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Vaccines and Neuroinflammation

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Abstract

Background: Post-vaccination adverse reactions (AEs) are a reason of strong debate among scientists. Unfortunately, we often make the mistake of discussing just the epidemiology but not the molecular biology. The action mechanism of the vaccines is still not fully known despite the fact that aluminum adjuvants have been used for about 100 years.

Hypothesis: We hypothesized a link between vaccinations and neuroinflammation. The peripheral pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), expressed after the injection of the vaccines can reach the brain and can cause neuroinflammation after microglia activation. Elevated pro-inflammatory cytokines, particularly TNF-α, have been described in studies regarding the cytokines profile in autistic children. IL-1β represents a cytokine that controls the local pro-inflammatory cascade and thereby affects the balance between protective immunity and destructive inflammation. A subgroup of children with ASD (Autism Spectrum Disorder) has developed neuroinflammation. Several postmortem studies have confirmed the activation of microglia and neuroinflammation. A recent study has shown the presence of aluminum in the brain of individuals with autism and this aluminum was also found in microglia cells. Aluminum from vaccines is redistributed to numerous organs, including brain, where it accumulates. Each vaccine adds to this tissue different level of aluminum. Aluminum, like mercury, activates microglia leading to chronic brain inflammation and neurotoxicity.

Conclusion: The molecular mechanisms presented here demonstrate how peripheral cytokines, expressed after vaccination, can cause neuroinflammation in some subjects, after microglia activation, depending on the immunogenetic background and the innate immune memory.

Keywords: Neuroinflammation; Vaccines; Microglia activation; ASD (Autism Spectrum Disorder); HPV vaccine AEs; Immune innate memory; Vaccines and aluminum; Peripheral cytokines

Introduction

Vaccines are an important health policy tool and have changed the history of infectious diseases. In recent years, the number of vaccines inject to infants has increased, and many doses are administered during the first year of life, when the immune system and the central nervous system have yet to complete their development. Furthermore, the efficacy of the vaccination program is affected by the the possible presence of maternal antibodies in the infant, and by the degree of environmental chemical pollution. Some vaccines contain the specific antigen associated with aluminum, such as *Infanrix-Hexa* (Combined Diphtheria-Tetanus-acellular Pertussis, Hepatitis B, inactivated Poliovirus and *Haemophilus influenzae* type b Vaccine), MenC (Meningococcal conjugate vaccines), MenB (Serogroup B meningococcal vaccines), and pneumococcal conjugate vaccine (*Prevenar*). Aluminum performs the essential task of activating the innate immune system. Other vaccines contain alive virus, such as MMR (Measles, Mumps, Rubella), and MMRV (Measles, Mumps, Rubella, and Varicella), and do not contain aluminum. Each injection of vaccine, regardless of the type of vaccine, is followed by the production of variable amounts of pro-inflammatory cytokines, which exert both local effects and at a distance from the production site. Post-vaccination adverse events (AEs) are a reason of strong debate among scientists. Unfortunately, we often make the mistake of discussing just the epidemiology but not the molecular biology. The action mechanism of the vaccines is still not fully known despite the fact that aluminum adjuvants have been used for about 100 years.

Since the peripheral cytokines, produced after the injection of the vaccines, are able to reach the central nervous system, we hypothesize that these cytokines can have effects on the microglia (macrophages of the central nervous system), and that these effects can be facilitated by repeated vaccinations to infants during the first year of life. In this paper, we studied the molecular biology of vaccines and present the putative mechanisms that link the injection of vaccines to neuroinflammation, which can occur as the effect of microglial activation and its subsequent pro-inflammatory orientation (M1).

Aluminum

Since equating the aluminum introduced into the body with food and water (essentially extracellular) to the one injected with the vaccines (exclusively intracellular) is not a matter of science (because of the total diversity in kinetics and bio-dynamics), the Mitkus et al. [1] estimates are not consistent with the significant study of Priest [2], and with the others mentioned in this paper. Indeed, Mitkus et al. [1] state that the burden of aluminium accumulated in the body from vaccines and diet, throughout an infant's first year of life, is significantly less than the corresponding safe level of aluminium burden modeled using the regulatory MRL. They conclude by stating that: Those episodic exposures to vaccines that contain aluminum adjuvant continue to be extremely low risk to infants and that the benefits of using vaccines

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containing aluminum adjuvant outweigh any theoretical concerns".

Unfortunately, many studies published on the hypothetical safety of aluminum injected with vaccines are not conclusive, and there are not randomized controlled trials (RCTs) on the safety of aluminum injected with vaccines. No significant change in levels of urinary or serum aluminum were seen after vaccination of preterm infants with vaccines containing a total of 1200 μg of aluminum [3]. Also this study confirms that the aluminum injected with the vaccines is not found in the serum of the vaccinated subjects, but does not show that the vaccine aluminum is safe. Mateusz et al. [4] have shown that infant blood-aluminum and hair-aluminum varied considerably but did not correlate with their immunization history. The aluminum injected with the vaccines cannot correlate with that of the blood and/or hair because it is not found free in the blood, as repeatedly said.

In 2018, it should already be quite clear that the aluminum injected with the vaccines cannot be measured in the serum because it is only found in the cells of the monocyte/macrophage lineage. Besides, intramuscular injection of alum-containing vaccine in mice was associated with the appearance of aluminium deposits in distant organs, such as spleen and brain, where they were still detected one year after the injection [5]. Nanomaterials can be transported by monocyte-lineage cells to draining lymph nodes, blood and spleen, and, similarly to HIV, may use CCL2-dependent mechanisms to penetrate the brain. In normal conditions, this occurs at a very low rate, thus explaining good overall tolerance of alum despite its strong neurotoxic potential. However, continuously escalating doses of this poorly biodegradable adjuvant in the population may become insidiously unsafe, especially in the case of over immunization or immature/altered blood brain barrier or high constitutive CCL-2 production [5]. Aluminum oxyhydroxide (Alhydrogel®) is a nano-crystalline compound forming aggregates that has been introduced in vaccine for its immunologic adjuvant effect in 1926. Although generally well tolerated on the short term, it has been suspected to occasionally cause delayed neurologic problems in susceptible individuals [6]. Concerns linked to the use of alum particles emerged following the recognition of their causative role in the so-called macrophagic myofasciitis (MMF), lesion detected in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). MMF revealed an unexpectedly long-lasting biopersistence of alum within immune cells in presumably susceptible individuals, thus stressing the fundamental misconception of its biodisposition [7].

With regard to aluminium introduced with food and water, available studies indicate that the oral bioavailability of aluminium in humans and experimental animals from drinking water is in the range of 0.3%, whereas the bioavailability of aluminium from food and beverages generally is considered to be lower, about 0.1% [8]. The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) for aluminium of 1 mg/kg body weight in 2006 [8]. Five years later, the committee re-evaluated the safety of aluminium and proposed a PTWI of 2 mg/kg body weight in June 2011. The PTWI applies to all aluminium compounds in food, including food additives [9]. Based on the maximum reported concentration of aluminium in breast milk, exposures of exclusively breastfed infants may be up to 6% of the PTWI of 2 mg/kg bw (2,000 μg/kg bw), with the highest exposure in high level consumers aged 0-3 months. In infants fed exclusively with ready-to-consume formulae, exposures to aluminium may be 4% of the PTWI. In those fed exclusively with powdered formulae, the exposure to aluminium could be up to 8, 11 and 21% of the PTWI, respectively from cows' milk, goats' milk and soya based products. In addition, the water used to reconstitute the infant formula could give further exposure of up to some 248 μg/kg bw/week aluminium (12% of the PTWI), resulting in total exposure of up to 33% of the PTWI.

The 33% of 2,000 μg/kg bw is 660 μg/kg bw. Since the absorption of aluminum from food is low, generally 0.5% or less [10], 0.5% of 660 μg/kg bw is equal to 3.30 μg/kg bw per week. As a year to 52 weeks, the total amount of aluminum theoretically absorbed in the first year of life should be 171.6 μg/kg (52 × 3.30 μg/kg). Moreover, assuming that at one year of life the weight of a child is on average 10 kg, it should have absorbed totally 1,716 μg of aluminum (Table 1).

As shown by the data presented in Table 1, the amount of aluminum injected in the first year of life, for Italian infants, is 2.52 times greater, compared to the maximum amount absorbed at one year from the diet [11-13]. However, most aluminium that enters the blood is excreted in urine within a few days or weeks and the gastrointestinal tract provides an effective barrier to aluminium uptake [2], while the aluminum administered with the vaccines is internalized by the cells of the monocyte/macrophage lineage, and for this reason it is intracellular and cannot be eliminated by the kidney.

All adjuvants modulated a common set of 168 genes and promoted antigen-presenting cell recruitment. Alum regulated 312 genes [14]. A number of *in vitro* experiments [15] have shown that alum activates the NLRP3 inflammasome in macrophages which, in turn, activates caspase-1 and consequent production of interleukin- (IL-) 1β (IL-1β). Upon activation, members of the Nod-Like Receptors (NLR) family, such as NLRP3, form complexes with ASC and pro-caspase-1. The complex formed by these molecules is referred to as the inflammasome. The NLRP3 inflammasome is activated by a number of materials, including alum. Whatever the cause of inflammasome activation, the consequences include production of active caspase-1 thus conversion of inactive precursor cytokines of the IL-1 family, including IL-1β, IL-18 and IL-33, to their active forms [16].

In summary, the aluminum salts injected as vaccine adjuvants are taken by the innate immunity cells (especially from dendritic cells), they engage a receptor called NLR (NLRP3), which together with other proteins is organized into an intracellular macromolecular complex that activates the enzyme caspase-1. This enzyme converts pro-IL-1β and pro-IL-18 into their active forms (IL-1β, and IL-18). The role of caspase-1 is not limited to the conversion of pro IL-1β to IL-1β alone, but it strongly affects the secretion of proinflammatory cytokines: IL-1β, IL-1α, IL-6, TNF-α, IL-18 and IFN-γ. IL-1 is a primary regulator of inflammatory and immune responses. *Via* its type I receptor it activates specific protein kinases, including the nuclear factor kappa-light-chain-enhancer (NF-κB) inducing kinase (NIK) and three distinct mitogen-activated protein (MAP) kinase cascades. These modulate a number of transcription factors including NF-κB, AP1 and CREB, each of which regulate a plethora of immediate early genes central to the inflammatory response [17]. Therefore, each injection of the vaccine produces a pro-inflammatory response. An immune response to the vaccine antigens

Vaccines	Aluminum	Doses	Total
Bexero [11]	500 μg	3	1,500 μg
Prevenar 13 [12]	125 μg	3	375 μg
Infanrix-Hexa [13]	820 μg	3	2,460 μg
Total aluminum inject	-	-	4,335 μg
Maximum amount absorbed at one year	For infant with 10 kg of body weight		1,716 μg

Table 1: Aluminum in vaccines (Italian schedula) and diet.

(to the quantities currently present in the vaccines), is non-possible without a pro-inflammatory response, which is produced by adjuvants.

Vaccines

Vaccines have drastically reduced infant death and disability caused by preventable diseases in the United States [18]. However, some vaccines may not achieve the desired goal, or they can cause serious AEs that alter the benefit-risk balance by shifting the balance in the direction of risk.

Vaccine 4CMenB (Bexero)

This vaccine has two major problems: First, it is strongly reactogenic; secondly, it provides little individual and collective long-term protection. The incidence of potentially vaccine-related, acute serious AEs in individuals receiving 4CMenB was low (5.4 per 1000 individuals), but was significantly higher than routine vaccines (1.2 per 1000 individuals). Long-term immunogenicity against strain NZ98/254 (Bexero) remains suboptimal [19]. Soeters et al. [20] have investigated MenB-FHbp impact on meningococcal carriage. Carriage prevalence on campus remained stable, suggesting MenB-FHbp does not rapidly reduce meningococcal carriage or prevent serogroup B carriage acquisition. In a university setting, the majority of meningococcal carriage was due to nongroupable strains, followed by serogroup B [21]. MenB-FHbp and MenB-4C do not have a large, rapid impact on meningococcal carriage and are unlikely to provide herd protection in the context of an outbreak response [22].

Human papillomavirus vaccines (HPV Vaccines)

HPV vaccines are neither safe nor effective as claimed by so much scientific literature. These vaccines are anti-virus vaccines, but they are not anti-tumor vaccines. In fact, they can prevent the infection (even if it is not always so), that before producing precancerous lesions (CIN2/3), it must become persistent. There are 3 licensed HPV vaccines (Gardasil 4, Gardasil 9 and Cervarix). All HPV vaccines are virus-like particles (VLPs) based on the major HPV capsid protein L1. Antigenically the vaccines are very similar but they are produced in different systems and contain different adjuvants. The Gardasil® vaccine is adjuvanted with aluminum hydroxyphosphate sulfate. The Cervarix® vaccine is formulated with AS04, which contains aluminum hydroxide salts and the Toll-Like Receptor 4 (TLR4) agonist MPL (3-O-desacyl-4'-monophosphoryl lipid A). Elevated levels of circulating plasma cytokine/chemokines were observed post first vaccination in Gardasil® recipients and proinflammatory cytokines were elevated following 1st and 3rd Cervarix® vaccinations [23]. The new Cochrane Review [24] on the HPV vaccine for cervical cancer prevention in girls and women, included studies that were not truly randomized, double-blind, placebo-controlled studies (RCTs) because in several paper they labelled aluminium salts as a placebo. As previously reported, aluminium regulates 312 genes [14] and for this reason it is not a placebo. This can be considered a scientific oxymoron.

In the VRBPAC Background Document [25] two important concerns were identified during the course of the efficacy review of this BLA. One was the potential for Gardasil™ to enhance disease among a subgroup of subjects who had evidence of persistent infection with vaccine-relevant HPV types at baseline. The other concern was the observations of CIN 2/3 or worse cases due to HPV types not contained in the vaccine. The results of exploratory subgroup analyses for study 013 suggested a concern that subjects who were seropositive and PCR-positive for the vaccine-relevant HPV types had a greater number of CIN 2/3 or worse cases [25]. Fischer et al. [26] have hypothesized that there may be a continuous change in the prevalence of HPV types following vaccination. Vaccinated young women had a higher prevalence of high-risk non-vaccine types [27]. Ultimately, the vaccination can induce evolutionary responses of viruses to vaccines, and they may appear several years after the introduction of such control measures.

Post-vaccination AEs can be added to this complicated scenario. In Japan, the period of HPV vaccination overlapped with that of the development of HPV vaccine-related symptoms in the vaccinated patients, including chronic regional pain syndrome (CRPS) and autonomic and cognitive dysfunctions. Moreover, 28 months have passed since the recommendation for HPV vaccination was withdrawn, and new HPV vaccine-related symptoms have not been observed during 14-month follow-up period. The sequence of these events suggests that HPV vaccination is temporally related to the development of these symptoms in Japanese adolescent girls [28].

Post-vaccination inflammatory syndrome: A new syndrome?

Giannotta [29] hypothesized that several vaccine AEs may be determined by the excessive production of proinflammatory cytokines, determined by the injection of these vaccines. He hypothesized that these post-vaccination reactions fall into the ASIA syndrome, but represent a sub-group of clinical syndromes determined by the excessive expression and secretion of pro-inflammatory cytokines. To elaborate this hypothesis, he started from two considerations: 1- all the girls affected by important adverse reactions, all around the world, experience an almost identical neurological symptomatology after the injection of the vaccines; 2 - if these AEs are attributable to vaccines it is necessary to understand how the vaccines is able to produce symptoms, essentially neurological in nature. In 2013, a number of safety signals arose for HPV vaccines: CRPS in Japan, postural orthostatic tachycardia syndrome (POTS) in Denmark, and long-lasting fatigue in the Netherlands [30-32]. The European Medicines Agency (EMA) reported a review of the safety concerns of POTS and CRPS in November 2015. The conclusion was that the current evidence does not suggest a causal association between HPV vaccines and POTS or CRPS [33]. Anyway, high levels of circulating plasma cytokine/chemokines were observed in post-vaccination time with HPV vaccines [23].

Once you have arrived at this point, the question arises: Can a peripheral immune stimulation produce a central nervous system response? Actually, there is a natural condition that certifies its existence: Sick behavior. Elevated levels of proinflammatory cytokines drive most if not all aspects of the sickness response either directly or indirectly [34]. Proinflammatory cytokines, including IL-1, IL-6, and tumor necrosis factor-α (TNF-α) activate the production and/or release of secondary inflammatory mediators [35], such as prostaglandins (PGs) and nitric oxide (NO). Proinflammatory cytokines directly stimulate numerous neurohormonal systems. Proinflammatory cytokines can directly interact with microglia and astrocytes in the glia limitans. Once microglia are activated, astrocytes are recruited leading to further activation of neuroinflammatory signals [36].

Having verified that peripheral cytokines can produce microglial activation, only the putative link between some AEs and the cytokines released after injection of these vaccines remains.

CRPS type I in Japan

Every pain syndrome has an inflammatory profile consisting of the inflammatory mediators that are present in the pain syndrome. The biochemical mediators of inflammation include cytokines, neuropeptides, growth factors and neurotransmitters. Irrespective

of the type of pain whether it is acute or chronic pain, peripheral or central pain, nociceptive or neuropathic pain, the underlying origin is inflammation and the inflammatory response [37]. Table 2 lists a number of neurological syndromes (painful and painless) and their relationship to proinflammatory cytokines.

The immune system *via* peripheral and central release of pro-inflammatory cytokines contributes significantly to pain modulation [38]. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α were involved in the process of pathological pain [39] IL-1β was one of the first cytokines to be implicated in peripheral nerve injury-induced neuropathic pain mechanisms in rodents, and TNF-α is critical for the development of neuropathic pain [40]. For instance, nociceptors are known to be IL-1β sensors and IL-1β can directly activate nociceptors to generate action potentials and induce hyperalgesia [41]. CRPS describes an array of painful conditions (nociceptive pain in CRPS type I) that are characterized by a continuing (spontaneous and/or evoked) limb pain that is seemingly disproportionate in time or degree to the usual course of any known trauma or other lesion. The pain is regional (not in a specific nerve territory or dermatome) and usually has a distal predominance [42]. Symptoms of CRPS-I include spontaneous pain ("burning" pain referred to the skin, and "aching" pain referred to deep tissues), and a variety of stimulus-evoked abnormal pain sensations, including mechano-hyperalgesia, mechano-allodynia, cold-allodynia and sometimes heat-hyperalgesia. Other symptoms include disorders of vasomotor and sudomotor regulation; trophic changes in skin, hair, nails, and bone; and dystonia and other motor abnormalities [43]. Thus, the most prominent mechanism appears to be the inflammatory process because all the classic signs of inflammation (oedema, redness, hyperthermia, and impaired function) are conspicuous in the early stages of CRPS [44].

High levels of the proinflammatory cytokines (TNF-*α* and IL-6) have been found in skin blister fluid of the affected limbs versus the unaffected limbs of CRPS patients [45]. In patients with CRPS, the levels of IL-1β and IL-6 were significantly increased in cerebrospinal fluid (CSF), compared to other subjects [46,47]. In the blood of subjects with painful neuropathy, TNF-α levels were doubled, compared to healthy subjects and those with non-painful neuropathy [48]. IL-1β can modulate the transmission of sensory neurons because it increases the release of substance P [49,50].

Thus, CRPS type I is associated with high levels of IL-1β and IL-6 in CSF, and high levels of TNF-α in the blood. Furthermore, these proinflammatory cytokines are strongly expressed after the injection of HPV vaccines (Figure 1). It is now evident that the pro-inflammatory response to the injection of the vaccine is identical, under the common citokines substrate, to the inflammatory profile of the CRPS type I. Certainly, individual predisposition and other possible interfering factors have determined who should get sick and who did not, while expressing (both categories of subjects) high levels of proinflammatory cytokines after injection of HPV vaccines.

Neurologic clinic syndrome	Pro-inflammatory cytokines				
	IL-1β	TNF-α	IL-6	IFN-γ	IL-8
Pathological pain [39]	Yes	Yes	Yes	Yes	-
Peripheral neuropatic pain [40]	Yes	Yes	-	-	-
Hyperalgesia [41]	Yes	-	-	-	-
CRPS [46-48]	Yes	Yes	Yes	-	Yes
Chronic fatigue [55,56]	Yes	Yes	Yes	-	-
CFS/ME [55,56]	Yes	Yes	-	-	-
POTS [54]	-	-	Yes	-	-

Table 2: Neurologic clinic syndrome and pro-inflammatory cytokines.

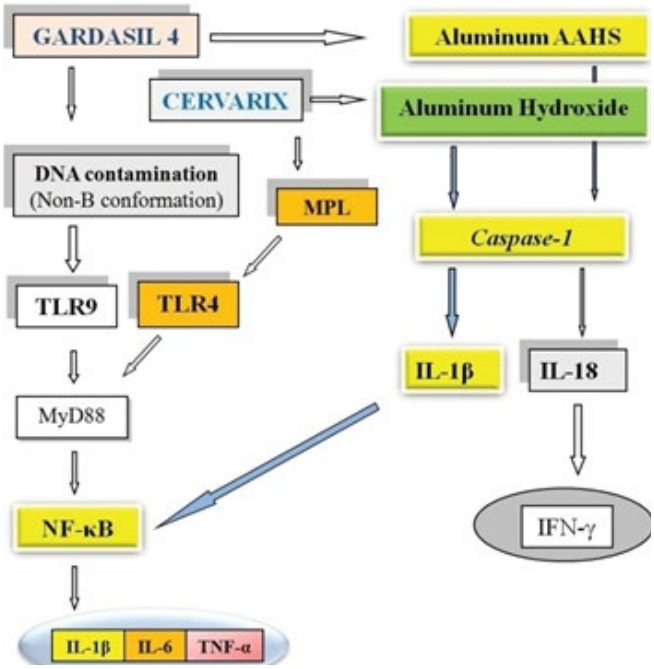


Figure 1: Gardasil and Cervarix vaccines contain aluminum, which activates caspase-1 enzyme, *via* NLRP3 inflammasome. The caspase-1 enzyme converts the pro-interleukins 1β and 18 in their active forms. IL-18 determines the production of IFN-γ. IL-1β represents a cytokine that controls the local pro-inflammatory cascade and contributes to activate the transcription factor NF-κB. The Cervarix adjuvant AS04 contains Aluminum Hydroxide and MPL. The second one stimulates the TLR4. Gardasil 4 vaccine is contaminated with foreign DNA in non-B conformation [145], which activates TLR9. TLRs act through the adapter protein MyD88 that acts by increasing the activity of NF-κB, which then increases the expression and secretion of IL-1β, IL-6 and TNF-α.

POTS in Denmark

A number of safety signals, CRPS, POTS, and chronic fatigue syndrome (CFS), have emerged with HPV vaccines, which share a similar pattern of symptomatology [51]. POTS is a heterogeneous disorder of the autonomic nervous system [52] in which a change from the supine position to an upright position causes an abnormally large increase in heart rate or tachycardia (30 bpm within 10 minutes of standing or head-up tilt). Brinth et al. [53] report the characteristics of a number of patients with a syndrome of orthostatic intolerance, headache, fatigue, cognitive dysfunction, and neuropathic pain starting in close relation to HPV vaccination. Furthermore, the diagnosis CFS/ME may be suitable in patients with suspected side effects to the Q-HPV vaccine [31]. Sympathetic activation and parasympathetic withdrawal in POTS patients were associated with increased serum IL-6 [54].

Long-lasting fatigue in the Netherlands

Lareb [32] has received a substantial number of reports concerning long-lasting AEs after vaccination with Cervarix®. The most frequently reported long-lasting AE was fatigue. The relation between elevated proinflammatory cytokines and fatigue and fatiguability is well documented [55]. In patients with CFS/ME, proinflammatory cytokines, including IL-1β and TNF-α, are elevated and are significantly associated with the severity of fatigue, a flu-like malaise, sadness and

autonomic symptoms [55]. Furthermore, other authors have found high levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, in CFS/ME patients [56]. IL-1β, IL-6 and TNF-α are strongly expressed after the injection of HPV vaccines, and in patients with CFS/ME, the same proinflammatory cytokines are elevated [55,56].

Microglia

It is now accepted that microglia are derived from mesodermal/mesenchymal tissues, primarily myeloid cells from the bone marrow. These cells migrate to the Central Nervous System (CNS) during the first trimester of pregnancy and throughout the early part of the second trimester in humans [57]. A recent study by Paolicelli et al. [58] demonstrated a central role of microglia in synaptic pruning and circuit development in the developing embryonic brain. Microglia cells are heterogenous in their distribution [59]. Microglia cells along brain–blood vessels are often found in an activated state and form a particular immunological barrier for the brain in conjunction with the blood–brain barrier (BBB). Microglia are also concentrated in sites of incomplete BBB function, such as the circumventricular organs (CVOs), or the organum vasculosum of the lamina terminalis, subcommissural organ, subfornical organ, area postrema, posterior pituitary, median eminence, pineal, and choroid plexus, as these are entry sites of blood-borne invaders and even larger molecules [60]. Microglia contain receptors for a number of cytokines, both proinflammatory and antiinflammatory. One of the critical types of cytokine receptors for microglia activation is the IL-1β receptors, which includes the subtypes IL1RI, IL-1RII, and IL-RIII [61]. IL-1β activates brain microglia. The BBB has an energy-dependent, saturable, carrier-mediated transport system for cytokines, primarily IL-1, IL-6, and TNF-α [62,63]. When endothelial cells making up the BBB come into contact with these peripheral cytokines, they secrete various immune molecules into the brain parenchyma, including NO, prostaglandin E2, IL-1, and IL-6, all proinflammatory cytokines known to affect neurological function [64]. It is now accepted that peripheral inflammation and immune activation secondarily effect brain function during the infectious process [65]. Microglial activation is quite rapid following systemic immune activation, usually within minutes and results in immunoexcitotoxicity. This secondary immune process has been named sickness behavior and is characterized by anorexia, hypersomnia, lethargy, reduced social interaction, reduced cognitive function, and weakness.

Microglia primed

Microglia are very long-living cells [66,67]. Microglia can switch from a resting phenotype to a primed state by an initial immune stimulus that is not excessively intense. For example, a mild head injury or episode of hypoxia can switch microglia from its resting state to a functional condition in which the enzymes and genetic activation is up-regulated, but the active immune molecules, primarily proinflammatory cytokines and chemokines, are not released [57]. NADPH oxidase is essential for microglial priming. NADPH oxidase primarily produces oxygen radicals and inducible nitric oxide synthase (iNOS) generates nitrogen radicals, which when combined forms the very powerful reactive nitrogen species (RNS) peroxynitrite [68].

With a second immune stimulus, these primed microglia began to release proinflammatory cytokines and chemokines in much higher concentrations than that of not primed microglia. Systemic immune stimulation can prime brain microglia, which means that either subsequent brain disturbances or systemic immune activation would trigger a magnified immune response within the brain. Immune events throughout life, exposure to neurotoxic metals, exposure to pesticides/herbicides and fungicides, head injury, and other factors, can cause episodes associated with microglia priming and activation, leading to a progressive loss of neurons in the most vulnerable parts of the CNS, such as the hypothalamus, temporal lobes (hippocampus, striatal area, amygdala, and entorhinal cortex) and prefrontal cortex [57].

In the infant or small child, the priming event may come from a number of sources, such as vaccination of the mother during pregnancy or with intrauterine or early post birth infections [69,70]. In other instances, the priming event may occur with the first vaccine inoculation, usually at birth (hepatitis B). Once primed, subsequent vaccinations, especially within months of the previous inoculation, will trigger full microglial activation and in the developing brain can result in abnormal pathways development [71-74]. While natural infections can also produce this neurodestructive response, vaccinations produce higher levels of immune activation and the immune response can persist longer than natural infections – sometimes lasting years [57].

It is well-established that inflammation in the periphery can prompt immune responses in the brain [75]. Contrary to the long-held assumption that immunological memory exists only in cells of the adaptive immune system, recent evidence has indicated that also myeloid cells display memory effects [76,77]. For example, certain immune stimuli train blood monocytes to generate enhanced immune responses to subsequent immune insults [78,79]. By contrast, other stimuli induce immune tolerance/suppression of inflammatory responses to subsequent stimuli [78,80]. Innate immune memory lasts for several days *in vitro* and for up to three months in circulating monocytes *in vivo* and is mediated by epigenetic reprogramming in cultured cells, with chromatin changes also apparent *in vivo* [78,81,82]. However, while training may be beneficial in the periphery, owing to enhanced pathogen elimination [82-84], and tolerance may be detrimental owing to higher rates of infection resulting from immune suppression [80], training promotes, while tolerance alleviates neuropathology [85].

In summary, innate immune memory is a vital mechanism of myeloid cell plasticity that occurs in response to environmental stimuli and alters subsequent immune responses. Two types of immunological imprinting can be distinguished, training and tolerance. These are epigenetically mediated and enhance or suppress subsequent inflammation, respectively. Peripherally applied inflammatory stimuli induce acute immune training and tolerance in the brain and lead to the differential epigenetic reprogramming of microglia that persists for at least six months. Individual cytokines applied peripherally may also elicit immune memory effects in the brain [85].

Autism Spectrum Disorders (ASD) and Neuroinflammation

ASD is a pervasive neurodevelopmental condition characterized by variable impairments in communication and social interaction as well as restricted interests and repetitive behaviors. The latest estimate of the prevalence of autism in the US refers to those born in 2006 [86], for whom the overall prevalence of ASD was 16.8 per 1,000 (one out of 59). In recent years, many studies indicate that children with an ASD diagnosis have brain pathology suggestive of ongoing neuroinflammation or encephalitis in different regions of their brains. Evidence of neuroinflammation or encephalitis in ASD includes: Microglial and astrocytic activation, a unique and elevated proinflammatory profile of cytokines, and aberrant expression of NF-κB of activated B cells. A conservative estimate based on the research suggests that at least 69% of individuals with an ASD diagnosis have microglial activation or neuroinflammation [87]. For a subgroup of children, parents report that their child had normal or near-normal

development for their first 15 to 24 months and then experienced a regression in their communication skills and/or social skills. Estimates of the prevalence of this "regressive" pattern range from 20% to 47% among children with autism [88-94].

Investigations of immune system problems in ASD, including aberrations in cytokine profiles and signaling, have been increasing in recent times and are the subject of ongoing interest [95]. The immune system and the nervous system are intricately interconnected. The functional status of the immune system affects a multitude of biological processes, including brain function and development, which can be affected when the innate and adaptive immune responses are dysregulated [96]. In addition, a systematic review evaluating pro-inflammatory markers in almost 4,000 children and adolescents with neuropsychiatric and neurodevelopmental disorders, including ASD, identified preliminary evidence of the role of inflammation in these conditions and an association with a pro-inflammatory state [97]. Altered cytokine profiles have been consistently linked to ASD in children in the postnatal period [98]. Cytokines may influence behavior through effects on neurotransmitter function, neuroendocrine activity, neurogenesis, and alterations to brain circuitry [99]. For example, cytokines have shown to increase release and decrease reuptake of the excitatory neurotransmitter glutamate, which can result in the pathological process of excitotoxicity [100]. This evidence for abnormal cytokine profiles in ASD suggests that immune system disturbances may be active and continuous contributors to the presentation of ASD. This accumulation of evidences has acted as the catalyst for efforts to characterize possible subgroups of ASD patients who are presented with immune system abnormalities or dysfunction and altered patterns of symptom presentation [101,102].

Peripheral cytokine signals are thought to access the brain through three pathways: Humoral (with antibody involvement), neural, and cellular [95,99]. These communication pathways involve at least five mechanisms: (1) passage of cytokines through leaky regions of the blood-brain barrier; (2) active transport *via* saturable cytokine-specific transport molecules on brain endothelium; (3) activation of endothelial cells, which release second messengers within the brain parenchyma; (4) transmission of cytokine signals *via* afferent nerve fibers, including the vagus; and (5) entry into the brain parenchyma of peripherally-activated monocytes which release cytokines. An alternate communication pathway has recently been proposed. It is based on the groundbreaking work by Louveau and colleagues [103] who identified functional lymphatic vessels, in the CNS, that carry fluid and immune cells from the cerebrospinal fluid, and in doing so, they discovered a pathway for immune cells to exit the CNS.

The entry of peripheral cytokines into the brain determines different effects.The brain recognizes cytokines such as the pro-inflammatory cytokines IL-1α, IL-1β, TNF-α, and IL-6 as molecular signals of sickness [104]. Furthermore, TNF-α, IL-6, and IL-1β can cross the blood-brain barrier and act on the hypothalamus where they promote fever and sickness behavior [105]. Elevated IL-1β and IL-6 have been associated with increased stereotypical behaviors [106]. Dysregulation of IL-1β, a pro-inflammatory cytokine expressed early in an immune response, is implicated in impairments in memory and learning [107]. IL-1β induces and inhibits neural progenitor cell proliferation in the CNS, which can contribute to region-specific deviant brain growth in ASD [108]. Children and adults with autism have increased plasma IL-1β levels [109-111]. Compared to monocytes of control subjects, monocytes from subjects with ASD produce excessive amounts of IL-1β after exposure to lipopolysaccharides [112,113]. IL-1β induces proliferation of neural progenitor in some brain areas, while inhibiting it in others [114]. This ability may contribute to the genesis of the observed areas of excessive growth and reduced growth in the brains of individuals with autism [107]. IL-1 is involved in the most sophisticated brain processes, its induction occurs in the hippocampus during learning processes and is essential to maintain long-term potentiation (LTP), but at higher doses, as encountered in pathological conditions, IL-1 inhibits LTP [115]. Both hypersecretion and reduced secretion of IL-1β are associated with impaired memory and language [116-118].

In summary, IL-1β participates in neurological processes and plays key role in the pathology and healing of the central nervous system. Normal levels of IL-1β and its IL-1ra receptor antagonist are necessary to achieve normal development and normal brain function.

TNF-α is a central regulator of inflammation and is elevated in the cerebrospinal fluid of children with ASD [119].

High level of IL-6 in ASD, both centrally and peripherally, has been frequently reported [106,120,121]. IL-6 is typically regarded as a pro-inflammatory cytokine and has been identified as a cytokine the brain recognizes as a molecular signal of sickness [104]. However, it also has regenerative or anti-inflammatory activity, and is involved in the regulation of metabolic and neural processes [122]. In a mouse model with elevated IL-6 in the brain, Wei and colleagues [123] have shown that IL-6 can modulate autism-like behaviors through impairments of synapse formation, dendritic spine development, and neuronal circuit balance. Immunocytochemical studies have identified marked activation of microglia and astroglia associated with the increased production of two cytokines by neuroglia, macrophage chemoattractant protein (MCP)-1, and TGF-β1 [124]. In addition, a unique profile of pro-inflammatory cytokines has been identified in cerebrospinal fluid [124]. Another postmortem study demonstrated also significant increases in pro-inflammatory and Th1 cytokines relative to matched controls [120].

Altogether, ASD is recognized as having an inflammatory component. There is an association between ASD and neuroinflammation in anterior regions of the neocortex [124-126] resulting from activation of microglia and astrocytes [127]. Gene networks involved in immune processes are overexpressed in the brain of individuals with ASD [128,129]. Postmortem brain samples from patients with ASD display neuroglial activation and inflammatory markers in cerebrospinal fluid.

NF-κB mediates regulation of immune response by inducing the expression of inflammatory cytokines and chemokines, establishing a feedback mechanism that can produce chronic or excessive inflammation. NF-κB is aberrantly expressed in orbitofrontal cortex in patients with ASD, as part of a putative molecular cascade leading to inflammation, especially of resident immune cells in brain regions associated with the behavioral and clinical symptoms of ASD [130]. The implication of the NF-κB signalling pathway in ASD further supports a potential role for neuroinflammation [131]. Immune pathways are activated by proinflammatory cytokines such as TNF-α and IL-6 that stimulate the nuclear translocation of various transcription factors, including NF-κB that subsequently results in the potentiation of the immune response [132]. Cytokines, chemokines, and reactive oxygen species are among a number of key mediators that induce NF-κB by activating IκB kinases [133]. These phosphorylate IκBα, leading to its poly ubiquitination and degradation [134], allows NF-κB to migrate to the nucleus, where it activates the transcription of various proinflammatory genes.

Aberrant levels of proinflammatory cytokines (IL-6, TNF-α, and

MCP-1), not only in brain specimens and cerebrospinal fluid [135], but also in amniotic fluid [136], indicate an active inflammatory process both in children and adults with ASD. Cytokines and chemokines are pleiotrophic proteins that coordinate the host response to infection as well as mediate normal, ongoing communication between cells of non-immune tissues, including the nervous system [137]. As a consequence of this dual role, cytokines induced in response to adverse stimuli (i.e. maternal infection or prenatal hypoxia) can profoundly impact fetal neurodevelopment. Microglia play a critical role in the pruning of synapses, thus providing a potential bridge between the atypical synaptic pruning and the immune dysregulatory hypotheses of ASD [138].

Ultimately, the pro-inflammatory cytokines produced by the activation of the peripheral immune system, also determine effects in central nervous system and in many cases of ASD there is evidence of neuroinflammation. Pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, appear to be at the forefront in the communication between the immune and the nervous system, playing dual roles in mediating physiological and neuroprotective roles in normal brain function or being detrimental and associated with brain diseases, especially when present at elevated concentrations [139].

Since in the first year of life, in the industrialized countries, the immune system is activated more by the incoming number of vaccinations than by the number of infections, we must begin to think that the pro-inflammatory cytokines released after vaccine injections can produce microglia activation which can cause neuroinflammation.

Discussion

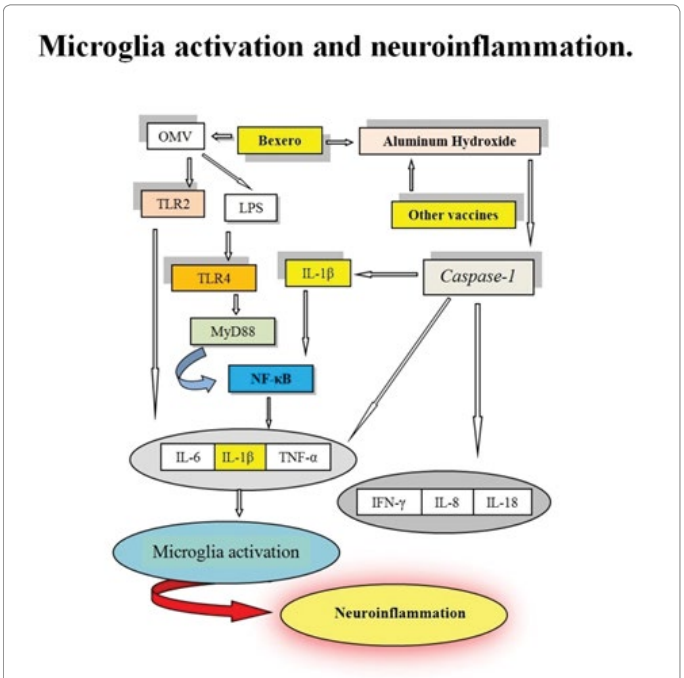
Cytokines, together with neurotransmitters and hormones, are signaling molecules possessing unique immunomodulatory functions. Virtually, they can influence every physiological system including neuroendocrine interactions, neurotransmitter metabolism and neuroplasticity, thereby affecting behavioral and cognitive functioning [140]. Cytokines take center stage in orchestrating immune responses [141]. Injection of the vaccines results in a strong expression of pro-inflammatory cytokines. Macrophages secrete pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, when activated. It seems that variations in vaccine-induced cytokine responses are modulated by genetic polymorphisms in many cytokine and cytokine receptor genes [142]. Cytokines are cell-to-cell messangers similar to hormones with the stronger activity in the microenvironmental of the cells that secrete them [143]. They act in most cases at shorter distances (with exceptions such as IL-1, IL-6 and TNF). However, cytokines penetrate most tissues, being delivered by migration of white blood cells of the hematopoietic tissue, which virtually permeates all other tissues in vertebrates. Furthemore, the immune competent cells are one of the largest sources of cytokines, that being capable to migrate in almost all tissues of the body, represent moving regulators of the local microenvironment [143].

We hypothesized a link between vaccinations and neuroinflammation. The peripheral pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), expressed after the injection of all vaccines, can reach the brain and can cause neuroinflammation after microglia activation. Depending on the age and type of vaccines, neuroinflammation may produce AEs, such as those following HPV vaccination. Elevated pro-inflammatory cytokines, particularly TNF-α, have been described in studies regarding the cytokines profile in autistic children. IL-1β represents a cytokine that controls the local pro-inflammatory cascade and thereby affects the balance between protective immunity and destructive inflammation. A subgroup of children with ASD have developed neuroinflammation. Several postmortem studies have confirmed the activation of microglia and neuroinflammation. A recent study shows the presence of aluminium in brain tissue in ASD. Besides, aluminium was also found in microglia cells [144]. Aluminium from vaccines is redistributed to numerous organs including brain, where it accumulates. Each vaccine adds to this tissue different level of aluminium. Aluminum, like mercury, activates microglia leading to chronic brain inflammation and neurotoxicity.

Gardasil and Cervarix vaccines (Figure 1) contain aluminum, which activates caspase-1 enzyme, *via* NLRP3 inflammasome. The caspase-1 enzyme converts the pro-interleukins 1β and 18 in their active forms. IL-18 determines the production of IFN-γ. IL-1β represents a cytokine that controls the local pro-inflammatory cascade and contributes to activate the transcription factor NF-κB. The Cervarix adjuvant AS04 contains Aluminum Hydroxide and MPL. The second one stimulates the TLR4. Gardasil 4 vaccine is contaminated with foreign DNA in non-B conformation [145], which activates TLR9. TLRs act through the adapter protein MyD88 that acts by increasing the activity of NF-κB, which then increases the expression and secretion of IL-1β, IL-6 and TNF-α.

Thus, there is a strong immune stimulation and a strong production of pro-inflammatory cytokines, including IL-1β IL-6 and TNF-α, which are capable of exerting effects at a distance from the production site.

In Figure 2, the mechanism of action of the aluminum is always represented, but a new anti-meningococcal B vaccine produces the activation of the TLR- 2 and 4. The OMV vesicles contain lipoproteins that activate the TLR2, and LPS that activate TLR4. The strong production of peripheral pro-inflammatory cytokines is capable of producing microglia activation and neuroinflammation.



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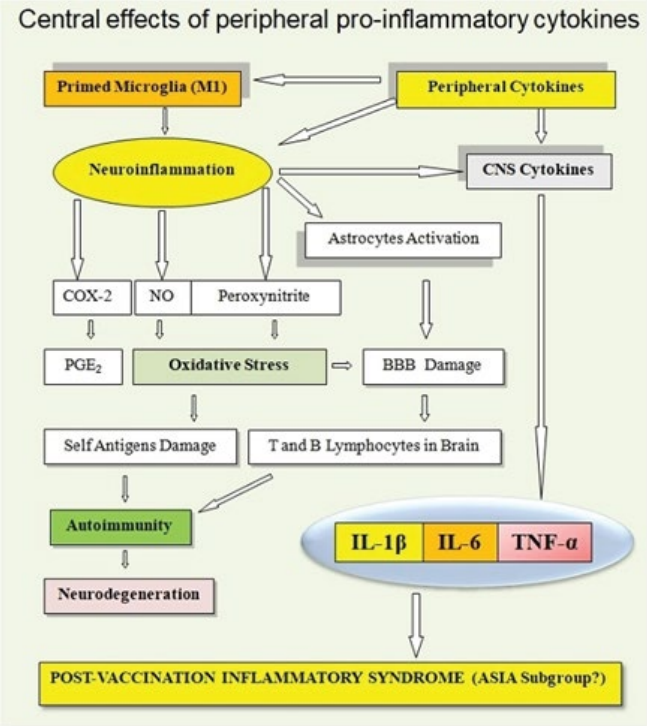

Figure 3: On the right side, you can see that the peripheral pro-inflammatory cytokines, expressed after the injection vaccines, can reach the brain and, apart from neuroinflammation, can cause a post-vaccination inflammatory syndrome [29], as in the case of HPV vaccines. If a neuroinflammation is present, it could be followed by autoimmune reactions and neurodegeneration. Peripheral cytokines can produce primed microglia and the inflammatory phenotype M1 participating in the neuroinflammation. The neuroinflammation increases production of pro-inflammatory cytokines, activates astrocytes, produces an oxidative stress and increases the production of prostaglandins in the brain. Oxidative stress and astrocytes activation cause a rupture of the BBB that eases the entry of the T and B lymphocytes in the brain. Oxidative stress also produces damage to self-antigens and may help to produce autoimmunity and neurodegenerative diseases.

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Conclusion

The existence of extensive lines of communication between the nervous system and immune system represents a fundamental principle underlying neuroinflammation. Immune memory in the brain is an important modifier of neuropathology. Systemic inflammation generates signals that communicate with the brain and lead to changes in metabolism and behavior, with microglia assuming a pro-inflammatory phenotype. Two types of immunological imprinting can be distinguished: Training and tolerance. These are epigenetically mediated and enhance or suppress subsequent inflammation, respectively. Peripherally applied inflammatory stimuli induce acute immune training and tolerance in the brain and lead to differential epigenetic reprogramming of brain-resident macrophages (microglia) that persists for at least six months.

The molecular mechanisms presented here demonstrate how peripheral cytokines, expressed after vaccination, can cause neuroinflammation in some subjects, after microglia activation, depending on the immunogenetic background and the innate immune memory.

The effects produced by the activation of the microglia, and the subsequent neuroinflammation, are diversified according to age: before the first two years of life they can contribute to producing ASD (in some subjects with ASD there is neuroinflammation and aluminium accumulation in the brain); while a different neurological symptomatology can arise in girls vaccinated with HPV vaccines. A post-vaccination inflammatory syndrome can explain the adverse reactions to HPV vaccines (ASIA Subgrup?). Indeed, IL-1β causes pathological pain and fatigue and is elevated in peripheral neuropathic pain and CRPS type I. Increased levels of IL-1β, IL-6, and TNF-α in the brain cause cognitive impairment, sleep disorders, and reduced motor activity.

Regarding the possible relationships between HPV vaccines and CRPS type I, at the molecular level, the cytokines typical of CRPS type I environment, with high levels of IL-1β, IL-6, and TNF-α [46-48], it is faithfully reproduced by the injection of HPV vaccines. Whereas in patients with POTS, sympathetic activation and parasympathetic withdrawal were associated with increased serum IL-6 [54]. IL-1β, IL-6 and TNF-α are strongly expressed after the injection of HPV vaccines, and in patients with CFS/ME, the same proinflammatory cytokines are elevated [55,56].

Suzuki and Hosono [146] did not find an association between HPV vaccine and reported post-vaccination symptoms in Japanese young women. The survey tool was an anonymous postal questionnaire.

As is evident, epidemiology uses different investigative tools than molecular biology. In the case of HPV vaccine AEs, molecular biology demonstrates what epidemiology does not detect.

Disclosure of Potential Conflicts of Interest

The authors declare that there are no competing interests regarding the publication of this paper.

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