Impaired Behavior in Young Female Mice Following Administration of Aluminum Adjuvants and the Human Papillomavirus (HPV) Vaccine Gardasil

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Abstract
Vaccine adjuvants and vaccines may induce autoimmune and inflammatory manifestations in susceptible individuals. To date most human vaccine trials utilize aluminum (Al) adjuvants as placebos despite much evidence showing that Al in vaccine-relevant exposures is toxic to humans and animals. We thus sought to evaluate the effects of Al adjuvant and whole vaccine formulation versus the true placebo on behavioral and inflammatory parameters in young female mice. Six week old C57BL/6 female mice received three injections of either, HPV vaccine Gardasil, Gardasil + pertussis toxin (Pt), Al hydroxide, or, vehicle control (19.12 mg/mL NaCl, 1.56 mg/mL L-histidine). The amount of injected Al and the HPV vaccine were the equivalent of human exposure. The behaviour of mice was evaluated at three and six months of age. At six months of age, both Gardasil and Al-injected mice showed a significant increase in depression-like behavior in comparison to vehicle subjected mice (Gardasil, p=0.025; Al p=0.009; and Gardasil + Pt, p=0.005). Notably, the increase in depression was already highly significant at three months of age for the Gardasil and Gardasil + Pt group (p≤0.0001). Additionally, at three months of age, compared to control mice, Al -injected mice showed a significantly decreased preference for spatial novelty in the Y-maze test (p=0.03), an indicative of cognitive impairment. Immunohistochemistry analysis revealed an increase in microglial activation in the CA1 area of the hippocampus of Gardasil-injected mice compared to the control. Moreover, anti-HPV antibodies from the sera of Gardasil and Gardasil + Pt-injected mice showed cross-reactivity with the mouse brain protein extract. Thus, it is likely that Gardasil via its Al adjuvant and it HPV antigen can trigger neuroinflammation and autoimmune reactions, further leading to untoward behavioral changes.

Keywords: Gardasil, aluminum, ASIA syndrome, autoantibodies, autoimmunity, neuroinflammation

Abbreviations Al: aluminum; APS: anti-phospholipid syndrome; β2-GPI: β2-glycoprotein I; FST: forced swimming test; HPV: Human papilloma virus ; Pt: pertussis toxin; U. S FDA: United States Food and Drug Administration
1.0 Introduction

Like other drugs, vaccines can cause adverse events, but unlike conventional medicines, which are prescribed to people who are ill, vaccines are administered to healthy individuals. Hence there is an added concern regarding risks associated with vaccinations. While most reported side effects from vaccines are mild and transient, serious adverse events do occur and can even be fatal (1-7).

There are currently major bottlenecks in our understanding of the exact mechanisms by which such events can be triggered. The main reason for this is the poor methodological quality of clinical studies that evaluate vaccine safety and the lack of in-depth research into adverse phenomena (4, 8-10). In addition, adverse events may not fit into a well-defined category of an autoimmune disease but rather, present themselves as a constellation of non-specific symptoms (i.e., arthralgia, myalgia, fatigue, nausea, weakness, paraesthesia, depression, mild cognitive disturbances etc.). Another complicating factor in researching vaccine-related adverse events is that the latency period between vaccination and the development of an overt and diagnosable autoimmune and/or neurological disease can range from days to many months (11-15), likely depending on individuals’ genetic makeup and other susceptibility factors (i.e., previous history of autoimmune disease or previous history of adverse reactions to vaccines).

From the above it is clear that establishing a definite causal link between vaccinations and disease manifestations in humans remains a complex task. Thus the potential risks from vaccines remain currently ill-understood and controversial. A further obfuscation to our understanding of potential risks from vaccinations stems from the persistent use of aluminum (Al) adjuvants-containing placebos in vaccine trials (16). Indeed, contrary to popular assumptions of inherent safety of Al in vaccines is compelling data from both human and animal studies which has consistently implicated this most widely used adjuvant in the pathogenesis of disabling neuroimmuno-inflammatory conditions (17-24).

Due to their capability of enhancing the immune response to foreign antigens, substances with adjuvant properties have been used for decades to enhance the immunogenicity of human and animal vaccines (25-29). Because of their immune-potentiating capacity, adjuvants enable the usage of smaller amount of antigens in vaccine preparations and are thus attractive from a commercial standpoint. Nonetheless, enhanced immunogenicity also implies enhanced reactogenicity. Indeed, although Al acts as an effective vehicle for the presentation of antigens, this process is not always benign since the adjuvant itself is intrinsically capable of stimulating pathological immune and neuro-inflammatory responses (18, 19, 21-24, 30-38). In spite of these data, it is currently maintained by both the pharmaceutical industry and drug regulating agencies that the concentrations at which Al is used in vaccines does not represent a health hazard (39, 40).

Apart from potential hazards associated with adjuvant use, other ingredients in vaccines also have the capacity of provoking undesirable adverse events. Indeed, since the mechanisms by which the host’s immune system responds to vaccination resemble the ones involved in the response to infectious agents, 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 (41-44). Molecular mimicry (whereby the vaccine foreign antigen resembles a host antigen) and subsequent cross-reactivity (binding of vaccine-induced antibodies to host antigens) is one of such mechanism by which both infections as well as vaccines can trigger...
autoimmune diseases (41, 45-47). Acute rheumatic fever, which presents several weeks after infection with Streptococcus pyogenes is one of the well-recognized examples whereby breakdown of self-tolerance and autoimmunity results from molecular resemblance between the bacterial M-protein and human glycoproteins (48). Another well-known example of molecular mimicry is found in the etiology of the anti-phospholipid syndrome (APS), an autoimmune multi-systemic disease associated with recurrent fetal loss, thromboembolic phenomena, thrombocytopenia as well as neurological, cardiac and dermatological involvement. APS is characterized by the presence of pathogenic autoantibodies against the β2-glycoprotein I (β2-GPI). The infectious etiology of APS was well established (46, 49). Likewise, a link between vaccination such as tetanus toxoid may trigger antibodies targeting tetanus toxoid and β2-GPI, due to molecular mimicry between the two molecules (46). Most recently Ahmed et al (45) have found that the peptide in influenza nucleopeptide A (the antigenic component of the Pandemrix influenza vaccine) shares protein residues with human hypocretin receptor 2, which has been linked to narcolepsy. The findings of Ahmed et al. (45) may explain the increased number of reports of narcolepsy which followed the influenza pandemic vaccination campaign in countries where Pandemrix had been used (50-52). These and other examples of molecular mimicry phenomena (45, 47, 53-62), show that overly vigorous and/or aberrant immune responses to either infections or vaccinations, while protective, can also be detrimental to the host.

The HPV vaccine Gardasil is one of many vaccines currently on the market that is adjuvanted with Al. Since the licensure by the U.S. Food and Drug Administration (FDA) and subsequent introduction on the market in 2009, the HPV vaccine has been linked to a variety of serious neurological and autoimmune manifestations (Table 1) including Guillain-Barré syndrome (63). Notably, out of 152 total cases identified via pubmed 129 (85%) are related to neuro-ophthalmologic disorders (Table 1). It should be noted that the pattern of adverse manifestations emerging from HPV vaccine case reports, matches that reported through various vaccine safety surveillance systems worldwide, with nervous system and autoimmune disorders being the most frequently reported (64).

Like most other vaccine safety trials, the trials for the HPV Gardasil vaccine utilized an Al-containing placebo (65-72) and hence the safety profile of the vaccine remains obscured by the use of a potentially toxic placebo. Thus, in order to investigate better the safety profile of Gardasil, as well as the Al adjuvant, in the current study, we evaluated and compared the effects of Al and whole HPV vaccine formulation versus that of a true placebo on behavioral, neurohistological and autoimmune parameters in young female C57BL/6 mice.

Materials and Methods

2.1 Mice husbandry

Six-week old C57BL/6 female mice, were obtained from Harlan Laboratories (Jerusalem, Israel), and were housed in the animal facility at Sheba Medical Center. The mice were raised under standard conditions, 23 ± 1°C, 12-hour light cycle (6:30 am to 6:30 pm) with ad libitum access to food and water. The Sheba Medical Center Animal Welfare Committee approved all procedures.

2.2. Injection procedures and experimental design
Six-week old C57BL/6 female mice received three injections (spaced 1 day apart) of either a) quardivalent HPV vaccine Gardasil, b) Gardasil + pertussis toxin (Pt), c) Al hydroxide or d) vehicle control (19.12 g/mL NaCl, 1.56 g/mL L-histidine). The number of injected animals was 19 per experimental group. Gardasil, Al and vehicle were injected intramuscularly (i.m.), while the Pt was given intraperitoneally (i.p.). The amount of injected Al and the HPV vaccine were the equivalent of human exposure. In particular, each mice in the Gardasil and Gardasil + Pt group received 0.25 μl of Gardasil (dissolved in 20 μl of vehicle solution). 0.25 μl of Gardasil is the equivalent of a human dose since the average weight of a 6-week old mice is approximately 20g. Gardasil is given as a 0.5 mL dose to teenage girls of cca 40 kg. Thus a 20 g mouse receives cca 2000 x less of the vaccine suspension than a human. Similarly, each mouse in the Al adjuvant group received 5.6 μg/kg body weight Al hydroxide dissolved in 20 μl vehicle solution. A single Gardasil dose contains 225 μg of Al and is given to a cca 40 kg female. This equates to 5.6 mcg Al hydroxide/kg body weight. The mice in the Pt group received 250 ng of Pt with each injection of Gardasil. Pt was added to this group for the purpose of damaging the blood-brain barrier.

The behaviour of mice was evaluated at three and six months post immunization for 1) depression by the forced swimming test (FST), 2) locomotor and explorative activity by the staircase test, 3) cognitive functions by the novel object recognition and Y-maze tests and 4) strength and motor function by the rotarod test. Following the first round of behavioral testing at three months of age, 5 mice from each of the four experimental groups were sacrificed and brain tissues were collected and processed for histological examinations. Blood specimens were also collected at this time for serological analysis.

2.3 Behavioral tests

2.3.1 Forced swimming test

This test is based on Porsolt et al.’s description (73). Mice were placed in individual glass beakers (height 39cm, diameter 21.7 cm) with water 15 cm deep at 25°C. On the first day, mice were placed in the cylinder for a pretest session of 10 minutes, and later were removed from the cylinder, and then returned to their home cages. Twenty-four hours later (day 2), the mice were subjected to a test session for six minutes. The behavioral measure scored was the duration (in seconds) of immobility, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the six minute test. A depression-like behavior was considered as an increased immobility time.

2.3.2 Staircase test

Locomotor, explorative activity and anxiety were evaluated by the staircase test, as described previously by Katzav et al. (74). In this test, stair-climbing and rearing frequency are recorded as measures of general locomotor function, exploratory activity and anxiety/attention. The staircase maze consisted of a polyvinyl chloride enclosure with five identical steps, 2.5 × 10 × 7.5 cm. The inner height of the walls was constant (12.5 cm) along the whole length of the staircase. The box was placed in a room with constant lighting and isolated from external noise. Each mouse was tested individually. The animal was placed on the floor of the staircase with its back to the staircase. The number of stairs climbed and the number of rears were recorded during a three-minute 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 the stair or against the wall. The number of stairs descended was not taken into account. Before each test, the animal was removed and the box cleaned with a diluted alcohol solution to eliminate smells.

2.3.3 Novel object recognition test

This is a visual recognition memory test based on a method described by Tordera et al. (75). The apparatus, an open field box (50 × 50 × 20 cm), was constructed from plywood painted white. Three phases (habituation, training and retention) were conducted on three separate test days. Before the training session, the mice were individually habituated by allowing them to explore the box for 10 minutes (day 1). No data were collected at this phase. During training sessions (day 2), two identical objects were placed into the box in the northwest and southeast corners (approximately 5 cm from the walls), 20 cm away from each other (symmetrically) and then the individual animal was allowed to explore them for 5 minutes. Exploration of an object was defined as directing the nose to the object at a distance of ≤1 cm and/or touching it with the nose and rearing at the object; turning around or sitting near the object was not considered as exploratory behavior. The time spent in exploring each object was recorded as well as the number of interactions with both objects. The animals were returned to their home cages immediately after training. During the retention test (day 3), one of the familiar objects used during the training session was replaced by a novel object. Then the animals were placed back into the box, and allowed to explore the objects for 5 minutes. The same parameters were measured as during the training session, namely, the time spent in exploring each of the two objects and the number of interactions with them. All objects were balanced in terms of physical complexity and were emotionally neutral. The box and the objects were thoroughly cleaned by 70% alcohol before each session to avoid possible instinctive odorant cues. A preference index, a ratio of the amount of time spent exploring any one of the two items (old and new in the retention session) over the total time spent exploring both objects, was used to measure recognition memory.

2.3.4 Y maze test

The Y maze test was used to assess spatial memory and interest in novel environments. It was comprised of three arms, built of black Perspex. Each arm was 8 × 30 × 15 cm at an angle of 120° from the others. One arm was randomly selected as the start arm. Each mouse was placed twice in the start arm. On the first trial, lasting for 5 minutes, one of the other two arms was randomly chosen to be blocked whereas on the second trial, lasting for 2 minutes, both arms were open. The two trials were separated by a 2 minute interval, during which the mouse was returned to its home cage. The time spent in each of the arms was measured. Between each trial and between each mouse, the maze was cleaned with a 70% alcohol solution and dried. Discrimination of spatial novelty was expressed by a preference index: time in the new arm - time old arm/time in the new arm + time in the old arm, assessing spatial memory. A normal cognitively non-impaired mouse is expected to recognize the old arm as old and spend more time in the new arm.

2.3.5 Rotarod

The rotarod was used to test general motor function and motor learning (76). The time that a mouse could remain walking on a rotating axle (3.6 cm diameter; speed of rotation: 16
rpm) without either falling or clenching onto the axle was measured. Each mouse was tested three times for 60 seconds. A day prior to testing the mice were habituated to the rotarod.

2.3.6 Statistical analysis
Results are expressed as the mean ± SEM. The differences in mean for average immobility time in the FST, the staircase test parameters (number of rearing and stair-climbing events), novel object recognition and Y maze tests were evaluated by T-test. Significant results were determined as \( p < 0.05 \).

2.7 Brain perfusion and fixation
The mice were anesthetised by an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and sacrificed by transcardiac perfusion with phosphate buffered saline (PBS) followed by perfusion with 4% paraformaldehyde (PFA, Sigma-Aldrich Israel Ltd., Rehovot Israel) in phosphate buffer (PO\(_4\), pH 7.4). After perfusion, the brain was quickly removed and fixed overnight in 4% PFA (in PO\(_4\), pH 7.4) at 4°C. On the following day, the brain was cryoprotected by immersion in 30% sucrose in 0.1M PO\(_4\) (pH 7.4) for 24 to 48 hours at 4°C before brain cutting. Frozen coronal sections (30 to 50 μm) were cut on a sliding microtome (Leica Microsystems GmbH, Wetzlar, Germany), collected serially and kept in a cryoprotectant at −20°C until staining.

2.8 Detection of autoantibodies in the sera
The levels of autoantibodies in the mice sera were tested by a home-made ELISA one month post injection. The targeted antigens were: HPV L1 major capsid protein of HPV types 6, 11, 16 and 18 (Gardasil antigenic components), mouse brain protein extract, Al hydroxide, dsDNA, bovine serum albumin (BSA) and β2glycoprotein-I (β2GPI). ELISA plates were coated with the target proteins, dsDNA or Al hydroxide at 20 μg/ml PBS/well and incubated overnight at 4°C. Washed plates were blocked with 3%BSA in PBS 1hr at 37°C. Sera were added at dilution of 1:200 for 2hrs at room temperature. The binding was probed with goat anti-mouse IgG conjugated to alkaline phosphatase at concentration of 1:5000 for 1hr at 37C. Following appropriate substrate, the data were read by ELISA reader at 405nm.

2.9 Inhibition assay
Anti-brain protein extract antibody positive sera at 50% binding to brain protein extract, were incubated with the brain proteins (10-50 mg/ml) overnight at 4°C. The following day, the mixtures were subjected to ELISA plates coated with HPV L1 major capsid protein of HPV types 6, 11, 16 and 18. The ELISA was performed as described above. The percentage of inhibition was calculated as follows:

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100 - \{[\text{OD of tested sample without inhibitor} - \text{OD of tested sample with inhibitor}] / \text{OD of tested sample without inhibitor}\} \times 100
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2.10 Brain tissue immunostaining
Brain sections were stained free-floating, incubated with the first antibodies overnight at 4°C. The slices were then washed in PBS + 0.1% Triton X-100, and incubated at room temperature for one hour with the corresponding fluorescent chromogens-conjugated secondary antibody. Sections were stained for specific antigens with antibodies against activated microglia (anti-Iba-1, polyclonal, Abcam, Cambridge, UK) and astrocytes (anti-GFAP monoclonal, Dako,
Carpinteria, CA, USA). Counter staining was performed with Hoechst (Sigma-Aldrich Israel Ltd., Rehovot Israel).

2.10.1 Image acquisition, quantification and statistical analyses

Iba-1 and GFAP immunostaining was visualized using ×4/0.1 NA, ×10/0.25 NA and ×40/0.65 NA objective lenses on a Nikon eclipse 50i fluorescence microscope equipped with a Nikon DS Fi1 camera. In order to minimize bleaching of the fluorescence, images were obtained by serially moving the slide with no fluorescence and then acquiring the images in a standard manner. All sections were then studied quantitatively for differences in immunostaining density among the groups, using Image J software (NIH, USA). Region of interests (ROIs) were drawn manually using the 'Polygon selection' tool. Brain regions were identified using a mouse brain atlas. ROIs were chosen to represent anatomical regions previously shown to be involved in cognition and/or to exhibit variable sensitivity to neuroinflammation in other models. The mean intensity of the specific ROIs (×10 magnification) was recorded for each individual animal recorded (Analyze >> Measure) and data were analyzed using SPSS statistical software (version 15.0). Univariate analysis was conducted for each ROI/Antibody separately using 'group' as a fixed factor and 'experiment' as a Covariate. Post-hoc analysis, One-way ANOVA, Student’s t-test, simple regression, or correlation analysis were used when appropriate, according to the experimental design. Significance level was determined in 1-tailed and 2-tailed tests. The level of statistical significance of differences is p<0.05. 0.1<p<0.05 is defined as a trend.

3. Results

3.1 Behavioral tests

After the behavioral testing at three months of age, compared to other groups, Al-injected mice showed a significant increase in rearing frequency in the staircase test, a measure for increased anxiety (p=0.02; Fig. 1A). There were no significant differences in the overall locomotor function between the groups, as measured by the number of stairs climbed in the staircase apparatus (not shown). Moreover, compared to control mice, Al-injected mice showed a significantly decreased preference for spatial novelty in the Y-maze test, an indicative of cognitive impairment (p=0.03; Fig. 2A). Gardasil and Gardasil + Pt groups also showed a much lower preference index for the new arm in the Y maze than the control although the difference did not reach statistical significance. However, the two groups injected with Gardasil showed highly significant depressive behavior in the FST compared to both control mice and Al-injected mice (p<0.0001; Fig. 3A). No significant differences in behavior were observed in the novel object recognition test.

After the behavioral testing at six months of age, Al-injected mice showed a significantly lower frequency of rearing compared to controls in the staircase test (p=0.012; Fig. 1B). A lower frequency of rearing is an indication of a reduced exploratory response to a novel environment, and, it can also indicate a non-selective attention deficit. The number of stairs climbed in the staircase remained non-statistically different between the groups (not shown). In the Y maze test, the differences between treatment groups were no longer significant after the second round of behavioral tests, however the same trend was observed as previously, namely, the Al and the two Gardasil groups showed a much lower preference to explore the new arm of the Y maze compared to the control group (Fig. 2B). Given that after the first round of testing at three
months of age, we sacrificed 5 animals from each of the four experimental groups, it is possible that our experiment was insufficiently powered to detect milder adverse effects arising from the different treatments. In the FST however, the changes were much more pronounced and hence retained despite the lower number of animals. Namely, after the second round of testing at six months of age, all treatment groups showed highly significant differences in depressive behavior (including Al), compared to control animals. Al, Gardasil and Gardasil + Pt animals spent much more time floating (measure of increased depressive behavior) than controls (p=0.009, p=0.025 and p=0.005 respectively; Fig. 3B). No significant differences in behavior were observed in the novel object recognition test and the rotarod test. Since rotarod measures muscular strength and locomotor function, the latter results indicates that differences observed in the FST were not due to locomotor dysfunction, but indeed, due to depression.

3.2 Autoantibody profile

One month post injection of either Al, Gardasil and Gardasil + Pt, the profile of serum antibodies was analyzed at dilution of 1:200. Elevated levels of antibodies recognizing the-HPV L1 capsid protein of HPV types 6, 11, 16 and 18 (Gardasil antigenic constituents), as well as anti-dsDNA and anti-brain protein extract antibodies were observed in the two groups of mice that received the HPV vaccine (p<0.001; Fig. 4). The binding of sera from Gardasil-immunized mice to HPV L1 capsid proteins was significantly inhibited by the mouse brain protein extract in a dose dependent manner (p<0.003-0.02) in comparison to Al-injected mice as shown in Figure 5. The titers of anti-HPV L1 and anti-brain protein extract antibodies were reduced after two months (data not shown). No titers of anti-Al antibodies were detected in the sera of any of the four treatment groups of mice (Fig. 5).

3.3 Brain tissue immunostaining

Following the behavioral tests at three months of age, 5 animals were sacrificed from each of the four experimental groups and used for brain immunostaining procedures. With this relatively small group size, there were no clear changes (i.e. overt to the naked eye) between the groups in both astrocyte and microglia staining in any of the regions of interests we investigated (CA1, CA3, dentate gyrus and the striatum). On the other hand, there was a significant difference between the groups in the density of Iba-1 immunostaining using one-tailed analysis (p=0.046). Further post-hoc analysis revealed significant increase in Iba-1 density in the CA1 of Gardasil-immunized mice compared to Al-injected mice (p=0.017; Fig.6). Increase in the Iba-1 density in the CA1 of Gardasil-immunized mice was a trend compared to vehicle-injected control mice (p=0.06; Fig.6). These results suggest that the CA1 might be vulnerable to small changes in neuroinflammation as a result of Gardasil immunization.

4. Discussion

The present results show long-term alteration of behavioral responses and neuroinflammatory changes in mice as a result of Al and Gardasil vaccine injection in exposure doses which are equivalent to those in vaccinated human subjects. In particular, mice injected with Al and Gardasil showed increased depression (Fig. 3) and impaired spatial memory, the latter however only reaching significance in the Al group (Fig. 2A). In addition, Al-injected mice showed abnormal responses to a novel environment, which initially was manifested as increased anxiety (Fig. 1A) and later, as a non-selective attention deficit (Fig. 1B) as measured by the
staircase test. The number of stairs and rearing frequency in this test are normally used to provide measures of general physical motor abilities and level of interest in the novelty of the environment. Rearing in response to environmental change (i.e., removing a mouse from the home cage and placing the animal in an open box or a stair-case apparatus) is often considered an index of non-selective attention in rodents, while rearing during object investigation likely reflects selective attention (77). Abnormal responses to a novel environment in the Al group are also confirmed by the Y-maze test which apart from spatial memory, also provides a measure of the rodent’s interest in exploring the new arm of the maze apparatus (Fig. 2A). Finally, we observed inflammatory changes specifically in the Gardasil-injected mice, namely, the presence of activated microglia in the CA1 area of the hippocampus (Fig. 6).

4.1 Possible mechanisms of vaccine-induced injury

4.1.1 The role of adjuvants

It is interesting to note that, in our hands, the extent of adverse neurological manifestations was similar in the three treatment groups whose only common denominator was the Al compound. As we noted above, the clinical trials for both HPV vaccines, Gardasil and Cervarix, used an Al-containing placebo and the safety of the vaccines was thus presumed on the finding that there was an equal number of adverse events in the vaccine and the alleged placebo group (65-72). The HPV vaccines, like many other vaccines, are adjuvanted with Al in spite of well documented evidence that Al can be not neuro- and immuno-toxic (18-24, 27, 30, 32, 35, 36, 38, 78-82) and hence does not constitute an appropriate placebo choice.

The appearance of diverse adverse neurological and immuno-inflammatory manifestations following routine vaccinations is well documented in the medical literature (17, 24, 41, 55, 83-92). Although the classical explanations for these phenomena have largely centered on vaccine antigens, in recent years attention has shifted to Al adjuvants. Consequently, in the last decade, studies on animal models and humans have indicated that Al adjuvants have an intrinsic ability to inflict adverse immune and neuro-inflammatory responses (18, 19, 21-23, 30-33, 93). This research culminated in delineation of ASIA-“autoimmune/inflammatory syndrome induced by adjuvants”, which encompasses the wide spectrum of adjuvant-triggered medical conditions characterized by a misregulated immune response (79, 80, 94). Notably, the vast majority of adverse manifestations experimentally triggered by Al in animal models, and those associated with administration of adjuvanted vaccines in humans are neurological and neuropsychiatric (14, 17-20, 30, 95, 96). These observations should not be particularly surprising given Al’s well-established neurotoxic properties (97-105). What has however been argued, is that the concentrations at which Al is used in vaccines are not sufficient to cause neurotoxicity (39, 40). This argument however is not supported by recent evidence.

It should be noted that the long-term biodistribution of nanomaterials used in medicine is largely unknown. This is likewise the case with Al vaccine adjuvant, which is a nanocrystalline compound spontaneously forming micron/submicron-sized agglomerates. It has been recently demonstrated that Al adjuvant compounds from vaccines, as well as Al-surrogate fluorescent nanomaterials, have a unique capacity to cross the blood-brain and blood-cerebrospinal fluid barriers and incite deleterious immuno-inflammatory responses in neural tissues (22, 30, 106). In particular, following injection, antigen-presenting cells (APCs) avidly take up Al particles (107), and in so doing, become long-lived cells (108), impeding Al’s solubilisation in the interstitial fluid (78). Thus a proportion of Al particles escapes the injected muscle, mainly within immune
cells, travels to regional draining lymph nodes, then exits the lymphatic system to reach the bloodstream eventually gaining access to distant organs, including the spleen and the brain. Moreover, the Trojan horse-mechanism by which Al loaded in macrophages enters the brain, results in the slow accumulation of this metal, due to lack of recirculation (22, 106). The sustained presence of Al in central nervous system tissues is likely responsible for the myriad of cognitive deficits associated with administration of Al-containing vaccines in patients suffering from post-vaccination chronic systemic disease syndromes including macrophagic myositis (MMF (14, 18, 19, 23)). Of note, the delivery of foreign viral immunogenic material to the brain via the “Trojan horse” mechanism appears also to be the underlying pathway of central nervous system malfunction following HIV and hepatitis C infection (109-111).

Thus contrary to popular assumptions, Al in the adjuvant form is not rapidly excreted but rather, tends to persist in the body long-term. As demonstrated by Khan et al. (106), intramuscular injection of Al-containing vaccine in mice is associated with the appearance of Al deposits in distant organs, such as spleen and brain where they were still detected one year after injection. Similarly, Al-particle fluorescent surrogate nanomaterials injected into muscle were found to translocate to draining lymph nodes and thereafter were detected associated with phagocytes in blood and spleen. Particles linearly accumulated in the brain up to the six-month endpoint. They were first found in perivascular CD11b+ cells and then in microglia and other neural cells. The ablation of draining lymph nodes dramatically reduced the biodistribution of injected Al-fluorescent surrogate nanocompounds. In addition, the nanoparticle delivery into the brain was found to be critically dependent on the major monocyte chemoattractant protein MCP-1/CCL2 as intramuscular injection of murine rCCL2 strongly increased particle incorporation into intact brain while CCL2-deficient mice had decreased neurodelivery (106).

Notably, regarding the latter finding, the most recent publication by Cadusseau et al. (112) shows that selective elevation of the MCP-1/CCL2 chemikine may represent a biological marker relevant to the pathophysiology of the MMF syndrome. MMF is one of the conditions included in the ASIA syndrome (94), and characterized by highly specific myopathological alterations at deltoid muscle biopsy, first recognized in 1998, and subsequently shown to be due to long-term persistence of vaccine-derived Al adjuvant compounds within macrophages at the site of previous vaccination – up to 8 to 10 years post injection (15, 18, 19, 23, 78, 113). Patients diagnosed with MMF tend to be female (70%) and middle-aged at time of biopsy (median 45 years), and having received 1 to 17 intramuscular Al-containing vaccine administrations (mean 5.3) in the 10 years before MMF detection (14). Clinical manifestations in MMF patients include diffuse myalgia, arthralgia, chronic fatigue, muscle weakness and cognitive dysfunction. In particular, up to 93% of patients suffer from chronic fatigue (over six months in duration (114)) and up to 89% from chronic diffuse myalgias (over six months in duration) with or without arthralgias (14). Fatigue is disabling in 87% and affects patient’s physical and mental functioning in 53% of cases (114). Overt cognitive alterations affecting memory and attention are manifested in 51% of cases (14). In addition to chronic fatigue syndrome, 15–20% of patients with MMF concurrently develop an autoimmune disease, the most frequent being multiple sclerosis-like demyelinating disorders, Hashimoto’s thyroiditis, and diffuse dysimmune neuromuscular diseases, such as dermatomyositis, necrotizing autoimmune myopathy, myasthenia gravis, and inclusion body myositis (14). Even in the absence of overt autoimmune disease, low titers of various autoantibodies and increased inflammatory biomarkers are commonly detected (115).

The prolonged hyperactivation of the immune system and chronic inflammation triggered by repeated exposure and unexpectedly long persistence of Al adjuvants in the human body (up
to 8 to 10 years post vaccination (15, 78, 116) are thought to be the principal factors underlying the toxicity of these compounds. One of the reasons for the long retention of Al adjuvants in bodily compartments including systemic circulation is most likely due to its tight association with the vaccine antigen or other vaccine excipients (117). Even dietary Al has been shown to accumulate in the central nervous system over time, producing Alzheimer type outcomes in experimental animals feed equivalent amounts of Al to what humans consume through a typical Western diet (118, 119).

The ability of Al adjuvant nanoparticles to cross the blood-brain barrier via a macrophage-dependent Trojan horse mechanism may explain in part why vaccines have a predilection to affect the central nervous system (14, 17, 19, 21, 22, 95, 101). Another explanation comes from the fact that Al nanomaterials can on their own damage the blood-brain barrier and induce neurovascular injury (37, 120). Collectively, these studies (37, 106, 120) show that nano-Al can accumulate in brain cells, inducing nerve and blood vessel damage and protein degradation in the brain. Persistent accumulation of nano-Al compounds regardless the source (i.e., vaccines, dietary etc.) in the central nervous system may thus increase the likelihood of the development of acute and/or chronic neurological disorders.

With respect to the particular Al compounds used in HPV vaccines, it is of further interest to note that Merck’s proprietary AAHS adjuvant used in Gardasil and Glaxo’s proprietary AS04 adjuvant complex used in Cervarix both induce a much stronger immune response than conventional Al-based adjuvants used in other vaccines (i.e., Al hydroxide and Al phosphate) (121, 122). Stronger immunogenicity of an adjuvant formulation also implies by default stronger reactogenicity and risk of adverse reactions. Because of the differences in immune-stimulating properties between different Al adjuvant compounds, safety of a particular adjuvant formulation cannot be a priori assumed on the basis of the allegedly good historical track record of other formulations. Rather, they need to be thoroughly evaluated case by case.

According to the U.S. FDA, a placebo is, “an inactive pill, liquid, or powder that has no treatment value” (123). From the literature cited above as well as the present study, it is obvious that Al in adjuvant form is neither inactive nor harmless and hence cannot constitute as a valid placebo. Commenting on the routine practice of using Al-based adjuvants as placebos in vaccine trials Exley recently stated that it is necessary to make a very strong scientific case for using a placebo which is itself known to result in side effects and that no scientific vindication for such practice is found in the relevant human vaccination literature (16). Conceivably, there is even less justification for using a novel and more potent Al formulation (Merck’s AAHS and Glaxo’s AS04 adjuvants) than those that have been in standard use (Al phosphate and hydroxide). The only aim that this practice achieves is to give misleading data on vaccine safety. Moreover, it is unethical to give a placebo to healthy clinical trial subjects that has no benefit but rather, may cause harm.

### 4.1.2 The role of vaccine-induced antigens: molecular mimicry

We observed elevated levels of antibodies recognizing HPV L1 capsid protein of HPV types 6, 11, 16 and 18 (p<0.001), as well as significant elevation of antibodies targeting the mouse brain-protein extract and dsDNA (p<0.001; Fig. 4). The binding of anti-HPV L1 capsid protein antibodies from the two groups of mice that received Gardasil injection to HPV L1 antigens, was inhibited in a dose dependent manner by using mouse brain protein extract as the inhibitor (p<0.003-0.02) in comparison to the Al-injected mice (Fig.5). On the basis of these results it would appear that the anti-HPV L1 antibodies from Gardasil-vaccinated mice have the
capacity to target not only the HPV L1 antigens but also brain antigen(s), either directly or via negatively charged phospholipids.

This interpretation is consistent with the findings of Kanduc (124, 125) who has shown that antigen present in both HPV vaccines Gardasil and Cervarix (the major capsid L1 protein of HPV-16), shares amino-acid sequence similarity with numerous human proteins, including cardiac and neuronal antigens, human cell-adhesion molecules, enzymes and transcription factors. These observations suggests that possible immune cross-reactions derived from utilization of HPV L1 antigens in current HPV vaccines might be a risk for cardiovascular and neurological autoimmune abnormalities (124, 125). Our observation that nearly 85% (129/152) of HPV vaccine adverse case reports in the current scientific literature relate to neuro-ophthalmic abnormalities supports this conclusion (Table 1).

Moreover, such contention is also supported by a case of severe acute cerebellar ataxia (ACA) following HPV vaccination where combined immunosuppressive therapy with methylprednisolone pulse and intravenous immunoglobulin (IVIG) therapies as well as immunoadsorption plasmapheresis resulted in complete recovery of the patient (35). In this particular case, the patient (12 year old girl) developed symptoms of ACA, including nausea, vertigo, severe limb and truncal ataxia, and bilateral spontaneous continuous horizontal nystagmus with irregular rhythm, 12 days after administration of the HPV vaccine. Severe ACA symptoms did not improve after methylprednisolone pulse and IVIG therapies, but the patient recovered completely after immunoabsorption plasmapheresis. Although no significant antibodies were detected in this patient, the remarkable effectiveness of immunoabsorption plasmapheresis strongly suggested that some unidentified antibodies were involved in the pathophysiology of ACA. Citing the work of Kanduc (125), the authors of this case have stated that further research on molecular mimicry between human proteins and HPV16 L1-derived peptide is needed to determine the exact pathologic mechanism of ACA (35).

Finally, the above mentioned findings by Ahmed et al. (45) indicate that molecular mimicry between host neural antigens and vaccine antigens may be a more common phenomena, occurring with other vaccines besides HPV.

4.2 Conclusions

In summary, both Al and Gardasil vaccine injections resulted in behavioral and cognitive impairments in mice (Figs. 1-3). Furthermore, immunostaining analysis showed an increase in Iba-1 density in the CA1 area of the hippocampus in Gardasil-immunized mice in comparison to vehicle and Al-injected mice, thus suggesting that CA1 might be vulnerable to neuroinflammation as a result of Gardasil immunization (Fig. 4).

In addition, we observed that the brain protein extract significantly inhibited in a dose-dependent manner, the binding of anti-HPV L1 antibodies isolated from the sera of Gardasil-immunized mice to HPV L1 antigens. Therefore, it is likely that mice immunized with the HPV vaccine developed cross reactive anti-HPV antibodies which in addition to binding to the HPV L1 capsid protein, may also bind to brain auto-antigens. The putative target antigen(s) should be further identified by immunoprecipitation and proteomics analyses.

In light of these findings, this study highlights the necessity of proceeding with caution with respect to further mass-immunization practices with a vaccine of yet unproven long-term clinical benefit (64, 126-131) which is capable of inducing immune-mediated cross-reactions with neural antigens of the human host. Especially considering the continually increasing
number of serious disabling neurological adverse events linked to HPV vaccination reported in the current medical literature (Table 1) and vaccine surveillance databases (63, 64).

Finally, in light of the data presented herein, new guidelines should be requested on the use of appropriate placebos in vaccine safety trials.

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

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References

16


Table 1. Summary of cases of autoimmune and inflammatory manifestations following HPV vaccination reported in the peer-reviewed medical literature. Out of 152 reported cases, 129 (85%) relate to neuro-ophthalmic disorders. Abbreviations: ANA, antinuclear antibodies; ADEM, acute disseminated encephalomyelitis; CIS, clinically isolated syndrome; CRPS, complex regional pain syndrome; MS, multiple sclerosis; POF, primary ovarian failure; POTS, postural orthostatic tachycardia syndrome (disorder of the autonomic nervous system); SLE, systemic lupus erythematosus; TM, transverse myelitis.

<table>
<thead>
<tr>
<th>Number of case reports</th>
<th>Age</th>
<th>Symptoms/ main clinical features</th>
<th>Final diagnosis</th>
<th>Reference</th>
</tr>
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<tr>
<td>3</td>
<td>17</td>
<td>Visual impairments</td>
<td>ADEM</td>
<td>(132)</td>
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<tr>
<td></td>
<td>15</td>
<td>Headache, nausea, fever, vertigo, diplopia</td>
<td></td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Headache, nausea, vomiting, diplopia</td>
<td></td>
<td>(91)</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>Upper limb pseudoathetosis</td>
<td>CIS/MS/ Clinically definite MS</td>
<td>(84)</td>
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<tr>
<td></td>
<td>16</td>
<td>Acute hemiparesis</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>21</td>
<td>Incomplete TM, left optic neuritis</td>
<td></td>
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<td></td>
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<td>Headache, incomplete TM</td>
<td></td>
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<tr>
<td></td>
<td>26</td>
<td>Incomplete TM, brainstem syndrome</td>
<td></td>
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<td>2</td>
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<td>Leg numbness, mid-thoracic back pain Blurriness, paraesthesia, optic neuritis</td>
<td>Demyelinating disease unspecified</td>
<td>(133)</td>
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<tr>
<td>1</td>
<td>11</td>
<td>Mood swings, abnormal eye movements, dizziness, leg weakness, myoclonic jerks</td>
<td>Opsoclonus myoclonus</td>
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<td>4</td>
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<td>Back pain, progressing spastic paraparesis, right arm weakness, left eye visual loss</td>
<td>Neuromyelitis optica</td>
<td>(135)</td>
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<td></td>
<td>14</td>
<td>Back pain, right thigh dysesthasias, left optic neuritis</td>
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<td></td>
<td>13</td>
<td>TM with flaccid paraplegia</td>
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<tr>
<td></td>
<td>18</td>
<td>Back pain and leg weakness, complete loss of monocular vision</td>
<td></td>
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<tr>
<td>2</td>
<td>16</td>
<td>Visual loss, headaches, left hemiparesis Visual disturbances, demyelinating lesions</td>
<td>Optic neuritis</td>
<td>(136)</td>
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<tr>
<td></td>
<td>17</td>
<td></td>
<td></td>
<td>(137)</td>
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<td>Paraesthesia, demyelinating lesions Progressive paraesthesia, demyelinating lesions</td>
<td>TM fitting the criteria for MS</td>
<td>(137)</td>
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<td>15</td>
<td>Facial paralysis</td>
<td>Bell’s palsy</td>
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<td>Nausea, vertigo, severe limb and truncal ataxia, and persistent nystagmus</td>
<td>Cerebellar ataxia</td>
<td>(35)</td>
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<td>Chronic (three months) disabling shoulder pain</td>
<td>Brachial neuritis</td>
<td>(138)</td>
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<tr>
<td>53</td>
<td>12-39</td>
<td>Orthostatic intolerance, severe non-migraine-like headache, excessive fatigue, cognitive dysfunction, gastrointestinal discomfort, widespread neuropathic pain</td>
<td>Dysautonomia, POTS, orthostatic intolerance and CRPS</td>
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<td>Headaches, general fatigue,</td>
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<td>(139)</td>
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<td>coldness of the legs, limb pain and weakness, orthostatic intolerance, tremors, persistent asthenia</td>
<td>(140)</td>
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<tr>
<td>17</td>
<td>20</td>
<td>Weight loss, dizziness, fatigue, exercise intolerance</td>
<td></td>
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<td>22</td>
<td>22</td>
<td>Diarrhea, weight loss, fatigue, dizziness, syncope</td>
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<tr>
<td>12</td>
<td>12</td>
<td>Syncope, pre-syncope, dizziness, small fiber neuropathy</td>
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<tr>
<td>15</td>
<td>15</td>
<td>Dizziness, headache, pre-syncope, syncope, Paresthesia, tachycardia, fatigue, headache, diarrhea, weight loss</td>
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<tr>
<td>14</td>
<td>18</td>
<td>Paresthesia, leg pain, orthostatic intolerance, Fatigue, dizziness</td>
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<tr>
<td>4</td>
<td>16</td>
<td>Paresthesia, numbness, limb paralysis, pain</td>
<td>(141)</td>
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<tr>
<td>13</td>
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<td>Allodynia, numbness, severe pain</td>
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<td>Paresthesia, numbness, severe pain</td>
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<td>12</td>
<td>12</td>
<td>Paresthesia, muscle weakness, pain</td>
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<td>Headaches, dizziness, recurrent syncope, orthostatic intolerance, fatigue, myalgias, tachycardia, dyspnea, visual disturbances, phonophobia, cognitive impairment, insomnia, gastrointestinal disturbances, weight loss</td>
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<td>Widespread neuropathic pain, paresthesia, insomnia, profound fatigue</td>
<td>Fibromyalgia</td>
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<td>Widespread neuropathic pain and paresthesia</td>
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<td>Paresthesia, muscle twitching, myalgia, fatigue, hyperhidrosis, and tachycardia, exercise intolerance</td>
<td>Autoimmune myotonia</td>
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<td>Skin rash, fever, nausea, stomach aches, headache, insomnia, night sweats, arthralgia, anxiety, depression, amenorrhoea, elevated serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and low levels of estradiol Depression, sleep disturbance, light-headedness, tremulousness, anxiety, cognitive dysfunction, amenorrhoea, high serum levels of FSH and LH with undetectable estradiol</td>
<td>POF</td>
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<td>13</td>
<td>14</td>
<td>Amenorrhoea preceded by oligomenorrhoa, high serum levels of FSH and LH and low estradiol</td>
<td>(145)</td>
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<td>Case</td>
<td>Age</td>
<td>Presenting Symptom(s)</td>
<td>Diagnosis</td>
<td>Reference</td>
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<td>3</td>
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<td>5 months amenorrhoea preceded by 12 months oligomenorrhoea, hot flashes, low serum levels of estradiol and Anti-Müllerian hormone</td>
<td>(146)</td>
<td></td>
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<tr>
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<td>18</td>
<td>6 months amenorrhoea, low serum levels of estradiol and Anti-Müllerian hormone</td>
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<td>15</td>
<td>3 months amenorrhoea preceded by 9 months oligomenorrhoea, hot flashes, low serum levels of estradiol and undetectable Anti-Müllerian hormone</td>
<td></td>
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<tr>
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<td>Vasculitic rash, soft tissue swellings of ankles and forearms, arthralgia, lethargy, epistaxis</td>
<td>Vasculitis</td>
<td>(147)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Severe flare of cutaneous vasculitis</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>16</td>
<td>Fatigue associated with prolonged menorrhagia, antiplatelet autoantibodies</td>
<td>Thrombocytopenic purpura</td>
<td>(148)</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>Jaundice, hepatosplenomegaly elevated serum aminotransferases</td>
<td>Autoimmune hepatitis</td>
<td>(149)</td>
</tr>
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<td>Severe constant epigastric pain, vomiting, fever</td>
<td>Pancreatitis</td>
<td>(150)</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>Arthralgias, pruritic rashes on lower extremities, bipedal edema, livedo reticularis, proteinuria, positive ANA and anti-dsDNA antibodies</td>
<td>SLE</td>
<td>(2)</td>
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<td>45</td>
<td>Intermittent fever, generalized weakness, oral ulcers, alopecia, malar rash, photosensitivity, arthritis, intestinal pseudo-obstruction, ascites, positive ANA, anti-dsDNA, anti-Ro/SSA and anti-La/SSB antibodies</td>
<td></td>
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<td></td>
<td>58</td>
<td>Malar and scalp rashes, fever, easy fatigability, cervical lymph nodes, gross hematuria and pallor, severe anemia and thrombocytopenia, active nephritis, patient expired a day after hospital admission</td>
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<td>6</td>
<td>32</td>
<td>Fatigue, severe myalgia, polyarthritis, anorexia, severe skin rash, malar rash, aphthous stomatitis, pharyngodynia, cervical lymphadenopathy, alopecia, severe weight loss, anemia, positive ANA and anti-dsDNA antibodies</td>
<td></td>
<td>(151)</td>
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<td></td>
<td>29</td>
<td>Weakness, diarrhea, malar rash, photosensitivity, arthritis, alopecia, severe weight loss, proteinuria, positive ANA and anti-dsDNA antibodies</td>
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<td></td>
<td>16</td>
<td>High-grade fever, generalized asthenia,</td>
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<td>diffuse polyartralgia, multiple erythematous annular cutaneous lesions on the face, trunk, and lower limbs, positivie ANA and lupus anticoagulant Fever, pharyngodynia, erythematous skin lesions of elbows and knees, generalized asthenia, anorexia, polyartralgia, anti-cardiolipin and lupus anticoagulant</td>
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<tr>
<td>16</td>
<td></td>
<td>Mild arthralgia, dyspnea, cervical lymphadenopathy, skin rash, positive ANA and anti-dsDNA antibodies Erythematous facial rash, fever, periorbital edema, weight loss, malaise, fatigue, alopecia, cervical, axillary and inguinal lymphadenopathy, anemia, thrombocytopenia, positive ANA, anti-RNP, anti-Smith and anti-RO/SSA antibodies</td>
<td></td>
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<tr>
<td>19</td>
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<td>Myalgia, arthralgia, generalized weakness, oral ulcers, Raynaud’s phenomenon, alopecia, headache, dyspnea, tachycardia, positive ANA, anti-Sm, anti-Ro, anti-RNP, anti-dsDNA, leukopenia, and complement consumption</td>
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<td>1</td>
<td>19</td>
<td></td>
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<tr>
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<td></td>
<td>Erythematous facial rash, fever, periorbital edema, weight loss, malaise, fatigue, alopecia, cervical, axillary and inguinal lymphadenopathy, anemia, thrombocytopenia, positive ANA, anti-RNP, anti-Smith and anti-RO/SSA antibodies</td>
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<tr>
<td></td>
<td></td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>1</td>
<td>20</td>
<td>Myalgias, arthralgias, livedo reticularis, Raynaud’s phenomenon, headache, tinnitus, positive ANA, lupus anticoagulant and anti-CCP</td>
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<tr>
<td></td>
<td></td>
<td>Juvenile spondyloarthropathy</td>
<td></td>
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<tr>
<td>1</td>
<td>16</td>
<td>Knee joint swelling, low back, buttock and chest wall pain, elevated leukocyte count in the synovial fluid, elevated C-reactive protein</td>
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Figure legends

Figure 1. Effects of Al, Gardasil and Gardasil + Pt toxin injections on exploratory activity and anxiety in C57BL/6 female mice as evaluated by the staircase test. Results are presented as the number of rears (mean ± SEM) during a three minute testing period. (A) Three months post-injection; (B) Six months post-injection. Al-injected mice showed abnormal responses to a novel environment, which was manifested as significantly increased rearing frequency at three months of age, (indicative of increased anxiety) and later the opposite, significantly decreased rearing frequency (indicative of a non-selective attention deficit).

Figure 2. Effects of Al, Gardasil and Gardasil + Pt toxin injections on spatial memory in C57BL/6 female mice as evaluated by the Y-maze test. Results are presented as a mean ± SEM preference index for spending time in a new arm (time in the new arm - time old arm/time in the new arm + time in the old arm). (A) Three months post-injection; (B) Six months post-injection. Al-injected mice showed a decreased preference for the new arm at three months of age.

Figure 3. Effects of Al, Gardasil and Gardasil + Pt toxin injections on depressive behavior in C57BL/6 female mice as evaluated by the forced swimming test (FST). Results are presented as duration in seconds (mean ± SEM) of immobility, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the six minute test. A depression-like behavior was considered as an increased immobility time (A) Three months post-injection; (B) Six months post-injection. At six months of age all treated mice exhibited a significant increase in depressive-like behavior compared to control mice.

Figure 4. Titers of serum antibodies one month post injection with either Al (A), Gardasil (G), Gardasil + Pt toxin (Gp) and vehicle (V). A home-made ELISA was used to detect the levels of anti-HPV, anti-Al hydroxide (Alum), anti-mouse brain protein extract, anti-dsDNA, anti-BSA and anti-β2glycoprotein-I (β2GPI) antibodies in the sera of immunized mice. Data are presented as mean ± SEM of 3 experiments, n=5 per group.

Figure 5. Inhibition of the binding of anti-HPV L1 antibodies from the sera of Gardasil injected mice to HPV antigens by the mouse protein extract. Data are presented as mean ± SEM of 3 experiments, n=5 per group.

Figure 6. Iba-1 immunostaining in the CA1 area of the hippocampus of C57BL/6 female mice injected with Al, Gardasil and Gardasil + Pt toxin. Brain sections from 5 animals out of each group were examined quantitatively for differences in immunostaining density using Image J software (NIH, USA) as described in Materials and Methods. The data are presented as % Intensity (mean ± SEM).