Molecular Therapy
Methods & Clinical Development
Original Article

Robust hepatitis B vaccine-reactive T cell responses in failed humoral immunity

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While virus-specific antibodies are broadly recognized as correlates of protection, virus-specific T cells are important for direct clearance of infected cells. Failure to generate hepatitis B virus (HBV)-specific antibodies is well-known in patients with end-stage renal disease. However, whether and to what extent HBV-specific cellular immunity is altered in this population and how it influences humoral immunity is not clear. To address it, we analyzed HBV-reactive T cells and antibodies in hemodialysis patients post vaccination. 29 hemodialysis patients and 10 healthy controls were enrolled in a cross-sectional study. Using multiparameter flow cytometry, HBV-reactive T cells were analyzed and functionally dissected based on granzyme B, interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), and IL-4 expression. Importantly, HBV-reactive $CD4^+$ T cells were detected not only in all patients with sufficient titers but also in 70% of non-responders. Furthermore, a correlation between the magnitude of HBV-reactive $CD4^+$ T cells and post-vaccination titers was observed. In summary, our data showed that HBV-reactive polyfunctional T cells were present in the majority of hemodialysis patients even if humoral immunity failed. Further studies are required to confirm their *in vivo* antiviral capacity. The ability to induce vaccine-reactive T cells paves new ways for improved vaccination and therapy protocols.

INTRODUCTION

End-stage renal disease (ESRD) patients with different etiology are at high risk for numerous infectious diseases due to constant hemodialysis and frequent application of human blood products.[1,2] Moreover, these patients reveal secondary immune deficiency that leads to higher incidence, severe and protracted disease course, and, consequently, increased mortality and morbidity in this cohort.[3] Thus, it is logical that the appropriate vaccination against various pathogens is of utter importance for proper ESRD patient management.

Appropriate immunization against hepatitis B virus (HBV) is one of the therapeutic priorities in ESRD management. Its importance is emphasized by lower virus clearance and higher chronification rate in this patient group.[4,5] The chronic diseased course often results in liver fibrosis and higher incidence of liver malignancies. Moreover, other systemic complications are not uncommon in chronic HBV course. Last but not least, chronic HBV infection is a severe obstacle in kidney transplantation and subsequent posttransplant immunosuppression.[6]

ESRD patients show significantly lower HBV vaccination efficacy as compared to healthy individuals.[7] Despite intensified approaches, on average 60% of ESRD patients achieve sufficient anti-hepatitis B surface antibody (anti-HBs) titers as compared to 95% in healthy populations.[8–10] The exact underlying mechanisms for failed vaccination are not clear. Besides direct immunotoxic effects of numerous uremic metabolites, lack of growth factors and hormones and inadequate insulin availability resulting in impaired cellular and humoral immunity are discussed among reasons for failed vaccination.[11–13] Further, impaired cell function due to the membrane contacts and dialysis shear stress was suspected among reasons for immune deficiency in this patient cohort.[3,14,15]

Due to the lower vaccination efficacy, alternative protocols for HBV vaccination in hemodialysis patients and in posttransplant settings, as well as other clinical groups, were developed. With standard vaccine composition, these protocols exploit higher application doses and increased boosting. Further approaches imply alternative vaccine preparations with different adjuvants and whole-length HBs-antigen.[16–23]

Sufficient anti-HBs-specific post-vaccination titers are broadly considered as the marker of vaccination efficiency and a correlate

Received 18 September 2020; accepted 17 March 2021;
https://doi.org/10.1016/j.omtm.2021.03.012.

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Table 1. Clinical characteristics of the study population

Groups	HR	LR	NR	HCtrl	p value
Participant number	12	7	10	10	ns
Age (median, years)	60	64	66	54	ns
Gender (female/male)	3/9	4/3	4/6	6/4	ns
Number of injections (median)	3.5	5.0	3.5	3.0	ns
Time postvaccination (median, years)	4.4	2.9	2.2	N/A	ns
Primary diagnosis					
hypertensive nephrosclerosis	4	5	3	N/A	ns
diabetic nephropathy	1	–	3	N/A	ns
glomerulonephritis of varying etiology	2	1	1	N/A	ns
tubulointerstitial nephritis	1	–	–	N/A	ns
other	4	1	3	N/A	ns
Hemodialysis (median, years)	4.17	4.83	4.75	N/A	ns

ns, not significant; N/A, not applicable.

of HBV-specific protection.[24–26] However, the role of HBs-reactive T cells in vaccination cannot be simplified to B cell helper function alone. The high frequency of HBs-reactive T cells in resolving acute hepatitis B, as well as the lack of these cells in chronic HBV infections, provide evidence for the antiviral protection of these cells. Further, HBs-specific T cells might compensate for lacking antibody titers in HBV.[27,28]

Last but not least, HBV vaccination is a unique human *in vivo* infectious disease model and corresponding vaccine-based protection presents new insights into the function of immune system and generation of pathogen-specific protection under normal and pathological conditions. Furthermore, these insights allow identification of new predictive markers of vaccination efficiency with a broad translational potential in numerous vaccination settings, as well as immune-based cell therapies.

In the current cross-sectional study, a detailed immunoprofiling in ^^ESRD hemodialysis patients and healthy individuals after HBV-specific vaccination was performed. Among numerous analyzed immune cell subsets, HBs-reactive T cells appear as the main players of efficient HBV vaccination.

RESULTS

Study population

The clinical data on the ESRD study cohort are provided in Table 1. There were no statistically significant differences between the study groups in terms of age, gender, applied vaccine, and the number of booster vaccinations, time since last booster vaccination, and time since first hemodialysis. Overall, 29 ESRD individuals were recruited into the study and allocated into three groups according to anti-HBs titers documented within 3 months before study enrollment. 12 study participants developed anti-HBs titer >100 mIU/mL, 7 participants between 10 and 100 mIU/mL, and 10 other participants <10 mIU/mL. Accordingly, the study participants were allocated into high responder (HR), low responder (LR), and non-responder (NR) groups. Healthy control group (HCtrl) showed vaccination titers >1,000 mIU/mL and revealed no significant criteria differences to ESRD groups.

Prevalence of patients with detectable HBs-reactive T cell responses in study cohorts

After peripheral blood mononuclear cells (PBMCs) stimulation with HBs overlapping peptide pools (OPPs), $CD4^+$, and $CD8^+$ HBs-reactive T cells were identified based on the expression of antigen-specific activation markers, CD154 ligand (CD40L), and CD137 (4-1BB), correspondingly (Figure 1A). Events detected in the negative control were subtracted from the events detected in the stimulated experiment. Detection of at least 10 HBs-reactive T cells ($CD154^+$ for $CD4^+$ and $CD137^+$ for $CD8^+$ T cells) after background subtraction was considered as a truly HBs-specific response. Notably, HBs-reactive $CD4^+CD40L^+$ T cells were detected in 7 out of 10 in humoral NR (70%), 7 out of 7 in LR (100%), and 12 out of 12 of HR (100%) study groups (Figure 1). In HCtrl group, all participants revealed HBs-reactive $CD4^+$ T cells (100%). HBs-reactive $CD8^+$ T cells were detected in few study participants (Figure 1B). However, in line with published data the prevalence of these cells was negligible and, thus, they were excluded from further analysis.

Functional capabilities of HBs-reactive $CD4^+$ T cells were further analyzed based on the expression of interleukin-2 (IL-2), IL-4, interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), and granzyme B (GrB; Figure 1A). Among study participants with detectable cytokine producing HBs-reactive $CD4^+$ T cells, TNF-α secretion was the most prevalent, followed by IFN-γ and IL-2 production (Figure 1). Although the prevalence of IFN-γ-producing HBs-specific $CD4^+$ T cells among study groups was comparable to that of TNF-α (Figure 1B), the frequencies of these were lower as compared to TNF-α- and IL-2-producers (both as relative frequencies and absolute numbers, Figure 2). We could also identify HBs-reactive cytotoxic T helper (Th) cells based on GrB expression. However, the prevalence of GrB-producing T cells among the study cohorts was relatively low and showed no significant differences (Figure 1B). Altogether, we could not identify any significant differences in prevalence of HBs-reactive T cells among study groups, neither for single cytokine producers nor for cytokine-independent $CD154^+$ HBs-reactive Th cells. The latter was surprising as majority of NR group revealed HBs-specific $CD4^+$ Th cells.

Functionalities of HBs-reactive Th cells in vaccination cohorts

Analysis of complete and single cytokine producing HBs-reactive $CD4^+$ Th cells showed significant differences in responses among low or absent titer groups (LR and NR) and high titer groups (HR and HCtrl, Figure 2). These differences were surprisingly present for complete HBs-specific Th cells as absolute number but not relative frequencies of $CD4^+$ T cells. Single TNF-α- or IL-2-producing HBs-reactive Th cells showed a significant difference among

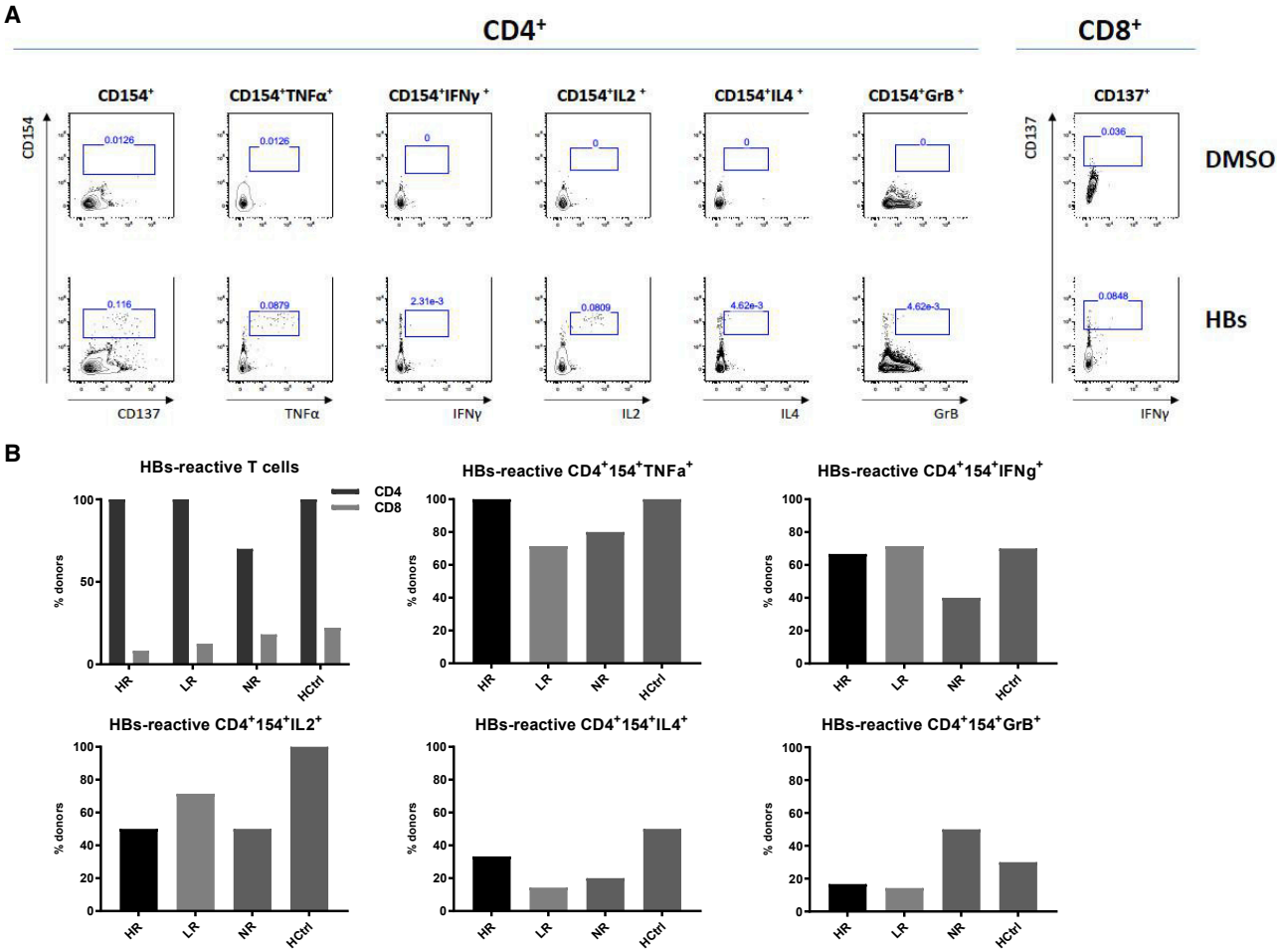

Figure 1. Prevalence of HBs-reactive responses among study groups

(A) HBs-reactive $CD4^+$ and $CD8^+$ T cells were analyzed using markers of antigen-specific stimulation (CD154 and CD137, respectively). For negative control, DMSO was added; for HBs-specific stimulations, HBs-OPP was used. Events detected in the negative control were subtracted from the events detected in the stimulated experiment. Detection of at least 10 HBs-reactive T cells ($CD154^+$ for $CD4^+$ and $CD137^+$ for $CD8^+$ T cells) after background subtraction was considered as a truly HBs-specific response. Further, $CD4^+$ cytokine producers were identified utilizing CD154 and corresponding cytokines or GrB. Gate values represent parent frequencies of corresponding subsets. (B) Prevalence of HBs-reactive T cells irrespective of cytokine-producing capacities for $CD4^+$ and $CD8^+$ T cells, as well as single cytokine producers and GrB for $CD4^+$ T cells, are presented. HR, high responder, LR, low responder, NR, non-responder, HCtrl, healthy controls.

study groups both as frequencies and absolute cell numbers per μL of peripheral blood. As stated above, the frequencies of HBs-reactive IFN-γ-producers were lower as compared to TNF-α and IL-2 producers, and we found no differences among IFN-γ-producing HBs-specific Th between groups, neither as relative frequencies nor absolute numbers.

Another interesting finding was the presence of IL-4-producing HBs-reactive Th2 cells in study groups. These cells were sporadically detected in ESRD patients; however, at significantly lower frequencies and absolute numbers as compared to HCtrls (Figure 2). Among ESRD groups, there were no statistically significant differences between HR, LR, and NR groups for IL-4-producing HBs-reactive Th2 cells.

GrB^+ Th cells were present at low numbers in some study participants, however, with no significant differences between the groups (Figure 2).

Polyfunctional HBs-reactive $CD4^+$ Th cells

T cells expressing more than one cytokine or effector molecule, the so-called polyfunctional T cells, have been previously described as a milestone of antiviral immunity cells and have been analyzed to delineate antiviral protection.[29–32] In order to further delineate the role of T cell polyfunctionality in HBV vaccination, multiple cytokine-producing HBs-reactive Th cells were analyzed in our study. Among combined cytokine producers, only differences for bi-functional $TNF\text{-}\alpha^+IL\text{-}2^+$ HBs-reactive Th cells could be identified. There were no differences in the prevalence of patients with detectable

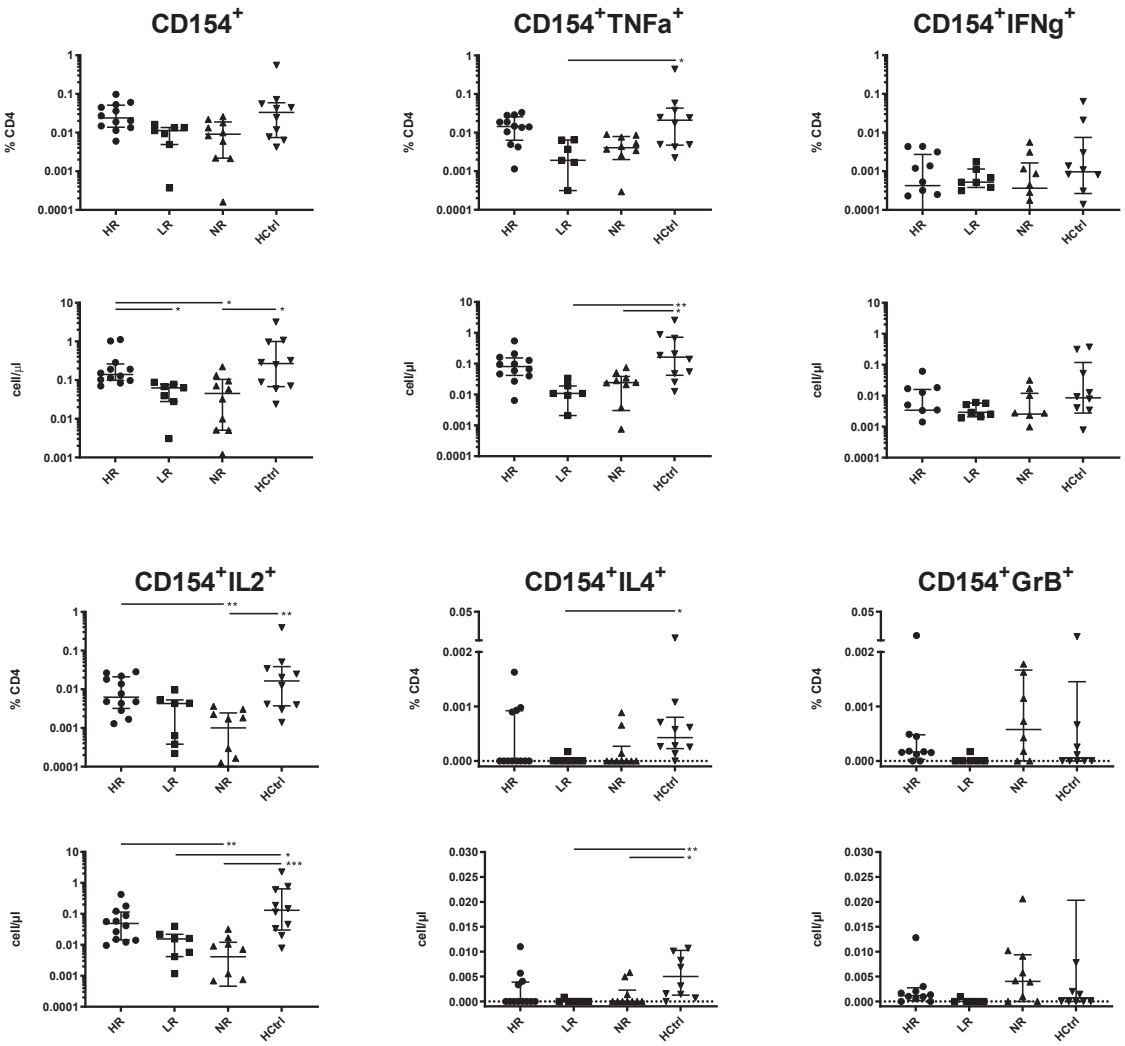

Figure 2. HBs-reactive CD4⁺ T cell responses among study groups

HBs-reactive Th cells, irrespective or cytokine-producing capacities and as single cytokine or GrB producers, are presented as relative frequencies and absolute cell numbers per µL of peripheral blood. Median with interquartile range is shown. *p < 0.05, **p < 0.01, ***p < 0.001. Only samples allowing reliable identification of HBs-reactive Th cells according to gating strategy were included into the analysis.Only samples allowing reliable identification of HBs-reactive Th cells according to gating strategy were included into the analysis. Thus, samples from the donors showing no HBs-reactive Th cells are not represented.

bi-functional T cells among study groups (data not shown). However, HCtrl and HR groups showed significantly higher frequencies and absolute numbers of these cells as compared to LR and NR groups (Figure 3).

Correlation between HBs-reactive T cell responses and post-vaccination titers

Next, we sought to analyze any correlation link between HBs-reactive Th cells and post-vaccination titers in ESRD patients. For this, complete HBs-specific Th cells and cytokine-producing subsets (single or combined) were analyzed as relative frequencies and absolute numbers. As in the NR study group, HBs-specific titers could not be determined (below detection level), and this cohort was assigned HBs-titer of 9. For HR cohort with titers > 1,000, the value of 1,001 was assigned. We could demonstrate significant correlation for HBs-reactive $CD154^+$ Th cells irrespective of cytokine-producing capacities (Spearman correlation coefficient r = 0.5; p = 0.0057 for relative frequencies and r = 0.59; p = 0.0021 for absolute cell numbers), $CD154^+TNF\text{-}\alpha^+$ (r = 0.58; p = 0.0014 for relative frequencies and r = 0.55; p = 0.0008 for absolute cell numbers), $CD154^+IL\text{-}2^+$ (r = 0.72; p < 0.001 for relative frequencies and r = 0.77; p < 0.001 for absolute cell numbers), and combined $CD154^+TNF\text{-}\alpha^+IL\text{-}2^+$ Th cells (r = 0.69; p < 0.001 for both relative frequencies and absolute cell numbers; Figure 4).

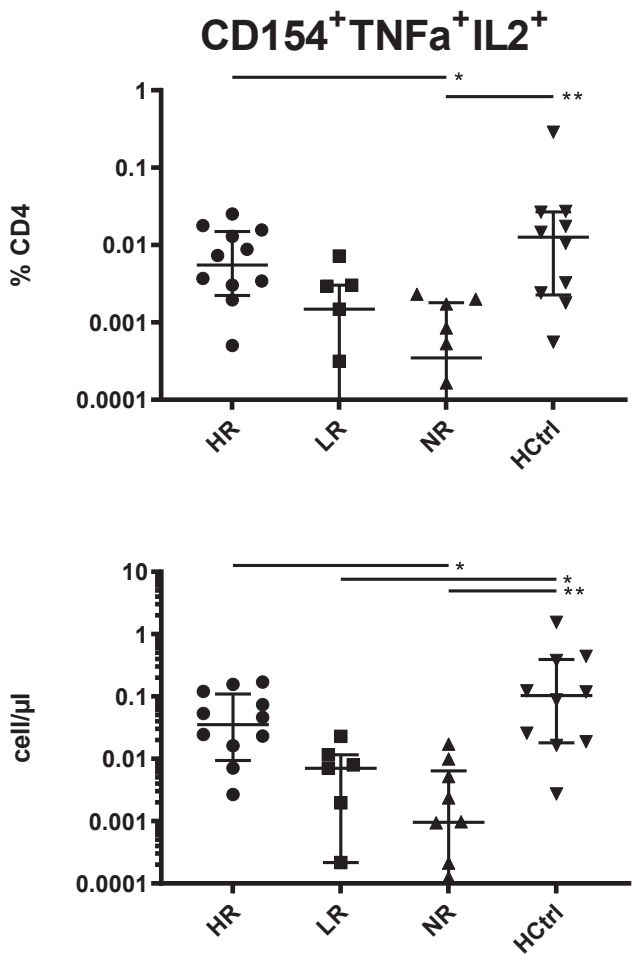

Figure 3. HBs-reactive polyfunctional CD4⁺ T cell responses among study groups

Polyfunctional HBs-reactive Th cells are presented as relative frequencies and absolute cell numbers per µL of peripheral blood. Median with interquartile range is shown. *p < 0.05, **p < 0.01, ***p < 0.001.

Absolute frequencies and differentiation status of peripheral blood T and B cells

In order to analyze the immunotoxic influence of uremia metabolites in ESRD study cohorts and vaccination outcome, we assessed absolute cell numbers of peripheral blood T and B cells. This analysis revealed no differences in absolute frequencies of T cells (Figure 5A). Absolute B cell frequencies, however, were significantly decreased in the NR group, as compared to HR and HCtrl (Figure 5A).

In order to rule out that the diminished number of B cells is responsible for the lack of HBs-specific antibody, concentrations of immunoglobulin (Ig) main classes were analyzed in the peripheral blood of recruited patients. Our analyses revealed that there were no statistical differences in either IgG, IgM, or IgA concentrations among the study groups (Figure S1).

Furthermore, to exclude differences in differentiation state/exhaustion of T cells as a further reason of the failed vaccination in NR group, we performed additional phenotypical profiling of CD4⁺ Th cells (Figure 5B). CD45RA and CCR7 are widely used to discriminate the differentiation status of T cell, allowing dissection of total Th pool into naive, central memory (CM), effector memory (EM), and terminally differentiated effector memory T cells expressing CD45RA (Temra). Expression analysis of these markers on total blood CD4⁺ Th cells showed higher relative frequencies of Temra in NR as comparted to HCtr. But comparing the absolute cell counts between the groups, the values were not significantly different (Figure 5C). Subsequent analysis of HBs-reactive Th cells in terms of differentiation status revealed no differences among study groups with slightly decreased Temra HBs-reactive CD4⁺ T cells in NR compared to HCtrl analyzed as absolute frequencies (Figure 5D).

DISCUSSION

ESRD patients are at high risk of developing numerous infectious diseases. On the one hand, this is due to uremia-associated secondary immune deficiency. On the other hand, hemodialysis and frequent human blood products administration significantly increases contraction of viral diseases, especially hepatitis B and C, HIV, and further infections. Furthermore, infectious diseases in uremic patients show protracted course and higher chronification rates.[4,5] Moreover, chronic viral infections pose a significant hurdle for kidney transplantation, the acknowledged therapeutic goal in ESRD hemodialysis patients. Thus, appropriate vaccination in ESRD patients is of vital importance to decrease morbidity and mortality in this patient cohort.[6]

The post-vaccination anti-HBs titers are broadly accepted as protection correlates and, thus, reflect vaccination efficacy.[24–26] As compared to healthy populations, ESRD patients, as well as kidney transplant recipients, show lower vaccination efficacy against HBV.[7–10] In posttransplant settings this is mostly due to, though not limited to, the immunosuppressive therapy. In ESRD patients, the underlying mechanisms are not exactly clear. More than 200 uremia associated metabolic products were described. However, the exact mechanisms or uremia-associated secondary immunosuppression are not properly defined. Among these, the direct cytotoxic effects on the bone marrow and hematopoiesis are broadly accepted. This leads to diminished absolute numbers of blood cell subsets in general and specifically immune cells (e.g., T and B cells). Thus, impaired T and B cell functions after vaccine application result in low vaccine-specific titers.[33–38] Furthermore, altered function of antigen-presenting cells due to hemodialysis procedure can also be responsible for the lower vaccination efficacy.[39] Further effects on CD4⁺ and CD8⁺ T cells and IL-8 plasma concentration after a single hemodialysis were reported.[40]

Here, using multiparameter flow cytometry HBs-reactive T cells were functionally analyzed and quantified in a cross-sectional study in an ESRD dialysis cohort and healthy population. Based on post-vaccination titers, three groups of HR, LR, and NRs were identified. One of

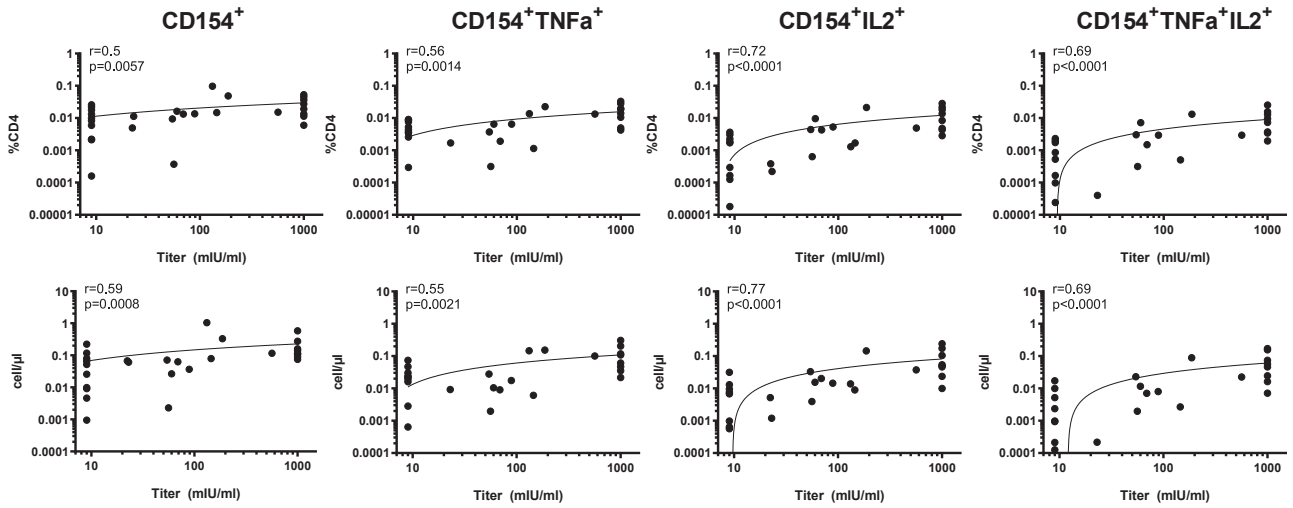

Figure 4. Correlation analysis between HBs-reactive CD4$^+$ T cells and postvaccination titers in ESRD patients
Non-linear fit with semilog line is presented. r, Spearman correlation coefficient.

the findings of the current study is that even in case of a failed vaccination, HBs-reactive T cells could be clearly identified in the vast majority of NR vaccinees. Functional assessment of vaccine-reactive T cells based on cytokine production revealed no differences between Th subsets among the ESRD groups. There were no differences in IFN-γ-producing Th1 cells, either among patients or healthy cohorts. However, there were significant differences in terms of vaccine-reactive Th cell secreting TNF-α and IL-2. These differences in prevalence of patients with detectable TNF-α, IL-2 single cytokine producers, and complete HBs-specific CD4$^+$ T cells was not significantly different among study groups. However, functional analysis of HBV-vaccine reactive T cells revealed that in addition to absolute numbers of single producers, bi-functional TNF-α$^+$IL-2$^+$ HBs-reactive Th cells were significantly higher in HR and HCtrl groups, as compared to LRs and NRs. These data were further explored in correlation analysis revealing a correlation link between post-vaccination titers and frequencies and absolute numbers of HBs-specific Th cells (either irrespective of cytokine production or as TNF-α- and IL-2-single and combined producers). Due to technical limitations, the post-vaccination titers >1,000 and <10 were assigned values of 1,001 and 9, correspondingly yielding highly statistical significance. If the absolute values of the titers outside measurement range were available, this would result in even higher statistical significance.

Taken together, our data demonstrate a robust HBs vaccine-reactive cellular immunity in patients with missing HBs-specific humoral immunity. Although the demonstration of the direct killing capacities was outside the scope of the study, the secretion of multiple cytokines by vaccine-reactive T cells suggests their killing functional potential as demonstrated in several previous works.[7,30–32,41–44]

TNF-α and IL-2 are secreted by T cells at early stages of immune response, as well as by antigen-reactive Th cells with advanced differentiation status (central memory Th cells as opposed to highly differentiated effector and Temra subsets).[45–47] The role of antigen-reactive Th cells with early differential status is of special interest. Recently, we showed that the absolute numbers of vaccine-specific central memory Th cells at baseline correlate with vaccination efficiency in settings of seasonal influenza vaccination.[44] Moreover, the diversity of the T cell receptor (TCR) repertoire of this subset further correlates with the post-vaccination titers. For this reason, it would be of interest to further analyze the differentiation status of HBs-specific T cells and their TCR repertoire composition in diseased and healthy subjects showing different vaccination outcomes in following studies.

As shown previously and confirmed in the current study, HBs-reactive T cells do not necessarily secrete IFN-γ, the well-accepted marker of antigen-reactive Th cells.[7] In fact, the frequencies of IFN-γ-producing Th cells, as well as the prevalence of patients with detectable IFN-γ-producing Th cells, were lower as compared to other cytokine or activation marker producing T cells. Therefore, IFN-γ-based ELISPOT assay relying on a single cytokine as only a readout parameter might significantly underestimate frequencies of antigen-specific Th response. Thus, analyses employing numerous cytokines or combination with cytokine-independent markers of antigen-specific stimulation are superior in term of qualitative and quantitative analysis.

Surprisingly, our data showed significantly lower numbers of HBs-specific IL-4-producing Th2 cells in ESRD groups as compared to the healthy cohort. However, we could not state any statistically significant correlation difference between IL-4-producing Th2 cells and post-vaccination titers. Th2 cells are known to promote B cell function and stimulate Ig class switch and antibody synthesis. The surprising fact is that in ESRD HR cohort, high post-vaccination titers could

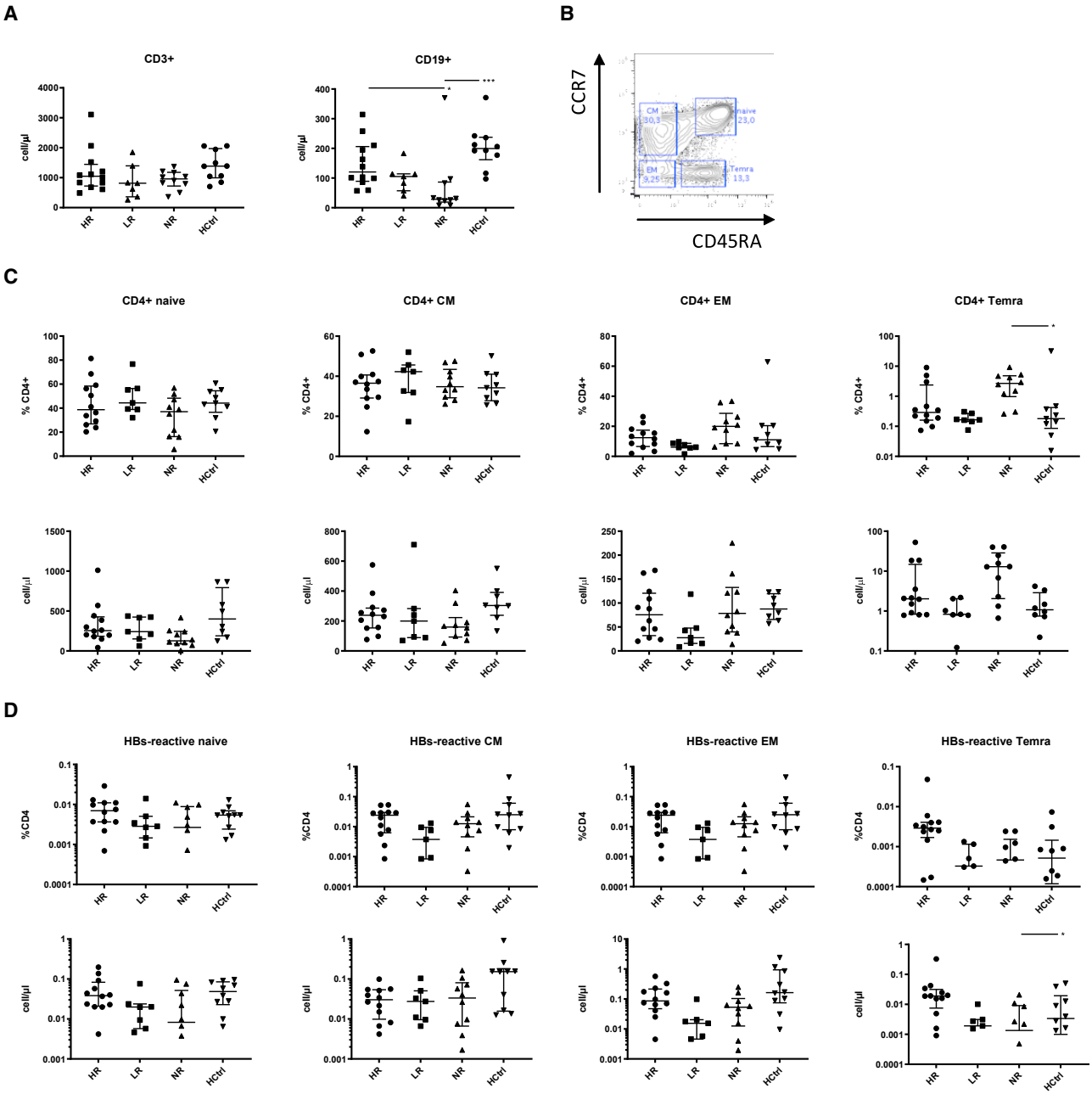

Figure 5. Absolute frequencies and differentiation status of peripheral blood T and B cells among study groups

(A) Absolute frequencies of peripheral blood T and B cells among the study cohorts. (B) Differentiation status of $CD4^+$ T cells was performed based on CD45RA and CCR7 expression allowing analysis of naive, central memory (CM), effector memory (EM), and terminally differentiated T cells expressing CD45RA (Temra). Gate values represent parent frequencies of corresponding subsets. Differentiation status of peripheral blood $CD4^+$ T cells (C) and HBs-reactive Th cells (D) among study groups presented as relative frequencies and absolute cell numbers. Median with interquartile range is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

be mounted regardless of the diminished numbers of HBs-reactive Th2 cells. The elucidation of compensatory mechanisms allowing achievement of sufficient vaccine-specific titers with diminished or absent Th2 cells would allow development of augmented vaccination protocols in low vaccination efficiency cohorts.

Our study has some limitations. One of the concerns is the specificity of the data on HBs-reactive T cells obtained by flow cytometric analysis, implying false-positive identification of cross-reactive T cells as HBs-reactive. However, no other methodologies including assays detecting seropositivity/seronegativity nor patient history can provide

100% reliability on the post-infection status. The lack of specific antibodies in patients recovered from certain infections (e.g., HBV, CMV, SARS-CoV-2), as well as in occult HBV infection, is also well-known. On the other hand, the applied flow-cytometry-based analysis of T cell antigen-reactivity is nowadays the most sensitive and specific for these purposes. In addition, the methodological approaches of negative stimulation control with subtraction of background noise from the specific stimulation decreases the chance of analysis bias. This approach is widely used in immunological studies assessing antigen-specific frequencies. Furthermore, applying the same methodology, we demonstrated previously HBsAg-reactive T cells despite the lack of HBs-specific antibodies.[7,48]

Moreover, circulating counterparts of follicular Th (cTfh) cells were recently identified among markers of successful vaccination.[48,49] These cells at baseline and at the peak of vaccine-induced immune response were reduced in chronic kidney disease settings and were attributed to failed vaccination. Due to technical limitations, we could not consider the analysis of cTfh in our current analysis. Furthermore, our analysis was performed in a cross-sectional manner. So, no time-dependent correlation between cellular and humoral immunity could be obtained. Therefore, further studies including a large marker panel and considering a prospective design will be required to identify correlates of efficient vaccination in ESRD patients preceding vaccine application or during early stages of vaccine-induced response.

In general, the role of pathogen-specific $CD4^+$ T cells cannot be reduced solitarily to B cell help. In addition, these cells possess cytotoxic capacities and can directly clear the infected cells.[50,51] Indirectly, the importance of HBV-reactive $CD4^+$ T cells is confirmed by high frequencies of these cells in resolving acute hepatitis and their lack of chronic HBV infection.[27] The versatile role of $CD4^+$ Th cells was also shown in varicella and BK virus settings.[52] At this time point, we can only speculate on the capacity of the detected polyfunctional T cells to protect against HBV infection in hemodialysis patients. Further studies are required to elucidate the exact role of the identified HBs-reactive T cells in preventing or combating HBV infection in absent of humoral immunity.

The main finding of our study is that the lack of HBs-specific antibody titers does not reflect the failed cellular immunity. The majority of HBV vaccination NRs was able to generate vaccine-reactive functional T cell immune response as demonstrated by the detection of single cytokine producing and polyfunctional HBs-reactive T cells. In addition, TNF-α and IL-2 production alone or in combination directly correlates with the HBs-specific post-vaccination titers. Present findings are in line with our previous study performed in kidney transplantation setting showing presence of HBs-reactive T cells in renal transplant recipients with failed antibody detection.[7] As previously reported, secondary immune deficiency in uremia patients proceeds in a graded pattern with B cell lineage being more susceptible than T cells. Indeed, our results showed decreased B cell numbers in NR group. To explore whether the B lymphopenia can be a cause for the failed vaccination efficacy in NR, we analyzed the general ability of NR patients to produce Ig main classes and compared these values to HR and LR. Our data showed that the total Ig concentrations in NR group did not differ from other study cohorts. Although our data cannot completely exclude that the decreased B cell counts is accounted for the failed post vaccination titers, data on the general Ig production do not support such an assumption. It would be of interest to analyze further functional B cell capacities (e.g., T cell stimulatory capacities, cytokine secretory capacities), as well as after polyclonal or antigen-specific stimulation in future studies. Further elucidation of factors responsible for successful or failed vaccination should help adjusting current vaccine composition, as well as vaccination protocols in low response cohorts, both diseased and healthy. Last but not least, insights from the HBV-vaccination studies can be successfully transferred into further vaccination settings, as well as cell-based immune therapies.

MATERIALS AND METHODS

Ethical approval

This study was approved by the ethical committee of the faculty of medicine at the Ruhr-University Bochum, Germany (ethic approval number 16-5649). Study participants have provided a written informed consent.

Study population

ESRD hemodialysis patients who fulfilled study inclusion criteria and had no exclusion criteria were recruited in an unbiased manner. Inclusion criteria were as follows: diagnosis of ESRD with constant therapeutic hemodialysis, completed HBV vaccination with accurately documented post-vaccination titers, and written informed consent. ESDR patients with laboratory signs of active or previous HBV and/or hepatitis C virus (HCV) infections, as well as HIV-positive individuals, were not included into the study. ESRD patients were allocated into the following groups according to the anti-HBs titers documented within a 3-month period before study enrollment: HR with anti-HBs titers >100 mIU/mL, LR with anti-HBs-titers of 10-100 mIU/mL, and NR with anti-HBs titers <10 mIU/mL. Further, healthy individuals with a completed HBV vaccination were enrolled as a control study group.

Study participants were vaccinated with one of the following vaccines: Engerix B Erwachsene, Twinrix Erwachsene, Gen HBVAX, and HBVAXPRO. Participants of the ESRD cohorts received 40 μg while healthy individuals received 10 μg vaccine dose. There were no statistical differences in the applied vaccines between the study groups.

Serological analyses

Complete and anti-HBs-specific Ig concentrations were measured on Cobas 800 (Roche).

Cell preparation and HBV-specific stimulation

PBMCs were isolated from EDTA anticoagulated peripheral venous blood by density gradient centrifugation using Biocoll Separating Solution (Biochrom, Germany). PBMCs were re-suspended at a final concentration of $5–10 \times 10^6$ cells/mL in complete RPMI 1640

medium (supplemented with 2.5% fetal bovine serum, penicillin, streptomycin, and L-glutamine).

HBs OPP was purchased from JPT, Germany (PepMix HBV [LEP] Ultra; amino acid composition and coverage of different HBV genotypes is available at the manufacturer webpage). HBs-OPP consisting of 15 amino acid long peptides with 11 amino acids overlapping between adjacent peptides was first dissolved in dimethyl sulfoxide (DMSO) and later in PBS according the manufacturer's protocol and used at a final concentration of 1 μg/mL. Cell were incubated for 6 h at 37°C and 5% CO_2. Brefeldin A (Sigma Aldrich, Germany) was added for the last 4 h of incubation at a final concentration of 5 μg/mL to block cytokine secretion. As a positive stimulation control, PBMCs were stimulated with Staphylococcus Enterotoxin B at a final concentration of 1 μg/mL (Sigma Aldrich, Germany). DMSO was used as a negative control in similar concentrations as HBs-specific stimulations.

Surface and intracellular marker staining

Stimulated cells were stained with fluorochrome-conjugated monoclonal antibodies to detect surface and intracellular markers. Intracellular staining was performed with Foxp3 Fixation/Permeabilization buffer kit (eBioscience, San Diego, CA, USA), and Fixable Viability eFluor 780 Dye (eBioscience) was used to exclude dead cells. The following antibodies were used: CD4-Alexa Fluor 700 (OKT4, BioLegend), CD8-V500 (RPA-T8, BD Biosciences, San Jose, CA, USA), GrB-Fitc (GB11, BioLegend), IL-2-Pe (MQ1-17H12, BioLegend), IL-4-PeDaz594 (MP4-25D2, BioLegend), CD137-Pe/Cy7 (4B4-1, BioLegend), CD154-Alexa Fluor 647 (24-31, BioLegend), TNF-α-eFl450 (MAb11, eBioscience), IFN-γ-BV650 (4S.B3, BioLegend), CD3-BV785 (OKT3, BioLegend), CD19-BV605 (HIB19, BioLegend), CD45RA-BV605 (HI100, BioLegend), CCR7-PerCP/Cyanine 5.5 (G043H7, BioLegend).

For the absolute quantification of immune cell subsets, total $CD19^+$, $CD3^+$, $CD4^+$, and $CD8^+$ T cells numbers were determined in 50 μL of the peripheral blood by flow cytometry as described below by means of CytExpress software (Beckman Coulter, USA). Absolute numbers of HBs-specific T cells were determined based on relative frequencies of HBs-specific T cells and absolute numbers of total $CD4^+$ T cells per μL of peripheral blood.

Multiparameter flow cytometry and data processing

Immune cell phenotyping and absolute cell numbers were determined by means of multiparameter flow cytometry on Cytoflex S immune analyzer using CytExpert Software (Beckman Coulter, USA). At least 5×10^5 $CD4^+$ T cells were recorded and analyzed.

Cytometric data analysis was performed using FlowJo 9.9.4 (Tree Star, USA). Events detected in the negative control were subtracted from the events detected in the stimulated experiment. Detection of at least 10 HBs-reactive T cells ($CD154^+$ for $CD4^+$ and $CD137^+$ for $CD8^+$ T cells) after background subtraction was considered as a truly HBs-specific response.

Flow cytometry data are freely available at http://flowrepository.org/ (submission ID FR-FCM-Z3FT) according to International Society for Advancement of Cytometry (ISAC) recommendation.[54]

Statistical analysis

Data analysis was performed using GraphPad Prism (GraphPad Software, USA). Matching of patient criteria was analyzed with a Kruskal-Wallis test. Kruskal-Wallis test with Dunn's correction for multiple comparisons was applied to analysis of HBs-reactive cell frequencies and absolute numbers. Difference in responder rates between study groups was determined using chi-square test. Correlations were performed with two-way Spearman test; as fitting method least-squares with non-linear regression was used. In all tests, a p value <0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.03.012.

ACKNOWLEDGMENTS

This work was supported by grants from Mercator Stiftung,Germany, German Research Foundation, Germany (DFG, grant 397650460), BMBF e:KID, Germany (01ZX1612A), and BMBF NoChro, Germany (FKZ 13GW0338B).

AUTHOR CONTRIBUTIONS

G.A. conducted the experiments, performed the experiments and analysis, and wrote the paper; T.R. supervised the work and performed the analysis; U.S. supervised the work and performed the analysis; S.K. performed the experiments; A.S. applied for funding and performed the analysis; J.H. provided clinical samples and designed the experiments; O.C. provided clinical samples and designed the experiments; H.A. provided clinical samples and designed the experiments; L.N. designed the experiment and performed analysis; L.G. designed the experiment and performed analysis; F.S. provided clinical samples and designed the experiments; F.B. provided clinical samples and designed the experiments; T.W. provided clinical samples and designed the experiments; M.N. designed the experiments, supervised the work, performed the experiments and analysis, and wrote the paper; N.B. designed the experiments, supervised the work, wrote the paper, and applied for funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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