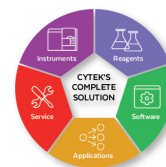


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Tick-Borne Encephalitis (TBE) and Hepatitis B Nonresponders Feature Different Immunologic Mechanisms in Response to TBE and Influenza Vaccination with Involvement of Regulatory T and B Cells and IL-10 ✓

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Tick-Borne Encephalitis (TBE) and Hepatitis B Nonresponders Feature Different Immunologic Mechanisms in Response to TBE and Influenza Vaccination with Involvement of Regulatory T and B Cells and IL-10

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Low responsiveness/nonresponsiveness is characterized by an insufficient immune response upon primary and/or booster vaccination and affects 1–10% of vaccinees. In the current study, we aimed to investigate whether nonresponsiveness is an Ag/vaccine-specific phenomenon and to clarify underlying immunological mechanisms. Nonresponders to tick-borne encephalitis (TBE) or hepatitis B Ag with a history of previous TBE vaccinations were booster vaccinated with TBE and influenza vaccine and compared with TBE high responders in terms of humoral and cellular immune response. Postboosters in TBE high responder existing TBE titers increased, and solid humoral responses to influenza vaccine were induced. In TBE nonresponders, low to undetectable prevaccination TBE titers remained low, whereas sufficient influenza Abs were induced. In both TBE groups, a positive correlation of humoral and cellular immune response was seen as high/low TBE titers were associated with sufficient/lack of Ag-specific T cell proliferation. Furthermore, responses to influenza were robust in terms of Abs and cytokine production. In contrast, in hepatitis B nonresponders, sufficient humoral responses to TBE and influenza Ags were induced despite lacking specific IL-2 and IFN- γ production. Importantly, these patients showed high IL-10 baseline levels in vitro. HLA-DR subtypes associated with hepatitis B nonresponsiveness were overrepresented in this group, and high IL-10 levels were linked to these subtypes. Whereas TBE and hepatitis B nonresponders had increased IL-10-producing FOXP3⁺ T regulatory cells upon vaccination, only in hepatitis B nonresponders, showing elevated prevaccination IL-10 levels, a prominent population of B regulatory cells was detected. We conclude that immunological pathways of nonresponsiveness follow different patterns depending both on vaccine Ag and genetic predisposition of the vaccinee. *The Journal of Immunology*, 2013, 191: 2426–2436.

Nonresponsiveness is currently defined as the inability of a vaccinee to produce sufficient protective Ab responses after a completed primary and/or booster vaccination. This phenomenon affects 1–10% of vaccinated individuals depending on the type of vaccination. A booster immunization with a subsequent check of the specific Ab titer is the current state of the art to distinguish nonresponders from individuals who show a decline in Ab levels that can be boosted. To date, the immunological background of vaccination failures remains largely unclear; it is unknown whether nonresponsiveness relates to only one or rather to several vaccine Ags, whether nonresponders have a higher susceptibility to infection and/or to a more severe course of the

disease, and whether vaccination breakthroughs occur in these individuals.

Vaccination failures have been observed and described in the context of several routine vaccinations. Best documented is nonresponsiveness to hepatitis B vaccine in which up to 10% of healthy, immune-competent vaccinees do not mount protective Ab responses to the most common hepatitis B vaccines containing the hepatitis B surface (HBs) Ag (1, 2). Measurement of anti-HBs Ab levels is used to determine protection against hepatitis B. The minimum level of Abs allowing for protection against HBV is defined as >10 mIU/ml (3). Because the number of hepatitis B nonresponders is significant and protection is of crucial importance for risk populations such as health care professionals, this specific type of vaccination failure has been investigated in more detail. Risk factors such as obesity, heavy smoking, or renal disease have been determined (4, 5), and, additionally, certain HLA class II subtypes (HLA-DRB1, HLA-DQB1) have been associated with nonresponsiveness to current hepatitis B vaccines (6). Also, the role of IL-10 in hepatitis B nonresponsiveness has been highlighted in several studies. Nonresponders frequently display a genotype of high TGF- β and IL-10 secretion (7) as well as a functional polymorphism in the IL-10 promoter that negatively influences humoral immune response to hepatitis B vaccine (8).

Similar to hepatitis B, nonresponders have also been detected among hepatitis A vaccinees. Vaccination against hepatitis A was established in the 1990s with a prediction of long lasting protection (9, 10). To determine the long-term Ab persistence 10 y after

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Abbreviations used in this article: GMT, geometric mean titer; HAI, hemagglutination inhibition; HBs, hepatitis B surface; NT, neutralization test; TBE, tick-borne encephalitis; T_{EM}, T effector memory; T-reg, T regulatory cell.

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primary vaccination, a clinical trial with >1000 participants was conducted at our institute (11). In the scope of this investigation, a small group of vaccinees (2%) with anti-hepatitis A virus Ab levels below protection level could be identified, and, in a booster study performed with these individuals, we could confirm a segment of true nonresponders whose Ab levels remained low after revaccination. Lack of Abs correlated with reduced cellular immune responses, for example, Ag-specific *in vitro* cytokine production and also with a decreased expression of the hepatitis A virus-specific receptor on T cells (12).

Tick-borne encephalitis (TBE) virus is presently highly endemic in Austria and in other Northern, Central, and Eastern European countries and parts of Russia. A rigorous vaccination campaign in Austria led to a sharp decline of TBE morbidity, and presently ~90% of the population is vaccinated against TBE (13). Our extensive studies on persistence of protective TBE immunity revealed that, 3 y after booster vaccination, 10% of the investigated individuals had anti-TBE titers below protection level, for example, neutralization test (NT) titers <1:10 (14). In half of these unprotected vaccinees, the NT titers could be boosted while remaining low in others, resulting in a non/low-responder rate of ~5% (15, 16). Notably, these non/low responders were most frequently found in the age group >50, indicating that immunosenescence possibly contributes to this type of nonresponsiveness.

From these observations, several questions arose, for example, whether nonresponsiveness is a general phenomenon of genetically predisposed persons or an Ag-specific effect; whether there is a correlation of humoral and cellular immune responses; and whether there are cellular prediction markers of nonresponsiveness. To answer these questions, we included two different types of nonresponders in the current study, that is, TBE nonresponders and hepatitis B nonresponders. Individuals with high Ab responsiveness to TBE served as a control group. All vaccinees included in the study had a history of previous TBE vaccination. Two different vaccines, namely TBE and influenza, were administered to the three groups, and the immune responsiveness of the study participants was compared at the humoral and cellular level. Vaccine-specific titer levels measured prior to and over a time period of 6 mo postvaccination were correlated with *in vitro* cytokine production of Ag-specific restimulated blood mononuclear cells. Additionally naive, memory, and regulatory subpopulations of lymphocytes were characterized to elucidate the underlying immunologic mechanisms responsible for the impaired immune responses to vaccination.

Materials and Methods

Study population

Healthy adults (>18 y, no upper age limit) of both sexes with a documented complete primary TBE vaccination plus one booster were included in the study. The individuals were recruited from a pool of vaccinees that participated in earlier studies on protective immunity to TBE and hepatitis B at our institute in cooperation with the affiliated outpatient clinic. After signing written informed consent, 67 participants were enrolled in the study, and three age- and gender-matched groups fulfilling the following inclusion criteria were established: TBE nonresponders with TBE NT titers <1:10 ($n = 23$); hepatitis B nonresponders with anti-HBs Ag-Ab levels <10 mIU/ml (despite primary hepatitis B immunization plus one booster) and a history of completed TBE vaccination ($n = 22$); and TBE high responders with NT >1:30 ($n = 22$), who served as an immunocompetent control group (Fig. 1A). After initial blood sampling (day 0), the study participants received a TBE booster and an influenza vaccination, and 1 wk, 8 wk, and 6 mo after the vaccinations, further blood samples were drawn. From the samples taken before and 1 wk after booster vaccination, both plasma and PBMCs were prepared to evaluate humoral and cellular parameters, 8 wk and 6 mo after booster only plasma was used for Ab kinetic analyses (Fig. 1B). The clinical trial was

conducted with the approval of the Ethical Committee of the Medical University of Vienna (registration number 474/2008) and the national regulatory authority.

Vaccines

Participants received the TBE vaccine FSME-IMMUN 0.5 ml, containing 2.4 μ g inactivated TBE virus (strain Neudörfl). Because the recruitment took place over the course of 2 y, two different seasonal influenza vaccines were used. Patients received either Inflflex V 2008/2009, 0.5 ml, containing 15 μ g hemagglutinin of A/Brisbane H1N1-like, of A/Uruguay H3N2-like, and of B/Florida B-strain or Inflflex V 2009/2010, 0.5 ml, containing identical A strains, but B/Brisbane B strain. Both types of vaccines are licensed in Austria, FSME-IMMUN (Baxter Innovations) since 1996 and Inflflex V (Berna Biotech Crucell) since 2002. All vaccines were stored at 2–6°C until usage.

Specific Ab titer levels to TBE and influenza Ag

Anti-TBE virus Abs were measured by NT in plasma samples obtained prior to and 1 wk, 8 wk, and 6 mo after booster (stored at –20°C until evaluation). Neutralization assays were carried out in microtiter plates using baby hamster kidney cells (American Type Culture Collection BHK-21), as described previously (17, 18). Twofold serial dilutions of polyclonal sera were mixed with 25 PFU virus (starting dilution of the serum in the mixture, 1:10) and incubated for 1 h at 37°C. BHK-21 cells were added, and incubation was continued for 3 d. The presence of virus in the supernatant was determined by four-layer ELISA. The virus neutralization titer was defined as the reciprocal of the serum dilution that gave a 90% reduction in the absorbance readout in the assay compared with the control without Ab.

Virus-specific Abs measured by hemagglutination inhibition (HAI) test are associated with protective immunity against influenza. According to the Centers for Disease Control and Prevention guidelines, a protective post-vaccination titer is either considered as HAI titer of >1:40 or as fold increase >2 of geometric mean titers (GMT) reaching a HAI titer of >1:40 (19). In the current study, we referred to fold increase of GMT in our study groups using the World Health Organization standard methodology for HAI tests (20). In brief, two serial 2-fold dilutions of the sera were prepared. The diluted sera were incubated either with the hemagglutinin Ag of the respective influenza strains present in the vaccines or with the solvent. Thereafter, a suspension of chicken erythrocytes was added. Hemagglutination occurred with virus Ag in the absence of sufficient inhibiting Abs. The HAI titer is the reciprocal value of the highest dilution inhibiting hemagglutination. The titer assigned to each sample is the geometric mean of two independent determinations. NT and HAI assay were carried out at the Department of Virology at the Medical University of Vienna.

Ag-specific *in vitro* restimulation of PBMC

PBMC were prepared by standard Ficoll-Paque centrifugation and stored, as previously described (12). For *in vitro* restimulation of PBMC, TBE virus Ag (strain Neudörfl) and influenza Ag (identical strains as in Inflflex V 2008/2009 and 2009/2010, respectively) were used. Ag TBE TICOVAC like was provided by Baxter Innovation (Vienna, Austria). The Ag concentration was equivalent to the used vaccine (2.4 μ g inactivated TBE virus, strain Neudörfl per 500 μ l solvent, final concentration 4.8 μ g/ml). Aluminum hydroxide, formalin, and stabilizing agents had been removed below detection levels by the manufacturer, and a final concentration of 0.048 μ g Ag/well was used. Inflflex V final product 2008/2009 was obtained from Berna Biotech Crucell (Berne, Switzerland) and comprised three influenza strains, as follows: A/Brisbane H1N1-like, A/Uruguay H3N2-like, and B/Florida B-strain. Product 2009/2010 contained identical influenza A strains, but B/Brisbane B strain. Inflflex V final product comprises 30 μ g/ml hemagglutinin of each strain, resulting in 90 μ g/ml total concentration; it was used for restimulation at 0.75 μ g/well.

For *in vitro* restimulation, frozen PBMC were thawed and re-established in complete RPMI 1640 medium. Cells in triplicates were plated in 96-well round-bottom plates at 8×10^5 /well. The respective Ag dilutions were added to a total culture volume of 200 μ l. The superantigen *Staphylococcus enterotoxin B* (0.2 μ g/well) was used as positive control, and cells incubated only with media provided baseline levels. Cultures were maintained for 48 h at 37°C, 5% CO₂, and 95% humidity; subsequently, the supernatants were harvested, pooled, and stored at –20°C until analysis.

Quantification of cytokine production in supernatants

Cytokines IL-2, IFN- γ , and IL-10 were quantified in culture supernatants via Luminex technology with Fluorokine MAP Human Base Kit A (R&D Systems) using fluorescently labeled microsphere beads and a Luminex

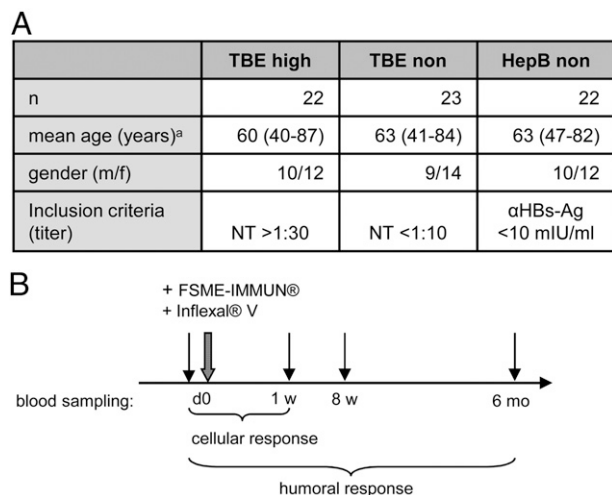


FIGURE 1. Study characteristics. **(A)** Demographic data and inclusion criteria of study participants enrolled in the three groups. ^aMean values and range. f, female; αHBs-Ag, anti-hepatitis B surface Ag-Abs; m, male; NT, NT titer. **(B)** Timeline of blood sampling, vaccinations, and immunological evaluations. FSME-IMMUN, TBE booster vaccination; Inflenza V, influenza booster vaccination.

reader. In a provided 96-well microplate, 50 μ l culture supernatants (duplicates in 1:2 dilutions) were incubated with 50 μ l fluorescently labeled microsphere beads for 3 h at 20°C. Then the plate was washed three times and incubated with 50 μ l biotin detection Ab for 1 h at room temperature. After 30 min of incubation with 50 μ l streptavidin-PE, the plate was run on the Luminex platform. The concentrations of cytokines were calculated via standard curves determined with known concentrations of cytokines provided by the manufacturer.

TGF- β levels in supernatants were assessed in duplicates with eBioscience Human/Mouse TGF- β 1 ELISA Ready-SET-Go! Kit (sensitivity 60 pg/ml).

Cytokine data sets were only included in the calculation of mean values if the restimulated PBMCs showed robust IFN- γ and/or IL-2 production in response to superantigen *Staphylococcus enterotoxin B* as positive control.

Data sets lacking a response to the positive control were excluded, indicating a poor viability of PBMC or methodological problems.

Immunofluorescent staining and cytometric analyses

PBMC preparations were washed once with PBS/0.5% BSA/0.5% sodium azide, diluted to 5×10^5 cells per microcentrifuge tube, and blocked with 20% human AB serum for 20 min. Surface staining was done with directly conjugated mAbs for 30 min at 4°C in the dark; staining of IgD required two steps, as follows: biotinylated anti-IgD Ab was incubated for 30 min, followed by streptavidin-allophycocyanin Ab for 30 min. For intracellular staining of IL-10 and granzyme B, cells with completed surface staining were incubated with fixation/permeabilization buffer (eBioscience) for 30 min in the dark, then washed and resuspended in permeabilization buffer (eBioscience) and stained with specific intracellular mAbs for 30 min at 4°C in the dark. After final washing with permeabilization buffer, cells were resuspended in FACS staining buffer (eBioscience) for analysis.

All stained PBMC were analyzed on FACSCalibur flow cytometer (BD Biosciences) by gating on cells with forward and side light scatter properties of lymphocytes using BD CellQuest software. Isotype-matched control Abs were used to verify the staining specificity of the experimental Abs.

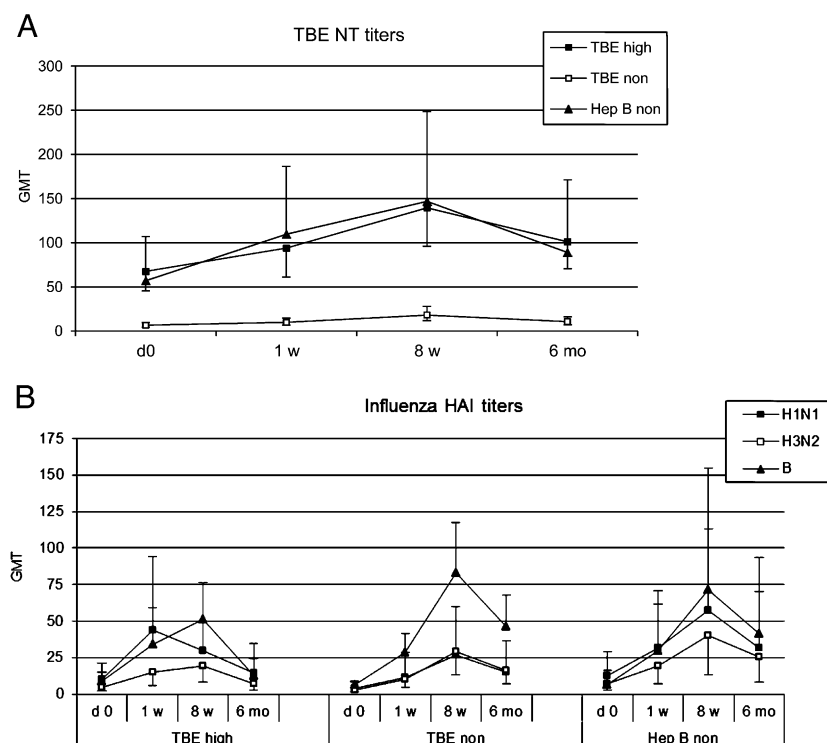
mAb for surface staining

The following mAbs were used: anti-human CD19 FITC (mouse IgG1 κ), anti-human CD27 PE (mouse IgG1 κ), anti-human IgD-biotin (mouse IgG2a κ), streptavidin-allophycocyanin; anti-human CD45RA PE (mouse IgG2b κ), anti-human CD4 PerCP (mouse IgG1 κ), anti-human CD8 allophycocyanin (mouse IgG1 κ), anti-human CD4 FITC (mouse IgG1 κ), anti-human CD28 PerCP (mouse IgG1 κ); all listed mAbs were purchased from BD Biosciences. Anti-human CCR7 FITC (IgG2a κ) was obtained from R&D Systems. CD24 PE (mouse IgG2a κ), CD38 PE/Cy5 (mouse IgG1 κ), and CD25 PE/Cy5 (mouse IgG1 κ) were purchased from eBioscience. Freshly isolated PBMC were used for flow cytometric analysis of surface markers.

mAb for intracellular staining

Human Regulatory T-Cell Staining Kit (eBioscience) was used according to manufacturer's instructions for the quantification of CD4⁺/CD25⁺/FOXP3⁺ T cells. Intracellular staining was done with anti-human IL-10 allophycocyanin (rat IgG2a κ ; BioLegend), anti-human granzyme B-PE (clone GB12 mouse IgG1; Invitrogen), and anti-human FOXP3 PE (rat IgG2a κ ; eBioscience). For intracellular staining of IL-10 and granzyme B, PBMC were seeded at 3×10^6 cells in 1 ml complete RPMI 1640 and stimulated with PMA (10 ng/ml) and ionomycin (1.25 μ M) for 4 h, with brefeldin A

FIGURE 2. GMT of NT and HAI test. **(A)** Anti-TBE virus Abs were measured by NT before and 1 wk, 8 wk, and 6 mo after booster immunization. Kinetics of neutralizing TBE Abs in TBE non-responders, TBE high responders, and hepatitis B non-responders are shown. Data points are GMT with 95% confidence interval. **(B)** HAI assay was used to determine influenza Ab titers. Kinetics of HAI test titers to the three influenza strains contained in the vaccine (influenza A H1N1 and H3N2, influenza B) from day 0 to 6 mo postbooster in the three investigated groups are depicted. Data points are GMT with 95% confidence interval. w, week.



added at 10 $\mu\text{g/ml}$ after 1 h. All intracellular staining was carried out with PBMC stored in liquid nitrogen.

HLA class II typing

HLA class II typing of the donor samples was done at the Department of Blood Group Serology, Medical University of Vienna. In the blood samples obtained for tissue typing, DNA was isolated either by salting-out method or GenoMTM-6 (GenoVision; VertriebsGesmbH, Vienna, Austria). HLA class II genes were typed at the high-resolution level by sequence-based typing and comprised HLA DRB1, DRB3/4/5, and DQB1 allele (21).

Statistical evaluation

Ab titers from NT and HAI assays were log transformed, and results are expressed as GMT with 95% confidence interval. Due to skewed distributions, cytokine data were also log transformed. Fractions of lymphocytes were arcsine transformed to obtain normality and to remove correlation between means and variances. Normality was assessed by Kolmogorov-Smirnov test with Lilliefors' correction. In all cases, no significant deviation from normality was found after transformation. Statistical significance was assessed by ANOVA and linear contrasts. Linear contrasts specified the a priori hypotheses, as follows: difference between pre- and postvaccination sera, and comparison between TBE responders and the two groups of nonresponders. For presentation of data in figures and tables, 95% confidence intervals were computed after log transformation of titers or concentrations and arcsine transformation of percentages. For all tests, p values <0.05 were considered significant.

Results

High TBE-specific Abs induced in TBE high responders and hepatitis B nonresponders; low TBE Abs in TBE nonresponders upon TBE revaccination

TBE-specific neutralizing Ab titers are measured as a correlate of protection, and NT titers $>1:10$ are considered protective (Fig. 1). In TBE high responders, protective GMT were present before booster (67.5 GMT [NT]) and increased significantly after booster with peak titers after 2 mo at 139.6 GMT (NT). Titer assessment 6 mo after booster revealed significantly higher titer levels compared with prebooster levels (101.1 GMT [NT]). In contrast, TBE nonresponders showing GMT below protection level prebooster (namely 6.5 GMT [NT]) displayed a slight increase to a GMT (NT) of 9.8 after 1 wk. GMT rose to 18.0 2 mo after booster and declined again (10.7 GMT [NT]) at 6 mo after booster vaccination. Also, in hepatitis B nonresponders, an adequate TBE immune response could be observed. GMT (NT) rose from 57.1 prebooster to 109.7 1 wk postbooster, increased further to 146.8 2 mo after vaccination, and then declined to 88.9 GMT (NT) after 6 mo (Fig. 2A).

To investigate whether nonresponders produce TBE Abs with altered avidity, we determined mean TBE IgG avidity indexes, as described elsewhere, in the three investigated groups (22). No significant differences were found between the groups, indicating that the quality of vaccine-induced IgG TBE Abs was not influenced in nonresponders (data not shown).

In all, TBE titers upon TBE booster remained low in TBE nonresponders, but were markedly enhanced in TBE high responders and also hepatitis B nonresponders.

Protective titers to influenza vaccine in all three groups

The HAI Ab responses against the three influenza virus strains contained in the vaccine were measured in all study participants before and at the three described time points after booster vaccination and are depicted as GMT (Fig. 2B). In TBE high responders, the measured HAI GMT to influenza A H1N1 and influenza B strain clearly increased, whereas titers to influenza A H3N2 remained rather low (GMT 19.6 8 wk postbooster). The titer increase to influenza H1N1 was very rapid in this group, and peak GMT were measured already 1 wk after booster. In the TBE nonresponder group, the HAI titers to both influenza A strains

showed only moderate increase, whereas strong humoral responses to influenza B strain were observed (peak titers 2 mo postbooster). In hepatitis B nonresponders, the humoral response to all three influenza strains was consistently robust with highest GMT measured 2 mo postbooster.

In conclusion, a marked increase of influenza Ab titers to at least one of the virus strains contained in Inflexal V was observed in all vaccination groups.

Different cytokine production profiles after Ag-specific restimulation of PBMC

Production of IL-2, IFN- γ , and IL-10 was measured in cultures of Ag-restimulated PBMC. Upon stimulation with TBE Ag, the

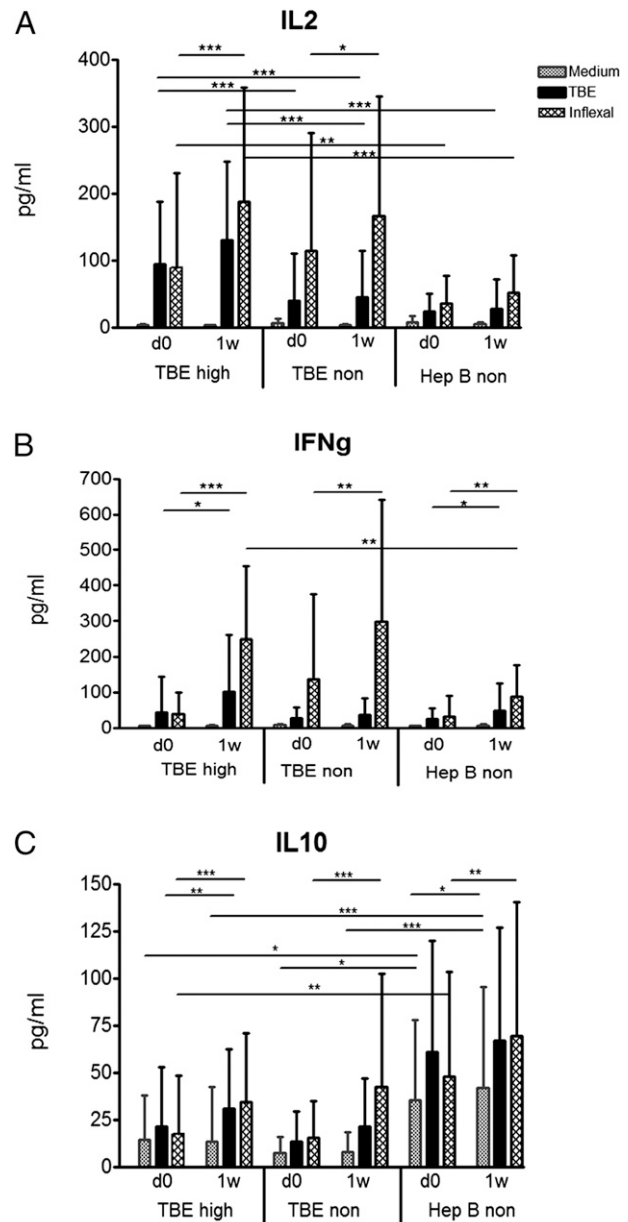


FIGURE 3. Quantification of cytokine secretion in PBMC after Ag-specific restimulation. IL-2 (A), IFN- γ (B), and IL-10 (C) production of PBMC incubated with medium (first bars from left), TBE Ag (full bars), and Inflexal Ag (third bars from left) for 48 h. IL-2, IFN- γ , and IL-10 concentrations in culture supernatants were assessed with Luminex technology. Cytokine levels in pg/ml before (d0) and 1 wk (1w) after booster. Mean values, SD, and p values are shown. ANOVA and linear contrasts, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table I. Distribution of T and B lymphocytes

T/B Cells As % of Total Lymphocytes	CD3		CD19	
	Day 0	1 wk	Day 0	1 wk
TBE high	73.75 (70.15–77.34)*	73.54 (69.62–77.47)	4.46 (3.54–5.37)	3.87 (3.20–4.55)
TBE non	66.50 (60.72–72.29)	68.85 (64.53–73.17)	4.78 (3.48–6.07)	5.29 (3.20–7.39)
Hep B non	72.57 (67.65–77.49)	73.31 (69.70–76.91)	5.13 (4.09–6.18)	4.86 (3.46–6.26)

Mean percentages of CD3 T cells and CD19 B cells before (day 0) and 1 wk after booster vaccination in the three study groups are depicted as percentages of total lymphocytes. Lower and upper 95% confidence intervals of mean percentages are indicated in parentheses. ANOVA and linear contrasts.

* $p < 0.05$, TBE high responders (TBE high) versus TBE nonresponders (TBE non).

Hep B non, Hepatitis B nonresponders.

levels of IL-2, which is a growth factor for Ag-activated T cells and measure of T cell proliferation, were significantly higher in TBE high responders compared with TBE nonresponders before booster (93.7 pg/ml versus 40.1 pg/ml). Measured 1 wk post-booster, the IL-2 levels markedly increased in TBE high responders while remaining low in TBE nonresponders (130.8 versus 45.3 pg/ml). In hepatitis B nonresponders, who showed high protective TBE Ab titers, very low IL-2 production before booster with only marginal increase postbooster was observed. When stimulated with influenza Ag containing the three respective strains, TBE high responders and TBE nonresponders showed high IL-2 levels before booster with a significant increase postbooster, this being in accordance with high humoral responses to influenza vaccine. In contrast, hepatitis B nonresponders displayed low Inflaxal-specific IL-2 levels before booster (significantly lower than in TBE groups) with nonsignificant increase postbooster despite the fact that high anti-influenza titers had been detected in this group (Fig. 3A).

The assessment of IFN- γ production in response to stimulation with TBE-Ag revealed a similar pattern as seen with IL-2. In TBE high responders, IFN- γ levels increased after booster from 43.9 to 101.1 pg/ml ($p < 0.05$), whereas in TBE nonresponders low prebooster IFN- γ levels remained low (26.4 versus 36.7 pg/ml) postbooster. In hepatitis B nonresponders, low IFN- γ levels to TBE-Ag with moderate increase after booster were observed (24.7 versus 49.3 pg/ml). The IFN- γ production upon stimulation with Inflaxal Ag increased significantly after booster in all three groups, yet on a significantly lower level in hepatitis B nonresponders (Fig. 3B).

Quantification of IL-10 in the three investigated groups revealed differing baseline levels in unstimulated PBMC. Compared with TBE high responders and nonresponders, unstimulated cells of hepatitis B nonresponders showed significantly higher IL-10 baseline levels before and after booster vaccination, and, also in restimulated PBMC, IL-10 levels were highest in this group. Postbooster increase of IL-10 in response to TBE Ag was observed only in TBE high responders, whereas, after restimulation with Inflaxal Ag, significant increase of IL-10 was observed in all three groups (Fig. 3C). Additionally, IL-10 production of restimulated cells was compared between the groups after subtraction of baseline

levels; this revealed that prebooster PBMC from hepatitis B nonresponders produced significantly more IL-10 upon TBE stimulation than both TBE groups (data not shown).

Also, TGF- β was quantified in supernatants of unstimulated and Ag-restimulated PBMC. Significantly elevated TGF- β levels were observed in unstimulated cells of TBE and hepatitis B nonresponders compared with TBE high responders; moreover, Ag-specific restimulation did not result in increased TGF- β production (data not shown).

In summary, the in vitro cytokine profiles revealed that high/low levels of IL-2 and IFN- γ were present in TBE high responders/nonresponders upon TBE stimulus, whereas Inflaxal induced high levels of IL-2 and IFN- γ in both TBE groups. In hepatitis B nonresponders, low IL-2 and IFN- γ levels were measured upon stimulation with both TBE and Inflaxal, which were accompanied by high IL-10 production in unstimulated as well as Ag-stimulated PBMC cultures.

Flow cytometric characterization and quantification of lymphocyte subpopulations

The magnitude of B and T lymphocyte populations, their respective naive/memory compartments, effector function of cytotoxic T cells, and regulatory immune cell populations were investigated in the three study groups.

Normal T and B cell distributions in the three study groups. The quantification of CD3 T cells and CD19 B cells as percentage of total lymphocytes revealed a normal distribution of the respective populations. Except for significantly more ($p < 0.05$) CD3 T cells before booster in TBE high responders compared with TBE nonresponders, no other significant differences between the three groups before/after booster were observed. Also, percentages of CD4 Th cells and CD8 CTL were in the normal range without significant differences between groups before/postbooster vaccinations (Tables I, II).

Naive and memory subpopulations of B cells. Distinction of naive and memory B cells was done via staining of CD27 and IgD on B cells. A smaller population of CD27⁺/IgD⁺ naive B cells was found in hepatitis B nonresponders compared with the TBE high-responder control group (45.3 versus 54.7% of total B cells). In turn, hepatitis B nonresponders showed an increased percentage of

Table II. Distribution of CD4 and CD8 T cells

Th/CTL As % of Total Lymphocytes	CD4		CD8	
	Day 0	1 wk	Day 0	1 wk
TBE high	53.25 (49.29–57.21)	53.81 (49.40–58.23)	18.54 (15.66–21.42)	17.81 (14.82–20.80)
TBE non	49.38 (43.73–55.03)	51.09 (46.42–55.76)	17.72 (14.98–20.47)	17.64 (14.86–20.42)
Hep B non	50.14 (45.47–54.81)	50.37 (45.90–54.83)	19.35 (15.85–22.86)	20.10 (16.65–23.56)

Mean percentages of CD4 T cells and CD8 cytotoxic T cells before (day 0) and 1 wk after booster vaccination in the three study groups as percentages of total lymphocytes. Lower and upper 95% confidence intervals of mean percentages are indicated in parentheses.

Hep B non, hepatitis B nonresponders; TBE high, TBE high responders; TBE non, TBE nonresponders.

switched B memory cells (CD27⁺/IgD⁻, 29.0 versus 24.1%); differences were not significant and remained unchanged after booster (data not shown).

Significantly different naive and memory subpopulations of T cells. Via expression of CCR7 and CD45RA on T cells, naive, lymph node homing T central memory, and peripheral tissue homing T effector memory (T_{EM}) populations of CD4 Th cell and CD8 cytotoxic T cells can be quantified (23). Results in CD4 T cells showed that both nonresponder groups had a lower mean percentage of naive T cells (39.9% in TBE nonresponders and 46.6% in hepatitis B nonresponders versus 52.9% in TBE high responders). In contrast, T memory populations were increased in nonresponders, especially T_{EM} percentages were significantly higher with 16.7% in TBE nonresponders ($p < 0.01$) and 11.6% in hepatitis B nonresponders ($p < 0.05$) versus 8.8% TBE high responders. These differences were observed both before and after booster vaccination (Table III).

In contrast to Th cells, CD8 T cells contain a CD45RA⁺ T_{EM} subset termed T_{EMRA} of ~30% (24). As seen in CD4 Th cells, TBE and hepatitis B nonresponders had less naive CD8 T cells than TBE high responders (22.7 and 25.3 versus 30.0% of CD8), but only TBE nonresponders showed increased T_{EM} cells (31.9 versus 25.6% in TBE high responders) before booster. After booster, a significant difference in T_{EM} population between TBE nonresponders and high responders was observed ($p < 0.05$, Table IV).

TBE nonresponders have more CTLs with progressed effector function. Coexpression of CD27 and CD28 on CD8 T cells can be used to distinguish three functionally different subsets of CD8 effector T cells. Early differentiated CD8 T cells express both CD27 and CD28, intermediates have a CD27⁺/CD28⁻ phenotype, and late differentiated cells are CD27⁻/CD28⁻ (25). Downregulation of these surface markers corresponds with a progressed/higher differentiated effector function of CTL (Fig. 4A). The quantification of these subsets in our study population showed that TBE nonresponders had clearly less CTL with early differentiated effector function compared with TBE high responders (52.3 versus 60.2%) before booster. In turn, more highly differentiated CTL were present in TBE nonresponders (33.9 versus 25.5%); this difference became even more pronounced (34.7 versus 23.5%; $p < 0.05$) after booster vaccination (Fig. 4B).

TBE nonresponders and hepatitis B nonresponders show increased FOXP3⁺ T regulatory cells. Both nonresponder groups displayed a significant increase of the CD4⁺/CD25⁺/FOXP3⁺ T regulatory cell (T-reg) population after booster (3.97–4.44% in TBE nonresponders, and 4.03–4.65% in hepatitis B nonresponders), whereas in the control group T-regs significantly decreased after vaccination (mean 5.04–4.59% of CD4 T cells; Fig. 5A). In representative nonresponder and high-responder vaccinees, a combined staining of intracellular IL-10 and FOXP3 was performed, revealing that IL-10-secreting FOXP3⁺ T-regs increase after booster in both nonresponder groups (Fig. 5B, 5C). In these donors, also type 1 T-regs (Tr1) were analyzed via intracellular staining of granzyme B and IL-10 in CD4 T cells (26), yet no induction of this adaptive T-reg population was observed (data not shown).

The described representative hepatitis B nonresponders were individuals, who featured high NT and HAI titers after booster, but lacked IL-2 and IFN- γ production upon Ag-specific in vitro restimulation. Additionally, these donors had high IL-10 basal levels in unstimulated PBMC and were HLA class II typed, showing both alleles associated with hepatitis B nonresponsiveness (HLA-DRB1*07, HLA-DQB1*02).

Representative TBE nonresponders lacked humoral and cellular responses to TBE vaccine. Humoral response to influenza was

Table III. Flow cytometric characterization of CD4⁺ T cells

CD4 T Cell Subpopulations As % of CD4	Naive		CM		EM		EMRA	
	Day 0	1 wk	Day 0	1 wk	Day 0	1 wk	Day 0	1 wk
TBE high	52.89 (47.42–58.35)	52.98* (47.17–58.80)	36.98 (31.87–42.10)	36.63 (31.18–42.08)	8.75*** (7.36–10.15)	8.8*** (7.24–10.51)	1.37 (0.72–2.03)	1.29 (0.76–1.81)
TBE non	39.87 (30.93–48.81)	40.56 (31.49–49.63)	40.04 (34.30–45.78)	40.41 (34.61–46.20)	16.67 (11.51–21.84)	15.45 (10.87–20.03)	3.28 (1.60–4.97)	3.58 (1.76–5.41)
Hep B non	46.62 (39.23–54.02)	47.20 (39.96–54.44)	38.73 (32.51–44.95)	37.05 (30.95–43.14)	11.30 (9.08–13.52)	12.63 (9.99–12.27)	3.33 (1.29–5.38)	2.89 (1.15–4.65)

Significantly different naive and memory subpopulations in CD4 T cells. Expression of CCR7 and CD45RA was used to quantify T_{naive} (Naive), T_{CM} (CM), and T_{EMRA} (EMRA) populations of Th cells. Percentages of subpopulations as percentages of total CD4 T cells; lower and upper 95% confidence intervals of mean percentages are indicated in parentheses. Statistical significance was assessed by ANOVA and linear contrast.

* $p < 0.05$, TBE high responders (TBE high) versus TBE nonresponders (TBE non).

** $p < 0.05$, TBE high versus hepatitis B nonresponders (Hep B non).

*** $p < 0.01$, TBE high versus TBE non.

Table IV. Flow cytometric characterization of CD8⁺ T cell subsets

CD8 T Cell Subpopulations As % of CD8	Naive		CM		EM		EMRA	
	Day 0	1 wk	Day 0	1 wk	Day 0	1 wk	Day 0	1 wk
TBE high	29.97 (22.87–37.07)	31.10 (23.41–38.78)	16.82 (13.12–20.53)	17.56 (13.68–21.44)	25.64 (21.94–29.34)	23.51* (19.69–27.34)	27.57 (23.03–32.11)	27.87 (21.89–33.86)
TBE non	22.73 (15.12–30.34)	22.81 (14.65–30.98)	14.95 (11.33–18.5)	15.22 (11.68–18.76)	31.90 (24.98–38.81)	31.71 (24.63–38.80)	30.35 (22.43–38.28)	29.79 (21.93–37.65)
Hep B non	25.31 (18.33–32.29)	25.00 (18.42–31.58)	18.11 (14.04–22.17)	15.59 (11.73–19.45)	25.41 (21.03–29.79)	25.90 (21.94–29.85)	30.66 (23.68–37.63)	33.52 (27.00–40.04)

Significantly different naive and memory subpopulations in CD8 T cells. Expression of CCR7 and CD45RA was used to quantify T_{naive} (Naive), T_{CM} (CM), T_{EM} (EM), and T_{EMRA} (EMRA) populations of CTL. Percentages of subpopulations as percentages of total CD8 T cells; lower and upper 95% confidence intervals of mean percentages are indicated in parentheses. Statistical significance was assessed by ANOVA and linear contrasts. *p < 0.05, TBE high responders (TBE high) versus TBE nonresponders (TBE non).

observed to at least one of three strains, and in vitro IL-2 and IFN-γ production upon restimulation with influenza Ag was present. These donors had no detectable IL-10 in unstimulated cultures of PBMC.

The representative TBE high responders had clearly increased Ab titers to both vaccine Ags after the booster vaccinations and also showed high in vitro IL-2 and IFN-γ production in Ag-restimulated PBMC. No IL-10 in unstimulated PBMC was present.

Large population of immature transitional B cells, precursors of B regulatory cells in hepatitis B nonresponders. Immature transitional CD19⁺/CD24^{high}/CD38^{high} B cells have been shown to possess regulatory capacity in healthy donors via IL-10 production (27). This B cell subtype was quantified in selected nonresponder and high-responder donors as percentage of total B cells (Fig. 6A). Hepatitis B nonresponders showed the highest numbers of this cell type constituting 34% of B cells before booster and remaining so postbooster. In TBE nonresponders, this potential B regulatory cell population significantly increased postbooster from lower prebooster numbers (from 10.5 to 18.4% and from 21.2 to 25.2%). In contrast, TBE high responders had medium percentages of CD19⁺/CD24^{high}/CD38^{high} B cells prebooster, which clearly decreased after vaccination (from 12.3 to 9.9% and from 20.9 to 7.6%) (Fig. 6B).

In conclusion, flow cytometric quantification of T cell subpopulations showed increased percentages of CD4 T_{EM} populations (versus decreased naive cell subsets) in TBE and hepatitis B nonresponders. In TBE nonresponders, also an increased CD8 T_{EM} subset along with highly differentiated effector function of CD8 T cells was present. Foxp3⁺Tregs producing IL-10 were increased postbooster in both nonresponder groups. In hepatitis B nonresponders, a consistently high percentage of immature transitional B cells was also observed.

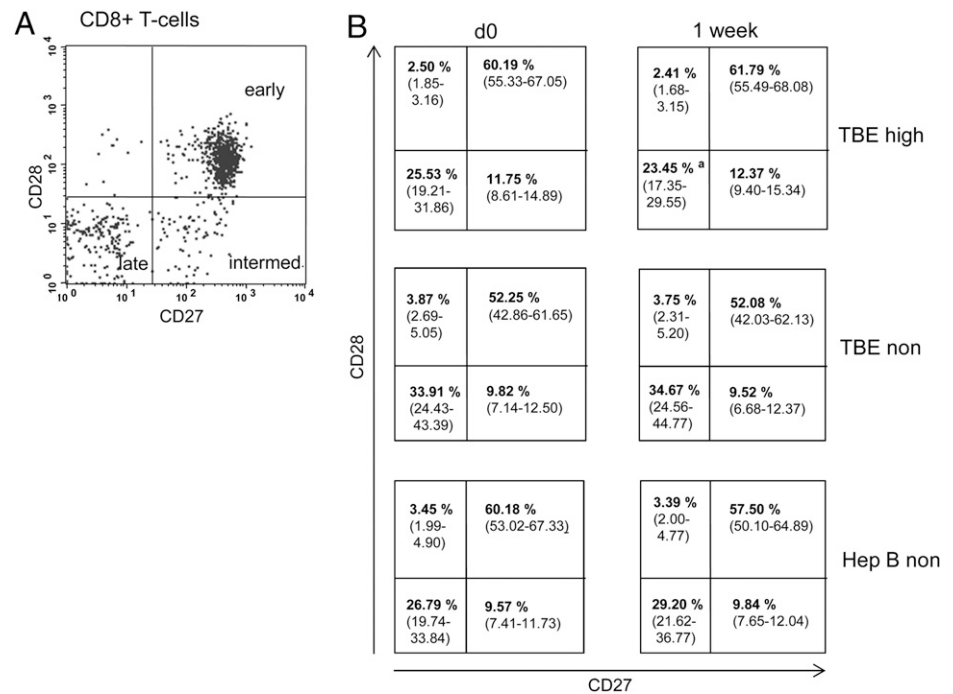
Confirmed HLA class II subtypes in hepatitis B nonresponsiveness

Certain HLA class II subtypes (HLA-DRB1*07 and HLA-DQB1*02) have been described to be associated with nonresponsiveness to current hepatitis B vaccines (6). These subtypes were also in our study overrepresented in the hepatitis B nonresponder group with 14 of 22 donors having at least one of these alleles. In contrast, the increased presence of these alleles was not seen in TBE nonresponders (7 of 23), similar as in the TBE high-responder control group (8 of 22). When calculating the total number of HLA-DRB1*07 and HLA-DQB1*02 alleles per group, we found 29 alleles in hepatitis B nonresponders versus 9 in TBE nonresponders (11 in TBE high responders). Thus, our data confirm the described HLA associations in hepatitis B nonresponsiveness and indicate that TBE nonresponsiveness is not correlated with these subtypes. We also observed that no other DR-B1, B3/4/5, or DQ-B1 subtypes were present with increased frequency in the TBE nonresponder group, suggesting that this type of nonresponsiveness probably involves no HLA class II association.

Discussion

In the current study, we asked whether nonresponsiveness is a general phenomenon and tried to identify important immunologic pathways. The study featured the comparison of TBE nonresponders/low responders versus a TBE high-responder control group and additionally included habitual hepatitis B nonresponders. Immune responsiveness at humoral and cellular level to TBE and influenza booster vaccination was evaluated in detail, and our results provide a new perspective on the immunologic background of this immune dysfunction.

FIGURE 4. TBE nonresponders have more CTLs with progressed/late effector function than TBE high responders. **(A)** Gating of the early ($CD27^+/CD28^+$), intermediate ($CD27^+/CD28^-$), and late ($CD27^-/CD28^-$) differentiated subpopulation of $CD8^+$ cytotoxic T cells. **(B)** Distribution of the three subsets as percentages of total $CD8^+$ CTLs before (d0) and 1 wk after booster vaccination in the three investigated groups. Lower and upper 95% confidence interval of mean percentage is indicated in parentheses. Statistical significance was assessed by ANOVA and linear contrasts; $^a p < 0.05$, TBE high responders versus TBE nonresponders.



As expected, in TBE nonresponders/low responders, TBE-specific titers increased only marginally until week 8 and then again declined below protective levels after 6 mo. In TBE high responders, an increase of already high TBE titers was observed

with peak titers 8 wk after booster. After 6 mo, Ab levels were still higher than prebooster titers. Hepatitis B nonresponders mounted TBE titers that were similar in magnitude and time profile to the high-responders control group, indicating that in these subjects

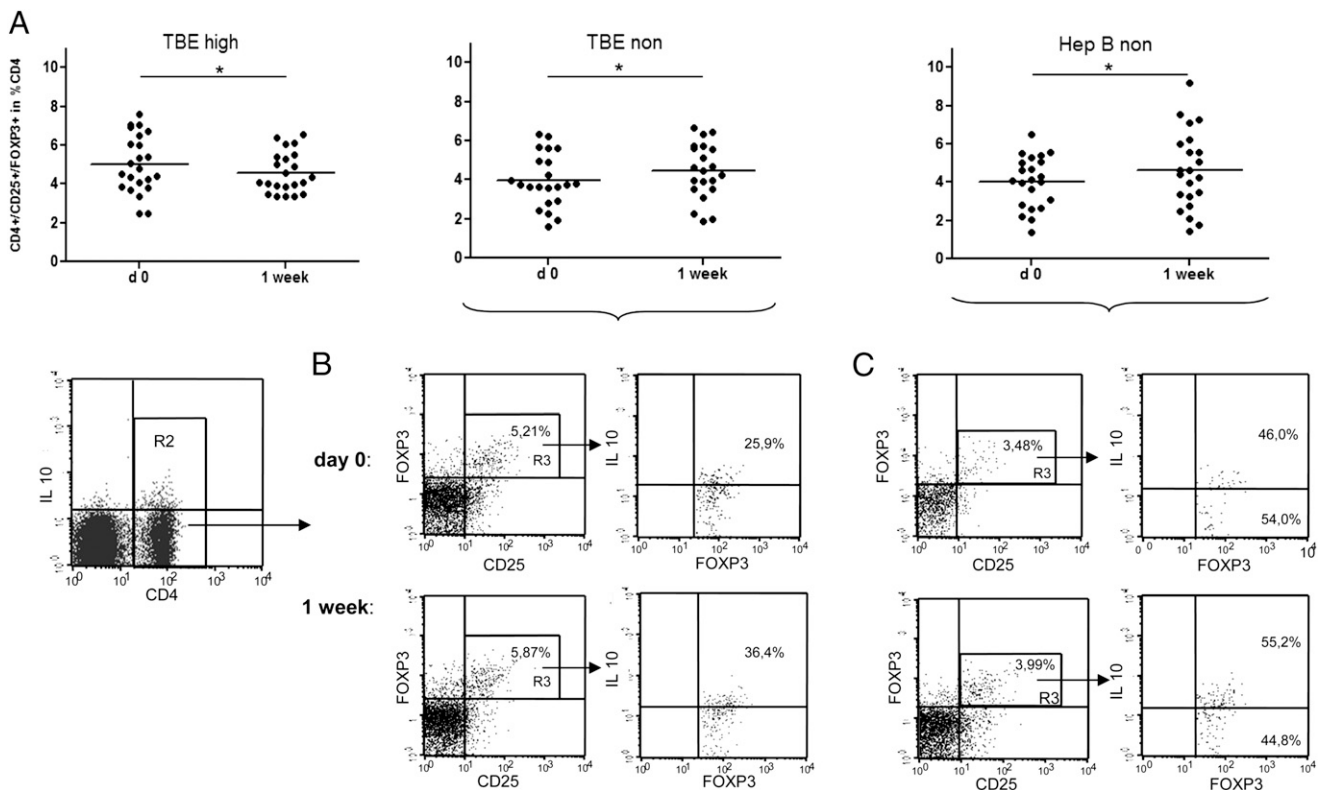


FIGURE 5. Increased Tregs in TBE and hepatitis B nonresponders. **(A)** T-reg distributions ($CD4^+/CD25^+/FOXP3^+$). Percentage of Tregs ($CD25^+/FOXP3^+$) as percentage of total $CD4^+$ T cells before (d0) and 1 wk after booster in the three investigated groups. Statistical significance was assessed by ANOVA and linear contrasts, $^a p < 0.05$. **(B and C)** Percentage of IL-10-positive $FOXP3^+$ Tregs before and after booster. Representative example of increased Tregs ($CD25^+/FOXP3^+$) as percentage of $CD4^+$ T cells, R3, and increased IL-10⁺ Tregs (as percentage of total Tregs) 1 wk after booster vaccination in a TBE nonresponder (B) and a hepatitis B nonresponder (C).

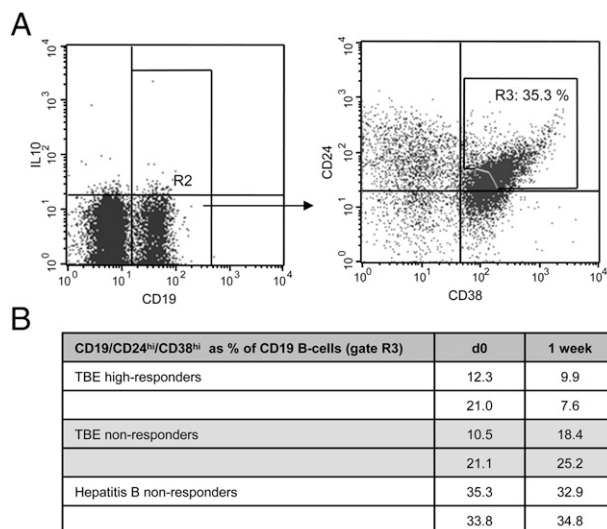


FIGURE 6. Large population of immature transitional B cells in hepatitis B nonresponders. **(A)** Gating of CD19⁺/CD24^{high}/CD38^{high} B cells. Expression of CD24 and CD38 on CD19⁺ B cells; the CD24^{high}/CD38^{high} subset constitutes immature transitional B cells. **(B)** Table with percentages of CD19⁺/CD24^{high}/CD38^{high} as percentage of total CD19⁺ B cells in selected donors of the three investigated groups.

nonresponsiveness at the humoral level seems to be restricted to hepatitis B Ag (Fig. 2A). This was further confirmed by sufficient humoral immune responses to the influenza vaccine in all three investigated groups (Fig. 2B). Taken together, the observed TBE and influenza titers indicate that both types of nonresponsiveness are Ag specific at the humoral level because high responsiveness (Ab production) to unrelated vaccine Ags is present in these vaccinees.

Cytokine levels in both TBE groups confirm that cellular responsiveness correlates with Ab titers. Upon in vitro stimulation with the TBE Ag, TBE high responders showed an increase of already higher IL-2 and IFN- γ production, whereas a lack of TBE-specific Abs in TBE nonresponders was accompanied by consistently low cytokine levels. Stimulation with the three influenza strains resulted in a significant increase of IL-2 and IFN- γ production postbooster in both TBE groups, which was in accordance with the observed high influenza-specific humoral responses. We recently described a similar positive correlation between Abs and cytokine production in hepatitis A high responders and nonresponders (12). In contrast, in hepatitis B nonresponders, humoral and cellular immune responses to unrelated vaccine Ags were not positively correlated. In these donors, high protective titers to TBE and influenza vaccine were detected, whereas restimulation with both vaccine Ags resulted in low IL-2 and IFN- γ levels (Fig. 3A, 3B).

In contrast, the measurement of immune-suppressive IL-10 in this group showed significantly higher IL-10 baseline levels already present in unstimulated PBMC before and after booster compared with both TBE groups. IL-10 levels after antigenic stimulation were also highest in hepatitis B nonresponders (Fig. 3C). Interestingly, highest IL-10 levels in unstimulated cells were observed in donors that had HLA class II subtypes associated with hepatitis B nonresponsiveness. We hypothesize that in these vaccinees elevated baseline IL-10 levels could be present due to genetic polymorphisms leading to higher IL-10 production as described for hepatitis B nonresponders (8). Alternatively, multiple hepatitis B boosters in individuals with specific MHC class II subtypes possibly resulted in prolonged low-avidity Ag presentation leading to induction of tolerance and IL-10 production.

According to our findings, the presence of IL-10 probably abrogates T cell proliferation upon TBE and influenza vaccine Ag stimulation in vitro in hepatitis B nonresponders. Yet, in vivo these donors are capable of generating TBE and influenza-specific Abs in a secondary/memory immune response. According to recent studies, secondary Ab responses require IL-2 production from proliferating, Ag-specific CD4 T cells, leading to induction of IgG-producing plasma cells and cognate T cell help (CD40/CD40L interaction) for Ig class switching (28, 29). These observations raise the question of how hepatitis B nonresponders are capable of producing Ag-specific Ab titers, if abrogated T cell proliferation is not only an in vitro phenomenon but also present in vivo. An increased B memory cell pool present in this group allows speculation that higher numbers of memory cells could enable sufficient Ab production despite lacking T cell proliferation. It certainly remains questionable whether these individuals are able to mount a robust primary immune response when encountering an unknown/new vaccine Ag. This is an issue to be addressed in further studies.

In addition to IL-10, also TGF- β baseline levels were increased, though in both nonresponder groups (data not shown). TGF- β is a prerequisite for peripheral induction of T-regs, which is in line with expanded T-reg populations found after booster vaccination in TBE and hepatitis B nonresponders.

For a more complete investigation of cellular immune response to vaccine Ags, we quantified different T and B cell subsets to observe whether nonresponsiveness is associated with altered naive and memory compartments and/or regulatory cell populations, possibly accounting for the observed high base levels of IL-10 in hepatitis B nonresponders.

Only limited data on the role of T-regs in vaccine responsiveness are available (30, 31). Mostly, their number and function have been investigated in relation to autoimmune diseases, in graft-versus-host reactions and in cancer (30, 32, 33). We have previously demonstrated that FOXP3⁺ T-regs appear to relatively decrease postbooster in normal responders, which was shown upon hepatitis A vaccination in healthy donors and in breast cancer patients receiving a Her-2/neu vaccine (34). Also, the application of a prostate cancer vaccine resulted in lower T-reg numbers postvaccination in investigated patients; additionally reduced T-reg activity postbooster correlated positively with the overall survival of these patients (35). The decrease of T-regs as percentage of CD4 T cells postbooster is most likely not in absolute numbers, but rather a result of Ag-specific T-effector cell expansion postbooster.

The quantification of FOXP3⁺ T-regs in our study population revealed that, in TBE high responders, representing immune-competent controls, FOXP3⁺ T-regs significantly decreased after booster vaccination, which is in accordance with the above-mentioned studies. In contrast, in both nonresponder groups, FOXP3⁺ T-regs were significantly expanded after booster (Fig. 5A). Also, Tr1 cells, which are peripherally induced upon chronic Ag stimulation in the presence of IL-10 (36), were characterized in selected donors, yet no induction was observed in the three investigated groups.

In TBE nonresponders, the expansion of existing TBE-Ag-specific natural T-regs (37) might have led to the observed increase of FOXP3⁺ T-regs, whereas high TGF- β levels indicate that, in parallel, the induction of inducible T-regs could also have occurred. The role of T-regs in nonresponsiveness to hepatitis B vaccine has been investigated by Yang et al. (38, 39), who showed higher numbers of FOXP3⁺ T-regs after a hepatitis B booster vaccination along with an upregulation of programmed cell death-1 molecule in nonresponders. A more recent study demonstrated higher FOXP3⁺

T-reg/CD4 T cell ratios in hepatitis B nonresponders as well as increased FOXP3 mRNA levels upon stimulation with HBs Ag or unspecific PHA stimulus (40). It is important to note that in our study context the expansion/induction of FOXP3⁺ T-regs in hepatitis B nonresponders occurred upon encounter of unrelated vaccine Ags, which indicates that induction of inducible T-regs in the presence of TGF- β rather than expansion of Ag-specific T-regs might account for the increase of FOXP3⁺ T-regs after booster in this group. In contrast, an induction of hepatitis B virus-specific T-regs might have occurred earlier in the context of repeated hepatitis B vaccinations, which now expand upon unspecific stimulus and lead to decreased immune responses to unrelated Ags.

Furthermore, we could demonstrate that, in selected TBE and hepatitis B nonresponders, more IL-10–producing Foxp3⁺ T-regs are present in the expanded T-reg populations after booster vaccination, possibly indicating an active suppressive function of this cell population (Fig. 5B, 5C).

The observations on T-reg populations in our study groups indicate that high IL-10 levels present in hepatitis B nonresponders prior to vaccination cannot solely be attributed to FOXP3⁺ T-regs because similar frequencies are present in TBE nonresponders, who do not show high IL-10 baseline levels. Thus, we characterized other potentially regulatory immune cell populations, firstly B cells. Immune regulatory function of human B cells via secretion of IL-10 has been described in the context of immune-related diseases such as multiple sclerosis (41, 42) and systemic lupus erythematosus (27, 43) as well as in natural tolerance of renal transplants (44). Blair et al. (27) have shown that human CD19⁺CD24^{high}CD38^{high} immature B cells possess regulatory capacity via production of IL-10 upon stimulation with anti-CD40, for example, abrogation of Th1 differentiation via IL-10 but not TGF- β was observed. Very recently, a role for suppressive B regulatory cells was demonstrated in chronic hepatitis B carriers; the numbers of IL-10–producing CD19⁺CD24^{high}CD38^{high} immature B cells correlated with disease flares, and sorted cells could suppress HBV-specific CD8 T cell responses in vitro (45). Additionally, these potential B regulatory cells have been shown to favor the induction of FOXP3⁺ T-regs (46).

In line with these findings, quantification of CD19⁺CD24^{high}CD38^{high} immature B cells in selected donors of our study population revealed the highest percentage of this potentially B regulatory cell type in hepatitis B nonresponders, remaining high after booster vaccination. A lesser percentage of CD19⁺CD24^{high}CD38^{high} immature B cells was found in both TBE groups, which increased postbooster in TBE nonresponders, but decreased in TBE high responders (Fig. 6B). We hypothesize that this B regulatory cell population might contribute to the increased baseline levels of IL-10 in hepatitis B nonresponders and also lead to an induction of FOXP3⁺ T-regs in both nonresponder groups.

In addition to polymorphic regulatory cell populations, the nonresponder groups showed variation also in other T cell compartments. Analysis of naive and memory CD4 T cell subsets revealed that especially TBE, and to a lesser extent also hepatitis B nonresponders, have less naive CD4 T cells (CCR7⁺/CD45RA⁺), and in turn significantly more T_{EM} cells (CCR7⁺/CD45RA⁺) before and after booster vaccination (Table III). The T_{EM} compartment represents circulating, Ag-experienced T cells homing to peripheral tissues with limited proliferative capacity, but readily available T cell effector function via production of effector cytokines and molecules (23, 47). Cytotoxic CD4 T cells in vaccine-induced protection in humans have been described previously (48), and thus, a higher frequency in nonresponders indicates that in these vaccinees increased CD4 T cell effector function might be present, possibly in compensation for lacking Abs.

Subsets of CD8 T cells show similar distribution patterns as CD4 Th cells. Naive CD8 T cells are less frequent in both non-responder groups, whereas a higher percentage of T_{EM} cells is found in TBE nonresponders before and after booster vaccination (Table IV). This indicates a particular role for CTL in TBE non-responsiveness, which is further supported by an increased presence of CTL with highly progressed effector function in these vaccinees. Expression of CD27 and CD28 on CTL allows the assessment whether virus-specific CTL effector function has been recalled repeatedly, this leading to downregulation of these surface markers (25). Quantification of the early, intermediate, and late subset shows that TBE nonresponders have less CTL, with early and more with progressed/late effector differentiation compared with TBE high responders (Fig. 4B). The fact that CD27 and CD28 are downregulated indicates that CTL have been repeatedly activated, possibly due to repeated TBE immunizations, which via cross-presentation induce Ag-specific CD8 T cells (49).

In summary, our study on vaccine nonresponsiveness shows that different types of nonresponsiveness are based on varying immunologic mechanisms. TBE nonresponsiveness is strictly Ag specific at the humoral and cellular level, and, unlike hepatitis B nonresponsiveness, seems to involve no HLA class II association. Increased CD8 T_{EM} populations and also progressed CTL differentiation indicate a possible compensatory mechanism on CD8 T cell level in this group. FOXP3⁺ T-regs are induced after booster vaccination, which potentially abrogates T cell proliferation to TBE Ag (at least in vitro) and/or directly suppresses IgG production of B cells. In contrast, hepatitis B nonresponsiveness displays a completely different immunologic setting. The humoral immune response to unrelated, but previously encountered, vaccine Ags is unimpaired, whereas T cell proliferation to these Ags is reduced in vitro, most likely due to high baseline levels of IL-10. Increased IL-10 is correlated with presence of HLA class II subtypes associated with hepatitis B nonresponsiveness, indicating a role for irregularities in hepatitis B Ag presentation. Whether this suppressive cytokine impairs immune function in other ways in these individuals remains to be investigated. Increased numbers of potential B regulatory cells are present in hepatitis B nonresponders, possibly contributing to a priori high IL-10 levels and in parallel inducing suppressive FOXP3⁺ T-regs that exert broader suppressive function.

The different immunologic pathways leading to vaccine non-responsiveness that were outlined in this work need to be further clarified; thus, more detailed in vitro studies on Ag presentation and functionality of involved regulatory cell subsets are being planned.

Disclosures

The authors have no financial conflicts of interest.

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