Chronic hepatitis C liver microenvironment: role of the Th17/Treg interplay related to fibrogenesis

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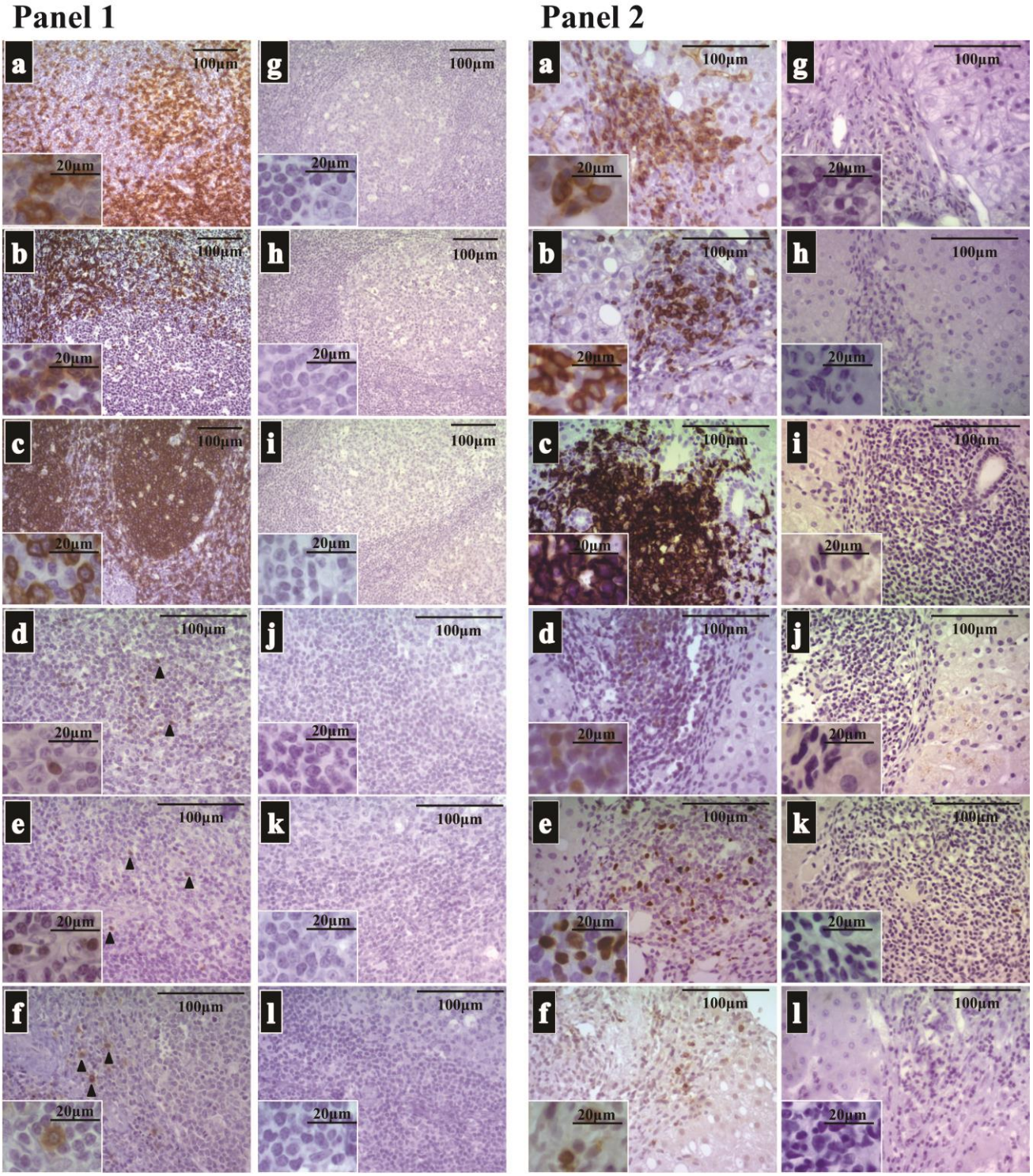
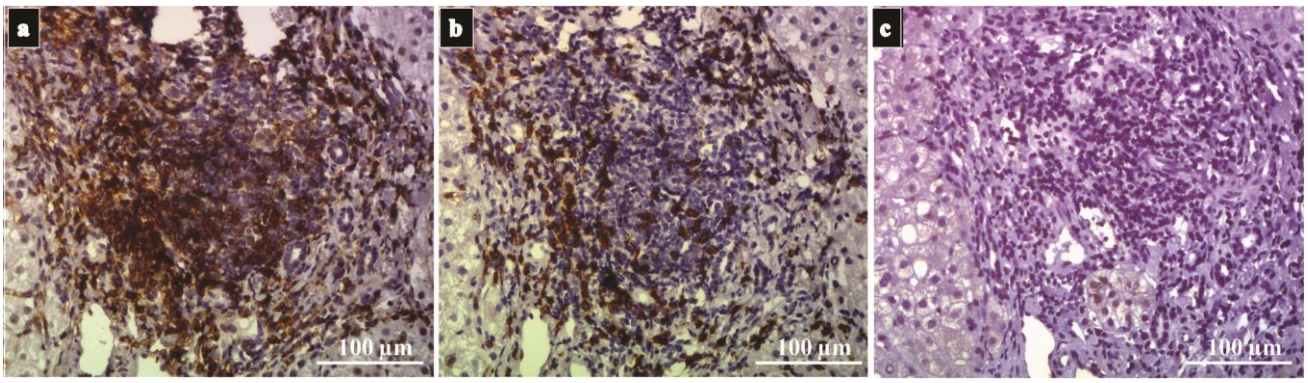
Supplementary table S1: Primer sequences used in SybrGreen qRT-PCR

Target	Primer Sequences (5'-3')	Product Length (nt)
IL-10	F GCTGTCATCGATTTCTTCCC R ACAAAGCCATGAGTGAGTTTGA	111
IL-17A	F AACGATGACTCCTGGGAAGAC R CCTGGATTTCGTGGGATTGTG	99
TGF-β	F CTTCCAGCCGAGGTCCTT R CCCTGGACACCAACTATTGC	92
IL-6	F AGTGAGGAACAAGCCAGAGC R GTCAGGGGTGGTTATTGCAT	99
IFN-γ	F GAGTGTGGAGACCATCAAGGA R GTATTGCTTTGCGTTGGACA	127
TNF-α	F CTGCTGCACTTTGGAGTGAT R AGATGATCTGACTGCCTGGG	93
HPRT	F ATGGGAGGCCATCACATTGT R ATGTAATCCAGCAGGTCAGCAA	77
β-actin	F CCACACTGTGCCCATCTACG R CCGTGGTGGTGAAGCTGTAG	131

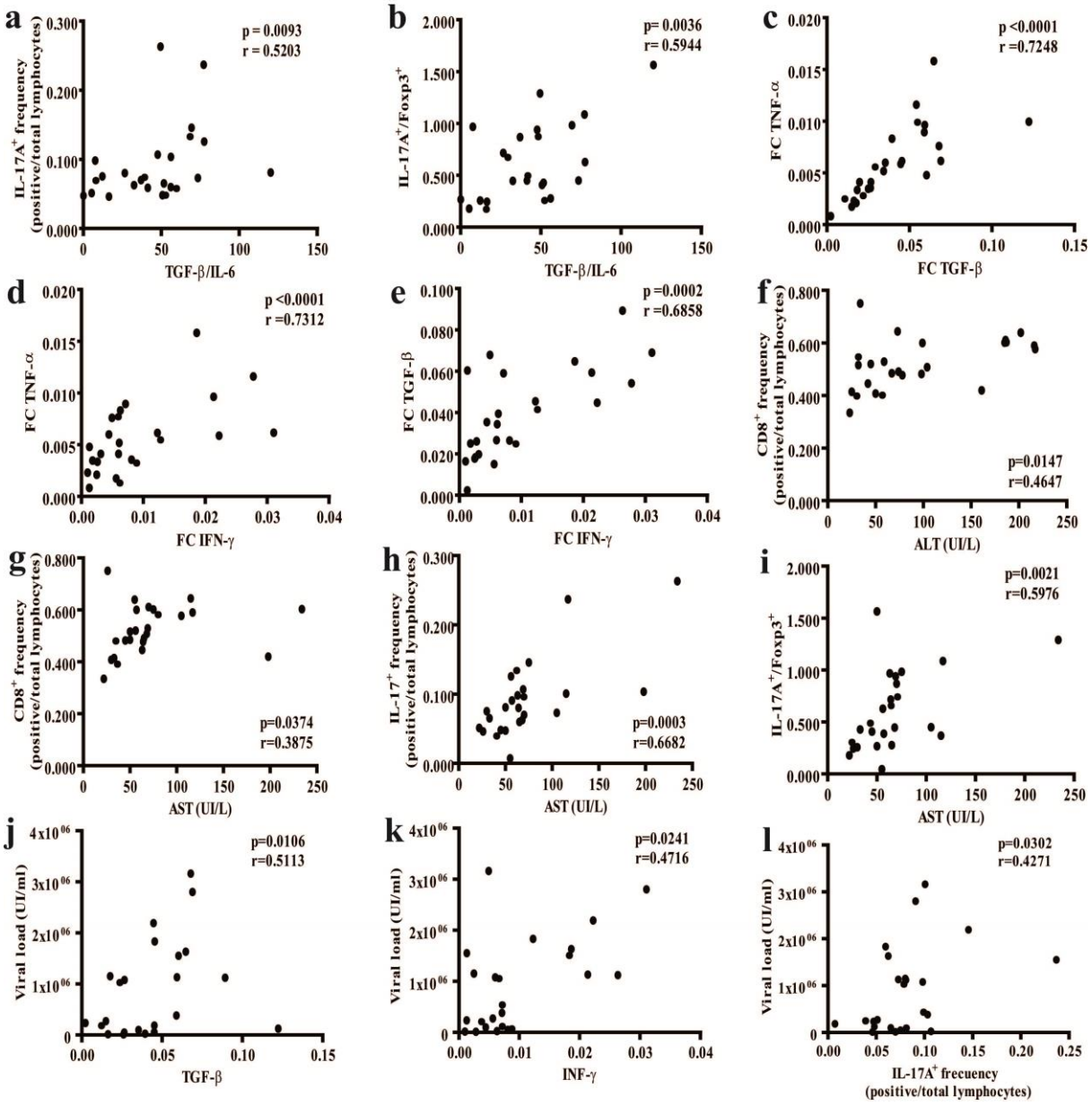
F: forward primer; R: reverse primer



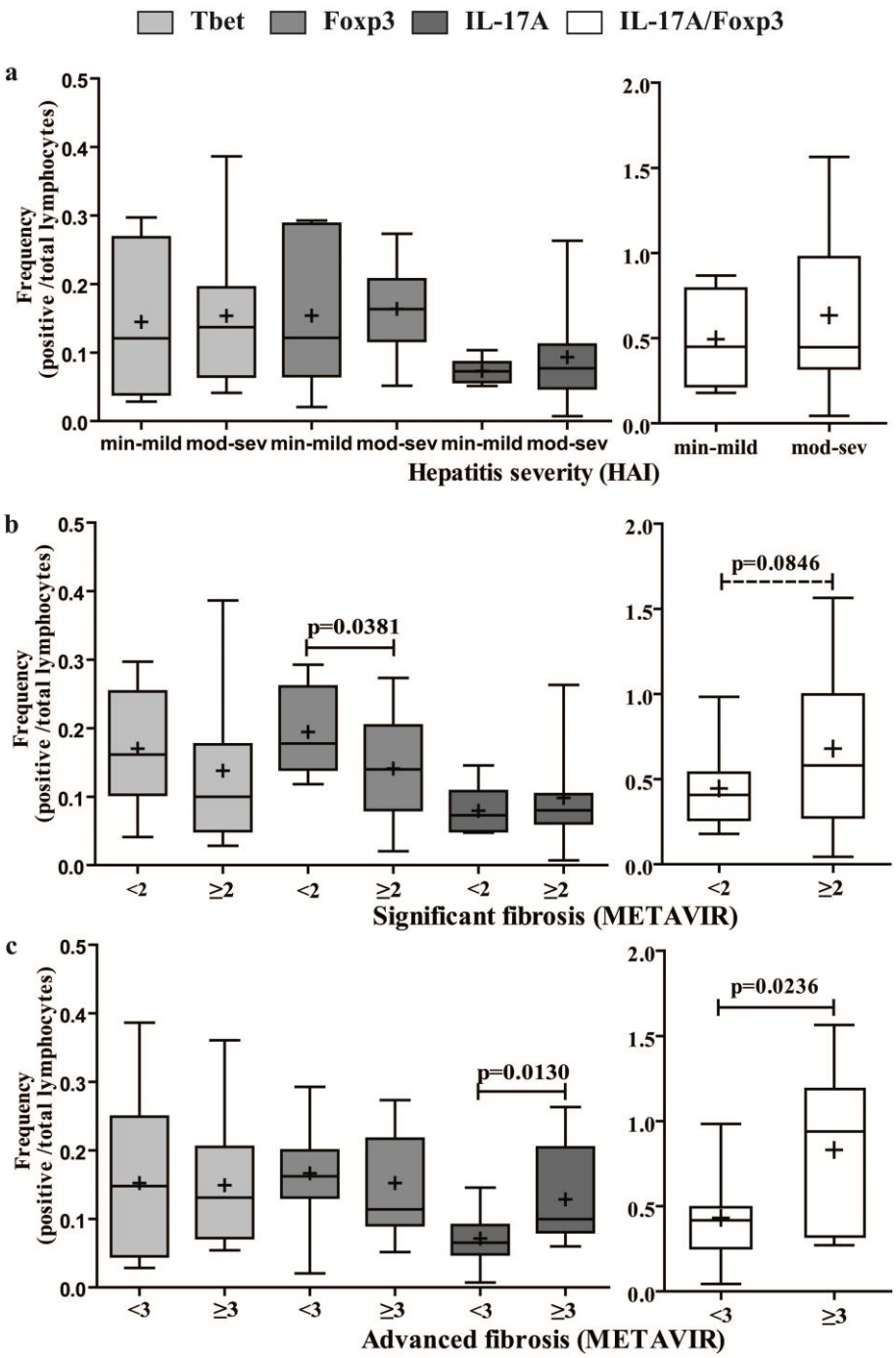
Supplementary Fig. 1 CD4$^+$, CD8$^+$, CD20$^+$, Tbet$^+$, Foxp3$^+$, IL-17A$^+$ lymphocytes immunostaining. Panel 1 shows positive and isotype controls on tonsil sections. Panel 2 shows positive and isotype controls on liver sections. a) CD4$^+$, b) CD8$^+$, c) CD20$^+$, d) Tbet$^+$, e) Foxp3$^+$, and f) IL-17A$^+$ lymphocytes. g), h), i), j), k) and l) show the isotype controls for a), b), c), d), e) and f), respectively.



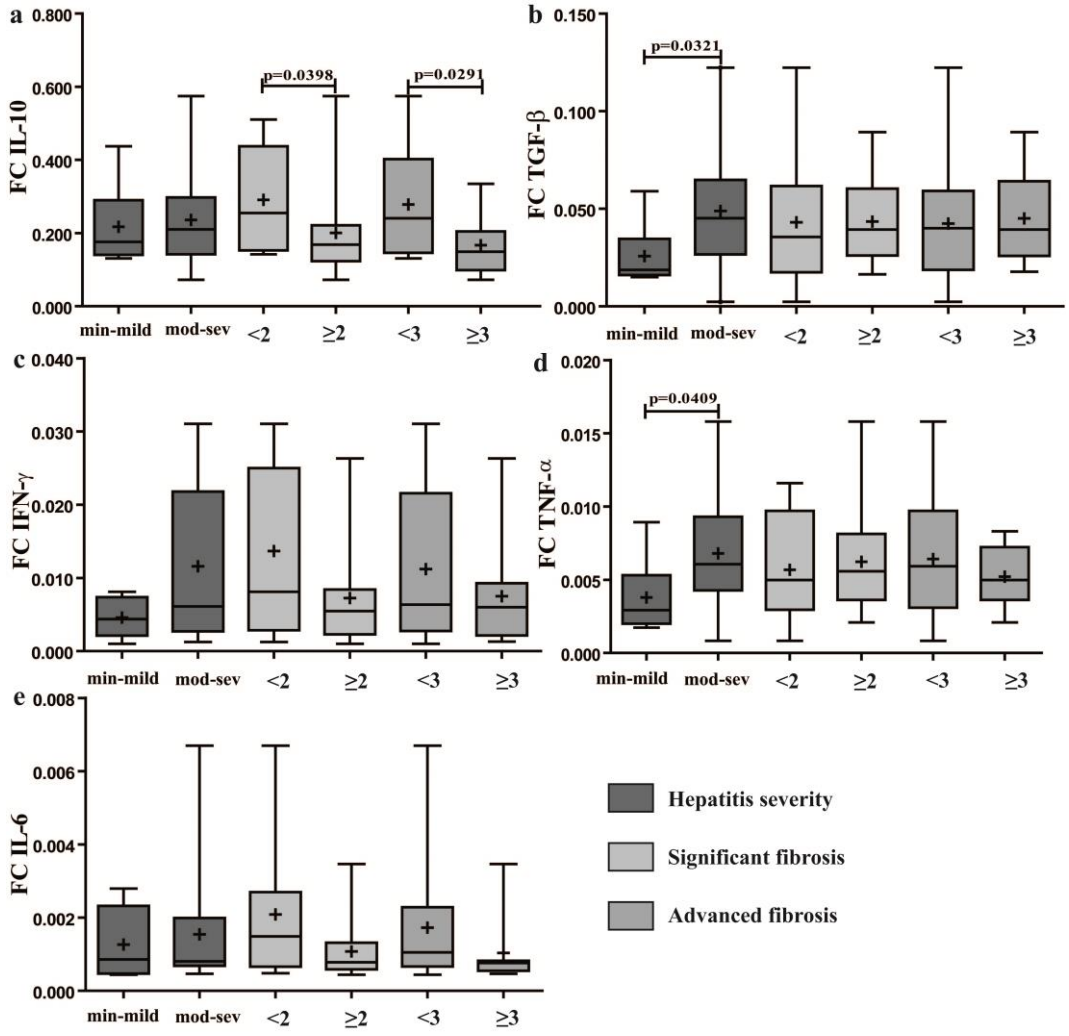
Supplementary Fig. 2 $CD4^+$ and $CD8^+$ lymphocytes distribution in P-P/I areas. A portal infiltrate showing a) $CD4^+$ lymphocytes in the centre and interface area b) $CD8^+$ lymphocytes with peripheral localization within the lymphoid aggregate and c) isotype control.



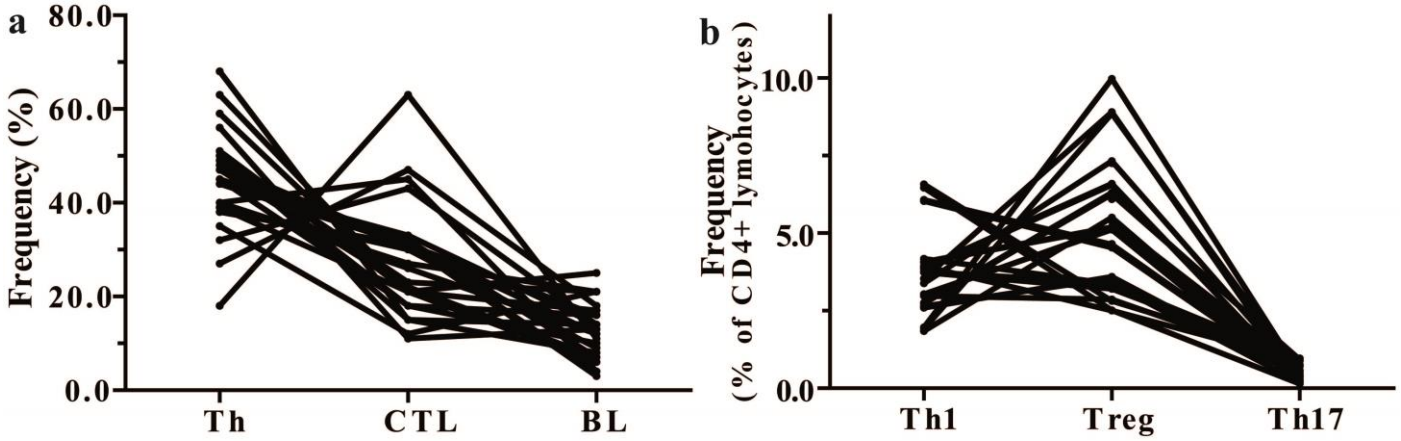
Supplementary Fig. 3 Correlation among cytokine expression levels, intrahepatic lymphocyte frequency, clinical, and virological parameters of CHC patients. a) Correlation between IL-17A$^+$ lymphocyte frequency and TGF-β/IL-6 ratio; b) Correlation between IL-17A$^+$/Foxp3$^+$ lymphocytes ratio and TGF-β/IL-6 ratio, c-e) correlation among TNF-α, IFN-γ and TGF-β expression level; f- i) correlation between transaminase level and CD8$^+$ lymphocyte frequency, IL-17A$^+$ lymphocyte frequency and IL-17A$^+$/Foxp3$^+$ lymphocytes ratio, respectively; j-l) correlation between viral load and TGF-β, IFN-γ, and IL-17A$^+$ lymphocyte frequency, respectively. Frequencies were calculated as immunostained P-P/I lymphocytes/ total P-P/I lymphocytes in all portal tracts of the tissue section (400×). FC: fold change. Spearman's nonparametric correlation (a, d-l) and Pearson's correlation coefficient (b and c) were used to measure the degree of association between the studied parameters.



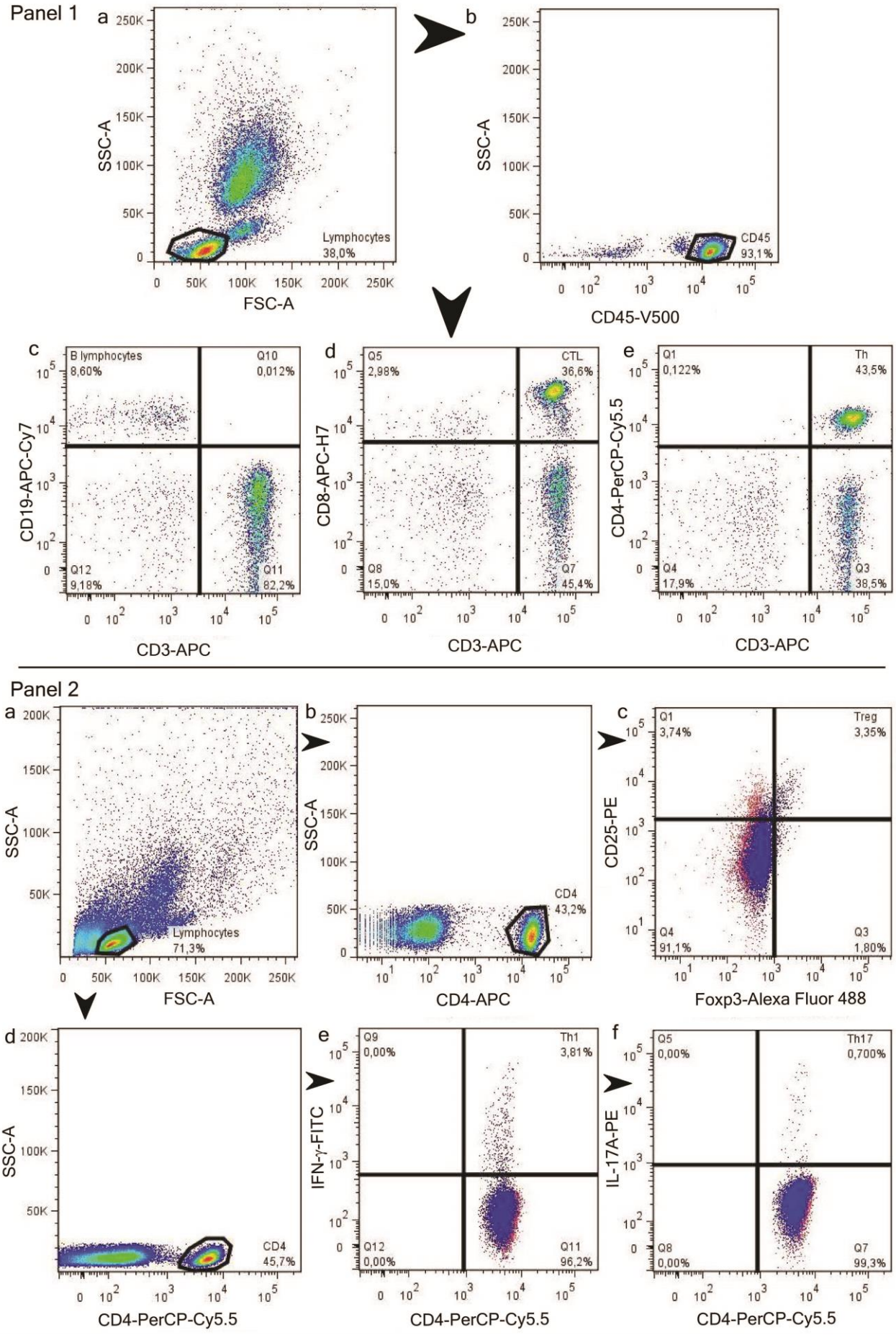
Supplementary Fig. 4 Intrahepatic Th subsets frequency related to histological liver damage parameters. Tbet$^+$, Foxp3$^+$, IL-17A$^+$ lymphocyte frequency as well as IL-17A$^+$/Foxp3$^+$ratio related to a) hepatitis severity, b) significant and c) advanced fibrosis. Hepatitis severity (min: minimal; mod: moderate, sev: severe) according to HAI. Significant (F ≥2) and advanced (F ≥3) fibrosis according to METAVIR. The results are depicted in box plots. Horizontal lines within boxes indicate medians. Horizontal lines outside the boxes represent the 5 and 95 percentiles. Mean is indicated as +. Frequencies were calculated as immunostained P-P/I lymphocytes/ total P-P/I lymphocytes in all portal tracts of the tissue section (400×). The Mann-Whitney U-test and unpaired t-test were used to compare sets of data..



Supplementary Fig. 5 Cytokine expression levels in the liver milieu of CHC patients related to liver damage. a) IL-10, b) TGF-β, c) IFN-γ, d) TNF-α, and e) IL-6 expression levels related to hepatitis severity, significant and advanced fibrosis. Hepatitis severity (min: minimal; mod: moderate, sev: severe) according to HAI. Significant (F ≥2) and advanced (F ≥3) fibrosis according to METAVIR. The results are depicted in box plots. Horizontal lines within boxes indicate medians. Horizontal lines outside the boxes represent the 5 and 95 percentiles. Mean is indicated as +. FC: fold change. The Mann-Whitney U-test and unpaired t-test were used to compare sets of data.



Supplementary Fig. 6 Peripheral lymphocyte populations in CHC patients. a)Th, CTLs and B lymphocytes frequency for each patient, b) Th1, Treg and Th17 lymphocytes frequency for each patient.


Panel 1
a
SSC-A
FSC-A
Lymphocytes
38,0%
b
SSC-A
CD45-V500
CD45
93,1%
c
CD19-APC-Cy7
CD3-APC
B lymphocytes
8,60%
Q10
0,012%
Q12
9,18%
Q11
82,2%
d
CD8-APC-H7
CD3-APC
Q5
2,98%
CTL
36,6%
Q8
15,0%
Q7
45,4%
e
CD4-PerCP-Cy5.5
CD3-APC
Q1
0,122%
Th
43,5%
Q4
17,9%
Q3
38,5%
Panel 2
a
SSC-A
FSC-A
Lymphocytes
71,3%
b
SSC-A
CD4-APC
CD4
43,2%
c
CD25-PE
Foxp3-Alexa Fluor 488
Q1
3,74%
Treg
3,35%
Q4
91,1%
Q3
1,80%
d
SSC-A
CD4-PerCP-Cy5.5
CD4
45,7%
e
IFN-γ-FITC
CD4-PerCP-Cy5.5
Q9
0,00%
Th1
3,81%
Q12
0,00%
Q11
96,2%
f
IL-17A-PE
CD4-PerCP-Cy5.5
Q5
0,00%
Th17
0,700%
Q8
0,00%
Q7
99,3%

77

Supplementary Fig. 7 Gating strategies for flow cytometry analysis. Panel 1: B lymphocytes, CTLs and Th frequency on fresh heparinized blood. Lymphocyte selection by a) SSC-A and FSC-A, and b) CD45 expression. Lymphocyte characterization by c) CD3-CD19^{+} (B lymphocytes), d) CD3^{+}CD8^{+} (CTL) and e) CD3^{+}CD4^{+} (Th cells). The percentage of each population is indicated. Panel 2: Th subpopulations on PBMCs sample. a) lymphocyte selection by SSC-A and FSC-A, b) CD4^{+} lymphocytes selection for Treg characterization, c) CD4^{+}CD25hiFoxp3^{+} (Treg) cells identification (blue) (isotype control staining is also shown in red), d) CD4^{+} lymphocytes selection for Th1 and Th17 characterization; e) CD4^{+}/ IFN-γ^{+} (Th1) lymphocytes and f) CD4^{+}/IL-17A^{+} (Th17) lymphocytes identification after anti-CD3/IL-2 stimulation (blue) (unstimulated PBMCs are also shown in red). The percentage of each population is indicated.