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Full Length Article

Cytokines profile in neonatal and adult wild-type mice post-injection of U. S. pediatric vaccination schedule

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ABSTRACT

Introduction: A recent study from our laboratory demonstrated a number of neurobehavioral abnormalities in mice colony injected with a mouse-weight equivalent dose of all vaccines that are administered to infants in their first 18 months of life according to the U. S. pediatric vaccination schedule.

Cytokines have been studied extensively as blood immune and inflammatory biomarkers, and their association with neurodevelopmental disorders. Given the importance of cytokines in early neurodevelopment, we aimed to investigate the potential post-administration effects of the U. S. pediatric vaccines on circulatory cytokines in a mouse model.

In the current study, cytokines have been assayed at early and late time points in mice vaccinated early in postnatal life and compared with placebo controls.

Materials and methods: Newborn mouse pups were divided into three groups: i) vaccine (V1), ii) vaccine × 3 (V3) and iii) placebo control. V1 group was injected with mouse weight-equivalent of the current U. S. pediatric vaccine schedule. V3 group was injected with same vaccines but at triple the dose and the placebo control was injected with saline. Pups were also divided according to the sampling age into two main groups: acute- and chronic-phase group. Blood samples were collected at postnatal day (PND) 23, two days following vaccine schedule for the acute-phase group or at 67 weeks post-vaccination for the chronic-phase groups. Fifteen cytokines were analyzed: GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, MCP-1, TNF-α, and VEGF-A. Wilcoxon Rank Sum test or unpaired Student's t-test was performed where applicable.

Results: IL-5 levels in plasma were significantly elevated in the V1 and V3 group compared with the control only in the acute-phase group. The elevation of IL-5 levels in the two vaccine groups were significant irrespective of whether the sexes were combined or analyzed separately. Other cytokines (VEGF-A, TNF-α, IL-10, MCP-1, GM-CSF, IL-6, and IL-13) were also impacted, although to a lesser extent and in a sex-dependent manner. In the acute-phase group, females showed a significant increase in IL-10 and MCP-1 levels and a decrease in VEGF-A levels in both V1 and V3 group compared to controls. In the acute-phase, a significant increase in MCP-1 levels in V3 group and CM-CSF levels in V1 and V3 group and decrease in TNF-α levels in V1 group were observed in treated males as compared with controls. In chronic-phase females, levels of VEGF-A in V1 and V3 group, TNF-α in V3 group, and IL-13 in V1 group were significantly decreased in contrast with controls. In chronic-phase males, TNF-α levels were significantly increased in V1 group and IL-6 levels decreased in V3 group in comparison to controls. The changes in levels of most tested cytokines were altered between the early and the late postnatal assays.

Conclusions: IL-5 levels significantly increased in the acute-phase of the treatment in the plasma of both sexes that were subjected to V1 and V3 injections. These increases had diminished by the second test assayed at week 67. These results suggest that a profound, albeit transient, effect on cytokine levels may be induced by the whole vaccine administration supporting our recently published observations regarding the behavioral abnormalities in the same mice. These observations support the view that the administration of whole pediatric vaccines in a neonatal period may impact at least short-term CNS functions in mice.

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1. Introduction

Vaccines are generally considered as very safe and effective prophylactic agents against infectious diseases. For this reason, the risks of side effects, if not entirely overlooked, are often discounted and/or deemed insignificant. However, growing evidence from the peer-reviewed scientific literature suggests that this "safe" assertion is not as fully substantiated as often claimed. For instance, vaccine trials may not have an adequate study design and true placebo controls, may be subject to various sources of bias, and lack realistic reporting outcomes. These deficiencies bring into question some conventional assertions about vaccine safety (Demicheli et al., 2005; Tomljenovic and Shaw 2013).

Another reason for increasing doubts concerning vaccine safety could be attributed to the fact that regulatory agencies have historically not viewed vaccines as inherently toxic (FDA 2002). Furthermore, a large volume of studies have reported the occurrence of serious immune and autoimmune disorders post vaccination, including those affecting the nervous system (Bardage et al., 2011; Karussis and Petrou 2014; Guimaraes et al., 2015; Soriano et al., 2015; Toussirot and Bereau 2015; Vadala et al., 2017; Segal and Shoenfeld 2018; Herve et al., 2019; Sirbu et al., 2020). Finally, there appear to be legitimate reasons regarding the veracity of the conclusions on vaccine safety typically drawn from the Vaccine Adverse Events Reporting System (VAERS). Indeed, the proportion of adverse reactions following specific vaccinations may be vastly underestimated given that VAERS relies exclusively on self-reporting. Moreover, many of the more serious immune-related adverse outcomes are not acute in nature, and the awareness of the possibility of their occurrence post-vaccination in the general public is very low, all of which decreases the chance of them being reported to VAERS (Lazarus 2010).

The current U. S. pediatric vaccination schedule recommended for newborns up to 18 months of age is shown in Table 1. This age window, in which children receive many of their vaccines, is critically important for the development of the central nervous system (CNS). Moreover, convincing research data suggest that high immune stimulation during this phase can significantly alter brain developmental processes (Custodio et al., 2018; Li et al., 2018; Bergdolt and Dunaevsky 2019; Carlezon et al., 2019), an outcome which in turn can result in lifelong impacts on brain functions. For example, various studies have shown that immune activation during early postnatal life leads to increased fear and anxiety-like behaviors, altered cognitive functions, and other abnormal behavioral responses, including impaired social interactions, deficits in object recognition memory and sensorimotor gating deficits in later life (Shi et al., 2003; Hornig et al., 2004; Bilbo et al., 2005; Spencer et al., 2005; Ibi et al., 2009; Konat et al., 2011; Olczak et al., 2011; French et al., 2013). Together these adverse outcomes suggest that immune activation during critical periods of both immune and neurological development may be a serious risk factor for developing neurobehavioral disorders including those of the autism spectrum disorder (ASD) (Belmonte et al., 2004; Dietert and Dietert 2008; Hertz-Picciotto et al., 2008).

Some constituents that can be included in vaccine formulations are very well known to act as neurotoxins, in particular aluminum (Cohen and Shoenfeld 1996; Agmon-Levin et al., 2009; Israeli et al., 2009; Tomljenovic et al., 2012; Tomljenovic and Shaw 2012). Other additives that may act as toxicants and which are frequently found in vaccines include phenol red, formaldehyde, polysorbate 80, and phenoxyethanol (Eldred et al., 2006; CDC 2020). Though, these potentially toxic constituents are not considered harmful as their formulation quantity is very low (Offit and Jew 2003; Eldred et al., 2006), evidence from animal experiments show that when aluminum and mercury are administered individually in the same dosages as found in vaccines, they were both able to induce serious detrimental neuro-immunological outcomes (Hornig et al., 2004; Authier et al., 2006; Petrik et al., 2007; Shaw and Petrik 2009; Hewitson et al., 2010; Olczak et al. 2010, 2011; Dorea 2011; Duszczyk-Budhathoki et al., 2012). Furthermore, studies have shown that aluminum adjuvants in vaccines are strongly associated with CNS disorders and autoimmune/inflammatory conditions in human adults (Passeri et al., 2011; Shoenfeld and Agmon-Levin 2011; Tomljenovic and Shaw 2012; Terhune and Deth 2013; Cadusseau et al., 2014; Exley 2014; Rigolet et al., 2014; Shaw et al., 2014a,b; Gherardi et al., 2015).

Children may be at a greater risk for possible neuro-immunotoxic complications with vaccines as they receive aluminum and other potentially toxic additives from vaccines in much larger amounts per

Table 1
Administration of vaccines in mice according to the U. S. CDC 2018 recommended vaccination schedule for preschool children of 0–18 months old (CDC 2018).

Infant age (months)	0	2	4	6	12	15	18
Equivalent mouse age (days)	7	8	9	10	14	18	21
The details of the applied vaccines	1.Recombivax HB (hepatitis B) *i.m injection* Al hydroxide Merck	1.Recombivax HB (hepatitis B) *i.m* injection Al hydroxide Merck	1. Pentacel (DTaP, Hib, IPV) *i.m* injection Al phosphate Sanofi Pasteur Limited	1.Recombivax *HB* (hepatitis B) *i.m* injection Al hydroxide Merck	1. Varivax (varicella) *s.c* injection No Al adjuvant Merck	1. Recombivax HB (hepatitis B) *i.m* injection Al hydroxide Merck	1. Fluzone (Influenza) *i.m* injection No Al adjuvant Sanofi Pasteur Limited
	–	2. Pentacel (DTaP, Hib, IPV) *i.m* injection Al phosphate Sanofi Pasteur Limited	2. Rotateq (Rotavirus) Oral administration No Al adjuvant Merck	2. Pentacel (DTaP, Hib, IPV) *i.m* injection Al phosphate Sanofi Pasteur Limited	2. MMR II *s.c* injection No Al adjuvant Merck	2. Pentacel (DTaP, Hib, IPV) *i.m* injection Al phosphate Sanofi Pasteur Limited	*2. Vaqta* (hepatitis A) *i.m* Al hydroxide Merck
	–	3.Rotateq (Rotavirus) Oral administration No Al adjuvant Merck	3. Prevnar 13 (Pneumoc) *i.m* injection Al phosphate Wyeth Pharm Inc	3. Rotateq (Rotavirus) Oral administration No Al adjuvant Merck	3. Fluzone (Influenza) *i.m* injection No Al adjuvant Sanofi Pasteur Limited	3. Prevnar 13 (Pneumoc) *i.m* injection Al phosphate Wyeth Pharm Inc	–
	–	4. Prevnar 13 (Pneumoc) *i.m* injection Al phosphate Wyeth Pharm Inc	–	4. Prevnar 13 (Pneumoc) *i.m* injection Al phosphate Wyeth Pharm Inc	4. Vaqta (hepatitis A) *i.m* injection Al hydroxide Merk	–	–
Vaccines/day	1	4	3	4	4	3	2
Total (vaccinations)	21						

DTaP: diphtheria, tetanus, acellular pertussis; Hib: Heamophilus influenzae; Pneumoc: pneumococcal; IPV: inactivated polio; MMR: measles mumps rubella; *s.c*: subcutaneous; *i.m*: intramuscular.

Table 2
Total number of male and female mice in each treatment group in acute- and chronic-phase group.

Treatment groups	Acute-phase				Chronic-phase			
	Mice (total)	Litters	Females	Males	Mice (total)	Litters	Females	Males
Control	24	3	6	18	26	4	7	19
Vaccine (V1)	24	3	13	11	25	4	14	11
Vaccine × 3 (V3)	23	3	11	12	25	4	12	13
Total	71	9	30	41	76	12	33	43

body weight in comparison to adults (Tomljenovic and Shaw 2012; Shaw et al., 2014). Moreover, since vaccines are considered safe, the whole pediatric vaccine schedule has in fact, never been adequately studied for possible long-term adverse neurological and immune-related impacts, even though animal experimental studies investigating the effect of single constituents suggest a potential risk for such outcomes (e.g, ethyl mercury (Dorea 2011); aluminum (Petrik et al., 2007; Shaw et al., 2013); formalin (Moghaddam et al., 2006)). It would appear therefore that a comprehensive analysis of vaccine safety warrants a more thorough examination than has been provided to date, especially for CNS disorders that are not necessarily acute and easily diagnosable (Freed et al., 2010).

Various cytokines are well-studied for their roles in immune responses and their association with neurodevelopmental disorders. Results from animal experimental models have shown that maternal immune activation during infection can modulate the developing fetal brain by increasing circulating cytokine levels (Patterson 2002; Yamashita et al., 2003), thus indicating the involvement of cytokines in alteration of normal brain maturation. For instance, *Borna* disease virus infection in neonatal rats results in neuronal death in hippocampus, neocortex, and cerebellum along with behavioral abnormalities similar to autism (Hornig et al., 2001). These results are associated with major changes in the cytokine expression at various locations in the brain, suggesting a potential involvement of cytokines during CNS injury (Plata-Salaman et al., 1999; Sauder and de la Torre 1999). Elevated levels of cytokines and their association with severity of diagnostic features in ASD (Ashwood et al., 2011c; Hashim et al., 2013) have also been documented. In addition, cytokines play an important role in regulating immune responses via pro- and anti-inflammatory pathways. Cytokine expression in the brain tissues of autistic patients has been observed and association of marked activation of microglia and astrocyte in the cerebellum with increased cytokine profiling indicate microglial neuro-immune reactions (Vargas et al., 2005).

Elevated levels of pro-inflammatory cytokines were also detected in the cerebrospinal fluid of autistic patients and these results further point to the important role of cytokines in ASD (Vargas et al., 2005; Chez et al., 2007). Furthermore, several studies reported the association between changes in cytokine levels and the worsening of clinical behavioral outcomes in autism patients (Ashwood et al., 2008; Al-Ayadhi and Mostafa 2012; Hashim et al., 2013). Association between cytokines profile and immune activation in autistic subjects has also been explored extensively (Croonenberghs et al., 2002; Jyonouchi et al., 2005; Vojdani et al., 2008; Saresella et al., 2009; Ashwood et al., 2011b; Malik et al., 2011). Results from a study that screened cytokine profiles in neonatal dried blood samples of children diagnosed with ASD in later life obtained from The Danish Newborn Screening Biobank suggested a depressed or hypoactive immune cell function during the early neonatal phase in ASD (Abdallah, Larsen, Mortensen, et al., 2012). Results from human and animal studies indicate the potential role of both pro- (Patterson et al., 2008; Parker-Athill and Tan 2010) and anti-inflammatory cytokines (Molloy et al., 2006; Goines et al., 2011) in accelerating clinical outcomes associated with ASD. Pro- and anti-inflammatory cytokines regulate many normal biological functions including neural development and functions. Any disruption in this balance may result in abnormal brain development and behaviors (Yirmiya and Goshen 2011).

The first part of our vaccine study included the assessment of short and long-term behavioral outcomes in mice receiving the weight-equivalent doses of pediatric vaccines versus a saline placebo and is reported elsewhere (Eidi et al., 2020). The present report is the continuation of the same study protocol and includes the assays of circulatory cytokines in the same groups of animals tested at two age time points, namely at 23 postnatal days (PND) and at 67 postnatal weeks.

2. Materials and methods

2.1. Animals, breeding and experimental groups

The experimental protocols were in accordance with the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of British Columbia (protocol #A16-0125 and #A16-0052 for breeding and experimental procedures, respectively).

This study included two cohorts with breeding pairs purchased from Jackson Laboratories. The breeders for the first cohort (acute-phase group) consisted of fourteen female and seven male C57BL/6 mice. The breeders for the second cohort (chronic-phase group) had sixteen female and eight male C57BL/6 breeder mice. The female and male mice were aged eight and five weeks old, respectively. Upon arrival, the breeders were housed separately in a room with 14/10 h light/dark cycle and 22 °C temperature for one week of acclimatization period. Breeder mice were fed Purina chow diet and water available *ad libitum*. After the acclimatization period, breeding pairs were established for one week where one male with two female mice were housed together. Male mice were separated from female mice after impregnation and body weights of

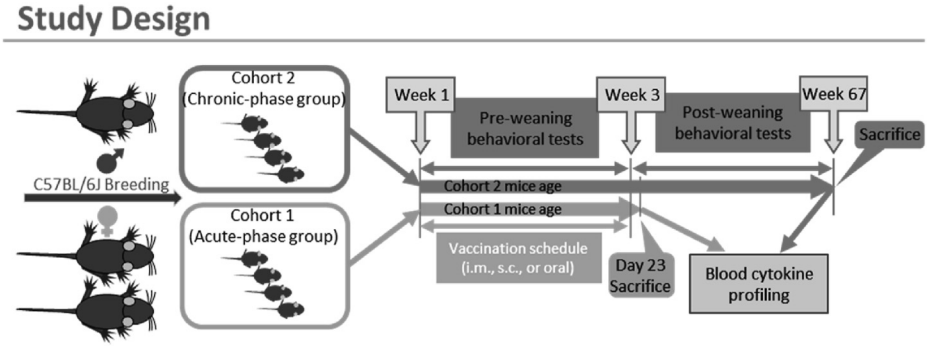

Fig. 1. Flow-chart of study design for acute- and chronic-phase group.

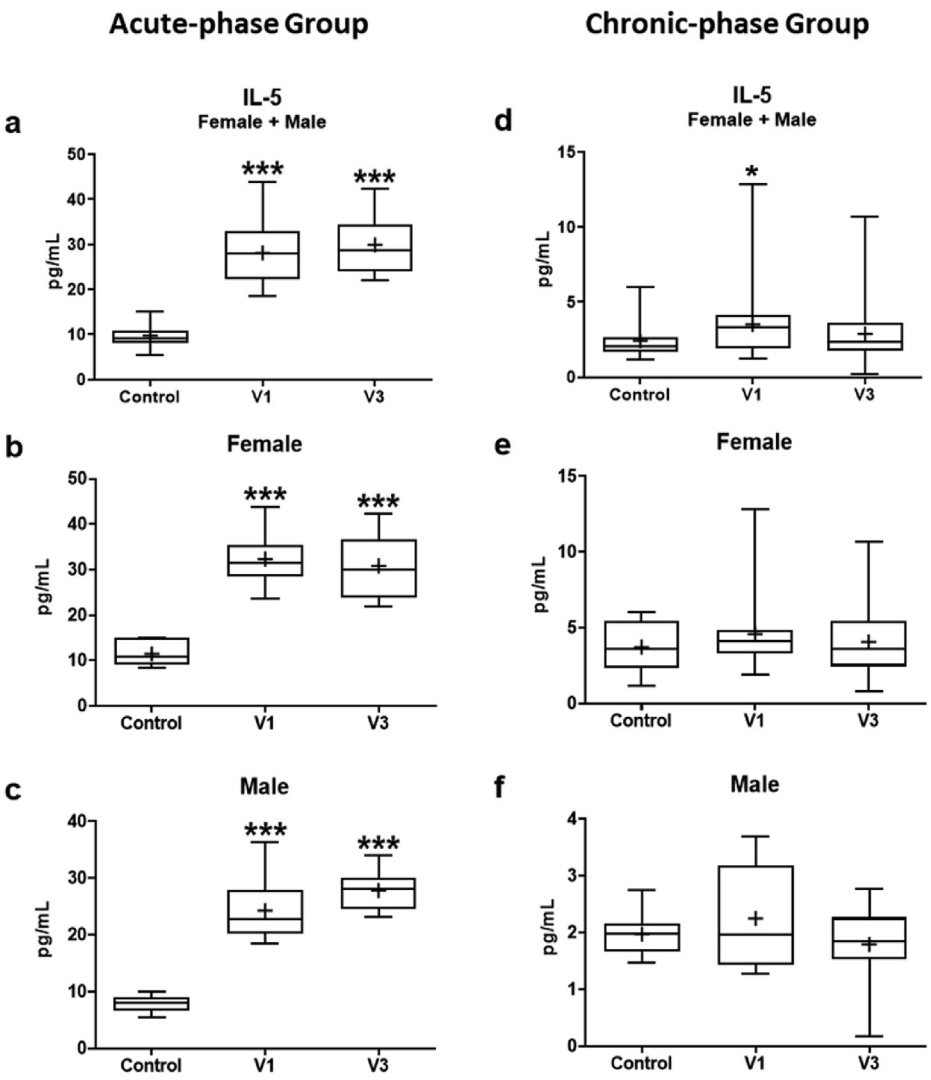

Fig. 2. IL-5 levels in the plasma obtained from the mice in the acute- and the chronic-phase groups. *p < 0.05 and ***p < 0.001 vs. control.

female mice were monitored closely.

During the second week of gestation, female mice were separated and housed individually. Newborn pups were assigned into three treatment groups having three litters in acute-phase and four litters in chronic-phase group. The treatment groups and number of mice in each group are described in Table 2.

The vaccine group was injected in a manner designed to model the U. S. pediatric vaccination schedule, with dosages adjusted to animal body weight to reflect the dosages received by human infants. The V3 group was injected with one shot of the triple dose vaccine each of the mice-weight equivalent doses given to the V1 group and the saline control group was injected with sterile saline buffer (PBS) (Table 1). The injection volumes were adjusted to 10 μl for all groups to minimize variability.

Mice in the acute-phase group were sacrificed two days after the last injections, namely at PND 23 and mice in the chronic-phase group went through several behavioral tests and were monitored for 67 weeks before sacrifice (Eidi et al., 2020). (See Fig. 1 for study design of acute- and chronic-phase group).

2.2. Plasma and organs sample preparation

In all cases, mice were deeply anesthetized with 4% isoflurane and 2 L/min oxygen and later maintained at 2% isoflurane and 1 L/min oxygen. Blood was collected immediately by cardiac puncture with a syringe coated with heparin and kept on ice. Blood samples were centrifuged at 4000×*g* for 14 min at 4 °C to isolate plasma and kept at −80 °C until ready to analyze. Following blood collection, the mice were perfused transcardially with PBS or 10% formalin and the brains, spinal cords, and other organs were collected for molecular or histological analysis.

2.3. Cytokine analysis

Plasma concentrations of 15 cytokines were determined using the Meso Scale Discovery (MSD) platform (Meso-Scale Diagnostics, LLC, Rockville, MD, USA, Cat. #K15069L-2). The MSD U-PLEX platform is an electrochemiluminesence-based multiplex assay capable of detecting quantity as low as picogram per milliliter (pg/mL). The U-PLEX Biomarker Group 1 (ms) Assays were used to determine the concentrations of GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, MCP-1, TNF-α, and VEGF-A. MSD assays were carried out according to the manufacturer's instructions. Briefly, individual U-PLEX Coupled Antibody Solution was prepared by coupling each biotinylated antibody to a specific linker in separate Eppendorf tubes. This solution was mixed by vortexing and incubated at room temperature for 30 min. After that, stop solution was added and mixed by vortexing followed by incubation at room temperature for 30 min. All the U-PLEX Coupled Antibody Solution were pooled in a falcon tube and vortexed. 96-well U-PLEX plates were coated with U-PLEX Coupled Antibody Solution and

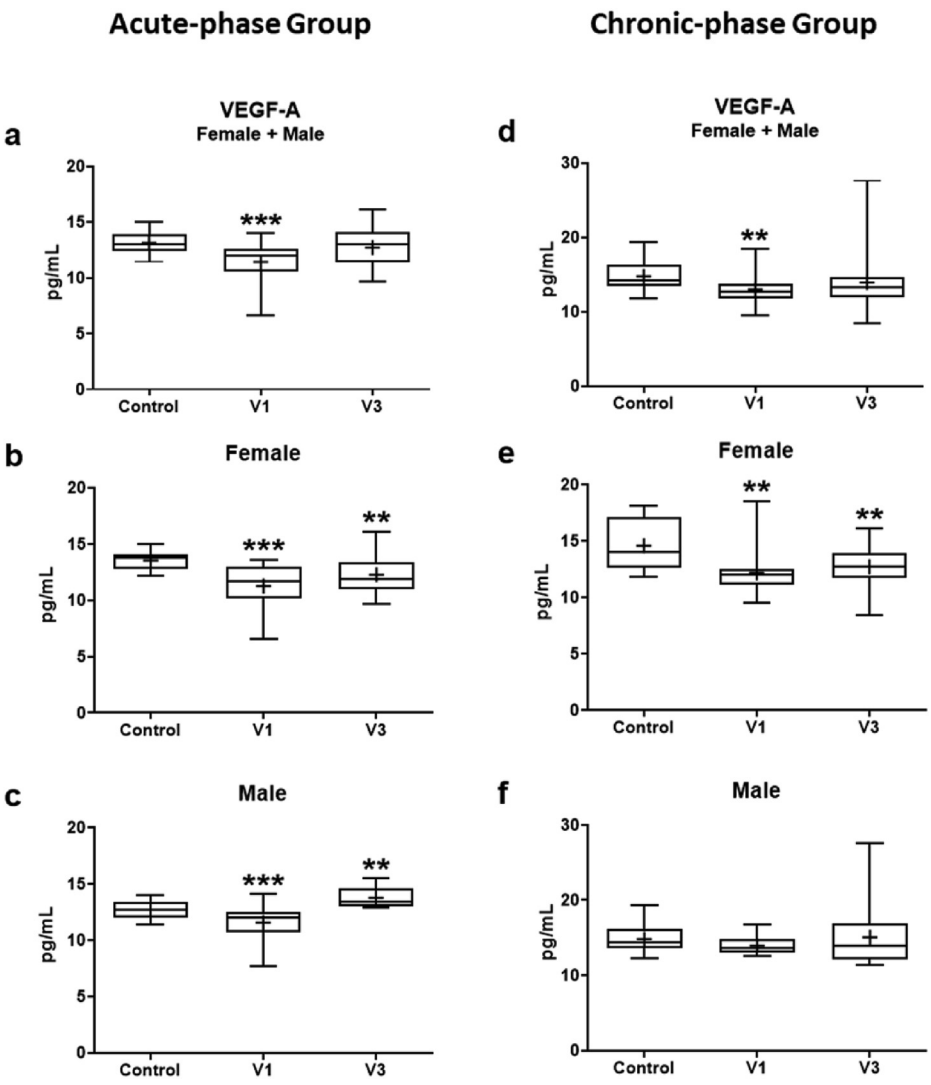

Fig. 3. VEGF-A levels in the plasma obtained from the mice the acute- and the chronic-phase groups. **p < 0.01 and ***p < 0.001 vs. control.

incubated at room temperature on a shaker for 1 h. Plates were washed three times with PBS-T. In the U-PLEX plate, Diluent was added to each well followed by Calibrator Standards or plasma samples. All the standards and plasma samples were loaded in duplicates. The Calibrator Standards were prepared by reconstituting the provided Calibrator vial with Diluent. After inverting the reconstituted Calibrator at least 3 times, this solution was kept for 30 min at room temperature. This Calibrator Standard is now Calibrator Standard 1 that gives highest point in the standard curve. After brief vortexing, Calibrator Standard 1 was 4-fold serially diluted with Diluent to make Standard Calibrator 2–7. Tubes were vortexed between each serial dilution. To make the Standard Calibrator 8, only the Diluent was used. The plates were sealed with an adhesive plate seal and incubated at room temperature on a shaker for 1 h. Plates were washed 3 times with PBS-T and detection antibody solution was added to each well. Plates were sealed again with an adhesive plate seal and incubated at room temperature on a shaker for 1 h. Plates were washed again 3 times with PBS-T and Read Buffer was added to each well and were read on MSD QuickPlex SQ 120. Cytokine concentrations were measured and presented in pg/mL. The experimenter was blinded to the identity of the plasma samples. This MSD kit and method has been used and validated by a number of scientific studies (Soh et al., 2016; Wiehagen et al., 2017; Xu et al., 2018; Burmeister et al., 2019; Santos et al., 2019; Song et al., 2019; Pang et al., 2020; Unger et al., 2020).

2.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Wilcoxon rank-sum test was performed to compare cytokines profile among treatment groups. Unpaired Student's t-test was used to compare between controls from acute and chronic-phase groups of both sexes combined and separated. The box plots display the range of cytokines from minimum to maximum. Table data are presented as mean ± standard error (SE). Sample size was 6–24 mice per group. A p-value of <0.05 was considered statistical significant.

3. Results

Acute-phase plasma levels of IL-5 were significantly higher in the V1 and V3 groups (Fig. 2a, b and 2c: $p < 0.001$) compared to the controls for both sexes combined and separated. In the chronic-phase group, IL-5 levels were significantly higher only in the V1 group in comparison with the control group for both sexes combined (Fig. 2d: $p < 0.05$). No other significant difference in IL-5 levels was detected in the chronic-phase group in any of the treatment groups compared with the control when both sexes were separated (Fig. 2e and f).

In the acute-phase group, VEGF-A levels were significantly lower in the V1 group for both sexes combined and separated compared with the

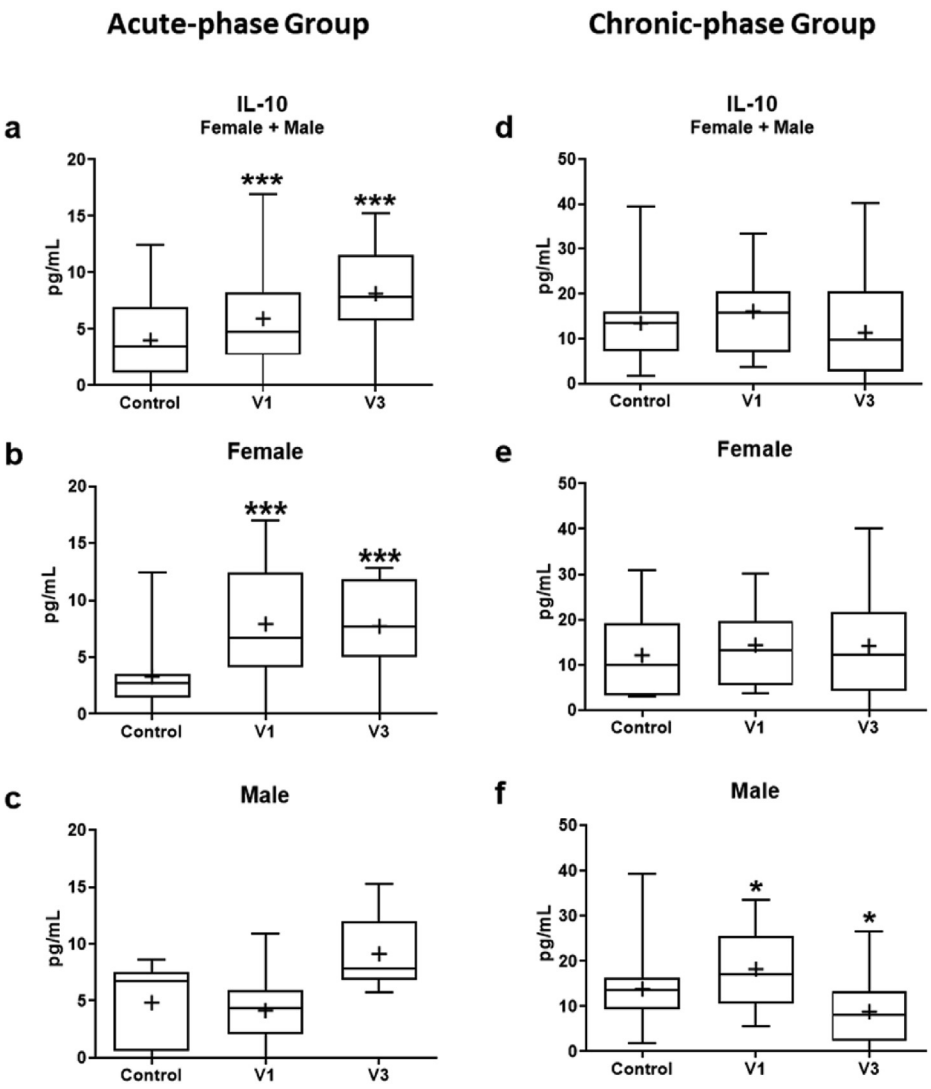

Fig. 4. IL-10 levels in the plasma obtained from the mice in the acute- and the chronic-phase groups. *$p < 0.05$ and ***$p < 0.001$ vs. control.

corresponding control animals (Fig. 3a, b, and 3c: $p < 0.001$). In addition, VEGF-A levels in V3 group were significantly decreased in females and increased in males (Fig. 3b and c: $p < 0.01$). Chronic-phase VEGF-A levels were significantly reduced in the V1 group for both sexes combined and in females compared to corresponding controls (Fig. 3d and e: $p < 0.01$). V3 group females showed significant lower levels of VEGF-A compared with control females (Fig. 3e: $p < 0.01$). No significant changes in VEGF-A levels were observed in male mice (Fig. 3f).

Acute-phase IL-10 levels werer significantly higher in the V1 and V3 group for both sexes combined and in females alone compared to corresponding control animals (Fig. 4a and b: $p < 0.001$). Chronic-phase IL-10 levels were significantly altered only in the male animals where they were increased in the V1 group and decreased in the V3 group (Fig. 4f: $p < 0.05$).

Acute-phase TNF-α levels were significantly lower in the V1 group for both sexes combined and in males (Fig. 5a: $p < 0.01$, Fig. 5c: $p < 0.001$). V3 group females showed significantly increased TNF-α levels compared with controls (Fig. 5b: $p < 0.01$). In chronic-phase group, TNF-α levels were significantly increased in the V1 group males (Fig. 5f: $p < 0.05$). The V3 group for both sexes combined had significantly decreased levels of TNF-α levels, as did female animals separately when compared with corresponding controls (Fig. 5d and e: $p < 0.05$).

In the acute-phase mice, MCP-1 levels were significantly higher in V1 group for both sexes combined and in females (Fig. 6a and b: $p < 0.01$). Similarly, V3 group had significantly increased MCP-1 levels for both sexes combined and separated compared to respective controls (Fig. 6a: $p < 0.001$, Fig. 6b and c: $p < 0.05$). In chronic-phase, MCP-1 levels in the V1 group were significantly decreased compared with controls (Fig. 6d: $p < 0.05$).

GM-CSF levels in the V3 group were significantly elevated in the acute-phase when both sexes were combined and in males (Fig. 7a and c: $p < 0.05$) while a decrease was observed in females (Fig. 7b: $p < 0.05$). In the acute-phase, V1 group GM-CSF levels were significantly increased in males (Fig. 7c: $p < 0.001$). Chronic-phase CM-CSF levels were not found to be significantly different in any of the vaccine groups when compared with controls.

Acute-phase group IL-6 levels were significantly elevated in the V1 group when both sexes were combined and compared with control animals (Fig. 8a: $p < 0.001$). In chronic-phase mice, IL-6 levels were significantly decreased in the V3 group for both sexes combined (Fig. 8d: $p < 0.05$) and in males (Fig. 8f: $p < 0.001$) compared with corresponding controls.

Chronic phase IL-13 levels were significantly decreased in V1 group females and in the V3 group for both sexes combined (Fig. 9d and e: $p < 0.05$).

Acute- and chronic-phase IFN-γ, IL-4, IL-2, IL-17A, IL-9, IL-12p70, and IL-1β were not significantly different in any of the treatment groups compared to the controls (supplementary data, Tables 1–6).

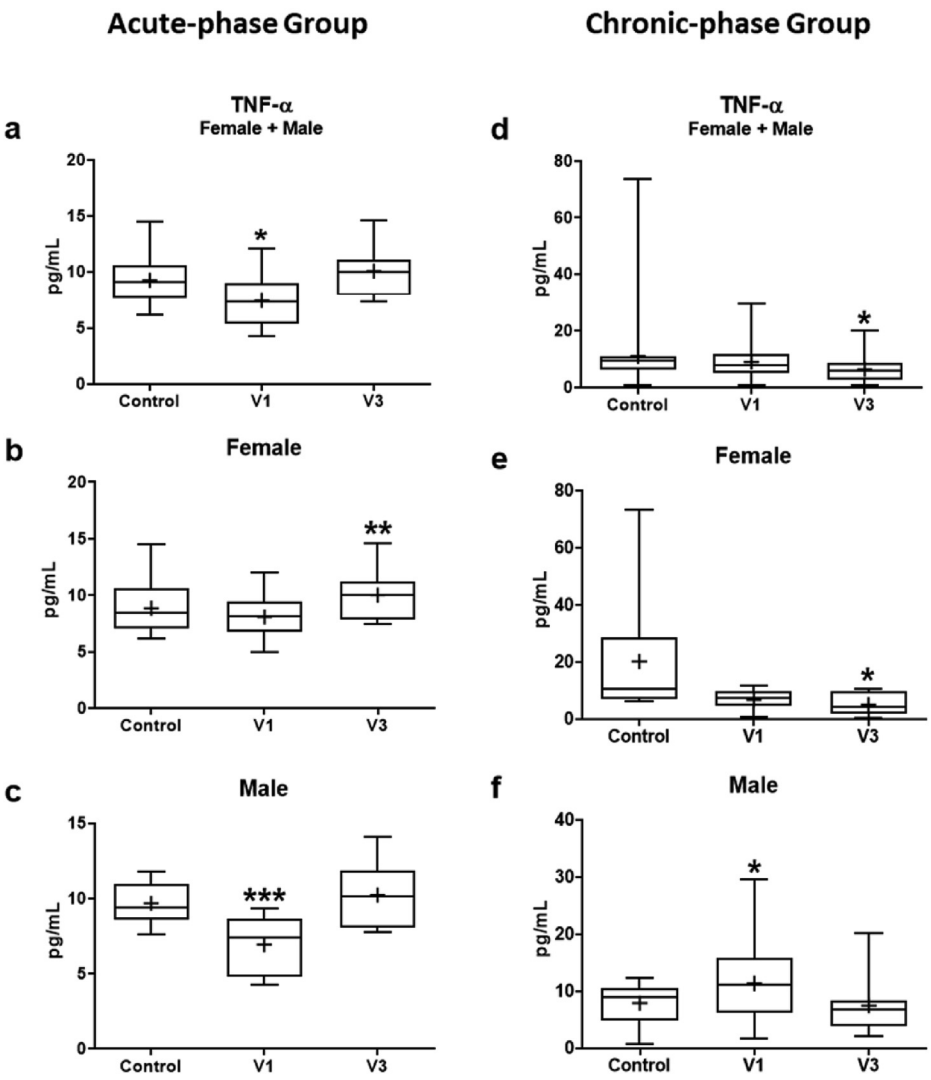

Fig. 5. TNF-α levels in the plasma obtained from the mice in the acute- and the chronic-phase groups. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. control.

Furthermore, IL-2, IL-17A, IL-9, IL-12p70, and IL-1β levels were not detected by the MSD assay kit in the plasma samples of the acute-phase animals. Analysis of cytokines in control groups between acute- and chronic-phase groups for both sexes combined and separated show an age-dependent cytokine level modulation (Tables 3–5). Overall qualitative assessment of cytokines is summarized in Tables 6 and 7.

4. Discussion

Difference in pathophysiological levels of circulating pro- and anti-inflammatory cytokines depends on various factors such as treatment, sex, and age (Klingstrom et al., 2008; Prather et al., 2009; Cruz-Almeida et al., 2015; Cai et al., 2016; Surcel et al., 2017; Toft et al., 2018; Zou et al., 2018). Studies have shown that peripheral cytokines modulate CNS functions by crossing the blood-brain barrier (BBB) via active protein transport mechanisms and by recruiting immune cells to the brain parenchyma (Banks 2005; Erickson et al., 2012; Fung et al., 2012). These cytokines act as mediators between neurons and microglia and regulate neuronal cell migration, proliferation, and differentiation (Biber et al., 2008; Patterson 2009).

In our recently published study (Eidi et al., 2020), we detected decreased sociability, increased anxiety-like behaviors, and alteration of visual-spatial learning and memory in male and female mice vaccinated according to the U. S. pediatric vaccine schedule. We also detected a slower acquisition of some neonatal reflexes in vaccinated female mice compared to vaccinated males and controls. The neurodevelopmental alterations observed in this study seemed to be sex-dependent and for most part – and consistent with the results presented herein – transient with age. In particular, the majority of treated mice at the end of the experimental period did not significantly differ from the control population such that, most of the abnormalities detected did not persist until the final evaluations at 67 weeks of age, although some persisted into adulthood.

In the current study, the observed alterations of certain cytokines can be seen as sex-, age- and vaccine dose-dependent (Tables 6 and 7). For example, in the acute phase, while the MCP-1 alterations were observed only in the female mice of V1 group, it was increased in females and males of the group V3. Moreover, in the chronic phase, there was no difference in the MCP-1 levels between males and females (Tables 6 and 7).

No sex-dependent effect has been observed in the case of IL-5 in the V1 and V3 groups in both acute and chronic phase (Tables 6 and 7).

In this current study, we analyzed fifteen cytokines (see Tables 3–7) in the plasma samples taken from mice following acute- and chronic-phase post V1, V3, and saline injections. To determine the differences among groups, individual mice (not the litters) were treated as the experimental unit for sample size given that no litter effect was observed in our current and previous studies (Eidi et al., 2020). Our observation revealed eight

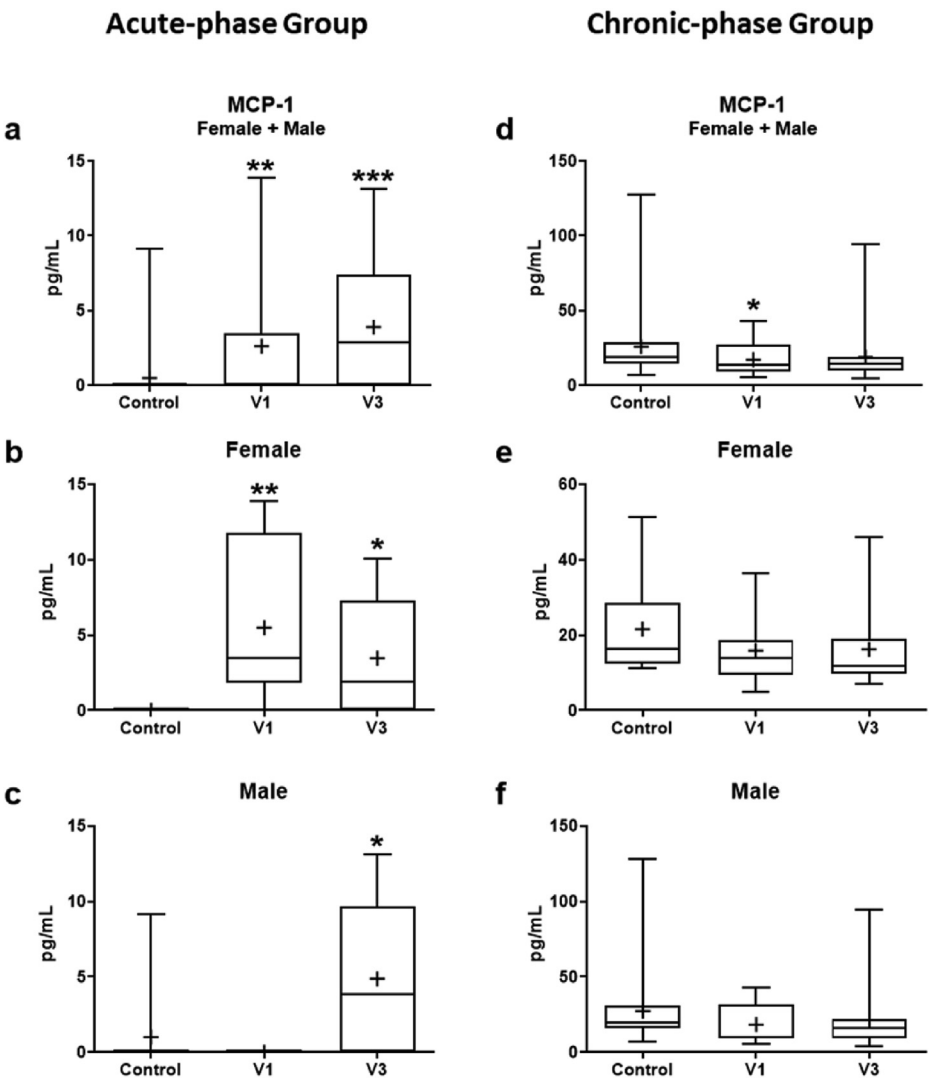

Fig. 6. MCP-1 levels in the plasma obtained from the mice in the acute- and the chronic-phase groups. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. control.

cytokines (IL-5, VEGF-A, IL-10, TNF-α, MCP-1, GM-CSF, IL-6, and IL-13) that were altered significantly in the acute- and/or chronic-phase of the experiment. Of these, the most striking change were elevated levels of IL-5 in the plasma of acute-phase in V1 and V3 groups compared to controls (see Fig. 2).

IL-5 is known as anti-inflammatory cytokine and is produced by T helper 2 (Th2) cells. Th2 cells produce anti-inflammatory cytokines which play a major role in regulating humoral immunity. For example, IL-5 acts against foreign pathogens by activating B cells and antibody production (Spellberg and Edwards 2001; Wills-Karp 2001; Shinkai et al., 2002). In a murine model, IL-5 has been found to be produced by brain cells such as astrocytes and microglia, suggesting an association between specific neural cells and immune cell interaction (Sawada et al., 1993).

In this current study, this robust increase in plasma IL-5 levels in V1 and V3 groups could be due to the Th2 cells activation in response to vaccine injections as a counter mechanism to minimize vaccine-induced effects, in turn increasing anti-inflammatory cytokines like IL-5. The plasma levels of IL-5 were similar in both V1 and V3 group, a pattern that was not dose-dependent and may indicate peaked production of IL-5 at the "regular" vaccine dose. Control group IL-5 levels were significantly reduced in the chronic-phase compared to the acute-phase, suggesting age-dependent reduction in circulating IL-5 production (see supplementary data, Tables 1–6). We also observed that plasma samples obtained from adult mice did not show any significant difference in IL-5 levels in any group compared with control, supporting our behavior data obtained from adult mice (Eidi et al., 2020) which likewise suggested a transient nature of the observed changes. Furthermore, our recent preliminary histological examination of IL-5 presence in the brain supports these findings (data not shown).

IL-10 and MCP-1 levels were elevated significantly in acute-phase mice but no difference in levels was seen in the chronic-phase group. Again, this may suggest an age-depended effect.

MCP-1 functions as a chemoattractant during tissue injury and it mediates activation and recruitment of monocytes and T-cells into damaged areas (Conti and DiGioacchino 2001). Immunocytochemical staining and confocal microscopy studies of brain indicated that astrocytes were the main source for MCP-1 (Vargas et al., 2005).

In the present study, we observed a significant reduction in the VEGF-A levels in both acute- and chronic-phase V1 and V3 animals (Fig. 3a, b, 3c, 3d and 3e). The only exception to this pattern were V3 males, which showed increased levels of VEGF-A (Fig. 3c). VEGF is an important angiogenic growth factor that is crucially involved in the embryogenesis and pre- and post-natal brain development and repair (Jesmin et al., 2004). It is a key signaling molecule in the CNS due to its role in neuroprotection, neuronal survival, and axonal outgrowth (Yasuhara et al., 2004). Critical reduction in VEGF levels is associated with hypoxia and leads to degeneration of cerebral cortex and neonatal death (Haigh et al., 2003; Virgintino et al., 2003; Skaper 2008).

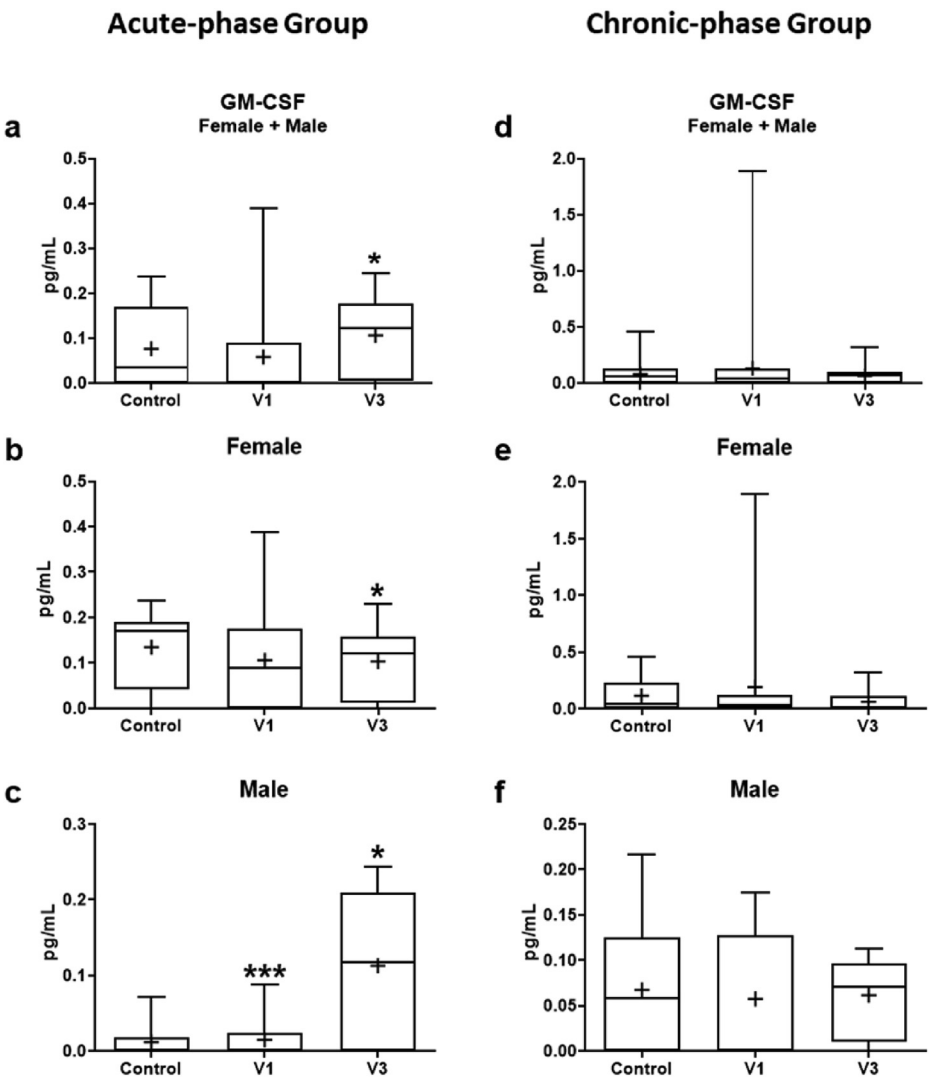

Fig. 7. GM-CSF levels in the plasma obtained from the mice in the acute- and the chronic-phase groups. *$p < 0.05$ and ***$p < 0.001$ vs. control.

In the present study, we observed both a reduction and increase in TNF-α levels in both acute- and chronic-phase group animals. In particular, TNF-α was significantly increased in acute-phase V3 females (Fig. 5b: $p < 0.01$) and chronic-phase V1 males (Fig. 5f: $p < 0.05$). TNF-α is known for its involvement in systemic inflammation and it is produced primarily by cells of monocytic lineage such as macrophages, microglia, astroglia, and alveolar macrophages (Pfeffer et al., 1993; Flynn et al., 1995).

Our study further showed significant increases in GM-CSF but only in the acute-phase animals, in V1 group males and V3 group for both sexes combined and in males (Fig. 7a and c).

Although the main purpose of our study was to examine the impact of whole vaccine formulation on blood markers of inflammation, it is possible that some of the observed outcomes in the vaccinated animals reflect primarily the impact of the Al adjuvants which are the constituents of many of the vaccines administered under the pediatric schedule (Table 2). Adjuvants are known to amplify the immune response and increase the reactogenicity of vaccine antigens. Indeed, with the exception of attenuated viruses, in the absence of Al most antigenic compounds fail to launch an adequate immune response (Dillon et al., 1992; Seubert et al., 2008). It is therefore precisely because of its powerful immunomodulatory properties that Al has been, and remains, the most commonly used vaccine adjuvant. The immune enhancing effects of Al were discovered in 1926 and Al has been used in vaccines ever since (Glenny et al., 1926). The adjuvant-mediated immune-enhancing effect is accomplished via mechanisms that impinge on both the innate and adaptive immune systems (Eisenbarth et al., 2008; Exley et al., 2010). In this context, research shows that Al adjuvants activate 312 genes, 168 of which play a role in immune activation and inflammation (Mosca et al., 2008). At least 13 cytokines and chemokines are produced within 4 h of Al adjuvant injection, including pro-inflammatory IL-1β and IL-6 (McKee et al., 2009). Although historically vaccine Al adjuvants have been portrayed as inherently safe (Eickhoff and Myers 2002; Offit and Jew 2003), studies in animal models and humans have demonstrated their ability to inflict inflammatory manifestations and immune-mediated diseases (Gherardi et al. 2001, 2015bib_Gherardi_et_al_2001; Shaw and Petrik 2009; Zivkovic et al., 2012bib_Gherardi_et_al_2015). Moreover, studies by Khan et al. and other research groups showed that administration of Al or an Al-containing vaccine was associated with Al deposits in distant organs such as lymph nodes, spleen, liver, and brain (Wen and Wisniewski 1985; Redhead et al., 1992; Flarend et al., 1997; Khan et al., 2013) where in some cases they were still detected up to one year after injection (Khan et al., 2013). Khan et al. also found that Al adjuvant particles once injected are sequestered by monocyte-lineage cells in MCP-1 dependent fashion, and were thus carried to draining lymph nodes, and from there reach other organs including the brain (Khan et al., 2013). Notably, Al translocation into the brain was significantly elevated after systemic and/or cerebral increase of the MCP-1/CCL2 signaling

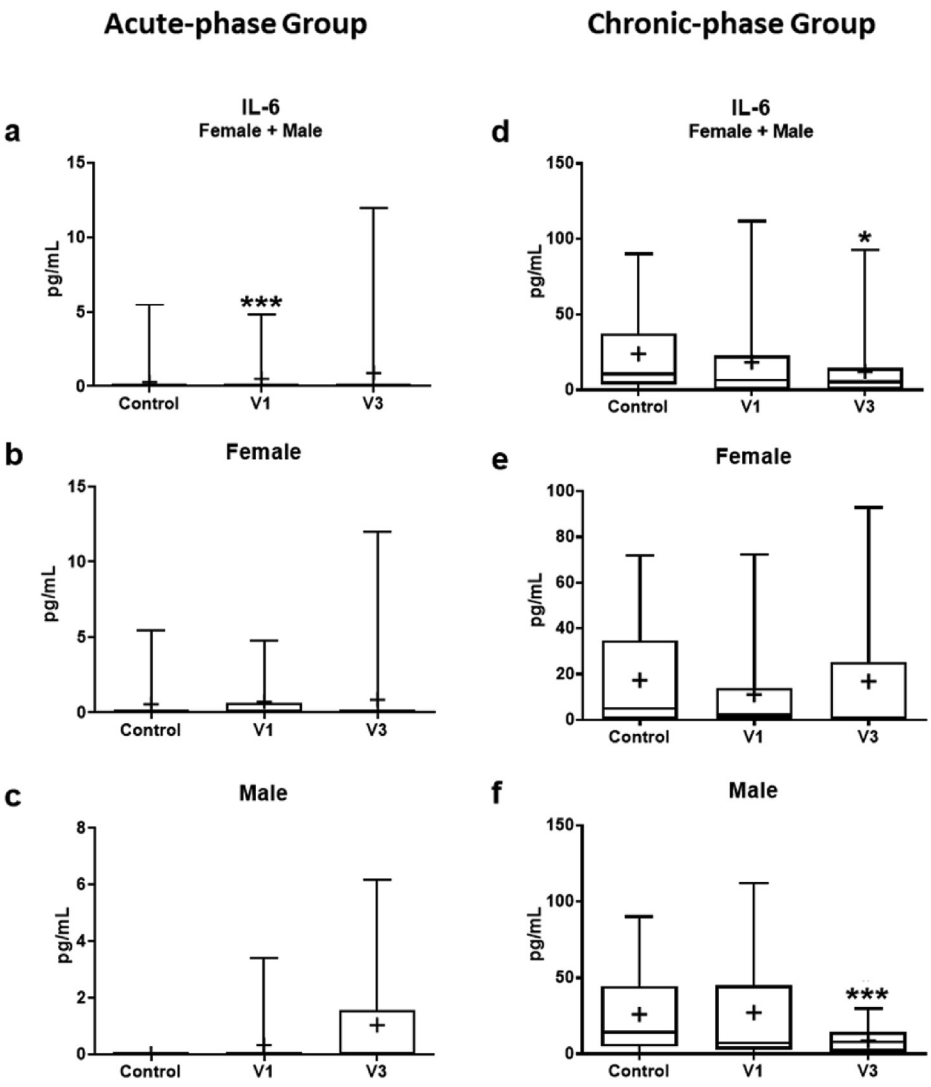

Fig. 8. IL-6 levels in the plasma obtained from the mice in the acute- and the chronic-phase groups. *p < 0.05 and ***p < 0.001 vs. control.

(Khan et al., 2013). These findings reveal a biologically plausible mechanism to explain cognitive impairments which are well known to be associated with administration of Al-adjuvanted vaccines and the persistence of Al in the human body (Gherardi and Authier 2012; Rigolet et al., 2014; Gherardi et al., 2015).

Our results show that MCP-1 levels are increased transiently in the systemic circulation post pediatric vaccination schedule, as elevated levels of this cytokine were almost exclusively observed in acute-phase animals (Fig. 6). It is possible nonetheless, that during this time some Al translocated to the brain of the animals since it was shown that Al particles translocate from muscle to brain within 3 weeks post injection (Eidi et al., 2015).

We fully recognize that any model system attempting to study human disease requires a careful consideration as to whether or not the resulting outcomes truly apply to humans. For example, the study by Seok et al., (2013) showed that genomic responses from the mouse models correlating human inflammatory disease may not closely resemble those in humans. We further realize that in a subject area that is as fraught with controversy as vaccination, extra care needs to be taken so as to not over interpret data to the human condition.

Even though our aim in the current study is to mimic the effects of vaccines on human children using a rodent model, it is still not clear whether the human corresponding cytokines could be affected by the pediatric vaccines in the same manner in mice as observed in the present study. In the following paragraphs, some examples regarding the involvement of the said cytokines in certain human neuro-developmental disorders are discussed.

A recent meta-analysis found that serum IL-5 was slightly increased and plasma IL-10 levels were moderately decreased in those with ASD compared with controls (Saghazadeh et al., 2019). Plasma taken from individuals who were high-functioning ASD showed that concentration of IL-5 and IL-13 were significantly higher along with abnormal immune responses in ASD subjects compared with matched controls (Suzuki et al., 2011). Furthermore, a case-control study found that women with higher IL-5 levels in serum at midgestation were significantly more likely to give birth to a child who later would more likely be diagnosed with ASD (Goines et al., 2011). Taken together, these studies suggest that elevated levels of IL-5 in the circulation may cross the BBB or induce cytokine release in the brain which may play some significant role in the etiology of autism.

In the literature, it has been shown that decreased levels of IL-10 in autistic patients compared with controls without autism (Manzardo et al., 2012; El-Ansary et al., 2016; Saghazadeh et al., 2019) while other studies found no significant association (Ashwood et al., 2011a; Suzuki et al., 2011; Tostes et al., 2012; Napolioni et al., 2013; Inga Jacome et al., 2016; Guloksuz et al., 2017). Vargas et al. however detected elevated IL-10 along with other pro-inflammatory cytokines in the anterior cingulate gyrus of patients with autism (Vargas et al., 2005).

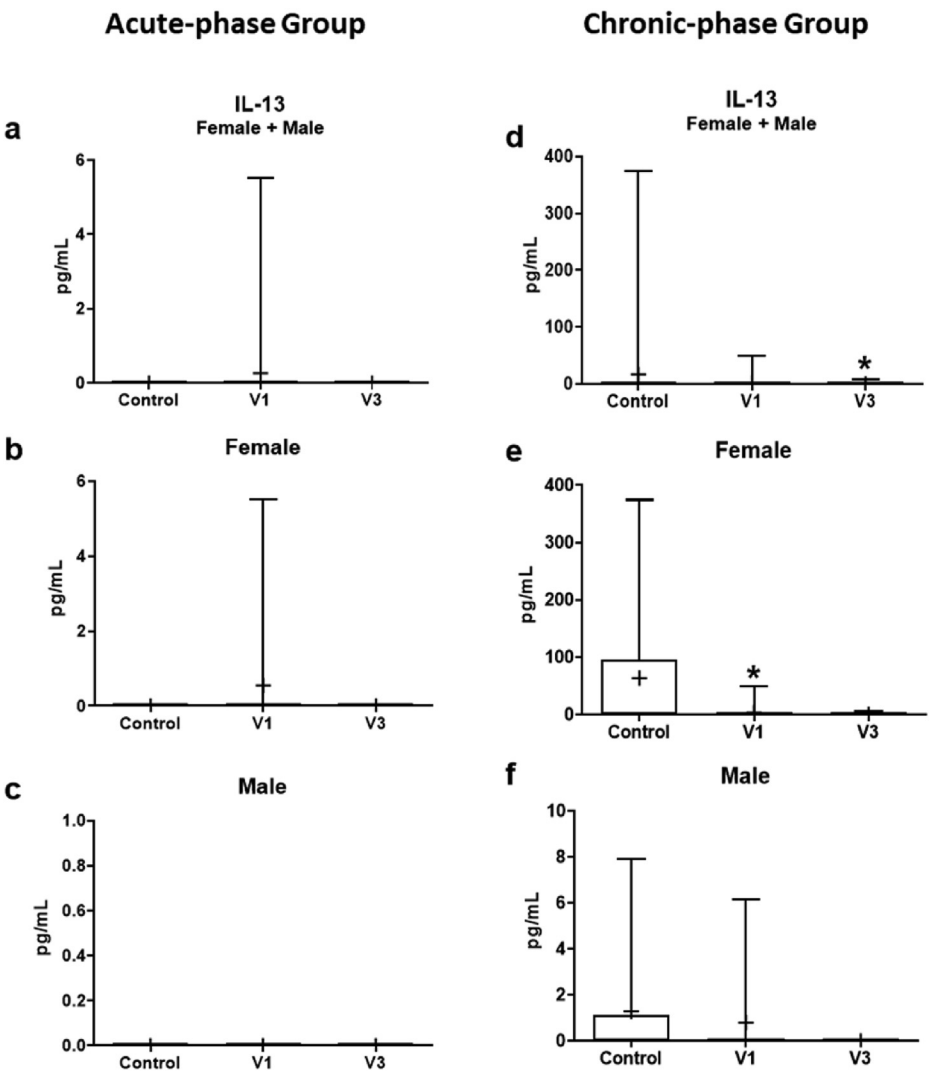

Fig. 9. IL-13 levels in the plasma obtained from the mice in the acute- and the chronic-phase groups. *p < 0.05 vs. control.

Table 3
Comparison between the acute- and chronic-phase controls (both sexes combined).

Cytokines	Control - acute-phase (Mean ± SE) pg/mL	Control - chronic-phase (Mean ± SE) pg/mL
GM-CSF	0.08 ± 0.02	0.08 ± 0.02
IL-5	9.73 ± 0.57	2.41 ± 0.24***
MCP-1	0.48 ± 0.43	25.79 ± 4.88***
VEGF-A	13.14 ± 0.19	14.75 ± 0.39***
IL-4	0.00 ± 0.00	0.12 ± 0.09
IL-13	0.00 ± 0.00	16.87 ± 15.55
IFN-y	0.25 ± 0.14	10.21 ± 4.31*
IL-10	4.01 ± 0.71	13.43 ± 1.72***
IL-6	0.29 ± 0.26	23.86 ± 5.69***
TNF-α	9.23 ± 0.42	10.96 ± 2.81

*p < 0.05 and ***p < 0.001 vs. control – acute-phase group.

Table 4
Comparison between the acute and chronic-phase control females.

Cytokines	Control - acute-phase (Mean ± SE) pg/mL	Control - chronic-phase (Mean ± SE) pg/mL
GM-CSF	0.13 ± 0.04	0.12 ± 0.07
IL-5	11.46 ± 1.08	3.73 ± 0.73***
MCP-1	0.00 ± 0.00	21.58 ± 6.14*
VEGF-A	13.53 ± 0.37	14.58 ± 0.98
IL-4	0.00 ± 000	0.35 ± 0.35
IL-13	0.00 ± 0.00	63.61 ± 62.14
IFN-y	0.00 ± 0.00	6.67 ± 3.45
IL-10	3.27 ± 1.42	12.11 ± 4.35
IL-6	0.55 ± 0.70	17.31 ± 11.39
TNF-α	8.84 ± 1.02	20.20 ± 10.71

*p < 0.05 and ***p < 0.001 vs. control – acute-phase group.

IL-6, which in our case was found to be highly significantly elevated in the acute-phase V1 group animals (Fig. 8a: $p < 0.001$) is another pro-inflammatory cytokine strongly implicated in maternal immune activation associated neurological disorders, including those of the autistic- and schizophrenia-like behaviors (Smith et al., 2007; Hsiao and Patterson 2011). In the fetal brain, studies have found elevated levels of IL-6 mRNA and protein and phosphorylation of STAT3 after maternal immune activation (Gilmore et al., 2005; Meyer et al., 2006), which strengthen the possibility that IL-6 acts on the developing brain in order to induce neurogenesis, microglial activation, astrogliosis and synaptic pruning (Conroy et al., 2004; Gilmore et al., 2004).

With respect to MCP-1, we note that elevated levels of this pro-inflammatory cytokine have been consistently detected in a number of neurological diseases. For example, plasma MCP-1 levels were found to be elevated in Alzheimer's disease patients and this increase was associated with greater severity and faster cognitive decline (Lee et al., 2018). Increased MCP-1 levels were also found in autism patients (Vargas et al.,

Table 5
Comparison between the acute and chronic-phase control males.

Cytokines	Control - acute-phase (Mean ± SE) pg/mL	Control - chronic-phase (Mean ± SE) pg/mL
GM-CSF	0.01 ± 0.01	0.07 ± 0.02**
IL-5	7.81 ± 0.34	1.97 ± 0.08***
MCP-1	1.01 ± 0.72	27.19 ± 0.6.23***
VEGF-A	12.71 ± 0.19	14.81 ± 0.42***
IL-4	0.00 ± 0.00	0.04 ± 0.02
IL-13	0.00 ± 0.22	1.29 ± 0.60*
IFN-y	0.52 ± 0.83	11.38 ± 5.66
IL-10	4.84 ± 0.00	13.86 ± 1.86***
IL-6	0.00 ± 0.00	26.04 ± 6.67**
TNF-α	9.67 ± 0.33	7.88 ± 0.87

*p < 0.05, **p < 0.01, and ***p < 0.001 vs. control – acute-phase group.

Table 6
Qualitative summary of cytokine results from acute-phase group.

Cytokines	V1			V3		
	F + M	F	M	F + M	F	M
GM-CSF	N	N	Inc	Inc	Dec	Inc
IL-5	Inc	Inc	Inc	Inc	Inc	Inc
MCP-1	Inc	Inc	N	Inc	Inc	Inc
VEGF-A	Dec	Dec	Dec	N	Dec	Inc
IL-13	N	N	N	N	N	N
IL-10	Inc	Inc	N	Inc	Inc	N
IL-6	Inc	N	N	N	N	N
TNF-α	Dec	N	Dec	N	Inc	N

F & M represent Females and Males, respectively.
N, Inc and Dec represent, "normal", "increased" (significantly) and "decreased" (significantly) as compared to control, respectively.

Table 7
Qualitative summary of cytokine results from chronic-phase group.

Cytokines	V1			V3		
	F + M	F	M	F + M	F	M
GM-CSF	N	N	N	N	N	N
IL-5	Inc	N	N	N	N	N
MCP-1	Dec	N	N	N	N	N
VEGF-A	Dec	Dec	N	N	Dec	N
IL-13	N	Dec	N	Dec	N	N
IL-10	N	N	Inc	N	N	Dec
IL-6	N	N	N	Dec	N	Dec
TNF-α	N	N	Inc	Dec	Dec	N

F & M represent Females and Males, respectively.
N, Inc and Dec represent, "normal", "increased" (significantly) and "decreased" (significantly) as compared to control, respectively.

2005). MCP-1 also modulates the recruitment of myeloid cells to inflammation or injury sites and is increased during ischemia, Alzheimer's disease, and experimental autoimmune encephalomyelitis (Ashwood et al., 2011c). Experimental studies have also found that individuals with autism have higher MCP-1 levels in plasma, brain, and cerebrospinal fluid. In particular, Vargas et al. found that in comparison to non-autistic controls, autistic patients had significantly increased MCP-1 protein levels in the cerebellum and the anterior cingulate gyrus (Vargas et al., 2005), both of which are regions involved in dysfunctional brain activity in autism (Mundy 2003). The observed increase in MCP-1 production in autistic patients was significantly associated with aberrant behavior scores and impairments in cognitive and adaptive functions (Vargas et al., 2005; Ashwood et al., 2011c). Examination of chemokine levels in amniotic fluid samples obtained from autistic individuals revealed that increase risk for ASD was associated with increase in MCP-1 levels in contrast with controls (Abdallah, Larsen, Grove, et al., 2012). MCP-1 has the ability to enhance synaptic transmission and neural excitability in hippocampal neurons and can modulate neuronal physiology (Melik-Parsadaniantz and Rostene 2008; Zhou et al., 2011).

Samuelsson et al. found that prenatal exposure to IL-6 increased both circulatory IL-6 levels and hippocampal expression of IL-6 mRNA. In addition, all exposed offspring showed neuronal loss, astrogliosis and impaired learning ability (Samuelsson et al., 2006). In addition, elevated serum levels of IL-6 was more common for women who subsequently gave birth to a child diagnosed with a neurodevelopmental delay but without autism (Goines et al., 2011). Other studies have found increased IL-6 levels in the plasma of ASD-affected children compared with typically developing children (Ashwood et al., 2011a). Autistic patients were also found to have higher levels of IL-6 in the brain compared to individuals without autism (Vargas et al., 2005; Li et al., 2009). In particular, Vargas et al. observed significantly elevated IL-6 levels in both the cerebrospinal fluid and the brain tissues of autistic patients. Increase in IL-6 co-localized with the increase in MCP-1 and IL-10 in the anterior cingulate gyrus of autistic brains when compared with controls (Vargas et al., 2005).

It has been suggested that disruptions in VEGF signaling are linked with cerebral cortex degeneration in patients of attention-deficit/hyperactivity disorder (Jesmin et al., 2004a,b;). VEGF has been shown to play an important role in mental disorders such as major depressive disorders (Clark-Raymond et al., 2017). VEGF associated neurovascular dysfunction has been well studied in autism, schizophrenia, and mood disorders and these disorders are associated with hypoxic conditions during early development phase leading to cognitive dysfunction (Newton et al., 2013; Howell and Armstrong 2017). Experimental studies report that serum VEGF levels in autistic patients are significantly decreased suggesting the association of serum VEGF levels and increased severity of presentation (Emanuele et al., 2010; Masi et al., 2017). Decreased mRNA of VEGF in dorsolateral prefrontal cortex of schizophrenia subjects compared with controls have been observed (Fulzele and Pillai 2009). However, studies also indicate inconsistent association of plasma VEGF levels with autism (Zakareia et al., 2012) or schizophrenia (Emanuele et al., 2010; Lee et al., 2015; Pillai et al., 2016; Misiak et al., 2018; Nguyen et al., 2018).

A study by Singh showed that plasma levels of TNF-α were not significantly different in autistic subjects compared with controls (Singh 1996). However, Jyonouchi et al. studied autistic children and compared them with their healthy siblings and other controls and found that TNF-α levels were elevated in ASD children (Jyonouchi et al., 2001). These latter authors also noted that ASD peripheral blood mononuclear cells produced higher levels of TNF-α and IL-6 when activated by lipopolysaccharide, an innate immunity stimulant. This study concluded that these ASD children had aberrant innate immune response. In addition, Li et al. found that TNF-α, along with IL-6, and GM-CSF, was significantly increased in the brains of ASD subjects compared with control subjects (Li et al., 2009). Furthermore, TNF-α was also found elevated in cerebrospinal fluid (Chez et al., 2007) and peripheral blood mononuclear cells of autistic subjects (Ashwood et al., 2011b). These studies suggest that TNF-α likely plays an important role during neurodevelopment.

Finally, a role for increased GM-CSF activity in autism is suggested by the previously mentioned study by Li et al., (2009).

Given the abovementioned caveats in the animal models, we note that the current results merely warrant further follow up studies. In the present case, future studies including histological examination and gene expression analysis such as through RT-PCR analysis of brain samples from both acute- and chronic-phase animals may provide further evidence for vaccine-induced effects in the brain and how they might relate to cytokines at the levels observed.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2021.100267.

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