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Multiple inflammasome complexes are activated in autistic spectrum disorders

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ABSTRACT

Background: Inflammasomes are multimeric protein platforms involved in the regulation of inflammatory responses whose activity results in the production of proinflammatory cytokines. Because neuroinflammation is observed in autistic spectrum disorders (ASD), a neurologic condition of childhood resulting in a complex behavioural impairment, we analyzed the inflammasomes activity in ASD. Additionally we verified whether alterations of the gastrointestinal (GI) barriers might play a role in inflammasomes activation.

Methods: The activity of the inflammasomes, the concentration of the inflammasomes-derived proinflammatory cytokines interleukin (IL)-1β and IL-18, and serum parameters of GI damage were analyzed in 25 ASD children, 23 healthy siblings (HS) and 30 unrelated age-matched healthy controls (HC).

Results: A significant upregulation of the AIM2 and the NLRP3 inflammasomes and an increased production of IL-1β and IL-18 that was associated with a consistent reduction of IL-33, an anti inflammation cytokine were observed in ASD alone. Notably, in a possible immune-mediated attempt to dampen inflammation, IL-37, a suppressor of innate inflammatory responses, was significantly augmented in these same children. Finally, intestinal fatty acid binding protein (IFABP), an index of altered GI permeability, was significantly increased in serum of ASD and HS.

Conclusions: These results show that the inflammasomes are activated in ASD and shed light on the molecular mechanisms responsible for ASD-associated neuroinflammation. The observation that GI alterations could be present as well in ASD offers a possible link between such alterations and neuroinflammation. Therapeutic strategies targeting inflammasome activation could be useful in ASD.

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1. Introduction

Autistic spectrum disorders (ASD) is described in DSM 5 as a neurodevelopmental disorder characterized by persistent deficits in social communication and social interaction across multiple contexts, and restricted, repetitive patterns of behavior, interests, or activities (DSM 5). This definition replaces the previous denomination of "Pervasive Developmental Disorders", found in DSM-IV and not universally accepted (Chiappedi et al., 2010). ASD is associated with psychiatric and non-psychiatric comorbidities that include immune alterations, such as an increased production of proinflammatory cytokines by peripheral blood immune cells and mucosal lymphocytes from the ileal lymphoid tissue (Ashwood and Wakefield, 2006). Notably, proinflammatory cytokines may be responsible for many features of autism, such as mood and sleep disturbances.

Inflammation was recently shown to be modulated by the activity of the inflammasomes: intracellular multimeric protein

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complexes whose aggregation leads to the generation of proinflammatory cytokines (Schroder and Tschopp, 2010). Inflammasomes activation is initiated by the ligation of Nod-like receptors (NLRs), cytoplasmic pattern recognition receptors, by antigens or by non-microbial danger signals (DAMPs) released by damaged cells (Jin and Flavell, 2010; Lamkanfi, 2011). At this point NLRs oligomerize to form multiprotein complexes that serve as platforms for the recruitment, cleavage, and activation of inflammatory caspases. Four inflammasome complexes (NLRP1, NLRP3, IPAF, and AIM2) have been partially characterized. These complexes contain an NLR family protein or AIM2; the PYCARD (*aka* ASC) and/or Cardinal adaptor proteins; and pro-caspases-1, 5 and 8 (Gurung et al., 2014; Gurung and Kanneganti, 2015). NLRP3, the best-characterized inflammasome, exists in a signaling incompetent conformation which changes upon a second signal that leads to the assembly of a multimolecular complex with PYCARD and caspase 1. NLRP3 is activated by many signals including exogenous ATP (Bauernfeind et al., 2011a, 2009; Lamkanfi and Dixit, 2009; Mariathasan et al., 2006; Schroder and Tschopp, 2010). The AIM2 inflammasome on the other hand, includes AIM2, PYCARD, and caspase-1 and is activated by cytosolic dsDNA from viruses or the host itself (Bauernfeind et al., 2011b; Hornung et al., 2009; Muruve et al., 2008; Schroder and Tschopp, 2010). Multiple signals, thus, trigger the formation of an active inflammasome leading to the cleavage and release of bioactive cytokines including IL-1β and IL-18 (Agostini et al., 2004; Bryant and Fitzgerald, 2009; Halle et al., 2008; Horvath et al., 2011; Kanneganti et al., 2006; Lamkanfi and Dixit, 2014; Martinon et al., 2009; Martinon et al., 2004a; Martinon and Tschopp, 2004b; Wen et al., 2013). Although these cytokines have a beneficial role in promoting inflammation and eliminating infectious pathogens, mutations that result in constitutive inflammasome activation and overproduction of IL-1β and IL-18 were linked to inflammatory and autoimmune disorders (Master, 2013; Pollard and Kono, 2013; Rubartelli, 2012). Notably, monocytes of ASD children were described to be hyper responsive to signaling *via* specific TLRs (Enstrom et al., 2010) but no data are available on the possible involvement of the inflammasome in ASD.

Gastrointestinal (GI) alterations, including an increase in GI permeability was also observed both in ASD children and in their first-degree relatives, suggesting the presence of an intestinal (tight-junction linked) hereditary factor in the families of subjects with autism (D'Eufemia et al., 1996; de Magistris et al., 2010). Alterations in the GI permeability can be inferred by measuring specific proteins in serum. Lipopolysaccaride (LPS), a major component of the cell wall of gram-negative bacteria, translocates from the intestinal lumen to the peripheral circulation when the integrity of the gut barrier is altered. Soluble CD14 (sCD14) and intestinal fatty acid-binding protein (I-FABP) are endogenously produced reactive biomarkers that are indexes of microbial translocation. sCD14, in particular, is secreted by the liver and by the intestinal monocytes in response to LPS, whereas I-FABP is released into the systemic circulation in case of enterocyte damage and intestinal ischemia (Brenchley et al., 2006; Derikx et al., 2007; Steele et al., 2014).

Alterations of the GI permeability result in immune activation, directly in the case of LPS that binds to toll like receptor (TLR) 4, or indirectly *via* the release of DAMPs, including ATP, from damaged/necrotic enterocytes. In both cases NF-Kb is activated and the proinflammatory cytokines IL-1β and IL-18 are produced.

In the attempt to shed light on the pathogenesis of ASD-associated neuroinflammation we analyzed the inflammasome activity in ASD children; results indicated that multiple inflammasome complexes are indeed activated in ASD, and this possibly correlates with an increased GI permeability.

2. Materials and methods

2.1. Patients and controls

Seventy-eight children were enrolled in the study. Twenty-five children (median age = 7 years; range = 3–11 years; 4 females and 21 males) had a diagnosis of autistic spectrum disorders (ASD) according to the DSM 5 criteria (American Psychiatric Association, 2013). The diagnostic protocol included clinical neuropsychiatric examination, Autism Diagnostic Observation Schedule (ADOS), Autism Diagnostic Interview – Revised version (ADI-R) and Childhood Autism Rating Scale (CARS). These children were characterized by normal growth parameters, blood pressure, liver function, and ECG. None of them had undergone any therapy, including psychotropic drugs, for at least a month before the study. Twenty-three healthy, unaffected siblings of the ASD patients were also enrolled in the study (HS) (median age = 9 years; range = 4–11 years; 11 females and 12 males). Sub-threshold levels of social impairment, as evaluated by the Social Responsiveness Scale score were present in the HS, but all of them were classified clearly outside the autism spectrum as defined in the DSM 5. Anamnestic data were analyzed for all children; both groups were immunized according to the vaccination schedules of the Italian Ministry of Health, and no major infectious or inflammatory event was recorded in the clinical history of any of them. A third group of individuals included 30 healthy controls (HC) (median age = 7 years; range = 3–11 years; 10 females and 20 males) without familiarity for autism. Controls were healthy children who had a negative personal and family history for neurologic, psychiatric and gastrointestinal symptoms or disorders, and who were characterized by a normal neurologic and psychiatric development.

All blood samples were drawn in the early morning, after an overnight fast. The control samples were collected from a separate institution within the same city (Pediatric Clinic of the University of Milano), were hand-carried in biohazard containers to the centralized laboratory at the Don C. Gnocchi Foundation IRCCS, and were processed within 3 h to avoid any biases. Overt GI symptoms were present only in a small minority of ASD (3/25: 12%) and HS (1/23: 4%) children; as indicated above, no HC reported any GI condition. Written consent was obtained and ethical approval was granted by the Ethics Committee of the Don C Gnocchi Foundation IRCCS in Milano, Italy.

2.2. Blood sample collection and cell separation

Whole blood was collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson & Co., Rutherford, NJ, USA). PBMC were separated on lympholyte separation medium (Cedarlane, Hornby, Ontario, CA) and washed twice in PBS at 1500 RPM for 10 min; viable leukocytes were determined with a Scepter 2.0 Handheld Automated Cell Counter (Millipore, Billerica, MA). Serum samples were obtained from whole blood at the end of clotting time (60 min) by centrifugation (3400g× 10 min) and stored at −80 °C until use.

2.3. Cell cultures

PBMC (1×10^6/ml) were cultured in 12-wells cell culture plates (Greiner Bio-One GmbH Frickenhausen Germany) with RPMI 1640 supplemented with 10% human serum, 2 mM L-glutamine, and 1% penicillin (Invitrogen Ltd, Paisley, UK) alone (un-stimulated), or were incubated with: 1) 2 μg/ml Lipopolysaccharide (LPS) alone for 2 h (Sigma-Aldrich, St. Luis, MO, USA) or, 2) were primed with LPS (2 μg/ml) for 2 h before being stimulated with 200 mM ATP

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