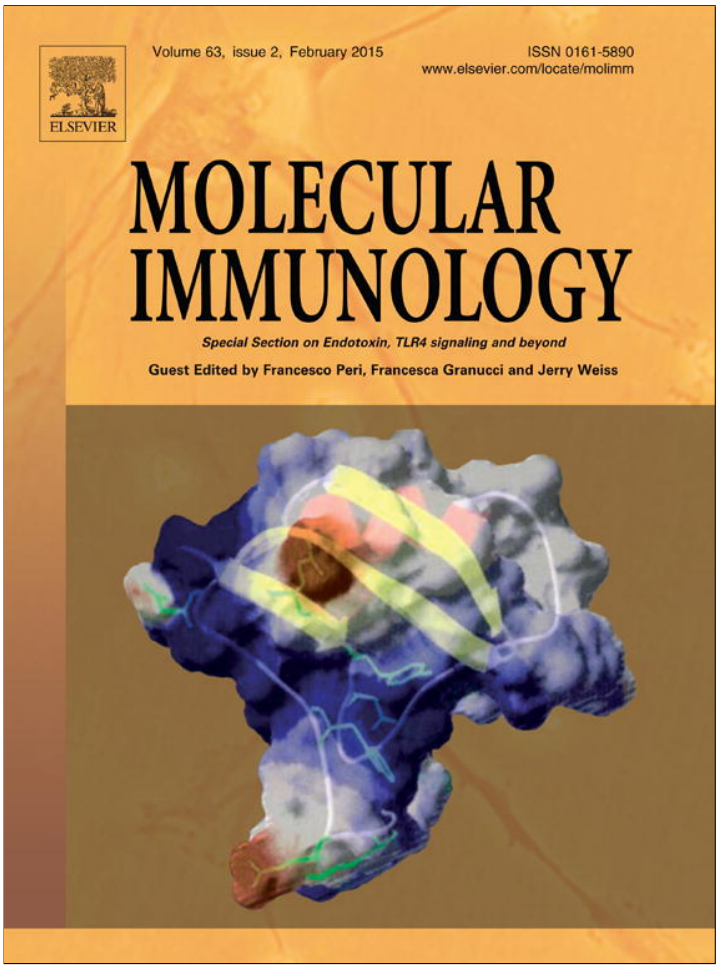

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In vitro stimulation with HBV therapeutic vaccine candidate Nasvac activates B and T cells from chronic hepatitis B patients and healthy donors

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ABSTRACT

Hepatitis B virus (HBV) chronic infections remain a considerable health problem worldwide. The standard therapies have demonstrated limited efficacy, side effects or need life-long treatments. Nowadays therapeutic vaccination is a promising option. Recently, we developed a new vaccine formulation called Nasvac, based on the combination of surface and core antigens from HBV. Clinical trials already performed showed good efficacy in virus control. However, the exact mode of action of Nasvac formulation remains unclear. So far the functional impairment of DCs during persistent HBV infection is a controversial issue. On the other hand, it is known that B cells may function as antigen presenting cells (APC) activating T cells. The hepatitis B core antigen contained in Nasvac vaccine is able to bind and activate a high frequency of naive human B cells. In the present study the surface expression of activation and exhaustion markers on B cells and the subsequent activation of T cells after *in vitro* stimulation with Nasvac antigens were evaluated in chronic HBV patients and healthy donors. B- and T-cell phenotype and proliferation were assessed by flow cytometry. Our results indicate that in contrast to exhaustions markers B cell activation markers were increased on both study groups after Nasvac stimulation. A shift toward an activation phenotype was observed for both B and T cells. The present work suggests that B cells could act as efficient APCs for Nasvac antigens in humans, which might suggest the use of activated B cells as immunotherapeutic strategy for chronic hepatitis B.

1. Introduction

Approximately an estimated 5% of the world population is chronically infected with the hepatitis B Virus. Chronic HBV infection (CHB) accounts for approximately 50% of the total cases of hepatocellular carcinoma (El-Serag, 2012).

Therapeutic options for CHB are limited, as the efficacy of antiviral treatment is moderate and the eradication of the virus is difficult to achieve. A new approach of therapy is based on the stimulation of the host's immune system by therapeutic vaccination. Several studies have been performed using different vaccine formulations. Despite none of these vaccines has demonstrated a sufficient level of clinical efficacy they have created great expectations (Michel and Mancini-Bourgine, 2005).

Recently, we developed a new formulation of HBV vaccine called Nasvac, which contains a combination of the HBV core (HBcAg) and surface (HBsAg) antigens. Several preclinical studies have been completed in mice (Aguilar et al., 2004; Lobaina et al., 2010). Until now four clinical trials were concluded, including a phase III trial (Aguilar-Betancourt et al., 2007; Al-Mahtab et al., 2013; Akbar et al., 2013). In this setting Nasvac was able to enhance viral control in CHB patients (Al-Mahtab et al., 2013; Akbar et al., 2013).

However, the mode of action of the Nasvac formulation is so far unknown. It has been shown that the antigen-presenting capacity of B cells may be important for the induction of optimal vaccine-induced responses (Crawford et al., 2006; Rodriguez-Pinto and Moreno, 2005). In the case of Nasvac vaccine the HBcAg antigenic

Abbreviations: HBV, hepatitis B virus; APC, antigen presenting cell; DCs, dendritic cells; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; CTL, cytotoxic T lymphocyte; HD, healthy donor; PBMC, peripheral blood mononuclear cell.

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component has a unique ability to bind and activate a high frequency of naive human and murine B cells (Lazdina et al., 2001, 2003). HBcAg-specific B cells from unprimed mice are able to take up, process and present HBcAg to naive T-helper cells *in vivo* 10^5 times more efficiently than classical APCs (Milich et al., 1997).

Considering this knowledge we decided to study the impact of this formulation on B cell activation and on stimulation of T cell responses *in vitro* in healthy individuals and CHB patients. The expression of activation and exhaustion markers on human PBMC stimulated with Nasvac or each separated antigen was evaluated. In addition, the proliferative capacity of different cell types after Nasvac stimulation was studied.

2. Materials and methods

2.1. Patient samples

Heparinized peripheral whole blood samples were obtained from a total of 34 individuals (both sexes) that attended to the Hannover Medical School (MHH) facilities, 18 healthy donors (HD) and 16 CHB patients. The HD had been vaccinated against HBV with the conventional prophylactic vaccine and developed protective anti-HBs titers (≥10 IU/L). In the CHB group 56% of the patients were without treatment at the moment of the analysis. Ethics approval for this study was obtained by the local ethics committees of MHH. Written informed consent was obtained from all patients involved in this study.

2.2. PBMC stimulation with Nasvac antigens

PBMCs were isolated from whole blood by standard Ficoll method. For *in vitro* stimulations cells were resuspended in complete RPMI-1640 culture medium (RPMI-1640 (Gibco), 5 mM HEPES (Gibco), 1 mM L-glutamine (Gibco), 1% Na-pyruvate (Gibco), 1% non-essential amino acids (Gibco), and 1% Penicillin/Streptomycin (Gibco), supplemented with 10% human AB serum (Lonza). 3×10^5 cells per well were incubated in U-bottom 96-well plates (Sarstedt AG, Germany). The cells were stimulated with the following conditions: 5 μg/ml of each separate antigen (HBsAg or HBcAg, obtained at CIGB facilities, Aguilar et al., 2004), 5 μg/ml of both antigens mixed (Nasvac formulation), 1 μg/ml of Staphylococcal Enterotoxin B (fragment 150–161) (SEB) as positive control, or complete media alone as negative control. All analyses were set up in duplicates for each condition. Cells were incubated at 37 °C and 5% CO_2 for 18–24 h.

2.3. Flow cytometry

A six-parameter flow cytometric analysis was performed using a FACS Canto II instrument equipped with Diva software (BD Biosciences). Cells were stained with different panels of monoclonal antibodies (mAbs): B cell activation panel: anti-CD19–APC-H7, anti-CD154–FITC, anti-CD69–PE, anti-CD86–PECy5, anti-CD40–APC, and anti-CD3–PECy7; T cell activation panel: anti-CD3–PECy7, anti-CD4–PECy5, anti-CD8–APC-H7, anti-CD25–FITC, anti-CD69–PE, and anti-CD279 (PD-1)-APC; B cell exhaustion panel: anti-CD19–APC-H7, anti-CD307d–PE, anti-CD273 (PD-1 ligand2)-FITC, and anti-CD274 (PD-1 ligand1)-PECy7. All antibodies were obtained from BD Pharmingen, except anti-CD307d and -PD-1 (BioLegend). In all the panels we also include Human BD Fc Block™ (BD). After 10 min of staining at 4 °C, cells were washed twice with PBS containing 2% fetal calf serum (FCS), 0.05% sodium azide, resuspended in the same solution and immediately analyzed. Data analyses were performed using FlowJo Software (TreeStar Inc.).

2.4. CFSE labeling and proliferation analysis by flow cytometry

1×10^7 PBMC were labeled with 4 μM of CFSE (Sigma–Aldrich) for 8 min at 37°. Staining was stopped by adding cold FCS and incubated for 5 min on ice. After two washes, PBMC were resuspended at concentration 3×10^6 ml^{-1} in RPMI 1640 complete medium. Cells were seeded into U-bottom 96-well plates (Sarstedt AG, Germany) at 3×10^5 cell/well and incubated at 37 °C for 5 days. On day 3–4 the cells were fed with 100 μl of IL2 medium (RPMI 1640 complete medium containing 5 U/ml rhIL2) by taking off 90 μl supernatant before. At the end of culture cells were harvested, washed with PBS containing 2% FCS, and stained with mAbs against CD3, CD4, CD8, CD19 and CD56 as described above. Data acquisition and analysis were performed by flow cytometry. Proliferation of B (CD19+), T (CD4+ and CD8+) and NK (CD56+) cells was analyzed as the percentage of cells that diluted the CFSE intensity at least once at the time of harvest.

2.5. Statistical analysis

GraphPad Prism version 5 was used for statistical analysis and graphics. Data expressed as means of duplicates were analyzed. A responder individual was defined as one showing an increment of the surface marker expression of more than 10% after antigenic stimulation compared to the negative control.

The normality of the data distribution was determined using Shapiro–Wilk test and a paired *t* test or a Wilcoxon matched-pairs test were done depending on the results of the normality test. A p value <0.05 was considered statistically significant.

3. Results

3.1. Activation of B cells

For the stimulation of human PBMC with Nasvac formulation and their individual antigens we found that the use of 5 μg/ml of each antigen, alone or combined, provided an optimal activation of B and T cells after 24 h (data not shown).

Gating strategies employed for analysis of B cells is shown in Fig. 1A. In HD the activation of total B cells could only be enhanced by stimulation with Nasvac and HBcAg, while the HBsAg was not able to induce activation (Fig. 1B and Supplementary Fig. 1). Only expression of CD86 significantly increased on B cells upon Nasvac stimulation, both at percentage of expression and mean fluorescence intensity (MFI) ($p = 0.0002$, and, $p = 0.0048$, respectively). No alterations of CD69 (medium: mean 34.1%, range 3.0–69.6%; Nasvac: mean 34.1%, range 4.9–64%) or CD40 expression (medium: mean 81.6%, range 59.4–93.7%; Nasvac: mean 81.2%, range 60.1–93.7%) could be observed, while expression of the latter was generally high both in percentage and expression intensity (MFI) (Fig. 1B). Also, expression of CD154 on B cells was not altered (data not shown).

In contrast, incubation of B cells from CHB patients with Nasvac resulted in a clear stimulation as the expression of activation markers on total B cells showed a significant increase (Fig. 1C). Here, also the frequency of CD69+ B cells was elevated in comparison to the negative control (medium: mean 18.4%, range 3.8–61.6%; Nasvac: mean 21.7%, range 4.7–61.1%; $p = 0.0001$) and similarly CD86 expression increase (medium: mean 32.2%, range 10.8–57.4%; Nasvac: mean 39.2%, range 15.2–61%; $p = 0.0001$). CD40 expression did not significantly change, but again levels were generally high (medium: mean 65.2%, range 22.9–93%; Nasvac: mean 64.9%, range 21.2–91%). Stimulation with the antigens separately induced slight but not significant increases (Supplementary Fig. 1). Generally, the

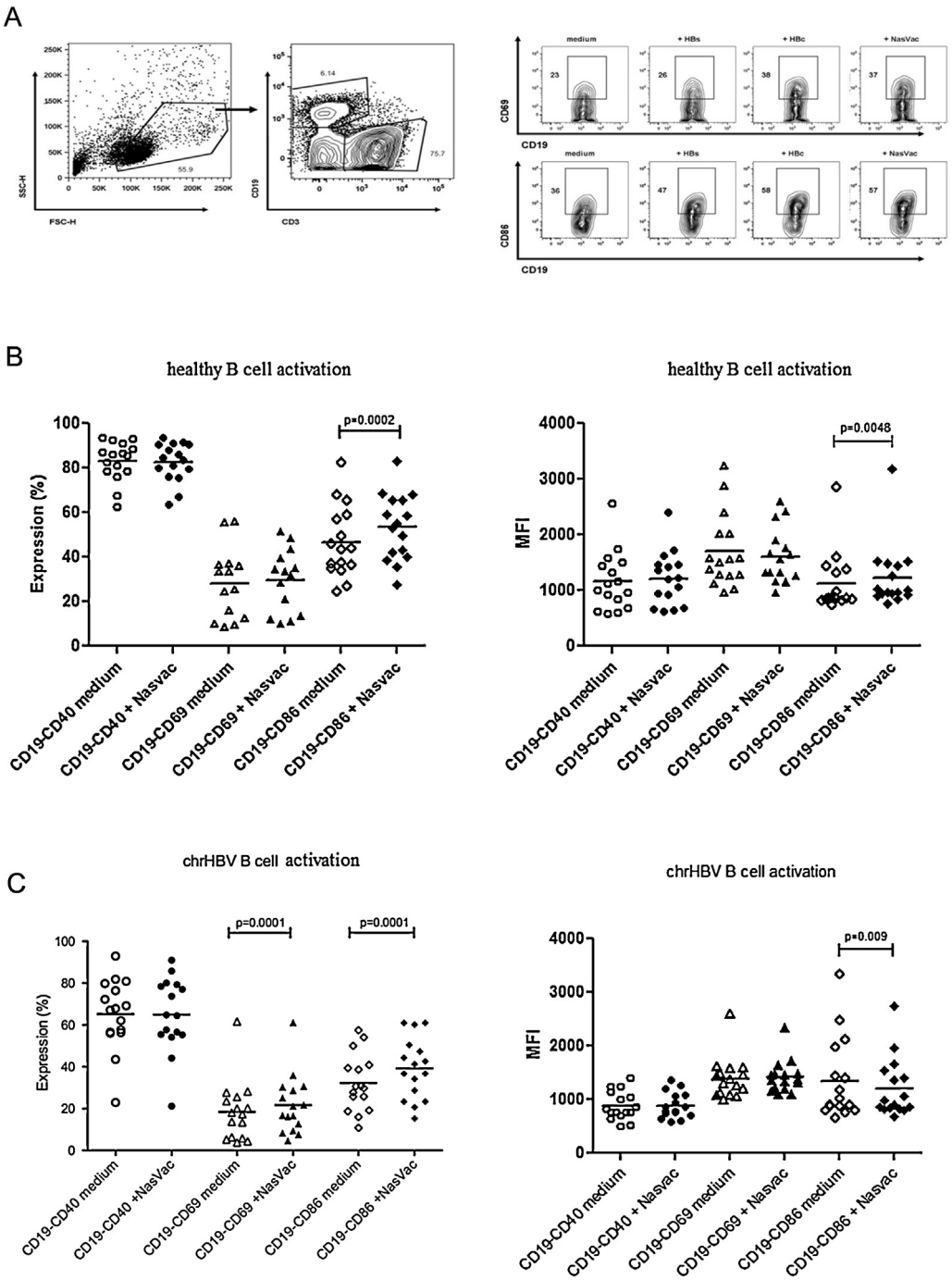

Fig. 1. Activation of B cells through Nasvac stimulation in vitro. (A) Gating strategy of lymphocyte population and of CD3−CD19+ B cells and representative FACS plots of CD69 and CD86 expression on CD19+CD3− B cells after stimulation with Nasvac or medium control. (B and C) Scatter plot summarizing the expression and mean fluorescence intensity (MFI) of CD40, CD69 and CD86 on CD3−CD19+ B cells after Nasvac stimulation in, healthy individuals, and CHB patients; respectively.

combination of both antigens as used in Nasvac formulation was more potent in activating B cells in both cohorts.

When we compared the basal expression levels of activation markers CD69, CD40 and CD86 on B cells between HD and CHB patients, we observed that non-stimulated cells from HD have higher expression of both markers than CHB patients (CD40: 82% *vs.* 65%, $p = 0.0014$; CD69: 34% *vs.* 18%, $p = 0.003$; CD86: 44% *vs.* 32%, $p = 0.021$; Supplementary Fig. 2). However and notably, stimulation of B cells from CHB patients was stronger as in HD, though expression level in CHB after Nasvac stimulation was still lower than the basal frequencies in HD.

3.2. Expression of exhaustion markers on B cells

Functional exhaustion of immune cells, commonly observed during persistent viral infections, can be characterized by the expression of different exhaustion markers. In the case of B cells we analyzed the expression of CD307d, as well as CD273 and CD274 (PDL-2 and PDL-1, respectively). Expression of PD-1 was analyzed on CD4+ and CD8+ T cells (see Section 3.3).

The basal levels of the exhaustion markers CD274, CD273 and CD307d on B cells showed no differences in CHB patients as compared to HD (CD274: mean 1.22% *vs.* 0.9%, CD273: mean 2.7% *vs.*

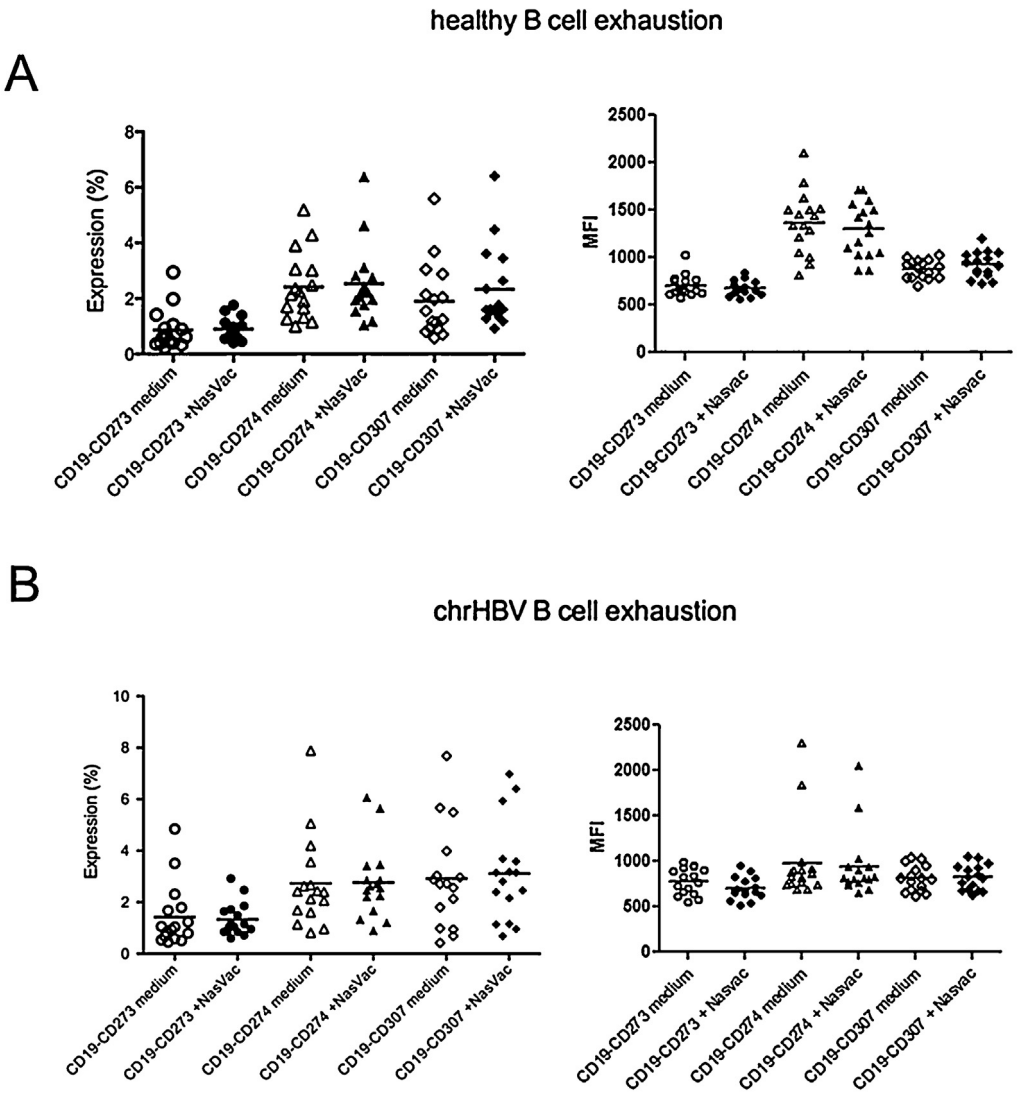

Fig. 2. Induction of exhaustion markers on CD3−CD19+ B cells after *in vitro* Nasvac stimulation, represented as percentage of expression and mean fluorescence intensity (MFI). (A) Healthy individuals ($n = 16$). (B) CHB patients ($n = 16$).

2.4%; CD307d: mean 2.9% *vs.* 1.9%, respectively; Supplementary Fig. 3). After Nasvac stimulation a slight increase in expression of these markers on B cells was detectable only in few HD (CD274: 5/16 (31.5%); CD273: 2/16 (12.5%); CD307d: 4/16 (25%)) and CHB patients (CD274: 1/16 (6.2%); CD273: 0/16 and CD307d: 0/16 individuals) (Fig. 2A and B). Both in HD as well as in CHB patients the change in expression (percentage and MFI) of these exhaustion markers upon Nasvac stimulation was not significant (Fig. 2A and B) except for a minor elevation in the percentage of expression of CD307d+ B cells in HD (medium: mean 1.9%, range 0.6–5.6%; Nasvac: mean 2.3%, range 0.9–6.4%; $p = 0.005$).

3.3. Activation of T cells

An important feature of B cells is their ability to activate antigen-specific T cell response. We analyzed the expression of activation and exhaustion markers on CD4+ and CD8+ T cells after PBMCs stimulation with Nasvac (Fig. 3) and each independent antigen (Supplementary Fig. 4). Overall, the increase of expression of the different markers analyzed was low. Still in HD significant increases in the expression of CD25 on CD4+ (medium: mean 2.3%, range 0.4–6.2%; Nasvac: mean 3.3%, range 1.0–8.0%; $p = 0.0015$; see Fig. 3A) could be observed. The activation of CD8 T cells was even more pronounced (Fig. 3B), here expression of both CD25 (medium: mean 1.9%, range 0.5–7.2%; Nasvac: mean 2.4%, range 0.5–9%; $p = 0.023$); and CD69 (medium: mean 12.4%, range 1.1–38.9%; Nasvac: mean 14.8%, range 3.9–44.3%; $p = 0.006$) could be seen. Further, a trend toward an increase of CD69+ CD4+ T cells was observed, although this did not reach statistical significance (mean 1.5% *vs.* 2.1%, $p = 0.055$). Expression of PD-1 was only altered on CD4T cells (medium: mean 13.3%, range 3.5–23.2%; Nasvac: mean 14.0%, range 3.6–22.5%; $p = 0.007$) and no change for CD8T cells could be observed after Nasvac stimulation. In general, for the CD4T cell population the obtained percentage of expression results are consistent with the data of MFI (Supplementary Fig. 5).

In CHB patients we observed induction of CD25 and CD69 expression in the majority of individuals analyzed. Similarly to the observations for B cells, also the strength of activation of T cells showed to be higher in CHB patients as compared to HD. A significant increase of CD25 expression on CD4T cells (medium: mean 1.6%, range 0.7–3.7%; Nasvac: mean 3.1%, range 1.3–5.7%; $p = 0.0001$; Fig. 3C) as well as both CD25 (medium: mean 1.2%, range 0.5–2.8%; Nasvac: mean 1.6%, range 0.7–3.4%; $p = 0.0001$) and CD69 (medium: mean 7.9%, range 3.1–20.2%; Nasvac: mean 10.2%, range 3.5–36.4%; $p = 0.038$; Fig. 3D) on CD8+ T cells. Interestingly, changes for PD-1 exhaustion marker could be observed

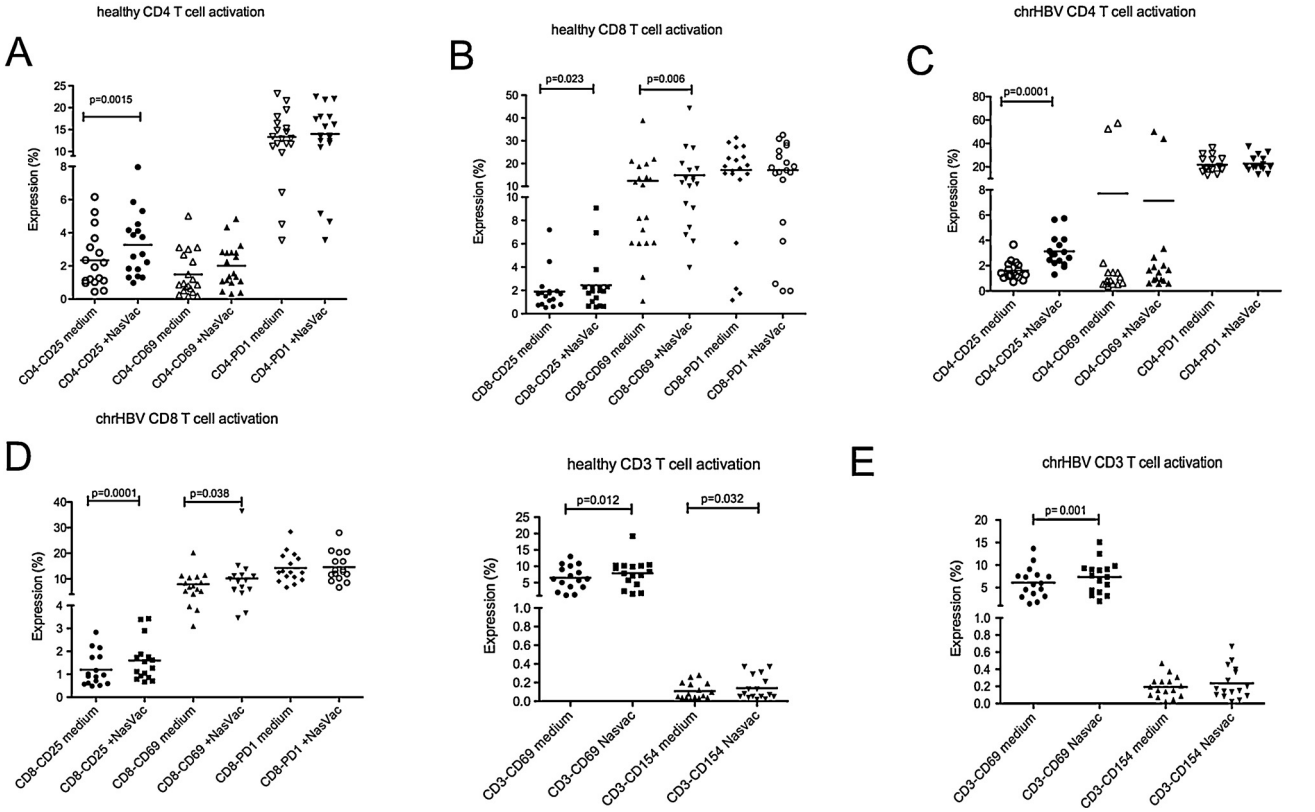

Fig. 3. Activation of T cells upon *in vitro* stimulation with Nasvac. Induction of expression of activation markers: (A) on CD4+ T cells from healthy individuals ($n = 17$); (B) on CD8+ T cells from healthy individuals ($n = 16$); (C) on CD4+ T cells from CHB patients ($n = 17$); (D) on CD8+ T cells from CHB patients ($n = 16$). (E) Change of expression of CD69 and CD154 on total CD3+ T cells in healthy individuals (right plot, $n = 16$) and CHB patients (left plot; $n = 17$).

on CD4+ (medium: mean 21.9%, range 12.8–36.2%; Nasvac: mean 22.8%, range 13.5–37.4%; $p = 0.0001$) only, but was absent for the CD8+ T cell population. On the other hand, the MFI data obtained only match in the significant increase detected for CD25 on CD8T cell, the rest of the markers did not showed important changes (Supplementary Fig. 5).

For practical reasons, related with the sample availability and the capacity of FACs instrument, we decided to evaluate the expression of CD154 only in total CD3+ T cells. In general, the expression observed for this marker was low in frequency. In HD a slight increase in CD154+ CD3+ T cells could be observed (Fig. 3E), which nevertheless was statistically significant. No alteration of expression was visible in CHB patients upon Nasvac stimulation.

On the other hand, after stimulation with each separate antigen we did not detect changes in the expression of CD154 on CD3+ cells (data no shown). Also, the separate antigens hardly induced activation of CD4+ and CD8+ T cells, which was only seen in few individuals (data not shown).

3.4. Proliferative response after in vitro Nasvac stimulation

We studied the proliferative capacity of different cells subsets (B, CD4+ and CD8+ T cells, and NK cells) derived from HD and CHB patients.

In HD only weak proliferative responses could be observed. Most frequently, proliferation of CD56+ NK cells (5/7 individuals; see Fig. 4A) and CD8+ T cells (4/7 individuals) were detected. Responses by B cells could not be detected in any individual and proliferation of CD4T cells was rare (2/7 individuals).

In CHB patients proliferative responses were similarly scarce and in the majority proliferation of NK cells could be observed (Fig. 4B). Here, 3/4 patients responded after Nasvac stimulation. For CD19+ B cells proliferation occurred in 2/4 individuals, but only in one case proliferation of CD4 and CD8T cells could be detected. For one individual analyzed a vigorous proliferation of all four cell populations was visible and the same individual also showed strong increase of B and T cell activation markers.

Summarizing, the *in vitro* stimulation of HD- or CHB patients-derived B cells with Nasvac formulation induce slight, but in some cases significant, increases in the expression of activation markers, mainly CD86 marker, who plays an important role as co-stimulatory signals to T cells. On the contrary, the exhaustion markers evaluated on B cells (CD274, CD273 and CD307d) shows only a slight increase detectable in few individuals. Furthermore, we observed a higher expression of CD25 on CD4+ and CD8+ T cells from HD and CHB patients after Nasvac stimulation, and also a significant increase for CD69 on CD8+ T cell from both studied groups. Interestingly, minor changes for PD-1 exhaustion marker were observed on CD4+ T cells, but were absent for the CD8+ T cell populations. On the other hand, for both groups HD and CHB patients, only a weak proliferative response could be observed after Nasvac stimulation, most frequently for the CD56+ NK cells. In general, our findings suggest that the *in vitro* stimulation with Nasvac induce a level of activation on peripheral blood B and T cells, more pronounced in the CD8+ T cells population.

4. Discussion

Therapeutic vaccination against HBV aims to induce functional virus-specific immune responses. Here, we show that the Nasvac vaccine candidate is able to stimulate HBV-specific immune responses by activating B cells rendering them into efficient APCs, which are in turn able to activate T cell responses.

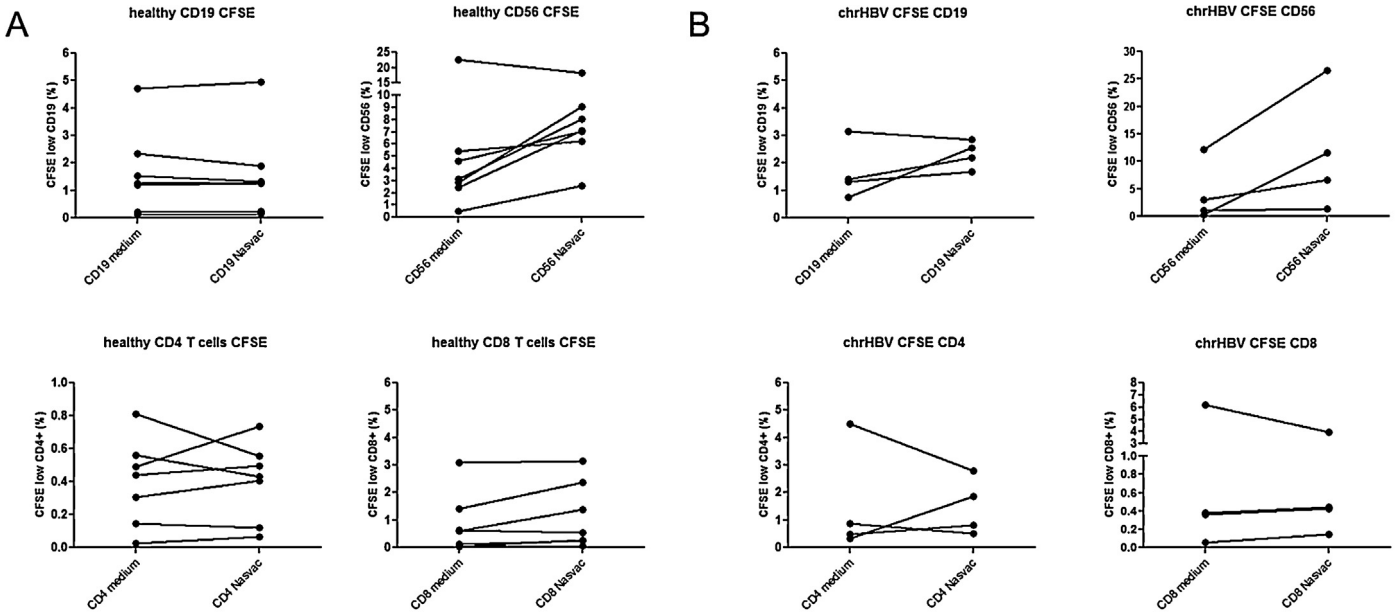

Fig. 4. Proliferation of lymphocytes upon *in vitro* Nasvac stimulation. (A) healthy individuals ($n = 7$). (B) CHB patients ($n = 4$). Frequencies of CFSE low cells gated on the respective cell populations are given. Proliferation of CD19+ B cells (upper left panel), CD56+ NK cells (upper right panel), CD4+ T cells (lower left panel) and CD8+ T cells (lower right panel) were analyzed.

The standards therapies for CHB infections have a limited efficacy and are accompanied by side effects and long treatment periods (Smith et al., 2008). Therapeutic vaccination is a promising approach (Inchauspe and Michel, 2007). As a novel vaccine candidate, Nasvac exploits the use of the intranasal administration route and its combination with systemic routes (Aguilar et al., 2004; Lobaina et al., 2010). This formulation is the first that employs the complete HBcAg as a recombinant protein. HBcAg obtained from *E. coli* constitutes a virus-like structure that encapsulates bacterial nucleic acid, which confers Th1 adjuvant properties (Riedl et al., 2002; Vanlandschoot et al., 2003). Nowadays Nasvac candidate is in phase III trial with promising results demonstrating its safety and efficacy (Al-Mahtab et al., 2013; Akbar et al., 2013). However, the mode of action of Nasvac formulation still remains unclear.

The *in vitro* activation of B cells by Nasvac formulation could be measured by the up-regulation of surface markers such as CD69, CD86 and CD40 (Abbas et al., 2012; Engel et al., 1994). After Nasvac stimulation we were able to detect an increase of CD86 on B cells from CHB patients and HD, and also an increase of CD69 in CHB patients; indicating the strength of the effect of this antigenic combination. The strongest stimulus was observed by Nasvac incubation, while responses to separated antigens were weaker or almost absent. This behavior is in agreement with previous reports indicating that the HBsAg is preferentially taken up and presented by dendritic cells and macrophages (Op den Brouw et al., 2008; Vanlandschoot et al., 2002). It was also reported in mouse studies that HBsAg does not induce costimulatory molecules on naive B cells (Milich et al., 1997; Scheerlinck et al., 1991). Our results are also in line with those obtained in mice by Akbar et al. (2010). They reported that HBsAg/HBcAg-pulsed DCs from C57BL/6J mice express significantly higher levels of CD86 and MHCII compared with unpulsed DCs. Additionally, in HBV-Tg mice, they found that the administration of HBsAg/HBcAg-pulsed DCs is capable to develop a higher antigen-specific humoral and proliferative immune response compared with HBsAg- or HBcAg-pulsed DCs. On the other hand, regarding the weaker signals of activation obtained for HB-vaccinated HD derived B cells after HBsAg *in vitro* stimulation we think that several factors may influence this behavior, the time last since vaccination, the proportion of HBs-specific memory B cells in peripheral blood, and the *in vitro* experimental conditions employed, among others.

In addition, we did not detect increases on CD40 expression on B cells from HD nor CHB patients after Nasvac stimulation, as expression of this molecule was generally high. The results obtained in HD resemble those reported by Negri et al. (2009), where strong increases in CD86 expression on B cells following treatment with different stimuli was observed but none of the treatments were able to induce an evident change in CD40 expression. Importantly however, stimulation with the Nasvac formulation neither induced a decrease of CD40 expression keeping the B cells able of delivering crucial co-stimulation to T cells.

T cell activation was measured by the expression of CD25, CD69 and CD154 surface markers (Abbas et al., 2012). In our study, CD154 expression on T cells from HD showed a marginal increase upon stimulation, which however was absent for CHB patients. Generally, the increase in frequencies of CD25 and CD69 positive T cells was comparatively low, which is due to our analysis of total T cells which includes cells specific for various other epitopes. In both groups, hepatitis B vaccinated HD and CHB patients; we could observe significant increases in CD25 on CD4+ and CD8+ T cells after Nasvac stimulation, and also an increase of CD69 on CD8+ T cells. On the other hand we suggest that the slight changes detected in PD1 expression on CD4+ T cells do not have a significant biological meaning. All these results together, suggest that B cells can in turn stimulate T cells. However, considering the variance observed for the surface markers, a higher number of patients would be evaluated to supports our findings. Furthermore as other authors had published (Oliviero et al., 2011) the surface expression of activation markers shows a high variability among individuals. This behavior could be increased by the presence of a persistent infection, like CHB, where the effect of the different patient characteristics counts.

On the other hand, we analyzed the proliferation of PBMC from HD and CHB patients after incubation with Nasvac. The results show that only few individuals respond. Although the reduced number of individuals evaluated in this experiment limits the strength of our

results, they are in line with similar reports from other researchers (Akbar et al., 2010; Oliviero et al., 2011). However, result of interest that in both study populations we found that the majority of the individuals evaluated develop a proliferative response for the NK cell subset. This data could be important considering that the cross talk between NK and antigen presenting cells influences the efficiency of the adaptive immune responses against virus, constituting a major link between innate and adaptive immune responses (Moretta, 2002). Taking into account the obtained results, and also knowing that Nasvac formulation contain some immunostimulatory motifs (both antigens are VLP, the HBsAg has a high lipid composition, and the HBcAg encapsulated bacterial nucleic acid (Aguilar et al., 2004)), we suggest that Nasvac is capable of stimulating the innate immune system *via* toll-like receptors (TLR). It is known that the activation of NK-cell induces release of IFN-γ, which in turn can cause maturation of DC and polarize T-cell responses (Caligiuri, 2008). IFN-γ production by NK cells shape the Th1 immune response, activate APCs to further up-regulate MHC class I expression (Caligiuri, 2008) and exert an antiviral effect on HBV-infected hepatocytes. It was recently shown that both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets from CHB patients display significantly lower INF-γ and TNF-α production (Oliviero et al., 2009). We do not know whether these functions could be restored in patients after Nasvac vaccination because this was not assessed so far. On the other hand, in a clinical trial evaluating a HBV DNA therapeutic vaccine candidate (Scott-Algara et al., 2010) the authors found that after vaccination occur modifications in the percentage and phenotype of the $CD56^{bright}$ NK cell population, some of them correlated with the HBV-specific T cell responses generated. All these information together, open new questions regarding the mechanisms of action of Nasvac formulation and its interaction with the innate immune system. A set of experiments are ongoing to evaluate if Nasvac preferentially induces cytokine secretion as effector mechanism.

Importantly, analyzing the expression of exhaustion markers on B and T cells no significant increase of PD-1 ligands or CD307d could be observed on B cells and only a weak increase of PD-1 expression on CD4 but not CD8T cells could be seen for both cohorts upon stimulation with the Nasvac. However, as comment before we suggest that the slight changes detected in PD1 expression on CD4+ T cells do not have a significant biological meaning. PD-1 is an inhibitory co-stimulatory receptor (Freeman et al., 2000). Sustained PD-1 expression on antigen specific CD8 T-cells is associated with impaired effector function in acute and chronic viral infections (Freeman et al., 2006). Our results suggest that B cells are not functionally impaired and likewise do not induce a subsequent exhaustion of T cells due to Nasvac stimulation. This data is in line with the reported by Oliviero et al. (2011), they found that B-cell activation, but not exhaustion is common in chronic viral hepatitis. The minor increase in PD-1 levels on CD4+ T cells after *in vitro* Nasvac stimulation and the increase of activation markers rather reflect a general activation of T cells instead of inhibitory responses.

5. Conclusions

Our results suggest that B cells could act as efficient APCs in spite of the tolerogenic environment established in CHB patients. Subsequently, also an activation of T cells occurred, strengthening this assumption. Even though the interaction of Nasvac formulation with the immune system cells should be further explored, the obtained results offer the first clues regarding its cellular mechanism of action and render new data demonstrating its capacity to stimulate CHB patient's immunity. These results also suggest that B cells have no limitations to become important antigen presenting cells during immunotherapy.

Conflict of interest

The authors have declared that no competing interests exits.

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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.molimm.2014.08.003.

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