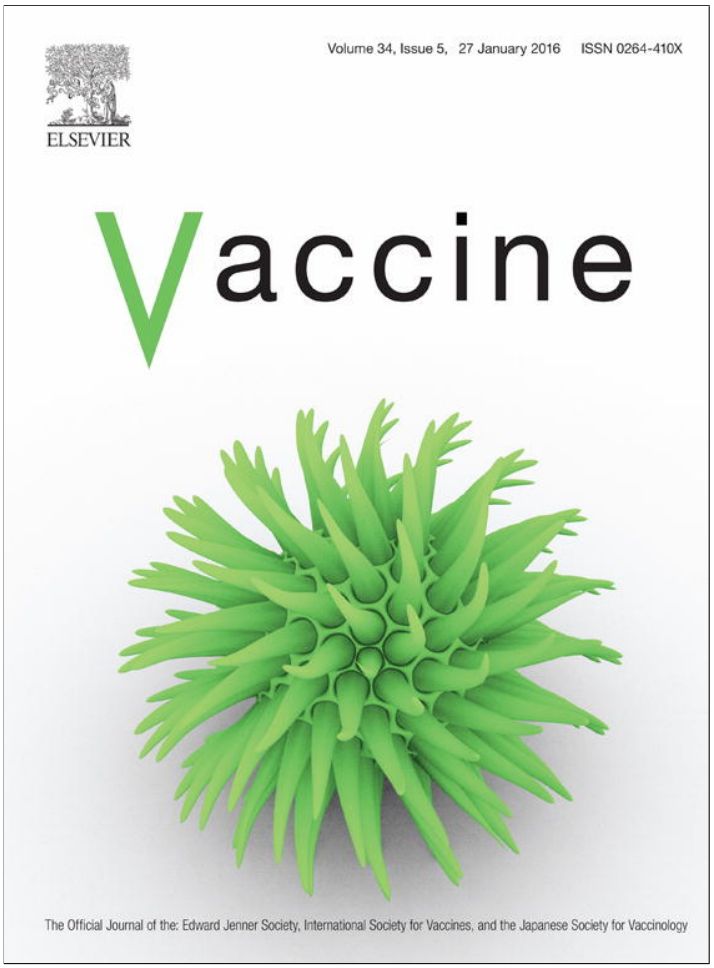

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# Characteristics of immune memory 10–15 years after primary hepatitis B vaccination

Irena Brunskole Hummel[a,*], Anita Zitzmann[a], Monika Erl[a], Jürgen J. Wenzel[b], Wolfgang Jilg[a]

[a] Institute of Medical Microbiology and Hygiene, University of Regensburg, 93053 Regensburg, Germany
[b] Institute of Clinical Microbiology and Hygiene, University Medical Centre Regensburg, 93053 Regensburg, Germany

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ABSTRACT

*Background and aims:* The definition of immune memory after hepatitis B vaccination is still under debate. Therefore, we analysed hepatitis B surface antigen (HBsAg)-specific memory in more detail by investigating the kinetics of humoral and cellular responses after hepatitis B booster vaccination.
*Methods:* The anti-HBs kinetics of 23 individuals with anti-HBs titres below 10 IU/l, who had been vaccinated 10–15 years ago, was monitored at day 0, 3, 7, 14 and 28 after booster vaccination. HBsAg-specific IFNγ- and IL5-secreting cells in enriched $CD4^+$ fraction were measured at day 0, 7 and 28 post-booster by enzyme-linked immunospot assay (ELISpot).
*Results:* 22 of 23 subjects showed similar anti-HBs kinetic curves, including 3 of 4 subjects who did not reach anti-HBs titres of 10 IU/l. The steep anti-HBs increase started between day 3 and 7 and peaked around day 14. A plateau or only minimal changes were visible between day 14 and 28. 17.4% of subjects showed pre-booster cellular responses, and this rate had increased to 47.8% and 56.5% after 7 and 28 days, respectively. The kinetic patterns of T cell responses differed considerably among subjects. A dominance of Th2 responses (IL5 secretion) over Th1 responses (IFNγ secretion) could be observed.
*Conclusions:* The presence of B cell memory could be shown by a typical anamnestic anti-HBs response curve after a booster dose in all but one individual. In contrast, T cell responses to booster vaccination, which occurred in approximately 50% of participants, were rather heterogeneous.


## 1. Introduction

Protection after hepatitis B vaccination is based on two immune mechanisms: specific antibodies against hepatitis B surface antigen (HBsAg) confer protection against infection with the hepatitis B virus (HBV), whereas HBsAg-specific B and T cell-mediated immune memory protects against HBV-disease after the disappearance of antibodies [1]. HBsAg-specific immune memory is usually shown by an anamnestic response to a booster dose of hepatitis B vaccine. A typical anamnestic response is characterised by a rapid 10–100-fold increase in specific antibodies, which starts 5–8 days after the re-exposure to the antigen and peaks after about 14 days [2–4]. HBsAg-specific memory has been shown to persist for at least 15–17 years after immunisation [5–7]. Long-term follow-up studies have shown that clinical HBV-disease or HBsAg-carrier status rarely occur among successfully vaccinated individuals, even in the case of anti-HBs titres <10 IU/l [8]. These observations led to the conclusion that protection against clinically significant breakthrough infection and chronic carriage is long-term rendering booster doses unnecessary [1,9–11]. However, over the past years, a number of studies have reported about waning immunity over time by showing a loss of the ability to respond to booster vaccination [12–16]. A recent meta-analysis has shown that, 20 years after infant vaccination, about 40% of individuals who had lost protecting antibodies did not respond to booster vaccination [17].

However, one problem in evaluating studies reporting on waning immunity is the lack of a clear definition of immune memory.

*Abbreviations:* ELISpot, enzyme-linked immunospot assay; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFNγ, interferon γ; IL5, interleukin 5; PBMC, peripheral blood mononuclear cells; SFC, spot forming cells; Th1, T helper 1; Th2, T helper 2.

* Corresponding author at: Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany. Tel.: +49 941 944 4620; fax: +49 941 944 6402.
*E-mail addresses:* irena.hummel@klinik.uni-regensburg.de (I. Brunskole Hummel), anita-zitzmann@t-online.de (A. Zitzmann), monika.erl@klinik.uni-regensburg.de (M. Erl), juergen.wenzel@klinik.uni-regensburg.de (J.J. Wenzel), wolfgang.jilg@klinik.uni-regensburg.de (W. Jilg).

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Anti-HBs antibodies can be reliably measured by standardised tests, but no standardised method is yet available for measuring specific immune memory. In studies in which immune memory was shown by means of the increase in anti-HBs after booster vaccination, anti-HBs titres were mostly measured only once and at rather different time points, ranging from 10 days to 2 months post-booster [7,18–21]. In addition, in some studies anamnestic response was defined as an increase in anti-HBs to ≥10 IU/l [21–25], in other studies as 4-fold increase in anti-HBs [5], and sometimes both criteria were applied [13,15,20,26]. Because of the differing definitions, it seems to be impossible to differentiate in all cases between anamnestic responses due to the presence of immune memory and primary responses after loss of immune memory.

In contrast to B cell memory, T cell memory has only been investigated in a few studies. In most of these few studies specific T cells were analysed after HBV booster vaccination using ELISpot assays with unfractionated PBMCs (peripheral blood mononuclear cells). However, this method is prone to failure because of the possible activation of nonspecific bystander cells. Moreover, HBsAg-specific T cell memory has been rather heterogeneously analysed in these studies and measured at different time points after booster vaccination [12,27–29].

In the present study we aimed to define optimal criteria for assessing HBsAg-specific immune memory. To assess B cell memory, we studied the kinetics of anti-HBs titres at day 0, 3, 7, 14 and 28 post-booster in subjects who had been vaccinated 10–15 years before and in whom anti-HBs had fallen below the critical value of 10 IU/l. In order to analyse the T cell memory as well, we enriched $CD4^+$ T cells and identified HBsAg-specific T helper 1 (Th1) and T helper 2 (Th2) cell responses in this fraction by measuring IFNγ- and IL5-secreting cells at day 0, 7 and 28 post-booster.

## 2. Methods

### 2.1. Study cohort

77 adolescents (33 male, 44 female) aged 14–18 years were included in this study. All subjects had obtained a full course of 3 vaccinations against hepatitis B 10–15 years ago. The interval between the 1st and the 2nd dose was 1–3 months (mean 1.4 months) and between the 2nd and the 3rd dose 6–20 months (mean 11.7 months). 66 subjects had received monovalent vaccines (63 Engerix B®, 3 GenHBvax®), 1 subject the hepatitis A/B combination vaccine Twinrix® and 1 the hexavalent vaccine Hexavac®. In 3 subjects, Engerix B® and GenHBvax® were mixed and in 6 subjects, the brand of at least one vaccine was unknown.

Anti-HBs and anti-HBc were determined using commercial enzyme immunoassays on the Architect System (Abbott, Sligo, Ireland). Subjects with anti-HBs values <10 IU/l were offered a single booster dose of monovalent Hepatitis B vaccine (Engerix B®, GSK, Rixensart, Belgium). The anti-HBs titre of revaccinated subjects was determined at day 0, 3, 7, 14 and 28 post-booster. Additionally, cellular immune responses were analysed by ELISpot at day 0, 7 and 28. Healthy non-vaccinated volunteers were studied as controls.

Written informed consent was obtained from each subject and at least one parent before enrolment in this study. The study protocol had been approved by the Ethics Committee of the University Regensburg before the study start (vote number 10-101-0166).

### 2.2. Isolation of $CD4^+$ T cells

PBMCs were isolated from heparinised blood on density gradients (Pancoll human, PAN Biotech, Aidenbach, Germany) and stored in liquid nitrogen until used. $CD4^+$ T cells were isolated from PBMCs by negative selection using magnetic cell sorting ($CD4^+$ T cell isolation kit, Miltenyi Biotech, Bergisch-Gladbach, Germany). Purity of the enriched $CD4^+$ fraction was assessed by flow cytometry using anti-CD3-VioBlue and anti-CD4(VIT4)-PerCP (Miltenyi) and usually exceeded 85%. Fewer than 3% of $CD3^+CD4^+$ cells were normally found in the CD4-free cell fraction. Propidium iodide (Miltenyi) was used for exclusion of dead cells.

Flow cytometric analyses were done with FACS Canto II (BD Biosciences, San Diego, CA). Data were analysed with FlowJo software Version X.0.7 (Tree Star, Ashland, OR, USA).

### 2.3. Detection of IFNγ- and IL5-secreting cells by ELISpot

ELISpot assays were conducted using the FluoroSpot kit for Human IFNγ/IL-5 (Mabtech, Nacka Strand, Sweden) according to the manufacturer's instructions [30,31]. A mixture of 54 peptides (15-mer with 11 amino acid overlap, purity ≥90%, JPT, Berlin, Germany), which covered the entire sequence of the small HBsAg [32], was used for 44 h-stimulation in a final concentration of 2 μg/ml per peptide. Routinely, each sample was analysed in 5 replicates. In 8% (22 of 276 cases), fewer replicates were tested because of the insufficient amount of PBMCs available (2 replicates in 1%, 3 replicates in 1%, 4 replicates in 6%). Cells stimulated with 5 μg/ml phytohaemagglutinin (Biochrom, Berlin, Germany) were tested in duplicates as a positive control. Anti-CD28 was added to each well at a final concentration of 0.1 μg/ml. Each well contained 0.16% of dimethyl sulfoxide. Spots were counted with an AID ELISpot Reader (Advanced Imaging Devices, Straßberg, Germany).

### 2.4. Definition of positive cellular responses

Spot forming cells (SFC) were determined in parallel for every sample in the following four setups:

(A) $2 \times 10^5$ cells of $CD4^+$ fraction and $1 \times 10^5$ cells of CD4-free fraction (needed for antigen presentation) without HBsAg peptides,
(B) $2 \times 10^5$ cells of $CD4^+$ fraction and $1 \times 10^5$ cells of CD4-free fraction with HBsAg peptides,
(C) $1 \times 10^5$ cells of CD4-free fraction without HBsAg peptides, and
(D) $1 \times 10^5$ cells of CD4-free fraction with HBsAg peptides.

The number of HBsAg-induced SFC/well was calculated for further data analysis as follows: (mean B − mean A) − (mean D − mean C).

Cellular immune responses in revaccinated subjects were defined as positive (HBsAg-specific) when:

(a) the number of SFC in setup B was ≥2-fold higher than the number of SFC in setup A and
(b) the number of HBsAg-induced SFC calculated as mentioned above was ≥4.0 for IL5-secretion and ≥5.2 for IFNγ-secretion (≥mean + 2 SD of the number of SFC measured in 9 non-vaccinated subjects).

### 2.5. Statistical analysis

Statistical analysis was done with GraphPad Prism 6.02 (GraphPad Software, LA Jolla, CA). Two-sided $p$ values of <0.05 were considered significant. Anti-HBs values <0.1 IU/l were set to 0.1 IU/l for further data analysis.

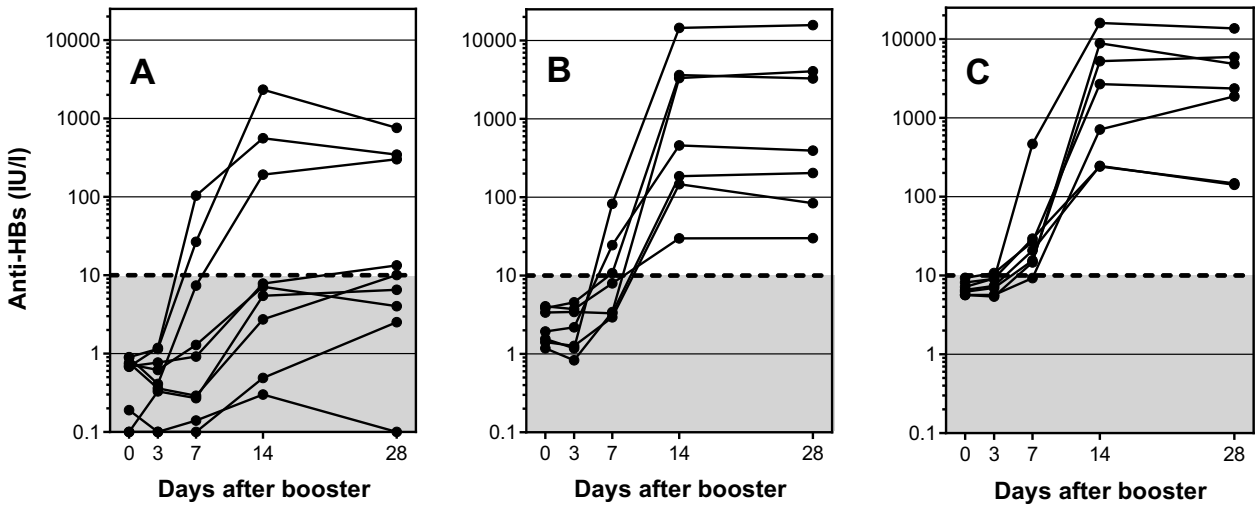


**Fig. 1.** Kinetics of anti-HBs response to hepatitis B booster vaccination. Kinetics of an anti-HBs response after booster in 23 study subjects with pre-booster anti-HBs-levels <1 IU/l (A), between 1 and 5 IU/l (B) and between 5 and 10 IU/l (C).

## 3. Results

### 3.1. Serological results 10–15 years after primary hepatitis B vaccination

All 77 participants were anti-HBc negative 10–15 years after primary immunisation. 51 subjects (66.2%) showed anti-HBs concentrations ≥10 IU/l (10–646 IU/l, median 52 IU/l). In 19 (24.7%), the anti-HBs titre was ≥100 IU/l. No correlation was found between the immunisation schedule (especially the interval between the 2nd and the 3rd dose) of primary vaccination and the anti-HBs levels 10–15 years later.

26 subjects (33.8%) had an anti-HBs titre <10 IU/l, of whom 21 had been vaccinated in infancy with Engerix B®, 2 with GenHBvax®, 1 with Twinrix® and 1 with Hexavac®. In 1 subject, the brand of the vaccine was unknown.

### 3.2. Anti-HBs response in revaccinated subjects

23 subjects with anti-HBs <10 IU/l were revaccinated, and anti-HBs responses were assessed at day 0, 3, 7, 14 and 28 post-booster (Figs. 1 and 2). The first response was detected at day 7 (1 subject showed an increase from 9.3 to 10.0 IU/l at day 3, which was not considered significant). At day 7, anti-HBs had increased above 10 IU/l in 11 subjects, at day 14 in 17 subjects and at day 28 in 2 additional subjects (Fig. 2). 12 subjects showed the highest anti-HBs titre at day 14 and 11 subjects at day 28. At the latter time point, 82.6% of participants had developed anti-HBs concentrations of ≥10 IU/l, 65.2% of ≥100 IU/l, 34.8% of ≥1000 IU/l and 8.7% of ≥10,000 IU/l.

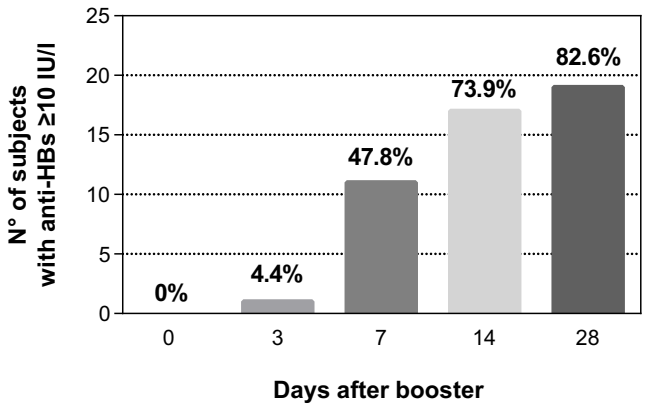


**Fig. 2.** Number (%) of subjects with anti-HBs ≥10 IU/l at different time points after booster vaccination.

A significant correlation was found between the anti-HBs titres pre-booster and 28 days post-booster ($p = 0.006$, Pearson's correlation analysis, supplementary Fig. 1). 6 of 9 subjects with a pre-booster anti-HBs titre <1.0 IU/l showed very poor responses (Fig. 1A). All subjects with pre-booster values between 5 and 10 IU/l had developed anti-HBs responses of ≥100 IU/l 4 weeks after booster vaccination (Fig. 1C). Again, there was no correlation between the response to the revaccination and the vaccine or the immunisation schedule used for the primary vaccination.

### 3.3. Cellular immune response in revaccinated subjects

HBsAg-specific cellular responses are shown in Table 1 and Fig. 3. 4 participants (17.4%) had positive pre-booster cellular responses. At day 7 post-booster, 11 participants (47.8%) showed specific cells, and, at day 28, the number of positive subjects had increased to 13 (56.5%). The frequency of IL5-producing cells per subject rose from a mean of 17 SFC/$2 \times 10^5$ $CD4^+$ T cells (range 0–123) at day 7 to a mean of 33 SFC/$2 \times 10^5$ $CD4^+$ T cells (range 0–232) at day 28; the corresponding numbers for IFNγ-secreting cells at day 7 and 28 were 8.7 SFC/$2 \times 10^5$ $CD4^+$ T cells (range 0–63) and 20 SFC/$2 \times 10^5$ $CD4^+$ T cells (range 0–317), respectively. In 1 subject, the number of IL5-secreting cells remained stable at the high level of about 55 SFC/$2 \times 10^5$ $CD4^+$ T cells at all three time points measured (Fig. 3B). The number of subjects with IL5-secreting cells was clearly higher than that of IFNγ-secreting cells (12 vs. 8 at day 28). A significant positive correlation could be found between IL5- and IFNγ-secreting SFC ($p < 0.001$, Spearman's rank correlation analysis, supplementary Fig. 2).

The calculation of mean fold changes at day 7 and 28 post-booster by using pre-booster responses as a baseline showed distinct kinetic profiles for IL5 and IFNγ secretion (Fig. 4). The mean fold change for IL5 secretion showed a relatively slow and steady increase from day 0 to day 28, whereas the mean fold change of IFNγ secretion increased steeply from day 0 to day 7, levelling off between day 7 and 28.

Looking for a relationship between anti-HBs values and T cell responses, we did not find any correlation between

**Table 1**
Hepatitis B surface antigen-specific IL5- and IFNγ-secreting cells after hepatitis B booster, 10–15 years after primary vaccination, determined by ELISpot.

| | Response after booster | Negative | Overall positive[b] | Weakly positive[b] (<10 SFC) | Moderately positive[b] (10–30 SFC) | Strongly positive[b] (>30 SFC) |
|---|---|---|---|---|---|---|
| IL5[a] | Day 0 | 20(86.9) | 3(13.1) | 0 | 2(8.7) | 1(4.4) |
| | Day 7 | 14(60.9) | 9(39.1) | 2(8.7) | 3(13.0) | 4(17.4) |
| | Day 28 | 11(47.8) | 12(52.2) | 2(8.7) | 4(17.4) | 6(26.1) |
| IFNγ[a] | Day 0 | 21(91.3) | 2(8.7) | 2(8.7) | 0 | 0 |
| | Day 7 | 18(78.2) | 5(21.7) | 2(8.7) | 1(4.4) | 2(8.7) |
| | Day 28 | 15(65.2) | 8(34.8) | 4(17.4) | 2(8.7) | 2(8.7) |
| IL5 and IFNγ[a] | Day 0 | 22(95.6) | 1(4.4) | – | – | – |
| | Day 7 | 20(87.0) | 3(13.0) | – | – | – |
| | Day 28 | 16(69.6) | 7(30.4) | – | – | – |
| IL5 and/or IFNγ[a] | Day 0 | 19(82.6) | 4(17.4) | – | – | – |
| | Day 7 | 12(52.2) | 11(47.8) | – | – | – |
| | Day 28 | 10(43.5) | 13(56.5) | – | – | – |

[a] Data are number (%) of revaccinated subjects.
[b] Criteria for a positive response are described in Section 2.4.

post-booster anti-HBs titres and IL5-secreting SFC ($p = 0.074$, Spearman's rank correlation analysis, Fig. 5A), but the post-booster anti-HBs titres positively correlated to the number of IFNγ-secreting SFC ($p = 0.002$, Fig. 5B). Interestingly, 3 participants showed a relatively strong IL5 response (15, 20 and 57 SFC/2 × $10^5$ $CD4^+$ T cells) after booster vaccination whereas their anti-HBs titres remained below 10 IU/l.

No correlation could be found between vaccination history and the quality of cellular responses after the booster dose.

## 4. Discussion

Immune memory is the crucial mechanism which establishes long-term protection after hepatitis B vaccination. It is particularly

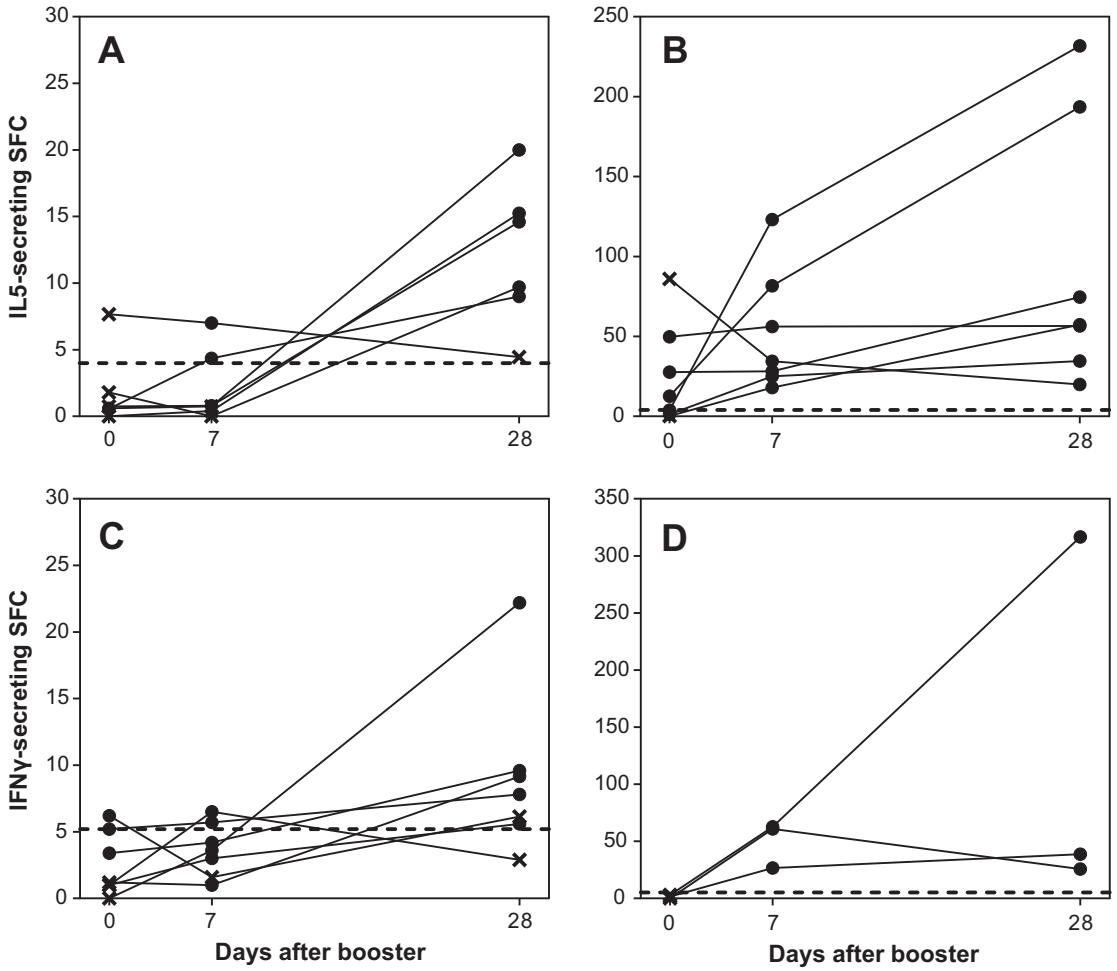


**Fig. 3.** Kinetics of cellular immune responses after hepatitis B booster vaccination. The amount of IL5-secreting cells with fewer (A) and more (B) than 30 spot forming cells (SFC)/2 × $10^5$ $CD4^+$ T cells and IFNγ-secreting cells with fewer (C) and more (D) than 30 SFC/2 × $10^5$ $CD4^+$ T cells are shown. For clarity, kinetics is shown only for participants with a positive cellular response at least at one of the three time points analysed. Criteria for a positive response were ≥2-fold increase above background (● – *more than 2-fold*, × – *less than 2-fold*) and ≥4.0 IL5-secreting or ≥5.2 IFNγ-secreting SFC/2 × $10^5$ $CD4^+$ T cells (*dashed lines*).

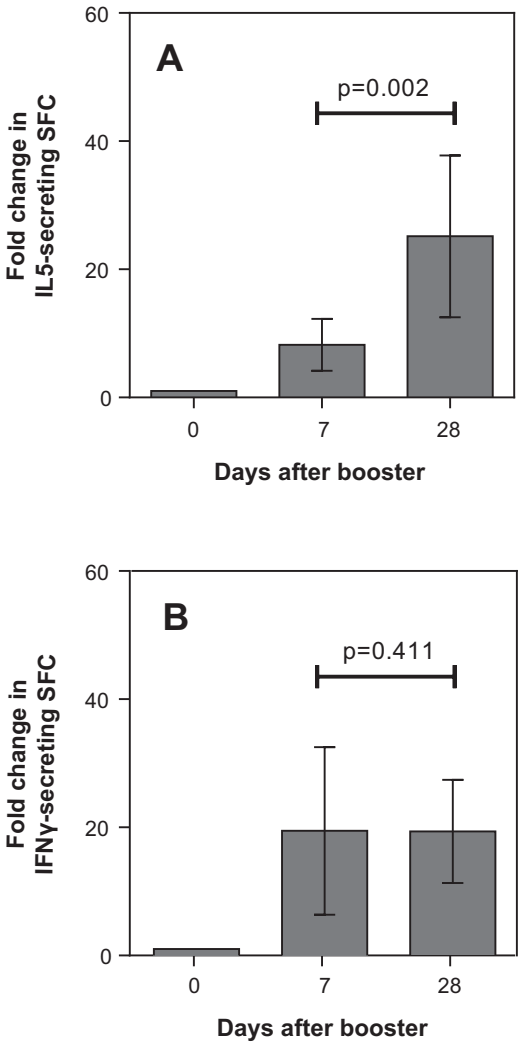


**Fig. 4.** Fold changes in T cell responses after stimulation with HBsAg. Mean ± standard errors of fold changes for IL5-secreting (A) and IFNγ-secreting (B) spot forming cells (SFC)/2 × $10^5$ $CD4^+$ T cells are shown. For each revaccinated subject ($n$ = 23), the fold change was calculated by dividing the number of SFC at day 7 and day 28 post-booster, respectively, by the number of SFC at day 0. Statistical analysis: Wilcoxon matched-paired signed rank test.

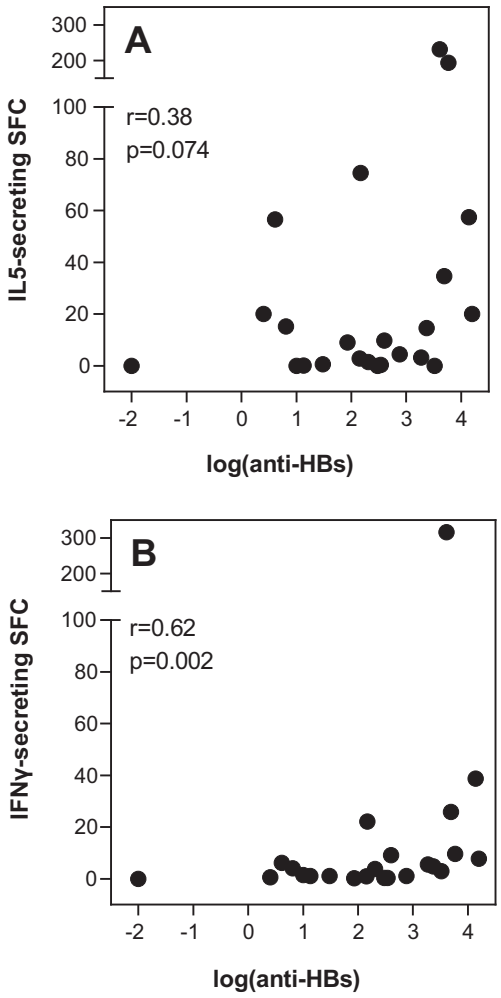


**Fig. 5.** Correlations between anti-HBs and cellular immune responses 28 days after hepatitis B booster vaccination. Correlations between anti-HBs titres and IL5-secreting spot forming cells (SFC)/2 × $10^5$ $CD4^+$ T cells (A) and between anti-HBs titres and IFNγ-secreting SFC/2 × $10^5$ $CD4^+$ T cells (B) are shown. $r$ – Spearman's rank correlation coefficient.

important in individuals vaccinated in early infancy, because in many of them neutralising antibodies decline below the protecting level of 10 IU/l within 10–15 years [17]. Hepatitis B specific immune memory has been described in several papers [12,29,33,34], but no consensus on a clear definition of immune memory has yet been found. To characterise immune memory in more detail, we analysed the kinetics of humoral and cellular responses to a challenge with hepatitis B vaccine in adolescents who had lost their protecting antibodies 10–15 years after infant vaccination.

19 (82.6%) of our 23 revaccinated subjects reached anti-HBs levels of ≥10 IU/l. All subjects showed a typical anamnestic response curve as similarly demonstrated earlier for tetanus and pertussis vaccine [2,4]: a steep increase in anti-HBs, which started between day 3 and 7, reached a peak around day 14, and showed a plateau or only minimal changes between day 14 and 28. Interestingly, 3 of 4 subjects with anti-HBs <10 IU/l also showed similar kinetics. Thus, it may be assumed that the shape of the curve is defined solely by the response of memory B cells irrespective of their frequency, which, on the other hand, is responsible for the resulting antibody level. A correlation between serum antibody levels and the frequency of corresponding IgG memory B cells was already shown by Bernasconi et al. for tetanus toxoid and measles virus [2].

According to our findings, peak anti-HBs values are usually reached between day 14 and 28 after booster vaccination. Consequently, no large differences should be found among studies measuring response between day 14 and 28 after booster vaccination [10,13,20,22,23].

In contrast to B-cell memory, which can easily be studied by means of standard anti-HBs assays, T-cell memory has to be assessed by direct analysis of T cells. First, this is complicated by the fact that the absolute number of specific T cells in peripheral blood is very low. Secondly, the most common method for monitoring T cell responses is the ELISpot, which is probably also the most sensitive assay. However, when this test is performed with unfractionated PBMCs, which is very often the case, its specificity may be impaired by the fact that bystander cells may also non-specifically produce cytokines. Finally, no standardised criteria exist for interpreting ELISpot results (applying more or less stringent criteria can drastically influence study results).

To overcome these problems at least partially, we used enriched $CD4^+$ T cells and analysed CD4-free cell fraction in parallel to detect non-specifically induced cells [35]. Moreover, to define a positive result, we used external and internal controls, which increased the specificity of our assay but probably at the expense of its sensitivity.

Before revaccination, 17.4% of our participants showed HBsAg-specific $CD4^+$ T cells. This rate had increased to 47.8% at day 7 and to 56.5% at day 28 after challenge. These numbers were substantially lower than those found in a similar study with adults [36], in which the cellular responses of each of the 15 subjects were already detectable before booster vaccination. However, these differences may have several reasons. First, in the latter study, $CD4^+$ cells were further separated into effector and memory cells, which increased the sensitivity of the test. Moreover, specific T cell responses may disappear over time after vaccination; the interval of 4–8 years between vaccination and evaluation was considerably shorter than that of 10–15 years in the present study. Finally, individuals vaccinated as adults preferentially show a dominance of Th1 cells [36,37] in contrast to the dominance of Th2 cells in newborns and infants [12,38], as evident also from the present study. Such age-dependent immune characteristics may also be responsible for our observation that T cell responses after challenge were more frequently detected at day 28 in contrast to responses of subjects vaccinated as adults, which peaked at day 10 [36]. Similar age-dependent kinetic patterns have already been observed for cellular responses after influenza vaccination [39].

A study more suitable for a comparison with our investigation was conducted in Taiwan, in which 92 subjects were tested 15–18 years after neonatal immunisation [12]. The authors found specific T cells in 34.8% of subjects before challenge and in 72.8% 28 days afterwards. The reason for our lower values is most probably the lower sensitivity of our assay as mentioned above. In two other studies with young adults conducted 18–20 years [29] and 20 years [28] after neonatal vaccination, T cell immunity was also analysed by IFNγ ELISpot. In the study by Chinchai et al., 50.6% of subjects showed a specific response without any booster [29], and in the study by Zhu et al., 93.2% 10–12 days after booster [28]. However, both studies were conducted in populations at a high risk for hepatitis B. In the study by Zhu et al. [28], 88.6% of subjects also had detectable HBeAg-specific cellular immunity. Thus, cellular reaction to HBsAg in that study and probably also in the study by Chinchai et al. [29] could be the result of HBV contact rather than of vaccination.

Our study gives a detailed insight into the kinetics of HBsAg-specific Th1 and Th2 cell responses, which were found to be rather heterogeneous. By analysing mean fold changes of SFC (Fig. 4), we could observe divergence in the kinetics of IL5- and IFNγ-secretion. However, focusing on the absolute number of HBsAg-specific cells, we observed an increase in about 80% of subjects between day 7 and 28 post-booster, irrespective of the cytokine analysed. Therefore, although effector memory T cells can be recruited within hours to days after antigen re-encounter [40], it seems to make sense to monitor HBsAg-dependent cellular immunity at a later time point, when also secondary effector T cells are already detectable, which were generated by the activation of central memory T cells.

In conclusion, T cell memory responses, detected in only about 50% of revaccinated adolescents, showed heterogeneous kinetic courses. In contrast, B cell memory was found by typical anamnestic anti-HBs response curves in almost all subjects, also in those with post-booster anti-HBs titres <10 IU/l. However, if immune memory still mediates protection in these individuals needs to be further investigated.

## Author contributions

Study concept and design: all authors
Acquisition of data: IBH, ME, AZ
Analysis and interpretation of data: IBH, WJ
Writing of the manuscript: IBH
Critical revision of the manuscript: all authors
Final approval of the manuscript: all authors

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## Conflict of interest

None declared.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.12.033.

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