Doctoral Thesis

Autoantibody and environmental damage to the brain

María Teresa Arango Guerrero

October, 2016

Autoantibody and environmental damage to the brain

María Teresa Arango Guerrero

Universidad del Rosario

Bogotá, Colombia

Professor Yehuda Shoenfeld MD, FRCP, MaACR

Professor Miri Blank PhD

Doctor Shaye Kivity MD

Zabludowicz Center for Autoimmune Diseases

Tel Aviv University

Sheba Medical Center

Tel Hashomer, Israel

CONTENT

Behavioral abnormalities and cognitive dysfunction may be present in patients with autoimmune diseases. These symptoms range from mild symptoms to acute life-threatening events. The mechanisms that responsible for these neuropsychiatric manifestations are still largely unknown, however several pathogenic pathways have been identified such as antibody-mediated neurotoxicity, vasculopathy induced by anti-phospholipid antibodies, cytokine-induced neurotoxicity, as well as external factors such as toxics, and medications. In order to further investigate some of these mechanisms we evaluated the effect of autoantibodies in animal models such as 16/6 idiotypic antibody and anti-ribosomal P antibodies in neuropsychiatric systemic lupus erythematosus , total IgG from narcoleptic patients in narcolepsy, and the effect of the human papillomavirus vaccine in naïve mice.

Using the passive immunization method through intracerebro-ventricular injection of the antibodies, we demonstrated histological and behavioral changes. Mice immunized with 16/6 idiotypic antibodies developed cognitive impairments while those immunized with anti-ribosomal P antibodies developed depression. The mice that received total IgG from narcoleptic patients developed sleep disturbances and brain histological changes consistent with the disease. Further analyses of the role of the human anti-ribosomal P autoantibody revealed that it can cross-react with the neuronal protein Gap43, thus interfering with cellular processes.

Immunization with the human papillomavirus vaccine caused the production of antibodies against brain components. Moreover, the mice immunized with the vaccine or with its adjuvant developed cognitive and behavioral deficiencies, which were ameliorated with dietary phospholipid supplementation.

Overall, herein we demonstrate that the behavioral and cognitive abnormalities can be part of the wide spectrum of clinical autoimmune manifestations. In addition, they can be caused by collateral damage due to the immune dysregulation caused by autoimmune conditions as well as by vaccination. We also suggest that different autoantibodies cause different symptoms based on different interactions with brain tissue.

Las anormalidades de comportamiento y disfunciones cognitivas pueden presentarse en pacientes con enfermedades autoinmunes. Estos síntomas pueden fluctuar de leves hasta eventos potencialmente mortales. En su gran mayoría los mecanismos responsables de estas manifestaciones neuropsiquiatricas siguen siendo desconocidas, sin embargo se han identificado varias vías patogénicas. Por ejemplo; neurotoxicidad mediada por anticuerpos, vasculopatía inducida por anticuerpos anti-fosfolípidos, neurotoxicidad inducida por citoquinas, así como factores externos que incluyen sustancias tóxicas y medicamentos. Para poder investigar algunos de estos mecanismos, evaluamos el efecto de auto-anticuerpos relacionados con el lupus eritematoso sistémico neuropsiquiátrico en modelos animales (Anticuerpo idiotípico 16/6 y el anticuerpo anti-ribosoma P) y narcolepsia (empleando IgG de pacientes narcolépticos), así como el efecto de la vacuna del virus del papiloma humano en ratones.

La inmunización pasiva por inyección intracerebro-ventricular de estos anticuerpos condujo a cambios histológicos, cognitivos y de comportamiento. En particular, los ratones inmunizados con el anticuerpo idiotípico 16/6 desarrollaron déficit cognitivo y los ratones inmunizados con el anticuerpo anti-ribosomal P desarrollaron depresión. Los ratones que recibieron IgG total de pacientes narcolépticos desarrollaron trastornos del sueño y cambios histológicos cerebrales consistentes con la enfermedad. Un análisis más profundo

del papel del anticuerpo anti-ribosomal P humano reveló que reacciona de manera cruzada con la proteína neuronal “Gap43” interfiriendo con procesos celulares.

Por otra parte, la inmunización con la vacuna del virus del papiloma humano causó la producción de anticuerpos contra componentes del cerebro. Adicionalmente, los ratones inmunizados con la vacuna, al igual que los inmunizados con su adyuvante desarrollaron deficiencia cognitiva y conductual, que fue mejorada con la suplementación alimenticia basada en fosfolípidos.

En general, aquí se demuestra que anormalidades del comportamiento y disfunciones cognitiva pueden ser parte de la amplia gama de manifestaciones clínicas autoinmunes. Además, nuestros resultados sugieren que estas pueden ser causadas por daños colaterales debido a la desregulación inmune causada por autoinmunidad, así como por la vacunación. Adicionalmente, sugerimos que diferentes auto-anticuerpos causan diferentes síntomas basados en diferentes interacciones con el tejido cerebral.

I would like to thank everyone and all the people than came by during all this process and helped me with their advice, their support, and their teachings. Every single person I have met during the last 5 years helped me to finalize this work.

First of all, I thank the *Universidad del Rosario* for the giving me the opportunity to do the doctoral program in such a prestigious academy, were I was granted a scholarship. I also, would like to thank the Zabludowicz Center for Autoimmune Disease, for opening their doors and embracing me as a family to develop my thesis work. In addition, I will be grateful all my life with the Federico and the Colton Foundations, especially with Mrs. Judy and Mr. Stewart Colton for founded my research period in Israel.

I would like to acknowledge all the members of the CREA – Centro de Estudio de Enfermedades Autoimmunes - where I started this journey; especially to Professor Juan-Manuel Anaya, for showing me a new path to follow in the field of autoimmunity and for introducing me to my mentor Professor Yehuda Shoenfeld.

Now it is the opportunity to give very special thanks to my mentor and guide during all this process, Professor Yehuda Shoenfeld. He gave me all his support in all possible and imaginable ways. I will never forget how he stood by me, all the time in the good times and in the bad ones, and how he helped me to grow as a researcher but also as a person. I would

also like to express my sincere gratitude for my supervisors Professor Miri Blank and Doctor Shaye Kivity for their invaluable guidance and support carrying out this project; as well as their great heart. I also would like to thank my coworkers at the laboratory and all the staff from the Zabludowicz Center for Autoimmune Diseases: My lovely Sabta Sara, Zipi, Nancy, Tomer, Boris, Luda, Smadar, Nina, and Hen and to all those student that were next to me. As well to Professor Joab Chapman and his team for the support every time I need it. Additionally I would like to give a special gratitude to Professor Eiji Matssura for giving me the opportunity to visit his laboratory in the Okayama University.

I would like to thank my Israeli Family, the Sehayek, who adopted me as one more daughter and taught me to love Israel. Additionally I would like to mention my Israeli sister Anna and her family, for her optimism, her permanent support and great human warmth. Also, the Traum Family, especially Itamar. And all the students I have had the great opportunity to meet and share amazing moments, especially: Angela, Paola, Paula, Shir, Sibel, Tomer, Giorgia, Mathilde, Luisa, Jasna, Mojca, Gali, Valeria, Jose Jiram, Lucja, Luiz, Elena, Michel, Sara, Andrés and his family.

I would never have gone through the PhD, without the support of my family and my friends, they deserve all my gratitude. My parents who are the driving motivation for all I have done in my life. My little sister, who believes in me and has always, supported me in her own particular way. My cousins Julian y Laura, and my aunts Maruja, Ligia, Gloria y Martha who

have been always unconditional. To my friends from the school and the university; especially Panda, Zulay, Natalia and Daniella, the sister I chose myself.

I dedicate this work to my parents for making me who I am, for teaching me all the values I have, and for being the best parents someone can have. Finally, I would like to mention my Uncle, Alberto who is accompanying me from Heaven and was an inspiration to start this research and teaching life.

Me gustaría dar las gracias a todas las personas que me acompañaron durante todo este proceso; que me ayudaron con sus consejos, su apoyo y sus enseñanzas. Cada persona que conocí durante los últimos 5 años me ayudó a finalizar este trabajo.

En primer lugar, doy las gracias a Dios. Segundo a la Universidad del Rosario por darme la oportunidad de realizar el programa de doctorado en una academia tan prestigiosa, además de concederme una beca. También me gustaría agradecer al *Zabludowicz Center for Autoimmune Disease*, por abrir sus puertas y recibirme como familia para desarrollar mi trabajo de tesis. Además, estaré agradecida toda mi vida con las Fundaciones Federico y Colton, especialmente con la Sra. Judy y el Sr. Stewart Colton por financiar mi período de investigación en Israel.

Quiero agradecer a todos los miembros del CREA - Centro de Estudio de Enfermedades Autoimmunes - donde comencé este viaje; especialmente al profesor Juan-Manuel Anaya, por mostrarme un nuevo camino a seguir en el campo de la autoinmunidad y por presentarme a mi mentor el profesor Yehuda Shoenfeld.

Que sea esta la oportunidad de dar un agradecimiento muy especial a mi mentor y guía durante todo este proceso, el Profesor Yehuda Shoenfeld. Él me dio todo su apoyo de todas las formas posibles e imaginables. Nunca olvidaré cómo estuvo conmigo, todo el tiempo en

los buenos tiempos y en los malos momentos, y cómo me ayudó a crecer como investigador, pero también como persona. También quiero expresar mi sincera gratitud a mis supervisores Miri Blank y al Doctor Shaye Kivity por su inestimable orientación y apoyo para llevar a cabo este proyecto, y su gran corazón. También me gustaría dar las gracias a mis compañeros de trabajo en el laboratorio y todo el personal del *Zabludowicz Center for Autoimmune Disease*: Mi hermosa Sabta Sara, Zipi, Nancy, Tomer, Boris, Luda, Smadar, Nina y Hen; y a todos los estudiantes que trabajaron a mi lado. También al profesor Joab Chapman y su equipo por haberme apoyado cada vez que lo necesité. Además, quisiera plasmar mi especial gratitud al profesor Eiji Matssura por darme la oportunidad de visitar su laboratorio en la Universidad de Okayama.

Me gustaría dar las gracias a mi familia israelí, los Sehayek, quienes me adoptaron como una hija más y me enseñaron a amar a Israel. Además, quiero también mencionar a mi hermana israelí, Anna y su familia, por su optimismo, su apoyo permanente y gran calidez humana. Por otra parte mi mejor amigo Shaye, por haberme apoyado tendiéndome su mano en cada situación por 5 años. A la Familia Traum, especialmente Itamar. Y a todos los estudiantes que tuve la increíble oportunidad de conocer y de compartir momentos increíbles, especialmente: Angela, Paola, Paula, Shir, Sibel, Tomer, Giorgia, Mathilde, Luisa, Jasna, Mojca, Gali, Valeria, José Jiram, Lucja, Luiz, Elena, Michel, Sara, Andrés y su familia.

Finalmente, quisiera mencionar que nunca habría recorrido el camino del doctorado, sin el apoyo de mi familia y mis amigos, quienes merecen toda mi gratitud. Mis padres que son la

motivación de todo lo que he hecho en mi vida. Mi hermana menor, quien siempre ha creído en mí, y me demostrado todo su apoyo de maneras muy particulares. A mis primos Julian y Laura, y mis tías Maruja, Ligia, Gloria y Martha quienes han sido siempre incondicionales. Y finalmente, a mis amigos del colegio y de la universidad; Especialmente Panda, Zulay, Natalia y Daniella, que al final se convirtió en la hermana que elegí.

Dedico este trabajo a mis padres por hacerme quien soy, por enseñarme todos los valores que tengo y por ser los mejores padres que alguien pueda tener. Por último, quisiera mencionar a mi tío Alberto, quien me acompaña desde el cielo y fue una inspiración para comenzar la carrera en investigación en mi vida.

ABBREVIATIONS

Anti-fibroblast antibodies (AFA)

Antigen-presenting cells (APCs)

Autoimmune/inflammatory syndrome induced by adjuvants (ASIA)

Blood-brain barrier (BBB)

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

Double stranded DNA (ds-DNA)

Fc receptors (FcR)

Human Hepatitis B vaccine (HBVv)

Human papilloma virus (HPV)

Human papilloma virus vaccine (HPVv)

Hypocretin (orexin) receptor 2 (HCRT2)

Intra-cerebra ventricular (ICV)

Intravenous immunoglobulins (IVIgs)

Melanin-concentrating hormone neurons (MCH)

Monoclonal antibody (mAb)

Neuromyelitis optica (NMO)

Neuropeptide glutamic acid-isoleucine (NEI)

Neuropsychiatric systemic lupus erythematosus (NPSLE)

N-methyl-D-aspartate receptor (NMDAR)

Nucleoprotein (NP)

Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal

(PANDAS)

Phosphatidylcholine (PC)

Rapid eye movement (REM)

Single stranded-DNA (anti-ssDNA)

Systemic lupus erythematosus (SLE)

Tight junctions (TJs)

Tribbles 2 (Trib2)

α–melanocyte-stimulating hormone (αMSH)

1.1 THE IMMUNE SYSTEM AND THE BRAIN

For a long time, the central nervous system (CNS) was considered an immune-privileged organ, in which antigens do not elicit an immune response. However, over the years this idea was challenged due to evidence demonstrating that while the access of different components of the peripheral immune system to the brain is tightly regulated, there is a presence of cells with immune function within the brain parenchyma. Examples of these cells are resident microglia and astrocytes which are found in the brain parenchyma, as well as from an extensive immune repertoire in the meninges and choroid plexus. Moreover, recent evidence shows that peripheral lymphocytes can migrate through the choroid plexus to the Cerebrospinal fluid (CSF) (1). In addition, new suggestions indicate that the immune system supports the proper function and development of the CNS.

The maintenance of the microenvironment of the CNS is essential for its normal function; ensuring the normal brain performance which has a critical role in cognition, regulation of metabolism and coordination of peripheral organs. Specific conditions are necessary to allow electrical and chemical signals to transit between neurons. This delicate balance is largely maintained by the presence of the blood-brain barrier (BBB), which regulates the communication and the entrance of different substances from the blood stream to the CNS (i.e. ions, oxygen, nutrients and cells). The BBB is one of the most selective and tightly

controlled barriers (2, 3). It is comprised of endothelial cells, pericytes, astrocytes, and neurons that collectively form a functional neurovascular unit. The BBB endothelial cells are the main contributor to the special characteristics of the BBB, and these cells express specific transporter and receptor proteins which control the entry and exit of metabolites across them (transcellular transport). In addition, they have high electrical resistance tight junctions (TJs), which limit the movement and traffic of substances between adjacent cells (paracellular transport). Interestingly, endothelial cells of the BBB have a lower amount of transcytotic vesicles and an absence of fenestraes when compared to endothelial cells from other tissues (2, 4).

1.2 ANTIBODIES AND THE BRAIN

Autoimmune diseases are immune-mediated inflammatory disorders characterized by loss of tolerance to self-antigens and the presence of auto-reactive T cells or various autoantibodies. Autoantibodies can be detected in ~5% of the healthy population, whereas approximately 2%-3% of them can potentially recognize brain structures *in-vitro* (5), but are not necessarily pathogenic. The presence of brain autoantibodies has been related to three conditions: First, they can appear as part of a systemic or organ specific autoimmune diseases (6, 7). Second, external factors such as infections or vaccination can induce the production of cross-reactive antibodies resulting in clinical manifestations associated with neurological damage (e.g. Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal –PANDAS) (8, 9). Third, in certain individuals the presence of malignancy

can induce the production of auto-antibodies, which can potentially recognize brain structures due to production and presentation of cryptic antigens (i.e. paraneoplastic disorders) (5, 10, 11). Understanding the role of autoantibodies in neurological diseases represents a challenge, since the access to this organ and its pathological analysis *in-vivo* is a difficult task. Therefore, most human studies have been performed *post-mortem*, which may create a bias due to tissue modifications that occur after death. Moreover, in cases when the disease lasted for a long period of time the original process could be masked and might not be identified at the time of the study (5, 12-14).

Several autoantibodies are directly responsible for inflammatory brain disorders such as, anti-N-methyl-D-aspartate receptor (NMDAR) in encephalitis (14), aquaporine-4 in neuromyelitis optica (NMO)(6) and anti-Hu in limbic encephalitis (14, 15). These antibodies are considered pathogenic because they can recognize a specific nervous system antigen, and were found to alter pathways related to the disease pathogenesis (16, 17). For instance, in NMO, autoantibodies against aquaporin-4, a protein expressed on the astrocytes membrane, causes complement activation leading to chronic inflammation and demyelination of the optic nerve and the spinal cord (6).

Four possible mechanisms of autoantibody-mediated brain damage were proposed: First, autoantibodies can be deposited in immune complexes within brain tissue activating the complement system (18). Second, autoantibodies may also induce necrosis or apoptosis *via* calcium influx into neurons. Third, auto-antibodies can interfere with neurotransmitter

signaling (19). Finally, the presence of autoantibodies can also impair the ability of brain cells to absorb nutrients. Another indirect explanation is that autoantibodies can interfere with pathways affecting the CNS from outside the brain (e.g. hypothalamic–pituitary–adrenal axis) (19).

1.2.1 Auto antibodies and systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a wide spectrum of clinical manifestations, which may affect almost every organ in the body. More than 180 autoantibodies have been described in SLE (20, 21). They can target numerous molecules of the human body, but are mainly directed to nuclear and intracellular components. Autoantibodies in SLE patients differ in their prevalence and may relate to different clinical manifestations (20, 22). For example, anti-double stranded DNA (ds-DNA) antibodies are most frequently associated with disease activity, mainly lupus nephritis (23). Several SLE autoantibodies can appear in other autoimmune diseases (e.g. anti-Ro, anti-La) (20, 22). However, not all the autoantibodies described in SLE are pathogenic or linked to disease activity, and can also appear in healthy individuals (24).

Neurologic and psychiatric symptoms are common manifestations in SLE, and above 50% of SLE patients suffer from these symptoms (25). They were collectively designated as neuropsychiatric SLE (NPSLE) manifestations. The American College of Rheumatology (ACR) established 19 case definitions in 1999 in an attempt to better classify and define NPSLE, especially for the purpose of clinical studies (26). Above 20 autoantibodies, systemic or

brain-specific, were detected in patients with NPSLE (7). Among the systemic antibodies, the anti-ribosomal P and the anti-DNA autoantibodies have been extensively studied with regard to NPSLE. Anti-ribosomal P antibodies recognize epitopes located in the large ribosome subunit, particularly in the P0, P1 and P2 proteins. This autoantibody is detected in 10-30% of NLSLE patients, mostly with psychosis or depression, but also in SLE patients with hepatitis and kidney involvement (25, 27-30). Several studies have shown the ability of anti-ribosomal P antibodies to bind brain tissue *ex-vivo* (31). Our group demonstrated that passive transfer of anti-ribosomal P antibodies to naive mice induces depression-like behavior by the force-swimming test (32). The 16/6 idiotype antibody, a human anti single stranded-DNA (anti-ssDNA) monoclonal antibody (mAb) (33) was also related to NPSLE. This particular autoantibody was found to cross-react with cytoskeletal proteins (vimentin), platelets, lymphocyte membranes, bacterial pathogens, glycoproteins, brain glycolipids and tumor cells, and, its deposits were found in the skin, kidney and brain tissue (34-36). The 16/6 autoantibody is present in 30% of SLE patients, and its levels were found higher in NPSLE patients (37).

1.2.2 Autoantibodies and Narcolepsy

Narcolepsy is a sleep disorder resulting from the lack of orexin, an essential neurotransmitter involved in the sleep-arouse equilibrium (38). *Post-mortem* analyses of narcoleptic patients demonstrate the loss of orexin producing neurons in the hypothalamus (39). Knockout mice for orexin also exhibit narcoleptic behavior (40). Narcoleptic patients suffer from uncontrollable sleep attacks, characterized by a rapid eye movement (REM)

sleep pattern, which is not preceded by a non-REM stage. In some patients, the sleep disturbance is accompanied with other symptoms such as cataplexy (loss of muscle tone), sleep paralysis and hallucinations (41, 42).

Currently, narcolepsy is considered a rare disease with a world prevalence ranging from 25 to 50 per 100,000 people (43). Its prevalence varies from one region to another, suggesting the importance of genetic and environmental factors (44-46). The orexin loss in narcolepsy resembles the specific destruction of insulin-producing β-cells in the pancreas of patients with diabetes mellitus type 1, which is an autoimmune disease (47, 48). There is a strong association between narcolepsy and specific polymorphisms of immune related genes. Several studies demonstrated that narcolepsy is highly associated with HLA risk polymorphisms, in particular *DQB1*06:02* and *DQA1*01:02*. The higher risk is reported for *DQB1*06:02* carriers as it is present in 82% to 99% of narcoleptic patients (49, 50), while only 12% to 38% of healthy individuals have this allele. Indeed, homozygote individuals for *DQB1*06:02* have an increased risk of developing narcolepsy (49-51). Other polymorphic associations have been described with other genes such as tumor necrosis factor alpha (42), tumor necrosis factor (ligand) superfamily member 4, T cell receptor alpha chain (52), Cathepsin H, DNA methyltransferase I (53, 54), among others (55). Many of the polymorphisms are in immune-related genes supporting the hypothesis of an immune-mediated mechanism, which may involve antigen presentation, including lymphocyte sub-populations as well as antigen presenting cells (13). Taken together, these clues suggest that narcolepsy may be an autoimmune disorder.

Due to the common mechanisms in autoimmune diseases (56), the presence of more than one autoimmune disease in one patient is common (57). A study in a Spanish cohort showed that approximately 16% of the narcoleptic patients had one or more immunopathological disorders including allergies and autoimmune diseases, such as systemic lupus erythematosus and multiple sclerosis. In addition, familial autoimmunity was recently demonstrated to be a frequent condition (58). In the case of narcolepsy, first-degree relatives of patients have a higher risk to develop the disease (59, 60), supporting the importance of genetic background. However, twin studies demonstrated that the concordance rate of narcolepsy in monozygotic twins is 20 – 35% suggesting that the development of the disease does not depend only on the genetic background but also on environmental factors (60).

1.2.3 Autoantibodies caused by environmental factors in narcolepsy

In genetically susceptible individuals autoimmune diseases can be triggered by exposure to external molecules or factors. As mentioned above, the concordance rate of narcolepsy in twins indicates the importance of environmental factors. Interestingly, the age of onset in narcolepsy is frequently during teen years, suggesting that hormonal changes in puberty might trigger the disease (46, 61-64). Few reports regarding the exposure to toxic substances have been done. For example, one study comparing narcoleptic patients with a group of match controls showed that exposure to heavy metals, woodwork, fertilizers and pesticides are a risk for narcolepsy in a particular population (65). Passive smoking has also

been related with the onset of the disease in HLA DQB1*06:02 carriers (66). In addition, other external stressors such as major changes in sleeping habits or changes in living style carried an additional risk (64). Unfortunately, all of this evidence is limited to small cohorts and specific populations making it necessary to clarify the role of these factors in the development of the disease. Nevertheless, the strongest and most discussed evidence regarding environmental factors and narcolepsy is its association with infections and vaccination. In 2007, the medical records analyses of narcoleptic *DQB1*06:02* carriers and matched controls as well as the evaluation of detailed questionnaires demonstrated the importance of infectious agents. Thus, measles infection and the presence of unexplained fever in the past history were reported to be associated with a higher risk of developing subsequent narcolepsy (64). It is widely recognized that infections can induce autoimmunity through different mechanisms such as molecular mimicry, epitope spreading, bystander activation and superantigens (67-71). In the narcolepsy scenario, it has been suspected that streptococcal and influenza A infections as well as the H1N1 vaccine play a role in the pathogenesis of the disease (72-74).

Despite recent descriptions of higher levels of inflammatory cytokines (i.e. G-CSF and IL-8) in the plasma of narcolepsy patients (75), there is no evidence of an inflammatory process. A major obstacle is the inability to analyze brain specimens of patients at early stages of the disease (5, 12-14). The genetic polymorphisms associated with the disease are related with the immune system, and two of them are related with antigen presentation: the HLA DQB1*06:02 and the TCRα polymorphism. This may indicate the importance of the T cell

response in the pathogenesis of narcolepsy, as it interacts directly with the HLA. It is possible that in predisposed individuals pathogenic T cells have escaped from the central tolerance process in the thymus. In consequence, these cells may have the ability to be stimulated by external factors and finally evolve into an autoimmune response against orexin neurons. In fact, functional analyses of $CD4^+$ lymphocytes from narcoleptic patients, but not from the controls, showed that these cells were able to recognize orexin peptides when they were presented by dendritic cells (homozygotic for DQA1*01:02/DQB1*06:02 haplotype), however the authors failed to reproduce these results (76-78).

1.2.3.1 Specific antibodies and narcolepsy

The importance of B-cell mediated response has also been evaluated in narcolepsy. Passive transfer of antibodies from narcoleptic patients to murine models has demonstrated either sleep behavioral disturbances or brain histological changes (79, 80). Moreover, sera from narcoleptic patients can bind brain or muscle structures (12, 80). However, the specific mechanisms by which the antibodies induce these changes are still unknown. So far, some attempts to identify a possible auto-antigen have been done based on the analysis of the specificity of antibodies from narcoleptic patients. In 2010, Cvetkovic-Lopes *et al*, described the presence of antibodies against Tribbles 2 (Trib2) in narcoleptic patients, which bound orexin producing neurons in mouse brains (81). These auto-antibodies were described originally in autoimmune uveitis (81) and they were found to have higher titers in a small group of narcoleptic patients (14%) when they were compared with healthy controls (81-83). A recent study also found that passive transfer of IgG from narcoleptic patients in rat

brains induced changes in different sleep parameters. Three different patterns in which sera of narcoleptic patients can bind to brain rat tissue were identified. In the first pattern, antibodies bound mainly hypothalamic melanin-concentrating hormone and pro-opiomelanocortin, but not orexin neurons. Interestingly, a more detailed analysis of the first pattern showed that antibodies recognized a common C-terminal epitope in the neuropeptide glutamic acid-isoleucine (NEI) and the α–melanocyte-stimulating hormone (αMSH) peptides. These two molecules are present in the hypothalamus in the melanin-concentrating hormone neurons (MCH) but not in orexin producing neurons (84). These results contrast Cvetkovic-Lopes report, which showed that sera from patients can bind orexin producing neurons (81). Unlike anti-Trib2, the presence of antibodies to MSH expressing neurons can potentially interfere in the process associated with narcolepsy, since MSH neurons can modulate orexin neurons functions (85, 86).

1.2.4 Adjuvants, autoimmunity and the brain

The development of an autoimmune condition is the result of the interaction between genetics and environmental triggers. Environmental factors, such as chemicals, drugs, alcohol and cigarette consumption, organic solvents, and infectious agents, have been associated with the development of autoimmune diseases (87-89). The study of the effect of environmental factors on patients with autoimmune diseases is limited to descriptive and epidemiological research, while animal models may provide a platform to investigate pathogenic mechanisms. Experimental models used for autoimmune diseases are

genetically engineered (i.e NZBxW F1) or induced by immune-boosting compounds (i.e. pristane induce arthritis) or by immunization of antigens and adjuvants (i.e CFA-arthritis), in susceptible mice strains. Interestingly, the development of some autoimmune diseases in animals does not depend on the administration of an antigen which is associated with the disease (90, 91).

1.2.4.1 Animal models and adjuvants

Many animal models of autoimmune diseases are developed based on the identification of a pathogenic antigen of the disease; thus the immunization of the specific antigen will develop a strong immune response resulting in autoimmunity (92). Usually, these immunizations are carried out together with adjuvants in order to potentiate the immune response (93). However, the immunization of susceptible individuals just with the common adjuvants has been found to induce the appearance of autoimmune characteristics. For instance, it has been shown that the adjuvants pristane, squalene, and Freund's adjuvant (complete or incomplete) can induce the appearance of antibodies related to SLE (94, 95), antiphospholipid syndrome (96) and Sjogren syndrome (97). Moreover, the treatment with the most common adjuvant, aluminum can accelerate or induce the exacerbation of autoimmune characteristics in animal models (97, 98).

These results in animal models suggest that there might be a risk of the use of certain adjuvant in vaccine preparations. In the particular case of aluminum, different forms of aluminum salts are used in vaccines against hepatitis B, hepatitis A, tetanus, and human

papilloma virus (HPV). Aluminum is a potent stimulator of humoral response trough of Th2 lymphocytes, however the mechanisms by which this activation occurs are still not well understood (99, 100). Different animal models have demonstrated that after the injection of aluminum adjuvant small particles can travel to distant organs, such as the spleen and the brain (101), inducing inflammation and immune response (102, 103). The ability of antigen-presenting cells (APCs) to take up the aluminum particles allows for some proportion of aluminum to move from the injected muscle, mainly within immune cells. In sequence, aluminum particles could then travel to the lymph nodes, exit the lymphatic system to reach the bloodstream, and eventually gain access to distant organs, including the spleen and the brain where aluminum deposits were still detected one year after injection (101). This is known as the Trojan horse-mechanism, which allows aluminum to enter and accumulate in the brain within macrophages. The toxic effect of the aluminum can result in neurocognitive adverse manifestations which have been previously reported after the administration of aluminum-containing vaccines (102, 103). In sheep it has been shown that continuous inoculation with aluminum adjuvanted vaccines can cause severe damage. For instance, the animals presented severe neurobehavioural outcomes such as restlessness, compulsive wool biting, generalized weakness, muscle tremors, loss of response to stimuli, ataxia, tetraplegia, stupor, coma and death. Post mortem analysis showed brain inflammatory lesions and the presence of aluminum in central nervous system tissues (102).

1.2.4.2 Autoimmune/inflammatory syndrome induced by adjuvants

So far, vaccines are the most successful approach in preventing infectious diseases. Global policies have been applied to maximize the immunization of different vaccines due to their effectiveness (104, 105). The main goal of a vaccine is to trick the immune system into thinking there is an infection and forces it to develop a response that will end in the development of a sustained memory immune response. The role of adjuvants is to assure the primary immune response against the particles of the pathogen within the vaccine (93, 106). However, side effects associated to vaccines have been reported, including different autoimmune conditions (107). Indeed, they can have a wide spectrum of side effects which can be related to the initiation or relapse of an autoimmune manifestation. They are currently known as the autoimmune/inflammatory syndrome induced by adjuvants (ASIA) (108-110). Vaccination-related ASIA is uncommon and it can be prevented if the individual risk is taken into account prior to the vaccination. Therefore, the specific background of a particular patient is considered (previous post-vaccination autoimmune phenomena, medical history of autoimmunity, allergies, family history of autoimmune diseases; presence of autoantibodies; and genetic background) (111). Interestingly, some of the main signs of ASIA syndrome are neurological manifestations (109, 110), such as chronic fatigue, depression, sleep disturbances, cognitive impairments, demyelination syndromes, optic neuritis and others (112-115). In the case of human papilloma virus vaccination, in particular, there seems to be a greater proportion of central nervous system-related autoimmune reactions, (for example, opsoclonus myoclonus, mood swings, depression and anxiety (116, 117).

In view of the evidence brought up above, we believe that specific autoantibodies as well as environmental factors such as adjuvants play a major role in the pathogenesis of different immune mediated manifestations such as NPSLE, narcolepsy and ASIA syndrome. Since the analyses of brain tissue from patients is not feasible, we use animal models in order to understand how autoantibodies and vaccine components can be involved in brain damage in these particular disorders.

2 PROJECT AIMS

- Evaluation of the effects of passive transfer of autoantibodies into the brain of naïve mice *via* intra-cerebra-ventricular injection. The following antibodies were injected
    - Anti-ssDNA 16/6 idiotypic antibody
    - Anti-ribosomal P antibody
    - Total IgG isolated from narcoleptic patients

- Evaluation of the effects of the administration of human papilloma virus vaccine in different autoimmune mouse models
    - Evaluation of the effect of aluminum adjuvanted vaccine in a healthy mouse strain (C57BL/6)
    - Evaluation of therapeutic effect of phospholipid supplementation

3.1 PAPER I:

16/6-idiotype expressing antibodies induce brain inflammation and cognitive impairment in mice: the mosaic of central nervous system involvement in lupus. Kivity S, Katzav A, Arango MT, Landau-Rabi M, Zafrir Y, Agmon-Levin N, Blank M, Anaya JM, Mozes E, Chapman J, Shoenfeld Y. BMC Med. 2013 Apr 4;11:90. (I.F 8.41). Describes how the 16/6-idiotype (anti-dsDNA human monoclonal autoantibody) induces brain inflammation and cognitive deficit in mice after passive transferred via intra-cerebra-ventricular injection (118).

3.2 PAPER II:

Anti-ribosomal-phosphoprotein autoantibodies penetrate to neuronal cells via neuronal growth associated protein, affecting neuronal cells *in vitro*. S Kivity, Y Shoenfeld, MT Arango, D J. Cahill,SL O'Kane, M Zusev, I Slutsky, M Harel-Meir, J Chapman, T Matthias and M Blank. Rheumatology Oxford. May 2016. pii: kew027. [Epub ahead of print]. (I.F 4.475) Describes how the human autoantibody anti-ribosomal P can cross-react with the brain protein Gap43, penetrating hippocampal cells and interfering with cellular processes (119).

3.3 PAPER III:

Passive transfer of narcolepsy: anti-TRIB2 autoantibody positive patient IgG causes hypothalamic orexin neuron loss and sleep attacks in mice. *Katzav A, *Arango MT, Kivity S, Tanaka S, Givaty G, Agmon-Levin N, Honda M, Anaya JM, Chapman J, Shoenfeld Y. J

Autoimmun. 2013 Sep; 45:24-30. (I.F 8.41). Describes how the passive transfer of total IgG from patients with narcolepsy induces clinical manifestations and histological changes in the brain similar to those presented by narcoleptic patients (79).

3.4 PAPER IV:

Behavioral abnormalities in female mice following administration of aluminum adjuvants and the human papillomavirus (HPV) vaccine Gardasil. Inbar R, Weiss R, Tomljenovic L, **Arango MT**, Deri Y, Shaw CA, Chapman J, Blank M, Shoenfeld Y.. Immunol Res. 2016 Jul 16. [Epub ahead of print] (I.F 3.098). The article describes how in a healthy mouse strains cognitive and behavioral deficit which are related to brain damage, as well as antibodies against brain components after they were immunized with the human papillomavirus vaccine (120).

3.5 PAPER V:

Phospholipid supplementation can attenuate vaccine-induced depressive-like behavior in mice. *Kivity S, *Arango MT, Molano-González N, Blank M, Shoenfeld Y. Immunol Res. 2016 Jul 27. [Epub ahead of print] (I.F 3.098). This paper describes how the behavioral impairment induced by the vaccine is attenuated by phospholipid supplementation (121).

4 BRIEF DESCRIPTION OF ADDITIONAL ACTIVITIES THAT WERE PERFORMED REGARDING NARCOLEPSY

4.1 METHODOLOGY

4.1.1 PET-CT scan and autoradiography

Total IgG from narcoleptic patients as well as commercial IgG (control), were labeled with radioactive isotopes (4). The mice were passively immunized by ICV injection with the labeled antibodies (79). After the immunization a PET-CT scan (positron emission tomography - computed tomography), was done daily for four days to follow the localization of the antibodies. After the final PET-CT scan the mice were sacrificed by cervical dislocation, their brains were dissected, and an autoradiography analysis was performed.

4.1.2 Active Immunization of peptides

C3H four weeks old mice were housed in the animal facility at Sheba medical center, and raised under standard conditions, temperature of (23±1ºC), 12-hour light cycles (6:30 AM–6:30PM) and free access to food and water. All procedures were approved by the Israeli Ministry of Health Animal welfare committee. The mice were actively immunized twice by footpath injection at 15 and 18 weeks of. Immunization was performed with 10ug of peptides per mouse in Complete Freund adjuvant (CFA). Three groups were immunized, the first one was immunized just with the adjuvant as control. The second one received the peptides from the human orexin that could potentially cause cross reaction at T cell level: Orx-1: AGNHAAGILTLGK, Orx-2: SGNHAAGILTMGR, Hg: ERNAGSGIIISDT (77, 78). Finally, the third group was immunized with the peptides from αMSH: EIGDEENSAKFPI-NH2 and NEI: SYSMEMFRWGKPV-NH2, which were identified as potential targets of autoantibodies in narcoleptic patients (84).

4.1.3 ELISA:

The production of antibodies to the different peptides was tested by home-made ELISA: mice sera at different time points and dilutions was added to ELISA plates coated with a specific peptide and blocked with 3% BSA for 2hrs. Sera was added at dilution of 1:200-1:2000 for 2 hrs. The binding was probed with anti-mouse IgG conjugated to alkaline phosphatase followed by substrate addition. The results were read at OD of 405nm.

4.1.4 Purification of specific antibodies:

The antibodies against the orexin peptides from a pool of the sera were purified on a peptide column constructed using a HiTrap column (GE Healthcare). The peptides used in the study comprised the following: Orx-1: AGNHAAGILTLGK, Orx-2: SGNHAAGILTMGR. HiTrapTM N-hydroxysuccinimide (NHS)-activated-SepharoseTM High Performance (GE Healthcare) was employed for the purification. The peptides were dissolved in coupling buffer (02 M NaHCO3, 05 M NaCl, pH 83), 5 mg/10 ml. Two ml of coupling buffer were added, followed by the peptides solution into the column using a pump for 4 h at room temperature. The non-bound peptides were washed and deactivated by 0.5 M ethanolamine 0.5 M NaCl, pH 8.3 buffer followed by 0.1 M acetate, 0.5 M NaCl, pH 4. The pH was adjusted using 0.05 M Na2HPO4, pH 7. Total fractionated IgG was loaded 2 mg/ml in PBS onto the peptides column. Following extensive washing, the bound antibodies were eluted with 0.2 M glycin-HCl, pH 2.5, neutralized immediately to pH 7 and dialysed with PBS (2). Afterwards, two additional groups of four weeks old C3H mice, were injected via ICV

with specific anti-orexin peptide antibodies or with total mouse IgG as we previously described (79).

4.1.5 Videotaping and scoring of narcoleptic episodes

All the groups of immunized mice were filmed and analyzed for immobility attacks every two weeks after ICV injection by using the EthoVision software (Noldus Information Technology, Netherlands). Each mouse was recorded individually for 18 h. After the end of the recording process the mouse was returned to its home cage. Narcoleptic episodes were defined as freezing episodes by using the EthoVision software and were strictly defined as we previously described (79).

4.2 RESULTS

4.2.1 Radiolabeled total IgG (Japanese collaboration)

The PET-CT scan did not show clear images due to the high intensity of the signal (Figure 1). However, the autoradiography found accumulation of signal in the hypothalamus for the narcoleptic IgG in more than three independent experiments compared to the control (Figure 2 and 3).

4.2.2 Peptide immunization

The group of mice immunized with orexin and hemagglutinin peptides developed antibodies against the three different peptides, however the mice immunized with the

peptides from αMSH and NEI did not develop antibodies (Figure 4A). Within this group of mice the highest antibody response was against the two peptides from orexin, while for the peptide from H1N1 hemagglutinin the response was lower (Figure 4B). After the establishment of the 50% binding capability of the sera samples, inhibition assays demonstrated that the antibodies produced against the orexin peptides can recognize both peptides, but not the peptide from the H1N1 hemagglutinin (Figure 4C). We did not detect any behavioral changes in the in any of the groups.

5 GENERAL DISCUSSION OF THE ARTICLES

This thesis summarizes the results of my work which has aimed to evaluate the effects of autoantibodies and vaccines on mice brain in terms of behavior, cognition and histological examination. In means of replicating the symptomatology associated to a particular disease, we passively transferred specific autoantibodies to mice brain: (Paper I (118) and (28, 119, 122)) or total IgG from patients (Paper III (79)). The results illustrate not only how is it possible to identify an alternative target for the antibodies in the brain, but also how they can interfere in cellular processes (Paper II (119)).

In addition, we demonstrated that environmental factors such as vaccines can induce behavioral changes in animal models (Paper IV (120)). Finally we tried an alternative for therapy which improved the depression-like behavior induce by the vaccine (Paper V (121)).

5.1 PASSIVE TRANSFER OF AUTOANTIBODIES TO THE BRAIN REPLICATE SPECIFIC SYMPTOMS

Neurological diseases such as multiple sclerosis, myasthenia gravis, Guillain-Barre syndrome, Neuromyelitis optica (NMO) and perhaps narcolepsy, are considered to be autoimmune-mediated (123). In addition, systemic autoimmune diseases such as SLE, Sjögren syndrome, arthritis, antiphospholipid syndrome and several vasculitis's may also involve the central nervous system, which usually indicates a severe disease (124). Due to the lack of consensus criteria for their diagnosis as well as to the wide spectrum of clinical manifestations, the importance of the neurological manifestations in these group of disease

is underestimated (125). In some cases it is not clear whether the neurological manifestation in systemic autoimmune diseases is the primary system to be effected or a secondary consequence of the disease (124, 126). in any case, various mechanisms to explain immune-mediated neuropsychiatric manifestations have been proposed, including ischemic vasculopathy, inflammatory vasculitis, cytokine-mediated inflammation, cytotoxicity, CNS invasion by mass-like lesions, and anti-neuronal or brain autoantibodies (124, 125).

During the years, hundreds of autoantibodies have been detected in the sera of patients with immune-mediated diseases, and a considerable number of them were shown to recognize brain structures (7, 125). Our results demonstrate that the 16/6 idiotypic antibody induced short and long memory deficiency in mice, while the anti-ribosomal P autoantibody induced depression. In addition injection of total IgG from narcoleptic patients to mice caused histological changes in the hypothalamus, as well as changes in the sleep behavior, hyperactivity, memory deficits and depression-like behavior. These results are examples of how the nervous system can be targeted by the immune system causing autoimmune phenomena, and how passive transfer of autoantibodies by intra-cerebra ventricular (ICV) injection can be used to overcome the BBB. The antibodies induce a disease of similar clinical manifestations in animal models to those presented by patients with SLE or narcolepsy (28, 79, 118, 122). Epidemiological studies in patient cohorts have suggested that pathologic autoantibodies which recognized neuronal targets, are associated with a

broad spectrum of symptoms including psychosis, catatonia, behavioral changes, memory loss, autonomic dysregulation, seizures, and abnormal movements. (127, 128).

5.2 SLE AND NEUROPSYCHIATRIC MANIFESTATIONS ASSOCIATED TO SPECIFIC ANTIBODIES

The link between NPSLE and anti–ribosomal P has been extensively described mostly with regard to depression and psychosis in SLE patients (25, 27-30). Previously, our group has shown the ability of anti-ribosomal P antibodies to bind brain tissue ex-vivo. In these studies, the passive transfer of anti-ribosomal P to naive mice induced depressive-like behavior (119). In the current study we showed the ability of anti-Ribosomal-P antibodies to penetrate, in-vitro, into live human neuronal cells. We demonstrated by confocal microscope that anti-ribosomal P antibodies (human and mouse) penetrate to the cytoplasm and localize near the nucleus of live neuronal cells. Interestingly, these autoantibodies affected signal transduction pathways, including the MAK kinase or Erk and Akt signaling pathways, and reduced the ability of proliferation of the cells. Moreover, we showed that anti-ribosomal P antibodies cross-react with the neuronal growth associated protein (GAP43) (Paper II).

It has been demonstrated before that other autoantibodies are able to penetrate cells through different pathways (129-132). Several natural anti-DNA antibodies can translocate across the plasma membrane and localize in the nucleus of mammalian cells, inducing caspase-mediated apoptosis through catalytic hydrolysis of DNA (131). Anti-fibroblast antibodies (AFA) have also the ability to penetrate living fibroblasts via the caveolin-linked pathway leading to the formation of cytoplasmic tubular invaginations and granules (133).

Other autoantibodies have shown the ability to enter living cells (134), such as anti-RNP which can penetrate to mononuclear cells, keratinocytes and lymphocytes or anti-Ro which penetrates skin cells. In the case of anti-Ribosomal P antibodies, we showed that the autoantibody can induce cell dysfunction and penetrate to several cell lines in-vitro (130, 132).

There are two possible ways in which the antibodies can interact with specific cells and be internalized. The first one implies the interaction with the Fc receptors (FcR). In this case, the antibody penetrates the cells through its constant region, and might recognize a specific intracellular antigen once it has already been internalized. For instance, the Fc-mediated penetration of anti-RNP (135) and anti-DNA antibodies into T-cells induce differential effects on cell cycle progression and DNA or RNA cell content (136). The second option, suggest that the interaction is defined by the most specific region of the antibody, the F(ab)2. In this case, the specific recognition of a cell surface molecule can lead to antibody penetration (133, 137). Our findings demonstrate that the internalization of anti-ribosomal P autoantibodies into neuronal cells depends on their specific interaction with the neuronal protein Gap43. Once inside the cells, the antibody induces alterations of the Erk and Akt phosphorylation, thus affecting signaling pathways, which are involved in neurogenesis, self-renewal and proliferation of neuronal cells (138-140). Gap43 is essential for the function of synapses and plays an important role in guiding the growth of axons and modulating the formation of new connections, explaining why it is an abundant protein in axonal growth cones of developing and regenerating neurons, as well as in pre-synaptic

terminals (141-144) (For a detailed discussion the reader is referred to discussion section in the Paper II).

5.3 NARCOLEPSY AS AN AUTOIMMUNE DISEASE TRIGGER BY H1N1 VACCINATION

Following the 2009 H1N1 vaccination campaign due to the preceding outbreak of influenza there was an increase in the diagnosis of narcolepsy in Finnish children, especially those vaccinated with Pandemrix (145-147). Several theories were aimed to explain the Pandemrix–narcolepsy relationship, such as the adjuvant squalene, the presence of alfa tocofarol and manufacturer process of the different vaccines (13, 62, 73, 147). Other studies suggested that a peptides from the H1N1 hemagglutinin of the Pandemrix vaccine can cross react, due to molecular mimicry, with different peptides of orexin (77, 78). Another study demonstrated that patients with post Pandemrix narcolepsy had anti-gangliosides antibodies detected in their blood (148). Here we demonstrate that antibodies against the peptides from the orexin, AGNHAAGILTLGK and SGNHAAGILTMGR, were not able to cross-react with the peptide from the H1N1 hemagglutinin ERNAGSGIIISDT, despite their similarities. These results suggest perhaps that the narcolepsy caused by the H1N1 vaccination is not mediated by antibodies against this particular peptide from the hemagglutinin.

The analysis of the vaccine as well as the affected population concluded that development of the disease in this particular group of children was the consequence of the interaction between different environmental and genetic factors (149-151): First, the manufacturing

process of Pandenrix® induced modifications of the viral components in the vaccine. Pandemrix ®, when compared to other vaccines has a higher amount of the nucleoprotein (NP), which constitutes the main protein in the H1N1A capsid (151, 152). Second, the higher frequency of the HLA allele *DQB1:06:02* within the patients that develop the disease after the vaccination suggested the importance of the genetic background. This may confer a unique ability to develop a stronger immune reaction against NP, particularly the development of anti-NP antibodies (150, 151). Supporting this idea, in one study, transgenic mice expressing the HLA *DQB1:06:02* developed higher levels of antibodies against NP, as compared to wild type mice (151). Another study demonstrated that the anti-NP antibodies from narcoleptic patients cross-react with the human hypocretin (orexin) receptor 2 (HCRT2), which suggests that the target cells in vaccine-induced narcolepsy are not the orexin producing neurons, but neurons that express HCRT2 (150). However, this mechanism is different than the classical findings in narcolepsy, where the lack of orexin is consequence of the absence of neurons that produce it.

5.4 INFLAMMATORY MECHANISMS IN NARCOLEPSY

Narcolepsy type I is considered an autoimmune disease due to the high association with polymorphisms in immune related genes, including the HLA *DQB1*06:02* allele (On the highest reported). This theory is also supported by the link to environmental factors which go further than the A H1N1 vaccination. For instance, streptococcal and AH1N1 infections themselves have been associated with the onset of the disease (The different theories have been summarized by us in the reference (147)). However, a clear mechanism has not been

described. Interestingly, a 2013 case report was described by Dauvilliers Y *et al* (153), the patient presented with orexin deficiency, cataplexy and a sleep disorder, as well as the presence of anti-Ma2 antibodies without evidence of neoplasia. Four months after the onset of the symptoms the patient died due to an unrelated complication and his brain was analyzed. The results showed inflammation and tissue injury in the hypothalamus, as well as the presence of different immune cells including antigen presenting cells, B lymphocytes and a strong cytotoxic inflammatory reaction characterized by the presence of CD8+ T lymphocytes and HLA I positive cells. The neuropathology analysis showed complete loss of orexin neurons. Interestingly, anti-Ma2 antibodies from the patient were able to recognize hypothalamic neurons of mice brains (153). Anti-Ma 2 antibodies are paraneoplastic antibodies which usually appear as a consequence of the presence of testicular cancer causing different paraneoplastic syndromes. Studies in patients with neurological paraneoplastic manifestations demonstrated that the presence of hypersomnia was associated with the Anti-Ma2 (154). Moreover the specific analysis of anti-Ma2 positive patients show that 32% of them suffer from excessive daytime sleepiness, which in some cases was diagnosed as narcolepsy with cataplexy (155). As mentioned before, due to the characteristics of the disease it is very difficult to directly analyze the brain tissue in order to understand the pathophysiology of the disease, therefore this case report gives precious information due to the proximity of disease onset. (153). We had checked the presence of different anti-paraneoplastic antibodies including anti-Ma2 in 24 Japanese narcoleptic patients, all positive or the HLA DQB1*06:02. One patient was positive for anti-Jo and Anti-

Ma1, one for Anti-Jo and one for anti CV2. In any of the patient we found the presence of anti-Ma2. (*Addendum, methodology ravo-Diagnostica by PNS-Blot: PNS 11 Line Assay).*

Thus, the presence of anti-Ma2 could be explained first as the result of a neoplasia in a very early stage. Second, it could be either the result of the destruction of the orexin neurons or third, the original cause of it. The presence of CD8+ in the damaged hypothalamus suggests that the target antigen is related to the orexin neurons, leading to cytotoxic activity causing neurotoxicity. This results from the exposure of cryptic antigens which might lead to the production of antibodies against intracellular components such as Ma2. On the other hand, anti-Ma2 could induce the immune reaction against an antigen related to the orexin neurons which cause the activation of auto reactive CD8+ cells leading to the tissue damage. These results may implicate the importance of cytotoxic activity during the onset of narcolepsy (153).

5.5 IMPORTANCE OF AUTOANTIBODIES IN NARCOLEPSY

The presence of an inflammatory activity in narcolepsy, perhaps antibody-mediated, could be supported by notion of improvement seen after the treatment with high-dose intravenous immunoglobulins (IVIgs) close to the disease onset (156, 157). Indeed, the importance of autoantibodies in narcolepsy has already been explored. Initially, Cvetkovic-Lopes et al., described the presence of antibodies against Tribbles 2 (Trib2) in narcoleptic patients. Trib2 is a protein involved in the cell cycle which was found to be expressed by orexin neurons at the same time that orexin was produced (81). It was found that titers of

Anti-Trib2 were higher in 14% narcoleptic patients compared with healthy controls (82, 83). Histological analysis showed that anti-Trib2 positive sera from narcoleptic patients bind orexin-producing neurons in mouse brains (81). A recent study, which used ataxin-3 to induce orexin neuronal cell toxicity in mice, detected the presence of antibodies against Trib2 after advanced neuronal destruction. In addition, the Trib2 antibodies were able to react with normal hypothalamic orexin neurons. These results suggest that the presence of anti-Trib2 antibodies in narcoleptic patients is an epiphenomena, the consequence of orexin cell destruction and not its cause (81). However, in our study the passive transfer by ICV injection of total IgG from narcoleptic patients to naïve mice induced sleep attacks similar to those observed in narcoleptic patients (79). In addition, brain histology showed decreased presence of orexin producing neurons in the lateral hypothalamus of the injected mice, as well as loss of NeuN (neuronal marker), and synaptophysin (synaptic marker) which did not necessarily proximal to the location of the orexin neurons (79). This may indicate that antibodies in the sera of narcoleptic patients might also affect other groups of neurons related to sleep regulation, contributing to the changes observed in the animals. This idea, was supported by a study which found that passive transfer of total IgG from narcoleptic patients into rat brains induced changes in different sleep parameters, as we previously reported (79, 84, 147). In addition, they evaluated the presence of autoantibodies by immunohistochemistry on healthy brain. Three different patterns were identified: 1) Antibodies bound hypothalamic melanin-concentrating hormone and pro-opiomelanocortin but not orexin neurons. 2) The antibodies recognized the GABAergic cortical inter-neurons. 3) The antibodies mainly bound *globus pallidus* neurons (84). A

deeper analysis of the first pattern found two possible epitopes in the C-terminal of the neuropeptide glutamicacid-isoleucine (NEI) and in the melanocyte-stimulating hormone (αMSH). These two proteins are expressed in the hypothalamus by the neurons in charge of the production of melanin-concentrating hormone (MSH), but not in orexin producing neurons. This corroborates with the damage we observed in areas which did not correspond to the regular positions of the orexin neurons, and thus may be the result of autoantibody action in the sera of narcoleptic patients (79, 84). Our results together with those from Bergman et al, provide some evidence for the importance of the humoral response in narcolepsy. However, we demonstrated that these peptides are not immunogenic in mice and therefore we cannot claim that autoantibodies against them have a major pathogenic role in narcolepsy.

5.6 ALUMINUM ADJUVANTED VACCINES AS RISK FACTORS FOR BEHAVIORAL CHANGES ASSOCIATED WITH AUTOIMMUNE MEDIATED PROCESSES

Our group has shown that vaccines and adjuvants can accelerate behavioral and cognitive changes in mice which are prone to develop autoimmune conditions. For example, in lupus prone mice the immunization with human hepatitis B vaccine or aluminum adjuvant aggravates kidney disease, affects blood counts, and neurocognitive functions (158). Consistently, here we showed how the immunization with human papilloma virus vaccine (Gardasil) and aluminum in a healthy mouse strain C57B/6 induces behavioral alteration as well as neuroinflammatory changes in mice, being the depressive-like behavior the strongest effect. In addition, the immunization with Gardasil induced an increment in levels

of anti-HPV antibodies, and antibodies targeting the brain-protein and the brain-phospholipid extract components in the mice. Suggesting that anti-HPV antibodies from Gardasil-vaccinated mice have the capacity to recognize also brain antigen(s) (120). Moreover we demonstrated that the mice depressive–like behaviour was ameliorated by dietary supplementation enriched with phosphatidylcholine (PC) (121).

We previously found similar results concerning the immunization with aluminum adjuvant regardless the vaccine (120, 121, 158). Aluminum, the most common used adjuvant, is known to be immune- and neurotoxic and was shown to be able to trigger both immune and inflammatory responses (159-162). Aluminum is reported to cause neurologic and psychiatric manifestations in animal models as well as humans (102, 163), in both oral and injectable exposures (164-167). Aluminum neurotoxicity was shown to impair learning, memory, concentration and speech skills, as well as effect psychomotor control, seizure threshold, and other mental aspects (168, 169). Moreover, aluminum was associated with several neurologic diseases, such as Alzheimer's disease (170), amyotrophic lateral sclerosis and Parkinsonism (171), Multiple Sclerosis (172, 173) and the autism spectrum (166, 170, 174).

Studies in animal models demonstrated that aluminum nanoparticles are capable of crossing the BBB and the CSF, and can incite neuro- inflammation (101, 161). Several mechanisms were proposed to mediate the stimulation of the immune system by aluminum: the aluminum can prolong antigen exposure; up-regulate antigen-presenting

function of macrophages and dendritic cells, thus improving their phagocytic skills; translocate the antigen to lymphoid organs, taking place in the activation of naïve T cells; increases the release of inflammatory cytokines and interacts with pattern-recognition receptors (PRRs) in the place of the injection and in the lymph nodes where it drains to, causing expansion of the local inflammatory process; it activates B cells in the spleen; and, finally, it activates the NLRP3 inflammasome pathway (99, 175). These aluminum properties are both responsible for the vaccine efficacy as well as for its toxicity, and the absent of severe side effects depend on a perfect balance (176). The activation of the NLRP3 inflammasome signaling cascade can illustrate this dual role of aluminum (99, 177, 178), at the same time it is the main pathway through which aluminum promotes adjuvant immune stimulation and it is typically related to the development of inflammatory and autoimmune diseases (179-183). The activation of the NLRP3 downstream components – especially IL-1beta and IL-18 – is correlated to CNS disorders that were already correlated to aluminum exposure, like, for instance Multiple Sclerosis, Alzheimer's disease and Parkinson's disease (180). Besides being neurotoxic (161, 169, 184, 185) and immunotoxic (176, 186), aluminum is also pro-oxidant (187, 188), interferes with body homeostasis(e.g. depresses glucose metabolism (184, 189) and can interfere cellular processes that involve calcium, ATP, membrane receptors signaling and mitochondrial function (169, 190, 191).

In addition, to the aluminum effect our results showed the production of antibodies against Gardasil, as well as antibodies targeting the mouse brain protein and brain phospholipid extracts in the sera of Gardasil-immunized mice (Paper IV). Interestingly, the binding of anti-

Gardasil antibodies from the sera of mice injected with Gardasil to components of the Gardasil was inhibited by mouse brain protein extract. These results indicate a cross-reaction with brain antigens. This suggestion is supported by the results of Kanduc (192, 193); this bioinformatics approach lead to the identification of 80 peptides of the major capsid L1 protein of HPV-16 which can be found in human proteins related to crucial cellular processes, such as adhesion molecules, peptides responsible for leukocyte differentiation and spermatogenesis, transcription factors, and neuronal antigens (193).

5.7 PHOSPHOLIPID SUPPLEMENTATION AMELIORATES DEPRESSION-LIKE BEHAVIOR INDUCE BY VACCINES

Our results showed that dietary supplementation with Phosphatidylcholine (PC) reduced depressive – like symptoms in mice which were immunized with Gardasil and aluminum (Paper V). Previous studies suggested a positive effect of phospholipids supplementation on depression and cognitive function in rodents (194) and humans (195-197). The beneficial effects of phospholipid supplementation can be explained by their ability to restore the membranes of damaged and weakened cells (198). For example, one study demonstrated that the PC supplementation to old rat myocytes resulted in the recovery of cellular functions, in particular heart-contractility (199). Studies in animal models indicate that PC attenuates depression like behavior (200). Phospholipid supplementation was found to be beneficial in different neurological conditions in humans, such as memory loss, cognitive decline, stroke, fatigue, movement disorders and alcohol ingestion (198, 201, 202). Similar results have been described in different patient's cohorts, as well as in patients with chronic fatigue syndrome (203-205). Phospholipids can cross the BBB and they can reverse

biochemical and structural changes in human nerve cells by restoring neuronal membranes (206, 207). Interestingly, depression can cause changes in brain lipids metabolisms by the release of glucocorticoid (GC) and activation of the hypothalamic–pituitary–adrenal axis. For instance, analysis of lipid content in brains of depressed rodents, showed that the phospholipid composition is altered (208).

It is believed that aluminum is neurotoxic through its ability to generate oxidative stress. This effect may be caused by the cellular dysfunction caused by mitochondrial oxidative damage from aluminum. In the current study the mice supplementation diet contained also omega-3. It should be noted that treatment with omega-3 was shown to ameliorate the adverse effects of aluminum on the brain. Ali HA et-al, showed that the supplementation of mice with omega 3 in addition to quercetin can ameliorate the oxidative stress which was induced by aluminum chloride in the mouse brain (209). Moreover, since phospholipid supplementation was shown to be effective restoring the mitochondrial activity in patients with CFS as well as in cancer patients after chemotherapy (201, 202, 210), it is possible to speculate that it might be also helpful reversing the effects of aluminum. In particular those related to the alteration of mitochondrial activity (191, 211, 212).

6 CONCLUSIONS AND PERSPECTIVES

In line with the results previously discussed, we conclude that specific autoantibodies can be directly responsible for specific symptoms in autoimmune mediated conditions, in particular behavioral and cognitive impairment. In the case of NPSLE, the 16/6-idiotypic antibodies an anti-ribosomal P antibodies caused cognitive impairments and histological evidence for brain inflammation and depression-like behavior (28, 118, 122). In addition, we have shown that anti-ribosomal P antibodies can penetrate neuronal cells in vitro by their interaction with Gap43, and can inhibit neuronal-cell proliferation *via* alteration of Akt and Erk signaling. The same methodological approach allowed the induction narcolepsy-like sleep changes in naïve mice. These results may implicate that antibodies in the sera of narcoleptic patients are involved in the mechanism of narcolepsy-like attacks.

These findings provide additional light on the diverse mosaic in the pathophysiology of autoimmune manifestations in the CNS. The identification of autoantibodies that are potentially harmful in this kind of conditions can help develop new treatment strategies: Blocking action of the pathogenic antibodies. For instance, the treatment with IVIG, can be an option considering the concept of anti-idiotypic antibodies. In fact, in the case of 16/6-idiotypic antibodies the IVIG itself may contain the anti-idiotype antibodies which mimics the brain antigen, and has shown some efficacy in the treatment of NPSLE patients (213) as well as in rheumatoid arthritis murine models (214). An additional therapeutic resource can be the implementation of inhibitory peptides based on the complementarity determining region of autoantibodies. Indeed, this approach has been explored in animal models as well as in a limited number of lupus patients (215-217) and in rheumatoid arthritis animal

models (218, 219). In the particular case of anti-ribosomal P antibodies it is possible to consider use of a peptide from the protein GAP43, but this will be only possible after the identification of the specific region that is recognized by these antibodies.

On the other hand we conclude that vaccines; in particular the HPV quadrivalent vaccine, together with its adjuvant aluminum can trigger neuroinflammation and autoimmune reactions, leading to similar behavioral changes to those induced by autoantibodies (i.e. depression). Indeed, the presence of inflammation and antibodies that potentially recognized brain components suggests that these adverse effects are the results of cross-reactions with the HPV proteins used in the vaccine. Interestingly, we have shown that the initial effect can be caused by aluminum itself. These results mimic our previous findings with regard to the Human Hepatitis B vaccine (HBVv) in a SLE mouse model. Previously, our group reported that immunization with the HBVv aggravated kidney disease in an animal model of SLE, and the immunization with the HBVv or the aluminum adjuvant affected blood counts, neurocognitive functions and brain gliosis (158). Finally, this study demonstrates the positive effect of phospholipid supplementation on post-HPVv depression-like behavior.

These findings highlight the necessity of moving forward to personalized medicine. Moreover, this is a call to consider the continually increasing number of seriously disabling neurological adverse events linked to HPV (220, 221). Finally, are results suggest an optional treatment for neurocognitive symptoms that might caused by the vaccination or the

aluminum adjuvant. Future studies aimed to evaluate whether PC supplementation is effective as a treatment for patients suffering from neurological manifestations after vaccination may be warranted.

7 LIST OF OTHER RELEVANT PUBLICATIONS

1. Arango MT, Perricone C, Kivity S, Cipriano E, Ceccarelli F, Valesini G, Shoenfeld Y. **HLA-DRB1 the notorious gene in the mosaic of autoimmunity**. Immunol Res. 2016 Jul 19. [Epub ahead of print] PubMed PMID: 27435705. (I.F 3.098) (111).
2. Arango MT, Kivity S, Shoenfeld Y. **Is narcolepsy a classical autoimmune disease?** Pharmacol Res. 2015 Feb;92:6-12. doi: 10.1016/j.phrs.2014.10.005. PubMed PMID: 25447795. (I.F 4.408)(147).
3. Tomijenovic L, Arango MT, Agmon-Levin N. **Vaccination in autoimmune animal models**. Isr Med Assoc J. 2014 Oct;16(10):657-8. PubMed PMID: 25438463. (I.F 1.013) (222).
4. Arango MT, Kivity S, Chapman J, Shoenfeld Y. **Narcolepsy-genes, infections and vaccines: the clues for a new autoimmune disease.** Isr Med Assoc J. 2014 Oct;16(10):636-7. PubMed PMID: 25438454. (I.F 1.013) (223).
5. Agmon-Levin N, Arango MT, Kivity S, Katzav A, Gilburd B, Blank M, Tomer N, Volkov A, Barshack I, Chapman J, Shoenfeld Y. **Immunization with hepatitis B vaccine accelerates SLE-like disease in a murine model.** J Autoimmun. 2014 Nov;54:21-32. PubMed PMID: 25042822. (I.F 8.41) (158).

8 REFERENCES

1. Strazielle N, Creidy R, Malcus C, Boucraut J, Ghersi-Egea JF. T-Lymphocytes Traffic into the Brain across the Blood-CSF Barrier: Evidence Using a Reconstituted Choroid Plexus Epithelium. PloS one. 2016;11(3):e0150945. PubMed PMID: 26942913. Pubmed Central PMCID: 4778949.
2. Keaney J, Campbell M. The dynamic blood-brain barrier. The FEBS journal. 2015 Nov;282(21):4067-79. PubMed PMID: 26277326.
3. Varatharaj A, Galea I. The blood-brain barrier in systemic inflammation. Brain, behavior, and immunity. 2016 Mar 16. PubMed PMID: 26995317.
4. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiology of disease. 2010 Jan;37(1):13-25. PubMed PMID: 19664713.
5. Diamond B, Honig G, Mader S, Brimberg L, Volpe BT. Brain-reactive antibodies and disease. Annual review of immunology. 2013;31:345-85. PubMed PMID: 23516983.
6. Wingerchuk DM, Weinshenker BG. Neuromyelitis optica (Devic's syndrome). Handbook of clinical neurology. 2014;122:581-99. PubMed PMID: 24507536.
7. Zandman-Goddard G, Chapman J, Shoenfeld Y. Autoantibodies involved in neuropsychiatric SLE and antiphospholipid syndrome. Seminars in arthritis and rheumatism. 2007 Apr;36(5):297-315. PubMed PMID: 17258299.
8. Dale RC, Heyman I. Post-streptococcal autoimmune psychiatric and movement disorders in children. The British journal of psychiatry : the journal of mental science. 2002 Sep;181:188-90. PubMed PMID: 12204920.
9. Dale RC. Post-streptococcal autoimmune disorders of the central nervous system. Developmental medicine and child neurology. 2005 Nov;47(11):785-91. PubMed PMID: 16225745.
10. Maraka S, Bulathsinghala CP, Grover P. Brain on fire: anti-N-methyl-D-aspartate-receptor encephalitis associated with ovarian teratoma. Connecticut medicine. 2013 Aug;77(7):389-92. PubMed PMID: 24195175.
11. Acien P, Acien M, Ruiz-Macia E, Martin-Estefania C. Ovarian teratoma-associated anti-NMDAR encephalitis: a systematic review of reported cases. Orphanet journal of rare diseases. 2014;9(1):157. PubMed PMID: 25312434. Pubmed Central PMCID: 4203903.
12. Ahmed SS, Schur PH, MacDonald NE, Steinman L. Narcolepsy, 2009 A(H1N1) pandemic influenza, and pandemic influenza vaccinations: what is known and unknown about the neurological disorder, the role for autoimmunity, and vaccine adjuvants. Journal of autoimmunity. 2014 May;50:1-11. PubMed PMID: 24559657.
13. Partinen M, Kornum BR, Plazzi G, Jennum P, Julkunen I, Vaarala O. Narcolepsy as an autoimmune disease: the role of H1N1 infection and vaccination. Lancet neurology. 2014 Jun;13(6):600-13. PubMed PMID: 24849861.
14. Martinez-Martinez P, Molenaar PC, Losen M, Stevens J, Baets MH, Szoke A, et al. Autoantibodies to neurotransmitter receptors and ion channels: from neuromuscular to neuropsychiatric disorders. Frontiers in genetics. 2013;4:181. PubMed PMID: 24065983. Pubmed Central PMCID: 3778371.

15. Honnorat J, Didelot A, Karantoni E, Ville D, Ducray F, Lambert L, et al. Autoimmune limbic encephalopathy and anti-Hu antibodies in children without cancer. Neurology. 2013 Jun 11;80(24):2226-32. PubMed PMID: 23658383.
16. Leypoldt F, Armangue T, Dalmau J. Autoimmune encephalopathies. Annals of the New York Academy of Sciences. 2014 Oct 14. PubMed PMID: 25315420.
17. Irani SR, Vincent A. Autoimmune encephalitis -- new awareness, challenging questions. Discovery medicine. 2011 May;11(60):449-58. PubMed PMID: 21616043.
18. Asgari N, Khorooshi R, Lillevang ST, Owens T. Complement-dependent pathogenicity of brain-specific antibodies in cerebrospinal fluid. Journal of neuroimmunology. 2013 Jan 15;254(1-2):76-82. PubMed PMID: 23031833.
19. Iseme RA, McEvoy M, Kelly B, Agnew L, Attia J, Walker FR. Autoantibodies and depression: evidence for a causal link? Neuroscience and biobehavioral reviews. 2014 Mar;40:62-79. PubMed PMID: 24480318.
20. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. Seminars in arthritis and rheumatism. 2004 Oct;34(2):501-37. PubMed PMID: 15505768.
21. Yaniv G, Twig G, Shor DB, Furer A, Sherer Y, Mozes O, et al. A volcanic explosion of autoantibodies in systemic lupus erythematosus: a diversity of 180 different antibodies found in SLE patients. Autoimmunity reviews. 2015 Jan;14(1):75-9. PubMed PMID: 25449682. Epub 2014/12/03. eng.
22. Gal Y, Gilad T, Dana B-AS, Ariel F, Yaniv S, Oshry M, et al. A volcanic explosion of autoantibodies in systemic lupus erythematosus: A diversity of 180 different antibodies found in SLE patients. Autoimmun Reviews. 2014.
23. Rekvig OP, Putterman C, Casu C, Gao HX, Ghirardello A, Mortensen ES, et al. Autoantibodies in lupus: culprits or passive bystanders? Autoimmunity reviews. 2012 Jun;11(8):596-603. PubMed PMID: 22041579.
24. Slight-Webb S, Lu R, Ritterhouse LL, Munroe ME, Maecker HT, Fathman CG, et al. Autoantibody-Positive Healthy Individuals Display Unique Immune Profiles That May Regulate Autoimmunity. Arthritis & rheumatology. 2016 Apr 5. PubMed PMID: 27059145. Epub 2016/04/10. Eng.
25. Hanly JG. Diagnosis and management of neuropsychiatric SLE. Nature reviews Rheumatology. 2014 Jun;10(6):338-47. PubMed PMID: 24514913.
26. 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 Aug;41(1):1-11. PubMed PMID: 20965549.
27. Bonfa E, Golombek SJ, Kaufman LD, Skelly S, Weissbach H, Brot N, et al. Association between lupus psychosis and anti-ribosomal P protein antibodies. The New England journal of medicine. 1987 Jul 30;317(5):265-71. PubMed PMID: 3496538.
28. 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 Mar;56(3):938-48. PubMed PMID: 17328071. Epub 2007/03/01. eng.
29. Eber T, Chapman J, Shoenfeld Y. Anti-ribosomal P-protein and its role in psychiatric manifestations of systemic lupus erythematosus: myth or reality? Lupus. 2005;14(8):571-5. PubMed PMID: 16175927.
30. Sato T, Uchiumi T, Ozawa T, Kikuchi M, Nakano M, Kominami R, et al. Autoantibodies against ribosomal proteins found with high frequency in patients with systemic lupus erythematosus with active disease. The Journal of rheumatology. 1991 Nov;18(11):1681-4. PubMed PMID: 1787488.

31. Bravo-Zehnder M, Toledo EM, Segovia-Miranda F, Serrano FG, Benito MJ, Metz C, et al. Anti-Ribosomal P Protein Autoantibodies from Neuropsychiatric Lupus Impair Memory. Arthritis & rheumatology. 2014 Oct 9. PubMed PMID: 25302407.
32. Katzav A, Ben-Ziv T, Blank M, Pick CG, Shoenfeld Y, Chapman J. Antibody-specific behavioral effects: intracerebroventricular injection of antiphospholipid antibodies induces hyperactive behavior while anti-ribosomal-P antibodies induces depression and smell deficits in mice. Journal of neuroimmunology. 2014 Jul 15;272(1-2):10-5. PubMed PMID: 24837568.
33. Shoenfeld Y, Hsu-Lin SC, Gabriels JE, Silberstein LE, Furie BC, Furie B, et al. Production of autoantibodies by human-human hybridomas. The Journal of clinical investigation. 1982 Jul;70(1):205-8. PubMed PMID: 7085884. Pubmed Central PMCID: 370244.
34. Blank M, Shoenfeld Y. The story of the 16/6 idiotype and systemic lupus erythematosus. The Israel Medical Association journal : IMAJ. 2008 Jan;10(1):37-9. PubMed PMID: 18300569.
35. Fanouriakis A, Boumpas DT, Bertsias GK. Pathogenesis and treatment of CNS lupus. Current opinion in rheumatology. 2013 Sep;25(5):577-83. PubMed PMID: 23836074.
36. Amital-Teplizki H, Avinoach I, Coates AR, Kooperman O, Blank M, Shoenfeld Y. Binding of monoclonal anti-DNA and anti-TB glycolipids to brain tissue. Autoimmunity. 1989;4(4):277-87. PubMed PMID: 2518831. Epub 1989/01/01. eng.
37. Amital-Teplizki H, Bearman JE, Miele PW, Jr., Sukenik S, Shoenfeld Y. A multidimensional autoantibody analysis specifying systemic lupus erythematosus patients with neuropsychiatric symptomatology. Israel journal of medical sciences. 1992 Jul;28(7):422-7. PubMed PMID: 1506165.
38. Rolls A, Schaich Borg J, de Lecea L. Sleep and metabolism: role of hypothalamic neuronal circuitry. Best practice & research Clinical endocrinology & metabolism. 2010 Oct;24(5):817-28. PubMed PMID: 21112028.
39. Thannickal TC, Nienhuis R, Siegel JM. Localized loss of hypocretin (orexin) cells in narcolepsy without cataplexy. Sleep. 2009 Aug;32(8):993-8. PubMed PMID: 19725250. Pubmed Central PMCID: 2717206.
40. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell. 1999 Aug 20;98(4):437-51. PubMed PMID: 10481909.
41. Sakurai T, Mieda M, Tsujino N. The orexin system: roles in sleep/wake regulation. Annals of the New York Academy of Sciences. 2010 Jul;1200:149-61. PubMed PMID: 20633143.
42. Fontana A, Gast H, Reith W, Recher M, Birchler T, Bassetti CL. Narcolepsy: autoimmunity, effector T cell activation due to infection, or T cell independent, major histocompatibility complex class II induced neuronal loss? Brain : a journal of neurology. 2010 May;133(Pt 5):1300-11. PubMed PMID: 20403960.
43. Longstreth WT, Jr., Koepsell TD, Ton TG, Hendrickson AF, van Belle G. The epidemiology of narcolepsy. Sleep. 2007 Jan;30(1):13-26. PubMed PMID: 17310860.
44. Wilner A, Steinman L, Lavie P, Peled R, Friedmann A, Brautbar C. Narcolepsy-cataplexy in Israeli Jews is associated exclusively with the HLA DR2 haplotype. A study at the serological and genomic level. Human immunology. 1988 Jan;21(1):15-22. PubMed PMID: 2896649.
45. al Rajeh S, Bademosi O, Ismail H, Awada A, Dawodu A, al-Freihi H, et al. A community survey of neurological disorders in Saudi Arabia: the Thugbah study. Neuroepidemiology. 1993;12(3):164-78. PubMed PMID: 8272177.
46. Akintomide GS, Rickards H. Narcolepsy: a review. Neuropsychiatric disease and treatment. 2011;7:507-18. PubMed PMID: 21931493. Pubmed Central PMCID: 3173034.
47. Blouin AM, Thannickal TC, Worley PF, Baraban JM, Reti IM, Siegel JM. Narp immunostaining of human hypocretin (orexin) neurons: loss in narcolepsy. Neurology. 2005 Oct 25;65(8):1189-92. PubMed PMID: 16135770.

48. Crocker A, Espana RA, Papadopoulou M, Saper CB, Faraco J, Sakurai T, et al. Concomitant loss of dynorphin, NARP, and orexin in narcolepsy. Neurology. 2005 Oct 25;65(8):1184-8. PubMed PMID: 16247044. Pubmed Central PMCID: 2254145.
49. van der Heide A, Verduijn W, Haasnoot GW, Drabbels JJ, Lammers GJ, Claas FH. HLA dosage effect in narcolepsy with cataplexy. Immunogenetics. 2014 Oct 3. PubMed PMID: 25277311.
50. Mignot E, Lin L, Rogers W, Honda Y, Qiu X, Lin X, et al. Complex HLA-DR and -DQ interactions confer risk of narcolepsy-cataplexy in three ethnic groups. American journal of human genetics. 2001 Mar;68(3):686-99. PubMed PMID: 11179016. Pubmed Central PMCID: 1274481.
51. Kornum BR, Faraco J, Mignot E. Narcolepsy with hypocretin/orexin deficiency, infections and autoimmunity of the brain. Current opinion in neurobiology. 2011 Dec;21(6):897-903. PubMed PMID: 21963829.
52. Hallmayer J, Faraco J, Lin L, Hesselson S, Winkelmann J, Kawashima M, et al. Narcolepsy is strongly associated with the T-cell receptor alpha locus. Nature genetics. 2009 Jun;41(6):708-11. PubMed PMID: 19412176. Pubmed Central PMCID: 2803042.
53. Faraco J, Lin L, Kornum BR, Kenny EE, Trynka G, Einen M, et al. ImmunoChip study implicates antigen presentation to T cells in narcolepsy. PLoS genetics. 2013;9(2):e1003270. PubMed PMID: 23459209. Pubmed Central PMCID: 3573113.
54. Winkelmann J, Lin L, Schormair B, Kornum BR, Faraco J, Plazzi G, et al. Mutations in DNMT1 cause autosomal dominant cerebellar ataxia, deafness and narcolepsy. Human molecular genetics. 2012 May 15;21(10):2205-10. PubMed PMID: 22328086. Pubmed Central PMCID: 3465691.
55. Han F, Lin L, Li J, Aran A, Dong SX, An P, et al. TCRA, P2RY11, and CPT1B/CHKB associations in Chinese narcolepsy. Sleep medicine. 2012 Mar;13(3):269-72. PubMed PMID: 22177342. Pubmed Central PMCID: 3288279.
56. Anaya JM. The autoimmune tautology. Arthritis research & therapy. 2010;12(6):147. PubMed PMID: 21092150. Pubmed Central PMCID: 3046506.
57. Anaya JM. The diagnosis and clinical significance of polyautoimmunity. Autoimmunity reviews. 2014 Apr-May;13(4-5):423-6. PubMed PMID: 24424171.
58. Cardenas-Roldan J, Rojas-Villarraga A, Anaya JM. How do autoimmune diseases cluster in families? A systematic review and meta-analysis. BMC medicine. 2013;11:73. PubMed PMID: 23497011. Pubmed Central PMCID: 3655934.
59. Ohayon MM, Okun ML. Occurrence of sleep disorders in the families of narcoleptic patients. Neurology. 2006 Aug 22;67(4):703-5. PubMed PMID: 16924030.
60. Mignot E. Genetic and familial aspects of narcolepsy. Neurology. 1998 Feb;50(2 Suppl 1):S16-22. PubMed PMID: 9484418.
61. Silber MH, Krahn LE, Olson EJ, Pankratz VS. The epidemiology of narcolepsy in Olmsted County, Minnesota: a population-based study. Sleep. 2002 Mar 15;25(2):197-202. PubMed PMID: 11902429.
62. 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(3):e33536. PubMed PMID: 22470453. Pubmed Central PMCID: 3314666.
63. Ohayon MM, Ferini-Strambi L, Plazzi G, Smirne S, Castronovo V. How age influences the expression of narcolepsy. Journal of psychosomatic research. 2005 Dec;59(6):399-405. PubMed PMID: 16310022.
64. Picchioni D, Hope CR, Harsh JR. A case-control study of the environmental risk factors for narcolepsy. Neuroepidemiology. 2007;29(3-4):185-92. PubMed PMID: 18043003.

65. Ton TG, Longstreth WT, Jr., Koepsell TD. Environmental toxins and risk of narcolepsy among people with HLA DQB1*0602. Environmental research. 2010 Aug;110(6):565-70. PubMed PMID: 20519130. Pubmed Central PMCID: 2930404.
66. Ton TG, Longstreth WT, Jr., Koepsell T. Active and passive smoking and risk of narcolepsy in people with HLA DQB1*0602: a population-based case-control study. Neuroepidemiology. 2009;32(2):114-21. PubMed PMID: 19039244. Pubmed Central PMCID: 2970624.
67. Rose NR. Infection, mimics, and autoimmune disease. The Journal of clinical investigation. 2001 Apr;107(8):943-4. PubMed PMID: 11306595. Pubmed Central PMCID: 199561.
68. Kivity S, Agmon-Levin N, Blank M, Shoenfeld Y. Infections and autoimmunity--friends or foes? Trends in immunology. 2009 Aug;30(8):409-14. PubMed PMID: 19643667.
69. Sfriso P, Ghirardello A, Botsios C, Tonon M, Zen M, Bassi N, et al. Infections and autoimmunity: the multifaceted relationship. Journal of leukocyte biology. 2010 Mar;87(3):385-95. PubMed PMID: 20015961.
70. Wucherpfennig KW. Mechanisms for the induction of autoimmunity by infectious agents. The Journal of clinical investigation. 2001 Oct;108(8):1097-104. PubMed PMID: 11602615. Pubmed Central PMCID: 209539.
71. Samarkos M, Vaiopoulos G. The role of infections in the pathogenesis of autoimmune diseases. Current drug targets Inflammation and allergy. 2005 Feb;4(1):99-103. PubMed PMID: 15720242.
72. Aran A, Lin L, Nevsimalova S, Plazzi G, Hong SC, Weiner K, et al. Elevated anti-streptococcal antibodies in patients with recent narcolepsy onset. Sleep. 2009 Aug;32(8):979-83. PubMed PMID: 19725248. Pubmed Central PMCID: 2717204.
73. Dauvilliers Y, Montplaisir J, Cochen V, Desautels A, Einen M, Lin L, et al. Post-H1N1 narcolepsy-cataplexy. Sleep. 2010 Nov;33(11):1428-30. PubMed PMID: 21102981. Pubmed Central PMCID: 2954689.
74. Viorritto EN, Kureshi SA, Owens JA. Narcolepsy in the pediatric population. Current neurology and neuroscience reports. 2012 Apr;12(2):175-81. PubMed PMID: 22213222.
75. Tanaka S, Honda M, Toyoda H, Kodama T. Increased plasma IL-6, IL-8, TNF-alpha, and G-CSF in Japanese narcolepsy. Human immunology. 2014 Aug;75(8):940-4. PubMed PMID: 24994458.
76. Underwood E. Immunology. Key narcolepsy-influenza vaccine findings retracted. Science. 2014 Aug 1;345(6196):498. PubMed PMID: 25082675.
77. De la Herran-Arita AK, Kornum BR, Mahlios J, Jiang W, Lin L, Hou T, et al. CD4+ T cell autoimmunity to hypocretin/orexin and cross-reactivity to a 2009 H1N1 influenza A epitope in narcolepsy. Science translational medicine. 2013 Dec 18;5(216):216ra176. PubMed PMID: 24353159.
78. De la Herran-Arita AK, Kornum BR, Mahlios J, Jiang W, Lin L, Hou T, et al. Retraction of the Research Article: "CD4+ T cell autoimmunity to hypocretin/orexin and cross-reactivity to a 2009 H1N1 influenza A epitope in narcolepsy". Science translational medicine. 2014 Jul 30;6(247):247rt1. PubMed PMID: 25080479.
79. Katzav A, Arango MT, Kivity S, Tanaka S, Givaty G, Agmon-Levin N, et al. Passive transfer of narcolepsy: anti-TRIB2 autoantibody positive patient IgG causes hypothalamic orexin neuron loss and sleep attacks in mice. Journal of autoimmunity. 2013 Sep;45:24-30. PubMed PMID: 23834844.
80. Smith AJ, Jackson MW, Neufing P, McEvoy RD, Gordon TP. A functional autoantibody in narcolepsy. Lancet. 2004 Dec 11-17;364(9451):2122-4. PubMed PMID: 15589310.
81. Cvetkovic-Lopes V, Bayer L, Dorsaz S, Maret S, Pradervand S, Dauvilliers Y, et al. Elevated Tribbles homolog 2-specific antibody levels in narcolepsy patients. The Journal of clinical investigation. 2010 Mar;120(3):713-9. PubMed PMID: 20160349. Pubmed Central PMCID: 2827962.

82. Lim AS, Scammell TE. The trouble with Tribbles: do antibodies against TRIB2 cause narcolepsy? Sleep. 2010 Jul;33(7):857-8. PubMed PMID: 20614841. Pubmed Central PMCID: 2894423.
83. Kawashima M, Lin L, Tanaka S, Jennum P, Knudsen S, Nevsimalova S, et al. Anti-Tribbles homolog 2 (TRIB2) autoantibodies in narcolepsy are associated with recent onset of cataplexy. Sleep. 2010 Jul;33(7):869-74. PubMed PMID: 20614846. Pubmed Central PMCID: 2894428.
84. Bergman P, Adori C, Vas S, Kai-Larsen Y, Sarkanen T, Cederlund A, et al. Narcolepsy patients have antibodies that stain distinct cell populations in rat brain and influence sleep patterns. Proceedings of the National Academy of Sciences of the United States of America. 2014 Sep 2;111(35):E3735-44. PubMed PMID: 25136085. Pubmed Central PMCID: 4156690.
85. Guan JL, Uehara K, Lu S, Wang QP, Funahashi H, Sakurai T, et al. Reciprocal synaptic relationships between orexin- and melanin-concentrating hormone-containing neurons in the rat lateral hypothalamus: a novel circuit implicated in feeding regulation. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2002 Dec;26(12):1523-32. PubMed PMID: 12461668.
86. Rao Y, Lu M, Ge F, Marsh DJ, Qian S, Wang AH, et al. Regulation of synaptic efficacy in hypocretin/orexin-containing neurons by melanin concentrating hormone in the lateral hypothalamus. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008 Sep 10;28(37):9101-10. PubMed PMID: 18784290. Pubmed Central PMCID: 2562258.
87. Barragan-Martinez C, Speck-Hernandez CA, Montoya-Ortiz G, Mantilla RD, Anaya JM, Rojas-Villarraga A. Organic solvents as risk factor for autoimmune diseases: a systematic review and meta-analysis. PloS one. 2012;7(12):e51506. PubMed PMID: 23284705. Pubmed Central PMCID: PMC3526640. Epub 2013/01/04. eng.
88. Anaya JM, Ramirez-Santana C, Alzate MA, Molano-Gonzalez N, Rojas-Villarraga A. The Autoimmune Ecology. Frontiers in immunology. 2016;7:139. PubMed PMID: 27199979. Pubmed Central PMCID: PMC4844615. Epub 2016/05/21. eng.
89. Floreani A, Leung PS, Gershwin ME. Environmental Basis of Autoimmunity. Clinical reviews in allergy & immunology. 2016 Jun;50(3):287-300. PubMed PMID: 25998909. Epub 2015/05/23. eng.
90. 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. Current medicinal chemistry. 2013;20(32):4030-6. PubMed PMID: 23992328. Epub 2013/09/03. eng.
91. Ruiz JT, Lujan L, Blank M, Shoenfeld Y. Adjuvants- and vaccines-induced autoimmunity: animal models. Immunologic research. 2016 Jul 14. PubMed PMID: 27417999. Epub 2016/07/16. Eng.
92. Taneja V, David CS. Lessons from animal models for human autoimmune diseases. Nature immunology. 2001 Sep;2(9):781-4. PubMed PMID: 11526385. Epub 2001/08/30. eng.
93. Apostolico Jde S, Lunardelli VA, Coirada FC, Boscardin SB, Rosa DS. Adjuvants: Classification, Modus Operandi, and Licensing. Journal of immunology research. 2016;2016:1459394. PubMed PMID: 27274998. Pubmed Central PMCID: PMC4870346. Epub 2016/06/09. eng.
94. Satoh M, Kuroda Y, Yoshida H, Behney KM, Mizutani A, Akaogi J, et al. Induction of lupus autoantibodies by adjuvants. Journal of autoimmunity. 2003 Aug;21(1):1-9. PubMed PMID: 12892730. Epub 2003/08/02. eng.
95. Reeves WH, Lee PY, Weinstein JS, Satoh M, Lu L. Induction of autoimmunity by pristane and other naturally occurring hydrocarbons. Trends in immunology. 2009 Sep;30(9):455-64. PubMed PMID: 19699150. Pubmed Central PMCID: PMC2746238. Epub 2009/08/25. eng.
96. 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 Feb;21(2):210-6. PubMed PMID: 22235055. Epub 2012/01/12. eng.

97. Bagavant H, Nandula SR, Kaplonek P, Rybakowska PD, Deshmukh US. Alum, an aluminum-based adjuvant, induces Sjogren's syndrome-like disorder in mice. Clinical and experimental rheumatology. 2014 Mar-Apr;32(2):251-5. PubMed PMID: 24739520. Pubmed Central PMCID: PMC3990870. Epub 2014/04/18. eng.
98. Ozaki A, Fukata K, Fukushima A, Yasuoka M, Yoshida O, Ueno H. Suppression or exacerbation of experimental autoimmune uveoretinitis in Lewis rats by pretreatment with or without an autoantigenic peptide in aluminum hydroxide. Japanese journal of ophthalmology. 2003 Jan-Feb;47(1):102-6. PubMed PMID: 12586187. Epub 2003/02/15. eng.
99. Exley C, Siesjo P, Eriksson H. The immunobiology of aluminium adjuvants: how do they really work? Trends in immunology. 2010 Mar;31(3):103-9. PubMed PMID: 20153253. Epub 2010/02/16. eng.
100. Gherardi RK, Aouizerate J, Cadusseau J, Yara S, Authier FJ. Aluminum adjuvants of vaccines injected into the muscle: Normal fate, pathology and associated disease. Morphologie : bulletin de l'Association des anatomistes. 2016 Jun;100(329):85-94. PubMed PMID: 26948677. Epub 2016/03/08. eng.
101. 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. PubMed PMID: 23557144. Pubmed Central PMCID: PMC3616851. Epub 2013/04/06. eng.
102. Lujan L, Perez M, Salazar E, Alvarez N, Gimeno M, Pinczowski P, et al. Autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA syndrome) in commercial sheep. Immunologic research. 2013 Jul;56(2-3):317-24. PubMed PMID: 23579772. Epub 2013/04/13. eng.
103. Petrik MS, Wong MC, Tabata RC, Garry RF, Shaw CA. Aluminum adjuvant linked to Gulf War illness induces motor neuron death in mice. Neuromolecular medicine. 2007;9(1):83-100. PubMed PMID: 17114826. Epub 2006/11/23. eng.
104. Bonanni P, Picazo JJ, Remy V. The intangible benefits of vaccination - what is the true economic value of vaccination? Journal of market access & health policy. 2015;3. PubMed PMID: 27123182. Pubmed Central PMCID: PMC4802696. Epub 2015/01/01. eng.
105. Rappuoli R, Miller HI, Falkow S. Medicine. The intangible value of vaccination. Science. 2002 Aug 9;297(5583):937-9. PubMed PMID: 12169712. Epub 2002/08/10. eng.
106. Fournie GJ, Mas M, Cautain B, Savignac M, Subra JF, Pelletier L, et al. Induction of autoimmunity through bystander effects. Lessons from immunological disorders induced by heavy metals. Journal of autoimmunity. 2001 May;16(3):319-26. PubMed PMID: 11334498. Epub 2001/05/04. eng.
107. Guimaraes LE, Baker B, Perricone C, Shoenfeld Y. Vaccines, adjuvants and autoimmunity. Pharmacological research. 2015 Oct;100:190-209. PubMed PMID: 26275795. Epub 2015/08/16. eng.
108. Cerpa-Cruz S, Paredes-Casillas P, Landeros Navarro E, Bernard-Medina AG, Martinez-Bonilla G, Gutierrez-Urena S. Adverse events following immunization with vaccines containing adjuvants. Immunologic research. 2013 Jul;56(2-3):299-303. PubMed PMID: 23576057. Epub 2013/04/12. eng.
109. 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 Dec;47:1-16. PubMed PMID: 24238833. Epub 2013/11/19. eng.
110. Shoenfeld Y, Agmon-Levin N. 'ASIA' - autoimmune/inflammatory syndrome induced by adjuvants. Journal of autoimmunity. 2011 Feb;36(1):4-8. PubMed PMID: 20708902. Epub 2010/08/17. eng.

111. Arango MT, Perricone C, Kivity S, Cipriano E, Ceccarelli F, Valesini G, et al. HLA-DRB1 the notorious gene in the mosaic of autoimmunity. Immunologic research. 2016 Jul 19. PubMed PMID: 27435705. Epub 2016/07/21. Eng.
112. Miravalle A, Biller J, Schnitzler E, Bonwit A. Neurological complications following vaccinations. Neurological research. 2010 Apr;32(3):285-92. PubMed PMID: 20406607. Epub 2010/04/22. eng.
113. Kavadas FD, Bitnun A, MacGregor D, Heurter H, Ford Jones EL. Acute neurological events associated with influenza vaccination: are the WHO criteria for assessing causality adequate? Scandinavian journal of infectious diseases. 2008;40(6-7):565-70. PubMed PMID: 18584549. Epub 2008/06/28. eng.
114. Fernandes GC, Camacho LA, Sa Carvalho M, Batista M, de Almeida SM. Neurological adverse events temporally associated to mass vaccination against yellow fever in Juiz de Fora, Brazil, 1999-2005. Vaccine. 2007 Apr 20;25(16):3124-8. PubMed PMID: 17316927. Epub 2007/02/24. eng.
115. Arnheim-Dahlstrom L, Pasternak B, Svanstrom H, Sparen P, Hviid A. Autoimmune, neurological, and venous thromboembolic adverse events after immunisation of adolescent girls with quadrivalent human papillomavirus vaccine in Denmark and Sweden: cohort study. BMJ (Clinical research ed). 2013;347:f5906. PubMed PMID: 24108159. Pubmed Central PMCID: PMC3805482. Epub 2013/10/11. eng.
116. McCarthy JE, Filiano J. Opsoclonus Myoclonus after human papilloma virus vaccine in a pediatric patient. Parkinsonism Relat Disord. 2009 Dec;15(10):792-4. PubMed PMID: 19447066. Epub 2009/05/19. eng.
117. Colafrancesco S, Perricone C, Tomljenovic L, Shoenfeld Y. HPV Vaccines and Primary Ovarian Failure: Another Facet of the Autoimmune/Inflammatory Syndrome Induced by Adjuvants (ASIA). American Journal of Reproductive Immunology. 2013;70(4):309-16.
118. Kivity S, Katzav A, Arango MT, Landau-Rabi M, Zafrir Y, Agmon-Levin N, et al. 16/6-idiotype expressing antibodies induce brain inflammation and cognitive impairment in mice: the mosaic of central nervous system involvement in lupus. BMC medicine. 2013;11:90. PubMed PMID: 23556432. Pubmed Central PMCID: 3616817.
119. Kivity S, Shoenfeld Y, Arango M, Cahill D, O'Kane S, Zusev M, et al. Anti-ribosomal-phosphoprotein autoantibodies penetrate to neuronal cells via neuronal growth associated protein (GAP43), affecting neuronal cells in-vitro. Rheumatology Oxford. 2016;Accepted for publication.
120. Inbar R, Weiss R, Tomljenovic L, Arango MT, Deri Y, Shaw CA, et al. Behavioral abnormalities in female mice following administration of aluminum adjuvants and the human papillomavirus (HPV) vaccine Gardasil. Immunologic research. 2016 Jul 16. PubMed PMID: 27421722. Epub 2016/07/17. Eng.
121. Kivity S, Arango MT, Molano-Gonzalez N, Blank M, Shoenfeld Y. Phospholipid supplementation can attenuate vaccine-induced depressive-like behavior in mice. Immunologic research. 2016 Jul 27. PubMed PMID: 27465467. Epub 2016/07/29. Eng.
122. Katzav A, Ben-Ziv T, Chapman J, Blank M, Reichlin M, Shoenfeld Y. Anti-P ribosomal antibodies induce defect in smell capability in a model of CNS -SLE (depression). Journal of autoimmunity. 2008 Dec;31(4):393-8. PubMed PMID: 18947972.
123. Shoenfeld Y, Cervera R, Gershwin M. Diagnostic Criteria in Autoimmune Diseases. Totowa, NJ USA: Humana Press; 2008.
124. Kampylafka EI, Alexopoulos H, Dalakas MC, Tzioufas AG. Immunotherapies for Neurological Manifestations in the Context of Systemic Autoimmunity. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics. 2016 Jan;13(1):163-78. PubMed PMID: 26510559. Pubmed Central PMCID: PMC4720664. Epub 2015/10/30. eng.

125. Liewluck T, Miravalle A. Immune-Mediated Neurological Disorders. Current neurology and neuroscience reports. 2015 Sep;15(9):61. PubMed PMID: 26202897. Epub 2015/07/24. eng.
126. Sofat N, Malik O, Higgens CS. Neurological involvement in patients with rheumatic disease. QJM : monthly journal of the Association of Physicians. 2006 Feb;99(2):69-79. PubMed PMID: 16434469. Epub 2006/01/26. eng.
127. Brenton JN, Goodkin HP. Antibody-Mediated Autoimmune Encephalitis in Childhood. Pediatric neurology. 2016 Jul;60:13-23. PubMed PMID: 27343023.
128. Libbey JE, Fujinami RS. Role for antibodies in altering behavior and movement. Autism research : official journal of the International Society for Autism Research. 2010 Aug;3(4):147-52. PubMed PMID: 20589715. Epub 2010/07/01. eng.
129. Koren E, Reichlin MW, Koscec M, Fugate RD, Reichlin M. Autoantibodies to the ribosomal P proteins react with a plasma membrane-related target on human cells. The Journal of clinical investigation. 1992 Apr;89(4):1236-41. PubMed PMID: 1313450. Pubmed Central PMCID: 442983.
130. Sun KH, Tang SJ, Lin ML, Wang YS, Sun GH, Liu WT. Monoclonal antibodies against human ribosomal P proteins penetrate into living cells and cause apoptosis of Jurkat T cells in culture. Rheumatology (Oxford, England). 2001 Jul;40(7):750-6. PubMed PMID: 11477279. Epub 2001/07/31. eng.
131. Lee EJ, Jang EJ, Lee E, Yu J, Chung HY, Jang YJ. Cell-penetrating autoantibody induces caspase-mediated apoptosis through catalytic hydrolysis of DNA. Bioorganic & medicinal chemistry. 2007 Mar 1;15(5):2016-23. PubMed PMID: 17234421.
132. Reichlin M. Cellular dysfunction induced by penetration of autoantibodies into living cells: cellular damage and dysfunction mediated by antibodies to dsDNA and ribosomal P proteins. Journal of autoimmunity. 1998 Oct;11(5):557-61. PubMed PMID: 9802943.
133. Ronda N, Gatti R, Giacosa R, Raschi E, Testoni C, Meroni PL, et al. Antifibroblast antibodies from systemic sclerosis patients are internalized by fibroblasts via a caveolin-linked pathway. Arthritis and rheumatism. 2002 Jun;46(6):1595-601. PubMed PMID: 12115191.
134. Alarcon-Segovia D, Ruiz-Arguelles A, Llorente L. Broken dogma: penetration of autoantibodies into living cells. Immunology today. 1996 Apr;17(4):163-4. PubMed PMID: 8871346. Epub 1996/04/01. eng.
135. Alarcon-Segovia D, Ruiz-Arguelles A, Fishbein E. Antibody to nuclear ribonucleoprotein penetrates live human mononuclear cells through Fc receptors. Nature. 1978 Jan 5;271(5640):67-9. PubMed PMID: 625325.
136. Alarcon-Segovia D, Llorente L. Antibody penetration into living cells. IV. Different effects of anti-native DNA and anti-ribonucleoprotein IgG on the cell cycle of activated T gamma cells. Clinical and experimental immunology. 1983 May;52(2):365-71. PubMed PMID: 6190600. Pubmed Central PMCID: PMC1535863. Epub 1983/05/01. eng.
137. Chizzolini C, Raschi E, Rezzonico R, Testoni C, Mallone R, Gabrielli A, et al. Autoantibodies to fibroblasts induce a proadhesive and proinflammatory fibroblast phenotype in patients with systemic sclerosis. Arthritis and rheumatism. 2002 Jun;46(6):1602-13. PubMed PMID: 12115192. Epub 2002/07/13. eng.
138. Peltier J, O'Neill A, Schaffer DV. PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. Developmental neurobiology. 2007 Sep 1;67(10):1348-61. PubMed PMID: 17638387.
139. Chan WS, Sideris A, Sutachan JJ, Montoya GJ, Blanck TJ, Recio-Pinto E. Differential regulation of proliferation and neuronal differentiation in adult rat spinal cord neural stem/progenitors by ERK1/2, Akt, and PLCgamma. Frontiers in molecular neuroscience. 2013;6:23. PubMed PMID: 23986655. Pubmed Central PMCID: 3753454.

140. Ma DK, Ponnusamy K, Song MR, Ming GL, Song H. Molecular genetic analysis of FGFR1 signalling reveals distinct roles of MAPK and PLCgamma1 activation for self-renewal of adult neural stem cells. Molecular brain. 2009;2:16. PubMed PMID: 19505325. Pubmed Central PMCID: 2700800.
141. Mizutani K, Sonoda S, Wakita H, Katoh Y, Shimpo K. Functional recovery and alterations in the expression and localization of protein kinase C following voluntary exercise in rat with cerebral infarction. Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology. 2014 Jan;35(1):53-9. PubMed PMID: 23793170.
142. Aigner L, Caroni P. Absence of persistent spreading, branching, and adhesion in GAP-43-depleted growth cones. The Journal of cell biology. 1995 Feb;128(4):647-60. PubMed PMID: 7860637. Pubmed Central PMCID: 2199882.
143. Iwata M, Shirayama Y, Ishida H, Kawahara R. Hippocampal synapsin I, growth-associated protein-43, and microtubule-associated protein-2 immunoreactivity in learned helplessness rats and antidepressant-treated rats. Neuroscience. 2006 Sep 1;141(3):1301-13. PubMed PMID: 16814933.
144. Law AJ, Pei Q, Walker M, Gordon-Andrews H, Weickert CS, Feldon J, et al. Early parental deprivation in the marmoset monkey produces long-term changes in hippocampal expression of genes involved in synaptic plasticity and implicated in mood disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2009 May;34(6):1381-94. PubMed PMID: 18615010. Pubmed Central PMCID: 2669475.
145. Kall A. The Pandemrix - narcolepsy tragedy: how it started and what we know today. Acta paediatrica (Oslo, Norway : 1992). 2013 Jan;102(1):2-4. PubMed PMID: 23016673. Epub 2012/09/29. eng.
146. O'Flanagan D, Barret AS, Foley M, Cotter S, Bonner C, Crowe C, et al. Investigation of an association between onset of narcolepsy and vaccination with pandemic influenza vaccine, Ireland April 2009-December 2010. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2014;19(17):15-25. PubMed PMID: 24821121. Epub 2014/05/14. eng.
147. Arango MT, Kivity S, Shoenfeld Y. Is narcolepsy a classical autoimmune disease? Pharmacological research. 2015 Feb;92:6-12. PubMed PMID: 25447795. Epub 2014/12/03. eng.
148. Saariaho AH, Vuorela A, Freitag TL, Pizza F, Plazzi G, Partinen M, et al. Autoantibodies against ganglioside GM3 are associated with narcolepsy-cataplexy developing after Pandemrix vaccination against 2009 pandemic H1N1 type influenza virus. Journal of autoimmunity. 2015 Sep;63:68-75. PubMed PMID: 26227560. Epub 2015/08/01. eng.
149. Ahmed SS, Steinman L. Mechanistic insights into influenza vaccine-associated narcolepsy. Human vaccines & immunotherapeutics. 2016 Mar 31:1-6. PubMed PMID: 27031682. Epub 2016/04/01. Eng.
150. Ahmed SS, Volkmuth W, Duca J, Corti L, Pallaoro M, Pezzicoli A, et al. Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2. Science translational medicine. 2015 Jul 1;7(294):294ra105. PubMed PMID: 26136476. Epub 2015/07/03. eng.
151. Vaarala O, Vuorela A, Partinen M, Baumann M, Freitag TL, Meri S, et al. Antigenic differences between AS03 adjuvanted influenza A (H1N1) pandemic vaccines: implications for pandemrix-associated narcolepsy risk. PloS one. 2014;9(12):e114361. PubMed PMID: 25501681. Pubmed Central PMCID: PMC4266499. Epub 2014/12/17. eng.
152. Jacob L, Leib R, Ollila HM, Bonvalet M, Adams CM, Mignot E. Comparison of Pandemrix and Arepanrix, two pH1N1 AS03-adjuvanted vaccines differentially associated with narcolepsy development. Brain, behavior, and immunity. 2015 Jul;47:44-57. PubMed PMID: 25452148. Epub 2014/12/03. eng.

153. Dauvilliers Y, Bauer J, Rigau V, Lalloyer N, Labauge P, Carlander B, et al. Hypothalamic immunopathology in anti-Ma-associated diencephalitis with narcolepsy-cataplexy. JAMA neurology. 2013 Oct;70(10):1305-10. PubMed PMID: 23939463. Epub 2013/08/14. eng.
154. Graus F, Dalmau J. Paraneoplastic neurological syndromes: diagnosis and treatment. Current opinion in neurology. 2007 Dec;20(6):732-7. PubMed PMID: 17992098. Epub 2007/11/10. eng.
155. Dalmau J, Graus F, Villarejo A, Posner JB, Blumenthal D, Thiessen B, et al. Clinical analysis of anti-Ma2-associated encephalitis. Brain : a journal of neurology. 2004 Aug;127(Pt 8):1831-44. PubMed PMID: 15215214. Epub 2004/06/25. eng.
156. Lecendreux M, Maret S, Bassetti C, Mouren MC, Tafti M. Clinical efficacy of high-dose intravenous immunoglobulins near the onset of narcolepsy in a 10-year-old boy. Journal of sleep research. 2003 Dec;12(4):347-8. PubMed PMID: 14633248. Epub 2003/11/25. eng.
157. Dauvilliers Y, Abril B, Mas E, Michel F, Tafti M. Normalization of hypocretin-1 in narcolepsy after intravenous immunoglobulin treatment. Neurology. 2009 Oct 20;73(16):1333-4. PubMed PMID: 19841387. Epub 2009/10/21. eng.
158. Agmon-Levin N, Arango MT, Kivity S, Katzav A, Gilburd B, Blank M, et al. Immunization with hepatitis B vaccine accelerates SLE-like disease in a murine model. Journal of autoimmunity. 2014 Nov;54:21-32. PubMed PMID: 25042822. Epub 2014/07/22. eng.
159. Couette M, Boisse MF, Maison P, Brugieres P, Cesaro P, Chevalier X, et al. Long-term persistence of vaccine-derived aluminum hydroxide is associated with chronic cognitive dysfunction. J Inorg Biochem. 2009 Nov;103(11):1571-8. PubMed PMID: 19748679. Epub 2009/09/15. eng.
160. Li X, Zheng H, Zhang Z, Li M, Huang Z, Schluesener HJ, et al. Glia activation induced by peripheral administration of aluminum oxide nanoparticles in rat brains. Nanomedicine. 2009;5(4):473-9

161. Shaw CA, Petrik MS. Aluminum hydroxide injections lead to motor deficits and motor neuron degeneration. J Inorg Biochem. 2009 Nov;103(11):1555-62. PubMed PMID: 19740540. Pubmed Central PMCID: 2819810. Epub 2009/09/11. eng.
162. Passeri E, Villa C, Couette M, Itti E, Brugieres P, Cesaro P, et al. Long-term follow-up of cognitive dysfunction in patients with aluminum hydroxide-induced macrophagic myofasciitis (MMF). J Inorg Biochem. 2011 Nov;105(11):1457-63. PubMed PMID: 22099155. Epub 2011/11/22. eng.
163. Zafrir Y, Agmon-Levin N, Paz Z, Shilton T, Shoenfeld Y. Autoimmunity following hepatitis B vaccine as part of the spectrum of 'Autoimmune (Auto-inflammatory) Syndrome induced by Adjuvants' (ASIA): analysis of 93 cases. Lupus. 2012 Feb;21(2):146-52. PubMed PMID: 22235045. Epub 2012/01/12. eng.
164. Rondeau V, Commenges D, Jacqmin-Gadda H, Dartigues JF. Relation between aluminum concentrations in drinking water and Alzheimer's disease: an 8-year follow-up study. American journal of epidemiology. 2000 Jul 1;152(1):59-66. PubMed PMID: 10901330. Pubmed Central PMCID: PMC2215380. Epub 2000/07/20. eng.
165. Rondeau V, Jacqmin-Gadda H, Commenges D, Helmer C, Dartigues JF. Aluminum and silica in drinking water and the risk of Alzheimer's disease or cognitive decline: findings from 15-year follow-up of the PAQUID cohort. American journal of epidemiology. 2009 Feb 15;169(4):489-96. PubMed PMID: 19064650. Pubmed Central PMCID: PMC2809081. Epub 2008/12/10. eng.
166. Bishop NJ, Morley R, Day JP, Lucas A. Aluminum neurotoxicity in preterm infants receiving intravenous-feeding solutions. The New England journal of medicine. 1997 May 29;336(22):1557-61. PubMed PMID: 9164811. Epub 1997/05/29. eng.

167. Rogers MA, Simon DG. A preliminary study of dietary aluminium intake and risk of Alzheimer's disease. Age and ageing. 1999 Mar;28(2):205-9. PubMed PMID: 10350420. Epub 1999/06/01. eng.
168. Shaw CA, Tomljenovic L. Aluminum in the central nervous system (CNS): toxicity in humans and animals, vaccine adjuvants, and autoimmunity. Immunologic research. 2013 Apr 23;56(2-3):304-16. PubMed PMID: 23609067. Epub 2013/04/24. Eng.
169. Tomljenovic L. Aluminum and Alzheimer's disease: after a century of controversy, is there a plausible link? Journal of Alzheimer's disease : JAD. 2011;23(4):567-98. PubMed PMID: 21157018. Epub 2010/12/16. eng.
170. Tomljenovic L, Shaw CA. Do aluminum vaccine adjuvants contribute to the rising prevalence of autism? Journal of Inorganic Biochemistry. 2011 Nov;105(11):1489-99. PubMed PMID: ISI:000297565500018. English.
171. Perl DP, Moalem S. Aluminum, Alzheimer's Disease and the Geospatial Occurrence of Similar Disorders. Reviews in Mineralogy & Geochemistry. 2006;64:115-34.
172. Authier FJ, Cherin P, Creange A, Bonnotte B, Ferrer X, Abdelmoumni A, et al. Central nervous system disease in patients with macrophagic myofasciitis. Brain : a journal of neurology. 2001 May;124(Pt 5):974-83. PubMed PMID: 11335699. Epub 2001/05/04. eng.
173. Exley C, Mamutse G, Korchazhkina O, Pye E, Strekopytov S, Polwart A, et al. Elevated urinary excretion of aluminium and iron in multiple sclerosis. Mult Scler. 2006 Oct;12(5):533-40. PubMed PMID: 17086897. Epub 2006/11/08. eng.
174. Seneff S, Davidson RM, Liu J. Empirical Data Confirm Autism Symptoms Related to Aluminum and Acetaminophen Exposure. *Entropy* 2012;14:2227-53.
175. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nature reviews Immunology. 2009 Apr;9(4):287-93. PubMed PMID: 19247370. Pubmed Central PMCID: 3147301. Epub 2009/02/28. eng.
176. Batista-Duharte A, Lindblad EB, Oviedo-Orta E. Progress in understanding adjuvant immunotoxicity mechanisms. Toxicology letters. 2011 Jun 10;203(2):97-105. PubMed PMID: 21392560.
177. Li XB, Zheng H, Zhang ZR, Li M, Huang ZY, Schluesener HJ, et al. Glia activation induced by peripheral administration of aluminum oxide nanoparticles in rat brains. Nanomedicine. 2009 Dec;5(4):473-9. PubMed PMID: 19523415.
178. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature. 2008 Jun 19;453(7198):1122-6. PubMed PMID: 18496530. Pubmed Central PMCID: PMC4804622. Epub 2008/05/23. eng.
179. Bauer C, Duewell P, Mayer C, Lehr HA, Fitzgerald KA, Dauer M, et al. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. Gut. 2010 Sep;59(9):1192-9. PubMed PMID: 20442201.
180. Chakraborty S, Kaushik DK, Gupta M, Basu A. Inflammasome signaling at the heart of central nervous system pathology. J Neurosci Res. 2010 Jun;88(8):1615-31. PubMed PMID: 20127816. Epub 2010/02/04. eng.
181. Jha S, Srivastava SY, Brickey WJ, Iocca H, Toews A, Morrison JP, et al. The inflammasome sensor, NLRP3, regulates CNS inflammation and demyelination via caspase-1 and interleukin-18. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010 Nov 24;30(47):15811-20. PubMed PMID: 21106820. Epub 2010/11/26. eng.
182. Rajamaki K, Lappalainen J, Oorni K, Valimaki E, Matikainen S, Kovanen PT, et al. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol

metabolism and inflammation. PloS one. 2010;5(7):e11765. PubMed PMID: 20668705. Pubmed Central PMCID: 2909263.
183. Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, et al. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nature immunology. 2011 May;12(5):408-15. PubMed PMID: 21478880. Pubmed Central PMCID: 4090391.
184. Joshi JG. Aluminum, a neurotoxin which affects diverse metabolic reactions. BioFactors (Oxford, England). 1990 Jul;2(3):163-9. PubMed PMID: 2198876. Epub 1990/07/01. eng.
185. Walton JR. Functional impairment in aged rats chronically exposed to human range dietary aluminum equivalents. Neurotoxicology. 2009 Mar;30(2):182-93. PubMed PMID: 19109991. Epub 2008/12/27. eng.
186. Gherardi RK, Coquet M, Cherin P, Belec L, Moretto P, Dreyfus PA, et al. Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. Brain : a journal of neurology. 2001 Sep;124(Pt 9):1821-31. PubMed PMID: 11522584. Epub 2001/08/28. eng.
187. Verstraeten SV, Golub MS, Keen CL, Oteiza PI. Myelin is a preferential target of aluminum-mediated oxidative damage. Archives of biochemistry and biophysics. 1997 Aug 15;344(2):289-94. PubMed PMID: 9264541. Epub 1997/08/15. eng.
188. Exley C. The pro-oxidant activity of aluminum. Free radical biology & medicine. 2004 Feb 1;36(3):380-7. PubMed PMID: 15036357. Epub 2004/03/24. eng.
189. Singla N, Dhawan DK. Regulatory role of zinc during aluminium-induced altered carbohydrate metabolism in rat brain. J Neurosci Res. Mar;90(3):698-705. PubMed PMID: 22108899. Epub 2011/11/24. eng.
190. Walton JR. Aluminum Disruption of Calcium Homeostasis and Signal Transduction Resembles Change that Occurs in Aging and Alzheimer's Disease. Journal of Alzheimer's disease : JAD. 2012 Jan 1;29(2):255-73. PubMed PMID: 22330830. Epub 2012/02/15. eng.
191. Shafer TJ, Nostrandt AC, Tilson HA, Mundy WR. Mechanisms underlying AlCl3 inhibition of agonist-stimulated inositol phosphate accumulation. Role of calcium, G-proteins, phospholipase C and protein kinase C. Biochem Pharmacol. 1994 Apr 20;47(8):1417-25. PubMed PMID: 8185649. Epub 1994/04/20. eng.
192. Kanduc D. Potential cross-reactivity between HPV16 L1 protein and sudden death-associated antigens. J Exp Ther Oncol. 2011;9(2):159-65. PubMed PMID: 21699023. Epub 2011/06/28. eng.
193. Kanduc D. Quantifying the possible cross-reactivity risk of an HPV16 vaccine. J Exp Ther Oncol. 2009;8(1):65-76. PubMed PMID: 19827272. Epub 2009/10/16. eng.
194. Vickers MH, Guan J, Gustavsson M, Krageloh CU, Breier BH, Davison M, et al. Supplementation with a mixture of complex lipids derived from milk to growing rats results in improvements in parameters related to growth and cognition. Nutrition research (New York, NY). 2009 Jun;29(6):426-35. PubMed PMID: 19628110. Epub 2009/07/25. eng.
195. Rondanelli M, Giacosa A, Opizzi A, Pelucchi C, La Vecchia C, Montorfano G, et al. Long chain omega 3 polyunsaturated fatty acids supplementation in the treatment of elderly depression: effects on depressive symptoms, on phospholipids fatty acids profile and on health-related quality of life. The journal of nutrition, health & aging. 2011 Jan;15(1):37-44. PubMed PMID: 21267525. Epub 2011/01/27. eng.
196. Rondanelli M, Giacosa A, Opizzi A, Pelucchi C, La Vecchia C, Montorfano G, et al. Effect of omega-3 fatty acids supplementation on depressive symptoms and on health-related quality of life in the treatment of elderly women with depression: a double-blind, placebo-controlled, randomized clinical trial. Journal of the American College of Nutrition. 2010 Feb;29(1):55-64. PubMed PMID: 20595646. Epub 2010/07/03. eng.

197. Benton D, Donohoe RT, Sillance B, Nabb S. The influence of phosphatidylserine supplementation on mood and heart rate when faced with an acute stressor. Nutritional neuroscience. 2001;4(3):169-78. PubMed PMID: 11842886. Epub 2002/02/15. eng.
198. Kullenberg D, Taylor LA, Schneider M, Massing U. Health effects of dietary phospholipids. Lipids Health Dis.11:3. PubMed PMID: 22221489. Epub 2012/01/10. eng.
199. Yechiel E, Barenholz Y. Relationships between membrane lipid composition and biological properties of rat myocytes. Effects of aging and manipulation of lipid composition. J Biol Chem. 1985 Aug 5;260(16):9123-31. PubMed PMID: 3839508. Epub 1985/08/05. eng.
200. Kanno T, Jin Y, Nishizaki T. DL-/PO-phosphatidylcholine restores restraint stress-induced depression-related behaviors and spatial memory impairment. Behav Pharmacol. Sep;25(5-6):575-81. PubMed PMID: 25083573. Epub 2014/08/02. eng.
201. Nicolson GL, Ash ME. Lipid Replacement Therapy: a natural medicine approach to replacing damaged lipids in cellular membranes and organelles and restoring function. Biochim Biophys Acta. Jun;1838(6):1657-79. PubMed PMID: 24269541. Epub 2013/11/26. eng.
202. Borella E, Israeli E, Shoenfeld Y. Lipid Replacement Therapy: Is it a New Approach in Patients with Chronic Fatigue Syndrome? Journal of Autoimmune Diseases and Rheumatology. 2014;2:28-34.
203. Baumeister J, Barthel T, Geiss KR, Weiss M. Influence of phosphatidylserine on cognitive performance and cortical activity after induced stress. Nutritional neuroscience. 2008 Jun;11(3):103-10. PubMed PMID: 18616866. Epub 2008/07/12. eng.
204. Vakhapova V, Cohen T, Richter Y, Herzog Y, Korczyn AD. Phosphatidylserine containing omega-3 fatty acids may improve memory abilities in non-demented elderly with memory complaints: a double-blind placebo-controlled trial. Dement Geriatr Cogn Disord.29(5):467-74. PubMed PMID: 20523044. Epub 2010/06/05. eng.
205. Kato-Kataoka A, Sakai M, Ebina R, Nonaka C, Asano T, Miyamori T. Soybean-derived phosphatidylserine improves memory function of the elderly Japanese subjects with memory complaints. J Clin Biochem Nutr. Nov;47(3):246-55. PubMed PMID: 21103034. Epub 2010/11/26. eng.
206. Delwaide PJ, Gyselynck-Mambourg AM, Hurlet A, Ylieff M. Double-blind randomized controlled study of phosphatidylserine in senile demented patients. Acta Neurol Scand. 1986 Feb;73(2):136-40. PubMed PMID: 3518329. Epub 1986/02/01. eng.
207. Glade MJ, Smith K. Phosphatidylserine and the human brain. Nutrition. Jun;31(6):781-6. PubMed PMID: 25933483. Epub 2015/05/03. eng.
208. Oliveira TG, Chan RB, Bravo FV, Miranda A, Silva RR, Zhou B, et al. The impact of chronic stress on the rat brain lipidome. Mol Psychiatry. Jan;21(1):80-8. PubMed PMID: 25754084. Epub 2015/03/11. eng.
209. Ali HA, Afifi M, Abdelazim AM, Mosleh YY. Quercetin and omega 3 ameliorate oxidative stress induced by aluminium chloride in the brain. J Mol Neurosci. Aug;53(4):654-60. PubMed PMID: 24488531. Epub 2014/02/04. eng.
210. Nicolson GL, Conklin KA. Reversing mitochondrial dysfunction, fatigue and the adverse effects of chemotherapy of metastatic disease by molecular replacement therapy. Clinical & experimental metastasis. 2008;25(2):161-9. PubMed PMID: 18058028. Epub 2007/12/07. eng.
211. Kumar V, Bal A, Gill KD. Impairment of mitochondrial energy metabolism in different regions of rat brain following chronic exposure to aluminium. Brain Res. 2008 Sep 26;1232:94-103. PubMed PMID: 18691561. Epub 2008/08/12. eng.
212. Tomljenovic L. Aluminum and Alzheimer's Disease: After a Century of Controversy, Is there a Plausible Link? Journal of Alzheimers Disease. 2011;23(4):567-98. PubMed PMID: ISI:000288989600001. English.

213. Milstone AM, Meyers K, Elia J. Treatment of acute neuropsychiatric lupus with intravenous immunoglobulin (IVIG): a case report and review of the literature. Clin Rheumatol. 2005 Aug;24(4):394-7. PubMed PMID: 15662488. Epub 2005/01/22. eng.
214. Svetlicky N, Kivity S, Odeh Q, Shovman O, Gertel S, Amital H, et al. Anti-citrullinated-protein-antibody-specific intravenous immunoglobulin attenuates collagen-induced arthritis in mice. Clinical and experimental immunology. 2015 Dec;182(3):241-50. PubMed PMID: 26132809. Pubmed Central PMCID: PMC4636886. Epub 2015/07/02. eng.
215. Mozes E, Sharabi A. A novel tolerogenic peptide, hCDR1, for the specific treatment of systemic lupus erythematosus. Autoimmunity reviews. 2010 Nov;10(1):22-6. PubMed PMID: 20659591. Epub 2010/07/28. eng.
216. 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 Dec;60(12):3744-54. PubMed PMID: 19950283. Epub 2009/12/02. eng.
217. Sthoeger ZM, Sharabi A, Molad Y, Asher I, Zinger H, Dayan M, et al. Treatment of lupus patients with a tolerogenic peptide, hCDR1 (Edratide): immunomodulation of gene expression. Journal of autoimmunity. 2009 Aug;33(1):77-82. PubMed PMID: 19346102. Epub 2009/04/07. eng.
218. Gertel S, Serre G, Shoenfeld Y, Amital H. Immune tolerance induction with multiepitope peptide derived from citrullinated autoantigens attenuates arthritis manifestations in adjuvant arthritis rats. Journal of immunology. 2015 Jun 15;194(12):5674-80. PubMed PMID: 25964493. Epub 2015/05/13. eng.
219. Gertel S, Shoenfeld Y, Amital H. Tolerogenic citrullinated peptide for arthritis. Oncotarget. 2015 Aug 14;6(23):19344-5. PubMed PMID: 26305847. Pubmed Central PMCID: PMC4637280. Epub 2015/08/26. eng.
220. Gomez Y. Dos años del enigmático desmayo de niñas vacunadas contra el papiloma. In: Gomez Y, editor. El Tiempo: El Tiempo; 2016.
221. Anaya JM, Reyes B, Perdomo-Arciniegas AM, Camacho-Rodriguez B, Rojas-Villarraga A. Autoimmune/auto-inflammatory syndrome induced by adjuvants (ASIA) after quadrivalent human papillomavirus vaccination in Colombians: a call for personalised medicine. Clinical and experimental rheumatology. 2015 Jul-Aug;33(4):545-8. PubMed PMID: 25962455.
222. Tomijenovic L, Arango MT, Agmon-Levin N. Vaccination in autoimmune animal models. The Israel Medical Association journal : IMAJ. 2014 Oct;16(10):657-8. PubMed PMID: 25438463. Epub 2014/12/03. eng.
223. Arango MT, Kivity S, Chapman J, Shoenfeld Y. Narcolepsy--genes, infections and vaccines: the clues for a new autoimmune disease. The Israel Medical Association journal : IMAJ. 2014 Oct;16(10):636-7. PubMed PMID: 25438454. Epub 2014/12/03. eng.

9 FIGURES

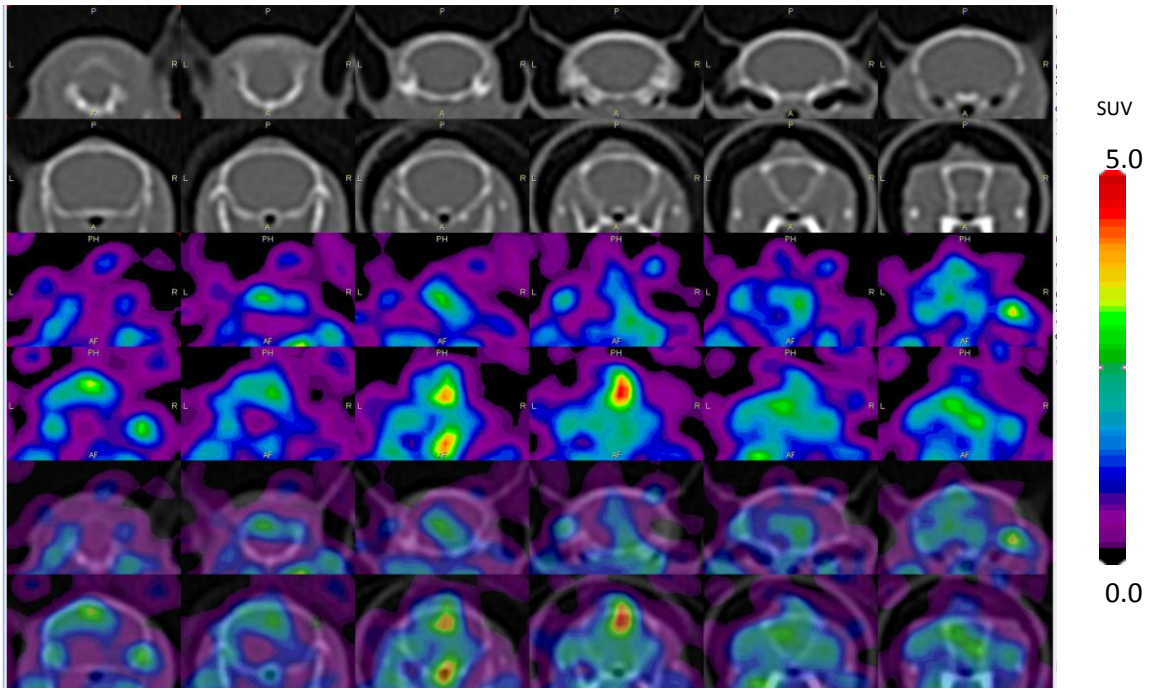


Figure 1. PET/CT Imaging of a mouse brain immunized by intracerebro ventricular (ICV) injection of raiolabeled total IgG from narcoleptic patients. Four days after immunization, axial images, slice thickness 0.9mm. Twelve images of posterior-to-anterior were displayed from the upper left.

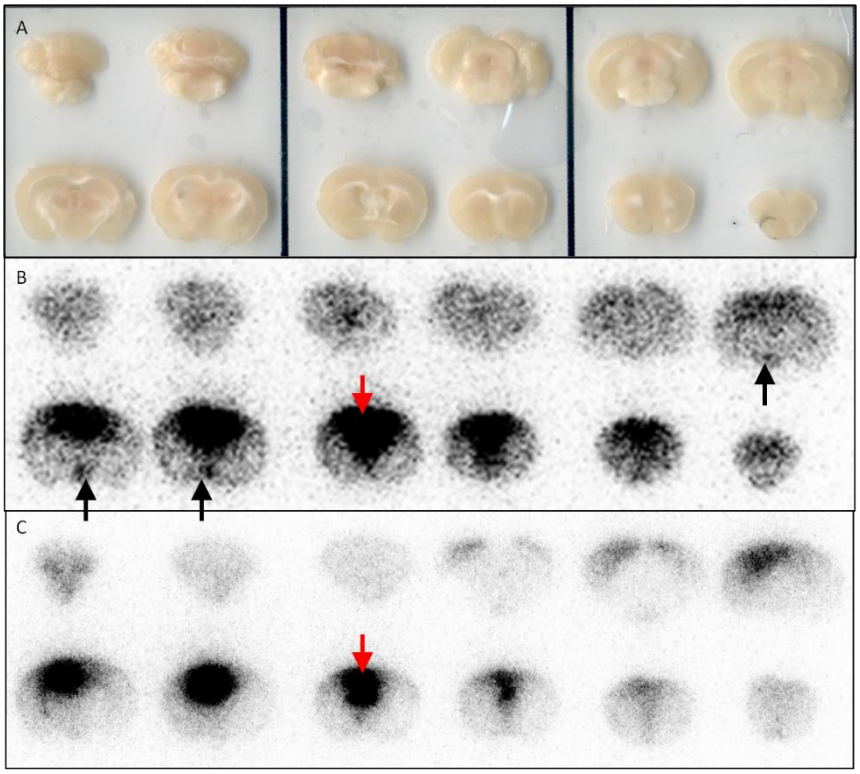


Figure 2. Autoradiography analysis. Four days after Intra cerebro-ventricular injection of IgG mice were sacrificed and their brains were divided into 12 segments (A). Black arrows indicate the accumulation of the signal in the hypothalamus after ICV injection of IgG isolated from narcoleptic patients (B) but not from healthy controls (C). Red arrows indicate the injection site which shows a higher intensity of the signal.

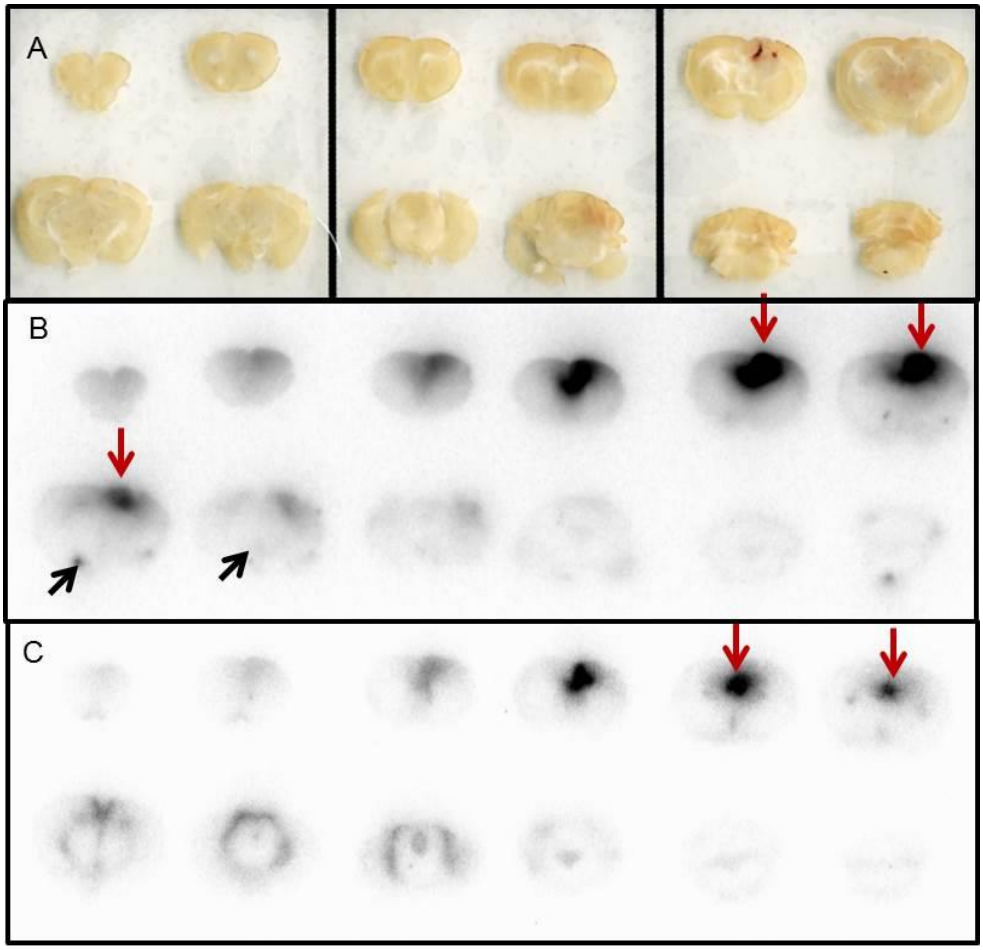


Figure 3. Autoradiography analysis. Four days after Intra cerebro-ventricular injection of IgG mice were sacrificed and their brains were divided into 12 segments (A). Black arrows indicate the accumulation of the signal in the amygdala after ICV injection of anti-ribosomal P purified antibodies (B) but not from healthy controls (C). Red arrows indicate the injection site which shows a higher intensity of the signal.

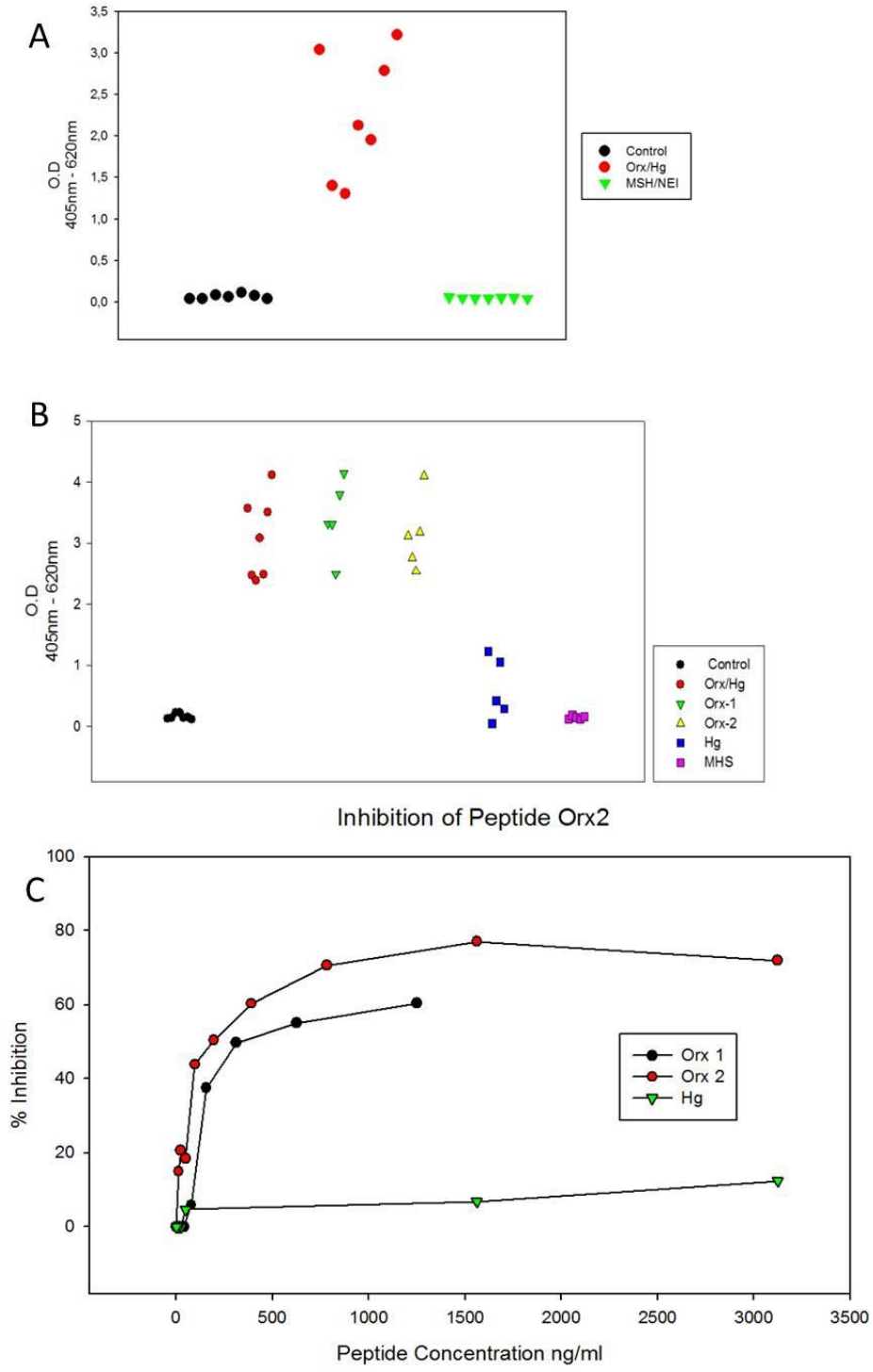


Figure 4. A. Antibody response against immunized peptides. Control: Immunized with CFA. Orx/Hg: Orexin peptides and H1N1 peptide with structural similarities. MSH/NEI: αMSH and NEI which

prevent biding of antibodies from narcoleptic patients to rats. B. Specific antibody response to peptides within the group immunized with Orexin peptides and H1N1 peptide with structural similarities. Antibodies against: Orx/Hg, the three different peptides Orexin peptides and H1N1 peptide with structural similarities. Orx1-1, the orx-1 peptide (AGNHAAGILTLGK). Orx-2. Orx-2 peptide (SGNHAAGILTMGR). Hg. Peptide from H1N1 hemagglutinin (ERNAGSGIIISDT). MSH: Control peptide. αMSH (SYSMEMFRWGKPV-NH2). C. Percentage of inhibition of antibodies against orx 2 Peptide. Black: Inhibition with Orx-1 peptide. Red: Inhibition with Orx-2 peptide. Green: Inhibition with H1N1 hemagglutinin peptide.

10 APPENDICES:

10.1 PAPERS AND COPYRIGHT PERMISSIONS:

Anti-ribosomal-phosphoprotein autoantibodies penetrate to neuronal cells via neuronal growth associated protein, affecting neuronal cells *in vitro*.

Kivity S(1), Shoenfeld Y(2), Arango MT(3), Cahill DJ(4), O'Kane SL(4), Zusev M(1), Slutsky I(5), Harel-Meir M(1), Chapman J(6), Matthias T(7), Blank M(1).

OBJECTIVE: Anti-ribosomal-phosphoprotein antibodies (anti-Ribos.P Abs) are detected in 10-45% of NPSLE patients. Intracerebroventricular administration of anti-ribosomal-P Abs induces depression-like behaviour in mice. We aimed to discern the mechanism by which anti-Ribos.P Abs induce behavioural changes in mice. METHODS: Anti-Ribos.P Abs were exposed to human and rat neuronal cell cultures, as well as to human umbilical vein

endothelial cell cultures for a control. The cellular localization of anti-Ribo.P Abs was found by an immunofluorescent technique using a confocal microscope. Identification of the target molecules was undertaken using a cDNA library. Immunohistochemistry and an inhibition assay were carried out to confirm the identity of the target molecules. Neuronal cell proliferation was measured by bromodeoxyuridine, and Akt and Erk expression by immunoblot. RESULTS: Human anti-Ribos.P Abs penetrated into human neuronal cells and rat hippocampal cell cultures in vitro, but not to endothelial cells as examined. Screening a high-content human cDNA-library with anti-Ribos.P Abs identified neuronal growth-associated protein (GAP43) as a target for anti-Ribos.P Abs. *Ex vivo* anti-Ribos.P Abs bind to mouse brain sections of hippocampus, dentate and amygdala. Anti-Ribos.P Abs brain-binding was prevented by GAP43 protein. Interestingly, GAP43 inhibited in a dose-dependent manner the anti-Ribos.P Abs binding to recombinant-ribosomal-P0, indicating mimicry between the ribosomal-P0 protein and GAP43. Furthermore, anti-Ribos.P Abs reduced neuronal cell proliferation activity in vitro ($P < 0.001$), whereas GAP43 decreased this inhibitory activity by a factor of 7.6. The last was related to Akt and Erk dephosphorylation. CONCLUSION: Anti-Ribos.P Abs penetrate neuronal cells in vitro by targeting GAP43. Anti -Ribos.P Abs inhibit neuronal-cell proliferation via inhibition of Akt and Erk. Our data contribute to deciphering the mechanism for anti-Ribos.P Abs' pathogenic activity in NPSLE.

Rheumatology (Oxford). 2016 May 6. pii: kew027.

http://rheumatology.oxfordjournals.org/content/early/2016/05/06/rheumatology.kew027.long

Kivity et al. BMC Medicine 2013, 11:90
http://www.biomedcentral.com/1741-7015/11/90

Autoimmunity

BMC Medicine

RESEARCH ARTICLE Open Access

16/6-idiotype expressing antibodies induce brain inflammation and cognitive impairment in mice: the mosaic of central nervous system involvement in lupus

Shaye Kivity[1,2*†], Aviva Katzav[1,3†], Maria Teresa Arango[1,4,5], Moran Landau-Rabi[1], Yaron Zafrir[1], Nancy Agmon-Levin[1], Miri Blank[1], Juan-Manuel Anaya[4], Edna Mozes[6], Joab Chapman[1,3] and Yehuda Shoenfeld[1,7]

Abstract

Background: The 16/6-idiotype (16/6-Id) of the human anti-DNA antibody was found to induce experimental lupus in naïve mice, manifested by production of autoantibodies, leukopenia and elevated inflammatory markers, as well as kidney and brain involvement. We assessed behavior and brain pathology of naive mice injected intra-cerebra-ventricularly (ICV) with the 16/6-Id antibody.

Methods: C3H female mice were injected ICV to the right hemisphere with the human 16/6-Id antibody or commercial human IgG antibodies (control). The mice were tested for depression by the forced swimming test (FST), locomotor and explorative activity by the staircase test, and cognitive functions were examined by the novel object recognition and Y-maze tests. Brain slices were stained for inflammatory processes.

Results: 16/6-Id injected mice were cognitively impaired as shown by significant differences in the preference for a new object in the novel object recognition test compared to controls ($P = 0.012$). Similarly, the preference for spatial novelty in the Y-maze test was significantly higher in the control group compared to the 16/6-Id-injected mice (42% vs. 9%, respectively, $P = 0.065$). Depression–like behavior and locomotor activity were not significantly different between the16/6-Id-injected and the control mice. Immunohistochemistry analysis revealed an increase in astrocytes and microglial activation in the hippocampus and amygdala, in the 16/6-Id injected group compared to the control.

Conclusions: Passive transfer of 16/6-Id antibodies directly into mice brain resulted in cognitive impairments and histological evidence for brain inflammation. These findings shed additional light on the diverse mosaic pathophysiology of neuropsychiatric lupus.

Keywords: Systemic lupus erythematosus, 16/6 idiotype, Anti-DNA, Neuropsychiatric lupus, Cognitive impairment

Background

Neuropsychiatric systemic lupus erythematosus (NPSLE) refers to a complex set of syndromes involving the central nervous system (CNS) in up to 56% of lupus patients [1-5]. Due to the varied diagnostic criteria applied to define NPSLE, the American College of Rheumatology has proposed a standard nomenclature of case definitions, reporting standards and diagnostic testing recommendations for the 19 neuropsychiatric Systemic lupus erythematosus (SLE) syndromes [6]. While some of the focal manifestations (for example, stroke) can be explained by vasculitic or thrombotic lesions, the pathogenicity of more diffuse manifestations of NPSLE (for example, cognitive impairment, depression and psychosis) remains relatively obscure. Nevertheless, studies have demonstrated the importance of various factors involved in the development of diffuse neuropsychiatric manifestations, such as the presence of autoantibodies, inflammatory mediators (for

* Correspondence: kivitys@gmail.com
†Equal contributors
[1]The Zabludovicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer, 2 Derech Sheba St., Ramat Gan 52621, Israel
[2]Rheumatic Disease Unit, Sheba Medical Center, Tel-Hashomer, 2 Derech Sheba St., Ramat Gan 52621, Israel
Full list of author information is available at the end of the article

example, cytokines, matrix metalloproteinases), neuropeptides and endocrine factors [7-10]. Other factors, such as medications and primary neurologic and psychiatric disorders, may play a major role as well.

More than 20 brain specific and non-specific autoantibodies have been proposed to be involved in the mechanism of NPLSE [11], including anti-neuronal [12], anti-ribosomal–P [13,14], anti-phospholipid [15] antibodies, as well as anti NR2/anti-DNA antibodies that cross react with N-methyl-D-aspartate (NMDA) receptors [3,16]. During the last two decades, anti-DNA idiotypes were characterized, and found to play an important role in systemic lupus erythematosus and NPSLE [17]. The 16/6 idiotype (Id) antibody is a human anti-single-stranded-DNA (anti-ssDNA) monoclonal antibody (mAb) originated from a patient with cold agglutinin disease [18]. The 16/6-Id was found to be polyspecific [19], cross reacting with cytoskeletal proteins (vimentin), platelets, lymphocyte membranes, pathogens such as *Klebsiela* polysaccharides and *Mycobacterium tuberculosis* glycoproteins, brain glycolipids and tumor cells [20-22]. The presence of 16/6-Id was detected in 30% of lupus patients, and their levels were found to correlate with disease activity [23,24]. Elevated titers of 16/6-Id were also detected in NPSLE patients [25]. Deposits of 16/6-Id were found in the skin, kidney and brain tissue [21,26,27], and were found to bind human cortical brain tissue sections *ex vivo*. The presence of circulating 16/6-Id was detected in patients with other autoimmuine diseases as well (for example, polymyositis, systemic sclerosis) [28,29]. Immunization of naïve mice with the human anti-DNA 16/6-Id mAb was shown to induce experimental lupus manifested both serologically and clinically. A wide profile of mice autoantibodies (for example, mouse 16/6-Id, and antibodies against dsDNA, ssDNA, Ro, La, RNP, Sm, histones, cardiolipin and phosphatydilserine), were detected, as well as leukopenia, elevated erythrocyte sedimentation rate (ESR), proteinuria and the deposition of immunoglobulins in the kidney mesangium [30-32]. In addition, recent-preliminary data showed histological brain changes in mice with experimental SLE induced by active immunization with the 16/6-Id (A. Marom and E. Mozes, unpublished results). Therefore, we hypothesized that the 16/6-Ids have a pathogenic role in neuropsychiatric lupus. In the present study we investigated the effect of 16/6-Id on behavioral and cognitive functions, as well as on the brain pathology of naïve mice injected intra-cerebra -ventricularily (ICV) with the 16/6-Id.

Methods

Mice, antibody injection and experimental design

Mice

Three-month-old, female C3H 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.

Monoclonal 16/6-Id expressing antibodies

The human monoclonal anti–DNA antibodies were produced by a hybridoma derived from fusion of the GM4672 lymphoblastoid cell line and peripheral blood or splenic lymphocytes obtained from three lupus patients. The human mAb that bears the 16/6-Id (IgG1/k) has been characterized previously [33]. The mAb was secreted by hybridoma cells that were grown in culture and were purified by using a protein G-sepharose column (Pharmacia, Fine Chemicals, Uppsala, Sweden).

The injection process is based on a detailed protocol reported by Shoenfeld *et al.* [34]. Mice were anesthetized by intra-peritoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). The skull was carefully exposed, and a small hole was drilled with a 25-gauge needle above the right lateral ventricle (2 mm lateral to the midline and 2.5 mm posterior to the bregma). A 27-gauge needle attached to a Hamilton syringe was inserted at this point to a depth of 2 mm, where preliminary tests had confirmed accurate ICV placement by injection of dye. Then 1 μl of anti-DNA 16/6-Id mAb or control IgG was slowly infused, the needle was withdrawn and the skin over the scalp was sutured. All antibody solutions used contained 6 mg protein/ml. Each mouse received only a single injection.

Experimental design

Twenty-one CH3 mice were injected ICV to the right hemisphere, 11 with human 16/6-Id antibodies and 10 with commercial human IgG antibodies (control). The forced swimming test (FST) was performed at Days 16 and 23 after antibody injection, the staircase test at Days 14 and 26, the novel object recognition at Days 19 and 20 and the Y-maze test at Day 21. At Day 24, under anesthesia, a systemic perfusion was performed, and the brains were collected. Immunofluorescence staining was performed to detect markers of inflammation or neuronal degeneration (see below).

Cognitive and behavioral tests

Forced swimming test

This test is based on Porsolt *et al.*'s description [35]. Mice were placed in individual glass beakers (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 a pretest session of 15 minutes, and later were removed from the cylinder, and then returned to their home cages. Twenty-four hours later (Day 2), the mice were

re-exposed to the swimming condition in a similar environment, and then 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.

Staircase test

Locomotor and explorative activity was evaluated by the staircase test, as described previously by Katzav *et al.* [15]. This test analyzes locomotor and exploratory activities (stair-climbing) and anxiety (rearing). 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.

Novel object recognition test

This is a visual recognition memory test based on a method described by Tordera *et al.* [36]. 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 two separate test days. Before training, mice were individually habituated by allowing them to explore the box for one hour. No data were collected at this phase. During training sessions, 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 for five 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; turning around or sitting near the object was not considered as exploratory behavior. The time spent in exploring each object was recorded. The animals were returned to their home cages immediately after training. During the retention test, the animals were placed back into the same box after a four-hour interval, and allowed to explore freely for five minutes. One of the familiar objects used during training was replaced by a novel object. 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. Individual animals demonstrating insufficient task performance were excluded from later specific statistical analyses for the following reasons: (1) non-exploration, which was defined as no objection interaction or (2) technical malfunctions during data collection.

Y maze test

The Y maze test was used to assess spatial memory. 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 five minutes, one of the other two arms was randomly chosen to be blocked whereas on the second trial, lasting for two minutes, both arms were open. The two trials were separated by a two-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 assessed by a preference index [37]: time in the new arm - time old arm/time in the new arm + time in the old arm, assessing spatial memory. The mouse is expected to recognize the old arm as old and spend more time in the new arm.

Immunofluorescence staining

Brain perfusion and fixation

The mice were anesthetized 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 (PO4, pH 7.4). After perfusion, the brain was quickly removed and fixed overnight in 4% PFA (in PO4, pH 7.4) at 4°C. On the following day, the brain was cryoprotected by immersion in 30% sucrose in 0.1M PO4 (pH 7.4) for 24 to 48 hours at 4°C before brain cutting.

Brain cutting and preservation

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. Staining was performed as

follows. Six mice (three IgG control and three 16/6 Id) were used for immunohistochemistry. 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-Iba1, pAb, Abcam, Cambridge, UK) and astrocytes (anti-GFAP mAb, Dako, Carpinteria, CA, USA). Counter staining was performed with Hoechst (Sigma-Aldrich Israel Ltd., Rehovot Israel).

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 $P < 0.05$.

Results

Cognitive and behavioral performance

The results of cognitive performance in the novel object recognition test are presented as the proportion time spent near objects (new and old) in both groups (Figure 1). There was a significant preference for attention to the new object in the control group (64% time spent near the new object compared to 36% time spent near the old object, $P = 0.012$), while no difference in the preference was seen in the mice injected with 16/6-Id (56% vs. 44% time spent near the new object vs. old object, $P = 0.655$). This suggests a specific visual recognition memory impairment in the 16/6-Id mice. Similarly, cognitive performance in the Y-maze test is presented as a preference index for new (additional percent time spent in the novel arm) in both groups (Figure 2). The control IgG mice spent 46% additional time in the new lane while the mice injected with 16/6-Id spent 9% additional time in the new lane ($P = 0.015$ by *t*-test).

In the forced swimming test there was no significant difference between 16/6-Id injected and control mice in depression-like behavior at Days 16 and 24 after injection. Average immobility times of the control mice vs. 16/6-Id injected mice were 117.6 ± 65.9 vs. 160 ± 72.8 ($P = 0.159$ by *t*-test) and 182.5 ± 45.4 vs. 205.7 ± 42.7 sec ($P = 0.238$ by *t*-test) on Days 16 and 24, respectively.

In the staircase test, there was no significant difference between the average rearing and stair-climbing counts, among mice from control-IgG vs. 16/6-Id (23.7 ± 2.6 vs. 21.8 ± 2.5 rearings, and 24.5 ± 2.3 vs. 16.5 ± 4.4 stair-climbing events, respectively, $P > 0.016$). The results also did not change from Day 14 to 26.

Brain pathology

Brain sections were stained for activated microglia and astrocytes (as markers for inflammation). The 16/6-Id injected mice demonstrated increased microglial activation (Iba-1 staining), at the hippocampus (CA1, CA3, dentate gyrus, stratum radiatum) as well as the amygdala, compared to IgG control (Figure 3). The difference

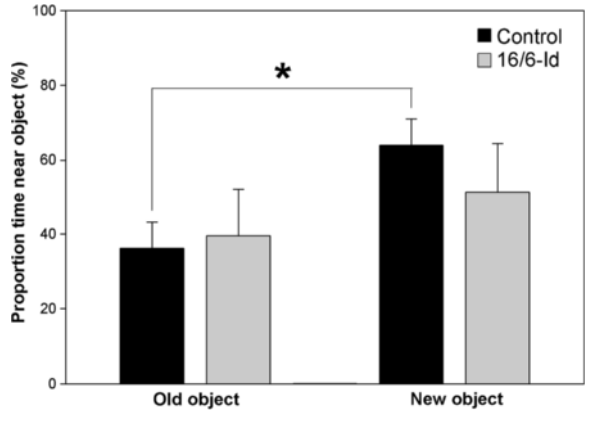


Figure 1 16/6-Id injected mice displayed impaired performance in the novel object recognition test. Results are presented as the proportion of time spent near the old and new objects by the 16/6-Id (gray bars) and IgG control (black bars) injected mice. The control mice (IgG) significantly preferred the new object (64% vs. 36% for the proportion time near the new vs. old objects respectively; $P = 0.01$), while the 16/6-Id injected mice had no significant preference to either objects (56% vs. 44% new vs. old; $P = 0.5$). Results presented as mean ± SEM. * Statistically significant ($P < 0.05$).

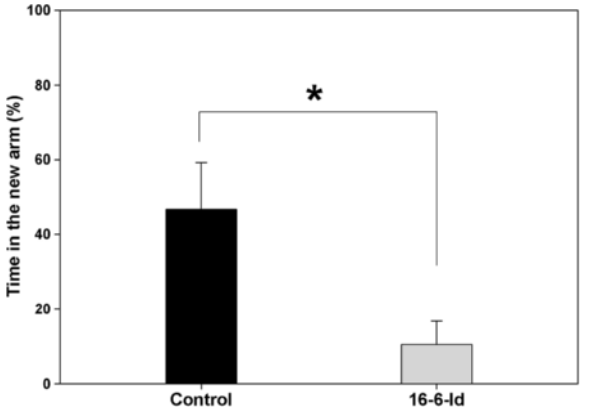


Figure 2 16/6-Id injected mice displayed impaired spatial memory in the Y-maze test. Results are presented as the proportion of time (mean ± SEM) spent in the new arm introduced by the 16/6-Id (gray bars) and IgG control (black bars) injected mice. In the figure it is shown that the control group (IgG injected) spent more time in the new lane as compared to the 16/6 injected group. They have recognized the old lane as known and preferred exploring the new lane, which means that their spatial memory is conserved. There was a significant difference in additional time spent in the new lane between the 16/6 and IgG group (0.46 vs. 0.09, $P = 0.02$ respectively). * Statistically significant ($P < 0.05$).

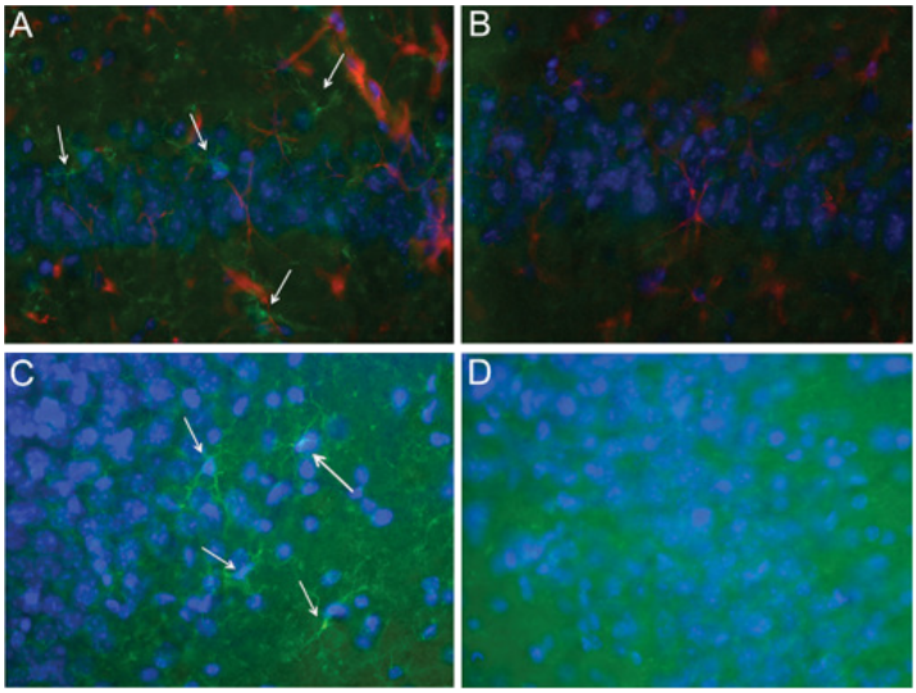


Figure 3 Increased brain inflammation (activated microglia) in 16/6-Id mice in the hippocampal regions (CA1, CA3). Staining of activated microglia (green, white arrows) was more prominent in the 16/6-Id injected mice brains (**A, C**) compared to control mice brains (**B, D**) in the hippocampal regions CA1 (**A, B**) and CA3 (**C, D**). Hoechst nucleus staining – blue, GFAP staining – red. Magnification ×40.

in microglial activation staining was not seen in the neucortex and piriform cortex, between 16/6-Id and control-IgG mice. Increased staining for astrocytes (GFAP staining) was also noted in the CA3 hippocampal region in the 16/6-Id injected mice compared to controls (Figure 4).

Discussion

In the present study we have observed that passive transfer of 16/6-Id antibodies directly to mice brains resulted in a selective cognitive impairment, expressed as visual recognition and spatial memory deficits. Depressive behavior (FST) and locomotor activity (staircase test) were not altered in the 16/6-Id injected mice, when compared to the control group. Our findings suggest that 16/6-Id antibodies may have a role in the pathogenesis of cognitive impairment observed in some patients with SLE [8].

Immunostaining of brain sections from both groups revealed increased presence of activated microglia and astrocytes, in the hippocampal region of the 16/6-Id injected mice, compared to the controls. The hippocampus has an important function in memory processing, therefore, its damage by an inflammatory processes may affect cognitive performance in the 16/6-Id injected mice. Astrocytes in steady state conditions are mainly responsible for biochemical support and several other chemical roles such as maintenance of extracellular ion balance. However, in special situations, astrocytes may increase in number as an inflammatory reaction aimed for scaring and repairing CNS tissue. Microglia serve as scavengers and are activated in an inflammatory reaction. The presence of more astrocytes (gliosis) or the activation of microglia in brain tissue can implicate an inflammatory state. Our hypothesis regarding the pathogenesis of 16/6-Id antibodies induced-brain impairment includes several mechanisms: 1) Neuronal degeneration may be caused by direct or indirect injury to hippocampal area. For example, recently Berry *et al*. demonstrated that anti-ATP synthase autoantibodies, purified from Alzheimer's disease patients, can lead to cognitive impairment and hippocampal neuron apoptosis in naïve mice [38]. Other neurotoxic autoantibodies, such as anti-phospholipid and anti-ribosomal P antibodies, were shown to penetrate living cells and cause functional cellular injury and apoptosis by inhibiting protein synthesis [39,40]. 2) Neuronal function modification. 16/6-Id antibodies may recognize and bind antigens expressed on neurons of the hippocampus and may affect brain cells by alter signaling, cell function and neurotransmitter pathways [41]. 3) Brain inflammation. Injection of 16/6-Id antibodies may lead to brain inflammation involving activation of microglia and astrocytes, and the production of pro-inflammatory cytokines. This inflammatory response can disrupt the

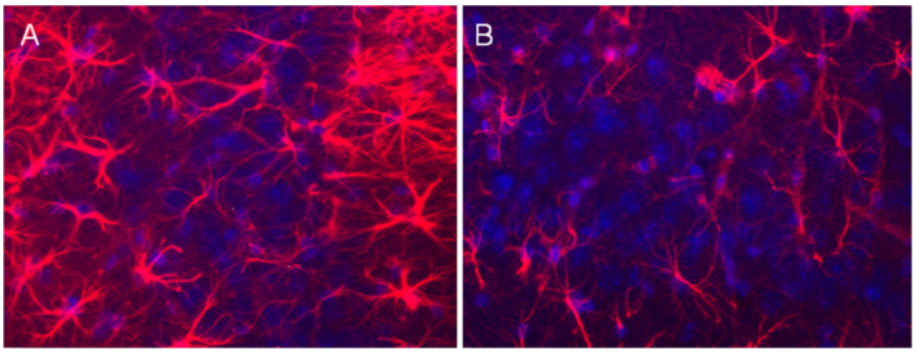


Figure 4 Increased brain inflammation (astrocyes) in 16/6-Id mice in the hippocampal region (CA3). Staining of astrocytes (red) in the hippocampal CA3 region was more prominent in the 16/6-Id injected mice brains (**A**) compared to control mice injected with commercial IgG (**B**). Hoechst nucleus staining - blue. Magnification ×40.

blood–brain barrier, facilitating entry into the brain by inflammatory factors, including circulating cells of the immune system, cytokines, immune-complex mediated small vessel inflammation, and complement components. The inflammatory reaction may induce cognitive changes observed in the injected mice.

We have extensively studied the pathogenesis of different autoantibodies and their influence on the brain. Injection of anti-ribosomal-P antibodies ICV to naïve mice resulted in depressive-like behavior in these mice [42,43]. In another study, we found that injection of antiphospholipid syndrome patients with antibodies induced memory deficits and hyperactivity [15,44]. This suggests that a certain antibody is linked with each specific disease manifestation. The presence of numerous autoantibodies, at least 174 in SLE and 20 in NPSLE, which might have a role in the mechanism of the disease were reported during the past years [11,45]. This may explain the diversity of 19 neuropsychiatric manifestations which can be demonstrated in more than 50% of SLE patients [46]. We propose a hypothesis, that in NPSLE patients different manifestations are the result of an interplay among various auto-antibodies and genetic and environmental factors. For this process to occur, auto-antibodies produced in the body must be able to cross the blood–brain barrier (BBB). It is presumed that the BBB can become transiently "unlocked" following an inflammatory insult, an immune complex damage or exposure to infectious endotoxins (for example, lipopolysaccharide, LPS), allowing antibody penetration. In addition, different auto-antibodies may attach to different epitopes, expressed unevenly in different brain areas or neuronal networks. In the studies of Diamond *et al.*, anti-DNA antibodies which can cross-react with the NR2 - anti-NMDA receptor were found in the sera, CSF and brains of SLE patients [16,47]. These antibodies were shown to alter brain cell function and to mediate apoptotic death *in vivo* and *in vitro* [16,47]. In their experiments, the BBB was breached temporarily by injection with LPS to imitate an infection [48], while others used noradrenalin to imitate a stressful condition; both conditions were implicated in triggering disease flare-ups in SLE and NPSLE patients. The studies of Diamond *et al.* added another layer to the current understandings regarding the role of different auto-antibodies in the pathogenesis of NPSLE. Another technique to bypass the BBB was used by us in several experiments. In the ICV technique, antibodies were injected directly into the lateral ventricle in the mouse brain, allowing antibody dispersal throughout the brain tissue. In our previous studies, an experimental NPSLE was induced by passive transfer of anti-ribosomal-P antibodies directly to mice brains [43]. The intra-cerebra-ventricularly injected mice exhibited a depression-like behavior, not associated with motor or cognitive deficits, and was significantly attenuated by prolong treatment with an anti-depressant (fluoxetine), but not with anti-psychotic drug (haloperidol). Interestingly, the anti-ribosomal-P antibody specifically stained neurons which are related to limbic and olfactory brain areas: the hippocampus, cingulate cortex and the primary olfactory piriform cortex [43]. The depressed mice also exhibited a decreased smell threshold capability [42], as well as olfactory and limbic imaging alterations, when manganese-enhanced-magnetic resonance imaging (MRI) was performed [49].

Another issue of this puzzle was stressed almost two decades ago when the importance of the idiotypic network in the induction of various autoimmune diseases was acknowledged [22,50]. One proposed mechanism of action of the 16 /6-Id is *via* the idiotypic network, in which injection of human anti-DNA 16/6-Id mAbs induces the generation of anti-Id, and anti-anti-Id, and so on. The production of 16/6-Id was found to be induced also by several infectious agents (for example, *Klebsiella pneumonia* [51,52] and *Mycobacterium tuberculosis* [53]); this could point to the role of infections in initiating the disease in a genetically susceptible individual [54].

The finding, that 16/6-Id antibodies were detected in other autoimmune diseases, such as PM/DM and scleroderma without them expressing central nervous symptoms is interesting. Perhaps, in some diseases (for example, SLE) a variety of systemic factors enable the altering of BBB permeability. These factors may include other circulating antibodies, inflammatory elements, as well as vasogenic agents, growth factors and free radicals. This phenomenon is not unusual in the autoimmunity field, for instance, anti-Ro antibodies are associated with myositis or sub-acute skin manifestations in some SLE patients and not in Sjogren patients.

The current finding, that the 16/6-Id is related to spatial novelty and visual recognition memory impairments in mice, may attest for immune-mediated damage to brain areas relevant for these functions. There is a wide agreement that spatial long-term memory and object recognition is dependent on the functioning of the hippocampal region [55]. Taken together, these concepts may promote the idea for a treatment for NPSLE via blocking or inhibiting the 16/6-Id. This can be done perhaps by treatment with intravenous gamma-globulin, which harbors anti-idiotypic antibodies itself, and has shown some efficacy in the treatment of NPSLE patients [56]. Other therapeutic means may involve the utilization of inhibitory peptides based on the complementarity determining region of anti-DNA antibodies. Indeed, such a peptide was shown to be effective in animal models and in a limited number of lupus patients [57-59].

Conclusions

Passive transfer of anti-DNA 16/6-idiotype directly to mice brains resulted in cognitive impairment, supported by cognitive testing impairments, and changes in brain histological analysis. Therefore, the 16/6-idiotype may have a role in cognitive decline, as well as other neuropsychiatric manifestations, which are found in lupus patients.

Abbreviations

anti-ssDNA: Anti-single-stranded-DNA; BBB: Blood–brain barrier; CNS: Central nervous system; FST: Forced swimming test; ICV: Intra-cerebra-ventricularly; i.p: Intra-peritoneal; LPS: Lipopolysaccharide; mAb: Monoclonal antibody; MRI: Magnetic resonance imaging; NMDA: N-methyl-D-aspartate; NPSLE: Neuropsychiatric systemic lupus erythematosus; PBS: Phosphate buffered saline; PFA: Paraformaldehyde; SLE: Systemic lupus erythematosus; 16/6-Id: 16/6-idiotype.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SK participated in the immunohistochemical and behavioral studies and drafted the manuscript. AK participated in the immunohistochemical and behavioral studies and helped to draft the manuscript. MTA participated in the immunohistochemical studies and performed the statistical analysis. MLR and YZ participated in the behavioral studies. NAL participated in the behavioral studies and helped to draft the manuscript. MB participated in the design and helped to draft the manuscript. JMA participated in the design and coordination of the study. EM and JC participated in the design and coordination of the study and helped to draft the manuscript. YS conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank Itzik Sehayek for his substantial contribution to this research.

Author details

[1]The Zabludovicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer, 2 Derech Sheba St., Ramat Gan 52621, Israel. [2]Rheumatic Disease Unit, Sheba Medical Center, Tel-Hashomer, 2 Derech Sheba St., Ramat Gan 52621, Israel. [3]Department of Neurology, Sagol Neuroscience Center, Sheba Medical Center, Tel-Hashomer, 2 Derech Sheba St., Ramat Gan 52621, Israel. [4]Center for Autoimmune Diseases Research (CREA), School of Medicine and Health Sciences, Universidad del Rosario, Kr 24 N°63C-69, Bogotá, DC 111221, Colombia. [5]Doctoral Program in Biomedical Sciences, Universidad del Rosario, Kr 24 N°63C-69, Bogotá, DC 111221, Colombia. [6]Department of Immunology, The Weizmann Institute of Science, 234 Herzl Street, Rehovot 76100, Israel. [7]Incumbent of the Laura Schwarz-Kip Chair for Research of Autoimmune Diseases, Sackler Faculty of Medicine, Tel-Aviv University, Haim Lebanon St., Ramat Aviv 69978, Israel.

Received: 28 September 2012 Accepted: 13 December 2012
Published: 4 April 2013

References

1. Ainiala H, Loukkola J, Peltola J, Korpela M, Hietaharju A: **The prevalence of neuropsychiatric syndromes in systemic lupus erythematosus.** *Neurology* 2001, **57:**496–500.
2. Brey RL, Holliday SL, Saklad AR, Navarrete MG, Hermosillo-Romo D, Stallworth CL, Valdez CR, Escalante A, del Rincon I, Gronseth G, Rhine CB, Padilla P, McGlasson D: **Neuropsychiatric syndromes in lupus: prevalence using standardized definitions.** *Neurology* 2002, **58:**1214–1220.
3. Lapteva L, Nowak M, Yarboro CH, Takada K, Roebuck-Spencer T, Weickert T, Bleiberg J, Rosenstein D, Pao M, Patronas N, Steele S, Manzano M, van der Veen JW, Lipsky PE, Marenco S, Wesley R, Volpe B, Diamond B, Illei GG: **Anti-N-methyl-D-aspartate receptor antibodies, cognitive dysfunction, and depression in systemic lupus erythematosus.** *Arthritis Rheum* 2006, **54:**2505–2514.
4. Greenwood DL, Gitlits VM, Alderuccio F, Sentry JW, Toh BH: **Autoantibodies in neuropsychiatric lupus.** *Autoimmunity* 2002, **35:**79–86.
5. Borchers AT, Aoki CA, Naguwa SM, Keen CL, Shoenfeld Y, Gershwin ME: **Neuropsychiatric features of systemic lupus erythematosus.** *Autoimmun Rev* 2005, **4:**329–344.
6. **The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes.** *Arthritis Rheum* 1999, **42:**599–608.
7. Rekvig OP, Putterman C, Casu C, Gao HX, Ghirardello A, Mortensen ES, Tincani A, Doria A: **Autoantibodies in lupus: culprits or passive bystanders?** *Autoimmun Rev* 2012, **11:**596–603.
8. Kozora E, Hanly JG, Lapteva L, Filley CM: **Cognitive dysfunction in systemic lupus erythematosus: past, present, and future.** *Arthritis Rheum* 2008, **58:**3286–3298.
9. Kapadia M, Sakic B: **Autoimmune and inflammatory mechanisms of CNS damage.** *Prog Neurobiol* 2011, **95:**301–333.
10. Oikonomou KG, Zachou K, Dalekos GN: **Alpha-actinin: a multidisciplinary protein with important role in B-cell driven autoimmunity.** *Autoimmun Rev* 2011, **10:**389–396.
11. Zandman-Goddard G, Chapman J, Shoenfeld Y: **Autoantibodies involved in neuropsychiatric SLE and antiphospholipid syndrome.** *Semin Arthritis Rheum* 2007, **36:**297–315.
12. Weiner SM, Klein R, Berg PA: **A longitudinal study of autoantibodies against central nervous system tissue and gangliosides in connective tissue diseases.** *Rheumatol Int* 2000, **19:**83–88.
13. Bonfa E, Golombek SJ, Kaufman LD, Skelly S, Weissbach H, Brot N, Elkon KB: **Association between lupus psychosis and anti-ribosomal P protein antibodies.** *N Engl J Med* 1987, **317:**265–271.

14. Toubi E, Shoenfeld Y: **Clinical and biological aspects of anti-P-ribosomal protein autoantibodies.** *Autoimmun Rev* 2007, **6:**119–125.
15. Katzav A, Pick CG, Korczyn AD, Oest E, Blank M, Shoenfeld Y, Chapman J: **Hyperactivity in a mouse model of the antiphospholipid syndrome.** *Lupus* 2001, **10:**496–499.
16. DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA, Volpe BT, Diamond B: **A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus.** *Nat Med* 2001, **7:**1189–1193.
17. Shoenfeld Y, Isenberg DA, Rauch J, Madaio MP, Stollar BD, Schwartz RS: **Idiotypic cross-reactions of monoclonal human lupus autoantibodies.** *J Exp Med* 1983, **158:**718–730.
18. Shoenfeld Y, Hsu-Lin SC, Gabriels JE, Silberstein LE, Furie BC, Furie B, Stollar BD, Schwartz RS: **Production of autoantibodies by human-human hybridomas.** *J Clin Invest* 1982, **70:**205–208.
19. Shoenfeld Y, Rauch J, Massicotte H, Datta SK, Andre-Schwartz J, Stollar BD, Schwartz RS: **Polyspecificity of monoclonal lupus autoantibodies produced by human-human hybridomas.** *N Engl J Med* 1983, **308:**414–420.
20. Andre-Schwartz J, Datta SK, Shoenfeld Y, Isenberg DA, Stollar BD, Schwartz RS: **Binding of cytoskeletal proteins by monoclonal anti-DNA lupus autoantibodies.** *Clin Immunol Immunopathol* 1984, **31:**261–271.
21. Amital-Teplizki H, Avinoach I, Coates AR, Kooperman O, Blank M, Shoenfeld Y: **Binding of monoclonal anti-DNA and anti-TB glycolipids to brain tissue.** *Autoimmunity* 1989, **4:**277–287.
22. Blank M, Shoenfeld Y: **The story of the 16/6 idiotype and systemic lupus erythematosus.** *Isr Med Assoc J* 2008, **10:**37–39.
23. Isenberg DA, Shoenfeld Y, Madaio MP, Rauch J, Reichlin M, Stollar BD, Schwartz RS: **Anti-DNA antibody idiotypes in systemic lupus erythematosus.** *Lancet* 1984, **2:**417–422.
24. Galeazzi M, Bellisai F, Sebastiani GD, Morozzi G, Marcolongo R, Houssiau F, Cervera R, Levy Y, George J, Sherer Y, Shoenfeld Y: **Association of 16/6 and SA1 anti-DNA idiotypes with anticardiolipin antibodies and clinical manifestations in a large cohort of SLE patients. European Concerted Action on the Immunogenetics of SLE.** *Clin Exp Rheumatol* 1998, **16:**717–720.
25. Amital-Teplizki H, Bearman JE, Miele PW Jr, Sukenik S, Shoenfeld Y: **A multidimensional autoantibody analysis specifying systemic lupus erythematosus patients with neuropsychiatric symptomatology.** *Isr J Med Sci* 1992, **28:**422–427.
26. Isenberg DA, Collins C: **Detection of cross-reactive anti-DNA antibody idiotypes on renal tissue-bound immunoglobulins from lupus patients.** *J Clin Invest* 1985, **76:**287–294.
27. Isenberg D, Dudeney C, Wojnaruska F, Bhogal BS, Rauch J, Schattner A, Naparstek Y, Duggan D: **Detection of cross reactive anti-DNA antibody idiotypes on tissue-bound immunoglobulins from skin biopsies of lupus patients.** *J Immunol* 1985, **135:**261–264.
28. Konikoff F, Isenberg DA, Kooperman O, Kennedy RC, Rauch J, Theodor E, Shoenfeld Y: **Common lupus anti-DNA antibody idiotypes in chronic liver diseases.** *Clin Immunol Immunopathol* 1987, **43:**265–272.
29. Shoenfeld Y, Livne A, Blank M, Argov S, Krup M, Fleishmakher E, Sukenik S, Teplizki H: **A human monoclonal anti-DNA antibody derived from a patient with polymyositis having the common lupus 16/6 idiotype.** *Immunol Lett* 1988, **19:**77–83.
30. Mendlovic S, Brocke S, Shoenfeld Y, Ben-Bassat M, Meshorer A, Bakimer R, Mozes E: **Induction of a systemic lupus erythematosus-like disease in mice by a common human anti-DNA idiotype.** *Proc Natl Acad Sci U S A* 1988, **85:**2260–2264.
31. Shoenfeld Y, Mozes E: **Pathogenic idiotypes of autoantibodies in autoimmunity: lessons from new experimental models of SLE.** *FASEB J* 1990, **4:**2646–2651.
32. Shoenfeld Y: **Idiotypic induction of autoimmunity: a new aspect of the idiotypic network.** *FASEB J* 1994, **8:**1296–1301.
33. Waisman A, Shoenfeld Y, Blank M, Ruiz PJ, Mozes E: **The pathogenic human monoclonal anti-DNA that induces experimental systemic lupus erythematosus in mice is encoded by a VH4 gene segment.** *Int Immunol* 1995, **7:**689–696.
34. Shoenfeld Y, Nahum A, Korczyn AD, Dano M, Rabinowitz R, Beilin O, Pick CG, Leider-Trejo L, Kalashnikova L, Blank M, Chapman J: **Neuronal-binding antibodies from patients with antiphospholipid syndrome induce cognitive deficits following intrathecal passive transfer.** *Lupus* 2003, **12:**436–442.
35. Porsolt RD, Bertin A, Jalfre M: **Behavioral despair in mice: a primary screening test for antidepressants.** *Arch Int Pharmacodyn Ther* 1977, **229:**327–336.
36. Tordera RM, Totterdell S, Wojcik SM, Brose N, Elizalde N, Lasheras B, Del Rio J: **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:**281–290.
37. 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.
38. Berry A, Vacirca D, Capoccia S, Bellisario V, Malorni W, Ortona E, Cirulli F: **Anti-ATP synthase autoantibodies induce neuronal death by apoptosis and impair cognitive performance in C57BL/6 mice.** *J Alzheimers Dis* 2013, **33:**317–321.
39. Sun KH, Tang SJ, Lin ML, Wang YS, Sun GH, Liu WT: **Monoclonal antibodies against human ribosomal P proteins penetrate into living cells and cause apoptosis of Jurkat T cells in culture.** *Rheumatology (Oxford)* 2001, **40:**750–756.
40. Koscec M, Koren E, Wolfson-Reichlin M, Fugate RD, Trieu E, Targoff IN, Reichlin M: **Autoantibodies to ribosomal P proteins penetrate into live hepatocytes and cause cellular dysfunction in culture.** *J Immunol* 1997, **159:**2033–2041.
41. Matus S, Burgos PV, Bravo-Zehnder M, Kraft R, Porras OH, Farias P, Barros LF, Torrealba F, Massardo L, Jacobelli S, Gonzalez A: **Antiribosomal-P autoantibodies from psychiatric lupus target a novel neuronal surface protein causing calcium influx and apoptosis.** *J Exp Med* 2007, **204:**3221–3234.
42. Katzav A, Ben-Ziv T, Chapman J, Blank M, Reichlin M, Shoenfeld Y: **Anti-P ribosomal antibodies induce defect in smell capability in a model of CNS-SLE (depression).** *J Autoimmun* 2008, **31:**393–398.
43. Katzav A, Solodeev I, Brodsky O, Chapman J, Pick CG, Blank M, Zhang W, Reichlin M, Shoenfeld Y: **Induction of autoimmune depression in mice by anti-ribosomal P antibodies via the limbic system.** *Arthritis Rheum* 2007, **56:**938–948.
44. Menachem A, Chapman J, Katzav A: **Hyperactivity induced by antiphospholipid syndrome serum.** *Ann N Y Acad Sci* 2009, **1173:**422–426.
45. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y: **Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients.** *Semin Arthritis Rheum* 2004, **34:**501–537.
46. Unterman A, Nolte JE, Boaz M, Abady M, Shoenfeld Y, Zandman-Goddard G: **Neuropsychiatric syndromes in systemic lupus erythematosus: a meta-analysis.** *Semin Arthritis Rheum* 2010, **41:**1–11.
47. Faust TW, Chang EH, Kowal C, Berlin R, Gazaryan IG, Bertini E, Zhang J, Sanchez-Guerrero J, Fragoso-Loyo HE, Volpe BT, Diamond B, Huerta PT: **Neurotoxic lupus autoantibodies alter brain function through two distinct mechanisms.** *Proc Natl Acad Sci U S A* 2010, **107:**18569–18574.
48. Kowal C, DeGiorgio LA, Nakaoka T, Hetherington H, Huerta PT, Diamond B, Volpe BT: **Cognition and immunity; antibody impairs memory.** *Immunity* 2004, **21:**179–188.
49. Kivity S, Tsarfaty G, Agmon-Levin N, Blank M, Manor D, Konen E, Chapman J, Reichlin M, Wasson C, Shoenfeld Y, Kushnir T: **Abnormal olfactory function demonstrated by manganese-enhanced MRI in mice with experimental neuropsychiatric lupus.** *Ann N Y Acad Sci* 2010, **1193:**70–77.
50. Shoenfeld Y, Ben-Yehuda O, Messinger Y, Bentwitch Z, Rauch J, Isenberg DI, Gadoth N: **Autoimmune diseases other than lupus share common anti-DNA idiotypes.** *Immunol Lett* 1988, **17:**285–291.
51. Isenberg DA, Shoenfeld Y: **An analysis of autoimmunity through studies of DNA antibody idiotypes.** *Autoimmunity* 1988, **1:**67–75.
52. El-Roiey A, Gross WL, Luedemann J, Isenberg DA, Shoenfeld Y: **Preferential secretion of a common anti-DNA idiotype (16/6 Id) and anti-polynucleotide antibodies by normal mononuclear cells following stimulation with Klebsiella pneumoniae.** *Immunol Lett* 1986, **12:**313–319.
53. Shoenfeld Y, Vilner Y, Coates AR, Rauch J, Lavie G, Shaul D, Pinkhas J: **Monoclonal anti-tuberculosis antibodies react with DNA, and monoclonal anti-DNA autoantibodies react with *Mycobacterium tuberculosis*.** *Clin Exp Immunol* 1986, **66:**255–261.
54. Kivity S, Agmon-Levin N, Blank M, Shoenfeld Y: **Infections and autoimmunity–friends or foes?** *Trends Immunol* 2009, **30:**409–414.
55. Broadbent NJ, Squire LR, Clark RE: **Spatial memory, recognition memory, and the hippocampus.** *Proc Natl Acad Sci U S A* 2004, **101:**14515–14520.

56. Milstone AM, Meyers K, Elia J: **Treatment of acute neuropsychiatric lupus with intravenous immunoglobulin (IVIG): a case report and review of the literature.** *Clin Rheumatol* 2005, **24:**394–397.
57. Mozes E, Sharabi A: **A novel tolerogenic peptide, hCDR1, for the specific treatment of systemic lupus erythematosus.** *Autoimmun Rev* 2010, **10:**22–26.
58. Lapter S, Marom A, Meshorer A, Elmann A, Sharabi A, Vadai E, Neufeld A, Sztainberg Y, Gil S, Getselter D, Chen A, Mozes E: **Amelioration of brain pathology and behavioral dysfunction in mice with lupus following treatment with a tolerogenic peptide.** *Arthritis Rheum* 2009, **60:**3744–3754.
59. Sthoeger ZM, Sharabi A, Molad Y, Asher I, Zinger H, Dayan M, Mozes E: **Treatment of lupus patients with a tolerogenic peptide, hCDR1 (Edratide): immunomodulation of gene expression.** *J Autoimmun* 2009, **33:**77–82.

doi:10.1186/1741-7015-11-90
Cite this article as: Kivity *et al.*: 16/6-idiotype expressing antibodies induce brain inflammation and cognitive impairment in mice: the mosaic of central nervous system involvement in lupus. *BMC Medicine* 2013 **11**:90.

Journal of Autoimmunity 45 (2013) 24–30

Contents lists available at SciVerse ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Passive transfer of narcolepsy: Anti-TRIB2 autoantibody positive patient IgG causes hypothalamic orexin neuron loss and sleep attacks in mice

Aviva Katzav [a,b,1], Maria T. Arango [b,c,d,1], Shaye Kivity [b], Susumu Tanaka [e], Gili Givaty [a,b], Nancy Agmon-Levin [b], Makoto Honda [e], Juan-Manuel Anaya [c], Joab Chapman [a,b], Yehuda Shoenfeld [b,f,*]

[a] Dept. Neurology and Sagol Neuroscience Center, Sheba Medical Center, Tel-Hashomer, Israel
[b] Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer, Affiliated to Sackler Medical School, Tel Aviv University, Israel
[c] Center for Autoimmune Diseases Research – CREA, Universidad del Rosario, Bogota, Colombia
[d] Doctoral Program in Biomedical Sciences, Universidad del Rosario, Bogota, Colombia
[e] The Sleep Control Project, Tokyo Metropolitan Institute of Psychiatry, Tokyo, Japan
[f] Incumbent of the Laura Schwarz-Kip Chair for Research of Autoimmune Diseases, Tel Aviv University, Israel

ARTICLE INFO

Article history:
Received 17 June 2013
Accepted 18 June 2013

Keywords:
Narcolepsy
Anti-Tribbles homolog 2 (TRIB2) antibodies
Passive transfer
Orexin
Behavioral deficits

ABSTRACT

Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness and cataplexy (a sudden weakening of posture muscle tone usually triggered by emotion) caused by the loss of orexin neurons in the hypothalamus. Autoimmune mechanisms are implicated in narcolepsy by increased frequency of specific HLA alleles and the presence of specific autoantibody (anti-Tribbles homolog 2 (TRIB2) antibodies) in the sera of patients with narcolepsy. Presently, we passively transferred narcolepsy to naïve mice by injecting intra-cerebra-ventricularly (ICV) pooled IgG positive for anti-TRIB2 antibodies. Narcolepsy-IgG-injected mice had a loss of the NeuN (neuronal marker), synaptophysin (synaptic marker) and orexin-positive neurons in the lateral hypothalamus area in narcolepsy compared to control-IgG-injected mice and these changes were associated with narcolepsy-like immobility attacks at four weeks post injection and with hyperactivity and long term memory deficits in the staircase and novel object recognition tests. Similar behavioral and cognitive deficits are observed in narcoleptic patients. This is the first report of passive transfer of experimental narcolepsy to naïve mice induced by autoantibodies and supports the autoimmune pathogenesis in narcolepsy.

1. Introduction

Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness and cataplexy (a sudden weakening of posture muscle tone usually triggered by emotion) caused by the loss of orexin neurons in the hypothalamus [1–4]. Autoimmune mechanisms are implicated in narcolepsy by increased frequency of specific HLA alleles [5] and the presence of specific autoantibody (anti-Tribbles homolog 2 (TRIB2) antibodies) in the sera of patients with narcolepsy [6–8]. In addition to sleep disorders and cataplexy, behavioral and cognitive deficits are also observed in narcoleptic patients [9–11].

The prevalence of narcolepsy with cataplexy is between 25 and 50 per 100,000 people dependent on environmental and genetic factors [12] with the highest prevalence in Japan (0.16%) [1]. It has been suggested that the cause of narcolepsy is a lack of orexin (hypocretin), an important neurotransmitter in the regulation of the sleep/wake cycle [2]. Though there are genetic animal models, such as orexin knockout mice and dogs with mutations in the orexins receptors [13–15], the etiology of narcolepsy in humans remains unclear. The hypothalamus of postmortem brains from narcolepsy patients show a loss of orexin neurons and the levels of orexin in the cerebrospinal fluid of narcolepsy patients are low or undetectable compared with healthy subjects [16–19]. Orexin is required for body processes such as feeding, cardiovascular

* Corresponding author. Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer, affiliated to Sackler Medical School, Tel Aviv University, Israel. Tel.: +972 3 5308070; fax: +972 3 5303673.
E-mail address: shoenfel@post.tau.ac.il (Y. Shoenfeld).
[1] Both first authors contributed equally to this study.

0896-8411/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jaut.2013.06.010

regulation, emotions, and locomotion. Orexin and histamine act synergistically to promote and maintain arousal behavior [2,20]. Removing orexin neurons sets up a mutually inhibitory cycle, which can cause unwanted abrupt transitions to rapid eye movement (REM) sleep and an excessive daytime sleepiness [21,22]. Human genetic data suggests immune factors in narcolepsy are highly associated with risk polymorphisms in the HLA system including the allele DQB1*06:02 in 82–99% of patients, in contrast to 12–38% of the healthy population, and the allele DRB1*15:01 [23,24]. Protective alleles have been found, such as DQB1*06:01 and DQB1*05:01 [23]. Interestingly, polymorphisms in TCR α chain gene and P2RY11 are also associated with narcolepsy [25,26]. Recent evidence has shown the presence of auto-antibodies against TRIB2, of the tribbles proteins family, found in orexin neurons [6–8]. Using transgenic mice and sera of narcolepsy patient, Cvetkovic-Lopes and coworkers found that the anti-TRIB2 antibodies present in the patients bound directly to the orexin neurons [6]. Our studies in Japanese patients have shown that the titers of anti-TRIB2 antibodies are higher in narcolepsy patients [8] especially if measured close to disease onset [6,7].

In the present study we passively transferred narcolepsy to naïve mice by injecting intra-cerebra-ventricularly (ICV) pooled IgG positive for anti-TRIB2 antibodies. These mice had a selective loss of orexin-positive neurons in the lateral hypothalamus and narcolepsy-like immobility attacks four weeks post injection. Control mice were injected with pooled matched healthy control IgG. This is the first report of passive transfer of experimental narcolepsy to naïve mice induced by autoantibodies and supports the autoimmune pathogenesis in narcolepsy.

2. Materials and methods

2.1. IgG samples

Blood samples and data related to sleep conditions were collected at the Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan) and Neuropsychiatric Research Institute (Tokyo, Japan). A total of 10 IgG samples from narcolepsy and matched healthy controls purified by using Ab-Capcher (Protenova Co., Ltd. Japan). Narcolepsy patients had also cataplexy, and all of them were positive for anti-TRIB2 by radioligand binding assay. Healthy controls were negative for anti-TRIB2, and they did not have excessive daytime sleepiness or any signs of immunologic abnormalities. Additionally all subjects were positive for the allele DQB1*06:02.

2.2. Mice

Thirteen female C3H mice 4 months old (Harlan Laboratories, IS) were used. The mice were housed in the animal house facility at the Sheba Medical Center, and were maintained at 23 ± 1 °C, and 12 h light cycle with free access to food and water. Two groups were injected, the first ($n = 6$) was injected with a total IgG purified from narcoleptic patients, the second group ($n = 7$) was injected with a total IgG from matched healthy controls. The Sheba Medical Center Animal Welfare Committee approved all procedures.

2.3. Passive transfer ICV

The passive transfer process was performed according to the protocol described previously [27]. Briefly, C3H naïve mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). After the skull was carefully exposed, 1 μl of the narcoleptic IgG extract or control was slowly infused intra cerebral ventricularly (ICV). The antibody solutions used contained 6 mg protein/ml. Each mouse received one single injection.

2.4. Videotaping and scoring of narcoleptic episodes

Mice were filmed and analyzed for immobility attacks weekly before and after ICV injection by using the NoldusPhenoTyper® cage and EthoVision software® (Noldus Information Technology, Netherlands). Under the same environmental conditions that were mentioned previously, each naïve mouse was recorded individually for 24 h. After the end of the recording process the mouse was injected ICV to with total IgG purified from narcoleptic patients. In parallel, another naive mouse was injected ICV with a total IgG from healthy controls. This protocol was repeated under the same conditions for seven days, after that each mouse that was ICV injected with IgG from narcolpetic patients were recorded serially every week, at days 7, 14, 21 and 28 post injections. Narcoleptic episodes were defined as freezing episodes by using the EthoVision software® and were strictly defined by the following features: (1) abrupt transition from an obvious motor activity; (2) a sustained change in posture maintained throughout the episode; and (3) an abrupt end to the episode with the resumption of obvious purposeful motor activity (essentially a switch between “off” and then “on” states). The exact time recorded on the video for the start and end of each episode was recorded along with the following additional observations: the predominant activity for the 5 seconds (s) preceding and the 10 s following an episode were categorized as feeding, drinking, ambulating, grooming, burrowing, climbing, or other.

2.5. Behavioral studies

To evaluate behavioral changes, the mice were tested in behavioral and cognitive tests including: staircase test, novel object recognition test, Y-maze test, and forced swimming test (FST). The tests were performed at week 5 after ICV administration.

2.5.1. Staircase test

This test evaluated exploratory activity and “anxiety −like” behavior. The staircase was placed in a room with constant lighting and isolated from external noise. Each mouse was tested individually. The number of stairs climbed and the number of rears were recorded for 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, 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.

2.5.2. Novel object recognition test

This test was used to evaluate a long term memory deficit. The apparatus, an open field box ($50 \times 50 \times 20$ cm), was constructed from a plywood painted white. Three phases (habituation, training, and retention) were conducted on 2 separate test days. Before training, mice were individually habituated by allowing them to explore the box for 1 h. No data were collected in this phase. During the training session, 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 mouse was allowed to explore for 5 min. Exploration of an object was defined as follows: directing the nose to the object at a distance of $\leq$1 cm and/or touching it with the nose. Turning around or sitting near the object was not considered as exploratory behavior. The time spent to explore each object was recorded. The animals were returned to their home cages immediately after training. During the retention test one of the familiar objects used during training was replaced by a novel object. The mice were placed back into the same box again after a 4-h interval and allowed

to explore freely for 5 min. All objects were balanced in term of physical complexity and were emotionally natural. The box and the objects were thoroughly cleaned by 70% alcohol after each session to avoid possible 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 [28]. Individual animals demonstrating insufficient task performance were excluded from later specific statistical analyses for the following reason: non-exploration, which is defined as no object interaction.

2.5.3. Y-maze test

The Y maze test was used to study the spatial short term memory, a task requiring hippocampal function and spatial memory. One arm was randomly selected as the "start" arm, and the mouse was placed twice in this arm. On the first trial, lasting for 5 min, one of the other two arms was randomly chosen to be blocked, whereas for 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 his 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% ethanol and dried. Discrimination of spatial novelty was assessed by a preference index: time in the new arm − time in the old arm/time in the new arm + time in the old arm.

2.5.4. Forced swimming test (FST)

This test is based on the method of Porsolt et al. to detect depression-like behavior [29]. Depression-like behavior was defined as an immobility (floating) and it was measured in seconds during the 6 min of test, when there was no presence of escape-oriented behaviors such as swimming, jumping, rearing, sniffing, or diving.

2.6. Histological studies

At 6 weeks post ICV passive transfer, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and underwent transcardiac perfusion with phosphate buffer saline followed by perfusion with 4% paraformaldehyde in PBS. The brains were removed, fixed in 4% p-formaldehyde in PBS, and cryoprotected by immersion in 30% sucrose at 4 °C. Frozen coronal sections (50 μm) were then cut on a sliding microtome and collected serially and were preserved in a cryoprotectant solution.

For detection of histological changes, brain sections were stained free-floating incubated overnight at 4 °C with first

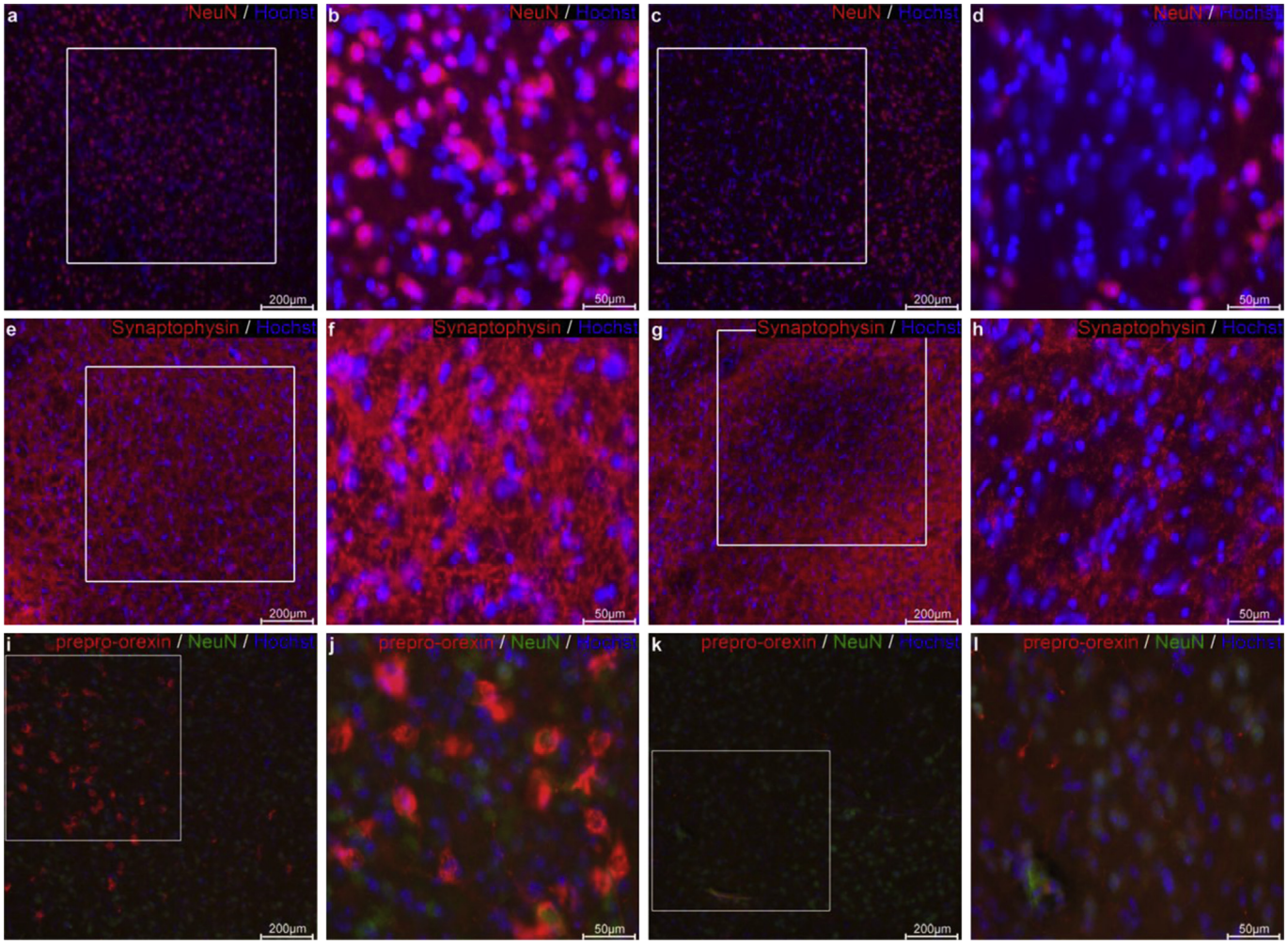


Fig. 1. Histopathological changes induced by narcolepsy-IgG. Coronal brain sections through the hypothalamus from mice injected ICV with IgG from narcolepsy patients and from healthy controls were stained for neuronal marker (NeuN) (top panel, a–d), synaptic marker (synaptophysin) (middle panel, e–h), and for orexin expressing neurons (prepro-orexin) (lower panel, i–l). (a–b, e–f, i–j) Representative images from control mice injected with control-IgG. (c–d, g–h, k–l) Representative images from mice injected with narcolepsy-IgG. First and third column images are at 10× magnification and scale bar 200 μm. Second and forth column images are at 40× magnification and scale bar 50 μm.

antibodies against neuronal marker (Neuronal Nuclei, NeuN, Chemicon, USA), synaptophysin (SigmaAldrich, USA) or Prepro-Orexin (Chemicon, USA). After washing, the sections were incubated at a room temperature for 2 h with the corresponding fluorescent chromogens-conjugated secondary antibody. Counter staining was performed with Hoechst.

2.7. Statistical analysis

The χ^2 test for categorical variable was applied for comparison between groups. Continuous values among groups were tested using ANOVA. The analysis was performed using the SPSS 17.0 software. Results are presented as averages ± standard error of the mean, and in percentages. Differences among the cases and controls in all the variables were established by Chi-square or Fisher's tests as appropriate. Continuous values among groups were tested using *T*-test or ANOVA. A level of 5% was used to define statistical significance ($p < 0.05$).

3. Results

3.1. Histopathological changes induced by narcoleptic IgG

Brain sections through the hypothalamus from narcolepsy and control mice were stained for neuronal and synaptic markers and for orexin expressing neurons and representative data are presented in Fig. 1. There was a loss of the neuronal marker (Neronal Nuclei, NeuN) (Fig. 1a–d) and the synaptic marker (synaptophysin) (Fig. 1e–h) in the lateral hypothalamus area in narcolepsy mice compared to controls. The most striking difference between the 2 groups was a significant loss of orexin-positive neurons (prepro-orexin staining) in the lateral hypothalamic area in narcolepsy-IgG compared to control-IgG mice (Fig. 1j–l).

3.2. Behavioral characterization of narcolepsy-like episodes

Mice were filmed and analyzed weekly before and after ICV injection of the Abs. For each mouse the 12 h of the dark phase were analyzed for immobility/freezing episodes. Narcolepsy-like episodes were not observed before the ICV injection of Abs. Narcolepsy/cataplexy-like immobility attacks were recorded in narcolepsy mice 4 weeks post injection but not before. The number and time of episodes for each mouse are summarized (Table 1).

3.3. Behavioral and cognitive performance

Narcolepsy mice and controls were tested in the staircase test and (Fig. 2a). Narcolepsy mice were significantly hyperactive as measured in both stair-climbing and rearing parameters ($p < 0.005$ by *t*-test) in the staircase test. The results of cognitive performance in the novel object recognition test are presented as discrimination index for novel object as a measure for long term recognition memory (Fig. 2b). There was a significant preference for attention to the new object in the control group compared to the narcolepsy group (34% vs. −16% additional percent time spent in near the novel object, $p = 0.00001$ by *t*-test). This suggests specific visual recognition memory impairment in the narcolepsy mice. Cognitive performance in the Y-maze test is presented as preference index for new (additional percent time spent in the novel arm) as a measure for short term spatial novelty memory (Fig. 2c). There was no significant difference between the narcolepsy and the control groups in the additional time spent in the new arm (45% vs. 18%, $p = 0.32$ by *t*-test). In the forced swimming test the narcolepsy mice performed poorly and exhibited significant depression-like behavior compared to controls (Fig. 2d). Average immobility times of the control mice vs. narcolepsy mice were 123.1 ± 28.1 vs. 219.4 ± 31.4 ($p = 0.00003$ by *t*-test).

Table 1
Characterization of the number and duration of sleeping attacks during the first 12 h of the light phase in mice injected with IgG from narcolepsy patients. The exact time recorded on the video for the start and end of each episode was recorded along with the following additional observations: the predominant activity for the 5 seconds (s) preceding and the 10 s following an episode were categorized as feeding, drinking, ambulating, grooming, burrowing, climbing, or other.

Mouse	Sleeping attacks *N*	Attack duration (average, sec)
1	4	88
2	3	66
3	0	–
4	1	210
5	2	464
6	2	188

4. Discussion

This study demonstrates the passive transfer of orexin neuron loss and narcolepsy-like behavioral changes by anti-TRIB2 positive narcolepsy patients IgG. The histological data showed a loss of both neuronal and synaptic markers demonstrating a neurodegenerative process in the hypothalamus and a loss of the prepro-orexin peptide in the mice injected with IgG from narcoleptic patients. Prepro-orexin is the precursor of the two kinds of orexins, A and B, and it is exclusively produced in the lateral hypothalamic orexin neurons [2,22]. This loss of orexin neurons is similar to what has been described in postmortem brains of narcoleptic patients [5,18,19]. Loss of the NeuN marker may indicate more extensive damage to neurons in this area. The loss of synaptophysin is compatible with a decreased neuronal activity and signaling. The loss of synapses in the lateral hypothalamic area is probably the consequence of the loss of orexin neurons in this area and may be related to with other neurotransmitters that are produced in the orexin neurons such as glutamate [30].

There are several animal models of narcolepsy, most of them are knockout mice with lack of orexin or orexin receptors, but the models that best resemble the symptoms characteristic of human narcolepsy are those with a loss of the orexin neurons [13,23,30–32]. The orexin/ataxin-3 hemizygous mice show the reduction of orexin neurons by ataxin-3 accumulations after birth, and these mice regarded as a good model for human narcolepsy [33]. This may indicate that the clinical signs and symptoms of human narcolepsy depend on the loss of orexin neurons and all their functions rather than just the loss of orexin. In 2004, Smith et al. described an animal model by intra-peritoneal injection with total IgG from narcoleptic patients to healthy mice [34]. They described the cessation of movements from a few seconds to 1 min during activities such as grooming in the injected mice, which are very similar to the behavioral pattern that we found in our narcolepsy mice. Mice injected with IgG from narcoleptic patients, develop cognitive and behavioral impairments compatible with narcoleptic patients that have alterations in their cognitive and behavioral functions [35]. The narcoleptic mice showed hyperactivity, long term memory deficits and depression. In humans, it has been reported that children with attention deficit hyperactivity disorder (ADHD) have a similar sleep pattern to narcolepsy patients, characterized by a period of sleepiness during the day and abnormal pattern of REM cycle [11,36,37]. In a case control-study Jara et al., showed that the frequency of depression in narcolepsy patients was higher than in healthy controls, and that it was not dependent on the presence of cataplexy [38]. Some authors suggest that in

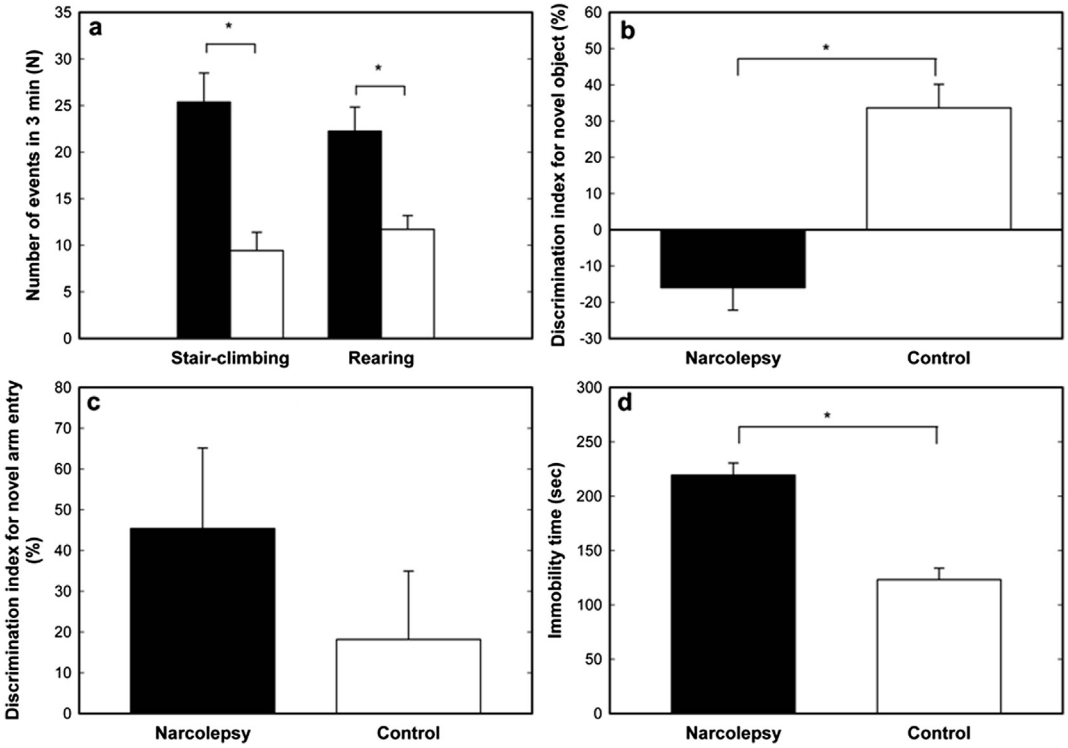


Fig. 2. Behavioral and cognitive changes induced by narcolepsy-IgG. (a) Behavioral measurements in the staircase test included activity (stair-climbing) and exploration (rearing). (b) Cognitive performance in the novel object recognition test is presented as discrimination index for novel object as a measure for long term recognition memory. (c) Cognitive performance in the Y-maze test is presented as preference index for new (additional percent time spent in the novel arm) as a measure for short term spatial novelty memory. (d) Depression-like behavior in the forced swimming test is presented as an immobility time. Results for narcolepsy mice (black bars, $n = 6$) and control mice (white bars, $n = 7$) are presented as mean ± SEM. $^{*}P < 0.05$ compared to controls (unpaired t test).

narcolepsy patients the depression and the associated anxiety may be a secondary outcome to the social consequences and the perception of a hostile environment [10,38]. However our results using a mouse model suggest that this manifestation may be a primary manifestation of the disease. The normal production of orexin is linked to the normal function of the limbic and dopaminergic systems related to the production of neurotransmitters such as norepinephrine, serotonin and histamine [2,22]. Burgess et al. described a change in the expression pattern of dopamine receptors in orexin knockout mice and they linked this alteration with the presence of sleep pattern changes and cataplexy [39]. However, the dopaminergic system is also important in the other processes such as making decisions and learning and deficits in these functions have been also demonstrated in narcolepsy [9]. In line with all these changes we also found memory impairments in the narcolepsy-IgG injected animals. There are several studies in narcolepsy patients that show alteration in attention and reduced learning, recognition and memory capacities [40]. There are reports of poor performance in the long term memory tests and a normal performance in the short term memory tests [37], similarly to what we found in the narcolepsy mice in the novel object recognition and Y-maze tests. As Lloyd et al. have described, there are several manifestations in children with sleep disorders that include learning difficulties, hyperactivity and mood changes [41].

5. Final comments

This paper is part of a dedicated issue of the Journal of Autoimmunity to honor the many contributions of Professor Abul Abbas. It is a particular pleasure for our group to contribute to this issue because of Abul's long commitment to the many Congresses of Autoimmunity. The Congress of Autoimmunity is an international meeting that occurs every two years. The goal of the Congress, like so many international meetings, has the broad mission of bringing together scholars to present their work and improve our understanding of autoimmunity. It has, however, served a much larger purpose which is that of widespread education in immunology to our younger colleagues and in this respect, Abul Abbas has graciously volunteered and has led with considerable enthusiasm a full-day meeting devoted just to an update on cutting-edge immunology. It is a session which is greeted with incredible enthusiasm. The success of the Congress which have included symposia dealing with geoepidemiology, new treatments, gender susceptibilities, genomics, pregnancy loss, differential diagnosis of unique and rare syndromes, epigenetics, twins, environmental factors and of course cellular immunology; all have succeeded in their own dedicated sessions that come after the initial introductory immunology course of Abul Abbas and for this we thank him [42–64]. The next Congress of Autoimmunity will be in Nice, France in March 2014 and we look forward to another cutting-edge presentation by Abul. It is therefore with humility and gratitude that this paper be included in this special issue.

6. Conclusions

We presently demonstrated that antibodies present in the sera of narcoleptic patients induce narcolepsy-like episodes in naïve mice after ICV injection. The narcoleptic patients' sera were positive for anti-TRIB2, which reinforces the hypothesis that TRIB2 may be a major auto-antigen in subgroup of narcolepsy that induces the destruction of orexin neurons in the brain of narcolepsy patients. It remains unclear how the antibodies responsible for the loss of

orexin neurons penetrate the blood brain barrier. The recognition that narcolepsy is an autoimmune disorder has important clinical implications. Similarly to type I diabetes, the time window in which to save orexin neurons from autoimmune destruction may be a very narrow as demonstrated by a single injection in the present study. Early detection and prompt therapy of narcolepsy are indicated.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgments

This study was supported by the Federico Foundation.

References

[1] Akintomide GS, Rickards H. Narcolepsy: a review. Neuropsychiatr Dis Treat 2011;7:507–18.
[2] Sakurai T, Mieda M, Tsujino N. The orexin system: roles in sleep/wake regulation. Ann N Y Acad Sci 2010;1200:149–61.
[3] Zeitzer JM, Nishino S, Mignot E. The neurobiology of hypocretins (orexins), narcolepsy and related therapeutic interventions. Trends Pharmacol Sci 2006;27:368–74.
[4] Dauvilliers Y, Arnulf I, Mignot E. Narcolepsy with cataplexy. Lancet 2007;369:499–511.
[5] Fontana A, Gast H, Reith W, Recher M, Birchler T, Bassetti CL. Narcolepsy: autoimmunity, effector T cell activation due to infection, or T cell independent, major histocompatibility complex class II induced neuronal loss? Brain 2010;133:1300–11.
[6] Cvetkovic-Lopes V, Bayer L, Dorsaz S, Maret S, Pradervand S, Dauvilliers Y, et al. Elevated Tribbles homolog 2-specific antibody levels in narcolepsy patients. J Clin Invest 2010;120:713–9.
[7] Kawashima M, Lin L, Tanaka S, Jennum P, Knudsen S, Nevsimalova S, et al. Anti-Tribbles homolog 2 (TRIB2) autoantibodies in narcolepsy are associated with recent onset of cataplexy. Sleep 2010;33:869–74.
[8] Toyoda H, Tanaka S, Miyagawa T, Honda Y, Tokunaga K, Honda M. Anti-Tribbles homolog 2 autoantibodies in Japanese patients with narcolepsy. Sleep 2010;33:875–8.
[9] Delazer M, Hogl B, Zamarian L, Wenter J, Gschliesser V, Ehrmann L, et al. Executive functions, information sampling, and decision making in narcolepsy with cataplexy. Neuropsychology 2011;25:477–87.
[10] Fortuyn HA, Mulders PC, Renier WO, Buitelaar JK, Overeem S. Narcolepsy and psychiatry: an evolving association of increasing interest. Sleep Med 2011;12:714–9.
[11] Miano S, Parisi P, Villa MP. The sleep phenotypes of attention deficit hyperactivity disorder: the role of arousal during sleep and implications for treatment. Med Hypotheses 2012;79:147–53.
[12] Longstreth Jr WT, Koepsell TD, Ton TG, Hendrickson AF, van Belle G. The epidemiology of narcolepsy. Sleep 2007;30:13–26.
[13] Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 1999;98:437–51.
[14] Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, et al. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 1999;98:365–76.
[15] Scammell TE, Willie JT, Guilleminault C, Siegel JM. A consensus definition of cataplexy in mouse models of narcolepsy. Sleep 2009;32:111–6.
[16] Mignot E, Lammers GJ, Ripley B, Okun M, Nevsimalova S, Overeem S, et al. The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. Arch Neurol 2002;59:1553–62.
[17] Nishino S, Ripley B, Overeem S, Nevsimalova S, Lammers GJ, Vankova J, et al. Low cerebrospinal fluid hypocretin (Orexin) and altered energy homeostasis in human narcolepsy. Ann Neurol 2001;50:381–8.
[18] Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med 2000;6:991–7.
[19] Thannickal TC, Siegel JM, Nienhuis R, Moore RY. Pattern of hypocretin (orexin) soma and axon loss, and gliosis, in human narcolepsy. Brain Pathol 2003;13:340–51.
[20] Anaclet C, Parmentier R, Ouk K, Guidon G, Buda C, Sastre JP, et al. Orexin/hypocretin and histamine: distinct roles in the control of wakefulness demonstrated using knock-out mouse models. J Neurosci 2009;29:14423–38.
[21] Huang W, Ramsey KM, Marcheva B, Bass J. Circadian rhythms, sleep, and metabolism. J Clin Invest 2011;121:2133–41.
[22] Ohno K, Sakurai T. Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. Front Neuroendocrinol 2008;29:70–87.
[23] Kornum BR, Faraco J, Mignot E. Narcolepsy with hypocretin/orexin deficiency, infections and autoimmunity of the brain. Curr Opin Neurobiol 2011;21:897–903.
[24] Mignot E, Lin L, Rogers W, Honda Y, Qiu X, Lin X, et al. Complex HLA-DR and -DQ interactions confer risk of narcolepsy-cataplexy in three ethnic groups. Am J Hum Genet 2001;68:686–99.
[25] Hallmayer J, Faraco J, Lin L, Hesselson S, Winkelmann J, Kawashima M, et al. Narcolepsy is strongly associated with the T-cell receptor alpha locus. Nat Genet 2009;41:708–11.
[26] Kornum BR, Kawashima M, Faraco J, Lin L, Rico TJ, Hesselson S, et al. Common variants in P2RY11 are associated with narcolepsy. Nat Genet 2011;43:66–71.
[27] 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 Rheum 2007;56:938–48.
[28] 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.
[29] Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 1977;229:327–36.
[30] Henny P, Brischoux F, Mainville L, Stroh T, Jones BE. Immunohistochemical evidence for synaptic release of glutamate from orexin terminals in the locus coeruleus. Neuroscience 2010;169:1150–7.
[31] Fronczek R, Baumann CR, Lammers GJ, Bassetti CL, Overeem S. Hypocretin/orexin disturbances in neurological disorders. Sleep Med Rev 2009;13:9–22.
[32] Chen L, Brown RE, McKenna JT, McCarley RW. Animal models of narcolepsy. CNS Neurol Disord Drug Targets 2009;8:296–308.
[33] Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron 2001;30:345–54.
[34] Smith AJ, Jackson MW, Neufing P, McEvoy RD, Gordon TP. A functional autoantibody in narcolepsy. Lancet 2004;364:2122–4.
[35] Wurtman RJ. Narcolepsy and the hypocretins. Metabolism 2006;55:S36–9.
[36] Peraita-Adrados R, Garcia-Penas JJ, Ruiz-Falco L, Gutierrez-Solana L, Lopez-Esteban P, Vicario JL, et al. Clinical, polysomnographic and laboratory characteristics of narcolepsy-cataplexy in a sample of children and adolescents. Sleep Med 2011;12:24–7.
[37] Walters AS, Silvestri R, Zucconi M, Chandrashekariah R, Konofal E. Review of the possible relationship and hypothetical links between attention deficit hyperactivity disorder (ADHD) and the simple sleep related movement disorders, parasomnias, hypersomnias, and circadian rhythm disorders. J Clin Sleep Med 2008;4:591–600.
[38] Jara CO, Popp R, Zulley J, Hajak G, Geisler P. Determinants of depressive symptoms in narcoleptic patients with and without cataplexy. J Nerv Ment Dis 2011;199:329–34.
[39] Burgess CR, Tse G, Gillis L, Peever JH. Dopaminergic regulation of sleep and cataplexy in a murine model of narcolepsy. Sleep 2010;33:1295–304.
[40] Fulda S, Schulz H. Cognitive dysfunction in sleep disorders. Sleep Med Rev 2001;5:423–45.
[41] Lloyd R, Tippmann-Peikert M, Slocumb N, Kotagal S. Characteristics of REM sleep behavior disorder in childhood. J Clin Sleep Med 2012;8:127–31.
[42] Oke V, Wahren-Herlenius M. The immunobiology of Ro52 (TRIM21) in autoimmunity: a critical review. J Autoimmun 2012;39:77–82.
[43] Chang C. Neonatal autoimmune diseases: a critical review. J Autoimmun 2012;38:J223–38.
[44] Chighizola C, Meroni PL. The role of environmental estrogens and autoimmunity. Autoimmun Rev 2012;11:A493–501.
[45] Seror R, Bootsma H, Bowman SJ, Dorner T, Gottenberg JE, Mariette X, et al. Outcome measures for primary Sjogren's syndrome. J Autoimmun 2012;39:97–102.
[46] Lee TP, Chiang BL. Sex differences in spontaneous versus induced animal models of autoimmunity. Autoimmun Rev 2012;11:A422–9.
[47] Ice JA, Li H, Adrianto I, Lin PC, Kelly JA, Montgomery CG, et al. Genetics of Sjogren's syndrome in the genome-wide association era. J Autoimmun 2012;39:57–63.
[48] Comarmond C, Plaisier E, Dahan K, Mirault T, Emmerich J, Amoura Z, et al. Anti TNF-α in refractory Takayasu's arteritis: cases series and review of the literature. Autoimmun Rev 2012;11:678–84.
[49] Carp HJ, Selmi C, Shoenfeld Y. The autoimmune bases of infertility and pregnancy loss. J Autoimmun 2012;38:J266–74.
[50] Shelly S, Boaz M, Orbach H. Prolactin and autoimmunity. Autoimmun Rev 2012;11:A465–70.
[51] Sawalha AH, Wang L, Nadig A, Somers EC, McCune WJ, Hughes T, et al., Michigan Lupus Cohort. Sex-specific differences in the relationship between genetic susceptibility, T cell DNA demethylation and lupus flare severity. J Autoimmun 2012;38:J216–22.
[52] Oertelt-Prigione S. The influence of sex and gender on the immune response. Autoimmun Rev 2012;11:A479–85.
[53] Amur S, Parekh A, Mummaneni P. Sex differences and genomics in autoimmune diseases. J Autoimmun 38:J254–65.
[54] Oertelt-Prigione S. Immunology and the menstrual cycle. Autoimmun Rev 2012;11:A486–92.
[55] Bogdanos DP, Smyk DS, Rigopoulou EI, Mytilinaiou MG, Heneghan MA, Selmi C, et al. Twin studies in autoimmune disease: genetics, gender and environment. J Autoimmun 2012;38:J156–69.
[56] Moroni L, Bianchi I, Lleo A. Geoepidemiology, gender and autoimmune disease. Autoimmun Rev 2012;11:A386–92.

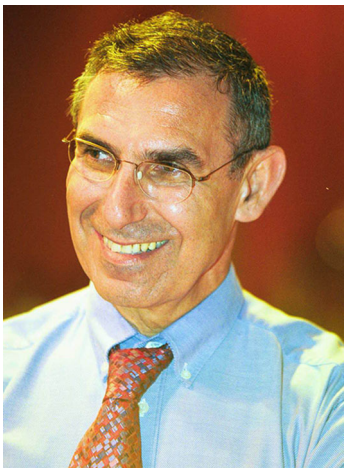
[57] Strickland FM, Hewagama A, Lu Q, Wu A, Hinderer R, Webb R, et al. Environmental exposure, estrogen and two X chromosomes are required for disease development in an epigenetic model of lupus. J Autoimmun 38:J135–43.
[58] Nussinovitch U, Shoenfeld Y. The role of gender and organ specific autoimmunity. Autoimmun Rev 2012;11:A377–85.
[59] Lidar M, Langevitz P. Pregnancy issues in scleroderma. Autoimmun Rev 2012;11:A515–9.
[60] Selmi C, Brunetta E, Raimondo MG, Meroni PL. The X chromosome and the sex ratio of autoimmunity. Autoimmun Rev 2012:A531–7.
[61] Palazzi C, Buskila D, D'Angelo S, D'Amico E, Olivieri I. Autoantibodies in patients with chronic hepatitis C virus infection: pitfalls for the diagnosis of rheumatic diseases. Autoimmun Rev 2012;11:659–63.
[62] Singh RP, Waldron RT, Hahn BH. Genes, tolerance and systemic autoimmunity. Autoimmun Rev 2012;11:664–9.
[63] Petrikova J, Lazurova I. Ovarian failure and polycystic ovary syndrome. Autoimmun Rev 2012;11:A471–8.
[64] Ostensen M, Villiger PM, Forger F. Interaction of pregnancy and autoimmune rheumatic disease. Autoimmun Rev 2012;11:A437–46.

Immunol Res
DOI 10.1007/s12026-016-8826-6

ENVIRONMENT AND AUTOIMMUNITY

Behavioral abnormalities in female mice following administration of aluminum adjuvants and the human papillomavirus (HPV) vaccine Gardasil

Rotem Inbar[1,2] · Ronen Weiss[3,4] · Lucija Tomljenovic[1,5] · Maria-Teresa Arango[1,6] · Yael Deri[1] · Christopher A. Shaw[5] · Joab Chapman[1,7] · Miri Blank[1] · Yehuda Shoenfeld[1,8]



Yehuda Shoenfeld

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 can be toxic to humans and animals. We sought to evaluate the effects of Al adjuvant and the HPV vaccine Gardasil versus the true placebo on behavioral and inflammatory parameters in female mice. Six-week-old C57BL/6 female mice were injected with either, Gardasil, Gardasil + pertussis toxin (Pt), Al hydroxide, or, vehicle control in amounts equivalent to human exposure. At 7.5 months of age, Gardasil and Al-injected mice spent significantly more time floating in the forced swimming test (FST) in comparison with vehicle-injected mice (Al, $p = 0.009$; Gardasil, $p = 0.025$; Gardasil + Pt, $p = 0.005$). The increase in floating time was already highly significant at 4.5 months of age for the Gardasil and Gardasil + Pt group ($p \leq 0.0001$). No significant differences were observed in the number of stairs climbed in the staircase test which measures locomotor activity. These results indicate that differences observed in the FST were unlikely due to locomotor dysfunction, but rather due to depression. Moreover, anti-HPV antibodies from the sera of Gardasil and Gardasil + Pt-injected mice showed cross-reactivity with the mouse brain protein extract. Immunohistochemistry analysis revealed microglial activation in the CA1 area of the hippocampus of Gardasil-injected mice. It appears that Gardasil via its Al adjuvant and HPV antigens has the ability to trigger neuroinflammation and autoimmune reactions, further leading to behavioral changes.

Keywords Gardasil · Aluminum · ASIA syndrome · Autoantibodies · Autoimmunity · Neuroinflammation

Abbreviations

Al	Aluminum
ASIA	Autoimmune/autoinflammatory syndrome induced by adjuvants
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

✉ Yehuda Shoenfeld
shoenfel@post.tau.ac.il

[1] Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel Hashomer, Sackler Faculty of Medicine, Tel-Aviv University, 52621 Israel, Israel

[2] Department of Obstetrics and Gynecology, Sheba Medical Center, 52621 Tel Hashomer, Israel

[3] Sagol School of Neuroscience, Tel Aviv University, 69978 Ramat Aviv, Israel

[4] Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, 69978 Ramat Aviv, Israel

[5] Neural Dynamics Research Group, Department of Ophthalmology and Visual Sciences, University of British Columbia, 828 W. 10th Ave, Vancouver, BC V5Z 1L8, Canada

[6] Doctoral Program in Biomedical Sciences, Universidad del Rosario, Bogota 111221, Colombia

[7] Department of Neurology and Sagol Neuroscience Center, The Sheba Medical Center, 52621 Tel Hashomer, Israel

[8] Incumbent of the Laura Schwarz-Kip Chair for Research of Autoimmune Diseases, Sackler Faculty of Medicine, Tel-Aviv University, 69978 Ramat Aviv, Israel

Published online: 16 July 2016

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, 2].

There are currently major stumbling blocks 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 many clinical studies that evaluate vaccine safety and the lack of in-depth research into adverse phenomena [3]. 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, paresthesia, depression, mild cognitive disturbances) [2]. 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 [4–6], likely depending on individuals' genetic predispositions 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 [7]. Indeed, contrary to popular *assumptions* of inherent safety of Al in vaccines, there is now compelling data from both human and animal studies which implicates this most widely used adjuvant in the pathogenesis of disabling neuroimmuno-inflammatory conditions [8–11].

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 [12]. 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 [9–11, 13–16]. 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 [17].

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 agent [18, 19].

The HPV vaccine Gardasil is one of many vaccines currently on the market that is adjuvanted with Al. Since the licensure by the US 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. 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 [20].

Like most other vaccine safety trials, the trials for the HPV Gardasil vaccine utilized an Al-containing placebo [21, 22] and hence the safety profile of the vaccine remains obscured by the use of a potentially toxic placebo [7]. 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

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-light cycle (6:30 am–6:30 pm) with ad libitum access to food and water. The Sheba Medical Center Animal Welfare Committee approved all procedures.

Table 1 Summary of cases of autoimmune and inflammatory manifestations following HPV vaccination reported in the peer-reviewed medical literature

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
2	17	Visual impairments	ADEM	[52]
	20	Headache, nausea, vomiting, diplopia		[53]
5	16	Upper limb pseudoathetosis	CIS/MS/	[54]
	16	Acute hemiparesis	Clinically definite MS	
	21	Incomplete TM, left optic neuritis		
	25	Headache, incomplete TM		
	26	Incomplete TM, brainstem syndrome		
2	19	Leg numbness, mid-thoracic back pain	Demyelinating disease unspecified	[55]
	18	Blurriness, paresthesia, optic neuritis		
1	11	Mood swings, abnormal eye movements, dizziness, leg weakness, myoclonic jerks	Opsoclonus myoclonus	[56]
4	17	Back pain, progressing spastic paraparesis, right arm weakness, left eye visual loss	Neuromyelitis optica	[57]
	14	Back pain, right thigh dysesthesias, left optic neuritis		
	13	TM with flaccid paraplegia		
	18	Back pain and leg weakness, complete loss of monocular vision		
2	16	Visual loss, headaches, left hemiparesis	Optic neuritis	[58]
	17	Visual disturbances, demyelinating lesions		[59]
2	27	Paresthesia, demyelinating lesions	TM fitting the criteria for MS	[59]
	26	Progressive paresthesia, demyelinating lesions		
1	15	Facial paralysis	Bell's palsy	[59]
1	12	Nausea, vertigo, severe limb and truncal ataxia, and persistent nystagmus	Cerebellar ataxia	[60]
1	19	Chronic (3 months) disabling shoulder pain	Brachial neuritis	[61]
53	12–39	Orthostatic intolerance, severe non-migraine-like headache, excessive fatigue, cognitive dysfunction, gastrointestinal discomfort, widespread neuropathic pain	Dysautonomia, POTS, orthostatic intolerance and CRPS	[62]
40	11–17	Headaches, general fatigue, coldness of the legs, limb pain and weakness, orthostatic intolerance, tremors, persistent asthenia		[63]
6	20	Weight loss, dizziness, fatigue, exercise intolerance		[64]
	22	Diarrhea, weight loss, fatigue, dizziness, syncope		
	12	Syncope, pre-syncope, dizziness, small fiber neuropathy		
	15	Dizziness, headache, pre-syncope, syncope		
		Paresthesia, tachycardia, fatigue, headache,		
	14	diarrhea, weight loss		
	18	Paresthesia, leg pain, orthostatic intolerance, Fatigue, dizziness		
4	16	Paresthesia, numbness, limb paralysis, pain		[65]
	13	Allodynia, numbness, severe pain		
	15	Paresthesia, numbness, severe pain		
	12	Paresthesia, muscle weakness, pain		
1	14	Headaches, dizziness, recurrent syncope, orthostatic intolerance, fatigue, myalgias, tachycardia, dyspnea, visual disturbances, phonophobia, cognitive impairment, insomnia, gastrointestinal disturbances, weight loss		[66]
2	11	Widespread neuropathic pain, paresthesia, insomnia, profound fatigue	Fibromyalgia	[67]
	14	Widespread neuropathic pain and paresthesia		
1	32	Paresthesia, muscle twitching, myalgia, fatigue, hyperhidrosis, and tachycardia, exercise intolerance	Autoimmune myotonia	[68]

Table 1 continued

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
3	14	Skin rash, fever, nausea, stomach aches, headache, insomnia, night sweats, arthralgia, anxiety, depression, amenorrhea, elevated serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and low levels of estradiol	POF	[69]
	13	Depression, sleep disturbance, light-headedness, tremulousness, anxiety, cognitive dysfunction, amenorrhea, high serum levels of FSH and LH with undetectable estradiol		[70]
	21	A menorrhea preceded by oligomenorrhea, high serum levels of FSH and LH and low estradiol		
3	16	5 months amenorrhea preceded by 12 months oligomenorrhea, hot flashes, low serum levels of estradiol and Anti-Müllerian hormone		
	18	6 months amenorrhea, low serum levels of estradiol and Anti-Müllerian hormone		
	15	3 months amenorrhea preceded by 9 months oligomenorrhea, hot flashes, low serum levels of estradiol and undetectable Anti-Müllerian hormone		
2	15	Vasculitic rash, soft tissue swellings of	Vasculitis	[71]
		ankles and forearms, arthralgia, lethargy, epistaxis		
	15	Severe flare of cutaneous vasculitis		
1	16	Fatigue associated with prolonged menorrhagia, antiplatelet autoantibodies	Thrombocytopenic purpura	[72]
1	11	Jaundice, hepatosplenomegaly elevated serum aminotransferases	Autoimmune hepatitis	[73]
1	26	Severe constant epigastric pain, vomiting, fever	Pancreatitis	[74]
3	17	Arthralgias, pruritic rashes on lower extremities, bipedal edema, livedo reticularis, proteinuria, positive ANA and anti-dsDNA antibodies	SLE	[75]
	45	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		
	58	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		
6	32	Fatigue, severe myalgia, polyarthralgia, anorexia, severe skin rash, malar rash, aphtous stomatitis, pharyngodynia, cervical lymphadenopathy, alopecia, severe weight loss, anemia, positive ANA and anti-dsDNA antibodies		[76]
	29	Weakness, diarrhea, malar rash, photosensitivity, arthritis, alopecia, severe weight loss, proteinuria, positive ANA and anti-dsDNA antibodies		
	16	High-grade fever, generalized asthenia, diffuse polyarthralgia, multiple erythematous annular cutaneous lesions on the face, trunk, and lower limbs, positivie ANA and lupus anticoagulant		
	16	Fever, pharyngodynia, erythematous skin lesions of elbows and knees, generalized asthenia, anorexia, polyarthralgia, anti-cardiolipin and lupus anticoagulant		
	19	Mild arthralgia, dyspnea, cervical lymphadenopathy, skin rash, positive ANA and anti-dsDNA antibodies		
	13	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		
1	19	Myalgia, arthralgia, generalized weakness, oral ulcers, Raynaud's phenomenon, alopecia, headache, dyspnea, tachycardia, positive ANA, anti-Sm, anti-Ro, anti-RNP, anti-dsDNA, leuko-penia, and complement consumption		[77]
1	20	Myalgias, arthral-gias, livedo reticularis, Raynaud's phenomenon, headache, tinnitus, positive ANA, lupus anticoagulant and anti-CCP	Rheumatoid arthritis	[77]

Table 1 continued

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
1	16	Knee joint swelling, low back, buttock and chest wall pain, elevated leukocyte count in the synovial fluid, elevated C-reactive protein	Juvenile spondyloarthropathy	[77]

Out of 152 reported cases, 129 (85 %) relate to neuro-ophthalmic disorders

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

Injection procedures and experimental design

Six-week-old C57BL/6 female mice received three injections (spaced 1 day apart) of either (a) quadrivalent HPV vaccine Gardasil, (b) Gardasil + pertussis toxin (Pt), (c) Al hydroxide or (d) vehicle control (19.12 mg/mL NaCl, 1.56 mg/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 (ip). The amount of injected Al and the HPV vaccine was the equivalent of human exposure. In particular, each mouse 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 six-week-old mice is approximately 20 g. Gardasil is given as a 0.5-mL dose to teenage girls of cca 40 kg. Thus, a 20-g mouse receives cca 2000 × less of the vaccine suspension than a human. Similarly, each mouse in the Al adjuvant group received 5.6 µg/kg body weights 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 µg 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. Since the actual adjuvant form used in Gardasil, amorphous Al hydroxyphosphate sulfate (AAHS), is a proprietary brand of the vaccine manufacturer and is not commercially available, we used Alhydrogel as a substitute.

Five out of 19 animals from each of the four experimental groups were used for sera collection purposes. These animals were not subjected to behavioral testing as sera were collected via retro-orbital bleeding which is a stressful procedure that in addition often leads to vision deficits. The behavior of mice was evaluated at three and 6 months post-immunization for (1) locomotor function and depression by the forced swimming test (FST), (2) locomotor and explorative activity by the staircase test and (3) cognitive functions by the novel object recognition test. Following the first round of behavioral testing at 4.5 months of age, five mice from each of the four experimental groups were killed and brain tissues were collected and processed for histological examinations. Blood specimens were also collected at this time for serological analysis.

Behavioral tests

Forced swimming test

The FST is the most widely used model of depression in rodents. It is commonly used for evaluation of antidepressant drugs, and experiments aimed at inducing and examining depressive-like states in basic and pre-clinical research [23, 24]. Nonetheless, it should be noted that increased floating time in the FST apart from being indicative of depressive behavior can also indicate locomotor dysfunction. For the purpose of this test, mice were placed in individual glass beakers (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 a pretest session of 10 min, 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 6 min. The behavioral measure scored was the duration (in seconds) of immobility or floating, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the 6-min test.

Staircase test

Locomotor, explorative activity and anxiety were evaluated by the staircase test, as described previously by Katzav et al. [25]. 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 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 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.

Novel object recognition test

This is a visual recognition memory test based on a method described by Tordera et al. [24]. 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 min (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 min. Exploration of an object was defined as directing the nose to the object at a distance of $\leq$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 min. 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.

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) and novel object recognition were evaluated by ANOVA and Tuckey for multiple comparisons in the post hoc analysis. Significant results were determined as $p < 0.05$.

Brain perfusion and fixation

The mice were anesthetized by an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and killed 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 0.1 M PO_4 (pH 7.4) for 24–48 h at 4 °C before brain cutting. Frozen coronal Sects. (30–50 μm) were cut on a sliding microtome (Leica Microsystems GmbH, Wetzlar, Germany), collected serially and kept in a cryoprotectant at −20 °C until staining.

Detection of autoantibodies in the sera

The levels of autoantibodies in the mice sera were tested by a homemade ELISA 1 month post-injection. Briefly, ELISA plates (M9410, Sigma-Aldrich) were coated separately with 20 μg/well of different antigens: Gardasil which contains the HPV L1 major capsid protein of HPV types 6, 11, 16 and 18, mouse brain protein extract, mouse brain phospholipid extract, Al hydroxide, dsDNA and β2glycoprotein-I (β2GPI). The plates were incubated overnight at 4 °C, washed and blocked with 3 %BSA in PBS 1 h at 37 °C. Sera were added at dilution of 1:200 for 2 h at room temperature. The binding was probed with goat anti-mouse IgG conjugated to alkaline phosphatase at concentration of 1:5000 for 1 h at 37C. Following appropriate substrate, the data were read by ELISA reader at 405 nm.

Inhibition assay

Brain protein extracts were prepared by lysis of brains from five healthy C57BL/6 mice, using ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1 mM EDTA,1 mM PMSF, 1 mM sodium vanadate, 0.1 % protease inhibitor mixture (Sigma-Aldrich L-4391 St Louis, MO, USA) for 30 min on ice and centrifuged at 13,000 rpm for 20 min. The lysate was dialyzed against PBS. Protein concentration was determined by BCA Protein Assay Kit (Pierce, Thermo scientific, Rockford, IL, USA).

ELISA plates were coated with the HPV vaccine Gardasil which contains the HPV L1 major capsid protein of HPV types 6, 11, 16 and 18. Following blocking with 5 % skim milk powder, sera from the immunized mice, at different dilutions 1:200–1:10,000, were added to the plates in order to define 50 % binding of the sera to the HPV. Next, dilutions of sera which showed 50 % binding to HPV were incubated overnight at 4 °C with different concentrations of mouse brain protein extract (10–50 μg/ml) as the inhibitor. The following day, the mixtures were subjected to ELISA plates coated with HPV for 2 h at room temperature. The binding of the antibodies which did not create complex with the brain protein extract was probed with anti-mouse IgG conjugated to alkaline phosphatase, followed by the appropriate substrate. The percentage of inhibition was calculated as follows: % inhibition = 100 − [(OD of tested sample without inhibitor − OD of tested sample with inhibitor)/(OD of tested sample without inhibitor)] × 100.

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 1 h 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).

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) was 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 was used when appropriate, according to the experimental design. Significance level was determined in one-tailed and two-tailed tests. The level of statistical significance of differences is $p < 0.05$.

Results

Behavioral tests

The ANOVA analysis showed significant differences in the performance of the mice in the forced swimming and the staircase tests 3 months after injection (Fig. 1). The specific differences were detected by the post hoc test which showed that the two groups injected with the Gardasil vaccine spent significantly more time floating compared to control mice and Al-injected mice (Fig. 1a). No significant differences were found between the groups in the overall memory skills (measured by the novel object recognition test), locomotor function, exploratory activity and anxiety which were measured in the staircase apparatus (Fig. 1c).

The analysis after the behavioral testing at 6 months post-injection demonstrated that the alterations in the FST performance were sustained in the group injected with Gardasil + Pt compared to control mice ($p = 0.024$; Fig. 1b), indicating that the effect of Gardasil + Pt exposure was long-lasting. Moreover, at 6 months post-injection, the Al-injected group likewise spent significantly more time floating compared to the control group ($p = 0.044$, Fig. 1b). Although the Gardasil group showed increased floating time compared to the vehicle-injected control group, the observed difference was not statistically significant. Given that after the first round of testing at 3 months post-injection, we killed five 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. Significant differences were also observed in the rearing frequency in the staircase test. Namely, the Al-injected mice showed a significantly lower frequency of rearing compared to the group injected with Gardasil + Pt in the staircase test ($p = 0.021$; Fig. 1d). 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. There was no statistically significant difference in the number of stairs climbed in the staircase test between the groups (not shown). In the FST,

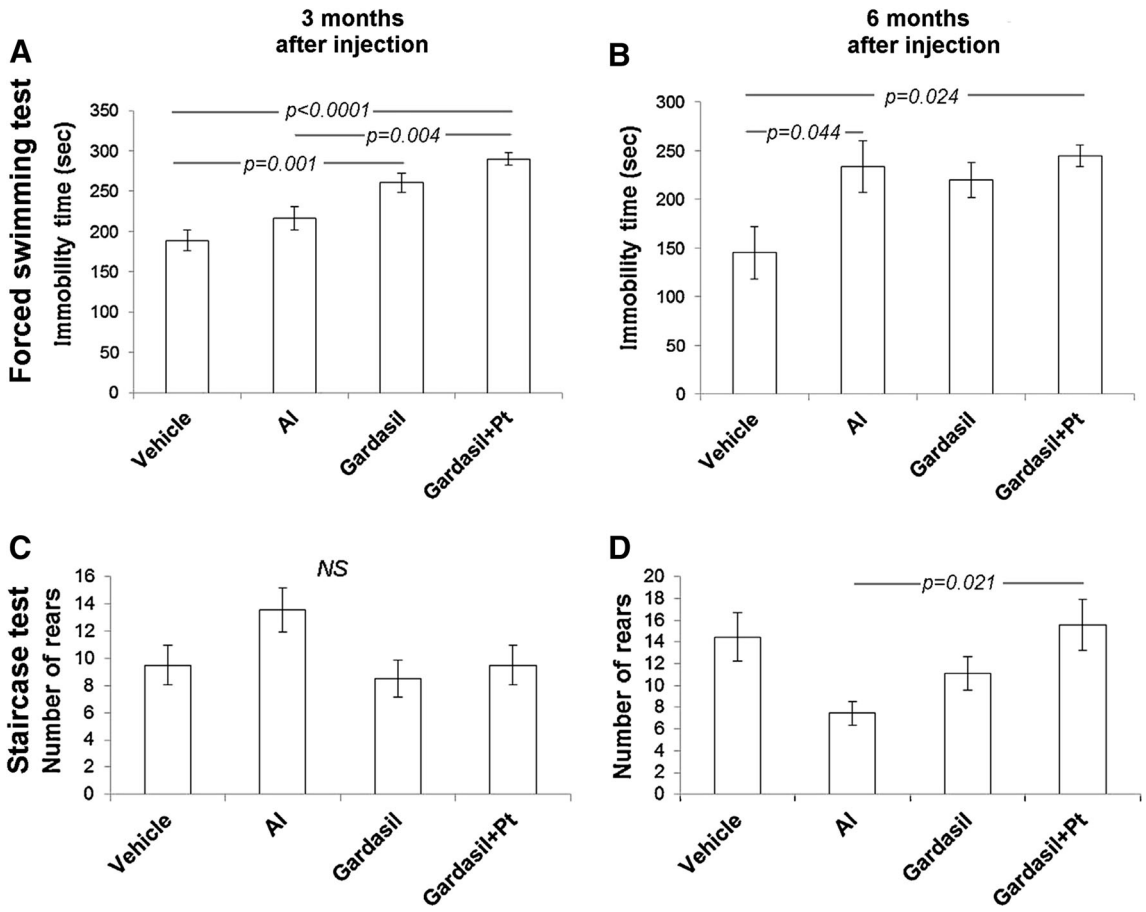


Fig. 1 Effects of Al, Gardasil and Gardasil + Pt toxin injections on behavioral tests. **a** and **b** show the floating time in C57BL/6 female mice as evaluated by the forced swimming test (FST). Results are presented as duration in seconds (mean ± SEM) of immobility, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the 6-min test. **a** Three months post-injection ($n = 14$ per treatment group); **b** Six months post-injection ($n = 9$ per treatment group). **b**, **c** show the reduced exploratory activity in C57BL/6 female mice as evaluated by the rearing frequency in the staircase test. Results are presented as the number of rears (mean ± SEM) during a 3-min testing period. **a** Three months post-injection ($n = 14$ per treatment group); **b** Six months post-injection ($n = 9$ per treatment group)

however, the changes were still significant despite the lower number of animals. No significant differences in behavior were observed in the novel object recognition test.

Autoantibody profile and inhibition assay

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 ($p < 0.002$), as well as anti-brain protein extract ($p < 0.002$) and anti-brain phospholipid extract antibodies ($p < 0.001$) were observed in the two groups of mice that received the HPV vaccine (Fig. 2). The titers of anti-HPV antibodies, anti-brain protein extract and anti-brain phospholipid extract antibodies were reduced after 2 months (data not shown). No elevation in the titers of anti-Al-hydroxide, anti-dsDNA and anti-β2GPI antibodies, was detected in the sera of any of the four treatment groups of mice (Fig. 2).

The binding of anti-HPV antibodies from the sera from the two treatment groups immunized with Gardasil to HPV L1 antigens was significantly inhibited by the mouse brain protein extract in a dose-dependent manner in comparison with Al-injected mice whose sera were negative for anti-HPV antibodies (Fig. 3).

Brain tissue immunostaining

Following the behavioral tests at 4.5 months of age, five animals were killed from each of the four experimental groups and used for brain immunostaining procedures. With this relatively small group size, there were no clear changes between the groups in both astrocyte and

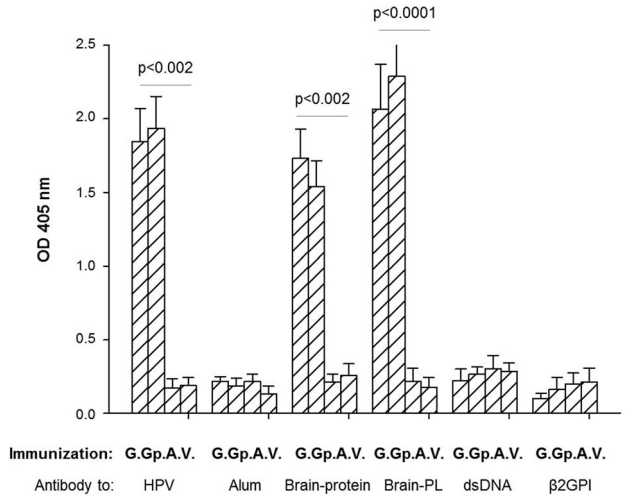


Fig. 2 Titers of serum antibodies 1 month post-injection with either Al (A), Gardasil (G), Gardasil + Pt toxin (Gp) and vehicle (V). A homemade ELISA was used to detect the levels of anti-HPV, anti-Al hydroxide (Alum), anti-mouse brain protein extract, anti-mouse brain phospholipid (PL) extract, anti-dsDNA and anti-β2glycoprotein-I (β2GPI) antibodies in the sera of immunized mice. Pools of sera ($n = 5$ per treatment group) were used as samples. All sera samples were assayed in triplicate. Data are presented as mean OD 405 ± SEM

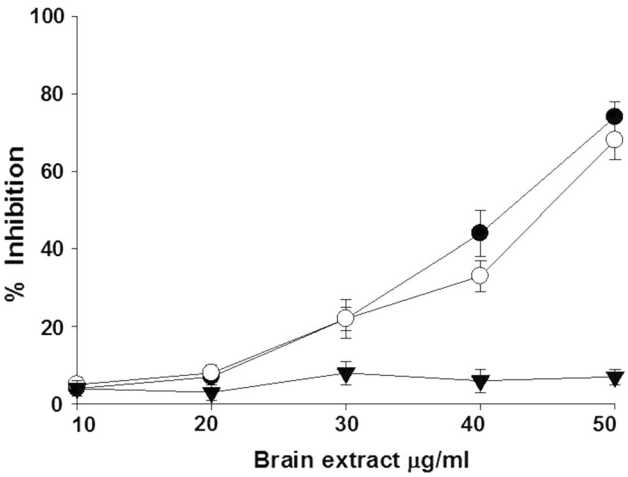


Fig. 3 Inhibition of the binding of antibodies from the sera of Gardasil-injected mice to components of the vaccine (presumably the HPV antigens) by the mouse protein extract. Pools of sera ($n = 5$ per treatment group) were used as samples. All sera samples were assayed by duplicates in independent experiments. Data are presented as mean (% Inhibition) ± SEM where % inhibition = 100 − [(OD of tested sample without inhibitor − OD of tested sample with inhibitor)/(OD of tested sample without inhibitor)] × 100 (*inverted triangle*) Al, (*filled balck circle*) Gardasil, (*open circle*) Gardasil + Pt

microglia staining in any of the regions of interests we investigated (CA1, CA3, dentate gyrus and the striatum). Nonetheless, 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

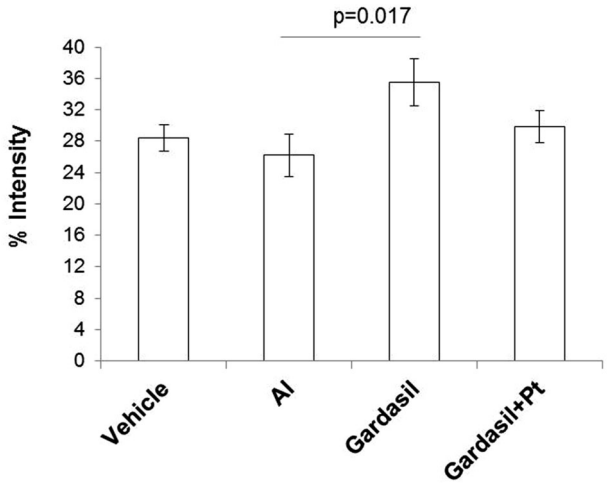


Fig. 4 Iba-1 immunostaining in the CA1 area of the hippocampus of C57BL/6 female mice injected with Al, Gardasil and Gardasil + Pt toxin. Brain sections from five animals out of each group were examined quantitatively for differences in immunostaining density using Image J software (NIH, USA) as described in "Materials and methods". The data are presented as % mean (% Intensity) ± SEM

Gardasil-immunized mice compared to Al-injected mice ($p = 0.017$; Fig. 4). These results suggest that the CA1 might be vulnerable to small changes in neuroinflammation as a result of Gardasil immunization.

Discussion

The present results show alteration of behavioral responses and neuro-inflammatory 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 spent significantly more time floating in the FST test (measure indicative either of locomotor dysfunction or depressive behavior), compared to control animals (Fig. 1a, b). In contrast, no significant differences were observed in the number of stairs climbed in the staircase test which is a measure of locomotor activity.

In addition, the Al-injected group showed abnormal responses to a novel environment, which was manifested in reduced rearing frequency in the staircase test, which indicates a reduction in exploratory behavior (Fig. 1d). The number of stairs and rears in this test is 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 staircase apparatus) is also considered an index of non-selective attention in rodents, while rearing

during object investigation likely reflects selective attention [26].

We further observed significant increase in levels of anti-HPV antibodies, and antibodies targeting the brain protein and the brain phospholipid extract components in the two groups of mice that received the Gardasil injection (Fig. 2). Moreover, the recognition of vaccine components (presumably the HPV L1 capsid protein species) by the antibodies from the sera of Gardasil-immunized mice was inhibited in a dose-dependent manner by the mouse brain protein extract (Fig. 3). On the basis of these results, it would appear that the anti-HPV antibodies from Gardasil-vaccinated mice have the capacity to target not only the HPV antigens but also brain antigen(s), either directly or via negatively charged phospholipids. Finally, we observed significant inflammatory changes in the Gardasil-injected mice, namely the presence of activated microglia in the CA1 area of the hippocampus (Fig. 4).

Possible mechanisms of vaccine-induced injury

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 [21, 22, 27–31]. The HPV vaccines, like many other vaccines, are adjuvanted with Al in spite of well-documented evidence that Al can be both neuro- and immuno-toxic [10, 11, 13, 32–35] 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 (Table 1). 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 [9–11, 13, 14, 33, 35–37]. 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 [2]. 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 [2]. These observations should not be particularly surprising given Al's well-established neurotoxic properties [38, 39]. What has, however, been argued is that the concentrations at which Al is used in vaccines are not sufficient to cause neurotoxicity [17, 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 the 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 [10, 13, 41]. 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 [10, 41]. 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 myofasciitis (MMF) [9, 11, 35].

Thus, contrary to prevalent 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. [41], intramuscular injection of Al-containing vaccine in mice is associated with the appearance of Al deposits in distant organs, such as spleen and brain, which were still detected 1 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 6-month end point. 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 [41].

In the ASIA syndrome, there could be a 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 years post-vaccination) [6, 42]. It is probable that one of the reasons why Al adjuvants are retained long-term in bodily compartments including systemic circulation is due to their tight association with vaccine antigens or other vaccine excipients [43]. Even dietary Al has been shown to accumulate in the central nervous system over-time, producing Alzheimer's disease type outcomes in experimental animals given dietary equivalent amounts of Al to what humans consume through a typical Western diet [44].

The ability of Al adjuvant nanoparticles to cross the blood–brain barrier via a macrophage-dependent Trojan horse mechanism may explain in part why some vaccines have a predilection to affect the central nervous system [8, 10, 33, 35, 39]. Another explanation comes from the fact that Al nanomaterials can on their own damage the blood–brain barrier and induce neurovascular injury [16, 45]. Collectively, these studies [16, 41, 45] 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) 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, AAHS in Gardasil and ASO4 (3-0-desacyl-4′-monophosphoryl lipid A (MPL) adsorbed onto Al hydroxide) in Cervarix, it should be noted that these new adjuvants induce a much stronger immune response than conventional Al adjuvants used in other vaccines (i.e., Al hydroxide and Al phosphate) [46]. 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 US FDA, a placebo is, '*an inactive pill, liquid, or powder that has no treatment value*' [47]. 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 [7]. Conceivably, there is even less justification for using a novel and more potent Al formulation than those that have been in standard use (Al phosphate and hydroxide). The only aim that this practice achieves is to give potentially 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.

The role of vaccine-induced antigens: immune cross-reaction

As noted above, we observed significant elevation of antibodies recognizing Gardasil components, most likely the HPV L1 capsid protein of HPV types 6, 11, 16 and 18 ($p < 0.002$) and of antibodies targeting the mouse brain protein ($p < 0.002$) and phospholipid extracts ($p < 0.001$) in the sera of Gardasil-immunized mice (Fig. 2). The binding of anti-HPV antibodies from the sera of mice injected with Gardasil to components of the HPV vaccine, presumably the HPV L1 antigens, was inhibited in a dose-dependent manner by using mouse brain protein extract as the inhibitor (Fig. 3). Taken together, these results suggest that 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 [48] who showed 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. 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. 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 immunoadsorption plasmapheresis [49]. Although no significant antibodies were detected in this patient, the remarkable effectiveness of immunoadsorption plasmapheresis strongly suggested that some unidentified antibodies were involved in the pathophysiology of ACA [49]. Citing the work of Kanduc [50], 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 [49]. Altogether, these observations suggest 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 [48, 50]. Our observation that nearly 85 % (129/152) of HPV vaccine adverse case reports in the current scientific literature relate to neuro-ophthalmic abnormalities may lend further support for this conclusion (Table 1).

Conclusions

In summary, both Al and Gardasil vaccine injections resulted in behavioral abnormalities in mice (Figs. 1, 2, 3). Furthermore, immunostaining analysis showed an increase in the Iba-1 density in the CA1 area of the hippocampus in Gardasil-immunized mice in comparison with 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 total IgG isolated from the sera of Gardasil-immunized mice to components of the vaccine, most likely, the HPV L1 capsid antigenic component (Fig. 3). 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 in cervical cancer prevention [20, 51] and which in the other hand is capable of inducing immune-mediated cross-reactions with neural antigens of the human host. This note of caution becomes even more relevant when considering the continually increasing number of serious disabling neurological adverse events linked to HPV vaccination reported in the current medical literature (Table 1) and in vaccine surveillance databases [20].

Finally, in light of the data presented in this manuscript, new guidelines should be requested on the use of appropriate placebos in vaccine safety trials [7].

Compliance with ethical standards

Conflict of interest Yehuda Shoenfeld has acted as a consultant for the no-fault US National Vaccine Injury Compensation Program. L.T. has served as an expert witness in cases involving adverse reactions following qHPV vaccine administration. The other co-authors declare no competing interests.

Funding This work was supported by the grants from the Dwoskin Foundation Ltd.

References

1. Marra F, et al. A meta-analysis of intradermal versus intramuscular influenza vaccines: immunogenicity and adverse events. Influenza Other Respir Viruses. 2013;7(4):584–603. doi:10.1111/irv.12000.
2. Perricone C, et al. Autoimmune/inflammatory syndrome induced by adjuvants (ASIA) 2013: unveiling the pathogenic, clinical and diagnostic aspects. J Autoimmun. 2013;47:1–16. doi:10.1016/j.jaut.2013.10.004.
3. Tomljenovic L, Shaw CA. No autoimmune safety signal after vaccination with quadrivalent HPV vaccine Gardasil? J Intern Med. 2012;272(5):514–5. doi:10.1111/j.1365-2796.2012.02551.x.
4. Blitshteyn S. Postural tachycardia syndrome following human papillomavirus vaccination. Eur J Neurol. 2014;21(1):135–9. doi:10.1111/ene.12272.
5. Poser CM, Behan PO. Late onset of Guillain-Barre syndrome. J Neuroimmunol. 1982;3(1):27–41.
6. Ryan AM, et al. Atypical presentation of macrophagic myofasciitis 10 years post vaccination. Neuromuscul Disord. 2006;16(12):867–9. doi:10.1016/j.nmd.2006.07.017.
7. Exley C. Aluminium-based adjuvants should not be used as placebos in clinical trials. Vaccine. 2011;29(50):9289. doi:10.1016/j.vaccine.2011.08.062.
8. Lujan L, et al. Autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA syndrome) in commercial sheep. Immunol Res. 2013;56(2–3):317–24. doi:10.1007/s12026-013-8404-0.
9. Couette M, et al. Long-term persistence of vaccine-derived aluminum hydroxide is associated with chronic cognitive dysfunction. J Inorg Biochem. 2009;103(11):1571–8. doi:10.1016/j.jinorgbio.2009.08.005.
10. Gherardi RK, et al. Biopersistence and brain translocation of aluminum adjuvants of vaccines. Front Neurol. 2015;6:4. doi:10.3389/fneur.2015.00004.
11. Rigolet M, et al. Clinical features in patients with long-lasting macrophagic myofasciitis. Front Neurol. 2014;5:230. doi:10.3389/fneur.2014.00230.
12. Marrack P, et al. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol. 2009;9(4):287–93. doi:10.1038/nri2510.
13. Shaw CA, Petrik MS. Aluminum hydroxide injections lead to motor deficits and motor neuron degeneration. J Inorg Biochem. 2009;103(11):1555–62. doi:10.1016/j.jinorgbio.2009.05.019.
14. Li X, et al. Glia activation induced by peripheral administration of aluminum oxide nanoparticles in rat brains. Nanomedicine. 2009;5(4):473–9.
15. Zhu Y, et al. Immunotoxicity of aluminum. Chemosphere. 2014;104:1–6. doi:10.1016/j.chemosphere.2013.10.052.
16. Chen L, et al. Manufactured aluminum oxide nanoparticles decrease expression of tight junction proteins in brain

vasculature. J Neuroimmune Pharmacol. 2008;3(4):286–95. doi:10.1007/s11481-008-9131-5.
17. Eldred BE, et al. Vaccine components and constituents: responding to consumer concerns. Med J Aust. 2006;184(4):170–5.
18. Shoenfeld Y, et al. Vaccination as an additional player in the mosaic of autoimmunity. Clin Exp Rheumatol. 2000;18(2):181–4.
19. Shoenfeld Y. Infections, vaccines and autoimmunity. Lupus. 2009;18(13):1127–8. doi:10.1177/0961203309351081.
20. Tomljenovic L, Shaw CA. Human papillomavirus (HPV) vaccine policy and evidence-based medicine: Are they at odds? Ann Med. 2013;45(2):182–93. doi:10.3109/07853890.2011.645353.
21. Garland SM, et al. (FUTURE) I Investigators) Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. N Engl J Med. 2007;356(19):1928–43. doi:10.1056/NEJMoa061760.
22. Munoz N, et al. Safety, immunogenicity, and efficacy of quadrivalent human papillomavirus (types 6, 11, 16, 18) recombinant vaccine in women aged 24–45 years: a randomised, double-blind trial. Lancet. 2009;373(9679):1949–57. doi:10.1016/S0140-6736(09)60691-7.
23. Can A, et al. The mouse forced swim test. J Vis Exp. 2012;59:e3638. doi:10.3791/3638.
24. Tordera RM, 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.
25. Katzav A, et al. Hyperactivity in a mouse model of the antiphospholipid syndrome. Lupus. 2001;10:496–9.
26. Brown JA, et al. Reduced striatal dopamine underlies the attention system dysfunction in neurofibromatosis-1 mutant mice. Human Mol Genet. 2010;19(22):4515–28.
27. Harper DM, et al. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet. 2006;367(9518):1247–55. doi:10.1016/S0140-6736(06)68439-0.
28. Villa LL, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol. 2005;6(5):271–8. doi:10.1016/S1470-2045(05)70101-7.
29. Mao C, et al. Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: a randomized controlled trial. Obstet Gynecol. 2006;107(1):18–27. doi:10.1097/01.AOG.0000192397.41191.fb.
30. Group TFIS. Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. N Engl J Med. 2007;356(19):1915–27. doi:10.1056/NEJMoa061741.
31. Verstraeten T, et al. Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines. Vaccine. 2008;26(51):6630–8. doi:10.1016/j.vaccine.2008.09.049.
32. Guimaraes LE, et al. Vaccines, adjuvants and autoimmunity. Pharmacol Res. 2015;100:190–209. doi:10.1016/j.phrs.2015.08.003.
33. Shaw CA, et al. Are there negative CNS impacts of aluminum adjuvants used in vaccines and immunotherapy? Immunotherapy. 2014;6(10):1055–71. doi:10.2217/imt.14.81.
34. Exley C. Aluminium adjuvants and adverse events in sub-cutaneous allergy immunotherapy. Allergy Asthma Clin Immunol. 2014;10(1):4. doi:10.1186/1710-1492-10-4.
35. Passeri E, et al. Long-term follow-up of cognitive dysfunction in patients with aluminum hydroxide-induced macrophagic myofasciitis (MMF). J Inorg Biochem. 2011;105(11):1457–63. doi:10.1016/j.jinorgbio.2011.08.006.
36. Zivkovic I, et al. Induction of decreased fecundity by tetanus toxoid hyper-immunization in C57BL/6 mice depends on the applied adjuvant. Innate Immun. 2012;18(2):333–42. doi:10.1177/1753425911407361.
37. Agmon-Levin N, et al. Immunization with hepatitis B vaccine accelerates SLE-like disease in a murine model. J Autoimmun. 2014;54:21–32. doi:10.1016/j.jaut.2014.06.006.
38. Exley C, Birchall JD. The cellular toxicity of aluminium. J Theor Biol. 1992;159(1):83–98.
39. Shaw CA, Tomljenovic L. Aluminum in the central nervous system (CNS): toxicity in humans and animals, vaccine adjuvants, and autoimmunity. Immunol Res. 2013;56(2–3):304–16. doi:10.1007/s12026-013-8403-1.
40. Offit PA, Jew RK. Addressing parents' concerns: Do vaccines contain harmful preservatives, adjuvants, additives, or residuals? Pediatrics. 2003;112(6 Pt 1):1394–7.
41. Khan Z, et al. Slow CCL2-dependent translocation of biopersistent particles from muscle to brain. BMC Med. 2013;11:99. doi:10.1186/1741-7015-11-99.
42. Gherardi RK, et al. Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. Brain. 2001;124(Pt 9):1821–31.
43. Lee SH. Detection of human papillomavirus L1 gene DNA fragments in postmortem blood and spleen after Gardasil® vaccination—a case report. Adv Biosci Biotech. 2012;3:1214–24.
44. Walton JR. A longitudinal study of rats chronically exposed to aluminum at human dietary levels. Neurosci Lett. 2007;412(1):29–33. doi:10.1016/j.neulet.2006.08.093.
45. Xiu C, et al. Aluminum chloride- and norepinephrine-induced immunotoxicity on splenic lymphocytes by activating beta-AR/cAMP/PKA/NF-kappaB signal pathway in rats. Biol Trace Elem Res. 2014;162(1–3):168–74. doi:10.1007/s12011-014-0149-7.
46. Caulfield MJ, et al. Effect of alternative aluminum adjuvants on the absorption and immunogenicity of HPV16 L1 VLPs in mice. Human Vaccin. 2007;3(4):139–45.
47. FDA. Food and Drug Administration. Inside clinical trials: testing medical products in people. (2009).
48. Kanduc D. Potential cross-reactivity between HPV16 L1 protein and sudden death-associated antigens. J Exp Ther Oncol. 2011;9(2):159–65.
49. Yonee C, et al. Association of acute cerebellar ataxia and human papilloma virus vaccination: a case report. Neuropediatrics. 2013;44(5):265–7. doi:10.1055/s-0033-1333873.
50. Kanduc D. Quantifying the possible cross-reactivity risk of an HPV16 vaccine. J Exp Ther Oncol. 2009;8(1):65–76.
51. van Bogaert L. Are the currently existing anti-human papillomavirus vaccines appropriate for the developing world? Ann Med Health Sci Res. 2013;3(3):306–12. doi:10.4103/2141-9248.117924.
52. Mendoza Plasencia Z, et al. Acute disseminated encephalomyelitis with tumefactive lesions after vaccination against human papillomavirus. Neurologia. 2010;25(1):58–9.
53. Wildemann B, et al. Acute disseminated encephalomyelitis following vaccination against human papilloma virus. Neurology. 2009;72(24):2132–3. doi:10.1212/WNL.0b013e3181aa53bb.
54. Sutton I, et al. CNS demyelination and quadrivalent HPV vaccination. Mult Scler. 2009;15(1):116–9.
55. Chang J, et al. Demyelinating disease and polyvalent human papilloma virus vaccination. J Neurol Neurosurg Psychiatry. 2011;82(11):1296–8. doi:10.1136/jnnp.2010.214924.
56. McCarthy JE, Filiano J. Opsoclonus Myoclonus after human papilloma virus vaccine in a pediatric patient. Parkinsonism Relat Disord. 2009;15(10):792–4. doi:10.1016/j.parkreldis.2009.04.002.
57. Menge T, et al. Neuromyelitis optica following human papillomavirus vaccination. Neurology. 2012;79(3):285–7. doi:10.1212/WNL.0b013e31825fdead.
58. DiMario FJ Jr, et al. A 16-year-old girl with bilateral visual loss and left hemiparesis following an immunization against human

papilloma virus. J Child Neurol. 2010;25(3):321–7. doi:10.1177/0883073809349322.
59. Alvarez-Soria MJ, et al. Trastornos neurológicos desmielinizantes y vacunación del papilomavirus humano. Rev Neurol. 2011;52(8):472–6.
60. Zhu YZ, et al. impact of aluminum exposure on the immune system: a mini review. Environ Toxicol Pharmacol. 2013;35(1):82–7. doi:10.1016/j.etap.2012.11.009.
61. Debeer P, et al. Brachial plexus neuritis following HPV vaccination. Vaccine. 2008;26(35):4417–9. doi:10.1016/j.vaccine.2008.06.074.
62. Brinth LS, et al. Orthostatic intolerance and postural tachycardia syndrome as suspected adverse effects of vaccination against human papilloma virus. Vaccine. 2015;33(22):2602–5. doi:10.1016/j.vaccine.2015.03.098.
63. Kinoshita T, et al. Peripheral sympathetic nerve dysfunction in adolescent Japanese girls following immunization with the human papillomavirus vaccine. Intern Med. 2014;53(19):2185–200.
64. Blitshteyn S. Postural tachycardia syndrome following human papillomavirus vaccination. Eur J Neurol. 2014;21:135–9. doi:10.1111/ene.12272.
65. Richards S, et al. Complex regional pain syndrome following immunisation. Arch Dis Child. 2012;97(10):913–5. doi:10.1136/archdischild-2011-301307.
66. Tomljenovic L, et al. Postural orthostatic tachycardia with chronic fatigue after HPV vaccination as part of the "autoimmune/autoinflammatory syndrome induced by adjuvants": case report and literature review. J Investig Med High Impact Case Rep. 2014;. doi:10.1177/2324709614527812.
67. Martinez-Lavin M. Fibromyalgia-like illness in 2 girls after human papillomavirus vaccination. J Clin Rheumatol. 2014;20(7):392–3. doi:10.1097/RHU.0000000000000165.
68. Cerami C, et al. Autoimmune neuromyotonia following human papilloma virus vaccination. Muscle Nerve. 2013;47(3):466–7. doi:10.1002/mus.23648.
69. Colafrancesco S, et al. HPV vaccines and primary ovarian failure: another facet of the autoimmune/inflammatory syndrome induced by adjuvants (ASIA). Am J Reprod Immunol. 2013;70(4):309–16.
70. Little DT, Grenville Ward HR. Adolescent premature ovarian insufficiency following human papillomavirus vaccination: a case series seen in general practice. J Investig Med High Impact Case Rep. 2014;. doi:10.1177/2324709614556129.
71. Melo Gomes S, et al. Vasculitis following HPV immunization. Rheumatology (Oxford). 2013;52(3):581–2. doi:10.1093/rheumatology/kes168.
72. Pugnet G, et al. Immune thrombocytopenic purpura following human papillomavirus vaccination. Vaccine. 2009;27(28):3690. doi:10.1016/j.vaccine.2009.04.004.
73. Della Corte C, et al. Autoimmune hepatitis type 2 following anti-papillomavirus vaccination in a 11-year-old girl. Vaccine. 2011;29(29):4654–6. doi:10.1016/j.vaccine.2011.05.002.
74. Das A, et al. Pancreatitis following human papillomavirus vaccination. Med J Aust. 2008;189(3):178.
75. Soldevilla HF, et al. Systemic lupus erythematosus following HPV immunization or infection? Lupus. 2012;21(2):158–61. doi:10.1177/0961203311429556.
76. Gatto M, et al. Human papillomavirus vaccine and systemic lupus erythematosus. Clin Rheumatol. 2013;32(9):1301–7. doi:10.1007/s10067-013-2266-7.
77. Anaya JM, et al. Autoimmune/auto-inflammatory syndrome induced by adjuvants (ASIA) after quadrivalent human papillomavirus vaccination in Colombians: a call for personalised medicine. Clin Exp Rheumatol. 2015;33(4):545–8.

Immunol Res
DOI 10.1007/s12026-016-8818-6

ENVIRONMENT AND AUTOIMMUNITY

Phospholipid supplementation can attenuate vaccine-induced depressive-like behavior in mice

Shaye Kivity[1,2,3,4] · **Maria-Teresa Arango**[1,2,5] · **Nicolás Molano-González**[6] · **Miri Blank**[1,2] · **Yehuda Shoenfeld**[1,2,7]

Yehuda Shoenfeld

Abstract Human papillomavirus vaccine (HPVv) is used worldwide for prevention of infection. However several reports link this vaccine, with immune-mediated reactions, especially with neurological manifestations. Our previous results showed that HPVv-Gardasil and aluminum-immunized mice developed behavioral impairments. Studies have shown a positive effect of phospholipid supplementation on depression and cognitive functions in mice. Therefore, our goal was to evaluate the effect of a dietary supplement on vaccine-induced depression. Sixty C57BL/6 *female* mice were immunized with HPVv-Gardasil, aluminum or the vehicle ($n = 20$ each group), and half of each group were fed 5 times per week with 0.2 ml of a dietary supplement enriched with phosphatidylcholine. The mice were evaluated for depression at 3 months of age, by the forced swimming test. Both the Gardasil and the aluminum-treated mice developed depressive-like behavior when compared to the control group. The HPVv-Gardasil-immunized mice supplemented with phosphatidylcholine significantly reduced their depressive symptoms. This study confirms our previous studies demonstrating depressive-like behavior in mice vaccinated with HPVv-Gardasil. In addition, it demonstrates the ability of phosphatidylcholine-enriched diet to attenuate depressive-like behavior in the HPVv-Gardasil-vaccinated mice. We suggest that phosphatidylcholine supplementation may serve as a treatment for patients suffering vaccine-related neurological manifestations.

Keywords Gardasil · Aluminum · Depression · Behavior · Autoimmunity

Abbreviations

HPVv	Human papillomavirus vaccine
HPV	Human papillomavirus
ASIA	Autoimmune/inflammatory syndrome induced by adjuvants
PC	Phosphatidylcholine
FST	Forced swimming test
MANOVA	Multivariate analysis of variance
CNS	Central nervous system
CFS	Chronic fatigue syndrome
PS	Phosphatidylserine
GC	Glucocorticoid

Shaye Kivity and Maria-Teresa Arango have contributed equally to this work.

✉ Yehuda Shoenfeld
shoenfel@post.tau.ac.il

[1] Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, 52621 Tel-Hashomer, Israel

[2] Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

[3] Rheumatic Disease Unit, Sheba Medical Center, Tel-Hashomer, Israel

[4] The Dr. Pinchas Borenstein Talpiot Medical Leadership Program 2013, Sheba Medical Center, Tel-Hashomer, Israel

[5] Doctoral Program in Biomedical Sciences, Universidad del Rosario, Bogotá, Colombia

[6] Center for Autoimmune Diseases Research (CREA)-Statistics, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia

[7] Incumbent of the Laura Schwarz-Kip Chair for Research of Autoimmune Diseases, Tel Aviv University, Tel Aviv, Israel

Published online: 27 July 2016

Introduction

Vaccines represent the most successful and sustainable approach to eradicate infectious diseases. Indeed, significant infectious diseases, such as small pox, have been eliminated thanks to worldwide vaccination programs. As a result, vaccination programs are the mainstay of preventive medicine in modern health systems. In order to efficiently design a vaccine, there is a need to unravel the life cycle of a specific pathogen and the disease it is related to. For instance, the discovery of the causal association between human papillomavirus (HPV) and few cervical cancers leads to the development of a prophylactic vaccine, the human papillomavirus vaccine [1]. Since 2006 the human papillomavirus vaccine (HPVv), mainly Gardasil, has been used worldwide for prophylactic protection of girls in puberty age, it has been suggested that Gardasil® can provide protection against persistent cervical HPV 16 and 18 strain infections for up to 8 years, which is the maximum time of research follow-up thus far [2, 3]. However, more evidence is needed to support this claim.

However, this vaccine and its adjuvant aluminum hydroxide have been reported to be associated with a variety of neurological and autoimmune manifestations [4–6]. It has been suggested that post-vaccination immune-mediated symptoms are part of the "Autoimmune/inflammatory syndrome induced by adjuvants" (ASIA) syndrome [7]. Worldwide vaccine safety surveillance systems describe numerous case reports regarding Gardasil's adverse effects, a large proportion of them related to the nervous system [5, 8]. Recently Inbar et al. [6] performed a review of the literature and summarized 152 cases of autoimmune and inflammatory manifestations following HPV vaccination. In out of 152 reported cases, 129 (85 %) were related to neuro-ophthalmic disorders. In addition, we have recently demonstrated that HPVv-Gardasil and aluminum-immunized young female mice exhibited behavioral and cognitive impairments, mainly depression and memory decline [6]. The strongest effect was found in mice at 3 and 6 months of age, in which Gardasil and aluminum injection lead to depressive-like behavior. It is hypnotized that Gardasil via its aluminum adjuvant and HPV antigens has the ability to trigger neuroinflammation and autoimmune reactions, further leading to behavioral changes [6]. This may be due to cross-reactivity between the vaccine and human proteins, as demonstrated by Kanduc [9, 10]. This bioinformatics approach demonstrated cross-reactivity between more than 80 peptides of serotype 16 HPV proteins and human proteins related to crucial cellular processes, such as adhesion molecules, peptides responsible for leukocyte differentiation and spermatogenesis, transcription factors, and neuronal antigens [10].

Phosphatidylcholine (PC) is the main constituent of the cell membrane, and being so, it is an essential phospholipid in mammalian cells and tissues. PC can be synthesized in nucleated cells or consumed through the diet. In neurons, PC is produced in the cell bodies, but also in the axons, where they contribute to neuron plasticity and axonal growth [11]. PC is also the precursor of other important molecules, such as other phospholipids (i.e. phosphatidylserine), and neurotransmitters (acetylcholine) [12, 13]. Previous studies showed a positive effect of phospholipids supplementation on depression and cognitive function in rodents [14] and humans [15–17]. Therefore, our goal was to evaluate the effect of a phospholipid dietary supplement on the depression-like behavior induced by Gardasil in immunized mice.

Materials and methods

Vaccine and adjuvant

In order to administrate equivalent doses of the Gardasil vaccine and its adjuvant, we normalized them according to mouse weight [6]. Briefly, we took in consideration the average weight of 40 kg of teenage girls. Therefore, a 20 g mouse received 2000 times less of the vaccine suspension. Secondly, considering that the adjuvant employed in the vaccine is not commercially available, we calculate the amount of aluminum hydroxide in a single dose of Gardasil. Therefore, each mouse received 5.6 μg/kg body weights aluminum which was dissolved in a vehicle reported in the vaccine consistent of 19.12 μg/mL NaCl, 1.56 μg/mL L-histidine.

Dietary supplement

Phospholipid supplement contained: Phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylinositol, minor glycolipids, linoleic and alpha linolenic acids, as well as minor fatty acids dissolved in ethanol. The solution included also Omega 3 and 6 in a ratio of 1:4. The phospholipid supplementation was given in a concentration of 600 mg/ml (BodyBio, Liquid Phosphatidylcholine, Millville, New Jersey, 08332, USA). The supplement was purchased through internet in its main website (http://www.bodybio.com/storeproduct426.aspx). We have no conflict of interests with the *BodyBio* Company.

Mice husbandry and experimental design

Female C57BL/6 mice at the age of 6 weeks (Harlan Laboratories Jerusalem, Israel) were kept under standard conditions, at 23 ± 1 °C, 12-h light cycle with ad libitum access

to food and water. They were housed in the animal facility at Sheba Medical Center where the Welfare Committee approved all procedures. The animals were divided in three different groups of 20 animals each, and they received three intramuscular (i.m.) injections (1 day apart) as follows: Group 1: aluminum hydroxide; group 2: quadrivalent HPV vaccine Gardasil; and group 3: vehicle control (19.12 μg/mL NaCl, 1.56 μg/mL L-histidine). In addition, ten mice from each group were fed 5 times per week with 0.2 ml of the dietary supplement enriched with PC.

Behavioral tests

Mice were weighted, and their behavior was evaluated at 3 months of age for depression by the forced swimming test (FST), strength and motor function by the rotarod test.

Rotarod

The rotarod was used to test general motor function and motor learning according to the protocol of Katzav et al. [18]. 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 for 60 s. The mean of 3 consecutive trials was recorded for each mouse. A day prior to testing, the mice were habituated to the rotarod.

Forced swimming test

This test is based on Porsolt et al.'s description [18–20]. The mice were subjected to two sessions: first a pretest of 10 min and finally a test session for 6 min. The behavioral measure scored was the duration (in seconds) of immobility, defined as the absence of any escape-oriented behaviors. Depression-like behavior was considered as an increased immobility time compared with the control group in the last 4 min of the test.

Statistical analysis

Results are expressed as the mean ± SD. Multivariate analysis of variance (MANOVA) and multivariate regression analysis where used to assess the association of the different immunizations, the phospholipid supplementation and weight to the performance of the mice in the rotarod and FST tests. For the regression analysis, the immunization treatment was considered as an ordinal variable, giving the following order: vehicle < aluminum < Gardasil. Finally, different contrast levels were calculated in order to compare the effect of the immunization treatments and the phospholipid supplementation following De Rosario-Martinez et al. [21] instructions. All analyses were done using R (Version 3.0.2). Significant results were determined as $p < 0.05$.

Results

Six weeks after injection, when mice reached the age of 3 months, we assessed them for depressive-like behavior, motor function and body weight (Table 1). We assessed depressive-like behavior by the force swimming test; however, increased floating time also might indicate locomotor dysfunction. To discard this possibility, we performed rotarod to evaluate the locomotor ability of the animals.

The MANOVA demonstrated an interaction between phospholipid supplementation and immunization (vehicle, aluminum and Gardasil), which affects the performance of the mice in the different behavioral tests ($p = 0.00228$), while the weight of the mice did not have any effect on it ($p = 0.293$). The pairwise contrast analysis demonstrated significant differences in the FST performance but not in the rotarod test when each group of mice was analyzed considering they received or not the phospholipid supplement (Table 1; Fig. 1). In the case of FST, no differences were found in the mice that were immunized with the vehicle (48.43 ± 27.69 vs 42.75 ± 16.96; $p = 0.585$). However, the phospholipid supplement in the mice immunized with aluminum and Gardasil had a significant effect. The average immobility time of the aluminum-immunized group treated with the supplement was reduced by 25.868 s (80.37 ± 17.97 vs 54.51 ± 23.44; $p = 0.0358$) and that of the Gardasil was reduced by 63.117 s (145.65 ± 32.08 vs 82.53 ± 19.77; $p = 5.7 \times 10^{-7}$). As for the rotarod test, the phospholipid supplementation did not affect the performance of the mice immunized with the vehicle (55.03 ± 10.12 vs 54.43 ± 6.94, $p = 0.886$), aluminum (44.7 ± 12.55 vs 55.16 ± 5.41, $p = 0.052$) or Gardasil (57.76 ± 7.06 vs 53.1 ± 12.63, $p = 0.559$).

Multivariate regression analysis demonstrated a significant linear trend for the FST, indicating that the immobility time in the aluminum groups was higher than that of the vehicle, and the values for Gardasil were higher than the aluminum and vehicle groups. In addition, the linear regression analysis demonstrated that in the absence of phospholipid supplementation, there is a higher increment rate in the immobility time among the different groups. For instance, in Fig. 1, the slope among the groups who did not received the phospholipid supplementation is 40.61 higher than those that received supplementation ($p = 0.000285$) (Fig. 1).

Discussion

In our present study, we induced depression in C57BL/6 mice by injecting Gardasil or its adjuvant, aluminum. The mice depressive-like behavior, assessed by the FST, was not attributed to motor- weakness, assessed by rotarod test. Our

Table 1 Parameters evaluated in C57BL/6 mice

	Phospholipid Supplementation	Weight	Rota rod	FST
Vehicle	–	19.89 ± 1.12	55.03 ± 10.12	48.43 ± 27.69
	+	20.94 ± 1.04	54.43 ± 6.94	42.75 ± 16.96
Aluminum	–	20.54 ± 1.08	44.7 ± 12.55	80.37 ± 17.97
	+	19.87 ± 1.18	55.16 ± 5.41	54.51 ± 23.44
Gardasil	–	21.4 ± 0.69	57.76 ± 7.06	145.65 ± 32.08
	+	20.62 ± 0.79	53.1 ± 12.63	82.53 ± 19.77

FST forced swimming test

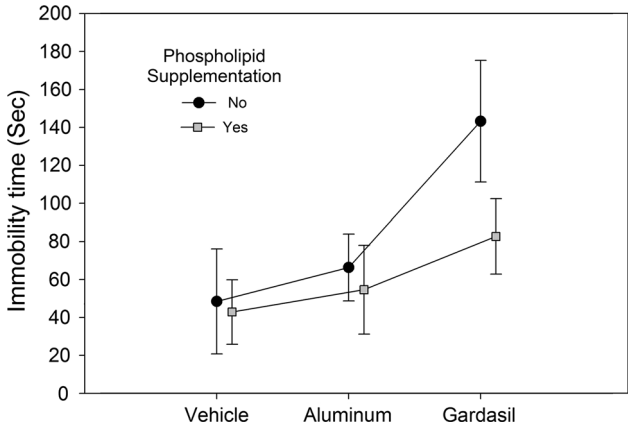


Fig. 1 Depressive-like behavior evaluation by forced swimming test and in C57BL/6 aluminum and Gardasil immunized mice after phospholipid supplementation. Results are presented as duration in seconds (mean ± SD) of immobility, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the 6-min test. A depression-like behavior was considered as an increased immobility time. All the mice that received the dietary supplement (*gray*) exhibited a significant decrease in depressive-like behavior ($p < 0.001$) compared the pair group which was not feed with the phospholipid supplement (*black*)

main finding is that dietary supplementation enriched with PC, ameliorated the depressive-like symptoms of the mice.

In recent years, there have been case reports describing post-vaccination side effects, which include neurological manifestations, such as chronic fatigue, depression, sleep disturbances, cognitive impairments, demyelination syndromes, optic neuritis and others [22–24]. In the case of HPV vaccination, in particular, there seems to be a greater proportion of central nervous system-related autoimmune reactions [6], (for example, opsoclonus myoclonus, mood swings, depression and anxiety [25, 26].

In this present study, HPV vaccine or aluminum both induced depressive-like behavior when injected to mice. It has been suggested that aluminum, which is used as a vaccine-adjuvant, is implicated in the mechanism of immune-mediated vaccine side effects. Aluminum is toxic on multiple levels; however, studies suggest the central nervous system (CNS) is its most sensitive target [27–32]. Several CNS manifestations were described following aluminum exposure, including: impaired learning processes, impaired memory and concentration, speech deficits, impaired psychomotor control, increased seizure activity, and altered behavior [33]. Moreover, aluminum has been linked to a different neurological diseases including Alzheimer's disease [33], amyotrophic lateral sclerosis and Parkinsonism dementia [34], multiple sclerosis [35, 36], as well as neurological impairments in children [29, 37–39]. Relatively small amounts of aluminum, even those equivalent to what is injected via vaccinations, can reach the brain [40, 41]. In one report, Lujan et al described a severe neurodegenerative syndrome in sheep, which was associated with the administration of aluminum-containing vaccines. The sheep displayed severe neurobehavioral outcomes, in addition to traces of aluminum in the brain, and inflammatory lesions in CNS biopsies [42]. It should be noted, however, that administration of higher doses of aluminum to experimental animals results in more severe neuropathological, biochemical and behavioral changes, which resemble Alzheimer's disease [43, 44]. These observations led researchers to focus on studies which examine the effect of aluminum on the nervous system of animals and humans. One of the main finding is that chronic aluminum exposure can alter mitochondrial energy metabolism and increase reactive oxygen species production in the brain [45]. In addition, aluminum exposure has also been shown to affect the endocrine system [46, 47], to interfere with cellular processes such as calcium homeostasis [48], and to affect ATP-dependent mechanisms and membrane receptor signaling [33, 49]. In our previous studies, we have demonstrated that the immunization with aluminum salts can induce histological changes such as activation of microglia in the brain, in particular in C57BL/6 mice [6] and also in NZB/WxF1 mice [50].

Animal studies and some human studies show that dietary phospholipids have a positive impact in several diseases including cancer, coronary disease and

inflammatory disease. This may be explained by the fact that phospholipids are highly effective in delivering their fatty acid residues for incorporation into the membranes of cells involved in different diseases [51]. For instance, in one study, supplementation of PC to old rat myocytes reversed their cellular functions to those of young myocytes [52]. Phospholipids were found effective in treating neurological conditions, such as memory loss, cognitive decline, stroke, fatigue, movement disorders and alcohol ingestion [51, 53, 54]. This was especially shown with phosphatidylserine (PS), which is synthesized from phosphatidylcholine or phosphatidylethanolamine, and is important for the maintenance of healthy nerve cell membranes and myelin. Dietary phosphatidylserine was shown to improve cognitive function in experimental animals and in humans [55, 56]. In an interesting study by Takeshi Kanno et al, mice subjected for depression by the chronic stress mice were treated with two forms of PC (1,2-dilinoleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine-reduced). In this study the depressive-like behavior was attenuated by PC treatment in a dose dependent manner, possibly by activating Akt and inhibiting GSK-3β [57]. In one study in humans, patients treated for 42 days with 200 mg of soy-based PS showed a more relaxed state before and after mental stress as measured by electro-encephalography [58]. PS also helped to improve memory in elderly patients in double-blinded controlled studies [59, 60]. In addition, phospholipid replacement therapy has been shown to be effective in the treatment of chronic fatigue syndrome (CFS). It has been proposed that one of the main causes of CFS might be alterations of the mitochondrial function due to oxidative stress [61–63]. However, some studies showed negative or no effects PLs on cognitive performance [64].

It is suggested that supplementary PS crosses the blood–brain-barrier and slows or reverses biochemical and structural changes in human nerve cells and mediated effects on neuronal membranes [65, 66]. The rational for the positive effect of phospholipids on the brain could be explained by the effect of stress and depression on brain lipid metabolism. Stress and depression both cause glucocorticoid (GC) release and activation of the hypothalamic–pituitary–adrenal axis, both of which may affect brain lipid metabolism. GCs have been shown to modulate brain lipid signaling via stimulation of phospholipase A2, a key lipid enzyme that cleaves membrane phospholipids into lysophospholipids and arachidonic acid. Mass spectrometry of brains of depressed rodents demonstrated that the brain phospholipid metabolism is altered; decreased phosphatidylethanolamine and PC levels yet an increased lysophosphatidylethanolamine levels were observed [67]. Other proposed mechanisms for PS-mediated cognitive improvement in experimental models are stimulation of dopamine-dependent adenylate cyclase activity [68], increasing the acetylcholine brain-levels [69, 70], and up-regulation of brain-derived neurotrophic factor and insulin-like growth factors [56].

In the current study, the mice supplementation diet contained also omega-3. It should be noted that treatment with omega-3 was shown to ameliorate the adverse effects of aluminum on the brain. Ali HA et al. showed that the supplementation of mice with omega 3 in addition to quercetin can ameliorate the oxidative stress which was induced by aluminum chloride in the mouse brain [71]. Moreover, since phospholipid supplementation was shown to be effective restoring the mitochondrial activity in patients with CFS as well as in cancer patients after chemotherapy [53, 54, 72], it is possible to speculate that it might be also helpful reversing the effects of aluminum, especially those related to the alteration of mitochondrial activity [33, 45, 49].

In conclusion, this study demonstrates the positive effects of phospholipid supplementation on post-HPVv depression-like behavior. Future studies should evaluate whether PC supplementation is effective as a treatment for patients suffering from neurological manifestations after vaccination.

Acknowledgments This work was supported by Judy and Stewart Colton as part of the Ph.D. Project of María-Teresa Arango.

Compliance with ethical standards

Conflict of interest 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.

References

1. Kash N, et al. Safety and Efficacy data on vaccines and immunization to human papillomavirus. J Clin Med. 2015;4(4):614–33. doi:10.3390/jcm4040614.
2. McCormack PL, Joura EA. Quadrivalent human papillomavirus (types 6, 11, 16, 18) recombinant vaccine (Gardasil(R)): a review of its use in the prevention of premalignant genital lesions, genital cancer and genital warts in women. Drugs. 2010; 70(18):2449–74. doi:10.2165/11204920-000000000-00000.
3. McCormack PL. Quadrivalent human papillomavirus (types 6, 11, 16, 18) recombinant vaccine (gardasil((R))): a review of its use in the prevention of premalignant anogenital lesions, cervical and anal cancers, and genital warts. Drugs. 2014;74(11):1253–83. doi:10.1007/s40265-014-0255-z.
4. Gatto M, et al. Human papillomavirus vaccine and systemic lupus erythematosus. Clin Rheumatol. 2013;32(9):1301–7. doi:10.1007/s10067-013-2266-7.
5. Geier DA, Geier MR. A case-control study of quadrivalent human papillomavirus vaccine-associated autoimmune adverse events. Clin Rheumatol. 2015;34(7):1225–31. doi:10.1007/s10067-014-2846-1.
6. Inbar R, et al. Behavioral abnormalities in female mice following administration of aluminum adjuvants and the human

papillomavirus (HPV) vaccine Gardasil. Immunol Res. 2016. doi:10.1007/s12026-016-8826-6.
7. Pellegrino P, et al. On the relationship between human papilloma virus vaccine and autoimmune diseases. Autoimmun Rev. 2014;13(7):736–41. doi:10.1016/j.autrev.2014.01.054.
8. Tomljenovic L, Shaw CA. Human papillomavirus (HPV) vaccine policy and evidence-based medicine: are they at odds? Ann Med. 2013;45(2):182–93. doi:10.3109/07853890.2011.645353.
9. Kanduc D. Potential cross-reactivity between HPV16 L1 protein and sudden death-associated antigens. J Exp Ther Oncol. 2011;9(2):159–65.
10. Kanduc D. Quantifying the possible cross-reactivity risk of an HPV16 vaccine. J Exp Ther Oncol. 2009;8(1):65–76.
11. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. J Lipid Res. 2008;49(6):1187–94. doi:10.1194/jlr.R700019-JLR200.
12. Tayebati SK, Amenta F. Choline-containing phospholipids: relevance to brain functional pathways. Clin Chem Lab Med. 2013;51(3):513–21. doi:10.1515/cclm-2012-0559.
13. Kim HY, et al. Phosphatidylserine in the brain: metabolism and function. Prog Lipid Res. 2014;56:1–18. doi:10.1016/j.plipres.2014.06.002.
14. Vickers MH, et al. Supplementation with a mixture of complex lipids derived from milk to growing rats results in improvements in parameters related to growth and cognition. Nutr Res. 2009;29(6):426–35. doi:10.1016/j.nutres.2009.06.001.
15. Rondanelli M, et al. Long chain omega 3 polyunsaturated fatty acids supplementation in the treatment of elderly depression: effects on depressive symptoms, on phospholipids fatty acids profile and on health-related quality of life. J Nutr Health Aging. 2011;15(1):37–44.
16. Rondanelli M, et al. Effect of omega-3 fatty acids supplementation on depressive symptoms and on health-related quality of life in the treatment of elderly women with depression: a double-blind, placebo-controlled, randomized clinical trial. J Am Coll Nutr. 2010;29(1):55–64.
17. Benton D, et al. The influence of phosphatidylserine supplementation on mood and heart rate when faced with an acute stressor. Nutr Neurosci. 2001;4(3):169–78.
18. Katzav A, et al. Induction of autoimmune depression in mice by anti-ribosomal P antibodies via the limbic system. Arthritis Rheum. 2007;56(3):938–48. doi:10.1002/art.22419.
19. Porsolt RD, et al. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther. 1997;229:327–36.
20. Katzav A, et al. Anti-P ribosomal antibodies induce defect in smell capability in a model of CNS -SLE (depression). J Autoimmun. 2008;31(4):393–8. doi:10.1016/j.jaut.2008.09.002.
21. De Rosario-Martinez H, et al. Post-Hoc interaction Analysis—package 'phia'. 0.2-1 ed2015. 2015. https://cran.r-project.org/web/packages/phia/phia.pdf
22. Alijotas-Reig J. Human adjuvant-related syndrome or autoimmune/inflammatory syndrome induced by adjuvants. Where have we come from? Where are we going? A proposal for new diagnostic criteria. Lupus. 2015;24(10):1012–8. doi:10.1177/0961203315579092.
23. Karussis D, Petrou P. The spectrum of post-vaccination inflammatory CNS demyelinating syndromes. Autoimmun Rev. 2013;13(3):215–24. doi:10.1016/j.autrev.2013.10.003.
24. Narcolepsy AS, et al. A(H1N1) pandemic influenza, and pandemic influenza vaccinations: what is known and unknown about the neurological disorder, the role for autoimmunity, and vaccine adjuvants. J Autoimmun. 2009;50:1–11. doi:10.1016/j.jaut.2014.01.033.
25. McCarthy JE, Filiano J. Opsoclonus Myoclonus after human papilloma virus vaccine in a pediatric patient. Parkinsonism Relat Disord. 2009;15(10):792–4. doi:10.1016/j.parkreldis.2009.04.002.
26. Colafrancesco S, et al. HPV vaccines and primary ovarian failure: another facet of the autoimmune/inflammatory syndrome induced by adjuvants (ASIA). Am J Reprod Immunol. 2013;70(4):309–16.
27. Shaw CA, Petrik MS. Aluminum hydroxide injections lead to motor deficits and motor neuron degeneration. J Inorg Biochem. 2009;103(11):1555–62. doi:10.1016/j.jinorgbio.2009.05.019.
28. Rogers MA, Simon DG. A preliminary study of dietary aluminium intake and risk of Alzheimer's disease. Age Aging. 1999;28(2):205–9.
29. Bishop NJ, et al. Aluminum neurotoxicity in preterm infants receiving intravenous-feeding solutions. N Engl J Med. 1997;336(22):1557–61. doi:10.1056/NEJM199705293362203.
30. Walton JR. Evidence that ingested aluminum additives contained in processed foods and alum-treated drinking water are a major risk factor for Alzheimer's disease. Curr Inorg Chem. 2012;2(1):19–39.
31. Rondeau V, et al. Relation between aluminum concentrations in drinking water and Alzheimer's disease: an 8-year follow-up study. Am J Epidemiol. 2000;152(1):59–66.
32. ATSDR. Toxicological profile for aluminum. Atlanta: Agency for toxic substances and disease registry; 2008.
33. Tomljenovic L. Aluminum and Alzheimer's disease: after a century of controversy, is there a plausible link? J Alzheimers Dis. 2011;23(4):567–98. doi:10.3233/Jad-2010-101494.
34. Perl DP, Moalem S. Aluminum, Alzheimer's disease and the geospatial occurrence of similar disorders. Rev Mineral Geochem. 2006;64:115–34.
35. Authier FJ, et al. Central nervous system disease in patients with macrophagic myofasciitis. Brain. 2001;124(Pt 5):974–83.
36. Exley C, et al. Elevated urinary excretion of aluminium and iron in multiple sclerosis. Mult Scler. 2006;12(5):533–40.
37. Melendez L, et al. Aluminium and other metals may pose a risk to children with autism spectrum disorder: biochemical and behavioural impairments. Clin Exp Pharmacol. 2013;3(1):120. doi:10.4172/2161-1459.1000120.
38. Tomljenovic L, Shaw CA. Do aluminum vaccine adjuvants contribute to the rising prevalence of autism? J Inorg Biochem. 2011;105(11):1489–99. doi:10.1016/j.jinorgbio.2011.08.008.
39. Seneff S, et al. Empirical data confirm autism symptoms related to aluminum and acetaminophen exposure. Entropy. 2012;14:2227–53.
40. Redhead K, et al. Aluminium-adjuvanted vaccines transiently increase aluminium levels in murine brain tissue. Pharmacol Toxicol. 1992;70(4):278–80.
41. Khan Z, et al. Slow CCL2-dependent translocation of biopersistent particles from muscle to brain. BMC Med. 2013;11:99. doi:10.1186/1741-7015-11-99.
42. Lujan L, et al. Autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA syndrome) in commercial sheep. Immunol Res. 2013;56(2–3):317–24. doi:10.1007/s12026-013-8404-0.
43. Vasudevaraju P, et al. Molecular toxicity of aluminium in relation to neurodegeneration. Indian J Med Res. 2008;128(4):545–56.
44. Obulesu M, Rao DM. Animal models of Alzheimer's disease: an understanding of pathology and therapeutic avenues. Int J Neurosci. 2010;120(8):531–7. doi:10.3109/00207451003760080.
45. Kumar V, et al. Impairment of mitochondrial energy metabolism in different regions of rat brain following chronic exposure to aluminium. Brain Res. 2008;1232:94–103. doi:10.1016/j.brainres.2008.07.028.
46. Agarwal SK, et al. Evaluation of the developmental neuroendocrine and reproductive toxicology of aluminium. Food Chem Toxicol. 1996;34(1):49–53. doi:10.1016/0278-6915(95)00088-7.
47. Singla N, Dhawan DK. Regulatory role of zinc during aluminium-induced altered carbohydrate metabolism in rat brain. J Neurosci Res. 2012;90(3):698–705. doi:10.1002/jnr.22790.
48. Walton JR. Aluminum Disruption of calcium homeostasis and signal transduction resembles change that occurs in aging and

Alzheimer's disease. J Alzheimers Dis. 2012;29(2):255–73. doi:10.3233/JAD-2011-111712.
49. Shafer TJ, et al. Mechanisms underlying $AlCl_3$ inhibition of agonist-stimulated inositol phosphate accumulation. Role of calcium, G-proteins, phospholipase C and protein kinase C. Biochem Pharmacol. 1994;47(8):1417–25. doi:10.1016/0006-2952(94)90342-5.
50. Agmon-Levin N, et al. Immunization with hepatitis B vaccine accelerates SLE-like disease in a murine model. J Autoimmun. 2014;54:21–32. doi:10.1016/j.jaut.2014.06.006.
51. Kullenberg D, et al. Health effects of dietary phospholipids. Lipids Health Dis. 2012;11:3. doi:10.1186/1476-511X-11-3.
52. Yechiel E, Barenholz Y. Relationships between membrane lipid composition and biological properties of rat myocytes. Effects of aging and manipulation of lipid composition. J Biol Chem. 1985;260(16):9123–31.
53. Nicolson GL, Ash ME. Lipid replacement therapy: a natural medicine approach to replacing damaged lipids in cellular membranes and organelles and restoring function. Biochim Biophys Acta. 1838;6:1657–79. doi:10.1016/j.bbamem.2013.11.010.
54. Borella E, et al. Lipid replacement therapy: is it a new approach in patients with chronic fatigue syndrome? J Autoimmune Dis Rheumatol. 2014;2:28–34.
55. Corwin J, et al. Behavioral effects of phosphatidylserine in the aged Fischer 344 rat: amelioration of passive avoidance deficits without changes in psychomotor task performance. Neurobiol Aging. 1985;6(1):11–5.
56. Park HJ, et al. Enhanced learning and memory of normal young rats by repeated oral administration of Krill Phosphatidylserine. Nutr Neurosci. 2013;16(2):47–53. doi:10.1179/1476830512Y.0000000029.
57. Kanno T, et al. DL-/PO-phosphatidylcholine restores restraint stress-induced depression-related behaviors and spatial memory impairment. Behav Pharmacol. 2014;25(5–6):575–81. doi:10.1097/FBP.0000000000000063.
58. Baumeister J, et al. Influence of phosphatidylserine on cognitive performance and cortical activity after induced stress. Nutr Neurosci. 2008;11(3):103–10. doi:10.1179/147683008X301478.
59. Vakhapova V, et al. Phosphatidylserine containing omega-3 fatty acids may improve memory abilities in non-demented elderly with memory complaints: a double-blind placebo-controlled trial. Dement Geriatr Cogn Disord. 2010;29(5):467–74. doi:10.1159/000310330.
60. Kato-Kataoka A, et al. Soybean-derived phosphatidylserine improves memory function of the elderly Japanese subjects with memory complaints. J Clin Biochem Nutr. 2010;47(3):246–55. doi:10.3164/jcbn.10-62.
61. Manuel Y Keenoy B, et al. Antioxidant status and lipoprotein peroxidation in chronic fatigue syndrome. Life Sci. 2001;68(17):2037–49.
62. Pall ML. Elevated, sustained peroxynitrite levels as the cause of chronic fatigue syndrome. Med Hypotheses. 2000;54(1):115–25. doi:10.1054/mehy.1998.0825.
63. Richards RS, et al. Blood parameters indicative of oxidative stress are associated with symptom expression in chronic fatigue syndrome. Redox Rep. 2000;5(1):35–41. doi:10.1179/rer.2000.5.1.35.
64. Tayebati SK, Amenta F. Choline-containing phospholipids: relevance to brain functional pathways. Clin Chem Lab Med. 2013;51(3):513–21. doi:10.1515/cclm-2012-0559.
65. Delwaide PJ, et al. Double-blind randomized controlled study of phosphatidylserine in senile demented patients. Acta Neurol Scand. 1986;73(2):136–40.
66. Glade MJ, Smith K. Phosphatidylserine and the human brain. Nutrition. 2015;31(6):781–6. doi:10.1016/j.nut.2014.10.014.
67. Oliveira TG, et al. The impact of chronic stress on the rat brain lipidome. Mol Psychiatry. 2016;21(1):80–8. doi:10.1038/mp.2015.14.
68. Leon A, et al. Effect of brain cortex phospholipids on adenylate-cyclase activity of mouse brain. J Neurochem. 1978;30(1):23–6.
69. Casamenti F, et al. Effect of phosphatidylserine on acetylcholine output from the cerebral cortex of the rat. J Neurochem. 1979;32(2):529–33.
70. Casamenti F, et al. Phosphatidylserine reverses the age-dependent decrease in cortical acetylcholine release: a microdialysis study. Eur J Pharmacol. 1991;194(1):11–6.
71. Ali HA, et al. Quercetin and omega 3 ameliorate oxidative stress induced by aluminium chloride in the brain. J Mol Neurosci. 2014;53(4):654–60. doi:10.1007/s12031-014-0232-8.
72. Nicolson GL, Conklin KA. Reversing mitochondrial dysfunction, fatigue and the adverse effects of chemotherapy of metastatic disease by molecular replacement therapy. Clin Exp Metastasis. 2008;25(2):161–9. doi:10.1007/s10585-007-9129-z.

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Nov 28, 2016

This Agreement between MARIA ARANGO ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	3997950646876
License date	Nov 28, 2016
Licensed content publisher	Oxford University Press
Licensed content publication	Rheumatology
Licensed content title	Anti-ribosomal-phosphoprotein autoantibodies penetrate to neuronal cells via neuronal growth associated protein, affecting neuronal cells in vitro:
Licensed content author	Shaye Kivity, Yehuda Shoenfeld, Maria-Teresa Arango, Dolores J. Cahill, Sara Louise O'Kane, Margalit Zusev, Inna Slutsky, Michal Harel-Meir, Joab Chapman, Torsten Matthias, Miri Blank
Licensed content date	05/06/2016
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	Autoantibody and environmental damage to the brain
Publisher of your work	n/a
Expected publication date	Dec 2016
Permissions cost	0.00 USD
Value added tax	0.00 USD
Total	0.00 USD
Requestor Location	MARIA ARANGO Cr 24 n 63c-69 Bogota , 111221 Colombia Attn: MARIA ARANGO
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	MARIA ARANGO Cr 24 n 63c-69 Bogota , Colombia 111221 Attn: MARIA ARANGO
Total	0.00 USD
Terms and Conditions	

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.
6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com
7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.
8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.
9. This license is personal to you and may not be sublicensed, assigned or transferred by you to any other person without Oxford University Press's written permission.
10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
12. Other Terms and Conditions:
v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Copyright information

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 28, 2016

This Agreement between MARIA ARANGO ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	3997951441084
License date	Nov 28, 2016
Licensed Content Publisher	Elsevier
Licensed Content Publication	Journal of Autoimmunity
Licensed Content Title	Passive transfer of narcolepsy: Anti-TRIB2 autoantibody positive patient IgG causes hypothalamic orexin neuron loss and sleep attacks in mice
Licensed Content Author	Aviva Katzav,Maria T. Arango,Shaye Kivity,Susumu Tanaka,Gili Givaty,Nancy Agmon-Levin,Makoto Honda,Juan-Manuel Anaya,Joab Chapman,Yehuda Shoenfeld
Licensed Content Date	September 2013
Licensed Content Volume Number	45
Licensed Content Issue Number	n/a
Licensed Content Pages	7
Start Page	24
End Page	30
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	full article
Format	electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Autoantibody and environmental damage to the brain
Expected completion date	Dec 2016
Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Requestor Location	MARIA ARANGO Cr 24 n 63c-69 Bogota , 111221

Colombia
Attn: MARIA ARANGO

Total 0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all

claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.
Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors:** the following clauses are applicable in addition to the above:
Preprints:
A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).
Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of

articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).
If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- after the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0.

Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.8

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

SPRINGER LICENSE
TERMS AND CONDITIONS

Nov 28, 2016

This Agreement between MARIA ARANGO ("You") and Springer ("Springer") consists of your license details and the terms and conditions provided by Springer and Copyright Clearance Center.

License Number	3997960186893
License date	Nov 28, 2016
Licensed Content Publisher	Springer
Licensed Content Publication	Immunologic Research
Licensed Content Title	Behavioral abnormalities in female mice following administration of aluminum adjuvants and the human papillomavirus (HPV) vaccine Gardasil
Licensed Content Author	Rotem Inbar
Licensed Content Date	Jan 1, 2016
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	1
Author of this Springer article	Yes and you are a contributor of the new work
Order reference number	
Title of your thesis / dissertation	Autoantibody and environmental damage to the brain
Expected completion date	Dec 2016
Estimated size(pages)	100
Requestor Location	MARIA ARANGO Cr 24 n 63c-69 Bogota , 111221 Colombia Attn: MARIA ARANGO
Billing Type	Invoice
Billing Address	MARIA ARANGO Cr 24 n 63c-69 Bogota , Colombia 111221 Attn: MARIA ARANGO
Total	0.00 USD
Terms and Conditions	

Introduction
The publisher for this copyrighted material is Springer. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions

established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License

With reference to your request to reuse material on which Springer controls the copyright, permission is granted for the use indicated in your enquiry under the following conditions:

- Licenses are for one-time use only with a maximum distribution equal to the number stated in your request.
- Springer material represents original material which does not carry references to other sources. If the material in question appears with a credit to another source, this permission is not valid and authorization has to be obtained from the original copyright holder.
- This permission
• is non-exclusive
• is only valid if no personal rights, trademarks, or competitive products are infringed.
• explicitly excludes the right for derivatives.
- Springer does not supply original artwork or content.
- According to the format which you have selected, the following conditions apply accordingly:
• **Print and Electronic:** This License include use in electronic form provided it is password protected, on intranet, or CD-Rom/DVD or E-book/E-journal. It may not be republished in electronic open access.
• **Print:** This License excludes use in electronic form.
• **Electronic:** This License only pertains to use in electronic form provided it is password protected, on intranet, or CD-Rom/DVD or E-book/E-journal. It may not be republished in electronic open access.

For any electronic use not mentioned, please contact Springer at permissions.springer@spi-global.com.

- Although Springer controls the copyright to the material and is entitled to negotiate on rights, this license is only valid subject to courtesy information to the author (address is given in the article/chapter).
- If you are an STM Signatory or your work will be published by an STM Signatory and you are requesting to reuse figures/tables/illustrations or single text extracts, permission is granted according to STM Permissions Guidelines: http://www.stm-assoc.org/permissions-guidelines/

For any electronic use not mentioned in the Guidelines, please contact Springer at permissions.springer@spi-global.com. If you request to reuse more content than stipulated in the STM Permissions Guidelines, you will be charged a permission fee for the excess content.

Permission is valid upon payment of the fee as indicated in the licensing process. If permission is granted free of charge on this occasion, that does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

-If your request is for reuse in a Thesis, permission is granted free of charge under the following conditions:

This license is valid for one-time use only for the purpose of defending your thesis and with a maximum of 100 extra copies in paper. If the thesis is going to be published, permission needs to be reobtained.

- includes use in an electronic form, provided it is an author-created version of the thesis on his/her own website and his/her university's repository, including UMI (according to the definition on the Sherpa website: http://www.sherpa.ac.uk/romeo/);
- is subject to courtesy information to the co-author or corresponding author.

Geographic Rights: Scope

Licenses may be exercised anywhere in the world.

Altering/Modifying Material: Not Permitted

Figures, tables, and illustrations may be altered minimally to serve your work. You may not

alter or modify text in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s).

Reservation of Rights

Springer reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction and (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

License Contingent on Payment

While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Springer or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received by the date due, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Springer reserves the right to take any and all action to protect its copyright in the materials.

Copyright Notice: Disclaimer

You must include the following copyright and permission notice in connection with any reproduction of the licensed material:

"Springer book/journal title, chapter/article title, volume, year of publication, page, name(s) of author(s), (original copyright notice as given in the publication in which the material was originally published) "With permission of Springer"

In case of use of a graph or illustration, the caption of the graph or illustration must be included, as it is indicated in the original publication.

Warranties: None

Springer makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.

Indemnity

You hereby indemnify and agree to hold harmless Springer and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License

This license is personal to you and may not be sublicensed, assigned, or transferred by you without Springer's written permission.

No Amendment Except in Writing

This license may not be amended except in a writing signed by both parties (or, in the case of Springer, by CCC on Springer's behalf).

Objection to Contrary Terms

Springer hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction

All disputes that may arise in connection with this present License, or the breach thereof,

shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.

Other conditions:

V 12AUG2015

SPRINGER LICENSE
TERMS AND CONDITIONS

Nov 28, 2016

This Agreement between MARIA ARANGO ("You") and Springer ("Springer") consists of your license details and the terms and conditions provided by Springer and Copyright Clearance Center.

License Number	3997960357482
License date	Nov 28, 2016
Licensed Content Publisher	Springer
Licensed Content Publication	Immunologic Research
Licensed Content Title	Phospholipid supplementation can attenuate vaccine-induced depressive-like behavior in mice
Licensed Content Author	Shaye Kivity
Licensed Content Date	Jan 1, 2016
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	1
Author of this Springer article	Yes and you are a contributor of the new work
Order reference number	
Title of your thesis / dissertation	Autoantibody and environmental damage to the brain
Expected completion date	Dec 2016
Estimated size(pages)	100
Requestor Location	MARIA ARANGO Cr 24 n 63c-69 Bogota , 111221 Colombia Attn: MARIA ARANGO
Billing Type	Invoice
Billing Address	MARIA ARANGO Cr 24 n 63c-69 Bogota , Colombia 111221 Attn: MARIA ARANGO
Total	0.00 USD
Terms and Conditions	

Introduction
The publisher for this copyrighted material is Springer. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions

established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License

With reference to your request to reuse material on which Springer controls the copyright, permission is granted for the use indicated in your enquiry under the following conditions:

- Licenses are for one-time use only with a maximum distribution equal to the number stated in your request.
- Springer material represents original material which does not carry references to other sources. If the material in question appears with a credit to another source, this permission is not valid and authorization has to be obtained from the original copyright holder.
- This permission
• is non-exclusive
• is only valid if no personal rights, trademarks, or competitive products are infringed.
• explicitly excludes the right for derivatives.
- Springer does not supply original artwork or content.
- According to the format which you have selected, the following conditions apply accordingly:
• **Print and Electronic:** This License include use in electronic form provided it is password protected, on intranet, or CD-Rom/DVD or E-book/E-journal. It may not be republished in electronic open access.
• **Print:** This License excludes use in electronic form.
• **Electronic:** This License only pertains to use in electronic form provided it is password protected, on intranet, or CD-Rom/DVD or E-book/E-journal. It may not be republished in electronic open access.

For any electronic use not mentioned, please contact Springer at permissions.springer@spi-global.com.

- Although Springer controls the copyright to the material and is entitled to negotiate on rights, this license is only valid subject to courtesy information to the author (address is given in the article/chapter).
- If you are an STM Signatory or your work will be published by an STM Signatory and you are requesting to reuse figures/tables/illustrations or single text extracts, permission is granted according to STM Permissions Guidelines: http://www.stm-assoc.org/permissions-guidelines/

For any electronic use not mentioned in the Guidelines, please contact Springer at permissions.springer@spi-global.com. If you request to reuse more content than stipulated in the STM Permissions Guidelines, you will be charged a permission fee for the excess content.

Permission is valid upon payment of the fee as indicated in the licensing process. If permission is granted free of charge on this occasion, that does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

-If your request is for reuse in a Thesis, permission is granted free of charge under the following conditions:

This license is valid for one-time use only for the purpose of defending your thesis and with a maximum of 100 extra copies in paper. If the thesis is going to be published, permission needs to be reobtained.

- includes use in an electronic form, provided it is an author-created version of the thesis on his/her own website and his/her university's repository, including UMI (according to the definition on the Sherpa website: http://www.sherpa.ac.uk/romeo/);
- is subject to courtesy information to the co-author or corresponding author.

Geographic Rights: Scope

Licenses may be exercised anywhere in the world.

Altering/Modifying Material: Not Permitted

Figures, tables, and illustrations may be altered minimally to serve your work. You may not

alter or modify text in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s).

Reservation of Rights

Springer reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction and (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

License Contingent on Payment

While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Springer or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received by the date due, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Springer reserves the right to take any and all action to protect its copyright in the materials.

Copyright Notice: Disclaimer

You must include the following copyright and permission notice in connection with any reproduction of the licensed material:

"Springer book/journal title, chapter/article title, volume, year of publication, page, name(s) of author(s), (original copyright notice as given in the publication in which the material was originally published) "With permission of Springer"

In case of use of a graph or illustration, the caption of the graph or illustration must be included, as it is indicated in the original publication.

Warranties: None

Springer makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.

Indemnity

You hereby indemnify and agree to hold harmless Springer and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License

This license is personal to you and may not be sublicensed, assigned, or transferred by you without Springer's written permission.

No Amendment Except in Writing

This license may not be amended except in a writing signed by both parties (or, in the case of Springer, by CCC on Springer's behalf).

Objection to Contrary Terms

Springer hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction

All disputes that may arise in connection with this present License, or the breach thereof,

shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.

Other conditions:

V 12AUG2015