European Journal of Immunology

Switched memory B cells maintain specific memory independently of serum antibodies: the Hepatitis B example

M. Manuela Rosado[1], Marco Scarsella[1], Elisabetta Pandolfi[2], Simona Cascioli[1], Ezio Giorda[1], Paola Chionne[3], Elisabetta Madonne[3], Francesco Gesualdo[2], Mariateresa Romano[2], Clara Maria Ausiello[4], Maria Rapicetta[3], Alessandro Remo Zanetti[5], Alberto Tozzi[2] and Rita Carsetti[1]

[1] Research Center Ospedale Pediatrico Bambino Gesù, IRCSS, Laboratory of Flow-cytometry and B cell development, [2]Epidemiology Unit, Piazza S.Onofrio 4, 00165 Roma, Italy; [3]Viral Hepatitis Section, Department of Infectious, Parasitic and immune-mediated Diseases, [4]Anti-Infectious Immunity Unit, Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome, Italy; [5]Dipartimento di Sanità Pubblica-Microbiologia-Virologia, Università degli Studi di Milano, Milan Italy

Corresponding author: Rita Carsetti

Research Center, Ospedale Pediatrico Bambino Gesù (IRCCS),

Piazza S.Onofrio 4

00165 Roma, Italy

Tel: +390668592647

Fax:+390668592904

e-mail: rita.carsetti@opbg.net

Received: October 21, 2010 / Revised: February 08, 2011 / Accepted: March 14, 2011

DOI: 10.1002/eji.201041187

European Journal of Immunology

Key Words: Hepatitis B, vaccination, B-cell memory, long-lived plasma cells, serum antibodies

Abbreviations: Hepatitis B surface antigen (HBsAg), CpG unmethylated oligodeoxynucleotides (CpG-ODN)

Abstract

Immunogenicity of a vaccine is conventionally measured through the level of serum antibodies early after immunization, but to ensure protection specific antibodies should be maintained long after primary vaccination. For hepatitis B protective levels often decline over time, but breakthrough infections do not seem to occur.

The aim of the study was to demonstrate whether after Hepatitis B vaccination, B cell memory persist even when serum antibodies decline. We compared the frequency of anti-hepatitis specific memory B cells that remain in the blood of 99 children 5 years after priming with Infanrix®-hexa (GlaxoSmithKline) (n=34) or with Hexavac® (Sanofi Pasteur, MSD) (n=65). These two vaccines differ in their ability to generate protective levels of IgG. Children with serum antibodies under the protective level, <10mIU/ml, received a booster dose of hepatitis B vaccine, and memory B cells and serum antibodies were measured two weeks later.

We found that specific memory B cells had a similar frequency in all children independently of primary vaccine. Booster injection resulted in the increase of memory B cells (from 11,3 in 10^6 cells to 28,2 in 10^6 cells, $p<0.01$) and serum antibodies (GMC from 2,9 to 284 mIU/ml) demonstrating that circulating memory B cells effectively respond to antigen challenge even when specific antibody fall under the protective threshold.

European Journal of Immunology

Introduction

Four subsets of B cells can be identified in the peripheral blood according to the expression of surface molecules. Transitional B cells, corresponding to recent bone marrow emigrants, are short-lived $CD19^{pos}CD24^{bright}CD38^{bright}$ and express high levels of IgM and IgD . They are unable to generate effective immune responses and in the periphery they differentiate into $CD19^{pos}CD24^{pos}CD38^{low}$ mature-naive B cells reducing the expression of IgM and IgD. Mature-naïve B cells have never been exposed to antigen and represent the bulk of circulating and resident follicular B cells. Memory B cells lose the CD38 marker, acquire CD27, and, depending on the presence of surface IgM, are sub-divided into IgM memory or switched memory B cells[1]. IgM memory B cells are thought to represent a primitive, low-affinity type of cells[2]. Switched memory B cells, instead, have been selected in germinal centers and are the effectors of the high-affinity adaptive response[3, 4]. B cells have the transitional phenotype in the neonate, whereas the development of mature, IgM and switched memory B cells starts with antigen exposure[1].

Switched memory B cells are generated in the germinal centers, specialized and complex structures triggered by exposure to infections or vaccinations. Here B cells, helped by T cells and antigen presenting cells, extensively proliferate, introduce somatic hypermutations (SHM) in their immunoglobulin genes, and are selected for their improved affinity to the stimulating antigen. The final products of the germinal center reaction are of two types: a) antibodies immediately used to terminate the infection and clear the pathogen; b) memory B cells and long-lived plasma cells that will prevent future re-infection[5]. Switched memory B cells are the effectors of anamnestic responses, because they have the remarkable ability to re-enact their previous encounters with specific antigens rapidly, producing large amounts of high-affinity antibodies[6]. It has been suggested that memory B cells may also contribute to preserve the diversity of serum

immunoglobulins by the constant secretion of small amounts of antibodies upon polyclonal stimulation by toll like receptor 9 (TLR 9)[3, 7, 8].

Recently it has been demonstrated that another population of B cells, the long-lived plasma cells, play an important role in protecting from infections. At the end of the germinal center reaction long-lived plasma cells migrate to the bone marrow, where they occupy a specialized niche and survive virtually forever[9], continuously producing immunoglobulins[10, 11] against previously encountered pathogens. Although both memory B cells and long-lived plasma cells contribute to protective immunity, their separate roles have not been elucidated yet[12]. It is also not known whether both populations are equally generated by the immune response and equally survive over the years.

Vaccination prevents infectious diseases by generating serum antibodies and memory B cells specific for the antigen used for immunization. In order to achieve protection against several diseases as soon as possible after birth, hexavalent vaccines have been developed. In Europe two products, Hexavac® (Sanofi Pasteur, MSD) and Infanrix®-hexa (GlaxoSmithKline) were licensed for use in October 2000. Both vaccines protect against diphtheria, tetanus, pertussis, poliomyelitis, Haemophilus influenzae type b and hepatitis B and were considered to have a comparable immunogenicity at the time of licensure, based on studies conducted shortly after the completion of primary immunization[13-16].

In September 2005, following the observation of a reduced immunogenicity of the hepatitis B component[17] in the Hexavac® (Sanofi Pasteur, MSD) and potential consequences on long-term protection the European Medicines Agency (EMA) recommended, as a precautionary measure, the withdrawal of Hexavac® (Sanofi Pasteur, MSD) from the market (for the scientific conclusions and grounds for the suspension of the marketing authorisation of Hexavac® http://ema.europe.eu/humandocs/PDFs/EPAR/Hexavac-H-298-Z-28-en.pdf).

As Hexavac® has been administered to a large number of children, a multicenter study funded by the Italian Drug Agency was carried out to investigate whether vaccinated children were still able respond to a booster dose of hepatitis B vaccine 5 years after primary immunization[18]. This study had the aim of evaluating whether the decline in antibody titers under the protective threshold (10mIU/ml), observed most frequently in children receiving Hexavac®, reflects a loss of immune memory. In this case re-vaccination would be advisable. The report included 833 children vaccinated with Hexavac® and 710 with Infanrix®-hexa. Specific antibodies concentrations had fallen under the protective level in 62% (512/831) of the children that had received Hexavac® and in 17% (119/590) of those vaccinated with Infanrix®-hexa. All children with HBsAg<10 mIU/ml received a booster vaccination with a monovalent Hepatitis B recombinant vaccine. The recall response was observed in both groups of children and comparable levels of anti-HBsAg antibody were measured in the sera. Thus, immunological memory persists in children 5 years after the primary cycle of vaccination even when specific antibodies wane in the serum.

Since serology for hepatitis B measured after 5 years from primary immunization was not predictive of the ability to respond to a recall vaccination, in a subgroup of children we studied the long-term response to hepatitis B focusing on B cell immunity with the aim of discriminating the persistence and function of the two components of immunological memory, i.e. memory B cells and serum antibodies.

RESULTS

Thirty-four children primed with Infanrix®-hexa (GlaxoSmithKline) (VacA) and sixty-five vaccinated with Hexavac® (Sanofi Pasteur, MSD) (VacB) were recruited in the central/south of Italy and included in the study (table 1).

As expected, antibody levels were significantly higher in the VacA than in VacB group (GMC

47mIU/ml vs 7.6 mIU/ml, p<.0001). The frequency of children with anti-HBsAg<10mIU/ml, considered non protective, was 17,6% (6/34) in the VacA group and 58,7% (38/65) in those who received VacB (Table 1 and Fig.1A). Moreover, although nearly 30% of all children had a level of anti-HBsAg antibodies between 10 and 100 mIU/ml (41,2% vaccinated with VacA versus 34,9% vaccinated with VacB), higher antibody concentrations were almost exclusively detected in children that received VacA (41,2% in VacA vs 6,3% in VacB, Table 1 and Fig.1A). In conclusion the serologic findings in our cohort of children are very similar to those previously reported [18] and confirm the higher efficacy of Infanrix®-hexa in the maintenance of antibody levels.

SEROLOGY AFTER BOOSTER DOSE

Among children who participated in the serology analysis and were recruited for the B cell immunity study, 45 had anti- HBsAg<10mIU/ml. 82% of them had primary vaccination cycle with VacB and 17% with VacA. Consent for booster vaccination was only obtained for 25 children, 20 previously vaccinated with VacB (80%) and 5 (20%) with VacA. A single dose of 10 µg monovalent Hepatitis B recombinant vaccine, Engerix-B® (GlaxoSmithKline), was administered and serum antibodies were measured 14-20 days after challenge. In 93% (23/25) of the children anti-HBsAg levels increased. In the 5 children of the VacA group the GMC increased from 3.5 to 1012.5, whereas in the VacB cohort the GMC went from 2.9 to 203.3 (table 2). High anti-HBsAg levels (>100mIU/ml) were detected in the majority of children, after the challenge dose, independently of the type of the vaccine used for priming (Fig.1B). The difference in the geometric mean titers of the two groups probably depends on the small size of the VacA cohort. Previous reports on a larger group did not show any significant difference between GMCs after boosting [18]. The high frequency of children with protective levels of antibodies early after a single dose of Engerix® suggests that we are observing a memory response. It has been shown before that only 8% of the vaccinees are seroprotected after a single dose [19].

Our results indicate that the concentration of specific antibodies detected in the serum several years after the primary immunization is not sufficient to assess the persistence of specific immunological memory, because the ability to respond to recall immunization remains even when serum antibodies fall under the protective threshold.

MEMORY POOL

Recall antibody responses are due to the rapid activation of memory B cells. As discordance between the frequency of memory B cells and the level of serum antibodies against hepatitis B has been reported before in HIV patients [20] and in adult care workers [21], we decided to evaluate the size of the total and specific memory pool in children before and after booster vaccination.

Memory B cells, absent at birth, slowly accumulate with antigenic experience over the years. We analyzed the frequency of $CD19^{pos}$ cells (Supplementary Fig.1A) and memory B cells (Supplementary Fig.1B) in the two groups of vaccinated children and we did not find significant differences. The frequency of total B cells was 10.3±4.4% in the VacA group and 10±3.5% in VacB. Among B cells, the frequency of memory cells was 26.3±8% and 24.6±7.5% in VacA and VacB recipients, respectively.

In order to explore the specificities contained in the B cell memory pool we polyclonally stimulated memory B cells with CpG-ODN in the presence of IL-21[22] and IL-4. We chose this method because of its ability to specifically stimulate and test the function of memory B cells [3, 23]. Our previous experiments performed with sorted cells from blood donors have demonstrated that only memory B cells proliferate and secrete antibodies upon CpG stimulation, whereas mature B cells survive without dividing and differentiating [3]. We repeated these experiments and tested by ELISpot the frequency and the Ig class of antibody secreting cells derived from purified mature and memory B cells. We found that mature B cells stimulated with CpG+IL4+IL21 for 5 days were

unable to generate plasma cells. In contrast, memory B cells were able to differentiate in antibody secreting cells in the same culture conditions, generating 3032±147 IgM and 2457,5±369 IgG secreting cells per 10^6 (Fig.2A and B). Antibodies specific for tetanus toxin were only detected in the wells with memory B cells and were of the IgG class (Fig.2A and B).

Based on these results we cultured with CpG+IL4+IL21 total PBMCs from children before and after the booster dose in order to measure the size and specificity of the memory pool. After 5 days in bulk culture, cells were counted and distributed in plates pre-coated with either antibodies or antigens and left to secrete for 5 hours. Frequency of anti-hepatitis B memory cells was calculated by counting the number of antibody secreting cells (ASC) that upon polyclonal stimulation were able to secrete IgG specific for hepatitis B. Because both vaccines used for primary immunization also contained the tetanus antigen, we tested the frequency of anti-tetanus IgG secreting cells and used it as our internal control (Supplementary Fig.2A). We also calculated the total number of IgM and IgG antibody forming cells in order to determine for each individual the size of the pool of memory B cells that we were able to stimulate *in vitro*.

The number of IgG and IgM ASC in VacA and VacB was not statistically different (3870±2564 IgG ASC/10^6 cells for VacA and 5277±3416 IgG ASC/10^6 for VacB, p=.08; 2518±1586 IgM ASC/10^6 cells for VacA and 3344±2153 IgM ASC/10^6 cells for VacB, p=.147) indicating that the two groups of children were comparable (Fig.3A). The homogeneity of the two groups was confirmed by the fact that there was no difference in the number of B cells secreting antibodies against the tetanus antigen (Fig.3B) (mean 8.5 ASC/10^6 plated cells for VacA and 8.6 ASC/10^6 plated cells for VacB, p>.12). The number of HBsAg specific spots in 1 million plated cells was 12 for VacA and 10.4 for VacB (p>.08) (Fig.3B). Thus, the two vaccines had a similar efficacy in generating memory B cells. In addition, when we grouped the children based on the serum level of antibodies we found no

difference in the number of HbsAg specific ASC independently of whether the serum level of anti-HBsAg IgG was <10, between 10 and 100 and >100 mIU/ml (Fig.3C).

We also calculated the frequency of antigen-specific memory B cells, by dividing the number of specific spots for the number of IgG spots, corresponding to the memory B cells which we are able to stimulate *in vitro*. The two groups of vaccinated children had the same frequency of anti-HBsAg IgG and of anti-tetanus toxin IgG secreting cells (Supplementary Fig.2 A and B). Roughly 4-5 in 1000 of the *in vitro* stimulated memory B cells were specific for either tetanus or Hepatitis B.

In order to prove that ASC detected *in vitro* derive from functional memory B cells able to react to the antigen also *in vivo*, we measured their frequency 2-3 weeks after the administration of a monovalent hepatitis B vaccine. Antibody titers rised in the serum in all but one child (VacB group pre-boost 0,9 mIU/ml and post-boost 11mIU/m) (Fig. 4A). The number of anti-HBsAg ASC (from 11,3 to 28,2/10^6 plated cells, $p<0.02$) and their frequency were also significantly increased (Fig.4 B and C), (from 0,46% to 0,89%, $p<.02$) whereas anti-tetanus ASC remained stable (Supplementary Fig.3, from 0,32% to 0,4%, $p>.4$).

Thus, memory B cells in children without protective antibodies were functional, because upon *in vivo* re-stimulation they increased in number and generated high antibody titers.

DISCUSSION

Immunological memory is the extraordinary property of the immune system to store, preserve, and recall previous antigenic experience and to use it in order to prevent or limit re-infection [24].

Vaccination, the most effective method to prevent infections, acts by creating an antigen-specific defense system composed of high-affinity antibodies and memory cells [25]. The combination of pre-formed antibodies and cells able to rapidly react to pathogen is a very effective system to

neutralize microbes and prevent the disease. Nonetheless, we do not know whether we would be protected from infections when only serum antibodies or only memory B cells were available.

The case of hepatitis B vaccines gives some important elements for discussion and further studies.

For hepatitis B a level of anti-HBsAg Ab above 10mIU/ml is considered protective[26], but 5 years after the vaccination the protective level is often not maintained [27]. However, notwithstanding the low levels of serum antibodies, breakthrough infections remain rare in vaccinated individuals, even in countries where hepatitis B is still endemic[28, 29]**.** This observation suggests that other mechanisms of defense generated by the vaccination, such as memory B cells, may play an important role in the prevention of overt disease.

In our study, memory B cells specific for the HBsAg were present in virtually all (63/65 for Hexavac® and 31/34 for Infanrix®-hexa) children regardless of the type vaccine received 5 years before and independently of whether the level of antibody was higher or lower than 10 mIU/ml. In the small group of children we were allowed to boost memory B cells were functional. In response to a challenge with a Hepatitis B monovalent vaccine, their frequency increased and high amounts of antibodies were detectable in the serum, independently of the type of primary vaccine and of the pre-challenge antibody levels.

From our studies two important conclusions can be drawn: a) the frequency of specific memory B cells is not related to the level of serum antibodies. Thus, serum antibodies cannot be used to predict a subsequent response to a specific challenge; b) vaccine components inducing the production and maintenance of serum antibody are different from those responsible for the generation of memory B cells, because VacA and VacB are equally able to generate memory B cells, but only VacA ensures the maintenance of protective levels of antibodies in the serum.

Each antigen experience leads to the production of specific memory B cells and plasma cells[30]. A fraction of the plasma cells is recruited to a dedicated, but yet unidentified, bone marrow niche[31] and survive for a long time, continuously secreting antibodies[32-34]. These antibodies are useful ready to use weapons against pathogen. As plasma cells are a terminal differentiated population that does not express a membrane-bound BCR and lacks most of the B-cell specific signaling elements[35], they cannot sense antigen and are, therefore, unable to respond to vaccination and/or infection. Their role is the maintenance of pre-formed high-affinity antibodies[10], whereas recall responses are carried out by memory B cells.

Our data show that specific memory B cells are rare in the peripheral blood. We found that 5 memory B cells in 1000 are specific for hepatitis B 5 years after vaccination. Memory B cells against tetanus are present at the same frequency. As the frequency of cells able to react to any specific antigen has been estimated to be 1 in 1 million in the population of mature naïve B cells, the enrichment due to a previous encounter is significant, especially if we consider that our pool of memory B cells must include all our past immunological experience in order to be effective. The rapid production of antibodies upon recall is due to memory B cells dividing and differentiating in new plasma cells, causing a peak of specific antibodies in the serum, followed by a progressive decline.

Our data confirm that in children, as in adults, the level of specific antibodies in the serum, measured years after vaccination, does not correlate with the frequency of memory B cells [21, 36-38].

This observation suggests that vaccine components generating long-lived plasma cells and memory B cells may be different. The comparison between Hexavac® and Infanrix®-hexa strongly confirms this observation, because even a vaccine that has been withdrawn from the market due

to its inefficiency in eliciting and maintaining high antibody level, generates normal numbers of effective and long-lived memory B cells. The most apparent difference between Hexavac® and Infanrix®-hexa is the amount of HB surface antigen in a vaccine dose, but both vaccines had complex hexavalent formulation. Further studies are necessary to identify the key element/s that modulate the frequency of either long lived plasma cells or memory B cells.

Although life-long immunity has been demonstrated after infection and vaccination, not all vaccines are able to induce protective Ab levels in the long term. The concept of waning immunity, as an example, has been proposed to explain outbreaks of pertussis in vaccinated children and adolescents[39, 40]. For hepatitis B, however, notwithstanding the low levels of serum antibodies, breakthrough infections remain rare in vaccinated individuals most probably due to the "re-activation" of the specific memory B cells[29].

The long incubation of hepatitis B may give sufficient time for the memory B cells to sense antigen, activate and differentiate into antibody secreting cells. Whereas ready to use antibodies may be indispensable for the control of other infections in which pathogens spreading is faster and should, when possible, remain confined in the small area of entry.

Material and Methods

Subjects: Children were eligible for the study if they were born in 2001-2002, children received the primary immunization cycle (including three doses administered at 3, 5 and 11 months of life) with Infanrix®-hexa (GlaxoSmithKline) (n=34) or Hexavac® (Sanofi Pasteur, MSD) (n=65), did not receive additional doses of hepatitis B vaccine, did not have chronic illness, or congenital, or acquired immune disorder, and their families agreed to have a blood sample drawn and signed an informed consent. The study was approved by the Ethical Committee of the Bambino Gesù Hospital.

Study design: A blood sample was collected at Ospedale Pediatrico Bambino Gesù, Rome from all

participants at baseline. Children with anti-HBsAg IgG levels < 10 mIU/ml were invited to receive a challenge dose of Hepatitis B vaccine (Engerix-B®, Glaxo SmithKline) and an additional blood sample collected 14-20 days after the recall. Ten ml of peripheral blood in sodium heparin were collected for antigen specific B memory analysis and plasma to measure the serological response to HBV.

Cell Sorting: Healthy donor's peripheral blood was donated and collected at Ospedale Pediatrico Bambino Gesù, Rome. Mononuclear cells were isolated by Ficoll Paque^{TM} Plus (Amersham Pharmacia Biotech) density-gradient centrifugation and stained with the appropriate combination of fluorescent labeled antibodies: monoclonal clone ML5 (anti-CD24) and clone M-T271 (anti-CD27) were obtained from BD Biosciences (San Diego, CA, USA). Mature cells, $CD24^{pos}CD27^{neg}$ and memory B cells, $CD24^{high}CD27^{pos}$, were purified using FACSAria II SE (BD Biosciences, San Diego, CA, USA). Cell purity post-sorting was >98%.

Cell Cultures: $4x10^6$ PBMCs were stimulated for 5 days in RPMI 1640 (Gibco BRL) supplemented with 10% heat inactivated foetal bovine serum (FBS, Hyclone Laboratories Logan UT), 2% L-glutamine (Gibco BRL), $5x10^{-5}$M 2-βmercaptoethanol (Sigma, St. Louis, USA) and 20mg/ml gentamycin (Gibco BRL), 2,5 μg/ml CpG-ODN (a 24 synthetic oligodeoxynucleotide sequence containing the proper CpG-DNA motif mimicking the immunostimulatory effects of bacterial DNA and the human optimal motif GTCGTT from Hycult Biotechnology, Uden, The Netherlands), 20ng/ml IL-21 (Peprotech, UK) and 20ng/ml IL-4 (Peprotech, UK).

ELISpot: 96 well plates (MultiScreen-HA, Millipore) were coated overnight with AffiniPure F(ab')2 Fragment Goat anti-human IgA+IgG+IgM (H+L) (Jackson Immuno Research Laboratories, Pennsylvania, USA), synthetic tetanus toxin peptide (C-term) (Acris-antibodies, Germany) and recombinant hepatitis B surface antigen (HBsAg adw), (ProSpec, Rehovot, Israel). After washing

with sterile PBS/0.05% Tween20, plates were blocked for 1hr at 37° with PBS/gelatine 1%. PBMC stimulated for 5days, as described before, were collected, counted and seeded in the pre-coated ELISA plates. Plates were left at 37°, 5% CO_2 for 5 hrs to allow antibody secretion. 1/2 serial dilution were done starting in the first well with: 2.5 $x10^5$ cells for IgG and $2x10^6$ cells for specific anti-tetanus IgG and anti-hepatitis B IgG, respectively. After incubation, plates were washed with dH_2O/0,05% Tween20 (once) and PBS/0,05% Tween20 (two times) and incubated overnight with anti-hIgG HRPO (1:2000) (Jackson Immuno Research Laboratories, Pennsylvania, USA) diluted in PBS+gelatin1%+0,05% Tween20 (Sigma). After washing twice with PBS/0,05% Tween20, TMB substrate (MABTECH-Elisaspot plus for human IgG kit) was used according to the manufacture instructions. Plates were left to dry before counting.

Analysis was performed blinded as for the type of vaccine.

Statistical analysis: Results are reported as mean ± standard error (SEM). Statistical analyses were carried out using the StatView statistical MacIntosh program (StatView Software, San Diego, CA). For comparing overall differences of results obtained with each assay between children receiving as primary vaccination Infanrix®-hexa or Hexavac®, the *t* Student Paired-Sample Test was performed. *p* values lower than 0.05 were considered to indicate statistical significance, and all reported *p* values are two-sides.

Acknowledgments: We thank all children and their families for their participation in the study as well as the study team and nurses of the Local Health Units whose enthusiastic collaboration made possible this study. This study was funded by AIFA (Agenzia Italiana del farmaco, Bando per la Ricerca Indipendente, anno 2006) with the registered code FAM67NFPN and by the EUROPAD NET (EC Grant Nr.201549).

Conflict of Interest: the authors declare no competing financial interest

European Journal of Immunology

References

1 **Carsetti, R., Rosado, M. M. and Wardmann, H.,** Peripheral development of B cells in mouse and man. *Immunol Rev* 2004. **197**: 179-191.

2 **Kruetzmann, S., Rosado, M. M., Weber, H., Germing, U., Tournilhac, O., Peter, H. H., Berner, R., Peters, A., Boehm, T., Plebani, A., Quinti, I. and Carsetti, R.,** Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med* 2003. **197**: 939-945.

3 **Capolunghi, F., Cascioli, S., Giorda, E., Rosado, M. M., Plebani, A., Auriti, C., Seganti, G., Zuntini, R., Ferrari, S., Cagliuso, M., Quinti, I. and Carsetti, R.,** CpG drives human transitional B cells to terminal differentiation and production of natural antibodies. *J Immunol* 2008. **180**: 800-808.

4 **Weller, S., Faili, A., Garcia, C., Braun, M. C., Le Deist, F. F., de Saint Basile, G. G., Hermine, O., Fischer, A., Reynaud, C. A. and Weill, J. C.,** CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc Natl Acad Sci U S A* 2001. **98**: 1166-1170.

5 **Dogan, I., Bertocci, B., Vilmont, V., Delbos, F., Megret, J., Storck, S., Reynaud, C. A. and Weill, J. C.,** Multiple layers of B cell memory with different effector functions. *Nat Immunol* 2009. **10**: 1292-1299.

6 **MacLennan, I. C. and Gray, D.,** Antigen-driven selection of virgin and memory B cells. *Immunol Rev* 1986. **91**: 61-85.

7 **Traggiai, E., Puzone, R. and Lanzavecchia, A.,** Antigen dependent and independent mechanisms that sustain serum antibody levels. *Vaccine* 2003. **21 Suppl 2**: S35-37.

8 **Bernasconi, N. L., Traggiai, E. and Lanzavecchia, A.,** Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002. **298**: 2199-2202.

9 **Manz, R. A., Thiel, A. and Radbruch, A.,** Lifetime of plasma cells in the bone marrow. *Nature* 1997. **388**: 133-134.

10 **Manz, R. A., Hauser, A. E., Hiepe, F. and Radbruch, A.,** Maintenance of serum antibody levels. *Annu Rev Immunol* 2005. **23**: 367-386.

11 **Mei, H. E., Yoshida, T., Sime, W., Hiepe, F., Thiele, K., Manz, R. A., Radbruch, A. and Dorner, T.,** Blood-borne human plasma cells in steady state are derived from mucosal immune responses. *Blood* 2009. **113**: 2461-2469.

12 **Good-Jacobson, K. L. and Shlomchik, M. J.,** Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. *J Immunol* 2010. **185**: 3117-3125.

13 **Heininger, U., Sanger, R., Jacquet, J. M. and Schuerman, L.,** Booster immunization with a hexavalent diphtheria, tetanus, acellular pertussis, hepatitis B, inactivated poliovirus vaccine and Haemophilus influenzae type b conjugate combination vaccine in the second year of life: safety, immunogenicity and persistence of antibody responses. *Vaccine* 2007. **25**: 1055-1063.

14 **Lim, F. S., Han, H. H., Jacquet, J. M. and Bock, H. L.,** Primary vaccination of infants against hepatitis B can be completed using a combined hexavalent diphtheria-tetanus-acellular pertussis-hepatitis B-inactivated poliomyelitis-Haemophilus influenzae type B vaccine. *Ann Acad Med Singapore* 2007. **36**: 801-806.

15 **Zepp, F., Schmitt, H. J., Cleerbout, J., Verstraeten, T., Schuerman, L. and Jacquet, J. M.,** Review of 8 years of experience with Infanrix hexa (DTPa-HBV-IPV/Hib hexavalent vaccine). *Expert Rev Vaccines* 2009. **8**: 663-678.

16 **Steiner, M., Ramakrishnan, G., Gartner, B., Van Der Meeren, O., Jacquet, J. M. and Schuster, V.,** Lasting immune memory against hepatitis B in children after primary immunization with 4 doses of DTPa-HBV-IPV/Hib in the first and 2nd year of life. *BMC Infect Dis* 2010. **10**: 9.

17 **Tichmann, I., Preidel, H., Grunert, D., Habash, S., Schult, R., Maier, R., Gildberg, P. K., Sengespeik, H. C., Meurice, F. and Sanger, R.,** Comparison of the immunogenicity and reactogenicity of two commercially available hexavalent vaccines administered as a primary vaccination course at 2, 4 and 6 months of age. *Vaccine* 2005. **23**: 3272-3279.

18 **Zanetti, A. R., Romano, L., Giambi, C., Pavan, A., Baitelli, G., Malchiodi, G., Valerio, A., Carnelli, V., Barale, A., Montù, D., Marchisio, P., Tozzi, A. and D'Ancona, F.,** Hepatitis B immune memory in children primed with hexavalent vaccines and given monovalant booster vaccines: an open-label, randomised, controlled, multicentre study. *Lancet Infectious disease* 2010. **10**: 755-761.

19 **Young, M. D., Schneider, D. L., Zuckerman, A. J., Du, W., Dickson, B. and Maddrey, W. C.,** Adult hepatitis B vaccination using a novel triple antigen recombinant vaccine. *Hepatology* 2001. **34**: 372-376.

20 **Tuaillon, E., Tabaa, Y. A., Baillat, V., Petitjean, G., Reynes, J. and Vendrell, J. P.,** Long-term persistence of memory B cells specific for hepatitis B surface antigen in HIV-1-infected patients. *Aids* 2007. **21**: 2343-2345.

21 **Valats, J. C., Tuaillon, E., Funakoshi, N., Hoa, D., Brabet, M. C., Bollore, K., Ducos, J., Vendrell, J. P. and Blanc, P.,** Investigation of memory B cell responses to hepatitis B surface antigen in health care workers considered as non-responders to vaccination. *Vaccine* 2010. **28**: 6411-6416.

22 **Konforte, D., Simard, N. and Paige, C. J.,** IL-21: an executor of B cell fate. *J Immunol* 2009. **182**: 1781-1787.

23 **Bernasconi, N. L., Onai, N. and Lanzavecchia, A.,** A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* 2003. **101**: 4500-4504.

24 **Ahmed, R. and Gray, D.,** Immunological memory and protective immunity: understanding their relation. *Science* 1996. **272**: 54-60.

25 **Castellino, F., Galli, G., Del Giudice, G. and Rappuoli, R.,** Generating memory with vaccination. *Eur J Immunol* 2009. **39**: 2100-2105.

26 **Plotkin, S. A.,** Correlates of protection induced by vaccination. *Clin Vaccine Immunol* 2010. **17**: 1055-1065.

27 **Milne, A., Allwood, G. K., Pearce, N. E., Lucas, C. R. and Krugman, S.,** Low dose hepatitis B vaccination in children. *N Z Med J* 1986. **99**: 47-49.

28 **Coursaget, P., Leboulleux, D., Soumare, M., le Cann, P., Yvonnet, B., Chiron, J. P., Coll-Seck, A. M. and Diop-Mar, I.,** Twelve-year follow-up study of hepatitis B immunization of Senegalese infants. *J Hepatol* 1994. **21**: 250-254.

29 **Pichichero, M. E., Blatter, M. M., Reisinger, K. S., Harrison, C. J., Johnson, C. E., Steinhoff, M. C., Senders, S. D., Rothstein, E. P., Willems, P. and Howe, B. J.,** Impact of a birth dose of hepatitis B vaccine on the reactogenicity and immunogenicity of diphtheria-tetanus-acellular pertussis-hepatitis B-inactivated poliovirus-Haemophilus influenzae type b combination vaccination. *Pediatr Infect Dis J* 2002. **21**: 854-859.

30 **Lanzavecchia, A., Bernasconi, N., Traggiai, E., Ruprecht, C. R., Corti, D. and Sallusto, F.,** Understanding and making use of human memory B cells. *Immunol Rev* 2006. **211**: 303-309.

31 **Tokoyoda, K., Zehentmeier, S., Chang, H. D. and Radbruch, A.,** Organization and maintenance of immunological memory by stroma niches. *Eur J Immunol* 2009. **39**: 2095-2099.

32 **Moser, K., Muehlinghaus, G., Manz, R., Mei, H., Voigt, C., Yoshida, T., Dorner, T., Hiepe, F. and Radbruch, A.,** Long-lived plasma cells in immunity and immunopathology. *Immunol Lett* 2006. **103**: 83-85.

33 **Tarlinton, D., Radbruch, A., Hiepe, F. and Dorner, T.,** Plasma cell differentiation and survival. *Curr Opin Immunol* 2008. **20**: 162-169.

34 **Amanna, I. J. and Slifka, M. K.,** Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. *Immunol Rev* 2010. **236**: 125-138.

35 **Manz, R. A., Lohning, M., Cassese, G., Thiel, A. and Radbruch, A.,** Survival of long-lived plasma cells is independent of antigen. *Int Immunol* 1998. **10**: 1703-1711.

36 **Pinna, D., Corti, D., Jarrossay, D., Sallusto, F. and Lanzavecchia, A.,** Clonal dissection of the human memory B-cell repertoire following infection and vaccination. *Eur J Immunol* 2009. **39**: 1260-1270.

37 **Leyendeckers, H., Odendahl, M., Lohndorf, A., Irsch, J., Spangfort, M., Miltenyi, S., Hunzelmann, N., Assenmacher, M., Radbruch, A. and Schmitz, J.,** Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen-specific IgG. *Eur J Immunol* 1999. **29**: 1406-1417.

38 **Bauer, T. and Jilg, W.,** Hepatitis B surface antigen-specific T and B cell memory in individuals who had lost protective antibodies after hepatitis B vaccination. *Vaccine* 2006. **24**: 572-577.

39 **Cherry, J. D., Gornbein, J., Heininger, U. and Stehr, K.,** A search for serologic correlates of immunity to Bordetella pertussis cough illnesses. *Vaccine* 1998. **16**: 1901-1906.

40 **Lugauer, S., Heininger, U., Cherry, J. D. and Stehr, K.,** Long-term clinical effectiveness of an acellular pertussis component vaccine and a whole cell pertussis component vaccine. *Eur J Pediatr* 2002. **161**: 142-146.

European Journal of Immunology

Figure Legends

Figure 1: Efficacy of Infanrix®-hexa (VacA, black dots) and Hexavac® (VacB, white dots) vaccines measured by serum antibodies. A) Graph shows the frequency of children grouped according to anti-HBsAg IgG serum concentrations <10, 10-100 or >100mIU/ml vaccinated with VacA (black) or VacB (white) vaccines. B) Graph shows the frequency of VacA and VacB children with anti-HBsAg IgG serum concentrations <10, 10-100 or >100mIU/ml 14-20 days boosting.

Figure 2: Antibody secreting cell analysis in total peripheral blood lymphocytes, in sorted mature B cells ($CD24^{pos}CD27^{neg}$) and in sorted memory B cells ($CD24^{pos}CD27^{pos}$) using spot ELISA. A) For total IgG and IgM determination, plates were coated with anti-IgG and anti-IgM, respectively, whereas tetanus toxin peptide (C-term) was used for specific anti-tetanus IgG determination. Spots were developed with anti-IgM (first line) or anti-IgG (second and third line). Images correspond to a representative example of 5 independent experiments. B) Bars represent the number of memory B cells (per million of PBLs) secreting either IgM, or IgG or specific IgG. The results for total cells (PBLs), FACS sorted mature and memory B cells are shown as means ± standard deviation of 5 independent experiments.

Figure 3: Number of memory B cells, per million of PBMCs, secreting antibodies after 5 days of stimulation with CpG, IL-4 and IL-21, measured by ELISpot. A) Box-plot represent the number of IgG and IgM antibody producing cells in one million of nucleated cells from children vaccinated with Infanrix®-hexa (VacA) and Hexavac® (VacB), respectively. B) Box-plot represent the number of antigen specific IgG secreting cells reactive to tetanus toxin peptide (Anti-T.Tox) and HBsAg (Anti-HBsAg) in children vaccinated with Infanrix®-hexa (Vac A) or Hexavac® (Vac B). C) Dots represents the number of anti-HBsAg IgG secreting cells in children vaccinated either with Infanrix®-hexa (Vac

European Journal of Immunology

A, n=34) or Hexavac® (Vac B, n=65) and grouped according to anti-HBsAg IgG serum concentration <10, 10-100 or >100 mIU/ml.

Figure 4: Anti-HBsAg IgG memory B cells and serum antibody analysis before (Pre) and after boosting (Post) with Engerix-B®. Note that all children (n= 25) were selected to have anti-HBsAg IgG serum levels lower than 10mIU/ml before boosting. A) Graph shows the rise in serum anti-HBsAg IgG 14-20 days after challenge in children previously vaccinated with Infanrix®-hexa (Vac A) or Hexavac® (Vac B). B) Number of anti-HBsAg IgG secreting cells per million of plated cells before (Pre) and 14-20 days after recall (Post) a single dose of Engerix-B®. C) Box-plot represent the frequency of anti-HBsAg IgG producing cells in the pool of IgG secreting cells in children before (Pre) and after boosting (Post), $p < 0.02$ (p value calculated using t student paired-sample test).

European Journal of Immunology

Table 1: Demographic and serologic characterization of the children enrolled in current study.

	Vaccine used for primary immunization	Number of subjects	Sex (F/M)	Age (y)	HBsAg carrier present in the family	Serum GMC (mIU/ml) (95%CI)	Number of children with anti-HBsAg IgG (mIU/ml)		
					Yes/No		<10	10-100	>100
VAC A	Infanrix®-hexa	34	14/20	6-7	1/33	47,0 (1,9-1122,8)	6 (17,6%)	14 (41%)	14 (41%)
VAC B	Hexavac®	65	27/38	6-7	2/63	7,62 (0,44-130,2)	38 (58,7%)	22 (34,9%)	5 (6,3%)

Table 2: Post-booster anti-HBsAg serum concentrations and anti-HBsAg IgG secreting cells in children primed with Infanrix®-hexa or Hexavac® 5 years before.

Vaccine primary immunization	Vaccine boost	Number of subjects	Serum GMC (mIU/ml) (95%CI)		Number of children with anti-HBsAg IgG (mIU/ml) after boost			Number of anti-HBsAg IgG spots/10^6 cells	
			pre	post	<10	10-100	>100	pre	post
Infanrix®-hexa	Engenrix®	5	3,5 (1,43-8,3)	1012,5 (199,2-5146,3)	0	1	4	15±18	23±25
Hexavac®	Engenrix®	20	2,9 (2,2-3,6)	203,3 (87,6-471,8)	1	7	12	10±7	29±13

European Journal of Immun

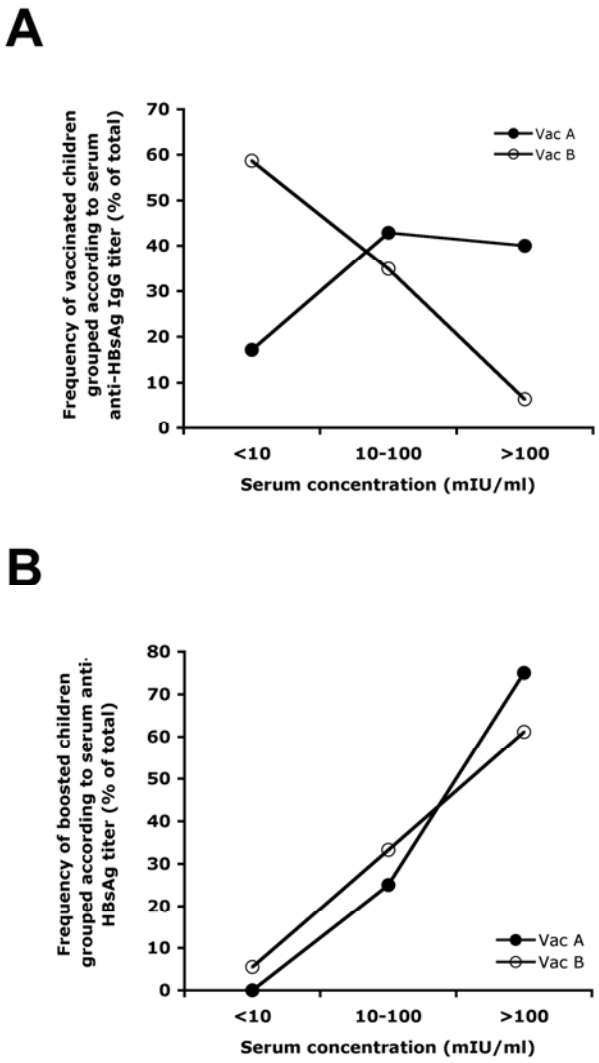

Figure 1

European Journal of Immunology

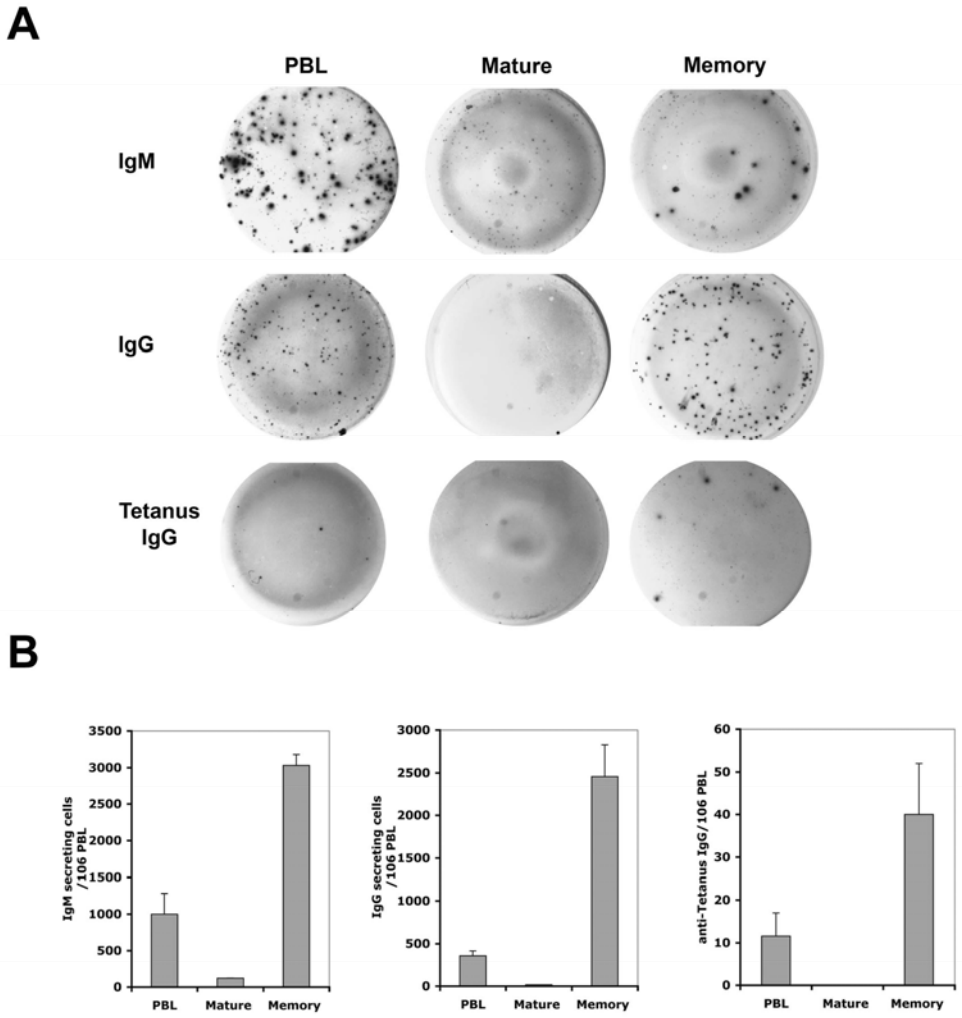

Figure 2

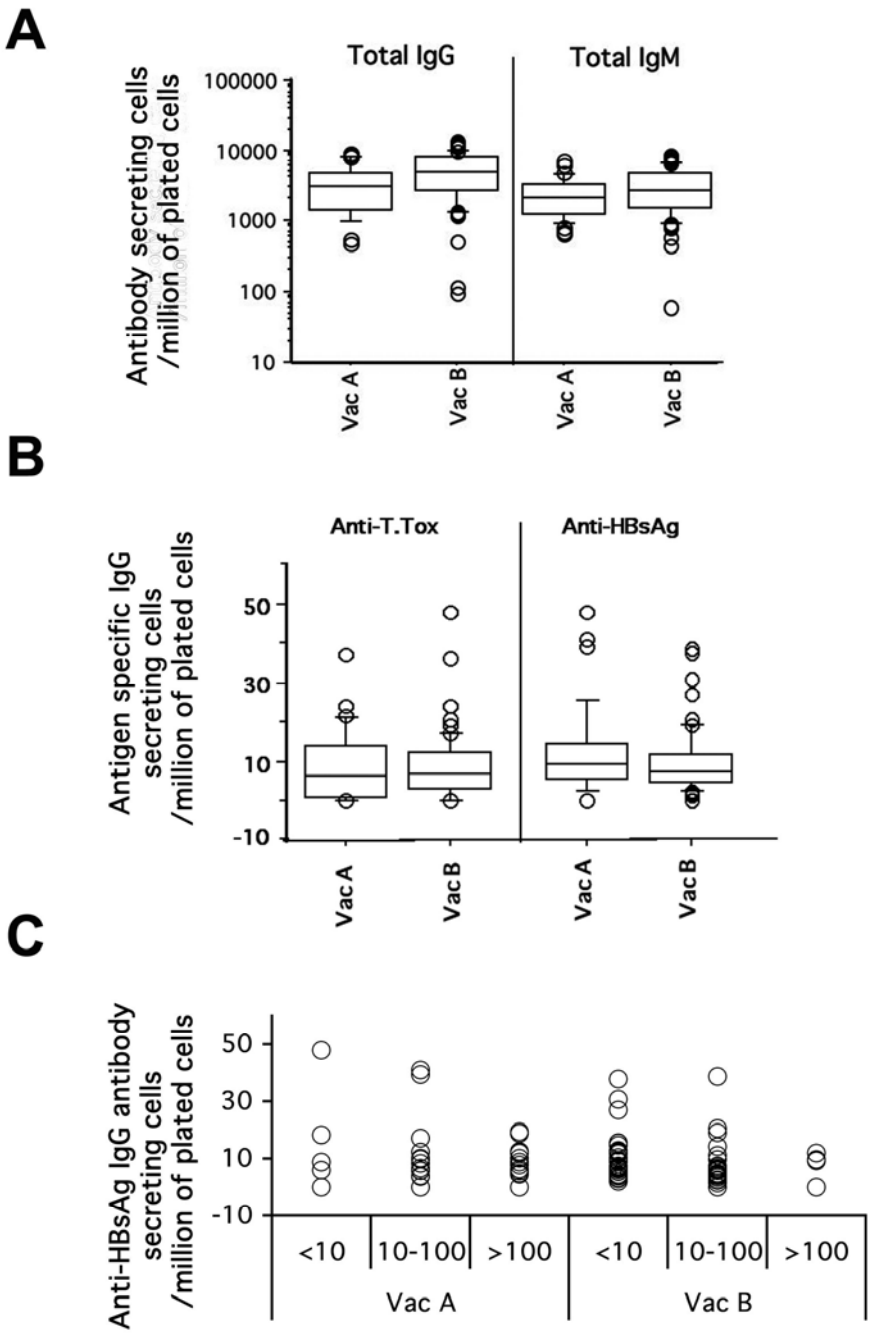

Figure 3

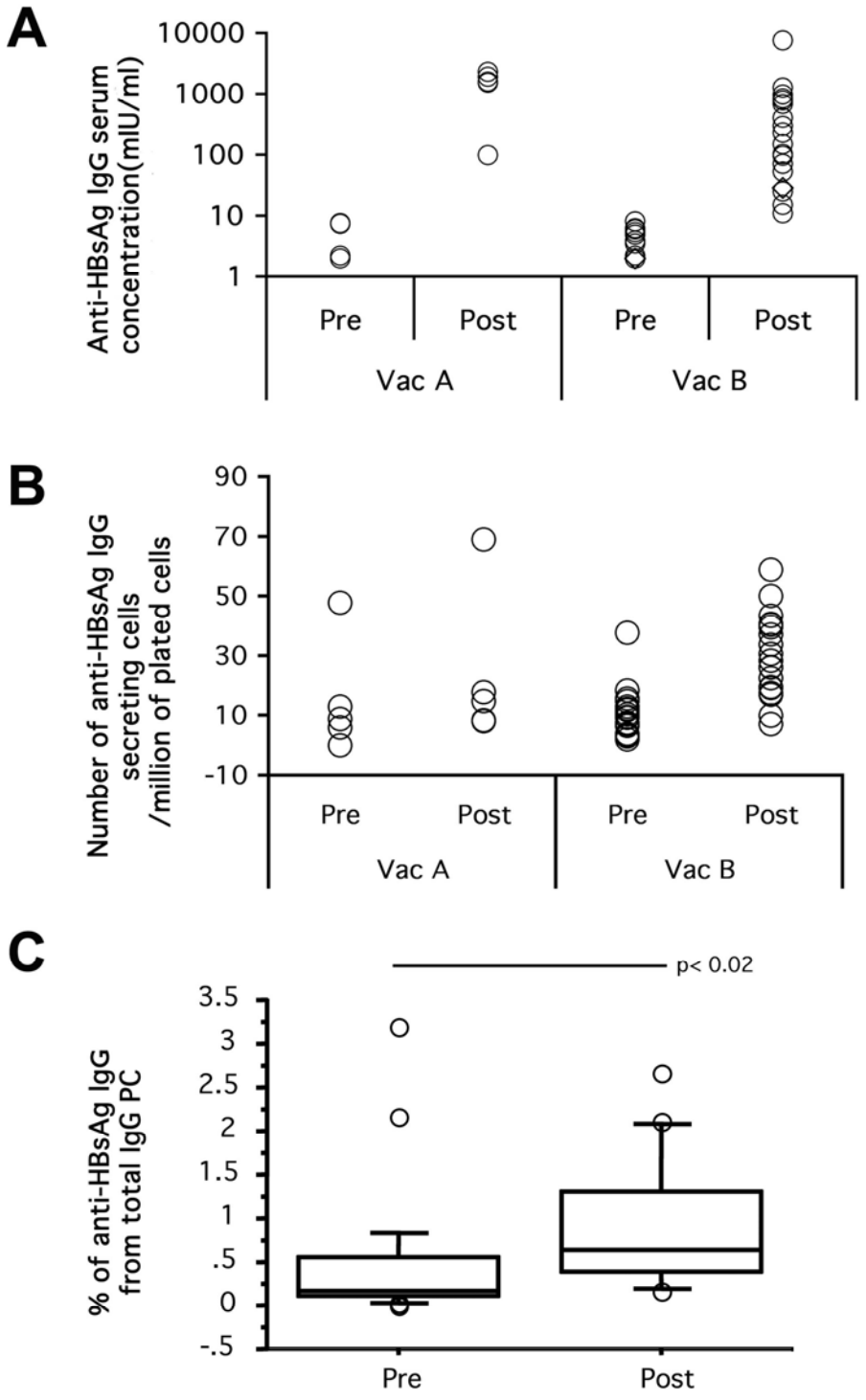

Figure 4