

Differential gene expression, irrespective of circulating Hepatitis B Surface Antigen levels, between Inactive Carrier and Nucleos(t)ide Analogue-Treated Hepatitis B Virus patients

Noé R. Montanari,^{1,a} Nádia Conceição-Neto,^{2,a} Ilse Van Den Wyngaert,³ Gertine W. Van Oord,¹ Zwier M. A. Groothuisink,¹ Sandra Van Tilburg,³ Robert A. de Man,¹ Jeroen Aerssens,² and André Boonstra¹

¹Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, the Netherlands, ²Infectious Diseases Biomarkers, Janssen Research and Development, Beerse, Belgium, and ³Computational Sciences, Janssen Research and Development, Beerse, Belgium

Long-term viremia control in chronic HBV patients occurs either spontaneously in inactive carrier (IC) patients or therapy-induced by nucleos(t)ide analogues (NUC). To better understand the characteristics of viremia control, we evaluated gene expression in purified leukocyte subsets from IC versus NUC-treated patients, and evaluated the putative modulatory effects of hepatitis B surface antigen (HBsAg). We observed that gene expression in NUC-treated patients differed markedly from IC patients, especially in dendritic cells, monocytes, and CD8⁺ T cells, while serum HBsAg levels had little effect. Nevertheless, based on our findings it cannot be excluded that HBsAg may act locally in the infected liver or preferentially affects HBV-specific cells.

Keywords. blood leukocytes; chronic HBV; transcriptome; inactive carriers; antiviral; HBsAg.

Chronic infection with hepatitis B virus (HBV) is a major global health burden. The World Health Organization estimates that 257 million individuals are living with chronic HBV, which can lead to liver fibrosis, cirrhosis, and ultimately hepatocellular carcinoma [1].

Chronic HBV patients can be categorized into 4 clinical phases, which vary in inflammatory activity and are highly variable in serum levels of alanine transferase (ALT) and viral replication (HBV-DNA). Patients in the inactive carrier (IC) phase (also known as chronic HBeAg-negative HBV infection) are characterized by the ability to naturally control viral replication, and exhibit low HBV-DNA and normal ALT levels throughout an indefinite period of time, likely via immune-mediated control [2, 3]. In contrast to IC patients, patients with active disease (elevated ALT and HBV-DNA) usually receive antiviral therapy consisting of nucleos(t)ide analogues (NUC) [3]. These medications are not curative but are highly effective in reducing HBV-DNA and normalizing ALT levels. However, even when viral replication is controlled and serum ALT levels are normalized during the IC phase and during NUC treatment, active translation of viral antigens, such as hepatitis B surface antigen (HBsAg), continues. High serum HBsAg has been postulated to be immunomodulatory, which is in line with phenomena seen in other chronic viral infections where continuous high antigen exposure induces a state of exhaustion, particularly in T cells [4]. A clear understanding of whether high concentrations of HBsAg in serum are responsible for the lack of an effective immune response to HBV in patients is still lacking.

In this study, we therefore examined the frequencies and transcriptome of blood-sorted monocytes, dendritic cells (DC), B cells, CD4⁺ and CD8⁺ T cells, natural killer (NK), and NKT cells of chronic HBV patients who controlled viral replication either spontaneously or by effective NUC treatment. In addition, the putative modulatory effect of HBsAg was evaluated by comparing HBsAg-high versus HBsAg-low IC patients.

METHODS

Patient Cohort

Cryopreserved peripheral blood mononuclear cells (PBMCs) from chronic HBV patients were selected from the Erasmus MC biobank. All patients tested negative for hepatitis D virus (HDV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV), and had minimal liver fibrosis (METAVIR ≤ F2). As a case-control comparison, 12 treatment-naive IC patients present in the HBsAg-high (median, 11 513 IU/mL; range, 5707–24 538 IU/mL) and 8 HBsAg-low (median, 99 IU/mL; range, 1–189 IU/mL) were included (Supplementary Table 1). The categorization of patients as HBsAg-high or HBsAg-low was based on the top and 40% serum HBsAg distribution (high, ≥ 1783 IU/mL; low, ≤ 989 IU/mL) of over 800 samples available in the Erasmus MC biobank. For testing of IC versus NUC patients, 24 treatment-naive IC patients were

Received 8 July 2020; editorial decision 23 September 2020; accepted 29 September 2020; published online October 3, 2020.

Correspondence: André Boonstra, PhD, Erasmus MC, University Medical Center Rotterdam, 3015 CE, Rotterdam, the Netherlands (p.a.boonstra@erasmusmc.nl).

^aN. R. M. and N. C.-N. contributed equally.

The Journal of Infectious Diseases® 2022;225:1471–6

© The Author(s) 2020. Published by Oxford University Press for the Infectious Diseases Society of America. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
DOI: 10.1093/infdis/jiaa614

tested against 16 NUC-treated patients with normalized ALT levels at least 12 months prior to sample collection who were on treatment for a median of 3.5 years (interquartile range, 3–5 years; [Supplementary Table 1](#)). The study and its protocols were approved by the institutional ethical review board. All patients provided informed written consent.

Flow Cytometry and Cell-Sorting Analysis

PBMCs were sorted for NK cells (CD3⁻CD56⁺), NKT cells (CD3⁺CD56⁺), CD4⁺ and CD8⁺ T cells (CD3⁺CD4⁺ and CD3⁺CD8⁺), monocytes (CD14⁺CD19⁻), B cells (CD19⁺CD14⁻), and DC (BDCA1⁺CD19⁻CD14⁻) using a fluorescence-activated cell sorting (FACS)-ARIA-II ([Supplementary Figure 1](#)). Five hundred sorted cells of each population were collected in Lyse-and-Go (ThermoFisher, discontinued) and stored at -80°C. Lysates were processed using the GeneChip HT Pico Kit (ThermoFisher).

Microarray Analysis

Fragmented biotin-labelled double-stranded cDNA was hybridized on a human Clariom GO Screen Assay (ThermoFisher) and scanning was done using GeneTitan. Gene expression values were normalized by robust multiarray average normalization on the microarray probe-level data [5], and downstream analysis was done in R version 3.4.2. Unsupervised analysis using spectral maps was performed and showed no batch effects. To validate that the sorting procedure yielded pure populations, we evaluated the expression levels of key marker genes for each leukocyte population such as CD19 for B cells, NKG7 for NK cells, and CD4 and CD8 for T-cell subsets ([Supplementary Figure 2](#)). A supervised analysis was performed using the *limma* package [6] for comparisons between patient groups within cell populations and corrected *P* values for multiple testing across genes $\leq .05$ were considered significant. The affected pathways were analyzed using MLP (mean log *P* analysis) and GO Biological Process [7]. The considered cutoffs for MLP were lower (5) and upper (100) threshold for gene set size where 7100 pathways from the Biological Process source were used.

Statistical Analysis

Group comparison of clinical parameters was performed using 2-way unpaired *t* testing, unless indicated otherwise. Group differences in cell frequencies were analyzed by Mann-Whitney testing using GraphPad Prism.

RESULTS

Diversity in Cell Frequencies and Gene Expression Profiles of Peripheral Sorted Blood Leukocytes Irrespective of Circulating HBsAg Levels

To determine the effect of ongoing HBsAg exposure, gene expression profiling was performed on FACS-sorted peripheral leukocytes obtained from HBsAg-high and HBsAg-low IC

patients ([Figure 1A](#)). IC patients with distinct HBsAg levels displayed no significant differences in leukocyte subset frequencies ([Supplementary Figure 3](#)). All IC patients were HBeAg negative, had low HBV-DNA, and normalized ALT levels ([Supplementary Table 1](#)). As shown in [Figure 1B](#), comparison of the 2 different groups yielded only few or no differentially expressed genes (DEG), using adjusted *P* value $\leq .05$ and 1.5 log fold-change (logFC) as cutoff, for sorted B cells (*GAS6-AS1*), T-cell subsets (CD4⁺ and CD8⁺ (*SLC33A1*, *OBSCN*), NK cells and NKT cells, and DC (*MOCOS*, *BPIFAF3*, *ZN665*, and *MAS4A4E*) ([Figure 1D](#)). In monocytes, the vast majority of DEG (22/35) were upregulated in the HBsAg-low group, although some immune-related genes (eg, *C6*, *ADA*, and *DEFB134*) were upregulated in the HBsAg-high group ([Figure 1B](#) and [1C](#)).

HBV Viremia Control in the IC Phase Versus During NUC Therapy Exhibits Altered Frequencies and Gene Expression Profiles in Blood Leukocytes

The identification of the underlying mechanisms that determine viral control are essential for the creation of new treatment strategies. Therefore, we compared blood leukocyte composition and transcriptome in 24 IC and 16 NUC-treated patients. Although the groups exhibited comparable liver enzymes (ALT and aspartate transaminase) and virological characteristics (HBV-DNA and HBsAg; [Supplementary Table 1](#)), higher frequencies of DC (median, 0.48% vs 0.25%, respectively; *P* value = .03) and CD8⁺ T cells (median, 31% vs 22%, respectively; *P* value = .001) were found in NUC-treated patients compared to IC patients, whereas the percentage of CD4⁺ T cells was higher in IC than NUC-treated patients (median 68% vs 51%; *P* value = .002; [Supplementary Figure 4](#)). These findings suggest an altered balance between the CD4⁺ and CD8⁺ T-cell populations in the IC versus NUC-treated groups.

Next, gene expression profiling on FACS-sorted blood leukocytes from IC and NUC-treated patients was conducted. Using adjusted *P* values $\leq .05$ and 1.5 logFC as cutoff, we observed that DC showed the highest number of DEG (805), followed by CD8⁺ T cells (189), monocytes (42), and B cells (13). In contrast, low numbers of DEG to no DEG were detected in CD4⁺ T cells, NK cells, and NKT cells (3, 1, and 0 DEG, respectively; [Figure 2A](#)).

Among the list of DEGs, DC from NUC-treated patients exhibited a higher expression of HLA-related genes (ie, *HLA-DQB1*, *HLA-C* or *HLA-DMB*), Interferon Stimulated Gene (ISG) (*NKAP*, *IFITM3*, *IRF2BP2*, *ISG15*, *IRAK3*, and *MAPK1*), caspases (*CASP1*, *CASP4*, and *CARD16*), chemokines and chemokines receptors (*CXCL16* and *CXCR4*), and also *TLR8* (lowest adjusted *P* value 7.2e-10; [Figure 2B](#)). On the other hand, expression of type I interferon (IFN) genes (IFN- ϵ and IFN- α 13) was higher in IC patients. All DEG identified for the CD8⁺ T cells showed higher expression in NUC-treated patients. Among these genes, we detected some related to cytotoxicity (ie, *KLRD1*,

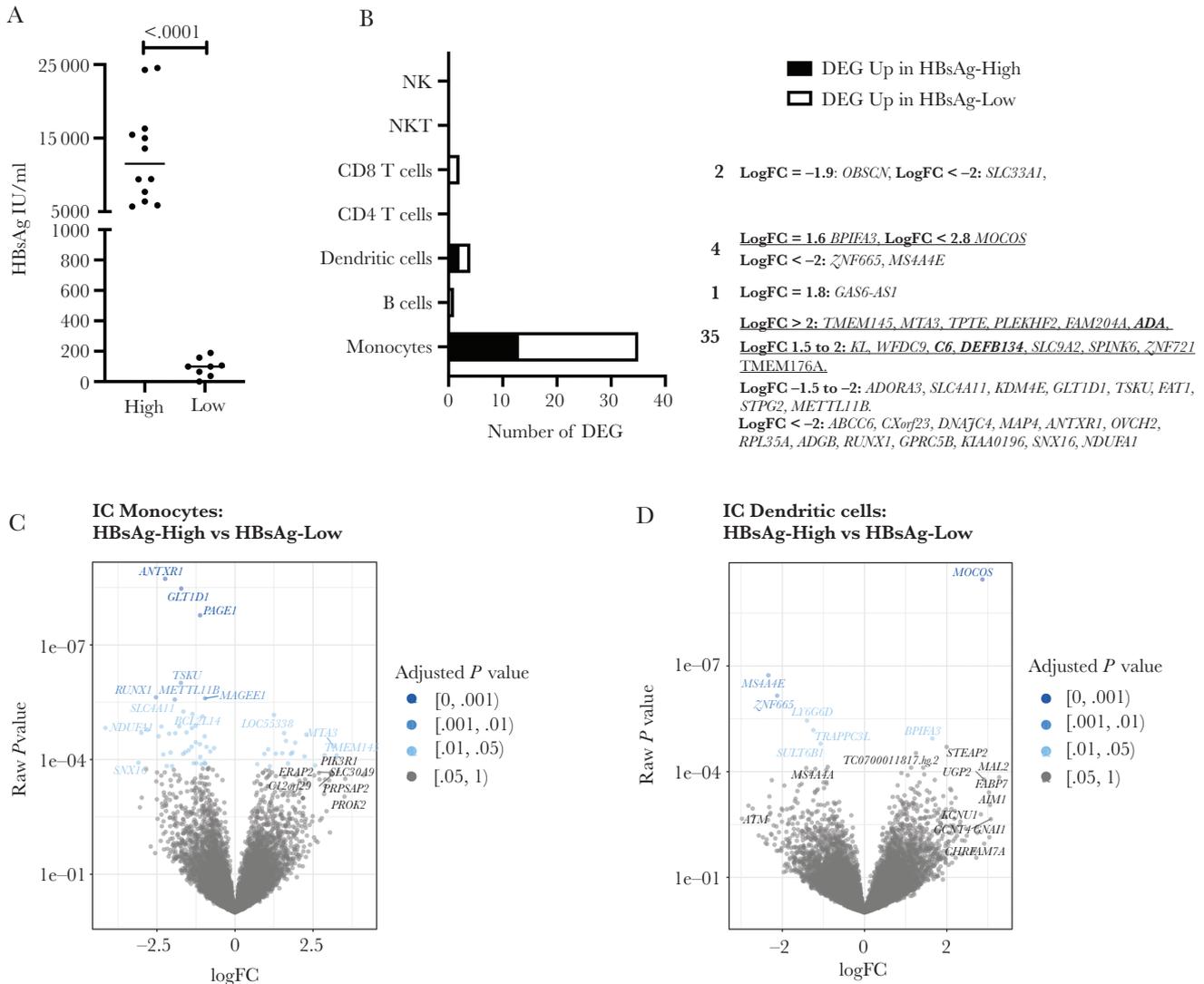


Figure 1. A, Serum HBsAg distribution in IC patients with high and low HBsAg levels (high mean 12 807 IU/mL, minimum 5707 IU/mL, maximum 24 538 IU/mL; low mean 94 IU/mL, minimum 1 IU/mL, maximum 189 IU/mL). Unpaired 2-way *t* test was conducted. B, Number of DEG (adjusted *P* value $\leq .05$ and 1.5 logFC) in sorted blood leukocytes from IC patients with distinct HBsAg levels. Black bars and underlined genes indicate DEG upregulated in the HBsAg-high group whereas white bars and nonunderlined genes indicate DEG upregulated in the HBsAg-low group. Genes are ordered by logFC increase. C and D, Volcano plot of DEG in monocytes and dendritic cells from IC patients with HBsAg-high versus HBsAg-low. Abbreviations: DEG, differentially expressed genes; HBsAg, hepatitis B surface antigen; IC, inactive carrier; logFC, log fold change; NKT, natural killer T cell.

KLRG1, *GZMH*, and *GZMB*), immune-cell trafficking (*S1PR1* and *CX3CR1*), and the transcription factor *IKZF5* from the IKAROS family. Moreover, monocytes exhibited an increased expression of Toll-like receptor (TLR)-related genes *LY6E* and *STK4* in NUC-treated patients compared to IC. Comparable to CD8⁺ T cells, most of the DEG in B cells were increased in NUC-treated patients. Among the genes with the highest fold-change, immunoglobulins were the primary component. The DEG list for all leukocyte populations is shown in [Supplementary Table 2](#). Finally, we conducted pathway analysis to identify the major biological processes driving the difference in gene expression profiles between IC and NUC-treated patients. As shown in [Figure 2C](#), in DCs

this was largely driven by signal recognition particle (SRP)-dependent protein targeting to membrane and endoplasmic reticulum pathways, followed by type I and II signaling, T-cell costimulation, and regulation of production of various interleukins (see also [Supplementary Table 3](#)). Moreover, CD8⁺ T cells were primarily driven by ubiquitin-related pathways (not shown) and monocytes by gap-junction assembly (not shown).

DISCUSSION

In this study, we observed that the gene expression profiles of patients with low/undetectable HBV-DNA and normalized ALT levels receiving NUC therapy differed markedly from IC

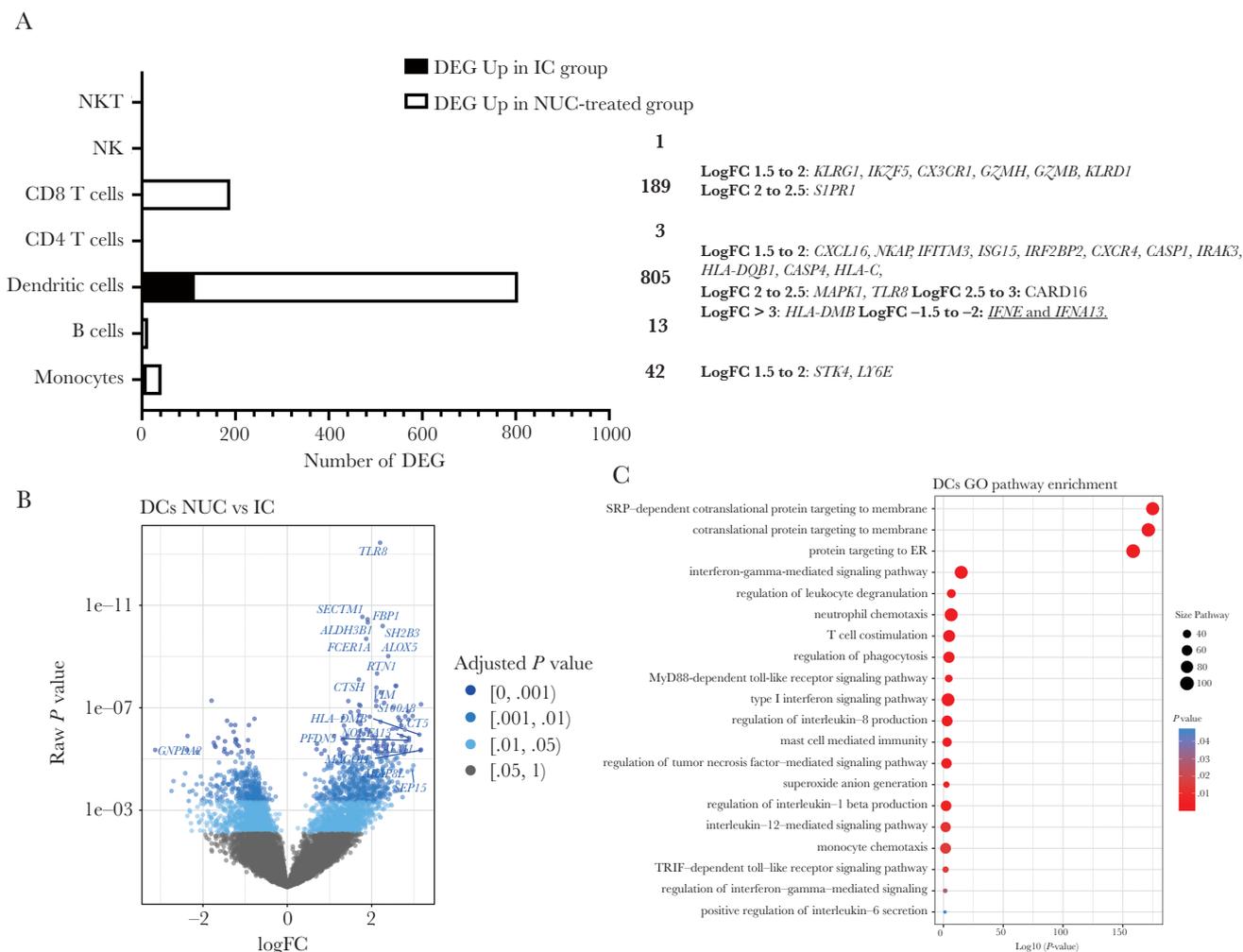


Figure 2. A, Number of DEG (adjusted *P* value $\leq .05$ and 1.5 logFC) in sorted blood leukocytes in IC vs NUC-treated patients. Black bars and underlined genes indicate DEG upregulated in the IC group whereas white bars and nonunderlined genes indicate DEG upregulated in the NUC-treated group. Genes are ordered by logFC increase. B, Volcano plot of DEG in DC in IC vs NUC-treated patients. C, Altered immune-related gene signaling from pathway analysis in DC from IC vs NUC-treated patients. Abbreviations: DEG, differentially expressed genes; IC, inactive carrier; logFC, log fold change; NKT, natural killer T cell; NUC, nucleos(t)ide analogue.

patients. However, distinct serum HBsAg levels only minimally affected gene expression profiles of blood leukocyte subsets in chronic HBV patients.

Our findings show that the transcriptome, particularly of DC, CD8⁺ T cells, and, to a lesser extent, monocytes are markedly different between IC and NUC-treated patients. This was unexpected because, although the mode of viral control differs, both groups of patients exhibit prolonged low/undetectable HBV-DNA and normalized ALT serum levels. The distinct gene expression profiles between IC and NUC-treated patients in DC and CD8⁺ T cells suggest that distinct regulatory processes are active. Indeed, we observed that the ISG expression levels in DC and cytotoxicity-related genes in CD8⁺ T cells are increased in NUC-treated patients as compared to IC patients. In contrast to DC, CD8⁺ T cells, and monocytes, differential gene expression was not observed for B, NK, NKT, and CD4⁺ T cells between IC and NUC-treated

HBV patients, and it does not lead to altered functioning of these cell types in the examined patient groups. Thus, it is tempting to speculate that although NUC-treated patients might experience a partial improvement in their immune response against HBV [8], it fails to reconstitute to an immune viral control state as seen in IC patients.

The continuous presence of high levels of HBsAg are generally considered to be an important factor impacting immune cell activity, with HBV-specific CD8⁺ T cells being examined most frequently with respect to the induction of T-cell exhaustion [4, 9, 10], thereby impeding the establishment of a long-lasting and effective immune response capable of eliminating or controlling the infection [11, 12]. To our surprise, transcriptomic characterization of highly pure FACS-sorted blood leukocytes collected from patients with highly distinct HBsAg serum levels exhibited only minimal modulation of gene expression levels. DEG were

only identified for monocytes between the HBsAg-high and HBsAg-low groups but not, or in very low numbers, for CD4⁺ and CD8⁺ T cells, NK, NKT, B cells, and DC (Supplementary Figure 5B). Importantly, the inclusion of a smaller cohort of NUC-treated patients (Supplementary Table 4; n = 15) with contrasting serum HBsAg level recapitulated the cell frequencies data and transcriptomic findings seen in the IC cohort with DEG identified solely in antigen-presenting cells (Supplementary Figure 5).

Interestingly, a previous study on sorted CD4⁺ and CD8⁺ T cells from IC patients found the gene expression of TLR-signaling (*MYD88*), cytotoxicity (*GZMA*, *GZMK*), and nuclear factor- κ B signaling (*BST2*) to be upregulated in patients with less than 1.5×10^3 versus more than 15×10^4 IU/mL HBsAg [13]. These discrepancies might be explained by differences in patient selection, methodology, or confounding factors—although limited information is available in the study. Moreover, assessment of the major blood leukocyte population revealed comparable frequencies irrespective of the HBsAg antigenemia level, as previously reported [14]. In light of these findings, studies evaluating the immunological effects—in the context of effective immune control—of HBsAg blockers should preferentially also address the intrahepatic compartment and HBV-specific responses.

In conclusion, our approach, using *ex vivo* evaluation of the transcriptome of sorted highly pure blood leukocytes, does not provide evidence that lower versus higher levels of peripheral HBsAg alters gene expression of immunomodulatory proteins with suppressive activity on the total polyclonal leukocyte population.

On the basis of our findings alone, it cannot be excluded that the HBsAg levels observed in this study might still be biologically excessive and thus capable of negatively modulating the immune response at the same level or even higher HBsAg levels. Moreover, it cannot be discounted that HBsAg may preferentially act by modulating the activity of HBV-specific cells and that more profound effects are delivered locally in the infected liver. In addition, NUC-treated patients may fail to reconstitute the peripheral immune response comparable to IC patients, which might explain HBV relapses during antiviral withdrawal.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank Heleen van Santen and Sara de Bil for help with collecting patient material and José Debes, MD, for his guidance in the writing process and critical thinking.

Author contributions. N. R. M. and A. B. designed research. G. W. O., Z. M. G., S. V. T., and I. V. d. W. contributed to acquisition of data. N. R. M., N. C.-N., A. B., and J. A. analyzed and interpreted the data. N. R. M. and A. B. wrote the manuscript. N. C.-N., N. R. M., and J. A. contributed to the statistical analysis. R. A. d. M., A. B., and J. A. provided essential research tools. A. B. and J. A. supervised the study. N. C.-N., A. B., and J. A. provided critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Financial support. This work was supported by the Foundation for Liver and Gastrointestinal Research.

Potential conflicts of interest. N. C.-N., I. V. d. W., and J. A. are employees of Janssen Pharmaceutica NV. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

- Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet* **2015**; 386:1546–55.
- Vanwolleghem T, Hou J, van Oord G, et al. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. *Hepatology* **2015**; 62:87–100.
- European Association for the Study of the Liver. EASL 2017 Clinical practice guidelines on the management of hepatitis B virus infection. *J Hepatol* **2017**; 67:370–98.
- McLane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T cell exhaustion during chronic viral infection and cancer. *Annu Rev Immunol* **2019**; 37:457–95.
- Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **2003**; 4:249–64.
- Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **2015**; 43:e47.
- Raghavan N, Verbeke T, ADBwcbJ C, Amaratunga D, Casneuf T, Ligtenberg W. *MLP: MLP*. R package version 1.36.0. **2020**.
- Boni C, Laccabue D, Lampertico P, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. *Gastroenterology* **2012**; 143:963–73.e9.
- Ye B, Liu X, Li X, Kong H, Tian L, Chen Y. T-cell exhaustion in chronic hepatitis B infection: current knowledge and clinical significance. *Cell Death Dis* **2015**; 6: e1694.

10. Utzschneider DT, Alfei F, Roelli P, et al. High antigen levels induce an exhausted phenotype in a chronic infection without impairing T cell expansion and survival. *J Exp Med* **2016**; 213:1819–34.
11. Kondo Y, Ninomiya M, Kakazu E, Kimura O, Shimosegawa T. Hepatitis B surface antigen could contribute to the immunopathogenesis of hepatitis B virus infection. *ISRN Gastroenterol* **2013**; 2013:935295.
12. Yeo YH, Ho HJ, Yang HI, et al. Factors associated with rates of HBsAg seroclearance in adults with chronic HBV infection: a systematic review and meta-analysis. *Gastroenterology* **2019**; 156:635–46.e9.
13. Gill US, Hansi N, Bert NL, et al. HBV-specific T cell responses in low replicating inactive carrier patients are independent of hepatitis B surface antigen load. *J Hepatol* **2018**; 68:S793–4.
14. Aliabadi E, Mix C, Manns MP, Kraft A, Cornberg M. FRI-131-Impact of HBsAg level on cellular immune responses in HBeAg negative patients with chronic hepatitis B virus infection. *J Hepatol* **2019**; 70:e445.