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Neuroimmune alterations in autism: a translational analysis focusing on the animal model of autism induced by prenatal exposure to valproic acid

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ABSTRACT (250 words)

Autism Spectrum Disorder (ASD) is a highly prevalent developmental disorder characterized by deficits in communication and social interaction and in stereotyped or repetitive behaviors. Besides the classical behavioral dyad, several comorbidities are frequently present in patients with ASD, such as anxiety, epilepsy, sleep disturbances and gastrointestinal tract dysfunctions. Although the etiology of ASD remains unclear, there is supporting evidence for the involvement of both genetic and environmental factors. Valproic acid (VPA) is an anticonvulsant and mood stabilizer that, when used during the gestational period, increases the risk of ASD in the offspring. The animal model of autism by prenatal exposure to VPA shows construct and face validity, since several changes seen in subjects with autism are also observed in the VPA animal model. Neuroimmune alterations are common both in autistic individuals and in animal models of autism. In addition, exposure to pathogens during the pregnancy is a known risk factor for ASD, and maternal immune activation can lead to autistic-like features in animals. Thus, immunological alterations in pregnancy could affect the developing embryo, since immune molecules can pass through the placental barrier. Here, we summarize important alterations in inflammatory markers, such cytokines and chemokines in patients with ASD and in the VPA animal model.

Keywords (3–9 key words): ASD, neuroimmune, cytokine, animal model, valproic acid

INTRODUCTION

Since the first descriptions, in the early 1940's by Leo Kanner and Hans Asperger, new data has been shared to the scientific community about Autism Spectrum Disorder (ASD) [1]. Currently, ASD is diagnosed by changes in two behavioral domains: a) communication and social interaction impairments in multiple contexts, including deficits in social reciprocity, non-verbal communication used for social interaction and in skills to initiate, maintain and understand relationships; and b) Repetitive behaviors, restricted and stereotyped activities [2].

There is no clinical marker or quantitative examination in peripheral tissues that can be used for an early diagnosis of this disorder [3]. Even though there are many well accepted surveys for behavioral diagnosis, ASD is a highly complex and heterogeneous disorder, presenting distinct manifestations, in which two individuals hardly share the same set of symptoms [4,5]. The large heterogeneity of the symptoms could potentially be explained by individual differences, for example in the immune system. Alterations in cytokines levels are common in autistic individuals, with a frequent observation of elevated levels of pro inflammatory cytokines [6,7].

Genome-wide association studies (GWAS) have already described interesting relations between immune system disruptions and neurological disorders like autism and schizophrenia [8]. Specifically in ASD, an interesting example is the dysregulated genes reported, as IL-1β and IL-12, both involved in cytokine-cytokine receptor interaction [9]. One study relating ASD and neuroimmune genetic disruption shows an alteration on glutamate receptor

metabotropic 5 (GRM5) single nucleotide polymorphisms (SNPs) [10], which is not exactly a neuroimmunological alteration, but this gene is highly expressed in many neuronal regions implicated in ASD, besides acting on synaptic plasticity, modulating innate immunity and microglia activation. When occurs a GRM5-positive allosteric modulation, several negative behaviors described in ASD are rescued, including stereotypies [10]. Taken together, the evidences showing genes interaction and ASD diagnosis demonstrate important genetic contribution in neuroimmunological imbalance in ASD. However, despite the data cited above, no gene was identified as an important actor in triggering this disorder.

According to the most recent epidemiological survey conducted in United States, the current incidence of ASD is 1:68 [11]. Although the etiology of ASD remains unknown, it is hypothesized that the onset of this disorder depends on the interplay between genetic and environmental factors. Epidemiological observations suggest that exposure to teratogens - especially in the first trimester of pregnancy - could be closely related to ASD development. An important example is the prenatal exposure to valproic acid (VPA) [12,13].

Valproic acid (VPA) and VPA animal model

The compound VPA is a drug widely used as an anticonvulsant and mood stabilizer in the treatment of epilepsy and bipolar disorder [13,14]. Although VPA is well tolerated and safe in adults, there is evidence of its teratogenicity [14]. Clinical studies over the years have shown that intrauterine exposure to VPA is associated with birth defects, cognitive impairments, and increased risk of autism [13]. In recent years, animal studies have investigated the anatomical, behavioral,

molecular, immunological and physiological outcomes related to exposure to VPA [13].

Epidemiological observations demonstrate a strong correlation between prenatal exposure to VPA and ASD [15–18]. Based on these observations, an animal model for study of autism prenatally induced by VPA was established [19–21]. Behavioral studies show that exposure to VPA in rats and mice leads to several autistic-like behaviors in male offspring, including social behavior deficits, increased repetitive behaviors, and communication deficits similar to those found in ASD subjects [19–23], pointing out the animal model's translationality, as the diagnosis of ASD is given through behavioral evaluation.

Since current diagnostic criteria for ASD are exclusively clinical and resulted from behavioral analyses, the study of ASD in humans prior to the onset of symptoms becomes a very challenging task. Animal models provide the opportunity for analyzing the developmental changes that can trigger ASD-like features [24,25]. They provide the possibility to study and manipulate biological pathways for understanding and even preventing or reversing the appearance of the morphological, functional and behavioral alterations found in ASD. In addition, studies with animals can reveal some new important factors involved in the etiology of this disorder.

Histone-deacetylases inhibitors (HDACi) and neuroimmune alterations

Autism and many other psychiatric disorders, like schizophrenia, bipolar disorder and major depression present not only susceptibility to environmental risk factors, but also a high genetic influence [26,27]. In the last years, there is

growing evidence indicating that epigenetic alterations may have an important role in several psychiatric disorders.

Epigenetic regulation includes long-term changes, as DNA methylation, and short-term changes, as modifications in histone proteins associated with DNA [28]. Histones are small basic proteins that act as spools around which DNA winds, regulating the packaging of DNA and allowing or inhibiting gene expression. When the histone is acetylated by histone acetyltransferases (HATs), this local alteration leads to chromatin decondensation, promoting gene expression by the activation of the transcription machinery. On the other hand, histone deacetylation - mediated by histone deacetylases (HDACs), results in inhibition of transcription promoting a controlled gene expression [28,29].

Substantial epigenetic alterations were found in the regulatory regions of many candidate genes for ASD, such as GABAergic genes, GAD67, Reelin, Oxytocin receptor, BDNF, showing that the epigenetic component in ASD has been widely studied [26]. The histone post-translational modifications, as acetylation and methylation, play a key role in the gene expression regulation [30]. These characteristics are crucial for important biological processes like the action of immune system, in which HDACs modulate gene expression of toll-like receptors and interferon signaling pathways [31]**.**

The HDAC inhibitors drugs play an important role in immune context. Studies showed an increased transcription of the major histocompatibility complex (MHC) class II, located in the tumor cell surface in mouse and humans [32], indicating an interesting effect on several immune cells. It leads to less viability of T CD4 cells and decreases the production of pro-inflammatory

cytokines, making the T CD8 cells increase the secretion of pro-inflammatory cytokines, modulating the activity of natural killer (NK), as well in cells and Treg cells [33].

Hence, several drugs used as antidepressants and mood stabilizer are characterized as HDAC inhibitors class. Valproate, a well-known HDAC inhibitor drug, induces important delays in the neuronal maturation [34], already described in ASD [35]. Moreover, VPA prenatal exposure alters the postnatal histone 3 (H3) acetylation levels in cerebellum [36], stimulates glial cell proliferation in the developing rat brain [37] and also induces changes in acetylation levels in astrocytes of hippocampus and cortex in cell culture, more than other antidepressants and mood stabilizer [38]. These unique effects of VPA, especially in comparison to similar HDAC inhibitor drugs, indicate that the VPA molecule might have exclusive properties which are still unclear, although some evidence indicates a possible VPA binding in the catalytic center of HDACs [39]. Those epigenetics alterations occur before the well described neuroimmune alterations, and, thus, epigenetics mechanisms may be involved in the immune disturbance [36]. These data highlight the role of the valproic acid and HDAC inhibitors as epigenetic modulators that could be underpinning the immunological alteration, as well as the neurological outcomes, in psychiatric disorders.

The intimate relationship between central nervous system and immune system

For a long time, immune and central nervous systems were considered compartments that operate separately and independently. However, recent studies demonstrate an active communication between these two systems,

modulating bi-directly each other with neurotransmitters and neuromodulators in periphery. In addition, in a landmark study, lymphatic vessels were discovered in central nervous system, putting in check the current view of the brain as an “immune privileged site” and raising new possibilities for the crosstalk between brain and immune system [40]. Anatomically the central nervous system (CNS) is bathed by the cerebrospinal fluid (CSF) and surrounded by the meninges, which contain lymphatic and blood vessels [41]. The brain parenchyma is separated from the circulating blood by a blood-brain barrier (BBB), which prevents the entry of pathogens, circulating immune cells, and other substances from the blood.

The BBB is defined as a semipermeable membrane that separates the circulating blood from the brain and extracellular fluid in the central nervous system [42]. CNS blood vessels interact with different peripheral and brain-resident immune cell populations, as perivascular macrophages and microglial cells, respectively. The BBB is formed by the concerted action of endothelial and glial cells. During development, at embryonic day 10 (E10), initial clues for angiogenesis lead to the early properties of BBB in CNS by activation of the Wnt/b-Catenin canonical pathway [43–45]. There is no consensus about the exact time when the BBB is fully formed [46]. Nevertheless, at E15, pericytes, which have crucial roles in BBB formation and maintenance, begin to interact intimately with endothelial cells (EC) in the capillary walls [47]. In postnatal life, endothelial cells from brain capillaries are covered up by mature pericytes, sharing their basement membrane with endothelial cells [48]. Moreover, the astrocytes project cellular terminations called “end feet” toward the capillaries, providing the outer layer of the BBB. Pericytes and astrocytes also secrete

proteins involved in extracellular matrix formation and deposition of the basement membrane [48,49].

The presence of this limiting barrier allows the CNS to control and fine tune the flow of a variety of molecules from periphery, regulating its permeability to seek homeostasis. In CNS physiology, there are extensive vessels where monocytes, granulocytes and dendritic cells circulate [50]. In addition, the brain parenchyma is populated with microglia, resident-cells from the immune lineage that play crucial roles in brain surveillance and response against multiple types of damage. Studies with rodents showed that, during neurodevelopment, specific monocytes emerge at E7 and infiltrate the CNS at E9.5 as pre-macrophages, expressing the chemotactic factor CX3C chemokine-receptor 1 (CRXCR1) [50]. The presence of cytokines as interleukin-1 beta (IL-1β) and tumor growth factor beta (TGF-β) allows the differentiation of pre-macrophages in early microglia at E14.5, which then generate mature microglia at P14. In fact, TGF-β seems to be crucial for microglial specification in CNS [51,52].

Microglial cells are capable to interact with almost all cell types in the CNS modulating cell maturation during development and promoting tissue repair and homeostasis. Moreover, in postnatal life, microglia play crucial roles in sensing perturbations in encephalic environment, actively responding to even minor pathological changes in CNS [53,54] by altering their shape and gene expression profile. The term “microglial activation” has been considered as a shift from a “resting” stage to an “activated” state when disturbance of tissue homeostasis is detected or upon experimental stimulation. However, this term implies the understanding of an “inactivated” phenotype when brain tissue is not facing any

changes in homeostasis. In fact, microglial cells are never inactive, showing highly dynamic surveillance functions in CNS [50,55,56]. Many authors are suggesting to rename this surveillance state of microglial cells to “surveying microglia”, instead of “resting microglia” [50]. These cells can shift from their “surveying” or “resting” state to “activated” or “alerted” state when facing chances in CNS homeostasis, as infections recognized by toll-like receptors [57], cell damage or trauma. Recent studies have demonstrated that the lipopolysaccharide (LPS) exposure downregulates the transcriptional factor Sal-like protein 1 (SALL1) and promotes several alterations in microglial identity, with a concomitant upregulation of genes associated to other resident macrophages, indicating that SALL1 might be important for maintenance of microglial identity in response to immune challenge [50,58]. Once activated, microglial cells can commit to different phenotypes called “reactive”, having a large functional and molecular diversity. These changes in microglia profile are correlated with the type of challenge faced by the CNS. They can shift to a pro-inflammatory state also called “M1 phenotype” [59] presenting highly phagocytic and neurotoxic activities and releasing pro-inflammatory chemokines and cytokines in response to an immune challenge, such as a microorganism invasion [60] or the presence of pro inflammatory signals [61–63]. Once the immune stimulator is controlled, microglial cells are able to shift to a more neuroprotective profile called “M2 phenotype” which involves anti-inflammatory responses [59,64]. Nonetheless, the activated pro-inflammatory profile can progress in pathological conditions. Although the immune challenge and the brain environment are responsible for the early microglial responses, signals from CNS resident and infiltrating immune cells can shape reactive profiles of microglial cells and play important roles in

many brain diseases [65–69]. All these stimuli could direct microglia's fate to alternative states, including microglial cell death, but there's still scarce information about the course of microglial activation, their reversibility to the surveying state [70] or the preservation of molecular memory of previous stimuli. Moreover, cells that infiltrate from the blood and differentiated into microglia could also return to the periphery [65,71].

There is a low basal entry of immune cells from blood periphery into the CNS in normal conditions. Studies have shown that, although microglial cells play major roles in brain surveillance, the perivascular macrophages represent a crucial immune regulator and sensor of perturbations in CNS and periphery. These cells are derived from bone marrow and are intimately associated with the bloodstream since they reside between endothelial cells and astrocyte's end feet [72–74]. This privileged location of perivascular macrophages allow them to simultaneously monitor the blood and the brain interstitial fluid, providing a fine control of brain homeostasis and BBB integrity [72,75]. Although macrophages display different locations, they can perform specific roles in these microenvironments. In addition to perivascular space, macrophages can be located within choroid plexus and meningeal space. In choroid plexus, which is considered the major site of CNS immune surveillance, there are tissue-resident macrophages called epiplexus cells disposed alongside the fourth ventricle with dendritic cells (DC), monocytes and mast cells [76,77]. Referred by many authors as the "immune regulatory gate", the choroid plexus is capable to induce specific immune responses and allows cell migration between blood and CSF [78,79]. The meningeal macrophages are positioned in the subdural meninges and act as sentinel cells for damage and infection in brain tissue, surveying the

cerebrospinal fluid (CSF) and the extracellular lumen of meningeal blood vessels [80,81]. Thus, macrophages play critical roles in CNS surveillance, homeostasis and disease. Nonetheless, there is a variety of other immune cell types in the brain environment. In physiological condition, studies have observed the presence of monocytes in meningeal spaces, although more evidence is still needed [82]. Granulocytes (neutrophils, mast cells, eosinophils, and basophils) can be found in meningeal spaces with mast cells also present in brain parenchyma [72,83]. These cells are highly phagocytic and play important roles in response to brain infections and tissue damage [72,84,85]. Dendritic cells (DC), the main antigen-presenting cells in periphery, can also be found in CNS. They are located in the choroid plexus, meningeal space, and are specially abundant in lymphatic vessels in meninges [86–88]. The presence around these vessels suggests important roles for DC in inflammatory diseases and brain infections [40].

During inflammatory condition, there is extensive infiltration of immune cells in the CNS. The barriers that regulate cellular entry are the blood-brain barrier (BBB) within the CNS parenchyma, and the blood-cerebrospinal fluid (blood-CSF) barrier within the choroid plexus" [89]. When brain homeostasis is compromised, immune cells can infiltrate from the periphery to the brain parenchyma due to the elevation in BBB permeability. This is generally observed and investigated in the context of a pathological CNS inflammatory response [90–92]. Under pathological conditions, microglia activation can lead to BBB disruption, allowing a substantial cellular infiltrate and amplifying the inflammatory response [93,94]. One of the key mediators in these processes is the release of cytokines and chemokines by periphery and brain-resident immune cells. This

novel view of the immune system as an active player in brain function is modifying our current view of neuropsychiatric disorders. Immune alterations are now seen as central for the pathophysiology of many brain diseases and further understanding of this neuroimmune axis can result in new therapies and diagnostic tools.

Neuroimmune alterations in ASD: from patients to the VPA animal model

In the last decade, the immune system has caught the attention of neuroscientists for the interplay between neurons and immune mediators, not only in disease, but also in the homeostasis of the brain. In the past, the central nervous system was called “an immune-privileged region”, once the blood brain barrier controls the cross talking between brain and the periphery. However, recent findings demonstrated that this privilege is not related to the absence of immune modulation in brain activity and homeostasis, but a time-dependent specific modulation in many regions during brain development [95]. Immune cells and immune molecules, such as cytokines and chemokines, can modulate cognitive, emotional and behavioral processes, triggering different responses in neuronal and glial cells [96]. Cytokines are small signaling-molecules acting as mediators of communication between immune cells. Their roles include stimulation and regulation of cell development, maturation and response against immune challenges [97,98]. Chemokines can be characterized as a vast group of 8-10 kDa molecules from the super family of cytokines that induce chemotaxis of immune cells. Once bound in their receptor, the complex chemokine-receptor can activate signaling cascades that induce immune cell trafficking to the target area. Also, this complex plays important roles as molecular signal in crosstalk

among neuronal and glial cells and immune resident cells in nervous system, as microglia [99,100]. Since chemokines are capable to target different types of receptor, they can modulate different cell processes, including cell adhesion, proliferation, phagocytosis, apoptosis, angiogenesis, cytokine secretion and T cell activation [101].

Lymphocytes are cells capable of recognizing any foreign antigens displayed by antigen-presenting cells, constituting the main cells of adaptive immunity [102]. Lymphocytes respond by proliferating and differentiating in effector cells, whose function is the elimination of the pathogen and creation of an immunological memory [103]. When naïve CD4+ T cells encounter specific antigens, they can differentiate into a range of effector subgroups. Several transcription factors are individually required for T-cell differentiation, generating a specific lineage that express characteristic cytokines. That is, once specific transcription factors are activated, they promote differentiation of naïve T cells, which differentiate into specific immunological responses: Th1, Th2 and Th17. In the presence of IFN-γ and IL-12, Signal transducer and activator of transcription (STAT) 1 and STAT4 signal for the expression of the transcription factor T box expressed in T cells (T-bet) and promotes response Th1. On the other hand, Th2 cell commitment occurs when IL-4 and STAT6 increase expression of GATA-binding protein (GATA3) transcription factor. The presence of TGF-β associated with IL-6 signaling via STAT3 generating the expression of retinoid-related orphan receptor (RORγt) transcription factor, results in the differentiation of Th17 cells. Also, TGF-β, with IL-2 signaling via STAT5 is known to generate, at least in vitro, inducible Treg cells, which express Foxp3 transcription factor (See Figure 1) [104].

The modulation of cytokine levels can alter significantly the brain physiology and behavior. Recent studies highlight a link between immune dysfunction and behavioral impairments [105]. For example, the relation between IL-6 and several altered behaviors has already been established in the literature [106–108]. Signs of neuroinflammation and altered inflammatory response are seen in ASD subjects throughout life [109]. Therefore, some authors hypothesize that the neuroimmune disturbances could be causal for ASD [110]. Below, we will detail the main neuroimmunological findings (summarized in Tables 1 and 2) in ASD subjects and in VPA animal model of autism:

IL-1β

IL-1β is a cytokine produced by fibroblasts, monocytes, tissue macrophages, dendritic cells (DCs), B lymphocytes, epithelial cells, and natural killer (NK) cells [111] that promotes inflammation by indirectly stimulating lymphocyte function and activating macrophages [112,113]. IL-1β has the ability to increase the expression of adhesion molecules such as VCAM-1 and ICAM-1, supporting the infiltration of inflammatory cells from the circulation into the tissue and resulting in chronic IL-1-induced inflammation [112,113]. IL-1β stimulates expression of inflammatory mediators and induces T-helper type 17 (Th17) response. Furthermore it can also play important roles as a mediator of the anti-inflammatory response [112,113].

Both elevation and reduction in IL-1β levels have already been reported in ASD subjects. Increased levels of IL-1β were found in plasma [114,115], serum [116,117], and peripheral blood mononuclear cells (PBMCs) [118–120] whereas decreased levels were described in neonatal dried blood samples (n-DBSS)

[121]. In VPA animal model, IL-1β was increased in hippocampus [122,123], in LPS-exposed hippocampus [109] and in whole brain homogenate [124]. Increased levels of this cytokine are associated with increased stereotypy [120], one of the main characteristics of ASD.

IL-2

Interleukin-2 has an important role in controlling the survival of immature and mature T cells [125] and is mainly secreted by CD8+ and CD4+ T cells after recognition of the antigen and co-stimulators [111]**.** IL-2 is the most important cytokine for promoting the clonal expansion of antigen-activated T cells [126]. The only report in ASD is a reduction of IL-2 levels in neonatal dried blood samples (n-DBSS) [121].

IL-4

IL-4 is the main cytokine of Th2 response and is primarily produced by T cells and mast cells. IL-4 promotes proliferation of B cells and cytotoxic T cells and stimulates IgG and IgE production [97], besides stimulating leukocytes recruitment and promoting the expression of adhesion molecules [127]. Increased levels of this cytokine were associated with greater impairments in non-verbal communication [120]. In ASD subjects, reduced level of IL-4 in n-DBSS [121] and elevated levels in amniotic fluid [128] have been reported.

IL-5

IL-5 is a cytokine produced by T cells that acts as an activator of eosinophils [129]. IL-5 promotes eosinophil proliferation and maturation,

stimulating IgA and IgM production [97]. In ASD patients, a decrease in IL-5 in n-DBSS [121] and an increase in plasma samples [115] were described.

IL-6

The main source of IL-6 are T-helper cells, macrophages and fibroblasts. IL-6 targets activated B-cells and plasma cells, promoting differentiation into plasma cells and IgG production [97]. IL-6 is also involved in induction of Th17 response and has a dual profile pro- and anti-inflammatory [112,113]. Studies have demonstrated essential involvement of IL-6 in triggering core symptoms related to pro-inflammatory response in autistic model of maternal immune activation (MIA) [130].

Increased levels of IL-6 are associated with increased stereotypy in ASD [120], impaired cognitive abilities, abnormal anxiety and decreased social interactions [107]. Here, we review the main findings about IL-6 levels in ASD: IL-6 is elevated in brain tissue (cerebellum, frontal cortex and anterior cingulated gyrus) [7,131,132], and in serum and PBMC [116–120], while it is reduced in plasma and n-DBSS [114,121]. In the VPA animal model of autism, higher levels of IL-6 were reported in hippocampus [123], hippocampus and spleen after LPS challenge [109] and whole brain homogenate [124].

IL-8

Interleukin-8 is a chemoattractant cytokine produced mainly by macrophages that specifically targets neutrophils, promoting their activation [133]. So, its major functions result from its chemotactic and pro-inflammatory activities [97]. Elevated levels of this cytokine were associated with increased

hyperactivity, stereotypy, and lethargy [120]. Higher levels of IL-8 was described in frontal cortex [132], plasma [115], cerebrospinal fluid (CSF) [134], PBMCs [120] and n-DBSS [121] of ASD subjects.

IL-10

This cytokine can be produced by several cellular types including DCs, macrophages, mast cells, NK cells, eosinophils, neutrophils and B cells [135], and is able to regulate growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells, exerting a primarily anti-inflammatory activity [97,135]. IL-10 is important to fine tune the immune response against invading pathogens, maintaining the homeostatic state [135]. In ASD patients, increased levels to IL-10 were found in anterior cingulated gyrus and amniotic fluid [128,134], while IL-10 levels is decreased in PBMCs [96].

IL-12

IL-12 is produced by T cell and acts in naïve T-cells and NK cells, activating them [97], and inducing IFNγ production, which is critical for the induction of Th1 cells [136]. Plasma, PBMCs and serum of ASD subjects show higher levels of IL-12 [115,117,120] whereas n-DBSS show lower IL-12 levels [121]. Increased IL-12 levels were associated with increased stereotypy and lethargy in ASD patients [120].

IL-13

Similarly to IL-4, IL-13 is involved in type-2 immunity and is produced by T-cells. However, basophils, eosinophils and NK cells can also produce IL-13 [137]. The only report in autistic patients shows increased plasma levels of IL-13 [115].

IL-17

Interleukin-17 has an important role in immunity against intra and extracellular pathogens [138]. IL-17-producing cells including natural killer T cells and innate lymphoid cells play crucial roles in inflammation-associated diseases, such as infection, autoimmunity and tumors [139]. Also was described the effector role of IL-17a in onset of offspring behavioral abnormalities of mothers MIA-induced, showing the important crosstalk between the neuroinflammatory state and behavioral manifestations [140]. Increase levels of IL-17 have been reported in plasma and serum [115,141] of patients with ASD.

IL-23

Considered a pro-inflammatory cytokine essential for the differentiation of Th17 lymphocytes [142], IL-23 is produced by macrophages, dendritic cells, keratinocytes and other antigen-presenting cells after recognition of microorganisms [143]. IL-23 is critically involved in autoimmune diseases responses [144]. In autistic patients, elevated IL-23 levels in serum samples were reported [117].

TNF-α

The tumor necrosis factor alpha (TNFα) is an endotoxin-induced serum factor promoting phagocyte cell activation [97], whose main targets and producers are macrophages. TNFα is in higher levels both in patients (frontal cortex [132], PBMC [96,118,119,145], serum [117] and amniotic fluid [128]) and in the VPA animal model of autism (hippocampus and spleen responding to LPS [109] and whole brain tissue [124]).

IFN-γ

Interferon-γ (IFN-γ) plays an important role in host defense against intracellular pathogens. It is produced by NK T cells, CD8+ T cells, and T-helper 1 (Th1) CD4+ T cells and its functions include supporting Th1 differentiation [146], and macrophage activation and increasing neutrophil and monocyte function [97]. Patients with ASD have increased levels of IFN-γ in frontal cortex [132], plasma [147], CSF [134] and PBMC [96] and reduced levels in n-DBSS [121].

TGFβ1

TGF-β is primarily secreted by T cells and B cells, and acts in activated T and B cells. The major function of this cytokine is to inhibit hematopoiesis and T and B cell proliferation [97]. Higher levels to TGFβ1 were reported in anterior cingulated gyrus and CSF [134] of ASD subjects.

MCP-1

Monocyte Chemoattractant Protein-1 (MCP-1) or C-C chemokine ligand 2 (CCL2) signals to cells that contain the specific CCR2 receptor, stimulating their migration to sites where CCL2 is produced and facilitating the amplification of

neuroinflammation [148]. Higher levels of MCP-1 were observed in plasma [149], CSF [134] and amniotic fluid [128] of autistic subjects. Increased levels in plasma were associated with greater impairments in visual reception, fine motor skills and expressive language [149].

GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by T cells, macrophages and fibroblasts and targets stem cells. Its major function is to stimulate production of granulocyte, monocyte and eosinophils [97]. Diminished levels of GM-CSF were described in n-DBSS of ASD pediatric subjects [121].

G-CSF

The main source of granulocyte colony-stimulating factor (G-CSF) are fibroblasts and endothelial cells and its targets are stem cells in the bone marrow. G-CSF has a hematopoietic function and stimulates granulocyte production [97]. Higher levels of this cytokine were described in plasma of autistic patients [114].

EGF

Epidermal growth factor (EGF) is a small chemoattractant peptide produced by activated T cells that is involved with wound healing by attracting fibroblasts and epithelial cells [114]. Higher levels of this chemokine were reported in plasma samples from autistic patients [114].

RANTES

Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) chemokine or CCL5 is involved in immune cell transport to the inflammation site, promoting polarization towards an Th1 response [150]. Higher levels were associated with increased severity of lethargy, stereotypy and hyperactivity [149] in ASD patients.

Eotaxin

The CC chemokine eotaxin/CCL11 is known to bind to the receptor CCR3 on eosinophils and Th2-type lymphocytes [151]. Increased levels of Eotaxin were associated with increased severity of lethargy, stereotypy and hyperactivity in ASD subjects [149].

Final considerations

Autistic Spectrum Disorder has a high prevalence and a growing incidence over the last few years. This has driven investments in public health and mobilized researchers and health professionals worldwide. There has been a significant progress in ASD research since the disorder was first described, but to date, its etiology remains unclear. An interesting hypothesis is that dysregulation of neuroimmune communication is involved in the onset of ASD. In this review, we summarized the main neuroimmune alterations found both in ASD subjects and in the VPA animal model of autism. Noticeably, several changes in the VPA model reflect the alterations found in patients with ASD (Figure 2). Animal models that present face and construct validity, such as the VPA model, can be an effective tool for the investigation of pathways and tissue alterations involved with the pathogenesis of ASD.

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Table 1. Main cytokines with altered levels in autism subjects

DSM	Severity	Described comorbidities	Age (years)	Source	Outcome	Analysis method	Reference
ASD	ND	ND	neonatal	amniotic fluid	↑ MCP-1, IL-4, IL-10, TNF-α and TNF-β	Flow cytometry	[128]
ASD	ND	ND	neonatal	n-DBSS	↓ IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ ↑ sIL-6Rα, IL-8	Flow cytometry	[121]
ASD (DSM-IV)	Mild, moderate and Severe	ND	2-21	Serum	↑ IL-1, IL-6, IL-12, IL-23, TNF-α	ELISA	[117]
ASD (DSM-5)	ND	ND	3-11	PBMCs	↓ CD4+, FOXP3+, T cells ↓ mRNA and protein expression FoxP3 ↑ Tbet, ↑ STAT3, ↑ GATA3	Flow cytometry, PCR and Western Blotting	[152]
ASD (DSM-5)	ND	ND	3-11	PBMCs	↑ ROR-γt in CD4	PCR and Western Blotting	[152]
ASD (DSM-IIIR/DSM-IV)	ND	GI issues	2-16	Duodenal Lamina Propria	↑ CD3+/TNFα+ ↓ CD3+/IL-10+	Flow cytometry	[153]
ASD (DSM-IIIR/DSM-IV)	ND	GI issues	2-16	Epithelium	↑ CD3+/TNFα+ ↓ CD3+/IL-10+	Flow cytometry	[153]
ASD (DSM-IV)	ND	ND	1-17	PBMCs	↑ TNF-α	ELISA	[145]
ASD (DSM-IV)	ND	GI issues	4-15	PBMCs	↑ TNF-α, IFN-γ ↓ IL-10	Flow cytometry	[96]
ASD (DSM-IV)	Severe (nonverbal adult pacients)	ND	18-44	Serum	↑ IL-1β, IL-6	ELISA	[116]

ASD (DSM-IV)	ND	ND	2.9-4.3	PBMCs	↑ IL-1β, IL-6, IL-8, IL-12 p40	Multiplexing bead immunoassays	[120]
ASD (DSM-IV)	ND	ND	2-14	PBMCs	↑ TNF-α, TNFRI, TNFRII, IL-6, IL-1β	ELISA	[119]
ASD (DSM-IV)	ND	ND	2.2-5	PBMCs	↑ 1L-1β, IL-6, TNF-α	Flow citometry	[154]
ASD (DSM-IV)	ND	ND	5-44	*post mortem* brain tissue	↑ IL-6, IL10, TGFβ1 (anterior cingulated gyrus)	Human cytokine array kits	[7]
ASD (DSM-IV)	ND	ND	5-44	CSF	↑ IFNγ, TGFβ2, IL-8, MCP1	Human cytokine array kits	[7]
ASD (DSM-IV)	ND	ND	4-37	*post mortem* brain tissue	↑ IFNγ, IL-6, IL-8, TNF-α (frontal cortex)	Multiplex Bead Immunoassays	[132]
ASD (DSM-IV)	ND	ND	4-14	*post mortem* brain tissue	↑ IL-6 (cerebellum)	Immunohistochemistry	[131]
ASD (DSM-IV)	ND	ND	7-15	Plasma	↑ IL-1β, IL-1RA, IL-5, IL-8, IL-12 (p70), IL-13, IL-17	ELISA	[115]
ASD (DSM-IV)	ND	ND	3-4.5	Plasma	↑ MCP-1, RANTES, Eotaxin	Multiplexing bead immunoassays	[149]
ASD (DSM-IV)	ND	ND	4.7-10.1	Plasma	↑ IFN-γ	ELISA	[147]
ASD (DSM-IV)	Mild to moderate and Severe	ND	6-11	Serum	↑ IL-17A (proportional increase to severity of autism)	ELISA	[141]
ASD (DSM-IV)	ND	ND	5-10	Plasma	↑ IL-1a ↓ IL-6, G-CSF, EGF	ELISA	[114]

DSM: Diagnostic and Statistical Manual of Mental Disorders; **CSF**: cerebrospinal fluid; **ELISA**: enzyme-linked immunosorbent assay; **IFN**: interferon; **IL**: interleukin; **ND**: not described; **n-DBSS**: neonatal dried blood samples; **PBMC**: peripheral blood mononuclear cells; **PCR**: polymerase chain reaction; **TNF**: tumor necrosis factor.

Table 2. Main cytokines with altered levels in the valproic acid animal model of autism

Animal	Dosage	Embryonic day	Administration via	Source	Age	Outcome	Analysis method	References
BALB/c	600 mg/Kg	E11	Subcutaneous	Dorsal hippocampus	P28	↑ IL-1β	PCR	[122]
BALB/c	400 mg/Kg and 600 mg/Kg	E12.5	Subcutaneous	Spleen	8-10 weeks	Only VPA did not onset inflammatory response, but showed exacerbated response to a LPS challenge: ↑ IL-1β, IL-6 and TNF-α expression	PCR	[109]
BALB/c	400 mg/Kg and 600 mg/Kg	E12.5	Subcutaneous	Hippocampus/ Cerebellum	8-10 weeks	↑ IL-6 and TNF-α expression in VPA animals exposed to a LPS challenge	PCR	[109]
Wistar	600 mg/Kg	E12.5	Intraperitoneal	Hippocampus	P40	↑ IL-6, ↑ IL-1β	ELISA	[123]
Wistar	800 mg/Kg	E12.5	Gavage	Whole brain	P21	↑ IL-1β, IL-6, TNF-α	ELISA	[124]

IL: interleukin; **PCR**: polymerase chain reaction.

Legend of figures

Figure 1. Th1, Th2, Th17 commitment lineage from naïve CD4+ T cells. The main functions of each immune response and the signature cytokine are highlighted in the boxes. APC: antigen-presenting cell; NK: natural killer cell; T-bet: T box expressed in T cells; GATA: GATA-binding protein; ROR: Retinoid-related orphan receptor; IL: Interleukin; IFN: Interferon; TGF: Transforming growth factor.

Figure 2. Main results of cytokines altered both in ASD subjects and in VPA animal model. At the interface of the columns and rows are shown the common findings both to humans and to animal model in different biological sources. The references are already cited in Table 1.

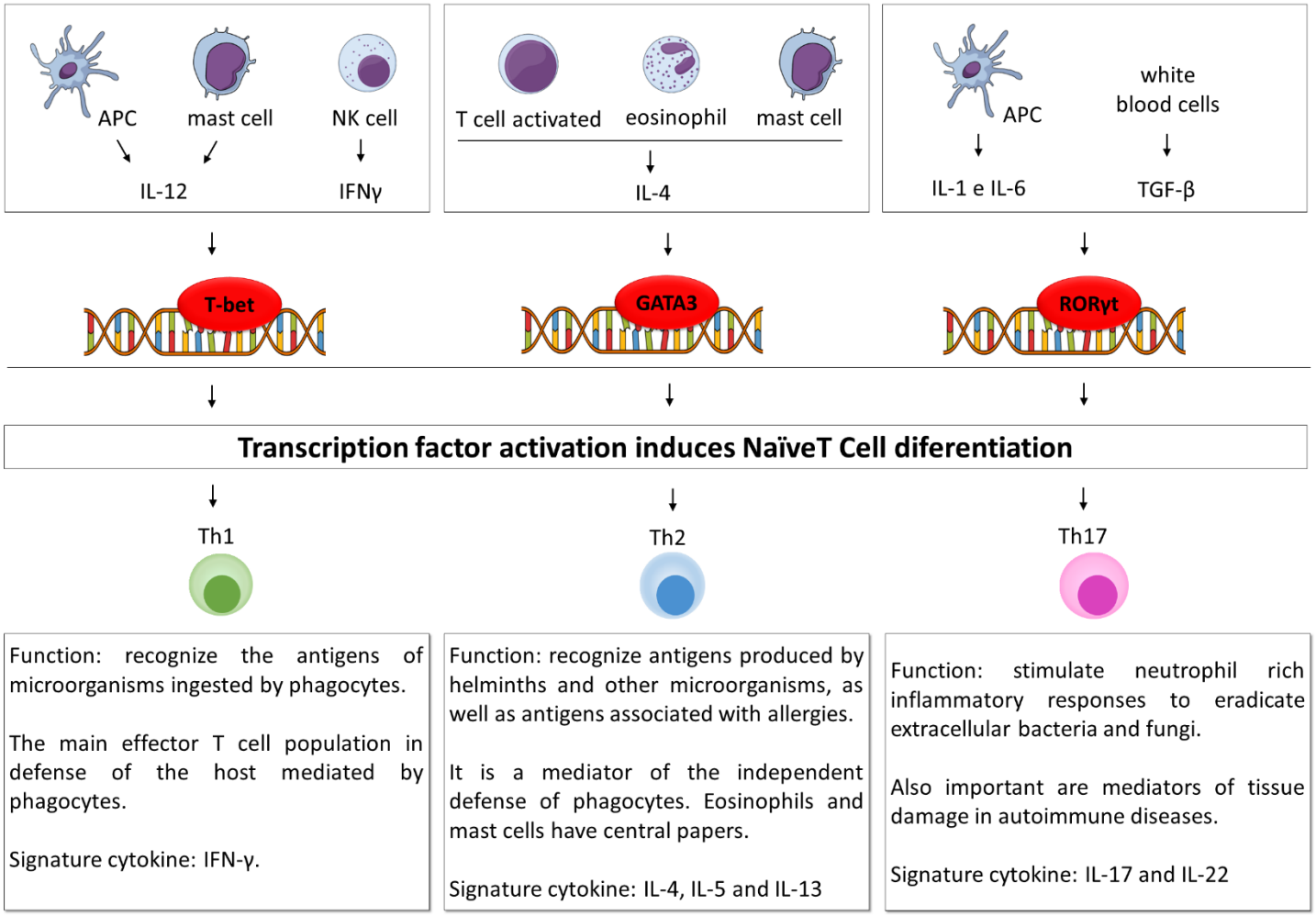

Figure 1. Th1, Th2, Th17 commitment lineage from naïve CD4+ T cells.

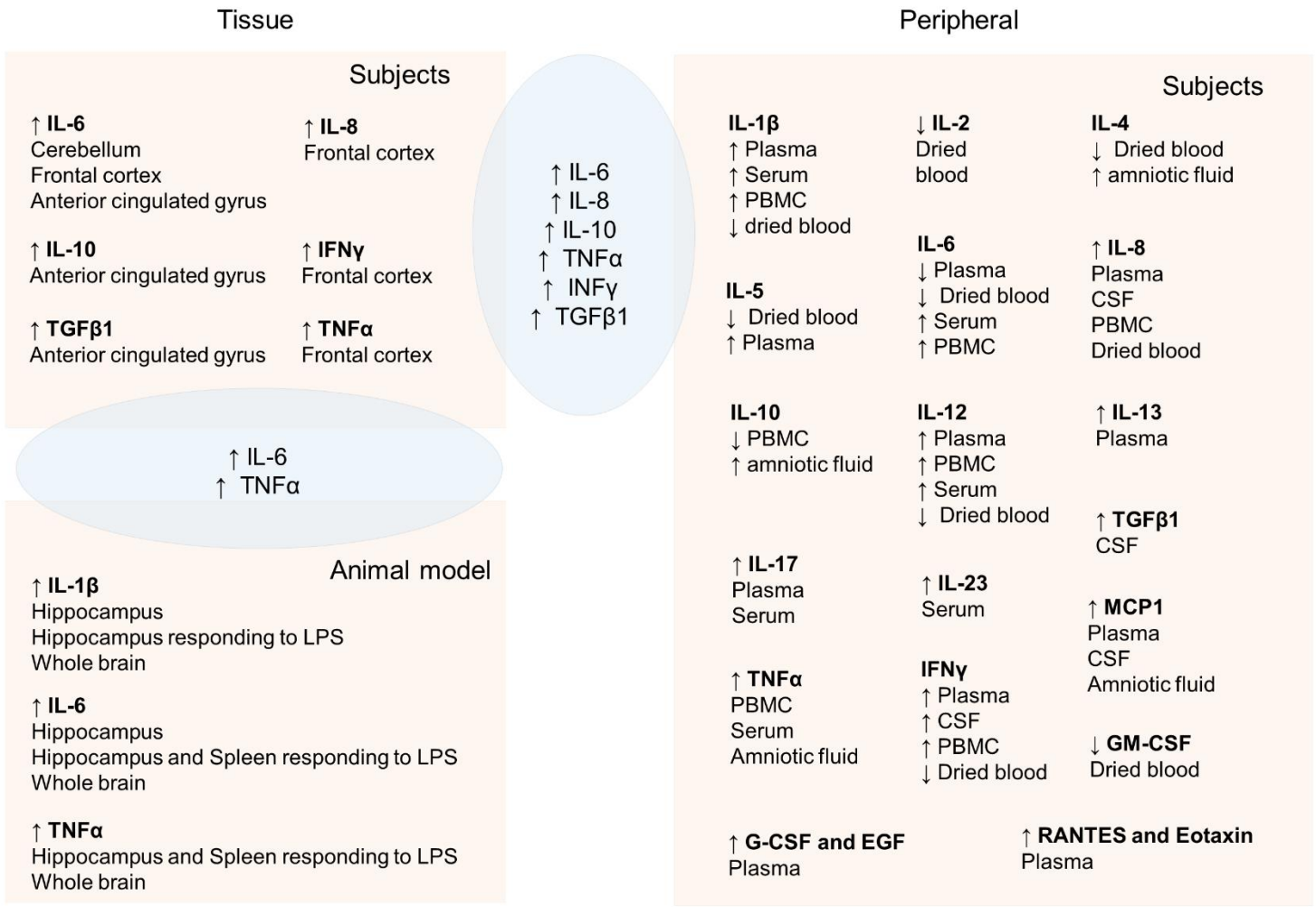

Figure 2. Main results of cytokines altered both in ASD subjects and in VPA animal model.