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MINI-SYMPOSIUM: Role of the Inflammasome in Brain Pathogenesis: A Potential Therapeutic Target?

Inflammasome activation in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)

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Abstract

The aptly named inflammasomes are powerful signaling complexes that sense inflammatory signals under a myriad of conditions, including those from infections and endogenous sources. The inflammasomes promote inflammation by maturation and release of the pro-inflammatory cytokines, IL-1β and IL-18. Several inflammasomes have been identified so far, but this review focuses mainly on the NLRP3 inflammasome. By still ill-defined activation mechanisms, a sensor molecule, NLRP3 (NACHT, LRR and PYD domains-containing protein 3), responds to danger signals and rapidly recruits ASC (apoptosis-associated speck-like protein containing a CARD) and pro-caspase-1 to form a large oligomeric signaling platform—the inflammasome. Involvement of the NLRP3 inflammasome in infections, metabolic disorders, autoinflammation, and autoimmunity, underscores its position as a central player in sensing microbial and damage signals and coordinating pro-inflammatory immune responses. Indeed, evidence in patients with multiple sclerosis (MS) suggests inflammasome activation occurs during disease. Experiments with the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), specifically describe the NLRP3 inflammasome as critical and necessary to disease development. This review discusses recent studies in EAE and MS which describe associations of inflammasome activation with promotion of T cell pathogenicity, infiltration of cells into the central nervous system (CNS) and direct neurodegeneration during EAE and MS.

MS/EAE AND INFLAMMASOME

Inflammasomes denote a specific set of damage- and stress-sensing supramolecular signaling complexes that function to induce maturation and secretion of the cytokines, IL-1β and IL-18, through caspase-1 activation and pyroptosis, a type of inflammatory cell death. These cytokines do not possess a secretion signal sequence and are initially translated in an inactive form as pro-IL-1β and pro-IL-18. As such, they need to be post-translationally processed into biologically functional forms. Classically, inflammasomes are composed of a protein that senses stimulation (NLRP3, AIM2, etc.), an adaptor molecule (ASC), and a catalytic protein (pro-caspase-1). These molecules form a complex upon activation, and quickly accumulate to form a large cytosolic oligomer. The presence of this oligomer allows the self-cleavage of pro-caspase-1 to the active form of caspase-1. Caspase-1 then cleaves pro-IL-1β and pro-IL-18 to their mature forms and induces (lytic) pyroptosis by activation of gasdermin D, which allows the release of cytokines in the cytoplasm (45). Inflammasome activation is thus a widely reactive, rapid and potent amplifier of inflammation that is integral to immune function. Traditionally associated with the sentinel behavior of phagocytes of the innate immune system, such as dendritic cells (DCs) and macrophages, inflammasomes have also been described in cells of glial (61), endothelial (95) and neuronal lineages (1, 44).

There are several known sensor molecules in inflammasomes—the most studied being NLRP1, NLRP3, NLRC4, AIM2 and pyrin. Each of these is activated by distinct stimuli. They respond to variety of pathogen-associated molecular patterns (PAMPs), such as anthrax lethal toxin (NLRP1) and flagellin (NLRC4), while AIM2 detects cytosolic dsDNA. Pyrin functions to sense pathogen modification and inactivation of Rho GTPases (96). NLRP3 is activated by a wide range of PAMPs and damage-associated molecular patterns (DAMPs). The NLRP3 inflammasome is the best studied of the inflammasomes and is activated by a wide range of conditions, both microbial and sterile. The NLRP3 inflammasome has been described in a number of autoimmune and autoinflammatory diseases. Involvement of the NLRP3 inflammasome in sterile inflammation is found in diseases such as gout, atherosclerosis, the cryopyrin-associated periodic syndromes (CAPS), and Alzheimer's disease (6, 20, 33, 71). Classically, secretion of IL-1β and IL-18 mediated by the NLRP3 inflammasome requires two distinct processes. The first process is induced by activation of gene transcription, including *Il1b* and *Nlrp3* by stimulating

receptors such as PRRs. Post-translational priming of the inflammasome components also occurs before activation. For example, de-ubiquitination of NLRP3 (41, 73) and linear ubiquitination of ASC (76) are such necessary steps to ready these components for NLRP3 inflammasome activation. Then, the second signal induces inflammasome activation—oligomerization of the inflammasome and activation of caspase-1. Inflammasome activation is induced by diverse stimuli such as; endogenous danger signals (extracellular ATP, cholesterol crystals, fibrillar β-amyloid), microbes (bacteria, viruses, fungi), and environmental/exogenous matter (asbestos, alum) (19). The mechanism of recognition of these molecular patterns by NLRP3 has yet to be fully elucidated. However, some intracellular factors are known to trigger NLRP3 inflammasome activation, including intracellular K^+ efflux, mitochondrial ROS production and the cathepsin release by "frustrated phagocytosis" (contents of the lysosome released but phagocytosis does not occur).

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) that presents in two general phenotypes, relapsing-remitting MS (RRMS), and progressive MS. Progressive MS is further subdivided into primary progressive MS (PPMS) and secondary progressive MS (SPMS). Patients with RRMS manifest "new or recurrent neurologic symptoms and signs with full or partial recovery and lack of disease progression between disease relapses" (59). PPMS is observed in patients with no history of RRMS, and is characterized by a steady loss of neurological function with occasional plateaus and temporary minor improvements. In contrast, SPMS indicates a worsening of RRMS to a progressive form of disease (59). Experimental autoimmune encephalomyelitis (EAE) is mediated by an autoimmune T cell response against antigens in the myelin sheath that insulates CNS neurons. While a causal relationship has not been defined for T cells in MS, myelin-reactive T cells are present in MS patients and possess an inflammatory signature (9, 65).

The innate immune system initially coordinates and carries out many effector functions of the adaptive immune system. As such, the inflammasomes are situated to exert a large amount of control over disease in MS and EAE. Indeed, caspase-1 and IL-1β are identified in MS plaques, and levels of caspase-1 and IL-18 are increased in MS patient peripheral blood mononuclear cells (PBMCs) (35, 60).

Our focus on the NLRP3 inflammasome comes from studies identifying its importance in development of EAE: Without NLRP3, mice were protected from the disease, and both ASC and caspase-1 were also found to be critical to EAE development (24, 29, 37). Mild EAE by the lack of NLRP3, ASC and caspase-1 points collectively to the involvement of the NLRP3 inflammasome in the disease. Our laboratory confirmed NLRP3 inflammasome activation in mice with EAE (38) and elucidated that the NLRP3 inflammasome exerted control of disease by promoting migration of inflammatory cells to the CNS (37). Additionally, IFNβ, a first line therapeutic for MS, functioned to suppress NLRP3 inflammasome activation and its effects in EAE, protecting mice from the disease (36, 38). In the following sections, we summarize recent findings concerning the role of inflammasomes and the inflammasome effector molecule, IL-1β, on MS and EAE.

NLRP3 inflammasome in EAE

In this section, we review relatively new findings on inflammasomes in EAE that have been published in the last several years.

Modulation of inflammasome activation affects EAE

Triggering NLRP3 inflammasome activation causes rapid oligomerization of NLRP3 with ASC, proteolytic activation of caspase-1, and release of IL-1β and IL-18. ATP is a well-studied endogenous simulator of this process (69, 89). Levels of extracellular ATP control NLRP3 inflammasome activation, thus detection of ATP is likewise an important process for cells to activate the NLRP3 inflammasome. Pannexin-1 is a large non-selective channel known to open in response to the engagement of extracellular ATP to the P2X7 receptor (68). Once open, pannexin-1 allows release of ATP into the extracellular space (74). Thus, preventing ATP flux through pannexin-1 blocks NLRP3 inflammasome activation through reduction of activating ligand availability. Indeed, in EAE, pannexin-1 blockade results in both a delay in disease onset and a lower peak score (53). Pannexin-1 knockout mice also show in a modest delay in EAE onset, but manifest a similar phenotype and score to WT at the peak of disease (53). Similarly to blocking its release, hydrolysis of extracellular ATP by the ectonucleotidase CD39 (encoded by *Entpd1*) expressed by conventional dendritic cells (cDCs) inhibits NLRP3 inflammasome activation in EAE (58). A recent study showed that the blockade of CD47 also ameliorates EAE through inhibition of the NLRP3 inflammasome (25). CD47 is also known as integrin associated protein (IAP). Based on decreased levels of active caspase-1 in ATP-stimulated macrophages in tissue culture and serum IL-1β in CD47-deficient EAE mice, CD47 signaling appears to support NLRP3 inflammasome activation during EAE (25). Thus, certain surface receptors modulate inflammasome activation and can be manipulated to mitigate disease in EAE.

Several cytokines present during EAE are also known to influence NLRP3 inflammasome activation. GM-CSF, whose expression in T cells is necessary for EAE development (14, 18, 23), is suggested to be involved in positive regulation of inflammasome activity in myeloid cells: GM-CSF increases IL-1β release by enhancing expression of ASC and pro-IL-1β in subsets of dendritic cells (DCs) (18, 47). Further, production of GM-CSF by T cells is dependent on their expression of IL-1R (18, 52). IL-1R signaling in T cells induces their production of GM-CSF, which primes DCs to enhance inflammasome activation (18). In contrast to the positive regulation of inflammasome by GM-CSF, type-I interferons (IFNα, IFNβ) were demonstrated to inhibit NLRP3 inflammasome activation in EAE (30, 38).

These molecular mechanisms of NLRP3 inflammasome regulation and their effects on EAE development are informative. However, the exact stimulatory conditions and spatiotemporal distribution of inflammasome activation in EAE remain elusive.

Cell types with active inflammasomes and their locations in EAE

Accumulated evidence distinctly shows that the NLRP3 inflammasome is involved in EAE pathogenesis (29, 30, 37, 38, 40, 75, 86). However, the timing, triggers and location and cell types with active inflammasomes are still largely unknown.

Recent studies demonstrated that inflammasome activation is involved in response to pertussis toxin (PTx), commonly injected on day 0 and day 2 after immunization to induce EAE, which enhances disease by poorly understood mechanisms. Mouse intraperitoneal PTx injections were found to induce IL-1β release by hematopoietic cells through an effect which was attributed to the pyrin inflammasome rather than the NLRP3 inflammasome (22). The pyrin inflammasome contains the sensor protein "pyrin," instead of NLRP3, and mutations in pyrin are known to be causative in Familial Mediterranean Fever (16, 17). In active EAE induced with PTx injection into 2D2 transgenic mice, pyrin deficiency significantly suppressed the development of disease, but pyrin was not necessary for passive EAE induced by T cell adoptive transfer (22). A separate investigation of PTx in EAE identified that PTx injections strongly induce IL-1β production by neutrophils and monocytes recruited to antigen-draining lymph nodes (dLNs), which results in promoting encephalitogenic Th17 responses (77). Thus it is possible in active EAE that PTx-induced monocytic pyrin inflammasome activation promotes IL-1β production to polarize T cells to a pathological phenotype, which then traffic to the CNS.

Inflammasome activation in myeloid cells in antigen-dLNs and the spleen is involved in the generation of the pathological T cells in EAE (38, 77). However, inflammasome activation in other cell types and *in vivo* sites may also contribute to the acquisition of a pathological phenotype by T cells and EAE progression. A recent report describes that CNS-resident mast cells, an often overlooked population known to be necessary for EAE development (13, 84), play a role in this process (81). T cells specific for a self-antigen traffic to the meninges and activate meningeal mast cells. Replacing these meningeal mast cells with inflammasome-incompetent caspase-1-deficient mast cells reduced disease score significantly when compared to reconstitution with WT mast cells (81). The result indicates that inflammasome activation in meningeal mast cells is also critical to EAE development.

In addition to the cell subsets of the innate immune system traditionally associated with inflammasomes and inflammasome-related genes, a recent publication indicated that ASC is necessary for neuropathological IL-17-producing T helper (T_H17) cell identity (57). In EAE, T_H17 cells are generated and participate in the disease development. T cell receptor signaling generates pro-IL-1β, which appears to be processed in a T_H17 cell-intrinsic, ASC-NLRP3–dependent fashion (57). However, instead of caspase-1, caspase-8 is responsible for this IL-1β maturation in Th17 cells (57). Involvement of caspase-8 in the NLRP3 inflammasome was initially shown in myeloid cells during infectious diseases (28), but not in T cells. This T cell-intrinsic IL-1β supports the survival of T_H17 cells in the CNS during EAE—without it, the cells do not survive to induce severe disease (57).

Collectively, these results indicate that inflammasome activation in EAE is neither restricted to a single location nor to a cell type.

IL-1β in EAE

IL-1β and IL-18 are the inflammatory effector cytokine processed by inflammasomes. IL-18 is necessary to EAE development (29). MS patients show increased levels of serum IL-18, as well as increased ex vivo production of IL-18 by PBMCs from MS patients (11, 43, 63). IL-18 enhances ex vivo Th1 and Th17 polarization as IL-1β does, particularly when combined with IL-23 (48). Despite this evidence that links IL-18 to MS and EAE, IL-18 has been less examined. Thus, in this section, we expand to discuss recent articles that primarily focus on IL-1β in EAE and MS.

IL-1β compromises CNS barrier integrity

The blood brain barrier (BBB) and blood–spinal cord barrier (BSCB) are barricades which serve to partition the tissues and extracellular fluid of the CNS from circulating leukocytes. IL-1β promotes the opening of these barriers, which allows entry of immune cells into the cerebrospinal fluid (CSF) and the CNS parenchyma (5). This step is an early event in the progression of MS and EAE (46, 67). Endothelial cells, including those of the CNS vasculature, directly respond to IL-1β to compromise BBB integrity (12). Congruent with this, *ex vivo* treatment of human endothelial cells from brain microvasculature with IL-1β increased adhesion molecule expression and neutrophil adherence, denoting a pathological phenotype (94). In addition to endothelial cells, CNS astrocytes also control the BBB and induce BBB permeability after exposure to IL-1β (4, 5). Recent work has connected these observations of IL-1β signaling in astrocytes on BBB integrity with known effects of Sonic hedgehog (SHH) signaling (3). IL-1β treatment causes a modest decrease in the expression and release of SHH from astrocytes, leading to BBB leakiness (93).

The impact of IL-1β was also demonstrated on the BSCB and inflammatory immune cells infiltrated in the CNS during EAE. IL-1R expression in endothelial cells (ECs) is necessary for the adhesion of neutrophils to endothelial cells of the BSCB (7), an early and necessary event in EAE. More recent finding demonstrated that IL-1β-dependent paracrine loop between ECs and infiltrated neutrophils and monocytes drives neuroinflammation (50). The finding suggests that IL-1β produced in the early stage of EAE signals through IL-1R expressed in luminal ECs to create a local site of leukocyte trafficking in the spinal cord vasculature, allowing myeloid cells to transmigrate into the CNS.

IL-1β is associated with CNS lesions in MS and EAE

MS and EAE are characterized by CNS lesions—foci of inflammation and tissue damage that accumulate lymphoid and myeloid cells. These lesions are now known to be present in both white and grey matter during acute and chronic phases of disease (26, 92). The cellular constituents of these lesions differ based on their location, but IL-1β expression is correlated similarly to both types (72). A combination of flow cytometric and immunohistochemical approaches demonstrated that infiltrating monocytes, rather than microglia, produce the IL-1β present in white matter spinal cord lesions in mouse EAE (91). In contrast, in rhesus macaques and human MS, IL-1β appears to be produced by lesion-associated microglia, as opposed to neutrophils or monocytes (8).

IL-1β promotes neurotoxicity in EAE

Neurodegeneration in the late stages of MS correlates to the frequency of inflammatory episodes in the early stages of the disease. The involvement of IL-1β in the inflammation-driven neurodegenerative process was studied by evaluating neuronal physiology and excitatory postsynaptic currents (EPSCs). EPSCs are induced in neurons by excitatory neurotransmitters, such as glutamate.

Excessive glutamate signaling and accumulation in the synapse can cause neuronal hyperactivation and excitotoxicity, and are associated with MS, as well as EAE (10, 70, 82, 87, 88). Congruently, an enhanced striatal neuron EPSC phenotype is correlated with neurodegeneration in EAE (10). CSF from MS patients increases EPSCs, along with signs of neuronal death, in mouse corticostriatal slice cultures; and this effect is mediated by IL-1β (78, 79). In the cerebellum, IL-1β increases EPSCs in Purkinje cells during EAE by impairing glutamate clearance by astroglia in neuronal synapses, an effect which is blocked by an IL-1R antagonist (56). IL-1β increases EPSCs in corticostriatal slice cultures, but this effect can be blocked by the adenosine deaminase inhibitor, Cladribine (2-chloro-2'-deoxyadenosine, 2CdA) (62), which was validated as a treatment for MS in the CLARITY clinical trial (27). Cladribine is known to deplete lymphocyte populations in the patient, although additional mechanisms may be relevant to its therapeutic effect (49). Thus, inhibition of neuronal response to IL-1β by Cladribine (62) potentially represents a new mechanism of disease suppression.

Inflammasomes in human MS

Genetic associations

Inflammasome-associated genetic mutations in humans are found in several diseases, such as the cryopyrin associated periodic disorders (CAPS) (2, 34). A previous report hinted at a connection between NLRP3 mutations and MS-like pathology in the brain in a patient with Muckle–Wells Syndrome (15). In line with this observation, a group of individuals with the CAPS-associated V198M and Q703K mutations in the NLRP3 protein showed a high comorbidity (53%) with MS (83). Thus, it is possible that inflammation in the NLRP3 autoinflammatory diseases also triggers CNS autoimmunity.

Focusing on IL-1R signaling, several SNPs in the *IL1RN* and *IL1A* genes were not found to have an association with MS. It was also reported that the rs16944 SNP in *IL1B* was also not MS-associated (32). However, another study which analyzed a different population of patients found that the heterozygosity of rs16944 in *IL1B* was associated with a significantly higher likelihood of early-onset MS, while homozygosity of rs16944 is protective in MS development (39). Turning to IL-18, one study suggested that the −607C/A SNP in the *IL18* gene promoter correlates susceptibility to MS (66). In contrast, a different study indicated that the −607C/A SNP is not significant but a SNP at position −137 might be a genetic risk factor for MS at least in the Turkish population (42).

Evaluation of IL-1β and IL-18 as possible biomarkers for MS

In addition to genetic approaches, the effort has been directed towards inflammasomes in MS as indicators predicting *in situ* response to treatment, disease severity, and progression. IL-1β and IL-18, secreted effectors of inflammasome activation, are obvious targets for such a biomarker. Elevated serum and CSF levels of IL-18 have been reported as associated with MS (11, 51). Though reports of IL-1β in the CSF of MS patients have conflicted (21, 31, 54, 90), IL-1β levels in CSF correlate with cortical pathology in very early MS (85). During a remission period in RRMS, IL-1β levels in the CSF also correlate with disease progression during treatment, but not with the likelihood of relapse (80). Taken together, it appears that further studies are necessary to determine the correlation between MS and IL-1β and IL-18, but examining these inflammasome-associated cytokines during specific phases of MS may yet prove valuable diagnostic tools.

Inflammasomes in response to IFNβ treatment

IFNβ is a first-line drug to treat RRMS. Multiple mechanisms are known describing the amelioration of MS and EAE by IFNβ. One of the mechanisms in EAE is inhibition of NLRP3 inflammasome activity (30, 38). Type-I IFN receptor (IFNAR) in mononuclear phagocytes, such as macrophages and dendritic cells, detects IFNβ and downregulates NLRP3 inflammasome activity through the SOCS-1-mediated breakdown of activated Rac-1, which enhances mitochondrial ROS production (38). IFNα, also as an IFNAR ligand, has a similar effect on the NLRP3 inflammasome (38). The inhibitory effect of type-I IFNs on other inflammasomes is not exerted at least to the NLRC4 inflammasome, implying specificity to the NLRP3 inflammasome (38). Although IFNβ is one of the most widely used treatments for RRMS, its use as a therapeutic is only partially effective, and not beneficial in 7%–49% of patients, depending on response criteria (75), which suggests that MS is a heterogeneous disease. Despite the efforts to identify genetic biomarkers associated with therapeutic response to IFNβ, currently no markers are available in clinics.

Responses to IFNβ in MS patients have been investigated with regards to inflammasomes (55, 64). One report showed that IFN-β treatment decreased mRNA expression levels of *NLRP3, NLRC4* and *AIM2* in PBMCs, as well as plasma IL-1β levels in MS patients (64). Another study was performed by breaking down a population of RRMS patients into groups of IFNβ responders and non-responders (55). Responder PBMCs exhibited reduced mRNA levels of *NLRP3* and dramatically less *IL1B* mRNA at baseline before treatment; however, the non-responders showed a distinct upregulation of *NLRP3* and *IL1B* mRNA 3 months after IFNβ treatment (55). The study illustrates a complex regulation of the genes with regards to disease involvement and response to IFNβ.

Our recent study showed that subtypes of disease with differential response to IFNβ exist in EAE, and their dependency on the NLRP3 inflammasome determines the response to IFNβ (36). The NLRP3-dependent EAE was responsive to IFNβ (36), congruent with the previous finding that the NLRP3 inflammasome is a suppressive target of IFNβ (38). Conversely, the NLRP3 inflammasome-independent EAE subtype does not respond to IFNβ (36). This NLRP3-independent IFNβ-resistant EAE subtype can be sufficiently induced with high doses of adjuvant (heat-killed Mycobacteria in EAE) (36). The finding suggests that bypassing the NLRP3 inflammasome to induce the pathology of EAE can be achieved by strong stimulation of innate immunity. Indeed, acute virus infection also "converted" NLRP3 inflammasome-dependent EAE to NLRP3 inflammasome-independent IFNβ-resistant disease (36). Thus, in addition to genetic predispositions, differential environmental stimuli may be important factors in MS heterogeneity. Interestingly, mice with such NLRP3 inflammasome-independent EAE can normally develop T_H17 cells, but they tend to accumulate inflammatory cells in the brain compared to the spinal cord (36). Also, NLRP3 inflammasome-independent EAE shows minimal remission due to irreversible neuronal damage (36). The study

identified the involvement of membrane-bound lymphotoxin (LT) and a chemokine receptor CXCR2 in the EAE subtype that is NLRP3 inflammasome-independent and IFNβ-resistant. PBMCs from patients with IFNβ-resistant RRMS showed higher relative expression of *LTBR* (encoding the receptor for mLT) and *CXCR2* than those from IFNβ-responders (36). In the future, testing gene expression in more defined immune cell populations will further clarify the pathology of MS regarding the heterogeneous involvement of the NLRP3 inflammasome, which appears to have an impact on the efficacy of IFNβ.

CLOSING REMARKS

Inflammasome activation is associated with MS and is necessary for the development of classical EAE which responds to IFNβ. The studies reviewed here continue to reinforce this concept and contribute significantly to a complete understanding of inflammasome activation in EAE and MS. They show not only that inflammasomes are involved in the pathogenesis of EAE, but that they promote or are associated with disease at multiple major steps in the disease development, such as initial inflammation, T cell skewing, CNS barrier breakdown and neurodegeneration. This highlights the continuing need to identify the locations and cellular sources of inflammasomes and IL-1β in EAE and MS to fully understand their impact on disease. The newly-defined role for pyrin in EAE additionally encourages investigation into inflammasome sensors other than NLRP3. Finally, the discovery of IFNβ-resistant, NLRP3-independent EAE which results in neurodegeneration in the brain may reflects the heterogeneity of disease found in RRMS patients, and might further recapitulate some aspects of progressive MS, such as lack of remission and neurite loss. Elaboration of this model alongside inflammasome-dependent EAE potentially opens new avenues for development of therapies for MS.

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