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Immunization with hepatitis B vaccine accelerates SLE-like disease in a murine model

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ABSTRACT

Hepatitis-B vaccine (HBVv) can prevent HBV-infection and associated liver diseases. However, concerns regarding its safety, particularly among patients with autoimmune diseases (i.e. SLE) were raised. Moreover, the aluminum adjuvant in HBVv was related to immune mediated adverse events. Therefore, we examined the effects of immunization with HBVv or alum on SLE-like disease in a murine model.

NZBWF1 mice were immunized with HBVv (Engerix), or aluminum hydroxide (alum) or phosphate buffered saline (PBS) at 8 and 12 weeks of age. Mice were followed for weight, autoantibodies titers, blood counts, proteinuria, kidney histology, neurocognitive functions (novel object recognition, staircase, Y-maze and the forced swimming tests) and brain histology.

Immunization with HBVv induced acceleration of kidney disease manifested by high anti-dsDNA antibodies ($p < 0.01$), early onset of proteinuria ($p < 0.05$), histological damage and deposition of HBs antigen in the kidney. Mice immunized with HBVv and/or alum had decreased cells counts mainly of the red cell lineage ($p < 0.001$), memory deficits ($p < 0.01$), and increased activated microglia in different areas of the brain compare with mice immunized with PBS. Anxiety-like behavior was more pronounced among mice immunized with alum.

In conclusion, herein we report that immunization with the HBVv aggravated kidney disease in an animal model of SLE. Immunization with either HBVv or alum affected blood counts, neurocognitive functions and brain gliosis. Our data support the concept that different component of vaccines may be linked with immune and autoimmune mediated adverse events.

1. Introduction

The development of vaccines and vaccination programs has proven to be a tremendously successful and cost-effective asset to human health [1,2]. One example of this achievement is the hepatitis B virus vaccine (HBVv), which had proven to be beneficial in preventing HBV-infection and associated liver diseases [3]. However, concerns regarding safety of HBVv were raised in recent years addressing both the general population as well as different sectors at high risk of adverse events such as patients with autoimmune diseases [4,5]. Several clinical studies and numerous case-reports documented the appearance of autoantibodies, autoimmunity, and overt autoimmune diseases following vaccination, in a small minority of subjects [1,6–11]. Hence, the dual sword of vaccination remains a concern, especially in genetically susceptible patients or

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those with immune mediated diseases such as systemic lupus erythematosus (SLE) [4]. In that particular case on the one hand viral and bacterial infections may favor an exacerbation of SLE thus supporting immunization while on the other aggravation of SLE or SLE-like disease was documented post immunization in some patients [9,12–17]. Establishing a causal link between immunization and autoimmunity in humans is difficult, as most post-vaccination events are rare. Moreover, these events may not be 'well defined' and may appear long after immunization took place, as was recently described and termed the Autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA) [18]. Even so, causality has been accepted by the medical community for certain vaccines and autoimmune conditions such as the outbreak of Guillain-Barré syndrome following the swine flu vaccination in 1976 [6] or transverse myelitis after oral polio-vaccination [9], thrombocytopenia and MMR [1] or narcolepsy after exposure to the H1N1 vaccine [19]. Similarly, HBVv has been allied with CNS demyelinating events, the appearance of multiple sclerosis and SLE [8,13,20–22].

The mechanisms by which the host's immune system responses to vaccination resemble the ones involved in the response to infectious agents namely, molecular mimicry, epitope spreading, polyclonal activation and others [23].Thus, a recombinant or a live attenuated infectious antigen used for vaccination, may inflict a range of immune and autoimmune responses similar to its parallel infectious agents [1]. In addition, different ingredients of a vaccine, especially adjuvants has been used for decades to enhance immunity [1,14,18,24]. Adjuvants enable usage of smaller amounts of antigen while maintaining an effective immune response. This is achieved *via* interaction of the adjuvant with Toll-like receptors, stimulation of the innate immune system, improving translocation of antigens, induction of cytokine and prevention of antigen destruction [18,25]. Lately, increased autoimmunity among recipients of vaccines was suggested to stem also from the adjuvant used in those vaccines, as adjuvants *per se* were found to induce autoimmunity [24,26,27].

Human studies aiming to test vaccines and/or adjuvant safety, especially in high risk groups are rare and yielded conflicting results. The many limitations of such studies may be partially overcome by animal models for which the follow-up period is shorter and immunization with different ingredient and doses of vaccines can be materialized [28,29]. In such studies, post-vaccination autoimmunity and adjuvants toxicity (e.g. hydrocarbon oil, squalene, or pristane) were documented in dogs, naïve mice, rats, farmed salmon fish and other models [29–31]. Evaluation of post-vaccination phenomena in animals that are genetically susceptible to autoimmunity may further enhance our knowledge regarding immunization of patients at high risk of autoimmunity [7,28,29]. Hence, in the current study we evaluated the effects of immunization with Hepatitis B vaccine or its adjuvant, alum, on NZBWF1 mice which are genetically prone to develop SLE-like disease.

2. Material and methods

2.1. Study protocol and mice

Female NZBWF1 mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained at 23 ± 1 °C, and 12 h light cycle with free access to food and drink. Sixty Mice were classified to three groups ($n = 20$), all of which were immunized twice *via* intramuscular injection, at the ages of 8 and 12 weeks. The three groups consisted of group 1: mice immunized with 0.4 ml phosphate buffered saline 1× (PBS1X), group 2: mice immunized with the 0.4 ml of HBVv (Engerix-B vaccine), as described in a former study [28], group 3: mice were immunized with aluminum hydroxide ($Al[OH]_3$) 0.2 mg in 0.4 ml of PBS, which is comparable to the quantity of aluminum in 0.4 ml of the Engerix vaccine. The Sheba Medical Center Animal Welfare Committee approved all procedures.

The following parameters of NZBWF1 lupus-like disease were followed in all mice: Mice weight was measured monthly, beginning at 16 weeks, blood samples (for antibodies titer, blood counts etc.) were analyzed at the ages of 10, 14, 18, 22 and 26 weeks; Urine protein was studied weekly between the ages of 14–25 weeks; Neuro-cognitive tests were performed at 21, 26, 30 weeks of age. Mice were sacrificed at 28–30 weeks of age, and organs were weighted during the procedure.

2.2. Blood analysis

Mice were bled by retro orbital puncture at the age of 10, 14, 18 and 22 weeks. Four samples of sera were used for antibody detection. The 5th sample was taken, at the age of 26 weeks, using anticoagulant for blood cell counts. Total Cell Blood Count (CBC) was performed using the Cell Dyn 1600 system® (Abbott Diagnostics, USA).

2.3. Autoantibodies measurement – anti double-stranded DNA (anti-dsDNA)

Autoantibodies measurement – anti double-stranded DNA (anti-dsDNA) were detected by homemade ELISA [32]. Briefly, polystyrene were coated sequentially with poly-L-lysine (50 ug/ml in water), ds-DNA-2.5 g/ml TBS (Tris base buffered saline) and poly-L-glutamate (50ug/ml). After blocking with 3% Bovine Serum Albumin (BSA), mice sera diluted 1:200 in 1% BSA were incubated for 2 h at room temperature. Bound antibodies were detected using 1:5000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma) and the addition of its substrate P-nitro-phenyl-phosphate. *Anti-phospholipids antibodies* were determined by homemade ELISA as was described previously [33]. Briefly, polystyrene were coated sequentially with phospholipid (cardiolipin, *β2 Glycoprotein I* and phosphorylcholine) (Sigma Chemical Co., St. Louis, MO) at a concentration of 50 ng/ml in ethanol. After blocking with 10% Bovine Serum (BS) in PBS1X, mice sera diluted 1:200 in 2% BS were incubated for 2 h at room temperature. Bound antibodies were detected using 1:5000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma) and the addition of its substrate P-nitro-phenyl-phosphate. *Anti Ro, La, Sm and RNP* antibodies were detected using commercial QUANTA Lite® kits for Sm, RNP, SSA and SSB according to the manufactured instructions (INOVA Diagnostics Inc, USA) with modifications. Bound antibodies were detected using 1:5000 dilution of goat anti-mouse IgG conjugated to peroxidase (Sigma) and the addition of its substrate 3,3′,5,5′-Tetramethylbenzidine (TMB). For all ELISA tests, Optic Density (O.D) was read in an ELISA reader using a 450 nm filter/620 nm reference filter.

2.4. Urine protein

Urine protein was measured weekly using Combur 10Test M strips (Roche Diagnostics GmbH, GE). Urine samples were graded, corresponding to the following approximate protein concentrations: 0 mg/dL, 30 mg/dL, 100 mg/dL and 500 mg/dL [31].

2.5. Neurocognitive and behavioral studies

Four neurocognitive studies were performed in which each mouse was tested individually.

2.5.1. The novel object recognition test (NOR)

The novel object recognition test (NOR) was done at the age of 21 weeks. The NOR test was performed using a long retention period (4 h) to evaluate deficiencies in long term memory. This visual recognition memory test is divided to three phases (habituation, training, and retention) [34]. In the habituation phase, the mice were placed in the arena (box 50 × 50 × 20 cm) for 20 min for free exploration. In the training phase, mice had 5 min to explore 2 identical objects in the arena. After this, mice were removed to the home cage for a 4 h retention interval, and finally in the retention phase, they were returned to the arena for 5 min after one object has been replaced with a novel object. Data from the retention phase were expressed as a preference index (time exploring novel object/total exploration). Exploration of the objects was defined as whisking, sniffing, rearing on or touching the object, and approach and obvious orientation to the object of ≤1 cm.

2.5.2. The Y maze test

The Y maze test was used to study the spatial memory and short term memory deficit, as the retention period in this test is 2 min. It was performed on week 21 and included two trials. In the first trial of the test, lasting for 5 min, one of the 2 arms of the Y maze was randomly chosen to be blocked whereas on the second trial, lasting for 2 min, both arms were open. The two trials were separated by a 2 min interval, during which the mouse was returned to its home cage. The time spent in each of the arms was measured in each trial. Discrimination of spatial novelty was assessed by a preference index [35]: time in the new arm - time old arm/time in the new arm + time in the old arm.

2.5.3. The staircase test

This test evaluated exploratory activity and "anxiety −like" behavior. It was performed at 21 weeks of age as previously described [36–39]. The staircase was placed in a room with constant lighting and isolated from external noise. The number of stairs climbed and the number of rears were recorded during a 3 min period. Climbing was defined as each stair on which the mouse placed all four paws; rearing was defined as each instance the mouse rose on hind legs (to sniff the air), either on a stair or leaning against the wall. The number of stairs descended was not taken into account. Before each test, the box was cleaned with a diluted alcohol solution to eliminate smells.

2.5.4. The forced swim test (FST)

This test is based on the description done by Porsolt to detect depressive-like behavior [39–41] and it was performed at the age of 26 weeks and at the age of 30 weeks of age. Each mouse was placed in a glass beaker (height 39 cm, diameter 21.7 cm) with water 15 cm deep at 25 °C. On the first day, mice were placed in the cylinder for 15 min training, and later were removed to the home cages. Twenty-four hours later, the mice were put for 6 min in the beaker under similar environmental conditions. Depression-like behavior was defined as immobility (floating) and is illustrated as the percentage time spent immobile [41]. The immobility time was measured in seconds, when there was not presence of escape-oriented behaviors such as swimming, jumping, rearing, sniffing, or diving.

2.6. Organ weight and histological studies

At the age of 28–31 weeks mice from each group were sacrificed for brain, kidney and spleen analyses. As for brain analysis, 5 mice from each group were anesthetized by intra-peritoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and sacrificed by trans-cardiac perfusion of PBS followed by perfusion with 4% p-formaldehyde in PBS. The brains were removed after perfusion, fixed in 4% p-formaldehyde, and cryoprotected by immersion in 30% sucrose for 24 h at 4 °C before cutting. Consequently, frozen coronal sections (50 μm) were cut on a sliding microtome and collected serially and, preserved in a cryoprotectant solution. In addition, the other 10 mice from each group were sacrificed by cervical dislocation; brains, spleens and kidneys were removed and weighed to calculate organ weight percentage (Organ weight × 100/Total mouse weight). Afterwards, brains and kidneys from 5 mice were fixed in p-formaldehyde and paraffin embedded; then 5 nm thick brain coronal and kidney sagittal sections slides were cut for histology. All the remaining animals were sacrificed by cervical dislocation at the age of 31 weeks.

2.6.1. Histological analysis of the kidneys

Paraffin sections kidneys were stained with hematoxylin and eosin (H&E). In addition, they were analyzed for detection of Ig deposits. In this case, direct immuno-fluorescence was performed with FITC conjugated anti-mouse $F(ab')_2$IgG (Sigma, St Louis, MO). Analysis of kidney damage was independently performed by an expert pathologist blinded to the clinical and experimental parameters.

Finally, for detection of Hepatitis B surface antigen (HBsAg), kidney paraffin sections were rehydrated with serial wash steps of xylene, ethanol 100%, ethanol 90%, ethanol 70% and ethanol 50% per 10 min each one. The sections were pretreated with VECTOR® M.O.M.™ Immunodetection Kit (Vector Laboratories, Burlingame, USA) to block all the endogenous mouse immunoglobulins within the tissue. Alter it; the sections were incubated with monoclonal antibodies (10 ug/ml) overnight at 4 °C against HBsAg (anti-HBsAgpAb, Millipore, USA). Then, sections were incubated at room temperature for 2 h with anti-mouse fluorescent 488- or 594 conjugated anti mouse secondary antibody. Counter staining was performed with Hoechst.

2.6.2. Histological analysis of the brain

For detection of inflammation markers, brain sections were stained free-floating were stained with three reagents: Hoechst was used to stain cell nuclei, anti-Iba1 for staining of activated microglia and anti-GFAP for astrocytes. For activated microglia (anti-Iba1 pAb, Abcam, UK) and astrocytes (anti-GFAP mAb, Dako, Carpinteria, USA) staining incubation with the first antibodies (10 ug/ml) was overnight at 4 °C. Then sections were incubated at room temperature for 2 h with the corresponding fluorescent chromogens-conjugated secondary antibody, according to the manufecter instructions.

Table 1
Blood cell counts in immunized mice.

Treatment	PBS	Alum	Engerix	*p*
	Mean ± SEM	Mean ± SEM	Mean ± SEM	
WBC ($x10^4$/μl)	**2.11 ± 0.171**	**1.48 ± 0.111**	1.82 ± 0.143	$p = 0.005^*$
LYM ($x10^4$/μl)	1.51 ± 0.147	1.19 ± 0.094	1.48 ± 0.109	NS
GRAN ($x10^4$/μl)	0.19 ± 0.023	0.13 ± 0.015	0.15 ± 0.027	NS
RBC ($x10^7$/μl)	**2.03 ± 0.089**	**1.64 ± 0.132**	**1.54 ± 0.083**	$p = 0.012^*$ $p = 0.002^{**}$
HCT (%)	**8.37 ± 0.383**	**6.72 ± 0.566**	**6.18 ± 0.356**	$p = 0.014^*$ $p = 0.002^{**}$
MCV (fL)	**41.2 ± 0.133**	40.9 ± 0.314	**40.1 ± 0.233**	$p = 0.003^{**}$
PLT ($x10^3$/μl)	163.1 ± 21.026	183.7 ± 10.535	196.4 ± 23.232	NS

WBC: White blood cell, LYM: Lymphocytes, GRAN: Granulocytes, RBC: Red blood cells, HCT: Hematocrit, MCV: Mean corpuscular volume, MCHC: Mean corpuscular hemoglobin concentration, PLT: platelet, NS: Not significant, SEM: Standard error of the mean. Comparisons made between treatments: PBS and alum*, and PBS and Engerix**. There were no differences between Alum and Engerix in anyone of the parameters (Parameters with significant differences are shown in bold).

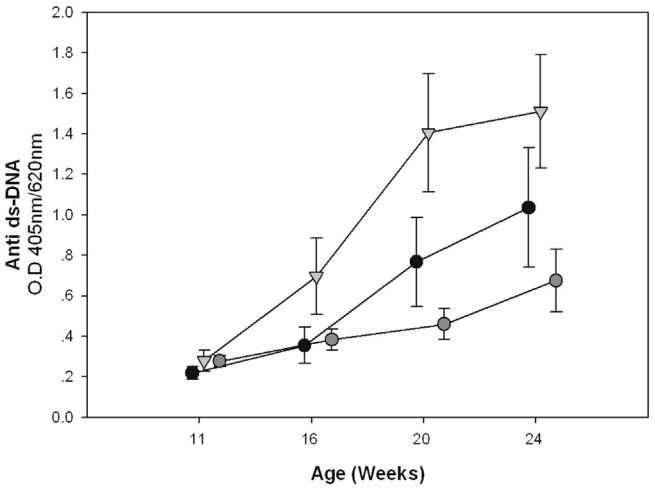

Fig. 1. Increment in ds-DNA levels were significantly higher in NZB/W F1 mice immunized with HBVv (Engerix) in comparison with the control group. After the age of 20 weeks differences in levels of ds-DNA auto-antibodies indicate a significant effect of the Engerix vaccine. Differences in the mean optical density values (OD 405 nm/600 nm) of anti-dsDNA antibody levels among groups was found ($p = 2.32 \times 10^{-4}$), pairwise comparisons showed significant differences between Engerix and PBS ($p = 0.004$) and Engerix and alum ($p < 0.001$). PBS (●), alum (●) and Engerix (▼).

In addition stating for alum deposition in the brain was performed. For detection of alum the brain paraffin sections were rehydrated with serial wash steps of xylene, ethanol 100%, ethanol 90%, ethanol 70% and ethanol 50% per 10 min each one. Then they were washed in a solution of chloridric acid 1% for 10 min. Sections were washed with PBS and they were placed in the Morin solution for staining during 10 min (0.2% Morin reagent in 85% ethanol containing 0.5% glacial acetic acid).

2.7. Statistical analysis

Differences among groups were tested using analysis of variance or covariance (ANOVA or ANCOVA) according to the data. Those analyses that were statistically significant were followed by LDS test to identify specific differences between groups. The analysis was performed using SPSS 17.0 software. Results are shown as averages ± standard error, and in percentages. A level of 5% was used to define statistical significance ($p < 0.05$), p reported values correspond to LDS test for specific group differences.

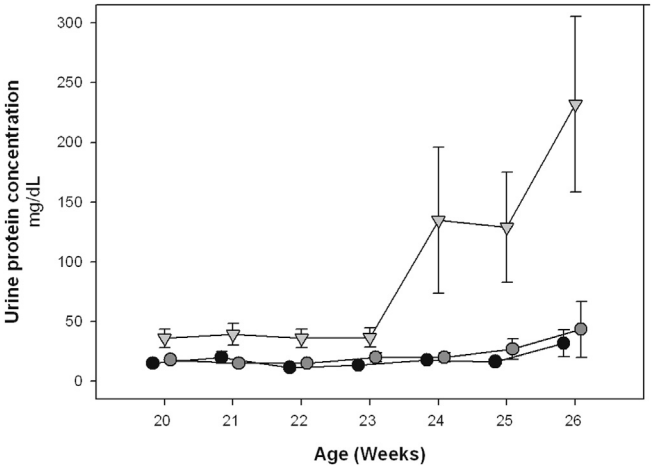

Fig. 2. Increment in proteinuria among NZB/W F1 mice immunized with Engerix in comparison with the PBS group. Protein urine levels showed differences among groups from the age of 24 weeks ($p < 0.001$), particularly an earlier appearance of proteinuria was noted from week 24 in the group of animals immunized with Engerix in comparison with the other groups. (Engerix and PBS $p < 0.004$ and Engerix and alum $p < 0.001$). PBS (●), alum (●) and Engerix (▼).

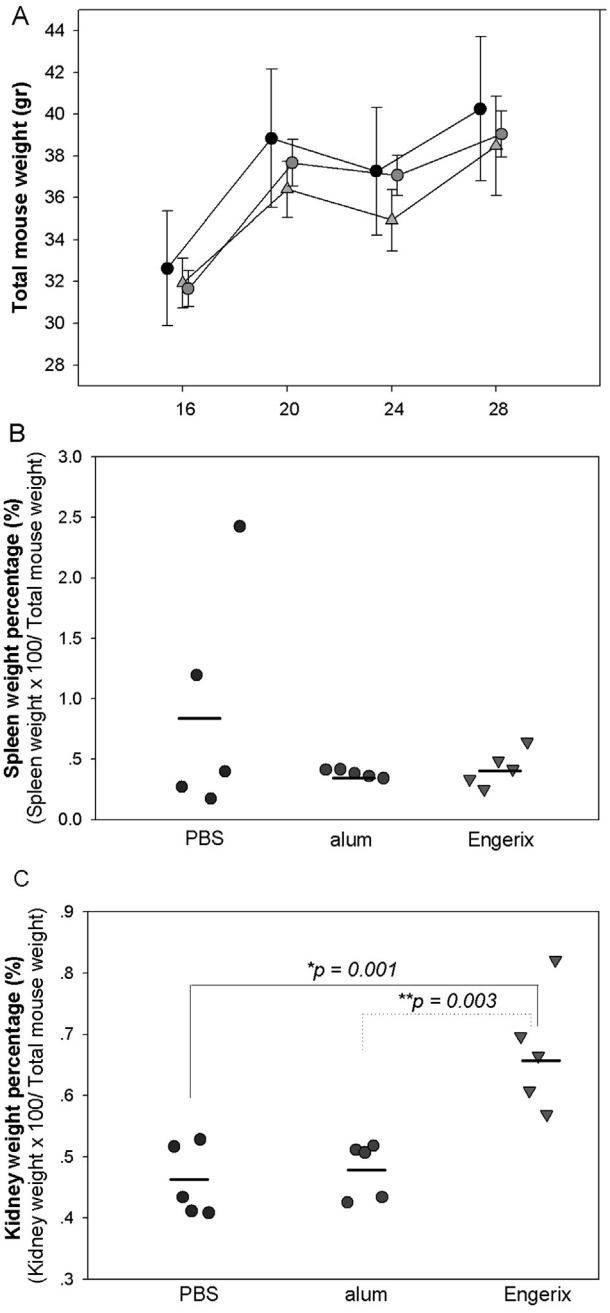

Fig. 3. NZB/W F1 mice total and organ weight analyses. (A) The total weight of all animals did not differ between groups along the experiment: Week 16 $p = 0.888$, week 20 $p = 0.231$, week 24 $p = 0.305$, week 28 $p = 0.375$. (B–C) Weight percentage of kidneys and spleen was calculated (Organ weight × 100/Total mouse weight). (B) Spleen weight percentage showed no differences among groups ($p = 0.262$). However kidney weight percentage (C) was significantly higher among mice immunized with Engerix compared with the other two groups (*Engerix vs PBS* $p = 0.001^{*}$ *and Engerix vs alum* $p = 0{,}003^{**}$). Weight is presented as mean ± SEM. PBS (●), alum (●) and Engerix (▼).

3. Results

In the current study major manifestations of SLE-like disease were followed in mice immunized with HBVv (Engerix), aluminum hydroxide (alum) and PBS.

3.1. Blood counts

Total blood cells counts differ between mice groups as specified in Table 1. In particular, total white blood cells (WBC) count was significantly lower in the group immunized with alum in comparison with mice immunized with PBS ($1.48 \pm 0.1 \times 10^4/\mu l$ *vs.* $2.11 \pm 0.17 \times 10^4/\mu l$; $p = 0.005$). Additionally, red blood cells (RBC), hemoglobin levels (HGB) and Hematocrit (HTC) percentage were significantly lower in alum and Engerix immunized animals in comparison with the control group (PBS) as specified in Table 1. Platelets counts did not differ between groups.

3.2. Autoantibodies

The presence of anti-Ro, anti-La, anti-Sm, anti-RNP, anti –cardiolipin (ACA), anti-phosphorilcholine (PC) and anti- β2 Glycoprotein-I auto-antibodies did not differ between groups. In contrast periodic analysis of serum samples for anti-dsDNA antibody levels showed faster increment in mice immunized with Engerix compare to those immunized with alum of PBS. These differences reached statistical significance at week 20 and remained so throughout the study (Fig. 1). These differences were

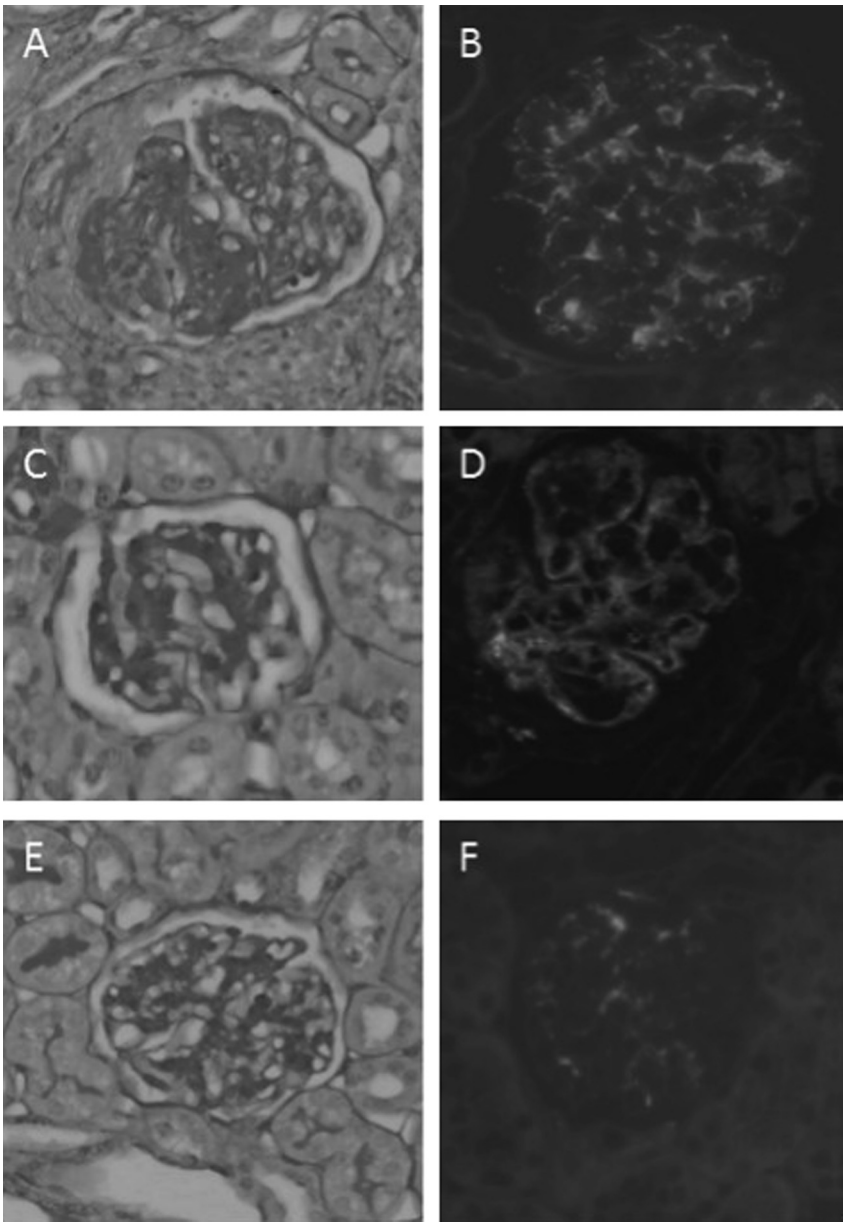

Fig. 4. Kidney histology of NZB/W mice sacrificed at the age of 26 weeks. Sections of representative slides images from each group (40× magnification) are shown: Engerix (top panel, A–B), alum (middle panel, C–D) and PBS (lower panel, E–F). First column (Fig. A, C, E) is hematoxylin and eosin (H&E) staining and second column (Fig. B, D, F) show detection of Ig deposits using FITC-conjugated anti-mouse F $(ab')_2$ IgG. Fig. A shows crescent proliferative glomerulonephritis with a significant staining of Ig deposits (B) following immunization with Engerix, alum and PBS immunization resulted in proliferative mesangial glomerulonephritis (C) and mild mesangial glomerulonephritis (E) respectively with less Ig deposits (D or F).

apparent by comparing mean optical density values (OD 405 nm/600 nm) of anti-dsDNA antibody levels among groups ($p < 0.001$) as well as using the pair wise comparisons for Engerix *vs.* PBS (1.41 ± 0.29 *vs.* 0.77 ± 0.22 respectively; $p = 0.004$) and Engerix *vs.* alum (1.41 ± 0.29 *vs.* 0.41 ± 0.08 respectively; $p < 0.001$).

3.3. Urine analysis

Similarly to anti-dsDNA antibodies protein urine levels showed differences between groups from the age of 23 weeks (Fig. 2). Particularly a rapidest appearance of proteinuria from week 24 was observed in the group of animals immunized with Engerix (average urine protein of 135 ± 61 mg/dL) in comparison with the other groups PBS and alum (135 ± 61 *vs.* 18 ± 2 mg/dL; $p < 0.004$ and 135 ± 61 *vs.* 20 ± 3.65 mg/dL; $p < 0.001$ respectively).

3.4. Mice and organs weights

Total body weight of animals did not differ between groups over the experiment period (Fig. 3A). Analyses of organs weight was performed using five animals from each group which were sacrificed by cervical dislocation. In these animals no differences in spleen weight percentage among the different groups was noticed: PBS 0.89 ± 0.42%, alum 0.38 ± 0.02% and Engerix 0.42 ± 0.07%; $p = 0.262$ (Fig. 3B). In contrast, kidney weight was significantly higher in the Engerix immunized group compared with the other two: Engerix *vs.* PBS 0.67 ± 0.04% *vs.* 0.46 ± 0.03%; $p = 0.001$ and Engerix *vs.* alum 0.67 ± 0.04% *vs.* 0.48 ± 0.02%; $p = 0.003$ (Fig. 3C).

3.5. Kidney histology

Analysis of kidneys biopsies showed SLE-like kidney damage in all animals. However, among mice immunized with Engerix severe and advanced nephropathy was documented in comparison with the other groups (Fig. 4). Hence, within the same period of time animals immunized with HBVv (Engerix) developed crescent proliferative glomerulonephritis (Fig. 4A) while the other groups showed mesangial disease with less Ig deposits. Additionally, using specific staining for hepatitis B surface antigen, the recombinant viral antigen used in the Engerix vaccine [42], we detected this particular antigen in the kidneys of animals immunized with the vaccine (Fig. 5).

3.6. Neuro-cognitive tests

3.6.1. Memory deficit

Memory deficit was assessed using two tests the novel object recognition (NOR) test evaluating long term memory and the Y maze test that reflects short term memory. In these tests results are presented as the preference index (time spent) with the new object or arm respectively (Fig. 6). In the current study control mice, immunized with PBS, had a significantly higher preference index for the new object in the NOR test compare to the other two groups: PBS *vs.* Engerix 0.43 ± 0.06 *vs.* −0.002 ± 0.06 ($p = 0.003$) and PBS *vs.* alum 0.43 ± 0.06 *vs.*0.03 ± 0.06 ($p = 0.006$). Similar results were observed in the Y maze test in which control mice (PBS) significantly preferred the new arm in the Y maze in comparison with the other two groups: PBS *vs.* Engerix, 0.64 ± 0.07 *vs.* 0.11 ± 0,09 ($p = 9.71 \times 10^{-5}$) and PBS *vs.* alum, 0.64 ± 0.07 *vs.* 0.34 ± 0.08 ($p = 0.026$). PBS immunized mice recognized the old object and the old arm as known and preferred exploring the new options while the other mice expressed less interest in the new object/lane respectively. Notably mice immunized with alum or Engerix actually spent more time with the old object/lane suggesting in addition to memory deficit a plausible behavioral change.

3.6.2. Anxiety-like behavior

Anxiety-like behavior was evaluated using the stair case tests analyzing both rearing and climbing indexes (Fig. 7). An increment in the number of rearing events indicates anxiety (which was documented in this study in mice immunized with alum in comparison with PBS (11.8 ± 1.23 *vs.* 7.8 ± 0.87; $p = 0.032$)). Increment in climbing stairs is indicative of hyperactivity, and although numerically alum immunized mice were more active than those

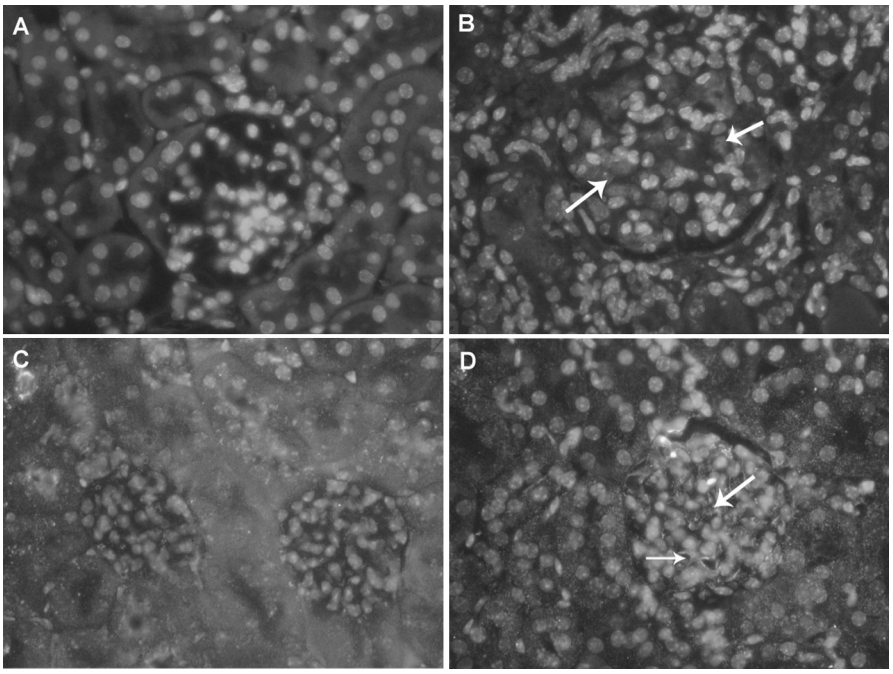

Fig. 5. Hepatitis B surface antigen detection in kidneys of NZB/W F1 immunized with PBS and Engerix. Representative slides of Hepatitis B surface antigen (HBs-Ag) staining (white arrows) using monoclonal mouse antibody detected with either 488-conjugated anti-mouse antibody (A–B) or 594-conjugated anti-mouse antibody (C–D). Hbs-Ag was detected in glomeruli of Engerix immunized mice (B–D) and was not detected in PBS injected mice (A–C).

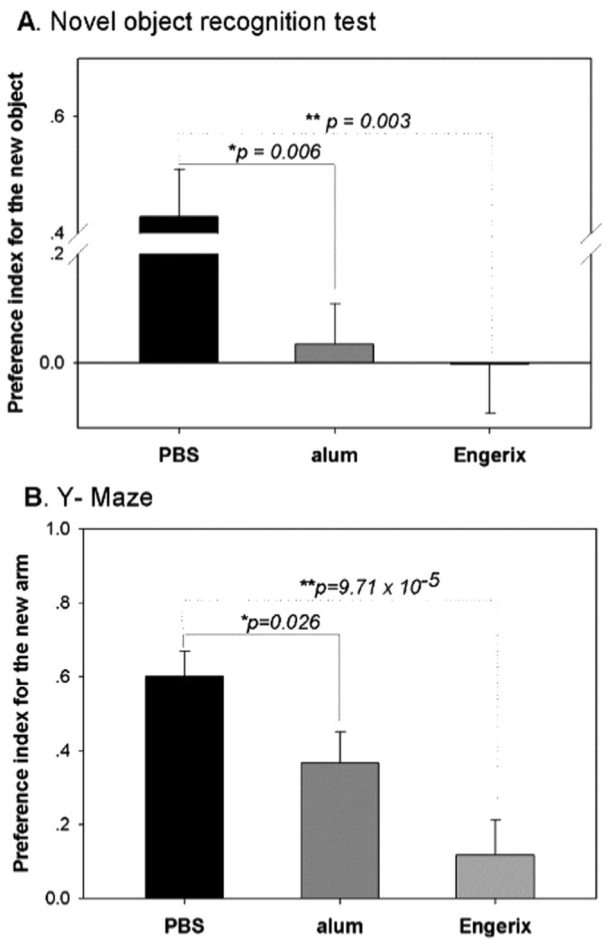

Fig. 6. Memory deficit in NZB/W mice immunized with Engerix or alum. Memory was assessed by two tests the Novel object recognition test (A) and Y maze test (B). Engerix and alum treated mice displayed impaired performance in both tests. In the novel object recognition test (A), results are presented as the preference time with the new objects. Notably control mice (PBS) significantly preferred the new object in comparison with the other two groups (*PBS vs Engerix* $p = 0.003$ *and PBS vs alum* $p = 0.006$). Similarly in the Y maze test control mice (PBS) significantly preferred the new arm in the Y maze in comparison with the other two groups (*PBS vs Engerix* $p = 9.71 \times 10^{-5}$ *and PBS vs alum* $p = 0.026$).

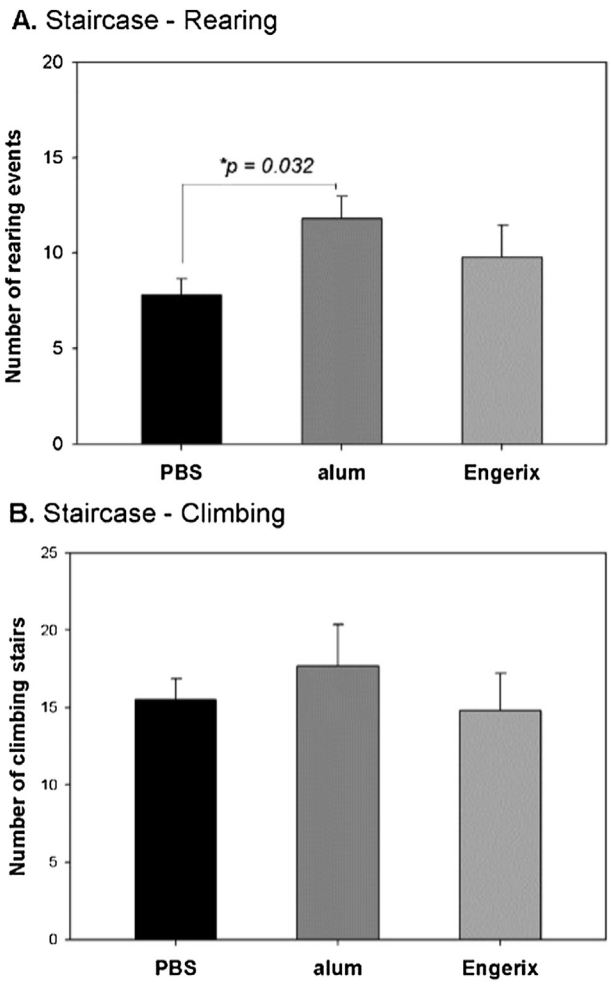

Fig. 7. NZB/W F1 mice treated with alum had anxious-like behavior. Differences between groups were evaluated using the stair case tests analyzing both rearing and climbing indexes analysis. An increment in the number of rearing events was observed in both immunized group in particularly in mice immunized with Alum (*PBS vs Alum* $p = 0.032$). Increment in climbing stairs (B) was also observed in mice immunized with alum although this difference did not reach statistical significance ($p = 0.631$).

immunized with PBS this did not reach statistical significance (17.7 ± 2.79 *vs.* 15.5 ± 1.34; $p = 0.631$).

3.6.3. *Depressive like-behavior*

Depressive like-behavior in immunized mice was assessed by the forced swimming test (FST). Immobility time in the FST indicates depressive behavior in mice (Fig. 8). At the age of 26 weeks no differences were observed between groups (PBS 133.±31.6 sec, alum 90 ± 30.6 sec and Engerix 140 ± 30.3; $p = 0.461$). Repeating this study at the age of 30 week revealed a lower immobility time representing less "depressive-like behavior" among alum immunized mice compare to mice immunized with Engerix, 183.6 ± 31.5 sec *vs.* 275.9 ± 20.3 sec ($p = 0.003$).

3.7. *Brain histology*

A variety of sections in mice brains were analyzed (e.g. cortex, amygdala, hypothalamus and hippocampus). In all regions and particularly those associated with the limbic system, memory and learning skills (i.e. hippocampus) a significant increase in staining for Iba-1 was found, indicating the presence of more activated microglia and inflammation in mice immunized with alum and Engerix (Fig. 9). In addition, the staining for GFAP, indicating the presence of astrocytes, reveled increment in the number of astrocytes particularly in the hippocampus of animals treated with alum and Engerix in comparison with those treated with PBS (Fig. 9). Moreover, detection of alum in brain ventricle of animal immunized with alum (data not shown) was noted and may suggest migration of this substance and deposition in mice brain.

4. Discussion

In the current study we report an acceleration of SLE-like disease, in NZBWF1 mice genetically prone to develop this disease, following immunization with the HBV vaccine (Engerix). Interestingly, we observed a differential effect of immunization with the whole vaccine (including the HBV surface antigen, alum and other ingredients) compare to immunization only with the vaccine adjuvant, alum. While the former aggravated kidney disease the latter was associated with hematological and neurological manifestations.

Kidney disease is a criterion of SLE, that is strongly linked with the presence of anti-dsDNA antibodies [43]. Lupus nephritis is characterized by mild to severe proteinuria as well as evidence of inflammation and deposition of immunoglobulin in the kidney.

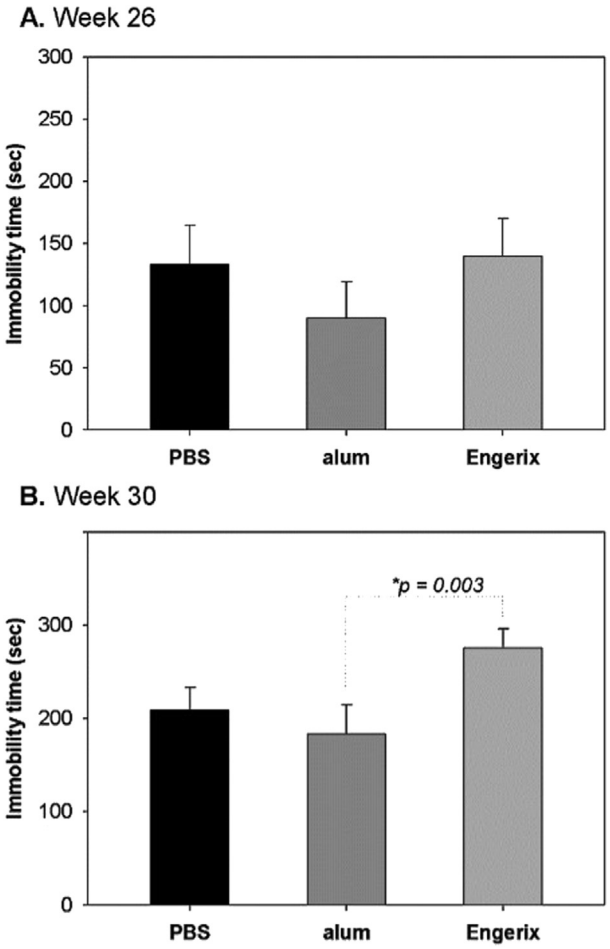

Fig. 8. NZB/W F1 mice immunized with alum showed less immobility time. Immobility time during the forced swimming test did not differ between groups at 26 weeks (A) although mice immunized with alum were less immobile ($p = 0.461$). At the age of 30 weeks (B) mice immunized with alum were significantly less immobilized compare to those immunized with Engerix ($p = 0.003$).

Herein we report early increment of anti-dsDNA antibodies levels and urine protein excretion among NZBWF1 mice, immunized with the whole HBV vaccine. In addition, we found, in this group of animals, advanced kidney damage with severe inflammation, the presence of crescents and higher deposition of immunoglobulin (Fig. 4). This aggressive disease was not apparent in mice immunized only with the vaccine adjuvant, aluminum hydroxide, or in control mice immunized with PBS at this early stage of NZBWF1-SLE disease. Furthermore, staining for anti-HBV surface antigen, demonstrated reactivity only in kidneys from animal exposed to the whole vaccine (Fig. 5). This supports the idea that the recombinant infectious antigen present in the vaccine is responsible for the fast progression of kidney damage either by being directly deposited in the kidney or by molecular mimicry and acceleration of anti-glomerular autoimmune process. This concept stands in agreement with a recent work performed by Wang et al. [42] who observed hepatitis B viral antigens (i.e. surface and core antigens) and HBV-immune complexes deposition in kidney biopsies from SLE patients with lupus nephritis. For this purpose Wang and colleagues studied renal biopsies from 166 patients with lupus nephritis and 384 controls for the presence of HBV antigen deposition. The latter were detected in 50% of tissue from SLE patients, about 20% of biopsies from patients with primary renal glomerular disease or allergic purpura and none in biopsies from patients with other diseases (i.e. diabetic nephropathy, hypertensive nephrosclerosis, thin basement membrane nephropathy, or Alport's syndrome). The recombinant antigen used for production of the Engerix vaccine is similar to the surface antigen of HBV detected in this study. In another study [44] lupus nephritis was triggered in a 27 year-old woman following inoculation with HBV vaccine. This event was linked with HLA haplotype related to this autoimmune disease, suggesting that post-vaccination immune mediated phenomenon may occur in genetically predisposed subjects. In this context, lately, the influence of pharmacogenomics biomarkers on the efficacy and toxicity of vaccines has been reported. For instance, a high rate of variability in immune response and toxicity towards hepatitis B vaccine was observed in relation to various HLA alleles as well as polymorphism of IL-10 and IL1β genes [45] .

Another line of evidence relating HBV antigens and kidney disease is the data accumulated in case-reports and case-series that documented the occurrence of nephritis following exposure to viral infection with HBV or exposure to the HBV vaccine. Moreover, response of this nephritis to anti-viral therapy was reported [46–48]. Therefore, hepatitis-B-associated nephritis was defined as a distinct entity that occurs mainly in hepatitis-B-prevalent areas of the world. This kidney disease affects both adults and children independently of liver involvement and is characterized by renal deposition of immune complexes, immunoglobulins, and complement components (C3, C4 and C1q). Moreover, hepatitis B viral antigens (HBsAg, HBcAg, HBeAg) were eluted from affected kidneys using acid elution techniques [49]. Similarities between Hepatitis B virus-related nephropathy and lupus nephritis have been reported as both present with proteinuria and glomerular lesions that may be at times indistinguishable [50]. However no autoantibodies or other SLE-related manifestations are expected in patients with HBV related nephropathy. Last but not least in the current study we have observed, for the first time to the best of our knowledge, an increment in the kidney size of mice injected with the HBV vaccine. Enlargement of diseased kidney was reported in acute glomerulonephritis, some cases of vasculitis (i.e. Goodpasture), infiltrative diseases (i.e. amyloidosis, sarcoidosis) infections (i.e. HIV related nephropathy) and other conditions (i.e. polycystic disease) [51]. Thus, one may suggest that in the current study the early damage or the type of immune mediated process induced by the vaccine is related to the kidney enlargement. Taking it all together, it seems that either by acceleration of the natural course of SLE-like disease (e.g. the presence of anti-dsDNA antibodies), or by a combination of SLE-like disease and HBV- related kidney damage, mice immunized with the Engerix suffered from early and more severe renal disease.

Cytopenia are common manifestations and a criterion of SLE disease [43,52]. In the current study we found immunization with alum only or HBV vaccine containing alum to correlate with the presence of anemia (e.g. low hemoglobin, RBC, and low MCV) and to a lesser extent with leucopenia. This observation is supported by data from humans and other animal models, as aluminum is known to be toxic (i.e. neurotoxicity) and cause anemia. In humans, increased aluminum levels in patients with chronic renal failure on hemodialysis were found to be associated with impaired erythropoiesis and iron metabolism. Comparably in several studies that evaluated the effects of aluminum on hematopoiesis in rats, anemia with microcytic feature was observed. For instance, exposure of young, male Wistar albino rats to aluminum sulfate resulted in significant decrease in mean corpuscular volume (MCV), red blood cell (RBC) deformability and hematocrit (Hct) [53]. In another study chronic exposure (weeks-months) to alum correlated with decreased hemoglobin concentration, hematocrit and serum iron in rats [54]. Alike, short term exposure to alum (days) also induced decreased hemoglobin, hematocrit, and increased reticulocytes counts [55]. In the latter study a mild degree of hypochromia and

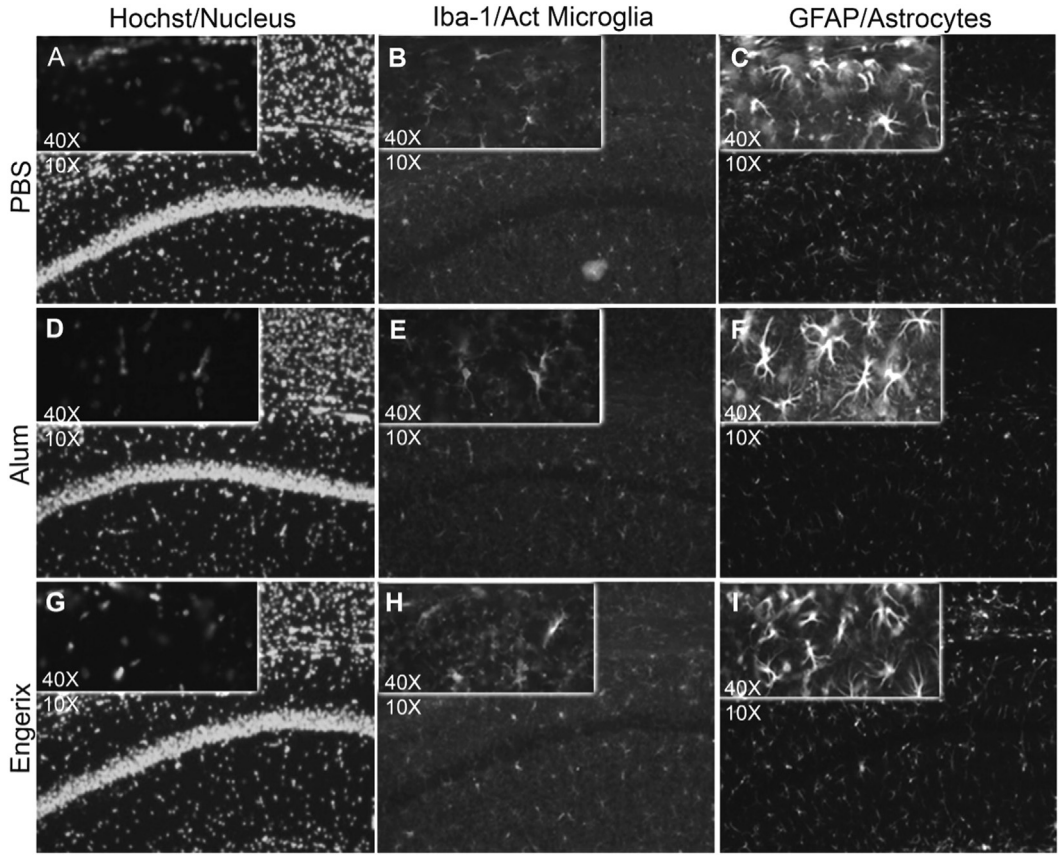

Fig. 9. Evidence of increased brain inflammation in NZB/W F1 mice immunized with alum and Engerix. Representative slides from the CA1 hippocampal region. Staining of activated microglia (Green, Iba-1) (B, E, H) and astrocytes (Red, GFAP) (C, F, I) were more prominent in the brain of alum (D, E, F) and Engerix treated mice (G, H, I) compared to PBS mice brains (A, B, C). Counting nuclei staining was performed using Hoechst (A, D, G). Magnification of 10× and 40×.

microcytosis as well as decrease in the index of erythroblasts maturation, and reduced plasma iron were documented. Indicating inhibited erythroblast maturation, erythropoiesis and iron metabolism in rats exposed to aluminum [55].

Neuropsychiatric manifestations are common in SLE (NPSLE) and have been reported in more than 50% of patients [56]. Of which 19 syndromes were defined as related to SLE disease by the American College of Rheumatology (ACR) [56]. Usually, neuropsychiatric events attributed to the disease itself are the primary manifestations of SLE rather than its complication [57]. NPSLE involvement is consider to be a consequence of the autoimmune process either *via* autoantibodies mediated processes (e.g. micro-vasculopathy and thrombosis due to anti-phospholipids antibodies, or psychosis due to anti-P-ribosomal antibodies) or *via* inflammation (e.g. SLE related seizures) [58–60]. SLE brain damage and behavioral changes were reported also in murine models of this autoimmune disease [61–64]. Remarkably among adult NZBW F1 mice (10–18 months old) neurological deficits and brain histological changes were reported regardless of other SLE-like manifestations (i.e. proteinuria and anti-dsDNA levels) [65]. In this strain of mice several mechanisms have been suggested to explain neurological damage. For instance anti-neuronal antibodies could be eluted from mice brains [66], and neuropeptides deficiency was reported in certain area of the brain of affected animal (e.g. hypothalamus) [67]. In addition, lymphocyte infiltration (i.e. CD3+), IgG and complement deposition, gliosis and neuronal loss were observed in hippocampal biopsies and linked with behavioral changes and memory deficit [68]. Furthermore histological evaluation confirmed the involvement of the 'middle section' of the brain in NZBWF1-NPSLE-like disease. This includes the neocortex, corpus callosum, hippocampus, dentate gyrus, ventricles, choroid plexuses, thalamus, and hypothalamus. These areas are unique for having more mononuclear and glial cell accumulation around blood vessels, suggesting that an inflammatory process of the limbic area/middle brain is perhaps the result of a higher number of blood vessels and a less efficient blood brain barrier [65].

In the current study, we observed neurological deficits of NZBWF1 mice, at a relatively younger age of 5–6 months, following immunization with either HBVv (Engerix) or alum compare to those immunized with PBS. This was exhibited by short and long memory shortages that were so pronounced that accompanying behavioral changes were suspected. Markedly, immunized animals did not only prefer the "old" object/lane but tended to ignore the new ones. In addition mice immunized with alum were found to be more anxious or "less depressed". These effects were not observed in mice immunized with the HBVv. This difference between groups and particularly the forced swimming test performed at week 30 may reflect the more advanced disease (i.e. kidney damage) of the HBVv inoculated group. The neurological deficits documented herein were also reflected on brain biopsies of immunized mice. Both HBVv and alum immunization induced inflammation and gliosis in many areas of the brain but particularly in the limbic area (part of the 'middle brain'). Such behavioral deficits and histological changes have been reported in patients diagnosed with NPSLE, supporting an aggravation of NZBWF1-NPSLE-like disease in immunized mice [57,69–71].

HBV vaccine was related to neurological damage in humans, such as development of demyelinating events [72], multiple

sclerosis and irritability [8,73,74]. Furthermore, evidence of cross reaction between antibodies against hepatitis B virus surface antigen and the myelin oligodendrocyte glycoprotein suggest molecular mimicry as the cause of HBVv related neuronal damage [75–77]. Intriguingly, in the current study we found quite similar effects following immunization only with the adjuvant of HBVv, alum. The toxic effects of alum have been well described and may interact with its efficacy as adjuvant [78,79]. In terms of adjuvancy aluminum bind lipids within the plasma membrane of dendritic cells, induce endocytosis [80], followed by activation of the inflammosome system *via* NALP3, and resulting in the production of pro-inflammatory signals and antibodies [81,82]. In parallel alum disturbs metal balance within cells, leading to failure of mitochondrial activity and generation of reactive oxygen species [83,84]. This is linked with morphologic and metabolic changes in cells, such us increased astrocytes in the brain [84]. Injection of alum to rats' brain (i.e. intra-cerebro-ventricular injection) induced severe degeneration of cholinergic neuronal terminals in the brain, especially in the cortex and hippocampus [85]. Perinatal exposure to alum affected brain development, and alter the dopaminergic system in mice [86]. In the current study we found alum to be linked with memory deficits and anxiety-like behavior. This stands in agreements with other studies that found joined exposure to alum and the development of Alzheimer disease and behavioral disorders in human and animals [79]. Such as, chronic feeding of alum that led to the development of Alzheimer-like disease, severely decreased learning and memory skills and increased anxiety in murine models [26,87,88]. Finally, we have injected both HBVv and Alum intramuscularly and not directly to the brain. Recent findings suggest that transportation of aluminum particles into mice brain is mediated by transmigration of monocytes through the blood brain barrier. This process was found to be monocyte chemotactic protein-1 protein (MCP-1/CCL2) dependent [89]. To conclude, we observed neurocognitive deficits and brain inflammation following HBVv or alum immunization. These effects may have been induced by the alum, present also in the HBVv, although some role of other vaccine ingredients can not be excluded.

There are some limitations to our study, such as the relatively high dose of HBVv (and in parallel alum) used compare to animal weight. This was based on former study evaluating HBVv in a genetically susceptible model [28] as well as other safety studies reported. Noticeably, the concordance of our results with numerous other studies as well as studies utilizing lower doses of HBVv/alum supports a link between exposure to these agents and the above mentioned effects. In addition as we have sacrificed most animal at 28–31 weeks of age, the effect of immunization on survival could not be asserted.

5. Conclusion

In this study we followed the effects of immunization with the HBV vaccine or its adjuvant, alum, on SLE-like disease in an autoimmune genetically prone mouse model. This model may be regarded as one representing the autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA) [18]. In other words we were able to demonstrate a differential and deleterious effects of the whole vaccine, probably due to its recombinant infectious ingredient on SLE-like kidney disease while immunization with either HBVv containing alum, or alum on its own induced cytopenia and NPSLE-like disease. These observations support the notion that each component of a vaccine, and particularly its adjuvant, may play a role in triggering autoimmunity, at least in susceptible animals. Finally, we should note that this paper is part of a special issue in the Journal of Autoimmunity that recognizes the distinguished careers of Michael Sela and Ruth Arnon, both of whom have contributed enormously in basic science, in teaching, and in translational research. It is part of the journal's efforts to recognize outstanding autoimmunologists, including in the past, Ian Mackay, Chella David, Noel Rose, Pierre Youinou and Harry Moutsopoulos, amongst others [90–93].

Declaration of competing interests

Y. Shoenfeld has acted as a consultant for the no-fault U.S. National Vaccine Injury Compensation Program. The other authors declare no competing interests.

References

[1] Agmon-Levin N, Paz Z, Israeli E, Shoenfeld Y. Vaccines and autoimmunity. Nature reviews Rheumatology 2009;5:648–52.
[2] Scalera NM, Mossad SB. The first pandemic of the 21st century: a review of the 2009 pandemic variant influenza A (H1N1) virus. Postgrad Med 2009;121:43–7.
[3] Keating GM, Noble S. Recombinant hepatitis B vaccine (Engerix-B): a review of its immunogenicity and protective efficacy against hepatitis B. Drugs 2003;63:1021–51.
[4] Bijl M, Agmon-Levin N, Dayer JM, Israeli E, Gatto M, Shoenfeld Y. Vaccination of patients with auto-immune inflammatory rheumatic diseases requires careful benefit-risk assessment. Autoimmunity reviews 2012;11:572–6.
[5] van Assen S, Agmon-Levin N, Elkayam O, Cervera R, Doran MF, Dougados M, et al. EULAR recommendations for vaccination in adult patients with autoimmune inflammatory rheumatic diseases. Ann Rheum Dis 2011;70:414–22.
[6] Lasky T, Terracciano GJ, Magder L, Koski CL, Ballesteros M, Nash D, et al. The Guillain-Barre syndrome and the 1992-1993 and 1993-1994 influenza vaccines. N Engl J Med 1998;339:1797–802.
[7] Classen JB, Classen DC. Clustering of cases of insulin dependent diabetes (IDDM) occurring three years after hemophilus influenza B (HiB) immunization support causal relationship between immunization and IDDM. Autoimmunity 2002;35:247–53.
[8] Mikaeloff Y, Caridade G, Suissa S, Tardieu M. Hepatitis B vaccine and the risk of CNS inflammatory demyelination in childhood. Neurology 2009;72:873–80.
[9] Agmon-Levin N, Kivity S, Szyper-Kravitz M, Shoenfeld Y. Transverse myelitis and vaccines: a multi-analysis. Lupus 2009;18:1198–204.
[10] Pellegrino P, Carnovale C, Pozzi M, Antoniazzi S, Perrone V, Salvati D, et al. On the relationship between human papilloma virus vaccine and autoimmune diseases. Autoimmunity reviews 2014;13:736–41.
[11] Lind A, Ramelius A, Olsson T, Arnheim-Dahlstrom L, Lamb F, Khademi M, et al. A/H1N1 antibodies and TRIB2 autoantibodies in narcolepsy patients diagnosed in conjunction with the Pandemrix vaccination campaign in Sweden 2009-2010. Journal of autoimmunity 2014;50:99–106.
[12] Older SA, Battafarano DF, Enzenauer RJ, Krieg AM. Can immunization precipitate connective tissue disease? Report of five cases of systemic lupus erythematosus and review of the literature. Seminars in arthritis and rheumatism 1999;29:131–9.
[13] Agmon-Levin N, Zafrir Y, Paz Z, Shilton T, Zandman-Goddard G, Shoenfeld Y. Ten cases of systemic lupus erythematosus related to hepatitis B vaccine. Lupus 2009;18:1192–7.
[14] Murdaca G, Orsi A, Spano F, Puppo F, Durando P, Icardi G, et al. Influenza and pneumococcal vaccinations of patients with systemic lupus erythematosus: current views upon safety and immunogenicity. Autoimmunity reviews 2014;13:75–84.
[15] Mok CC, Ho LY, Fong LS, To CH. Immunogenicity and safety of a quadrivalent human papillomavirus vaccine in patients with systemic lupus erythematosus: a case-control study. Ann Rheum Dis 2013;72:659–64.
[16] Soldevilla HF, Briones SF, Navarra SV. Systemic lupus erythematosus following HPV immunization or infection? Lupus 2012;21:158–61.
[17] Gatto M, Agmon-Levin N, Soriano A, Manna R, Maoz-Segal R, Kivity S, et al. Human papillomavirus vaccine and systemic lupus erythematosus. Clin Rheumatol 2013;32:1301–7.
[18] Shoenfeld Y, Agmon-Levin N. 'ASIA' - autoimmune/inflammatory syndrome induced by adjuvants. Journal of autoimmunity 2011;36:4–8.
[19] Nohynek H, Jokinen J, Partinen M, Vaarala O, Kirjavainen T, Sundman J, et al. AS03 adjuvanted AH1N1 vaccine associated with an abrupt increase in the incidence of childhood narcolepsy in Finland. PloS one 2012;7. 1e33536–9 e33536.
[20] Stubgen JP. Immune-mediated myelitis following hepatitis B vaccination. Autoimmunity reviews 2012;12:144–9.
[21] Zafrir Y, Agmon-Levin N, Paz Z, Shilton T, Shoenfeld Y. Autoimmunity following hepatitis B vaccine as part of the spectrum of 'Autoimmune (Autoinflammatory) Syndrome induced by Adjuvants' (ASIA): analysis of 93 cases. Lupus 2012;21:146–52.
[22] Bruzzese V, Zullo A, Hassan C. Connective tissue disease following hepatitis B vaccination. J Clin Rheumatol 2013;19:280–1.

[23] Kivity S, Agmon-Levin N, Blank M, Shoenfeld Y. Infections and autoimmunity–friends or foes? Trends in immunology 2009;30:409–14.
[24] Tomljenovic L, Shaw CA. Mechanisms of aluminum adjuvant toxicity and autoimmunity in pediatric populations. Lupus 2012;21:223–30.
[25] Israeli E, Agmon-Levin N, Blank M, Shoenfeld Y. Adjuvants and autoimmunity. Lupus 2009;18:1217–25.
[26] Shaw CA, Petrik MS. Aluminum hydroxide injections lead to motor deficits and motor neuron degeneration. Journal of inorganic biochemistry 2009;103:1555–62.
[27] Perricone C, Colafrancesco S, Mazor RD, Soriano A, Agmon-Levin N, Shoenfeld Y. Autoimmune/inflammatory syndrome induced by adjuvants (ASIA) 2013: Unveiling the pathogenic, clinical and diagnostic aspects. Journal of autoimmunity 2013;47:1–16.
[28] Sundberg JP, Silva KA, Zhang W, Sundberg BA, Edwards K, King LE, et al. Recombinant human hepatitis B vaccine initiating alopecia areata: testing the hypothesis using the C3H/HeJ mouse model. Vet Dermatol 2009;20:99–104.
[29] Cruz-Tapias P, Agmon-Levin N, Israeli E, Anaya JM, Shoenfeld Y. Autoimmune (auto-inflammatory) syndrome induced by adjuvants (ASIA)–animal models as a proof of concept. Curr Med Chem 2013;20:4030–6.
[30] Leiss H, Niederreiter B, Bandur T, Schwarzecker B, Bluml S, Steiner G, et al. Pristane-induced lupus as a model of human lupus arthritis: evolvement of autoantibodies, internal organ and joint inflammation. Lupus 2013;22:778–92.
[31] Xu Y, Zeumer L, Reeves WH, Morel L. Induced murine models of systemic lupus erythematosus. Methods Mol Biol 2014;1134:103–30.
[32] Shoenfeld Y, Rauova L, Gilburd B, Kvapil F, Goldberg I, Kopolovic J, et al. Efficacy of IVIG affinity-purified anti-double-stranded DNA anti-idiotypic antibodies in the treatment of an experimental murine model of systemic lupus erythematosus. International immunology 2002;14:1303–11.
[33] Bakimer R, Fishman P, Blank M, Sredni B, Djaldetti M, Shoenfeld Y. Induction of primary antiphospholipid syndrome in mice by immunization with a human monoclonal anticardiolipin antibody (H-3). J Clin Invest 1992;89:1558–63.
[34] Tordera RM, Totterdell S, Wojcik SM, Brose N, Elizalde N, Lasheras B, et al. Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). Eur J Neurosci 2007;25:281–90.
[35] Dix SL, Aggleton JP. Extending the spontaneous preference test of recognition: evidence of object-location and object-context recognition. Behav Brain Res 1999;99:191–200.
[36] Simiand J, Keane PE, Morre M. The staircase test in mice: a simple and efficient procedure for primary screening of anxiolytic agents. Psychopharmacology (Berl) 1984;84:48–53.
[37] Shrot S, Katzav A, Korczyn AD, Litvinju Y, Hershenson R, Pick CG, et al. Behavioral and cognitive deficits occur only after prolonged exposure of mice to antiphospholipid antibodies. Lupus 2002;11:736–43.
[38] Katzav A, Pick CG, Korczyn AD, Oest E, Blank M, Shoenfeld Y, et al. Hyperactivity in a mouse model of the antiphospholipid syndrome. Lupus 2001;10:496–9.
[39] Katzav A, Kivity S, Blank M, Shoenfeld Y, Chapman J. Adjuvant immunization induces high levels of pathogenic antiphospholipid antibodies in genetically prone mice: another facet of the ASIA syndrome. Lupus 2012;21:210–6.
[40] Katzav A, Solodeev I, Brodsky O, Chapman J, Pick CG, Blank M, et al. Induction of autoimmune depression in mice by anti-ribosomal P antibodies via the limbic system. Arthritis and rheumatism 2007;56:938–48.
[41] Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 1977;229:327–36.
[42] Wang Z, Li M, Zeng X, Liu X. Hepatitis B virus-associated antigen deposition in renal tissue from patients with systemic lupus erythematosus. The Journal of rheumatology 2012;39:974–8.
[43] Yu C, Gershwin ME, Chang C. Diagnostic criteria for systemic lupus erythematosus: A critical review. Journal of autoimmunity 2014;48–49C:10–3.
[44] Santoro D, Vita G, Vita R, Mallamace A, Savica V, Bellinghieri G, et al. HLA haplotype in a patient with systemic lupus erythematosus triggered by hepatitis B vaccine. Clinical nephrology 2010;74:150–3.
[45] Thomas C, Moridani M. Interindividual variations in the efficacy and toxicity of vaccines. Toxicology 2010;278:204–10.
[46] Santoro D, Stella M, Montalto G, Castellino S. Lupus nephritis after hepatitis B vaccination: an uncommon complication. Clinical nephrology 2007;67:61–3.
[47] Marinchev L, Antonov K, Peytcheva V, Kolarov Z. Outcome in a patient with systemic lupus erythematosus and concurrent chronic hepatitis B infection. BMJ case reports 2010;2010.
[48] Pennesi M, Torre G, Del Santo M, Sonzogni A. Glomerulonephritis after recombinant hepatitis B vaccine. The Pediatric infectious disease journal 2002;21:172–3.
[49] Venkataseshan VS, Lieberman K, Kim DU, Thung SN, Dikman S, D'Agati V, et al. Hepatitis-B-associated glomerulonephritis: pathology, pathogenesis, and clinical course. Medicine 1990;69:200–16.
[50] Lai FM, To KF, Wang AY, Choi PC, Szeto CC, Li PK, et al. Hepatitis B virus-related nephropathy and lupus nephritis: morphologic similarities of two clinical entities. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc 2000;13:166–72.
[51] Zagoria RJ, T G. Genitourinary Radiology: The Requisites; 1997. pp. 139–40. Chapter 4.
[52] Newman K, Owlia MB, El-Hemaidi I, Akhtari M. Management of immune cytopenias in patients with systemic lupus erythematosus - Old and new. Autoimmunity reviews 2013;12:784–91.
[53] Turgut S, Bor-Kucukatay M, Emmungil G, Atsak P, Turgut G. The effects of low dose aluminum on hemorheological and hematological parameters in rats. Archives of toxicology 2007;81:11–7.
[54] Farina M, Lara FS, Brandao R, Jacques R, Rocha JB. Effects of aluminum sulfate on erythropoiesis in rats. Toxicology letters 2002;132:131–9.
[55] Ganchev T, Dyankov E, Zacharieva R, Pachalieva I, Velikova M, Kavaldjieva B. Influence of aluminium on erythropoiesis, iron metabolism and some functional characteristics of erythrocytes in rats. Acta physiologica et pharmacologica Bulgarica 1998;23:27–31.
[56] Unterman A, Nolte JE, Boaz M, Abady M, Shoenfeld Y, Zandman-Goddard G. Neuropsychiatric syndromes in systemic lupus erythematosus: a meta-analysis. Seminars in arthritis and rheumatism 2011;41:1–11.
[57] Hanly JG. Diagnosis and management of neuropsychiatric SLE. Nature reviews Rheumatology 2014;10:338–47.
[58] Zandman-Goddard G, Chapman J, Shoenfeld Y. Autoantibodies involved in neuropsychiatric SLE and antiphospholipid syndrome. Seminars in arthritis and rheumatism 2007;36:297–315.
[59] Mahler M, Agmon-Levin N, van Liempt M, Shoenfeld Y, Waka A, Hiepe F, et al. Multi-center evaluation of autoantibodies to the major ribosomal P C22 epitope. Rheumatology international 2012;32:691–8.
[60] Kivity S, Tsarfaty G, Agmon-Levin N, Blank M, Manor D, Konen E, et al. Abnormal olfactory function demonstrated by manganese-enhanced MRI in mice with experimental neuropsychiatric lupus. Annals of the New York Academy of Sciences 2010;1193:70–7.
[61] Sherman GF, Galaburda AM, Behan PO, Rosen GD. Neuroanatomical anomalies in autoimmune mice. Acta neuropathologica 1987;74:239–42.
[62] Sakic B, Szechtman H, Keffer M, Talangbayan H, Stead R, Denburg JA. A behavioral profile of autoimmune lupus-prone MRL mice. Brain, behavior, and immunity 1992;6:265–85.
[63] Sakic B, Szechtman H, Denburg JA. Neurobehavioral alterations in autoimmune mice. Neuroscience and biobehavioral reviews 1997;21:327–40.
[64] Vogelweid CM, Wright DC, Johnson JC, Hewett JE, Walker SE. Evaluation of memory, learning ability, and clinical neurologic function in pathogen-free mice with systemic lupus erythematosus. Arthritis and rheumatism 1994;37:889–97.
[65] Kier AB. Clinical neurology and brain histopathology in NZB/NZW F1 lupus mice. Journal of comparative pathology 1990;102:165–77.
[66] Moore PM. Evidence for bound antineuronal antibodies in brains of NZB/W mice. Journal of neuroimmunology 1992;38:147–54.
[67] Bracci-Laudiero L, Aloe L, Lundeberg T, Theodorsson E, Stenfors C. Altered levels of neuropeptides characterize the brain of lupus prone mice. Neuroscience letters 1999;275:57–60.
[68] Lapter S, Marom A, Meshorer A, Elmann A, Sharabi A, Vadai E, et al. Amelioration of brain pathology and behavioral dysfunction in mice with lupus following treatment with a tolerogenic peptide. Arthritis and rheumatism 2009;60:3744–54.
[69] Zardi EM, Taccone A, Marigliano B, Margiotta DP, Afeltra A. Neuropsychiatric systemic lupus erythematosus: Tools for the diagnosis. Autoimmunity reviews 2014;13:831–9.
[70] Postal M, Costallat LT, Appenzeller S. Neuropsychiatric manifestations in systemic lupus erythematosus: epidemiology, pathophysiology and management. CNS drugs 2011;25:721–36.
[71] Trysberg E, Nylen K, Rosengren LE, Tarkowski A. Neuronal and astrocytic damage in systemic lupus erythematosus patients with central nervous system involvement. Arthritis and rheumatism 2003;48:2881–7.
[72] Hocine MN, Farrington CP, Touze E, Whitaker HJ, Fourrier A, Moreau T, et al. Hepatitis B vaccination and first central nervous system demyelinating events: reanalysis of a case-control study using the self-controlled case series method. Vaccine 2007;25:5938–43.
[73] Hernan MA, Jick SS, Olek MJ, Jick H. Recombinant hepatitis B vaccine and the risk of multiple sclerosis: a prospective study. Neurology 2004;63:838–42.
[74] Kulkarni PS, Sapru A, Bavdekar A, Naik SS, Patwardhan M, Barde P, et al. Immunogenicity of two diphtheria-tetanus-whole cell pertussis-hepatitis B vaccines in infants: a comparative trial. Human vaccines 2011;7:941–4.
[75] Bogdanos DP, Smith H, Ma Y, Baum H, Mieli-Vergani G, Vergani D. A study of molecular mimicry and immunological cross-reactivity between hepatitis B surface antigen and myelin mimics. Clinical & developmental immunology 2005;12:217–24.
[76] Israeli E, Agmon-Levin N, Blank M, Chapman J, Shoenfeld Y. Guillain-Barre syndrome–a classical autoimmune disease triggered by infection or vaccination. Clinical reviews in allergy & immunology 2012;42:121–30.
[77] Agmon-Levin N, Blank M, Paz Z, Shoenfeld Y. Molecular mimicry in systemic lupus erythematosus. Lupus 2009;18:1181–5.
[78] Exley C, Siesjo P, Eriksson H. The immunobiology of aluminium adjuvants: how do they really work? Trends in immunology 2010;31:103–9.
[79] Shaw CA, Tomljenovic L. Aluminum in the central nervous system (CNS): toxicity in humans and animals, vaccine adjuvants, and autoimmunity. Immunologic research 2013;56:304–16.
[80] Flach TL, Ng G, Hari A, Desrosiers MD, Zhang P, Ward SM, et al. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nature medicine 2011;17:479–87.

[81] Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nature immunology 2008;9:847–56.
[82] Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nature immunology 2011;12:509–17.
[83] Wu Z, Du Y, Xue H, Wu Y, Zhou B. Aluminum induces neurodegeneration and its toxicity arises from increased iron accumulation and reactive oxygen species (ROS) production. Neurobiology of aging 2012;33. 199 e1–12.
[84] Han S, Lemire J, Appanna VP, Auger C, Castonguay Z, Appanna VD. How aluminum, an intracellular ROS generator promotes hepatic and neurological diseases: the metabolic tale. Cell biology and toxicology 2013;29:75–84.
[85] Platt B, Fiddler G, Riedel G, Henderson Z. Aluminium toxicity in the rat brain: histochemical and immunocytochemical evidence. Brain research bulletin 2001;55:257–67.
[86] Abu-Taweel GM, Ajarem JS, Ahmad M. Neurobehavioral toxic effects of perinatal oral exposure to aluminum on the developmental motor reflexes, learning, memory and brain neurotransmitters of mice offspring. Pharmacology, biochemistry, and behavior 2012;101:49–56.
[87] Ribes D, Colomina MT, Vicens P, Domingo JL. Impaired spatial learning and unaltered neurogenesis in a transgenic model of Alzheimer's disease after oral aluminum exposure. Current Alzheimer research 2010;7:401–8.
[88] Walton JR. Cognitive deterioration and associated pathology induced by chronic low-level aluminum ingestion in a translational rat model provides an explanation of Alzheimer's disease, tests for susceptibility and avenues for treatment. International journal of Alzheimer's disease 2012;2012: 914947.
[89] Khan Z, Combadiere C, Authier FJ, Itier V, Lux F, Exley C, et al. Slow CCL2-dependent translocation of biopersistent particles from muscle to brain. BMC medicine 2013;11:99.
[90] Pillai S. Rethinking mechanisms of autoimmune pathogenesis. Journal of autoimmunity 2013;45:97–103.
[91] Gershwin ME, Shoenfeld Y. Abul Abbas: an epitome of scholarship. Journal of autoimmunity 2013;45:1–6.
[92] Invernizzi P. Liver auto-immunology: the paradox of autoimmunity in a tolerogenic organ. Journal of autoimmunity 2013;46:1–6.
[93] Jamin C, Renaudineau Y, Pers JO. Pierre Youinou: when intuition and determination meet autoimmunity. Journal of autoimmunity 2012;39:117–20.