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Inflammatory cytokine production in a mouse model of Aicardi-Goutieres syndrome and neuroinflammation

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Most neurological diseases are associated with a tissue injury that is detected by the innate immune response (IIR), leading to an inflammatory component. The IIR is activated through conserved Pattern Recognition Receptors, including membrane bound Toll like receptors (TLRs), intracellular nucleotide-binding oligomerization domain like receptors and the receptors for advanced glycation end-products (Amor et al., 2010; Heppnerrt et al., 2015). These receptors detect highly conserved structural motifs of damaged or stressed tissues (danger-associated molecular patterns (DAMP)). Cytosolic receptors of nucleic acids, such as cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) and melanoma differentiation associated gene 5 (MDA-5) can also trigger IIR activation leading to interferon (IFN) and IFN stimulated gene (ISG) expression through DNA/RNA sensing signaling pathways (Yang and Li, 2020). Once bound to their cognate receptor, the recognition complexes migrate into the nucleus and initiate different signaling cascades that eventually lead to central nervous system (CNS) inflammation.

The canonical cascade of nucleic acid sensing signaling pathway employs the production of interferons that trigger transcription and translation of ISG instrumental in initiating and propagating inflammation. However, not all ISG inductions are mediated through induction of IFN, leading to the oxymoron of non-interferon dependent stimulation of ISG. An example of the canonical pathway is Alzheimer disease where amyloid-β peptides (Aβ) produced by aberrantly processed amyloid-β precursor protein precipitate in the extracellular space to form Aβ plaques, while the hyperphosphorylated microtubule-associated protein Tau forms the neurofibrillary tangles in neurons (Heppner et al., 2015). These protein products act as DAMPs to stimulate TLR2, TLR3 and TLR4 to activate IIR and produce inflammatory cytokines that promote neurodegeneration (Sochocka et al., 2019). Examples of IFN independent ISG production include infection with human cytomegalovirus (Ashley et al., 2019).

Aicardi-Goutieres syndrome (AGS) is a neurological disease of the young associated with CNS inflammation and degeneration. Mutations in seven genes associated with nucleic acid metabolism have been linked to AGS (Crow and Manel, 2015; Rice et al., 2017). Production of aberrant nucleic acids detected by cGAS and MDA-5 is believed to account for the neuroinflammation and degeneration seen in AGS. We recently developed a knock-In mouse in which a single nucleotide replacement (G>T) was made to the catalytic domain of ADAR1 gene locus to recapitulate a genetic feature of human AGS, the ADAR1 K999N mutation (Guo et al., 2021). As expected the brains of K999N mice showed aberrant RNA processing and the RNA sensing signaling pathway was activated leading to elevated transcription and translation of ISGs, including *CXCL10*, *ISG-15*, *ifit-1*, *ifit-3*, *CCL-5*, *Oasl-2*, *Stat-1*, *Mx-2*, *PKR* and others. Therefore, this K999N mouse is unique compared to other models of AGS associated mutations, in which IIR was not activated in the brains. What was most surprising, however, was the brains of K999N mice show no increase in interferon transcription or translation and no inflammation. Equally surprising is that despite universal expression of the mutation only select brain regions demonstrate elevated ISGs. Additionally, within those regions, neurons and glia selectively express different sets of ISGs. These findings raise several questions regarding the pathogenesis of ADAR1 mutations specifically and the control of CNS inflammation in general.

In the presence of aberrant RNA metabolism, how are ISGs induced without IFN and does this offer a potential target for abating neurological inflammation in AGS? Interferon production is a common pathway induced by the DNA/RNA sensing pathway. However, some viruses (e.g., human cytomegalovirus) have developed defenses to IFN (Ashley et al., 2019). To combat this defense, host cells have developed an IFN-independent means of ISG induction. In human cytomegalovirus infection this second line of defense is absolutely dependent upon IRF3. In our mouse model of AGS, we found no evidence of increased IFN transcription or translation. This implies that detection of aberrant RNA metabolism by IIR utilizes an alternative non-canonical pathway for ISG induction. In addition, we found that IRF7, rather than IRF3 was highly expressed in our mutant mouse implying that IRF7 may participate in the IFN-independent ISG production besides the known IRF3 dependent pathway. This hypothesis can be readily tested by crossing our transgenic model with knockout IRF3 or IRF7 mice. Should ISG induction be suppressed in such a model, this raises the tantalizing therapeutic possibility that AGS could be treated with small molecules interfering with IRF3 or IRF7.

Why are RNA processing errors in the CNS prone to augmenting ISGs? AGS mutations including the ADAR1 K999N mutation causing RNA processing errors is expressed throughout the organism, yet the inflammatory injury is prone to the CNS in AGS patients. While RNA editing is a feature of all mammalian cell metabolism, brain metabolism is particularly notable for abundant RNA processing (Deng et al., 2020). Is it possible that neuron RNA metabolism makes them selectively prone to activating the IIR but they have evolved a means of suppressing secondary inflammation?

Why do different CNS cells containing the same genetic mutation express different ISGs and are these signals interacting? When RNA expression and translation are assessed by Northern and Western blots respectively, the cellular context of heterogenous organs like the brain is lost. When we performed in situ hybridization on the transgenic K999N model, we were surprised to note that different cells expressed different ISGs. For example, neurons and not microglia expressed abundant ISG15 while microglia and not neurons expressed abundant *CXCL10*. Even more striking was that the differentially expressing neurons and microglia were spatially interdigitated suggesting that they were responding to each other in a micro local network (Chhatbar et al., 2018).

Why is there no inflammatory response in the *ADAR1* mutant mice like that seen in AGS despite robust transcription and translation of ISGs? ISGs include several highly potent cytokines. Many of these cytokines have been expressed in the CNS of a variety of transgenic animal models, all of which are notable for an intense CNS inflammatory response (Akwa et al., 1998). Yet our K999N line produces a mixture of cytokines in very high levels with no inflammation. What is missing to develop

the expected inflammatory response or what is present that suppresses this response?

One possibility is that despite the intense ISG response, the intact neuronal environment is not signaling to the periphery to elicit the ingress of inflammatory components. This would be a bit surprising because the theoretical purpose of inflammatory cytokines is to do precisely that attract and support local inflammation. Nevertheless, in a sort of reverse "immune privilege" perhaps an intact BBB does not evoke expression of appropriate cytokine receptors on the surface of CNS cells or prevents any of these cytokines from reaching responsive cells in the periphery. It is difficult to know whether high level of ISGs are present in brain tissues of AGS patients before clinical symptoms appear, however, late-onset AGS has been observed following infection and vaccinations. This would imply that a "second hit" is required to drive this pro-inflammatory state into frank inflammation. To test such a hypothesis, one could introduce a variety of "second hits" like blood-brain barrier disruption, infection or possibly even aging, to see if these stimulants would drive the model into an inflammatory state. Such a discovery would have tremendous implications for our understanding of the control of CNS inflammation and methods to suppress it.

What does elevated ISG expression mean in the absence of cellular inflammation?

Given the robust character of ISG expression in the CNS of the K999N model, we were surprise to see essentially none of the histological hallmarks of inflammation. Immunohistochemical staining for glial fibrillary acidic protein demonstrated no astrocytosis while staining for ionized calcium binding adapter molecule 1 showed no microglial proliferation. Subjectively the microglia demonstrated a slight blunting and thickening of their processes but the only objective of microglial activation was strong expression of the ISG *CXCL10*. Why the ISG signaling does not progress further to include the classic signs of inflammation (e.g., microglial proliferation, disruption of blood brain barrier, extracellular edema, ingress of inflammatory cells) remains to be determined. Whatever the missing signals or factors are, they may hold the key to novel mechanisms of controlling CNS inflammation.

As with many important scientific breakthroughs, these experimental observations raise more questions than answers. In the case of mutant ADAR1 transgenics, the stimulation of ISGs in the absence of interferon production and the absence of inflammation may provide the key to suppressing neuroinflammation and abating many neurological diseases.

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