



# Subcutaneous injections of aluminum at vaccine adjuvant levels activate innate immune genes in mouse brain that are homologous with biomarkers of autism



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## ABSTRACT

Autism is a neurobehavioral disorder characterized by immune dysfunction. It is manifested in early childhood, during a window of early developmental vulnerability where the normal developmental trajectory is most susceptible to xenobiotic insults. Aluminum (Al) vaccine adjuvants are xenobiotics with immunostimulating and neurotoxic properties to which infants worldwide are routinely exposed. To investigate Al's immune and neurotoxic impact in vivo, we tested the expression of 17 genes which are implicated in both autism and innate immune response in brain samples of Al-injected mice in comparison to control mice. Several key players of innate immunity, such as cytokines *CCL2*, *IFNG* and *TNFA*, were significantly upregulated, while the nuclear factor-kappa beta (NF-κB) inhibitor *NFKBIB*, and the enzyme controlling the degradation of the neurotransmitter acetylcholine (*ACHE*), were downregulated in Al-injected male mice. Further, the decrease of the NF-κB inhibitor and the consequent increase in inflammatory signals, led to the activation of the NF-κB signaling pathway resulting in the release of chemokine *MIP-1A* and cytokines *IL-4* and *IL-6*. It thus appears that Al triggered innate immune system activation and altered cholinergic activity in male mice, observations which are consistent with those in autism. Female mice were less susceptible to Al exposure as only the expression levels of NF-κB inhibitor and *TNFA* were altered. Regional patterns of gene expression alterations also exhibited gender differences, as frontal cortex was the most affected area in males and cerebellum in females. Thus, Al adjuvant promotes brain inflammation and males appear to be more susceptible to Al's toxic effects.

## 1. Introduction

Autism spectrum disorders (ASD) is a heterogeneous group of neurodevelopmental disorders characterized by impairment in social interaction, verbal communication and repetitive/stereotypic behaviors [1,2]. A growing body of scientific literature shows that general immune dysfunction including various neuroimmune abnormalities (i.e., abnormal cytokine profiles, neuroinflammation and presence of auto-antibodies against brain proteins) are key pathological biomarkers in ASD patients [3–15]. Other key characteristics of autistic brains include abnormal neural connectivity [16–19], decreased number of cerebellar Purkinje cells [20–22], small cell size and increased cell packing density at all ages in the limbic system (the hippocampus, amygdala and entorhinal cortex) suggesting a curtailment in normal neuronal development [20].

It is also generally acknowledged that ASDs are complex disorders

resulting from the combination of genetic and environmental factors with multiple gene–gene and gene–environmental interactions, although there is still uncertainty about the exact proportions of each component [23]. Moreover, the molecular mechanisms of these gene–environmental interactions which result in autistic pathology remain to be discovered. Aluminum (Al) is an environmental toxin with demonstrated negative impact on human health, especially the nervous system, to which humans are regularly exposed. In particular, Al can enter the human body through various sources including food, drinking water, cosmetic products, cooking utensils and pharmaceutical products including antacids and vaccines [24–33]. In addition, Al is also present in many infant formulas [34]. However, compared to dietary Al of which only ~0.25% is absorbed into systemic circulation, Al from vaccines may be absorbed at over 50% efficiency in the short term [35] and at nearly 100% efficiency long-term [36]. Thus, vaccine-derived Al has a much greater potential to produce toxic effects in the body than

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**Table 1**  
Schedule of injections with Al hydroxide in treated mice.

Treatment group	Mouse age (days postnatal)																	Total Al injected (ug/kg body weight)
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
Aluminum		170		150	110				80		20					20	550	
Control (saline)		X		X	X				X		X					X	0	

that obtained through diet. Nonetheless, even dietary Al has been shown to accumulate in the central nervous system (CNS) over time, producing Alzheimer's disease type outcomes in experimental animals fed equivalent amounts of Al to those humans consume through a typical Western diet [26,37].

Unlike dietary Al, Al used in vaccines is specifically designed to produce a long-lasting immune response, and, in this context, rapid excretion of the adjuvant would nullify the very reason it is put in vaccine formulations. Another reason for the observed long retention of Al adjuvants in bodily compartments (i.e., 8–10 years following injection) [38,39] is due to their tight association with the vaccine antigen or other vaccine excipients (i.e., contaminant deoxyribonucleic acid - DNA) [40]. Notably, experiments in adult rabbits demonstrate that even in antigen-free form, the two predominant forms of approved and clinically used vaccine adjuvants, Al hydroxide and Al phosphate, are very poorly excreted. The cumulative amount of Al hydroxide and Al phosphate excreted in the urine of adult rabbits, as long as 28 days post intramuscular injection, was < 6% and 22% respectively as measured by accelerator mass spectrometry. It was also shown that within this timeframe, 17% of Al hydroxide and 51% of the Al phosphate was absorbed into the systemic circulation. In addition, the injected Al did not remain localized at the injection site but rather distributed to distant organs with highest amounts detected in the kidneys, followed by the spleen, liver, heart, lymph nodes and the brain [35]. In summary, the study in rabbits showed that absorption following intramuscular Al particulate injections into the blood is not instantaneous, and only some of the Al was absorbed from the injection depot over the first 28 days. These data are supported by the Khan et al. [41] study suggesting that the initial trajectory for Al hydroxide from the muscle is into the lymphatic system carried by circulating macrophages. In particular, in a series of experiments, the French group showed that Al injected in vaccine-relevant amounts into 8–10 week old mice (mimicking the amount that adult humans receive through vaccinations) is able to travel to distant organs including the spleen and the brain, where it can be detected one year after injection. The translocation of Al into the brain is dependent on the phagocytic macrophages which engulf the Al particles and carry them to the draining lymph nodes and thereafter across the blood-brain barrier, in a Trojan-horse like mechanism. This translocation is facilitated by a leaky blood-brain barrier as it was initially observed to occur in C57BL/6 mdx mouse strain (with leaky blood brain barrier) [41]. Subsequently however, it was also observed to occur in wild type mouse strains such as the CD-1 strain [42] which was also the model used in our study. Moreover, the subcutaneously injected Al appears to travel much faster to the brain than intramuscularly injected Al. Notably while no brain translocation of Al was observed by day 270 post-injection in CD-1 mice, subcutaneous injection showed early brain translocation at day 45 post Al-injection, at a dose of 200 µg Al/kg [42].

Collectively, these findings refute the notion that adjuvant nanoparticles remain localized and act through a “depot effect”. On the contrary, Al derived from vaccine formulations can cross the blood-brain and blood-cerebrospinal fluid barriers and incite immunoinflammatory responses in neural tissues [43–46]. These observations led Khan et al. [41] to suggest that repeated doses of Al hydroxide may be “insidiously unsafe”, especially in closely-spaced immune challenges presented to an infant or a person with damaged blood-brain or

cerebrospinal fluid barriers [41]. The problem with vaccine-derived Al is thus twofold: it drives a prolonged immune response even in the absence of a viral or bacterial threat and, it can make its way into various organ systems producing untoward effects. Some of the toxic actions of Al on the nervous system include: disruption of synaptic activity, misfolding of crucial proteins, promotion of oxidative stress, activation of microglia and the induction of neuroinflammatory responses [24,28,33,45,47,48]. Moreover, by its ability to stimulate macrophages to produce pro-inflammatory mediators [49,50], Al may trigger systemic inflammatory responses. Altogether, these observations show that the adjuvant form of Al has a unique potential to induce neuroimmune disorders, including those of the autism spectrum.

Given that infants worldwide are regularly exposed to Al adjuvants through routine pediatric vaccinations, it seemed warranted to reassess the neurotoxicity of Al in order to determine whether Al may be considered as one of the potential environmental triggers involved in ASD.

In order to unveil the possible causal relationship between behavioral abnormalities associated with autism and Al exposure, we initially injected the Al adjuvant in multiple doses (mimicking the routine pediatric vaccine schedule) to neonatal CD-1 mice of both sexes. The amount of the adjuvant was the equivalent to what children receive during the pediatric vaccination visits in their first year of life. The doses injected (Table 1) were also comparable to the dose used by the French group (200 µg Al/kg) in their experiment which demonstrated early brain translocation of Al adjuvant in adult 8 week old CD-1 mice following subcutaneous injection [42], the same route of exposure used by us. At six months of age, our male and female mice injected with Al in the early post-natal period, exhibited a range of altered behaviors [51].

In autism, the adverse neurobehavioral alterations are presumed to reflect underlying alterations in CNS structure and/or function. In the present study, those previously observed adverse behavioral outcomes appear to be confirmed at the molecular level. We detected an excessive activation of inflammatory factors in specific brain areas as a result of Al-injection both in male and female mice, though males were more severely affected. Our results are consistent with the observed male susceptibility bias in ASD [52]. Furthermore, the present findings underscore the well-established intrinsic connection between excessive immune stimulation (in our case induced by Al) and subsequent alteration of normal neurodevelopmental pathways, thus substantiating the notion that immunological alterations during critical periods of early development play a crucial role in the pathology of neurobehavioral disorders including those of the autism spectrum [4,9,53–56].

## 2. Materials and methods

### 2.1. Animals and breeding

Previous studies in our laboratory showed behavioral and motor deficits in CD-1 mice following Al adjuvant injection and thus for comparison purposes this same strain was chosen [44,45,51]. Male and female CD-1 breeders were obtained from Charles River (Wilmington, MA). All animals were housed at the Jack Bell Research Centre Animal Care Facility in Vancouver, BC, Canada. Females and males were housed separately (apart from for breeding purposes) at no more than five animals per cage and at an ambient temperature of 22 °C and a 12/

12 h light cycle. All mice were fed Purina mouse chow and water ad libitum. For the purposes of breeding, three female and three male mice of 16 weeks of age were housed together (total of four cages of breeders). Following impregnation, males were removed from the breeder's cage and housed separately and the females were monitored for the parturition date, which was taken as postnatal day (PND) 0. After birth, the pups from the four litters were distributed at PND3, so that each litter consisted of 14 pups. Injections were started at PND3 (Table 1). All mice were weaned at PND35 (five postnatal weeks) and were kept housed at three to five animals per cage until the end of the experiment. Mice were weighed every two days until they were 10 weeks of age and from then on they were weighed once a week. At 16 weeks of age the mice were euthanized and the brain tissues were collected for gene expression profiling experiments. The brain samples of five males and five females injected with AI and five males and five female control mice were randomly paired for gene expression profiling. One half of the brain sample from each mouse was used for semi-quantitative polymerase chain reaction (PCR) analyses, and the other half was used for Western blotting. The brain samples of five mice from each group were used for brain region-specific gene expression profiling. The experiments for each mouse were repeated three times for statistical purposes. All experimental procedures on animals were approved by the University of British Columbia (UBC)'s Animal Care Committee (protocol #A11-0042) and were in compliance with the Canadian Council on Animal Care regulations and guidelines.

## 2.2. Aluminum adjuvant

Alhydrogel®, an Al hydroxide (Al(OH)<sub>3</sub>) gel suspension, was used as a source of Al hydroxide. Alhydrogel is manufactured by Superfos Biosector a/s (Denmark) and was purchased from SIGMA Canada. Al hydroxide and Al phosphate are the two most commonly used adjuvants in clinically approved vaccines, although they differ in physicochemical properties as well as cytotoxicity [57]. In order to be able to make valid comparisons, we opted to use Al hydroxide as this is the form we used in our previous work [44,45,51].

## 2.3. Dosage and administration

We sought to mimic the U.S. vaccination schedules as closely as practically possible in our mouse model [51]. For this purpose, CD-1 mouse pups were divided in two groups: the U.S. vaccination schedule group, and saline control, each consisting of 5 males and 5 females. Since most pediatric vaccinations are given to children before the age of 2 years, we spread out the schedule of Al injections over the first three postnatal weeks which approximately corresponds to a human equivalent of 0–2 years of age (Table 1). The dosages of Al adjuvant administered to mice were approximately equivalent (μg/kg) to those administered to children in the U.S. The U.S. schedule received six injection of Al hydroxide (at 170, 150, 110, 80, 20 and 20 μg/kg body weight respectively), for a total of 550 μg/kg body weight (Table 1). The treated mice were injected subcutaneously into the loose skin behind the neck (the “scruff”) to minimize discomfort and for the ease of injection. We recognize that most pediatric vaccines are administered intramuscularly, but we aimed to follow as closely as possible the conditions of previous studies from our laboratory [44,45,51] in order to be able to make valid comparisons. Mice up to 12 days postnatal were injected with a micro-needle while older mice were injected with a standard 30 G needle. The total injection volume for each animal was 15 μL of either Al hydroxide in saline or saline alone.

## 2.4. Ribonucleic acid (RNA) extraction

RNA extractions were carried out with the PureLink RNA Mini kit manual (Invitrogen) according to the manufacturer's instructions. Briefly, 100 mg of each brain tissue was ground thoroughly to powder

in liquid nitrogen. 1.5 mL of lysis buffer prepared with 2-mercaptoethanol was added to the sample which was then homogenized for 45 s using a rotor-stator at maximum speed. The sample was then centrifuged at 12,000 × g for 2 min at room temperature and the supernatant transferred to a new ribonuclease-free tube. One volume of 70% ethanol was then added to the tissue homogenate which was mixed thoroughly and 700 μL of the sample transferred to a spin cartridge. The sample was centrifuged at 12,000 × g for 15 s at room temperature. 700 μL wash buffer I was then added to the spin cartridge and further centrifuged at 12,000 × g for 15 s at room temperature. 500 μL of wash buffer II with ethanol was added to the spin cartridge and centrifuged at 12,000 × g for 15 s at room temperature. An additional centrifuge step at 12,000 × g for 1 min at room temperature was performed to dry the membrane with attached RNA. Finally, 50 μL of ribonuclease-free water was added to the center of the spin cartridge and the sample was incubated at room temperature for 1 min. To elute the sample, a final centrifuge step was carried out, 2 min at ≥ 12,000 × g at room temperature.

## 2.5. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

1 μg of total RNA was heated at 65 °C for 5 min and used as a template for the first strand cDNA synthesis. The reaction mixture contained 3 μg of random hexadeoxynucleotide primers (Invitrogen), 1 mM of deoxynucleotide (dNTP) mix (Invitrogen), 40 units of ribonuclease Inhibitor (Invitrogen), 1 × reverse transcription buffer (Invitrogen) and 40 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Invitrogen) in a final volume of 20 μL. The reaction mixture was incubated at 42 °C for 1 h, heated to 92 °C for 10 min. Non-reverse transcribed RNAs were included in each PCR reaction to exclude the contamination of genomic DNA. Housekeeping gene β-Actin was used as an input control. The gene-specific primers used for RT-PCR reactions were all located in the first exon of each gene producing around 200 bp PCR products. The sequences of the specific primers are listed in Table 2. PCR was performed using 2 μL of 5 × diluted first strand cDNA on a Perkin Elmer 9600 thermocycler in a total volume of 20 μL with 1 unit of Taq polymerase (Invitrogen), 0.2 mM of dNTP mix (Invitrogen), 1 × PCR buffer (Invitrogen) and 0.20 μM of each primer. The standard program comprised 30 cycles of 45 s at 95 °C, 45 s at 55 °C and 30 s at 72 °C. PCR products were detected by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (EtBr) and visualized under ultraviolet light by video image system (Bio-Rad). The samples which were analyzed and compared to each other (control and Al treated tissues) were loaded on the same gel following the same settings of the image analyses. Densitometric analysis of EtBr-stained gel bands was performed using ImageJ software. *t*-tests were used to compare the means of two groups.

## 2.6. Western blot analysis and antibodies

The brain tissue samples designated for Western blot analysis were placed in liquid nitrogen and snap-frozen. For a ~5 mg piece of tissue, ~300 μL lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0) was added rapidly and the tissue homogenized with an electric homogenizer and then maintained at constant agitation for 2 h at 4 °C. The sample was then centrifuged for 20 min at 12000 rpm at 4 °C. The supernatant was aspirated and the pellet discarded. 2x sample buffer (62.5 mM Tris-HCl (pH 6.8 at 25 °C), 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol) was then added to the sample. The lysate was boiled at 100 °C for 5–10 min to denature the proteins in the sample. Protein for each sample was analyzed with 10% SDS-polyacrylamide resolving gels and 5% stacking gels using a Bio-Rad Gel electrophoresis system (Bio-Rad). The Bio-Rad Wet Transfer system (Bio-Rad) was then applied to transfer proteins from gels to

**Table 2**  
DNA primers used in semi-quantitative RT-PCR.

Genes	Forward primers	Reverse primers
KLK1	TGTTCTAGCCCTGTCCCTA	TCAGGGTGAGGGATGGCTTT
NFKB1B	GAGGTGGCTGGGGCCAT	GTGTCCCCATCCTCAGTGAC
NFKB1E	TCATCCGCTCCCGCGC	TCACACTGGCTATCATCCGC
SFTPB	CTATCAGTGGGCTCATCC	ACTTCCGGATTGCTTCTGG
ACHE	CGCCTCAGACACGAGCC	AAGCTGAGACTGGGCTCC
C2	CTCCCCTAGCTCTCCGTTCT	CTCTGACATTCTCTCCAGGCT
CCL2	CTCTCTCTCTCCACCACCAT	ACTACAGCTTCTTTGGGACACC
CEBPB	CGGGACGCAGCGAGAT	GCTCGTGCTCGCCAATG
CRP	TCCCAAGGAGTCAGATACTTC	ATCGTACTTCAGCACCAACC
IFNG	AGTTCTGGGCTTCTCTCTCT	TCCTTTTGCCAGTTCTCTCCA
LTB	GAGTCTGGATGGGGACA	TTTTCTGAGCTGTGCTCCT
MMP9	TGCGTTATAGCGGAGTCTTAGG	TGGATCTCAGGAGGATGAACAG
PACRG	AGACCAACAGCCTGACGGG	CCACAGAGCTGATTCCGAAGT
SELE	ACAACAATTCCACTGAACAGAA	ATGTGTGTAGTCCCGCTGAC
SERPINE1	AGCACACAGCCAACACAG	GGACCACCTGCTGAAACACT
STAT4	GTGTAAGCCTGTCTTCTCACA	CAAAGTCAGGCTGAAGCCCC
TNFA	AAATAGCTCCAGAAAAGCAAGC	GAAGAGGCTGAGACATAGGCA
IL-4	TGTCACTGCAAAATCGACACCT	AAAAGAGTGGCAGGGGAGAAG
IL-5	TGCTTCTGCATTGTGATTGTC	TACAGCACACCAGCATTCAT
IL-6	CCACCGGGAACGAAAGAGAA	TCCTCTCTGGGGGTACTGG
IL-1 $\beta$	CAGGCTGCTCTGGGATTCTC	GGAGCAGAGGCTTTGACACT
IL-8	TCTTGGCAGCCTTCTGTATTT	TGGAATTGTCTGAGTTACCTTG
RANTES	GTCAGACTCTGCCTAGAATGC	ATATGGCTGTCTCAGGGTCTC
MIP-1 $\alpha$	ATGTGAAGCAAAATCGCAGCC	GGTGACCGTCTGGCTTTTA
iNOS	ACCAAGGTGACCTGAAAAGAGG	CTGGGTCTCTGTGCAAACTC
COX-2	CAGGAGCATCTGAATGGGG	ACAAGCTTTCAAGCAACTGGA
PLA2	TGGATCACAGTTTAGGAAATGA	AGGAGACTTGCAAGGGTTTCG
VCAM-1	TCCTGGAGGAAGGCAGTTCT	TGACACTCTCAGAAGGAAAAGC
ICAM-1	TCTAACAGCAGAGCTGCCAGG	TGCACGTGATCATGAGGGGT
E-selectin	TGGCAACCTTTATAGGGTTATGT	GGCTGAACACTGGCAACAAC
$\beta$ -ACTIN	AGGCCAGAGCAAGAGAGGT	GTGACCCGCTCTCCGGAGTC

nitrocellulose membranes (Bio-Rad) by running at 100 V for 2 h in a cold room (4 °C). The membrane was blocked with 5% non-fat milk in Tris buffered saline tween 20 buffer (TBST) for 1 h and then probed with primary antibodies diluted in 3% bovine serum albumin/TBST at 4 °C overnight. The primary antibodies included: rabbit polyclonal actin, NF- $\kappa$ B p105/p50, phospho-NF- $\kappa$ B p65, phospho-I $\kappa$ B $\beta$ , phospho-I $\kappa$ B $\alpha$  antibodies (1:1000; Cell Signaling), rabbit polyclonal acetylcholinesterase (*ACHE*), nuclear factor-kappa beta (NF- $\kappa$ B) inhibitors *NFKB1B* and *NFKB1E* antibodies (1:500; Santa Cruz), goat polyclonal C-C motif chemokine ligand 2 (*CCL2*), tumor necrosis factor alpha (*TNFA*) antibodies (1:500; Santa Cruz), goat polyclonal *IFNG* antibody (1:1000; Cell Signaling). The membrane was washed three times with TBST for 15 min and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:1000; Cell Signaling) and HRP-conjugated donkey anti-goat secondary antibody (1:500; Santa Cruz) for 1 h at room temperature. The membrane was then washed three times for 15 min and results were visualized using an enhanced chemiluminescence reaction assay (PerkinElmer Life Sciences). Image J software was used to calculate and normalize the density of protein against the actin control.

### 3. Results

#### 3.1. Gene expression alterations in Al-injected female and male mice

In order to investigate the effect of early post-natal pediatric Al adjuvant exposure in a mouse model, we mimicked the U.S. vaccination schedules as closely as practically possible by subcutaneously administering Al to neonatal mice (Table 1). Following the completion of behavioral testing (results presented in a prior publication [51]), mice were euthanized, and the whole brain tissues were collected and applied to subsequent gene expression profiling. We sought to examine the effect of Al injection on the expression of specific immune markers in mice brains, in order to determine the potential causal link between

Al exposure and ASD through hyperactivation of immune markers in the brain. Herbert et al. [58] reported 46 inflammatory genes that overlapped with ASD susceptibility genes that served our purpose, yet this was too broad of a range to be experimentally investigated. Given that Al carried by circulating macrophages is expected to mainly induce an innate immune response, we focused our efforts on innate immune system-related molecules associated with macrophage function. We thus cross-checked the 46 inflammatory genes identified by Herbert et al. [58] with the Innate Immune Database (<http://www.innatedb.com/>) to identify those with innate immune function and on the basis of this, 17 genes were selected (Table 3) for experimentally assessing gene expression variation in response to Al injection. We also selected for the purposes of our investigation the gene encoding *ACHE*, the enzyme that catalyzes the breakdown of acetylcholine (ACh) and other choline esters that function as neurotransmitters, in addition to genes with specific function in the immunoinflammatory response. The gene product of *ACHE* is found predominantly at neuromuscular junctions and cholinergic synapses, where its activity serves to terminate synaptic transmission. The reason for this choice was two-fold: first, studies suggest that the activity of *ACHE* gene product (AChE) is altered in autism and that this alteration correlates with deficits in social functioning [59]; second, it is further known that many neurotransmitters play significant immunomodulatory roles and ACh in particular has been shown to dampen the immunoinflammatory response. Hence the inhibition of the degradatory activity of AChE in the CNS results in the suppression of the humoral immune response, while conversely, the inhibition of ACh synthesis causes the enhancement of the immune response [60,61].

We first examined the gene and protein expression changes in whole brain samples. The semi-quantitative RT-PCR analyses of the samples of Al-injected CD-1 male mice revealed 7 gene expression alterations in comparison to control male mice, including four genes which showed upregulation (*CCL2*, interferon gamma (*IFNG*), lymphotoxin beta (*LTB*), and *TNFA*) and three which were downregulated (*ACHE*, C-reactive



**Table 3**  
The molecular function of genes of interest.

Genes	Full names	Function
KLK1	Kallikrein 1	A subgroup of serine proteases implicated in carcinogenesis.
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	The protein encoded by this gene belongs to the NF-kappa-B inhibitor family, which inhibits NF-kappa-B by complexing with, and trapping it in the cytoplasm.
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	The protein encoded by this gene binds to components of NF-kappa-B, trapping the complex in the cytoplasm and preventing it from activating genes in the nucleus.
SFTPB	Surfactant protein B	This gene encodes the pulmonary-associated surfactant protein B (SPB), an amphipathic surfactant protein essential for lung function and homeostasis after birth.
ACHE	Acetylcholinesterase	Acetylcholinesterase hydrolyzes the neurotransmitter, acetylcholine at neuromuscular junctions and brain cholinergic synapses, and thus terminates signal transmission.
C2	Complement component 2	A serum glycoprotein that functions as part of the classical pathway of the complement system.
CCL2	Chemokine (C-C motif) ligand 2	Chemokines are a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes.
CEBPB	Enhancer binding protein (C/EBP), beta	Activity of this protein is important in the regulation of genes involved in immune and inflammatory responses.
CRP	C-reactive protein	It is involved in several host defense related functions based on its ability to recognize foreign pathogens and damaged cells of the host and to initiate their elimination by interacting with humoral and cellular effector systems in the blood.
IFNG	Interferon gamma	The protein encoded is a soluble cytokine with antiviral, immunoregulatory and anti-tumor properties and is a potent activator of macrophages.
LTB	Lymphotoxin beta	An inducer of the inflammatory response system and involved in normal development of lymphoid tissue.
MMP9	Matrix metalloproteinase 9	Involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling.
PACRG	PARK2 co-regulated	The parkin co-regulated gene protein forms a large molecular complex with chaperones, including heat shock proteins 70 and 90, and chaperonin components.
SELE	Selectin E	Found in cytokine-stimulated endothelial cells and is thought to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining.
SERPINE1	Serpin peptidase inhibitor, clade E	The principal inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), and an inhibitor of fibrinolysis.
STAT4	Signal transducer and activator of transcription 4	In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators.
TNFA	Tumor necrosis factor alpha	This cytokine is mainly secreted by macrophages, involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.

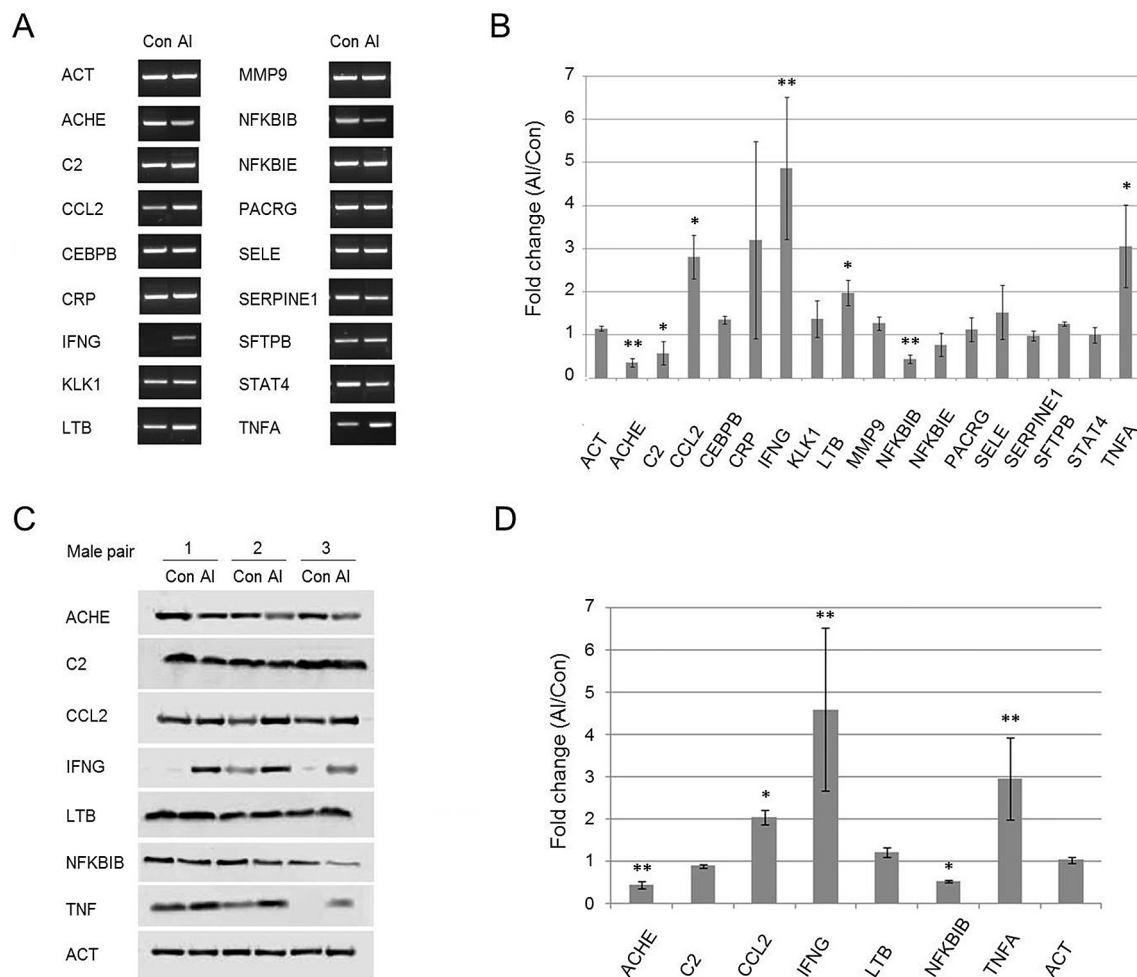
protein (C2), and *NFKBIB* (Fig. 1A, B). Five out of 7 gene expression alterations were verified at the protein level on Western blots (Fig. 1C, D), including *IFNG* and *TNFA* both of which are multifunctional proinflammatory cytokines and macrophage activators [62,63] that exhibited more than three-fold increase. Next in the sequence of significantly upregulated genes and its corresponding protein product was *CCL2*, a macrophage-secreted chemokine, also known as monocyte chemoattractant protein-1 (MCP-1) [64]. In contrast, the expression levels of both *ACHE* and *NFKBIB* were significantly decreased in Al-injected male mice. We next examined the expression pattern of genes of interest in female mice. Two gene expression alterations, namely, upregulation of *TNFA* and downregulation of *NFKBIE* were identified in whole brain samples of Al-injected female mice compared to control female mice (Fig. 2A, B). Both of these gene expression alterations were confirmed at the protein level by the Western blot analysis (Fig. 2C, D). Taken together, a number of changes indicative of the activation of the immune-mediated NF- $\kappa$ B pathway were observed in both male and female mice brains as a result of Al-injection, although females seemed to be less susceptible than males as fewer genes were found altered in female brains.

### 3.2. Deactivation of NF- $\kappa$ B inhibitor in Al-injected male mice

Having established that the NF- $\kappa$ B inhibitors in both male and female were downregulated by Al exposure, we sought to determine whether NF- $\kappa$ B signaling activity was affected by Al injection. NF- $\kappa$ B is composed of two subunits: p65 (RelA) and p50 (NF- $\kappa$ B1). p50 is synthesized as longer precursor molecules of p105 which is further processed to smaller, transcriptionally active forms. p65 activity requires enhancement by phosphorylation [65,66]. NF- $\kappa$ B exists in the cytoplasm in an inactive form associated with inhibitory proteins termed

I $\kappa$ B, of which the most important ones may be I $\kappa$ B $\beta$  encoded by *NFKBIB* gene, and I $\kappa$ B $\epsilon$  encoded by *NFKBIE* gene. Upon exposure to activation signal such as TNF- $\alpha$ , the I $\kappa$ B is phosphorylated, degraded and released from the NF- $\kappa$ B heterodimer [65–68]. Thus, the activation of NF- $\kappa$ B depends on three elements: processing of p50 from its precursor p105, and phosphorylation of p65 and I $\kappa$ B. To determine if NF- $\kappa$ B was activated in the brains of Al-injected mice, we analyzed p50, p65 and I $\kappa$ B in detail by using phosphorylation-specific antibodies of p65, I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$ , and p105/p50 antibody which detects endogenous levels of the precursor protein p105 and its cleavage product p50. As shown by the Western blotting results (Fig. 3A), in both male and female mice exposed to Al, the phosphorylation level of p65 was comparable between Al-injection group and control group. Similarly, the p105/p50 ratio of Al-injection group was comparable to that of the control group in both males and females (Fig. 3B).

At this point, we focused our investigation on the phosphorylation level of I $\kappa$ B $\beta$  in male mice and I $\kappa$ B $\epsilon$  female mice given that the transcription of *NFKBIB* declined in males and *NFKBIE* in females. The Western blotting results showed that the phosphorylation of I $\kappa$ B $\beta$  was activated in the brains of male Al-injected mice in contrast to control male mice (Fig. 3C), indicating the deactivation of NF- $\kappa$ B inhibitor  $\beta$ . However, I $\kappa$ B $\epsilon$  in Al-injected females remained unphosphorylated, as in control females (Fig. 3C). Overall, these results show that Al injection activated the NF- $\kappa$ B by deactivating the NF- $\kappa$ B inhibitor in the male brain, while it appeared to have no such influence on the female brain. In addition, the decreased transcription of *NFKBIB* and disabled I $\kappa$ B $\beta$  protein seem to form a negative feedback loop, in which less protein is required, and thus less protein is produced.



**Fig. 1.** Gene expression alterations in the brains of Al-injected male mice. A) Semi-quantitative RT-PCRs revealed that *ACHE*, *C2* and *NFKBIB* were downregulated, while *CCL2*, *IFNG*, *LTB* and *TNFA* were upregulated in the brains of Al-injected male mice compared with control mice.  $\beta$ -Actin was used as internal control. Al: Al-injected mice, Con: control mice. B) Quantification of gene expression level change. Values presented are the average of five independent experiments and were determined by densitometry. The results are expressed as mean  $\pm$  standard error of the mean (SEM). C) The downregulation of *ACHE* and *NFKBIB* and upregulation of *CCL2*, *IFNG*, and *TNFA* were validated at protein level, as measured by Western blotting.  $\beta$ -Actin was included as a loading control. D) Quantification of the data shown in C). The histograms show the mean  $\pm$  SEM of five independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

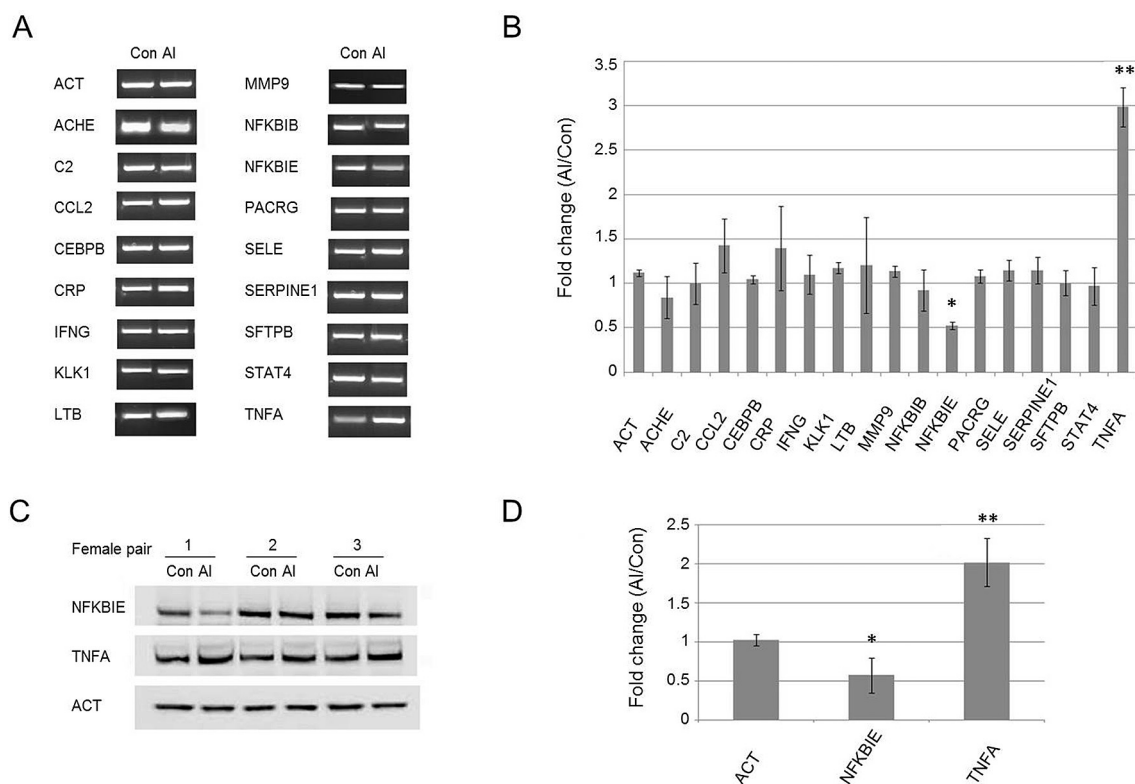
### 3.3. Activation of NF- $\kappa$ B pathway in Al-injected male mice

After activation, NF- $\kappa$ B induces the transcription of proinflammatory mediators of the innate immune response, including the cytokines *TNF- $\alpha$* , interleukins *IL-1 $\beta$* , *IL-4*, *IL-5*, *IL-6*, chemokines *IL-8*, *MCP-1*, macrophage inflammatory protein (*MIP*)-1 $\alpha$ , regulated on activation, normal T cell expressed and secreted (*RANTES*), enzymes cyclooxygenase (*COX*)-2, inducible nitric oxide synthase (*iNOS*), phospholipase A2 (*PLA*2), and adhesion molecules intercellular adhesion molecule (*ICAM*)-1, *E-selectin*, vascular cell adhesion molecule (*VCAM*)-1. These molecules are important components of the innate immune response to invading substance and are required for the migration of inflammatory and phagocytic cells to tissues where NF- $\kappa$ B has been activated in response to infection or injury [65,69]. As cited above, *TNF- $\alpha$*  acts, as a signal to launch the NF- $\kappa$ B pathway and simultaneously serves an inflammatory effector transcriptionally activated by NF- $\kappa$ B [69,70]. As already noted, *TNFA* gene expression was significantly upregulated in both male and female Al-injected mice (Fig. 1A, D; Fig. 2A, D). In an attempt to explore whether NF- $\kappa$ B activation led to the upregulation of the downstream components of the NF- $\kappa$ B signaling pathway besides *TNFA*, we conducted semi-quantitative RT-PCRs to measure the expression levels of all these NF- $\kappa$ B-dependent proinflammatory mediators in the brain tissues of both male and female mice. As anticipated, besides *TNFA*, other chemokine and cytokines

were identified with elevated expression levels in the brains of Al-injected male mice, including macrophage-inflammatory protein *MIP*-1 $\alpha$ , interleukins *IL*-4, and *IL*-6 (Fig. 4A–D). However, no further upregulation was detected in the female mice brains apart from *TNFA* (data not shown). Together, these results strongly suggest that the NF- $\kappa$ B pathway was activated in the brains of Al-injected male mice, resulting in the expression of excessive levels of immune and proinflammatory factors.

### 3.4. Brain region-specific gene expression in Al-injected female and male mice

We used whole brain tissues to obtain the above results of expression alterations in male and female mice. As it was of interest to further uncover if the detected alterations were region-specific, frontal cortex, hippocampus, thalamus, and cerebellum, the areas reported to be most affected in autism [6–8,71,72] were dissected and utilized in subsequent Western blot assays. In Al-injected male mice, 4 out of 5 expression alterations were enriched in frontal cortex (Fig. 5A). Most notably, the upregulations of *TNFA* and *IFNG* which exhibited the highest fold change and showed multiple concentration areas, while alterations of *ACHE*, *CCL2* and *NFKBIB* were accumulated in only one area (Fig. 5A). The Al-injected females predictably exhibited fewer changes, namely, the upregulation of *TNFA* and downregulation of



**Fig. 2.** Gene expression alterations in the brains of Al-injected female mice. A) Semi-quantitative RT-PCRs revealed that *NFKBIE* was downregulated, while *TNFA* was upregulated in the brains of Al-injected female mice compared with control mice.  $\beta$ -Actin was used as internal control. B) Quantification of gene expression level change. Values presented are the average of five independent experiments and were determined by densitometry. The results are expressed as mean  $\pm$  SEM. C) The gene expression alterations of *NFKBIE* and *TNFA* were validated at protein level, as measured by Western blotting.  $\beta$ -Actin was included as a loading control. D) Quantification of the data shown in C). The histograms show the mean  $\pm$  SEM of five independent experiments, as measured by western blotting. \* $p < 0.05$ , \*\* $p < 0.01$ .

*NFKBIE* in the cerebellum (Fig. 5B). In summary, gene expression alterations displayed a brain region-specific pattern in both male and female mice. Most of the expression alterations were concentrated in the frontal cortex of Al-injected male mice, while the cerebellum appeared to be the key structure showing expression alterations in Al-injected female mice.

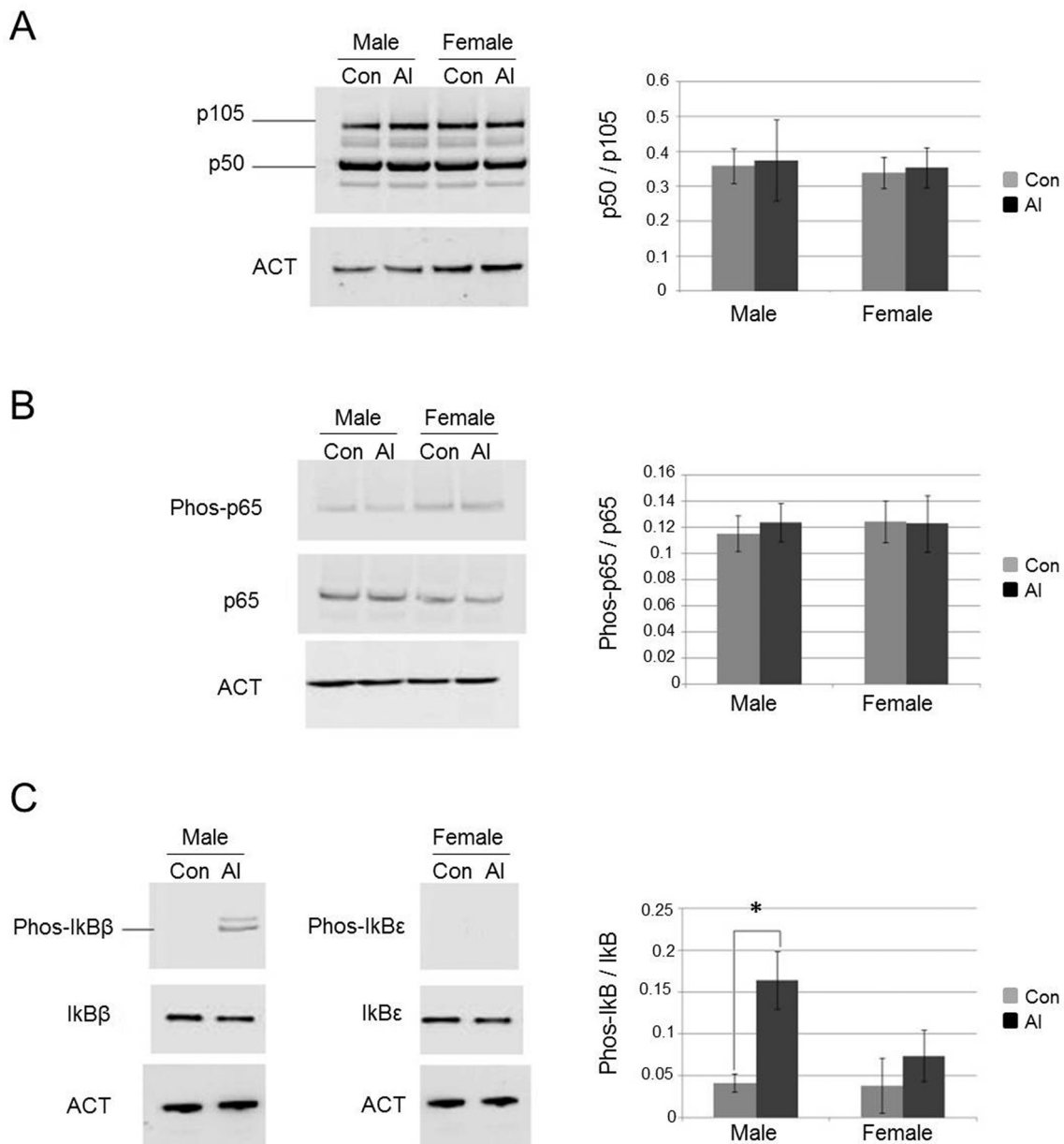
## 4. Discussion

### 4.1. Autism as a neuroimmune and neuroinflammatory disorder

Autism is widely recognized as a disease with an underlying immunoinflammatory component, although heretofore little has been known about the molecular mechanisms responsible for the observed immune abnormalities [4–15]. In our current mouse model, subcutaneous injection of the Al adjuvant induced the activation of several key players of the innate immune response driven by NF- $\kappa$ B activation in various brain regions, most prominently in the frontal cortex of males and in the cerebellum of females. Specifically the expression levels of *CCL2*, *IFNG* and *TNFA* were significantly upregulated, while the NF- $\kappa$ B inhibitor *NFKBIB* was downregulated in the brains of Al-injected male mice (Fig. 1B). In addition, the decrease of the NF- $\kappa$ B inhibitor and the consequent increase in inflammatory signals led to the elevation of NF- $\kappa$ B – responsive genes including *MIP-1A*, *IL-4* and *IL-6*. Female mice appeared to be less susceptible to Al's neuroinflammatory effects as only the expression levels of NF- $\kappa$ B inhibitor and *TNFA* were altered in Al-injected females (Fig. 2B). Some of the observed alterations in gene expression were also confirmed at the protein level, such as the increase of CCL2, IFN- $\gamma$  and TNF- $\alpha$  in male brains (Fig. 1D) and the corresponding increase in IFN- $\gamma$  and TNF- $\alpha$  in female brains (Fig. 2D). We further detected elevated levels of phosphorylated I $\kappa$ B $\beta$  in the brains of

Al-injected male mice (Fig. 3C), indicating the deactivation of the NF- $\kappa$ B inhibitor  $\beta$ . However, I $\kappa$ B $\beta$  in Al-injected females remained unphosphorylated, as in control females (Fig. 3C). Overall, these results show that Al injection activated NF- $\kappa$ B by deactivating the NF- $\kappa$ B inhibitor in the male brain, while it appeared to have less influence on the female brain. Furthermore, Al downregulated the gene expression of *ACHE*, the enzyme that controls the degradation of the neurotransmitter ACh in male mouse brain. In summary, Al triggered the innate immune response in the brain and altered cholinergic activity in male mice. Altogether, our results indicate that the Al adjuvant may impair brain function by interacting with neural and immune system mediators and by promoting inflammation and that males are more susceptible to this type of Al toxicity.

The ability of Al to influence gene expression in the brain has been previously well established by Lukiw et al. [27], in particular, at nanomolar concentrations, Al can bind to DNA and inhibit transcription from selected AT-rich promoters of human neocortical genes [27]. Al's repressive action on gene transcription is linked to its ability to 1) decrease the access of transcriptional machinery to initiation sites on DNA template by enhancing chromatin condensation [73,74]; and/or 2) interfere with adenosine triphosphate (ATP)-hydrolysis-powered separation of DNA strands either indirectly (by binding to phosphonucleotides and increasing the stability and melting temperature of DNA) or directly (by inhibiting the ATPase-dependent action of RNA polymerase) [27,73–76]. These effects were experimentally demonstrated at physiologically-relevant Al concentrations (10–100 nM) [27,77] and at levels that have been reported in Alzheimer disease patients' chromatin fractions [76]. Moreover, in addition to its direct and repressive action on gene expression, Al can directly promote transcription while indirectly promoting lipid peroxidation and oxidative stress. In this manner, Al can activate the reactive oxygen species (ROS)-sensitive



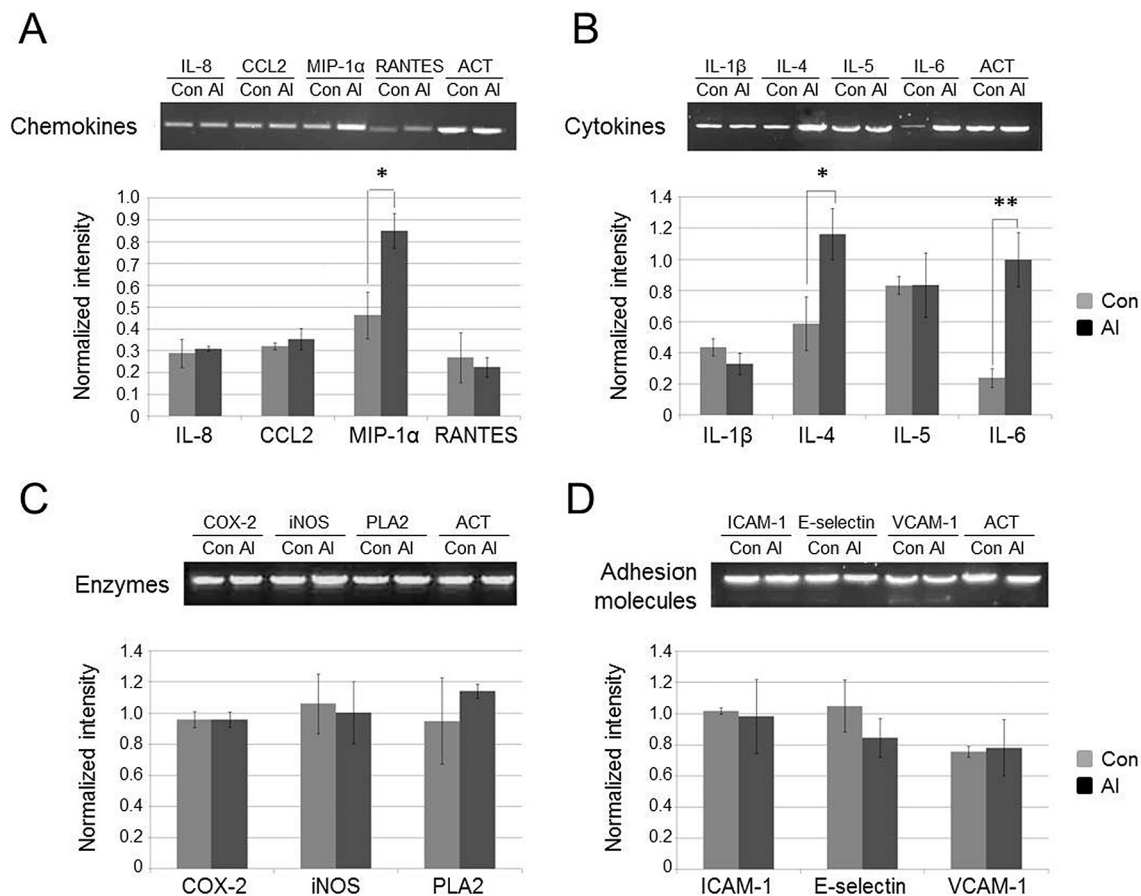
**Fig. 3.** Deactivation of NF- $\kappa$ B inhibitor in the brains of AI-injected male mice. A) Processing of p50 from its precursor p105 remained unchanged in both AI-injected male and female mice in comparison to control male and female mice.  $\beta$ -Actin was included as a loading control. Protein levels of p50 and p105 were quantified by normalization to  $\beta$ -Actin. The histograms show the mean  $\pm$  SEM of four independent experiments. B) Phosphorylation level of p65 was comparable between AI-injection group and control group in both female and male mice. Phosphorylated p65 (phos-p65) and total p65 were quantified by normalization to  $\beta$ -Actin. The histograms show the mean  $\pm$  SEM of four independent experiments. C) Phosphorylation of I $\kappa$ B $\beta$  (phos-I $\kappa$ B $\beta$ ) was dramatically increased in the brains of AI-injected male mice in contrast to the control male mice, while I $\kappa$ B $\epsilon$  in AI-injected female remained unphosphorylated as in the control female mice. Phosphorylated and total NF- $\kappa$ B inhibitors were quantified by normalization to  $\beta$ -Actin. The histograms show the mean  $\pm$  SEM of five independent experiments. \* $p < 0.05$ .

transcription factors, hypoxia inducible factor-1 (HIF-1) and NF- $\kappa$ B and augment specific neuroinflammatory and pro-apoptotic signaling cascades by driving the expression from a subset of HIF-1 and NF- $\kappa$ B-inducible promoters [78,79]. Both HIF-1 and NF- $\kappa$ B are upregulated in Alzheimer's disease where they fuel the proinflammatory cycle which leads to further exacerbation of oxidative stress and inflammation, culminating in neuronal death [80].

A prolonged inflammatory response and oxidative stress, as well as immune dysfunction, underlie behavioral impairments in autism [7,8,13,14,81–84]. Indeed, numerous studies showed elevated proinflammatory cytokine levels both in serum and brain specimens of autism patients [7,14,15,85] which were in some cases correlated with impaired behavioral outcomes [14]. Of specific interest in context to the present study are neuropathological post-mortem examinations on

autistic brains conducted by Vargas et al. [7] which showed evidence of an active neuroinflammatory process in the cerebral cortex and the cerebellum with extensive loss of cerebellar Purkinje cells. Vargas et al. [7] studied both male and female brain specimens and although their analysis did not separate between the sexes it should be noted that in our animal model, the frontal cortex was the most affected area in males and cerebellum in females (Fig. 5). The most prominent pathological findings by Vargas et al. [7] were marked reactivity of the Bergmann's astroglia in areas of Purkinje cell loss within the Purkinje cell layer, as well as marked astroglial reactions in the granule cell layer and cerebellar white matter. It should be further noted that the ability of adjuvant AI to induce dramatic activation of glial cells has been repeatedly demonstrated [45,47]. Moreover, the cytokine profiling conducted by Vargas et al. [7] indicated that MCP-1 (also known as CCL2)





**Fig. 4.** Activation of inflammatory genes involved in NF- $\kappa$ B signaling pathway in the brains of AI-injected male mice. The expression levels of all the NF- $\kappa$ B-dependent A) chemokines, B) cytokines, C) enzymes and D) adhesion molecules, were measured by semi-quantitative RT-PCRs in male brain tissues.  $\beta$ -Actin was used as internal control. The gene expression levels were quantified by normalization to  $\beta$ -Actin. Values presented are the average of five independent experiments and were determined by densitometry. Error bars show mean  $\pm$  SEM. \* $p$  < 0.05, \*\* $p$  < 0.01.

and tumor growth factor (TGF)- $\beta$ 1, derived from neuroglia, were the most prevalent cytokines in autistic compared to control brain tissues. The cerebrospinal fluid derived from autistic patients also showed a unique proinflammatory profile of cytokines, including a marked increase in MCP-1 [7]. Higher levels of IL-6 were also observed in the prefrontal cortex and anterior cingulate gyrus of autism brain specimens compared to controls. Similarly, CCL2/MCP-1 and IL-6 were two of the key cytokines found elevated in male mice in our study (Fig. 1B, D; Fig. 4A, B), validating our animal model. Altogether these observations suggest that the autistic brain is a result of a disease process that arises from altered activity of immune-related pathways in the brain. Other evidence in support of this interpretation is the frequent finding of autoimmune manifestations, particularly those affecting the CNS, in autistic individuals who appear to have more widespread biochemical changes [3]. Immune abnormalities in ASD are also present outside and beyond the nervous system. Indeed, a large body of data points to a role of systemic immune system dysregulation in the pathophysiology of ASD which is likely to precede the inflammatory and autoimmune manifestations in the brain [5,6,9,10,86,87].

#### 4.2. Cytokine imbalances in autism

Abnormalities in the levels of cytokines and chemokines are as mentioned, an important pathological feature in the autistic brain and it is proposed that they may be the result of both genetic and environmental factors. Furthermore, the cytokine aberrations may directly contribute to autistic neurological dysfunctions [7,8,14,15,88,89]. The immune system and the nervous system are in constant communication

and this communication is mainly mediated by immune cytokines. Thus, cytokines are known to influence both the development and the function of the nervous system. They influence cell differentiation and migration, establishment of synaptic connections, the release and biosynthesis of neurotransmitters, and are involved in diverse processes including cognition and memory, regulation of circadian rhythms, thermoregulation, endocrine and autonomic functions [4,15,90–92]. It is therefore not surprising to find aberrations in cytokine and chemokine levels in both neurodevelopmental and neurodegenerative disease states [93–96]. In autism, cytokine imbalances are thought to impair the proper structural development of the brain and consequently impact behavior [4,7,8,14,15,85,88,97]. Among the most notable cytokines and chemokines found to be deregulated in autism are IL-1 $\beta$ , IL-6, IL-4, MCP-1/CCL2, IFN- $\gamma$ , TGF- $\beta$  and TNF- $\alpha$ , all of which, with the exception of IL-1 $\beta$  and TGF- $\beta$  were found to be upregulated in our AI-injected mice. Notably, elevated levels of these cytokines and chemokines are likewise seen in autistic patients, while TGF- $\beta$  is decreased in autism [7,8,14,15,88,89].

#### 4.3. Aberrant activation of the NF- $\kappa$ B pathway in autism

The inducible transcription factor NF- $\kappa$ B is a central regulator of the immune response. The activation of the NF- $\kappa$ B signaling pathway induces the expression of numerous inflammatory cytokines and chemokines and leads to the innate immune response in mammals. NF- $\kappa$ B is present in almost all cell types where it mediates cellular responses to a variety of stress stimuli (such as oxidative stress and antigen exposure) and mediates the expression of a wide array of immunoregulatory genes

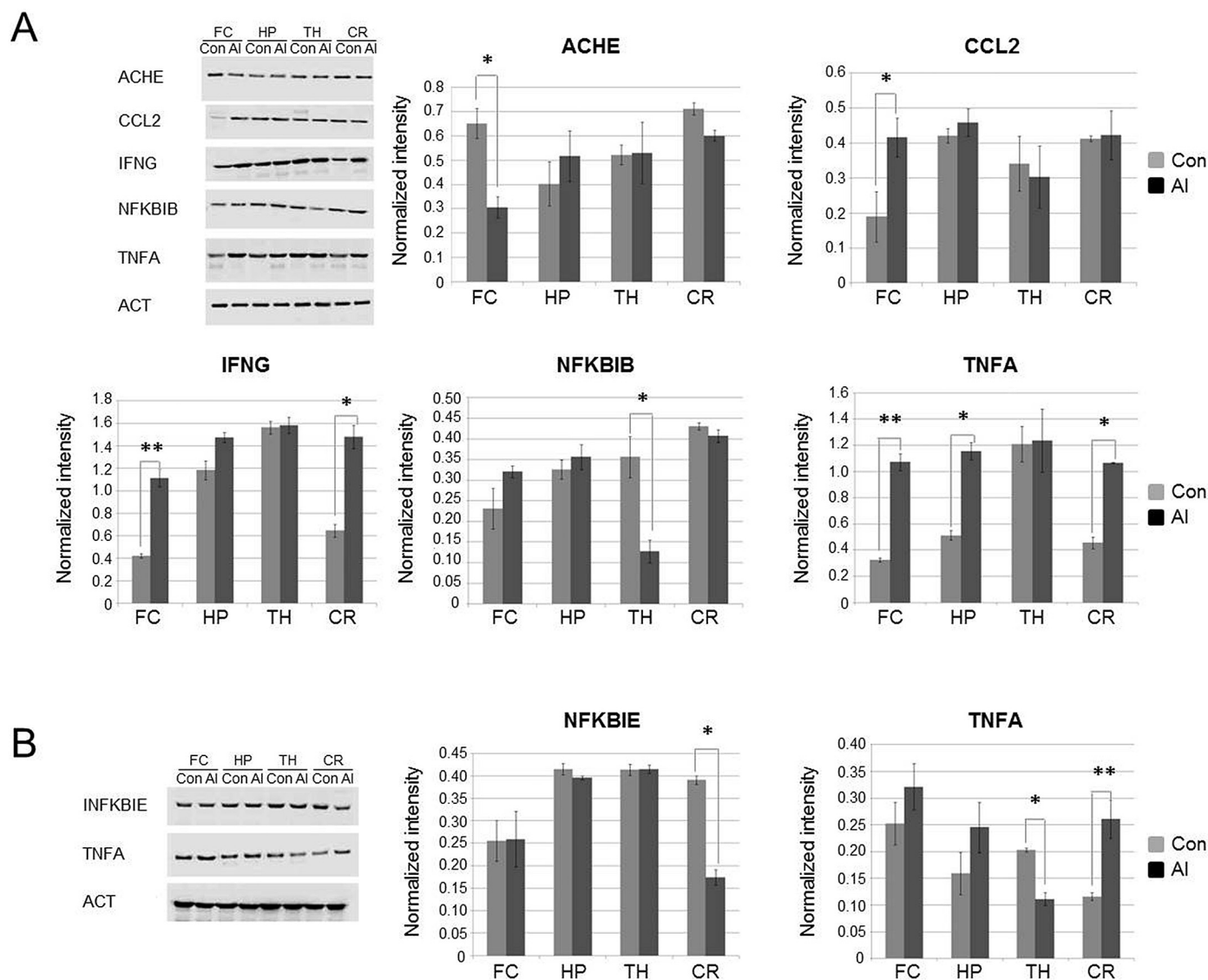


Fig. 5. Distinctive patterns of brain region-specific gene expression between AI-injected female and male mice. A) Brain region-specific gene expression in male mice examined by western-blotting. FC: frontal cortex, HP: hippocampus, TH: thalamus, CR: cerebellum. Quantification of protein levels was normalized to  $\beta$ -Actin. The histograms show the mean  $\pm$  SEM of five independent experiments. B) Brain region-specific gene expression in female mice examined by western-blotting. Quantification of protein levels was calculated by normalization to  $\beta$ -Actin. The histograms show the mean  $\pm$  SEM of five independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

[65,68,98–101]. Under normal conditions, NF- $\kappa$ B is present in the cytoplasm as an inactive heterotrimer, with its two subunits p65 and p50 associated with the inhibitory protein I $\kappa$ B. Stimulation with a proinflammatory cytokine such as TNF- $\alpha$  and IFN- $\gamma$ , activates the I $\kappa$ B kinase complex, triggering the degradation of I $\kappa$ B and allowing free NF- $\kappa$ B heterodimer to translocate into the nucleus and activate the expression of immune and inflammatory-related genes [65–68]. Since NF- $\kappa$ B is itself activated by the same inflammatory cytokines and chemokines which it induces [70], NF- $\kappa$ B is thus regulated via a positive feedback mechanism which becomes aberrantly active, resulting in a chronic inflammatory response [99]. Indeed, while NF- $\kappa$ B activity is essential for proper function of the immune system, its constitutive activation has been associated with numerous disease states such as aging-related diseases, malignancies and various inflammatory diseases [68,102,103], including those of the nervous system such as Alzheimer's disease [104,105], Parkinson's disease [106], multiple sclerosis [107] and also autism [5,6].

Although female mice appeared to be less susceptible to AI exposure than male mice, it is notable that markers of activation of the NF- $\kappa$ B pathway were observed in both sexes, Namely, AI reduced the

expression levels of the of NF- $\kappa$ B inhibitor proteins I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  (encoded by the *NFKB1B* and *NFKB1E* genes respectively) and increased the level of TNF- $\alpha$  both at the gene and protein level in males (Fig. 1B, D) and females (Fig. 2B, D). In addition, in the male brains AI increased the expression at the gene and protein level of chemokines CCL2/MCP-1 and IFN- $\gamma$  (Fig. 1B, D), both of which are NF- $\kappa$ B targets [108,109]. AI also activated the phosphorylation of the I $\kappa$ B inhibitor in the brains of male mice, indicating that AI exposure activated the NF- $\kappa$ B pathway by deactivating the NF- $\kappa$ B inhibitor (Fig. 3C). Consequently, the expression levels of other NF- $\kappa$ B downstream target genes were also elevated in the male brains, including *MIP-1A*, and the interleukins *IL-4*, and *IL-6* (Fig. 4A–D). Altogether, the current results demonstrate that AI exposure activated the NF- $\kappa$ B pathway in the brains of male mice, resulting in excessive levels of immune and inflammatory factors.

The aberrant activation of the NF- $\kappa$ B pathway has been demonstrated in two studies of autistic children. In particular, Naik et al. [5] showed a significant increase in NF- $\kappa$ B DNA binding activity in peripheral blood samples of children with autism ( $n = 67$ ) compared to healthy controls ( $n = 29$ ). Further commenting on their findings, Naik et al. [5] have concluded that children with autism could be in a “hyper

arousal” state of NF- $\kappa$ B due to the constant effect of environmental stressors. The adjuvant form of injected AI is engulfed by macrophages that can traverse the blood-brain-barrier, invade and accumulate in the CNS [38,41,42], thus AI could act as a constant stimulator of the immunoinflammatory and oxidative stress pathways via its activation of HIF-1 and NF- $\kappa$ B responsive genes [78,79]. Indeed, as Naik et al. [5] note, one of the key ways in which the NF- $\kappa$ B pathway is activated is through the production of reactive oxygen species (ROS) [100]. Moreover, ROS generation is related to stress which could be due to multiple environmental and behavioral factors. Although evidence of increased oxidative stress appears to be absent in mice exposed to AI, our analysis was limited to only three oxidative stress markers, COX-2, iNOS and PLA2. Subsequent work should include a more elaborate investigation including markers of lipid peroxidation which are shown to be increased in autism [81,82], especially given the fact that AI is a known inducer of oxidative stress and lipid peroxidation. Indeed, as noted above, it is in this way that AI activates the ROS-sensitive transcription factors, HIF-1 as well as NF- $\kappa$ B and augments specific neuroinflammatory and pro-apoptotic signaling cascades [78,79].

Other relevant findings regarding the aberrant activation of NF- $\kappa$ B in autism were supplied by Young et al. [6] who showed elevated expression of NF- $\kappa$ B in post-mortem brain samples of autistic patients compared to control samples. In particular, excessive NF- $\kappa$ B subunit p65 expression has been observed predominantly in the nuclear compartments of the orbitofrontal cortex. The immunofluorescence analysis further showed that these relative increases in expression localized to neurons, astrocytes, and microglia, but were particularly pronounced in highly activated microglia. The elevated levels of NF- $\kappa$ B in the nuclear fraction clearly suggest the activation of the molecule [6]. Previously, as mentioned above, Vargas et al. [7,8] showed extensive neuroglial and innate neuroimmune system activation in brain tissues of patients with autism, particularly in the cortical region. In our study, the frontal cortex was similarly the most affected area by the proinflammatory signaling in the male brain (Fig. 5A). Our work together with the report by Young et al. [6] indicates that excessive activation of the NF- $\kappa$ B pathway appears to be responsible for the observed immunoinflammatory response in the cortex of autistic patients, the brain regions involved in emotional and cognitive processing, learning, and social behavior, all of which are impaired in individuals with autism [110,111]. Moreover, current research supports the hypothesis that autism results from altered connections within or between regions of the cortex [72]. Indeed, abnormal neural connectivity is one of the key pathological features of the autistic brain. The term connectivity encompasses local connectivity within neural assemblies and long-range connectivity between brain regions. Similarly, there is also physical connectivity (“hard-wiring”), associated with synapses, tracts and functional connectivity (“soft-wiring”), associated with neurotransmission [112]. High local connectivity may develop in tandem with low long-range connectivity in the autistic brain [113]. In summary, abundant evidence supports our findings that abnormal activity of immune signaling in the brain interferes with the establishment of appropriate neuronal circuitry during development, thus contributing to the emergence of autistic phenotypes [4,56,97].

#### 4.4. AChE dysregulation in autism and the role of AChE in immunomodulation

Apart from the activation of immune and inflammatory markers in the brain, we observed a decreased expression of AChE as well as its protein product (AChE) in AI-injected male mice (Fig. 1B, D). Cholinergic activity is involved in numerous neurological functions relevant to autistic pathology including attention, social interactions, emotional responses, stereotypical behaviors, cognition, and memory [59,114,115]. Not surprisingly, deficits in cholinergic activity have been detected in autism, in particular, post-mortem observations showed altered expression of nicotinic ACh receptors and others

[114,116]. Thus pharmacological enhancement of cholinergic neurotransmission (and that includes the inhibition of the degradatory activity of AChE) appears to be beneficial in some autistic patients, resulting in improvement in cognitive abilities, attention and memory [117]. Consistent with this, AChE inhibition and the resultant ACh elevation appears to decrease cognitive rigidity, improve social preference and enhance social interaction in a mouse model of autism [115]. On the other hand, decreased activity of AChE in the hippocampus was also reported to be associated with increased anxiety, depression-like behaviors and decreased resilience to repeated stress in another rodent model [118]. Adults with autism exhibited reduced AChE activity in the fusiform gyrus but not in other cortical areas, presenting further evidence that this type of abnormality may be associated with social dysfunction [59]. It is well established that the region of the fusiform gyrus called the fusiform face area is consistently active during face viewing in normally developing individuals [119]. In contrast, autistic individuals lack fusiform face area activation in response to strangers' faces [120]. Fusiform gyrus activity including face recognition tasks are processes which critically depends on cholinergic signaling [121,122]. It is further thought that the hypofunction of the fusiform face area in autism may be due to neuropathological abnormalities in the fusiform gyrus and abnormal functional connectivity between the right fusiform gyrus and the left amygdala [59,123]. Because ACh is significantly involved in the regulation of both structural and functional maturation of cortical circuits and because the modulatory effect of ACh in turn depends on AChE activity, it is thought that reduced AChE activity in the fusiform gyrus may in part contribute to the reduction in the number of cholinergic neurons observed post-mortem in the fusiform gyrus of autistic patients [59,124].

Although the fusiform gyrus is not present in rodents, there is another explanation for the resultant pattern of AChE expression in our study; namely, the well-known immunoregulatory role of neurotransmitters [60,61]. ACh is the primary parasympathetic neurotransmitter and the receptors to which it binds (nicotinic and muscarinic cholinergic receptors) are found on numerous types of immune cells. Nicotinic receptors, in particular, are known to mediate cholinergic anti-inflammatory effects in macrophages. Notably, activation of the nicotinic ACh receptor on macrophages inhibits NF- $\kappa$ B signaling, thereby dampening the immunoinflammatory response [125,126]. The immunoregulatory cholinergic pathway also prevents excessive elevations of serum levels of TNF- $\alpha$  during toxic shock, through the release of ACh from the vagus nerve (and is consequently abolished by vagotomy), hence preventing excessive inflammatory responses [127,128]. Consistent with the attenuating effect of ACh on immunoinflammatory effects, the inhibition of ACh synthesis causes the enhancement of immune response [60,61]. Similarly, AChE potentially negates the cholinergic anti-inflammatory effects via its hydrolysis of ACh, not surprisingly, elevated expression of AChE is found in many inflammatory conditions. Conversely, inhibition of the degradatory activity of AChE via AChE inhibitors results in the suppression of the humoral immune response and the amelioration of inflammation not only in the periphery but also in the CNS [60,129,130]. As an example of the latter, in the brain AChE upregulation is associated with an enhanced immune response that facilitates the epileptogenic process in status epilepticus while ACh has the opposite effect [130]. It is notable in this respect that an estimated 5–40% of children with autism suffer from seizures and this association tends to be stronger in more severely affected patients [131].

In summary, the parasympathetic nervous system is activated by inflammatory cytokines and via cholinergic activity while providing a negative-feedback control of innate immune responses to restore homeostasis. Therefore it seems plausible that the degradatory activity of AChE was downregulated in AI-injected male mice in order to activate the ACh-mediated immunosuppressive mechanism that restores homeostasis.

#### 4.5. The mechanistic link between immune stimuli and adverse neurological outcomes: How vaccine adjuvants may contribute to autism

Extensive research has underscored the tight connection between development of the immune system and that of the CNS, thus substantiating the view that disruption of critical events in immune development may play a role in neurobehavioral disorders including those of the autism spectrum [4,9,15,86,90,91]. Thus, it has been proposed that the widespread manifestations of immune abnormalities in ASD may stem from deleterious effects of immune insults that occur during a narrow window of postnatal development which is characterized by extensive shaping of both the CNS and the immune system [9,56,86]. Indeed, early-life immune insults (both peri- and post-natal) have been shown to produce long-lasting, highly abnormal cognitive and behavioral responses, including increased fear and anxiety, impaired social interactions, deficits in object recognition memory and sensorimotor gating deficits [51,53–56,132–134]. These symptoms are typical of ASD and result from the heightened vulnerability of the developing immune system to disruption by immunomodulating environmental pollutants such as bisphenol A, polychlorinated biphenyls (PCBs), lead (Pb), mercury (Hg) and Al [9,56,86,133,135]. Of the later, although Hg and Al in particular can come from various sources, the one common source to which infants and pregnant women are universally exposed is through vaccinations. With respect to Al in the vaccine adjuvant form, over the last decade, studies on animal models and humans have indicated that Al adjuvants have an intrinsic ability to inflict adverse neurological and immunoinflammatory manifestations [45,47,136,137]. This research culminated in delineation of ASIA—“autoimmune/inflammatory syndrome induced by adjuvants”, which encompasses the wide spectrum of adjuvant-triggered medical conditions characterized by a misregulated immune response [138,139]. Notably, a large portion of adverse manifestations experimentally triggered by Al in animal models [45,140], and those associated with administration of adjuvanted vaccines in humans are neurological and neuropsychiatric [46,141]. The ability of Al adjuvants to cross the blood-brain barrier and blood-cerebrospinal fluid barrier [41–43,45,46] may in part explain the reason the adverse manifestations following vaccinations tend to be neurological with an underlying immunoinflammatory component [141–143]. Thus Al impacts on the CNS and immune system are reciprocally linked and not disparate actions [33,56,144,145].

It is important to note that Al or other agents with immunostimulating properties do not necessarily need to breach the blood-brain barrier in order to induce a neuroinflammatory response. Indeed, the principal mechanism by which peripheral (systemic) immune stimulation affects responses in the brain is critical to understanding the potential role of Al adjuvants in neurodevelopmental disorders of the autism spectrum. As noted above, an important advance in understanding the functions of the normal and diseased brains was the recognition that there is an extensive communication between the immune system and cells in the CNS [90,91]. As a result of this neuro-immune cross-talk, neural activity can be dramatically altered in response to a variety of immune stimuli [146–148]. Such peripheral immune stimuli lead to de novo production of proinflammatory cytokines within the brain by activated microglia, the brain's resident immune cells [147,149]. Importantly, this immunoinflammatory response in the brain occurs even when the offending agent does not cross the blood-brain barrier [90,150].

Numerous studies show that proinflammatory responses arising from a single peripheral immune stimulus early in the postnatal period are sufficient to disrupt normal neural development [53,151]. Moreover, such immune stimuli can increase CNS vulnerability to subsequent immune insults that can permanently impair CNS function [152–155]. For example, new born rodents exposed to peripheral immune stimuli with either bacterial s or viral antigen mimetics within the first two postnatal weeks, develop deficits in social interactions, altered

responses to novel situations, anxiety-like behaviors, impairments in memory, long-lasting increase in seizure susceptibility, abnormal immune cytokine profiles and increased extracellular glutamate in the hippocampus [53–55,155,156]. All of these abnormalities are observed in autistic children in various degrees [2,7,157,158].

Repeated exposure to bacterial and viral antigens (most of which are adsorbed to Al adjuvants) through current vaccination schedules is clearly analogous both in nature and timing to peripheral immune stimulation with microbial mimetics in experimental animals during early periods of developmental vulnerability of the CNS. In view of these clear analogies, pediatric vaccinations can no longer be dismissed as a plausible cause for the growing burden of neurodevelopmental and immune abnormalities in children [159].

Research data further show that many cytokines induced by an immune response (including adjuvant-mediated) can act as “endogenous pyrogens”. That is, cytokines can induce a rapid-onset fever by acting directly on the hypothalamus without requiring the formation of other cytokines (i.e., IL-1 $\beta$ , IL-6, TNF- $\alpha$  [90,160–162]). While transient fever is an essential component of the early immune response to infection, the prolonged febrile response is a hallmark of many inflammatory and autoimmune diseases [161]. Moreover, fever-promoting cytokines produced in peripheral tissues upon immune stimulation can enter the brain via the circumventricular organs [161], which are among the few sites in the brain devoid of a blood-brain barrier, and can thus promote brain inflammation. That persistent hyperinflammation of the CNS plays a prominent role in the development of autism is solidly established by the existing data [6–8]. At least 13 cytokines and chemokines are produced within 4 h of Al adjuvant injection, including pro-inflammatory IL-1 $\beta$  and IL-6 [163]. Since the very nature of peripheral immune stimulation can influence brain function, the possibility that such outcomes could also occur with administration of vaccines and vaccine adjuvants deserves consideration. In this context, research shows that Al adjuvants activate 312 genes, 168 of which play a role in immune activation and inflammation [164].

In humans, the best studied condition linked to adjuvant Al is the neuromuscular disorder macrophagic myofasciitis (MMF), a condition characterized by highly specific myopathological alterations in deltoid muscle biopsies due to long-term persistence of vaccine-derived Al hydroxide nanoparticles within macrophages at the site of previous vaccine injections [136,137,165]. Patients diagnosed with MMF tend to be female (70%) and middle-aged at time of biopsy (median age 45 years), having received 1 to 17 intramuscular Al-containing vaccines (mean 5.3) in the 10 years before MMF detection [39]. Clinical manifestations in MMF patients include diffuse myalgia, arthralgia, chronic fatigue, muscle weakness and cognitive dysfunction. Overt cognitive alterations affecting memory and attention are manifested in 51% of cases. In addition to chronic fatigue syndrome, 15–20% of patients with MMF concurrently develop an autoimmune disease [39].

The pathological significance of the MMF lesion has long been poorly understood because of the lack of an obvious link between persistence of Al agglomerates in macrophages at sites of previous vaccination and delayed onset of systemic and neurological manifestations. However, recent experiments in animal models have revealed that injected nano-Al adjuvant particles have a unique capacity to travel to distant organs including the spleen and the brain where they are detected up to one year following injection [41,42]. Moreover, the Trojan horse-like mechanism by which Al enters the brain, results in its slow accumulation and is likely responsible for cognitive impairments associated with administration of Al-containing vaccines [136,137]. The bioaccumulation of Al in the brain appears to occur at a very low rate in normal conditions, thus potentially explaining the presumably good overall tolerance of this adjuvant despite its strong neurotoxic potential. Nonetheless, according to Khan et al. [41], continuously increasing doses of the poorly biodegradable Al adjuvant may become insidiously unsafe, especially in cases of repetitive closely-spaced vaccinations and altered blood-brain barrier.



Yet, while an adult MMF patient may have received up to 17 vaccines in 10 years prior to diagnosis [39], an average child in the U.S. would have received about the same number of Al-adjuvanted vaccines in their first 18 months of life according to the current U.S. CDC vaccination schedule [166]. In humans, important aspects of brain development (i.e., synaptogenesis) occur during the first 2 years after birth [167,168], a period in which the immature brain is extremely vulnerable to neurotoxic and immunotoxic insults [9,86,167]. This is the time when children receive the majority of their pediatric vaccinations. In view of these observations, there should be concern about the potential risks of injected vaccine-derived Al for which total clearance from the CNS may be virtually impossible due binding with neural proteins, DNA and hence, its progressive accumulation [41].

Several recent studies support the possibility that Al may participate in the growing burden of ASD. For example, Melendez et al. [169] have recently shown an elevation of several metals including chromium, arsenic and particularly Al in the blood of autistic children in comparison to the reference values for normal children. These authors identified two important items of data regarding exposure to toxic metals. First, in 80% of cases the autistic children have used controlled drugs and 90% of them have received all of their vaccines. In addition, 70% of mothers had received vaccines. Hence, the results by Melendez et al. [169] suggest that cumulative exposure to Al from pharmaceutical sources (i.e., Al-containing drugs and vaccines) in early periods of developmental vulnerability (both pre- and postnatal) may contribute to the development of ASD. These findings indicate that Al is another environmental agent that can now be added to the list of xenobiotics associated with developmental immunotoxicity (as defined by Dietert and Dietert [9]) and thus an important, yet underappreciated, risk factor in disorders of the autism spectrum. A study by Yasuda and Tsutsui [170] likewise supports this conclusion. These authors examined hair concentrations of 26 trace elements in 1967 children with autistic disorders aged 0–15 years, and demonstrated that many of the patients, especially the infants aged 0–3 years-old, were suffering from marginal to severe zinc (Zn) and magnesium (Mg) deficiency and/or high burdens of several toxic metals. Moreover, the highest proportion of infants had Al-overload, followed by cadmium (Cd) and Pb, in this critical period of early neurodevelopmental vulnerability.

#### 4.6. Gender difference and female protective effect in ASD

Most neuropsychiatric diseases have a sex bias in their presentation. ASD affects males 4 times more than females [52]. Conversely, the frequency of depressive disorder and anxiety disorder are greater in females. The cause for these differences is not well understood. An important distinction between these two cohorts of disorders is that those which are male biased tend to occur early in development, whereas those that are more prevalent in females generally do not occur until after puberty. Sex differentiation in neuropsychiatric diseases was originally attributed to increased level of amino acid neurotransmitters,

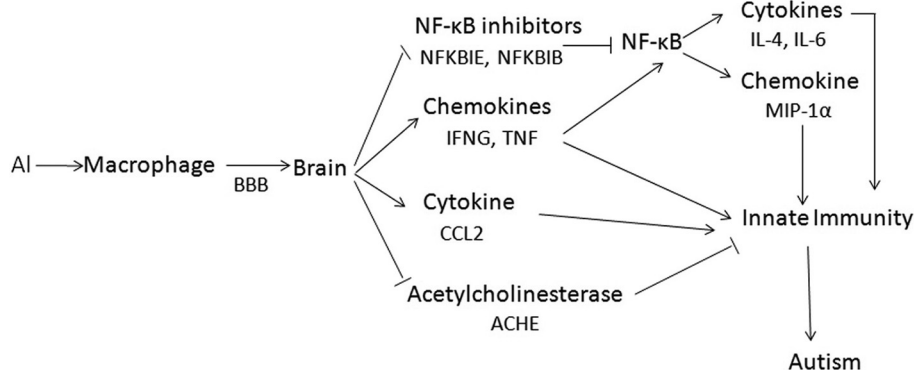
such as  $\gamma$ -aminobutyric acid (GABA), and glutamate in the male brain [171]. However, multiple attempts to identify a neurotransmitter system subject to hormonal regulation and serving as the final common denominator of steroid-hormone induced masculinization of the brain have largely failed. Newer research has demonstrated that the origins of many sex differences in the brain are outside the realm of neurotransmission but instead involving inflammatory and immune mediators such as microglia and mast cells, all of which are higher in males [172]. In our current study, we present evidence that the deleterious consequences of early post-natal Al exposure that seem to stem from over-activation of the innate immune system in brain, display sex differences. Male brains were more affected as more immune factors were activated in male mice in response to Al exposure. From this outcome it follows that the female nervous system is possibly more resistant to early stage Al toxicity. In this instance, *TNFA* decreased in the thalamus perhaps to offset the *TNFA* upregulation in the frontal cortex of Al-injected females (Fig. 5B).

ASD is a disorder that mainly manifests in early childhood. Sex-specific states may present as a window of vulnerability, where the normal developmental trajectory becomes most susceptible to reprogramming. The potential for early developmental insults, such as Al intake through neonatal vaccination, to alter this trajectory underlines the putative etiological role of Al in ASD. Based on the critical role for neuroimmune signaling in programming of the sexually dimorphic brain and neural toxicity of Al, it is possible that Al mediates the brain's inflammatory response and contributes to the disruption of “normal” sex differences related to neurodevelopmental disease susceptibility. Obviously, more detailed studies are required to test these ideas.

## 5. Conclusion

Immune activation is a prominent feature of ASD in autopsy material. Numerous immune system abnormalities have been described in individuals with autism. Chief among these is the increased activation of the innate immune response in autistic brain specimens [6–8]. However, the mechanism by which the innate immune system is activated remains far from clear. Although our Al-based model of ASD is still being developed and remains preliminary, the present study has shown extensive upregulation of innate immune activators and down-regulation of innate immune inhibitors via the NF- $\kappa$ B pathway stimulation in the CNS of Al-treated mice. In addition, the degradatory activity of AChE was downregulated in Al-injected mice, possibly to activate the ACh-mediated immunosuppressive mechanism and restore homeostasis.

Based on the data we have obtained to date, we propose a tentative working hypothesis of a molecular cascade that may serve to explain a causal link between Al and the innate immune response in the brain (Fig. 6). In this proposed scheme, Al may be carried by the macrophages via a Trojan horse mechanism similar to that described for the human immunodeficiency virus (HIV) and hepatitis C viruses [173,174],



**Fig. 6.** A model for molecular cascade of gene-toxin interaction. Al carried by a macrophage travels across the blood-brain-barrier and invades the central nervous system (CNS). Upon gaining access to the CNS, aluminum activates various inflammatory factors, and inhibits NF- $\kappa$ B inhibitors, which further leads to the activation of NF- $\kappa$ B signaling pathway dictating the innate immune response and the release of more immune factors. The increased immunoinflammatory signal downregulates the degradatory activity of AChE mice, possibly to activate the ACh-mediated immunosuppressive mechanism and restore homeostasis. If homeostasis is not restored, the unrestrained innate immune and inflammatory response will impair normal neurodevelopmental pathways, leading to abnormal connectivity of neural networks and aberrant structuring of the brain. The end result will ultimately manifest in abnormal behavioral and social functions.

travelling across the blood-brain-barrier to invade the CNS. Once inside the CNS, AI activates various proinflammatory factors and inhibits NF- $\kappa$ B inhibitors, the latter leading to activation of the NF- $\kappa$ B signaling pathway and the release of additional immune factors. Alternatively, the activation of the brain's immune system by AI may also occur without AI traversing the blood-brain barrier, via neuroimmuno-endocrine signaling. Either way, it appears evident that the innate immune response in the brain can be activated as a result of peripheral immune stimuli. The ultimate consequence of innate immune over-stimulation in the CNS is the disruption of normal neurodevelopmental pathways resulting in autistic behavior.

It may be argued that if the aim of the present study was to investigate an ASD risk factor, and ASD is a childhood disorder, why then were mice sacrificed at 16 weeks of age (adult age, given that female mice become sexually mature at 6 weeks after birth and males at 8 weeks) and not at earlier time-points? The answer to this is that our initial aim was to investigate whether AI had long-term consequences on neurodevelopment that would persist into adulthood since autistic symptoms obviously do not disappear in adult ages. Our following study aims to investigate an earlier time point, namely 22 days post-natal.

In conclusion, our data support a gene-environmental factor interaction model, which posits that complex diseases, such as ASD, are etiologically and biologically heterogeneous. The effect of the genes is conditional on the environment and a particular genotype may uniquely sensitize particular individuals to certain toxic environmental factors such as AI. It is plausible to further hypothesize that individuals already affected by ASD may react differently to the same environmental stimuli and may have less tolerance to the prenatal or postnatal immunotoxic exposures due to genetic predispositions. Our study highlights the additive contributions of immune genes and environmental toxins in a mouse model of early post-natal exposure.

The observed array of gene and protein expression changes in AI-injected mice suggests that the impact of AI may be broad and profound in the CNS, however, the toxicity may ultimately converge on highly specified biological pathways during brain development instead of being randomly distributed. One of the candidate biological pathways discovered in the present study is the NF- $\kappa$ B pathway. The toxicity of AI may thus lie in altering the signal flow through the NF- $\kappa$ B pathway. Upon the activation of the NF- $\kappa$ B heterodimer by AI, the gate of NF- $\kappa$ B signaling is open and excessive proinflammatory factors are released to mediate an aberrant immune response especially in the brains of male mice. The upregulated NF- $\kappa$ B-dependent immune factors could amplify the signal output by inducing NF- $\kappa$ B activation in return. Hence, the small input made by AI injection gives rise to a large magnitude of perturbation on the innate immune system in the brain through this positive feedback loop.

Finally, two limitations need to be noted with regard to the study methods. First, it should be noted that in this study we did not make separate controls for dietary AI ingestion. Although as noted above, only a small proportion of AI derived from dietary sources (food and water) is absorbed into the systemic circulation ( $\sim 0.25\%$ ) as opposed to injected AI (which is absorbed at over 50% efficiency in the short term [35] and at nearly 100% efficiency long-term [36]), even dietary AI has been shown to accumulate in CNS over time, resulting in Alzheimer's disease type outcomes. This particular neurotoxic effect of AI has been observed in experimental animals fed equivalent amounts of AI to what humans consume through a typical Western diet [26,37]. The amount of AI in Purina chow brands can vary and may well have significant effects in the long term. Nonetheless, all our animals, AI-injected and non-injected, received the same chow and the same water, yet there were significant differences in inflammatory gene and protein expression in the brain between the AI-exposed and control groups. Hence, these differences could not have been due to the dietary AI which was the same across all groups. Additionally, the AI-injected females were less affected by these inflammatory changes than AI-

injected males, which further agree with the observation that females are less susceptible than males to a range of neurotoxicant and neural-injury effects due to the protective effects of estrogen [175–178]. In an ideal situation, two additional groups of animals would have been included in order to control for the effect of dietary AI: 1) mice fed with AI-free Purina chow and AI-free high-performance liquid chromatography (HPLC)-treated water, and, 2) mice injected with AI and fed with AI-free Purina chow and AI-free HPLC-treated water. However the addition of such controls was beyond the scope of the current preliminary study.

The second limitation is that no controls were included to ascertain that AI, if perhaps present after RNA extraction from AI-treated mice, did not influence reverse transcriptase enzyme activity hence yielding a spurious result. Nonetheless, the fact that the majority of gene expression changes were confirmed at the protein level via Western blotting, makes this interpretation improbable. Moreover, there is no evidence that AI inhibits reverse transcriptase but rather the transcriptional machinery involving DNA polymerase, as cited above [27,73–76]. Indeed, Sabbioni et al. [179] tested 44 different metal ions for their ability to inhibit HIV reverse transcriptase, AI included. AI showed no activity against reverse transcriptase, but only platinum ( $\text{Pt}^{4+}$ ), silver ( $\text{Ag}^+$ ), rhodium ( $\text{Rh}^{3+}$ ),  $\text{Zn}^{2+}$  and  $\text{Hg}^{2+}$  decreased the reverse transcriptase activity in a dose-response manner.

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