a
Eotaxin-3
TARC
TNF-b
VEGF-A
MCP-1
CRP
IL-1b
IFN-g
IL-5
PlGF
VEGF-C
0
1
2
3
4
5
−log10 p
−log10 p
5
4
3
2
1

B


Pearson correlation between peripheral protein and PD-L1 (RNA) at baseline
Biomarker
PD-1
ICAM-1
IP-10
TNF-a
MIP-1b
IL-6
PD-L1
IL-4
MIP-1a
IL-7
VCAM-1
IL-12/IL-23p40
IL-10
Tie-2
IL-15
VEGF-C
IL-8
FGF (basic)
VEGF-A
GM-CSF
MCP-4
IL-2
IL-17A
IFN-g
SAA
PlGF
VEGF-D
IL-5
MCP-1
IL-12p70
Eotaxin
TARC
IL-1a
IL-13
CRP
IL-16
Eotaxin-3
Flt-1
TNF-b
MDC
IL-1b
0
1
2
3
−log10 p
−log10 p
2
1

C

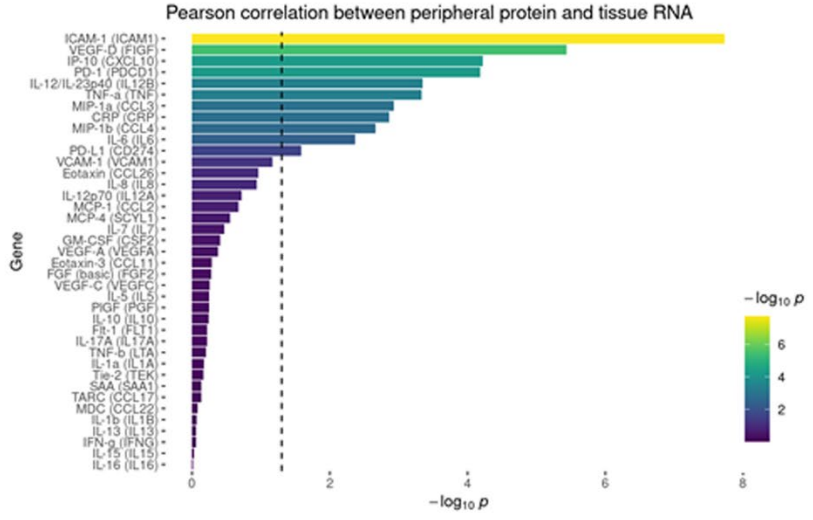

Pearson correlation between peripheral protein and tissue RNA
Gene
ICAM-1 (ICAM1)
VEGF-D (FIGF)
IP-10 (CXCL10)
PD-1 (PDCD1)
IL-12/IL-23p40 (IL12B)
TNF-a (TNF)
MIP-1a (CCL3)
CRP (CRP)
MIP-1b (CCL4)
IL-6 (IL6)
PD-L1 (CD274)
VCAM-1 (VCAM1)
Eotaxin (CCL26)
IL-8 (IL8)
IL-12p70 (IL12A)
MCP-1 (CCL2)
MCP-4 (SCYL1)
IL-7 (IL7)
GM-CSF (CSF2)
VEGF-A (VEGFA)
Eotaxin-3 (CCL11)
FGF (basic) (FGF2)
VEGF-C (VEGFC)
IL-5 (IL5)
PlGF (PGF)
IL-10 (IL10)
Flt-1 (FLT1)
IL-17A (IL17A)
TNF-b (LTA)
IL-1a (IL1A)
Tie-2 (TEK)
SAA (SAA1)
TARC (CCL17)
MDC (CCL22)
IL-1b (IL1B)
IL-13 (IL13)
IFN-g (IFNG)
IL-15 (IL15)
IL-16 (IL16)
0
2
4
6
8
−log10 p
−log10 p
6
4
2

Fig. S6: Correlation between peripheral cytokine and chemokine biomarkers with serum ALT and liver PD-L1 expression. 41 analytes quantified by MSD were compared to **(A)** ALT, **(B)** intrahepatic PD-L1 gene expression and **(C)** their own intrahepatic gene expression using Pearson's correlation and ranked by p value.

	Immune High (N=14)	Immune Low (N=27)	Total (N-41)	p value
Age				
N	14	27	41	0.4655
Mean (SD)	36 (6.1)	34 (11.2)	35 (9.7)	
Min, Max	25, 49	19, 61	19, 61	
Sex				
F	5 (35.7%)	10 (37.0%)	15 (36.6%)	0.9344
M	9 (64.3%)	17 (63.0%)	26 (63.4%)	
BMI				
N	14	27	41	0.8798
Mean (SD)	24.8 (5.53)	24.1 (4.61)	24.3 (4.88)	
Min, Max	17.9, 35.7	15.7, 34.1	15.7, 35.7	
Race				
Asian	12 (85.7%)	19 (70.4%)	31 (75.6%)	0.2839
White	2 (14.3%)	8 (29.6%)	10 (24.4%)	
Genotype				
A	0	2 (7.4%)	2 (4.9%)	0.2520
B	3 (21.4%)	8 (29.6%)	11 (26.8%)	
C	10 (71.4%)	11 (40.7%)	21 (51.2%)	
D	1 (7.1%)	6 (22.2%)	7 (17.1%)	
Baseline HBV DNA (log_{10} IU/ml)				
N	14	27	41	0.1413
Mean (SD)	7.1 (1.26)	6.6 (1.49)	6.8 (1.43)	
Min, Max	4.4, 9.0	3.8, 8.9	3.8, 9.0	
Baseline HBsAg (log_{10} IU/ml)				
N	14	27	41	0.7519
Mean (SD)	3.7 (0.59)	3.6 (0.60)	3.6 (0.50)	
Min, Max	2.1, 4.4	2.0, 4.8	2.0, 4.8	
Baseline ALT (IU/ml)				
N	14	27	41	0.1270
Mean (SD)	132 (78.1)	101 (74.4)	112 (76.2)	
Min, Max	49, 347	6, 341	6, 347	
Baseline HBeAg Status				
Non-Reactive	4 (28.6%)	12 (44.4%)	16 (39.0%)	0.3291
Reactive	10 (71.4%)	15 (55.6%)	25 (61.0%)	
Treatment Arm				
Screen Failure	3 (21.4%)	4 (14.8%)	7 (17.1%)	0.3589
PEG	3 (21.4%)	8 (29.6%)	11 (26.8%)	
TDF	4 (28.6%)	3 (11.1%)	7 (17.1%)	
TDF + PEG	3 (21.4%)	4 (14.8%)	7 (17.1%)	
[TDF + PEG] - TDF	1 (7.1%)	8 (29.6%)	9 (22.0%)	
Baseline HBV RNA (log_{10} copies/ml)				
N	11	21	32	0.8118
Mean (SD)	5.3 (1.52)	5.4 (1.62)	5.4 (1.56)	
Min, Max	2.6, 7.2	2.5, 8.2	2.5, 8.2	
Baseline HBcrAg (log_{10} IU/ml)				
N	10	21	31	0.9158
Mean (SD)	6.7 (1.36)	6.5 (1.74)	6.5 (1.61)	
Min, Max	4.2, 8.3	3.1, 8.3	3.1, 8.3	

Table S1: Patient Characteristics from the 174-0149 clinical trial liver biopsy sub-study. Baseline clinical parameters associated with immune high and immune low samples are shown. No clinical parameters or viral biomarkers differentiated immune high versus immune low subsets. P values were calculated using Cochran-Mantel-Haenszel test and Wilcoxon test for categorical data and continuous data, respectively.

Supplementary references

[1] Hennequin A, Derangere V, Boidot R, Apetoh L, Vincent J, Orry D, et al. Tumor infiltration by Tbet+ effector T cells and CD20+ B cells is associated with survival in gastric cancer patients. Oncoimmunology 2016;5:e1054598.

[2] Becht E, de Reynies A, Giraldo NA, Pilati C, Buttard B, Lacroix L, et al. Immune and Stromal Classification of Colorectal Cancer Is Associated with Molecular Subtypes and Relevant for Precision Immunotherapy. Clin Cancer Res 2016;22:4057-4066.

[3] Messina JL, Fenstermacher DA, Eschrich S, Qu X, Berglund AE, Lloyd MC, et al. 12-Chemokine gene signature identifies lymph node-like structures in melanoma: potential for patient selection for immunotherapy? Sci Rep 2012;2:765.

[4] Coppola D, Nebozhyn M, Khalil F, Dai H, Yeatman T, Loboda A, et al. Unique ectopic lymph node-like structures present in human primary colorectal carcinoma are identified by immune gene array profiling. Am J Pathol 2011;179:37-45.