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Pierre Tonnerre, Thomas F. Baumert

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2 **Unraveling the liver antiviral immunity in functional cure of chronic**  
3 **hepatitis B using scRNASeq**

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5 Pierre Tonnerre<sup>1\*</sup> and Thomas F. Baumert<sup>2,3,4,5\*</sup>

6  
7 <sup>1</sup> Institut de Recherche Saint-Louis, Université Paris-Cité, Inserm U976, Team ATIP-Avenir, Paris,  
8 France ; <sup>2</sup> University of Strasbourg, Inserm, Institute for Translational Medicine and Liver Disease,  
9 Inserm UMR\_S1110, Strasbourg, France ; <sup>3</sup>Gastroenterology and Hepatology Service,  
10 Strasbourg University Hospitals, Strasbourg, France ; <sup>4</sup>Institut Hospitalo-Universitaire (IHU),  
11 University of Strasbourg, France; <sup>5</sup> Institut Universitaire de France (IUF), Paris, France

12  
13  
14 \* Correspondence: Dr. Pierre Tonnerre, PhD, Institut de Recherche Saint-Louis, Université Paris-  
15 Cité, Inserm U976, Team ATIP-Avenir, 1 avenue Claude Vellefaux, 75010, Paris, France, email:  
16 [pierre.tonnerre@inserm.fr](mailto:pierre.tonnerre@inserm.fr) and Prof. Thomas F. Baumert, M. D, Institute for Translational  
17 Medicine and Liver Disease, Inserm UMR\_S1110, 3 Rue Koeberlé, Strasbourg, France, email:  
18 [Thomas.Baumert@inserm.fr](mailto:Thomas.Baumert@inserm.fr)

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25 **Editorial**

26 More than 250 million people are infected with hepatitis B virus (HBV) worldwide, despite  
27 availability of an effective prophylactic vaccine[1,2]. Although current standard of care such as  
28 nucleos(t)ide analogs (NA) and interferon-alfa (PEG-IFN) are successful in controlling viral  
29 replication, these therapies very rarely result in complete cure due to persistence of HBV  
30 covalently closed circular DNA (cccDNA) in infected hepatocytes[3]. Thus, CHB patients remain  
31 at high risk of developing cirrhosis and hepatocellular carcinoma[2]. Functional cure (FC), which  
32 is characterized by the loss of viral surface antigen (HBsAg), has emerged as a more feasible  
33 treatment endpoint[4,5]. Indeed, most adults with acute HBV infection can successfully control  
34 the virus. Moreover a fraction of patients with CHB can reach FC following a course of antiviral  
35 therapy[4,6]. Functional cure has been associated with lower risk of disease progression and  
36 HCC [2].

37         The antiviral immune response plays a crucial role for the control of HBV infection. In CHB,  
38 immune cells become dysfunctional due to prolonged exposure to high loads of antigens[6].  
39 Previous studies have pinpointed the important role for virus-specific T cells as a potential key  
40 cellular component of FC[7–9]. Others have highlighted a possible role for innate immune cells,  
41 such as Kupffer and NK cells[10–12]. However, the composition of the liver immune  
42 microenvironment in patients with FC remains largely elusive.

43         To address this important question, an elegant study from Drs. Balakrishnan C. Narmada,  
44 Atefeh Khakpoor, Nirranjan Shirgaonkar and colleagues [13], led by senior authors Profs. Seng  
45 Gee Lim, from the National University Hospital, Singapore, and Ramanuj DasGupta, from the  
46 Genome Institute of Singapore, performed a comprehensive analysis of the liver  
47 microenvironment and peripheral immune responses in patients achieving FC. The authors used  
48 single-cell RNA sequencing and multiparametric flow-cytometry analysis of core needle liver  
49 biopsies and matched-peripheral blood samples to capture 26 distinct major cell types/states,  
50 including hepatocytes, from 29 patients with CHB and 9 patients with FC (patients presenting

51 clearance of viral HBsAg, either upon treatment (n=7) or spontaneously (n=2), with biopsies being  
52 performed between 7 to 84 months after HBsAg loss). The study unraveled a unique liver  
53 microenvironment associated with FC characterized by altered innate and adaptive immune cell  
54 compositions and functional states, as compared to CHB.

55         The first key finding of the study was the association of remodeling of the liver innate  
56 immune microenvironment with FC (Fig. 1). Single cell transcriptomic analysis of hepatic needle  
57 biopsies revealed an altered innate immune signature in FC. In particular, the authors identified  
58 an increase in conventional and liver-resident NK cells in FC, marked by the emergence of a  
59 CXCR6<sup>+</sup> CD69<sup>+</sup> NK population expressing high level of activating receptors (NKp46, CD38,  
60 NKG2D and TRAIL) and lower level of inhibitory receptors (PD-1 and TIM-3)[13]. This was  
61 accompanied by a remodeling of the myeloid liver compartment, characterized by increased  
62 frequencies of pro-inflammatory CD14<sup>+</sup> monocytes, a subset of conventional dendritic cells (DC2)  
63 as well as FOLR2/VSIG4 Kupffer cells, in FC compared to CHB. The authors also reported a  
64 higher number of marginated neutrophils in FC. Interestingly, computational analysis of inferred-  
65 interactions identified high inferred-interactions between neutrophils and endothelial cells,  
66 through chemokine/receptor interactions, as a potential mechanism for increased neutrophil  
67 trafficking in the liver of FC patients [13]. Importantly, most of the above findings were validated  
68 at the protein level using flow cytometry and by comparison with a single-cell transcriptomic  
69 dataset of healthy liver tissues [14] suggesting an activation state of the intrahepatic innate  
70 immune microenvironment in FC. In addition, computational analysis of inferred-interactions  
71 highlighted potential interactions between innate cells, particularly DC2 and Kupffer cells, with  
72 CD4<sup>+</sup> and CD8<sup>+</sup> T cells, suggesting an important role for myeloid cells in eliciting T cell immune  
73 responses [13]. Interestingly, the authors pinpointed the similarities between the observed  
74 FOLR2/VSIG4 Kupffer cell gene profile and an IL2-dependent “KC2” subtype of Kupffer cells,  
75 recently reported by De Simone *et al*, with the ability to cross-present antigens and rescue  
76 exhausted T cells in mouse models of HBV infection[12].

77           The second important finding was the discovery of restored liver CD4+ T cell responses  
78 as a hallmark of FC (Fig. 1). Investigating T cell populations and associated transcriptomic  
79 signatures in patients with FC, the authors observed a marked reduction in FOXP3+ regulatory T  
80 cells (Tregs) and a lower frequency of exhausted CD8+ T cells, which were associated with  
81 decreased expression of inhibitory receptors encoding genes[13]. Importantly, the authors  
82 described an increased frequency of CD4+ T cells with an effector/cytotoxic gene signature  
83 (PRF1, GZMA, GZMK TNF, NCR3), referred as “CD4-CTLs” by the authors[13]. Again, these  
84 findings were further validated at the protein level by flow cytometry analysis of liver samples. It  
85 has to be noted that these signatures were not present in patient’s paired-blood samples,  
86 highlighting the local compartmentalization of the immune alterations into the liver of patients with  
87 FC, or the dilution of these alterations in the peripheral blood. Nevertheless, using peptide pools  
88 covering the different proteins of the virus (HBx, Core, Polymerase and HBs), the authors  
89 successfully identified HBV-specific T populations in the peripheral blood of patients with CHB  
90 and FC. Strikingly, in contrast to CHB, FC associated with an increase in the frequency of  
91 Polymerase- and HBs-specific T cell responses, but not Core and HBx, which were dominated by  
92 a CD4+ polyfunctional (IFN $\gamma$ +TNF $\alpha$ +) phenotype[13]. These results are in line with recent findings  
93 from Hoogeveen *et al*[9] and highlight the heterogeneity and functional plasticity of HBV-specific  
94 T cells, depending on the targeted viral protein. Concomitant with these findings, the authors  
95 identified a subset of hepatocytes with higher expression levels of MCH-II genes predominantly  
96 present in patients with FC. These results suggest that liver CD4-CTLs or HBV-specific CD4 T  
97 cells could directly recognize a subset of hepatocytes through TCR recognition, contributing to T  
98 cell priming and viral control in FC. Interestingly, using droplet digital PCR and single cell cDNA  
99 amplification of viral sequences, the authors provided evidence of transcriptionally active cccDNA  
100 in FC, albeit at much lower levels than in CHB, supporting low-level viral replication, hence T cell  
101 antigen stimulation.

102           The strength of the study is its reasonable numbers of patient samples, the study of the  
103 liver microenvironment as its focus area, including the analysis of the liver virological features in  
104 in patients with FC, the use of scRNASeq to study the cellular phenotypes, providing an invaluable  
105 patient-derived data resource for additional studies, and the phenotypic validation at the protein  
106 level of key findings. A potential limitation is the pending mechanistic and functional studies to  
107 understand the impact of the findings for the biology of FC.

108           Collectively, this study elegantly highlights the intrinsic link between intrahepatic immune  
109 cell activation and virus control, thereby providing novel biological insights into the liver immune  
110 composition and cell states in FC. Interestingly, since most of these changes - as compared to  
111 CHB - were not observed in paired-PBMCs, the study again confirms the need for more studies  
112 investigating the human hepatic microenvironment in FC and the complex interactions and cell-  
113 cell communication between infected hepatocytes and the liver immune ecosystem. Novel  
114 technologies such as spatial transcriptomics[15,16] or multiplexed imaging[17,18] will provide  
115 additional contributions to the questions in the future. The next steps of investigation will be the  
116 study of the molecular and cellular mechanisms leading to the re-emergence of HBV-specific  
117 CD4+ T cell responses in the liver, and whether CD4+ T cells directly target infected-hepatocytes  
118 expressing HLA class II-restricted antigens.

119           What are the clinical implications of the study by Narmada, Khakpoor and colleagues? A  
120 detailed understanding of the liver immunobiology associated or leading to FC, will be useful to  
121 contribute to develop urgently needed antiviral strategies for FC. The study may provide  
122 opportunities to identify blood-based biomarkers associated with FC. In combination with  
123 additional mechanistic data, the study of Narmada, Khakpoor colleagues will open new insights  
124 informing the design and development of immunotherapies harnessing innate immune response  
125 as well as the therapeutic potential of CD4+ T cells to achieve FC.

126

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136

137 **Competing interests:**

138 The authors declare no competing interests.

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140 **Author contributions:**

141 P.T. and T.F.B. conceptualized and wrote the manuscript.

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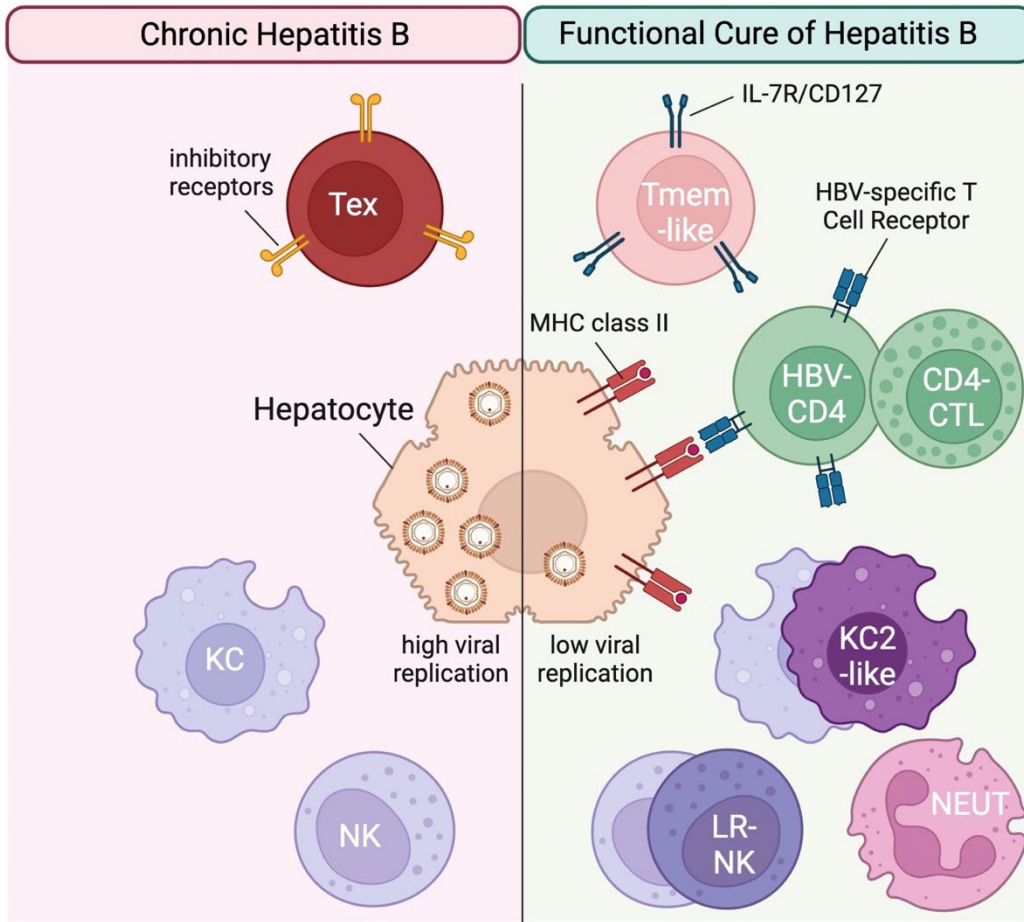
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221 **Figure 1: Remodeling of the liver antiviral immunity in functional cure (FC) of chronic**  
 222 **hepatitis B unraveled using single cell RNASeq of patient derived liver tissues [13].** Patients  
 223 with FC are characterized by *in situ* low-levels of HBV viral replication, the presence of MHC  
 224 class-II expressing hepatocytes, decreased frequency of intrahepatic exhausted T cells (Tex), the  
 225 presence of memory-like T cells expressing IL7R/CD127 (Tmem-like) and CD4 T cells with  
 226 cytotoxic functions (CD4-CTL), and the re-emergence of HBV-specific CD4 T cells targeting  
 227 Polymerase and HBs epitopes. This altered T cell response in FC is accompanied by a re-  
 228 enforced innate immune response characterized by increased Liver Resident-NK cells expressing  
 229 CXCR6 and CD69 (LR-NK), FOLR2/VSIG4 Kupffer cells (KC2-like) and marginated neutrophils  
 230 (NEUT) as described by Narmada, Khakpoor and colleagues [13]. Figure created with  
 231 BioRender.com