




Functional Deficiency in Endogenous Interleukin-1 Receptor Antagonist in Patients with Febrile Infection-Related Epilepsy Syndrome

Benjamin D. S. Clarkson, PhD ^{1,2}, Reghann G. LaFrance-Corey, MSc,^{1,2}
Robert J. Kahoud, MD,^{1,3} Raquel Farias-Moeller, MD ⁴, Eric T. Payne, MD,¹ and
Charles L. Howe, PhD ^{1,2,5,6}

Objective: We recently reported successful treatment of a child with febrile infection-related epilepsy syndrome (FIRES), a subtype of new onset refractory status epilepticus, with the recombinant interleukin-1 (IL1) receptor antagonist (IL1RA) anakinra. On this basis, we tested whether endogenous IL1RA production or function is deficient in FIRES patients.

Methods: Levels of IL1 β and IL1RA were measured in serum and cerebrospinal fluid (CSF). The inhibitory activity of endogenous IL1RA was assessed using a cell-based reporter assay. *IL1RN* gene variants were identified by sequencing. Expression levels for the secreted and intracellular isoforms of IL1RA were measured in patient and control cells by real-time polymerase chain reaction.

Results: Levels of endogenous IL1RA and IL1 β were elevated in the serum and CSF of patients with FIRES (n = 7) relative to healthy controls (n = 10). Serum from FIRES patients drove IL1R signaling activity and potentiated IL1R signaling in response to exogenous IL1 β in a cell-based reporter assay. Functional assessment of endogenous IL1RA activity in 3 FIRES patients revealed attenuated inhibition of IL1R signaling. Sequencing of *IL1RN* in our index patient revealed multiple variants. This was accompanied by reduced expression of intracellular but not secreted isoforms of IL1RA in the patient's peripheral blood mononuclear cells.

Interpretation: Our findings suggest that FIRES is associated with reduced expression of intracellular IL1RA isoforms and a functional deficiency in IL1RA inhibitory activity. These observations may provide insight into disease pathogenesis for FIRES and other inflammatory seizure disorders and may provide a valuable biomarker for therapeutic decision-making.

ANN NEUROL 2019;85:526–537

Febrile infection-related epilepsy syndrome (FIRES),¹ a subtype of new onset refractory status epilepticus (SE),² is a rare seizure disorder defined by the occurrence of refractory SE that lacks a structural, toxic, or metabolic cause starting between 2 weeks and 24 hours after febrile illness, with or without fever at the onset of SE.^{3,4} Most FIRES patients are treated with antiseizure drugs, ketogenic diet,⁵ immunomodulatory therapies, and/or medically induced coma,⁶ with low

overall therapeutic efficacy rates.^{7,8} The majority of FIRES cases have poor outcomes, including development of refractory focal epilepsy, cognitive decline, brain atrophy, vegetative state, and death.^{9,10} In a recent study, 12% of patients died during the acute phase of the disease, and 93% of survivors developed refractory epilepsy, with the majority showing significant cognitive impairment.⁸ Magnetic resonance imaging in chronic cases revealed cortical and hippocampal atrophy,¹¹

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.25439

Received Dec 11, 2018, and in revised form Feb 15, 2019. Accepted for publication Feb 15, 2019.

Address correspondence to Dr Howe, Translational Neuroimmunology Laboratory, Mayo Clinic, Guggenheim 1542C, 200 First Street SW, Rochester, MN 55905. E-mail: howe@mayo.edu

From the ¹Department of Neurology, Mayo Clinic, Rochester, MN; ²Translational Neuroimmunology Laboratory, Mayo Clinic, Rochester, MN; ³Department of Pediatrics, Mayo Clinic, Rochester, MN; ⁴Department of Neurology, Medical College of Wisconsin, Milwaukee, WI; ⁵Department of Immunology, Mayo Clinic, Rochester, MN; and ⁶Center for Multiple Sclerosis and Autoimmune Neurology, Mayo Clinic, Rochester, MN

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and brain biopsies indicated gliosis without overt immune cell infiltration. Investigations into genetic or infectious causes have generally been negative.¹² These findings emphasize how little is known regarding FIRES etiology and underscore the possibility of multiple distinct etiologies.¹³

Anakinra is a recombinant form of the endogenous interleukin-1 receptor antagonist (rIL1RA). We recently reported the successful use of anakinra across 3 separate treatment epochs in a developmentally normal 32-month-old girl who progressed to super-refractory SE secondary to FIRES following banal febrile respiratory infection.¹⁴ Based on 12-month follow-up clinical examination, her motor, verbal, and social development were within normal limits for her age, suggesting a remarkable neuroprotective potential of anakinra treatment in FIRES.

IL1R signaling is implicated in febrile seizures¹⁵ and epileptogenesis,¹⁶ and single dose IL1RA treatment has previously been shown to reduce spike frequency in animal models of SE.¹⁷ Several lines of evidence suggest that blocking IL1R signaling in the brain during severe acute inflammatory states may be beneficial. In a rodent model of sepsis, IL1RA administration blocked the proconvulsant effect of intraperitoneal injection with lipopolysaccharide.¹⁸ IL1R signaling blockade was also found to reduce blood–brain barrier disruption, inflammation, and forebrain neuron loss in rat models of SE,^{17,19} and to prevent progression in models of acquired epilepsy.²⁰ Likewise, transgenic overexpression of IL1RA in astrocytes resulted in potent antiseizure effects.²¹ More recently, rIL1RA was found to prevent seizure sensitization when acutely administered in a mouse model of post-traumatic epilepsy,²² and rIL1RA therapy successfully halted seizures in a child with persistent systemic inflammation and epilepsy unresponsive to multiple antiseizure drugs.²³

The mechanism of IL1RA-mediated antiseizure activity is unclear and has not been studied in the context of FIRES. Surprisingly, we found that FIRES patients had elevated levels of endogenous IL1RA in serum (n = 5) and cerebrospinal fluid (CSF; n = 7) prior to the initiation of anakinra treatment. Given this unexpected elevation, coupled to the anakinra responsiveness of our index case, we hypothesized that the endogenous IL1RA in these subjects is functionally compromised. To test this hypothesis, we established a cell-based reporter assay to measure IL1RA function in serum and CSF collected from healthy controls (HCs), FIRES patients, and patients with other seizure disorders.

Subjects and Methods

Patient Samples

The Mayo Clinic Institutional Review Board approved the use of all human materials; all experiments were conducted in compliance with the relevant regulations. Subjects provided written informed voluntary consent. Samples from patients with normal pressure hydrocephalus

(NPH; n = 4), electrical SE in sleep (ESES; n = 3), FIRES (n = 7), and other medically refractory epilepsy (MRE) conditions (n = 4) were obtained from the Mayo Clinic Neuroimmunology Laboratory and the Mayo Clinic Center for Multiple Sclerosis and Autoimmune Neurology biobank. HC serum (n = 10) or peripheral blood mononuclear cells (n = 4) were obtained directly from consenting volunteers (TABLE).

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Leukosep tubes. Fresh whole blood was overlaid on 16 ml Ficoll-Paque and spun at $1,000 \times g$ for 10 minutes. The buffy coat was collected and washed. Cells were stored in vapor phase liquid nitrogen in freezing media containing 10% dimethylsulfoxide (DMSO).

IL1RA Enzyme-Linked Immunosorbent Assay

The human IL1RA/IL1F3 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions. Serum and CSF samples were diluted (1:2–1:5,000 in assay diluent), and duplicate samples were measured against a standard curve (31.3–2,000 pg/ml in duplicate). After chromogen development, absorbance was measured on a Spectramax M3+ microplate reader (Molecular Devices, Sunnyvale, CA).

Multiplexed Cytometric Bead Array

A human inflammatory cytokine kit, human chemokine kit, and human enhanced sensitivity IL1 β flex set (all BD Biosciences, Franklin Lakes, NJ) were used according to the manufacturer's instructions. Clarified cell supernatants, serum, and CSF were diluted 1:2 to 1:10 in assay diluent, and values were measured against a standard curve using an Accuri C6 (BD Biosciences) flow cytometer.

HEK-Blue IL1R Cells

Human embryonic kidney (HEK) cells expressing IL1 receptor and secreted embryonic alkaline phosphatase under NF- κ B/AP1 transcriptional control (HEK-Blue IL1R; InvivoGen, San Diego, CA) were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, 100 μ g/ml Normocin, 200 μ g/ml hygromycin B, 1 μ g/ml puromycin, and 100 μ g/ml Zeocin. HEK-Blue IL1R cells were treated with recombinant human IL1 β (PeproTech, Rocky Hill, NJ). After 3 hours, saturating concentrations (10 μ g/ml) of anakinra were added to the cells to block further IL1R signaling, and endpoint supernatants were collected at 24 hours. Supernatants were mixed with QUANTI-Blue reagent, and absorbance at 655 nm was recorded every 5 minutes for 3 hours at 37°C in kinetic read mode.

Real-Time Polymerase Chain Reaction

Lysates in RLT Plus buffer (Qiagen, Valencia, CA) were homogenized with QIASHredder columns (Qiagen). RNA was isolated using RNeasy Plus Micro Kits (Qiagen). Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland) was used to synthesize cDNA from RNA samples using oligo-dT primers. Equal amounts of template RNA were used for each cDNA reaction. Reactions were incubated at 55°C for 45 minutes and inactivated by

heating at 85°C for 5 minutes. Real-time polymerase chain reaction (RT-PCR) was performed on 2 to 10 ng cDNA in SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), in triplicate, for 50 cycles on a Bio-Rad CFX Connect system with the following protocol: 15 seconds at 95°C, 45 seconds at 55°C, and 5 seconds at 65°C. Primers (Supplementary Table 1) were selected using PrimerBLAST (National Center for Biotechnology Information, Bethesda, MD) to have melt temperatures >60°C. Data were analyzed in Excel using the Pfaffl method based on an estimated amplification efficiency of 95%.²⁴ Expression across all samples was normalized to the *GAPDH* housekeeping gene.

IL1RN Sanger Sequencing

IL1RN amplicons were generated from the index FIRES patient’s DNA by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) and primers targeting ~4,000 bp segments of *IL1RN* (see Supplementary Table 1). Targeted long-read Sanger sequencing was performed on extracted amplicons by the Mayo Clinic Gene Expression Core facility and by Genewiz using amplicon-specific sequencing primers (see Supplementary Table 1). Trace data were analyzed and aligned with Mutation Surveyor software, and confirmed variants were compared to NG_021240.1 RefSeqGen cited variants.

Statistical Analyses

For multiple comparisons, 1-way analysis of variance (ANOVA) or nonparametric (Kruskal–Wallis) tests were performed where appropriate. Reported *p* values were corrected for multiple comparisons (Holm–Sidak correction for ANOVA; Dunn correction for Kruskal–Wallis). Unpaired 2-tailed Student *t* tests were used for comparisons made between 2 groups; Welch’s correction was applied when sample sizes differed substantially between groups.

Results

Patients

Blood was collected from 14 healthy volunteers. Control CSF was collected from 4 patients with NPH. For comparison to the blood and/or CSF collected from 7 FIRES cases, these same biospecimens were collected from 3 patients with ESES and 4 patients with MRE who did not have FIRES. The Table lists demographic information, clinical diagnosis, and the number of samples collected for each patient or control. Differences in the number of patient samples utilized across figure panels resulted from the limited availability of biospecimens for each assay. On this basis, the MRE and ESES groups were combined for analysis in the first figure but separated in the third figure.

Elevated Levels of IL1RA in Serum and Elevated IL1β and IL1RA in CSF of FIRES Patients

We sought to determine whether there was a diminished level of endogenous IL1RA in serum or CSF from our index anakinra-responsive FIRES patient. We measured IL1RA levels by ELISA in FIRES patient serum and CSF before and after initiation of anakinra treatment, as well as in serum from

HCs, CSF from NPH patients, and serum and CSF from patients with other MRE. Surprisingly, we found that prior to the initiation of anakinra therapy, IL1RA was marginally elevated in FIRES serum compared to HCs and it was strongly elevated in FIRES CSF compared to NPH CSF and CSF from patients with other medically refractory epilepsies (Fig 1). Serum IL1β was elevated in some patients with either FIRES or MRE, and CSF IL1β levels were elevated in all FIRES

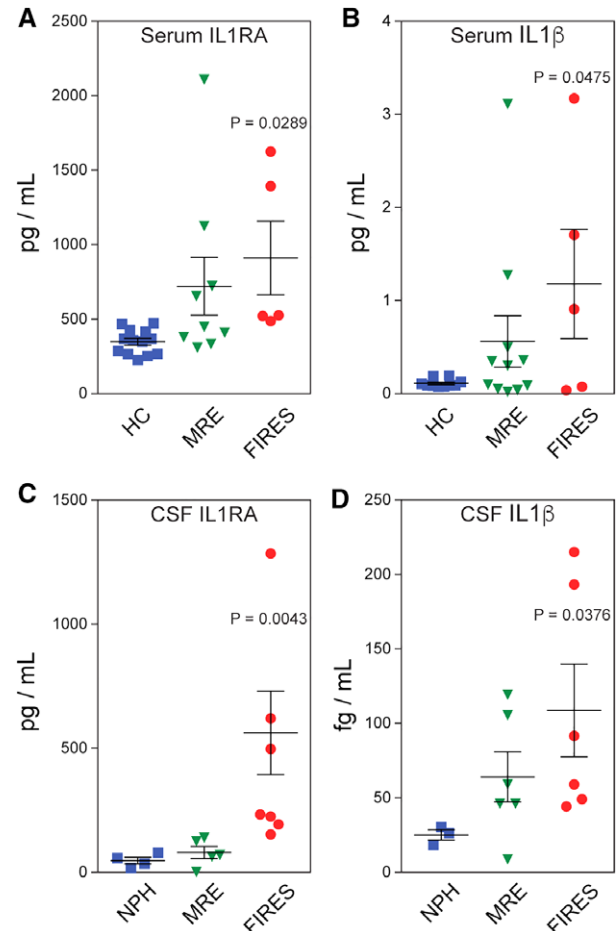


FIGURE 1: Serum and cerebrospinal fluid (CSF) levels of interleukin-1 receptor antagonist (IL1RA) and IL1β in febrile infection-related epilepsy syndrome (FIRES) and other seizure disorders. (A) Plot shows serum IL1RA level measured by enzyme-linked immunosorbent assay (ELISA) in healthy controls (HCs), patients with FIRES, and patients with other medically refractory epilepsies medically refractory epilepsy (MRE) including Landau–Kleffner syndrome or electrical status epilepticus during sleep (HCs, n = 13; MRE, n = 9; FIRES n = 5). (B) Plot shows serum IL1β (HCs, n = 10; MRE, n = 11; FIRES, n = 5). (C) Plot shows CSF IL1RA level detected by ELISA in patients with normal pressure hydrocephalus (NPH; controls) and patients with MRE or FIRES (NPH, n = 4; MRE, n = 5; FIRES n = 7). (D) Plot shows level of IL1β in CSF (NPH, n = 3; MRE, n = 6; FIRES, n = 6). Multiple comparison corrected 1-way analysis of variance probability values (Dunnnett) are indicated compared to HCs (A, B) or NPH (C, D). Each dot represents 1 patient or subject for all groups except MRE and HC serum measurements, which also include several biological replicates from different dates. Error bars indicate standard error of the mean.

patients and most MRE patients. Serum levels of IL1RA did not differ between FIRES patients and other MRE patients but were significantly different from HCs. In several FIRES patients, CSF levels of IL1RA exceeded serum levels, suggesting localized central nervous system (CNS) production. This finding is consistent with reports of elevated IL1RA following seizure activity.²⁵ Following anakinra treatment, both serum

and CSF levels further increased, reflecting detection of the exogenous IL1RA (data not shown), and the serum–CSF ratio was reversed, as expected.

Sensitive Cell-Based Assay for Measuring Serum IL1R Signaling Activity

Given that both IL1 β and IL1RA were elevated in FIRES patient serum, we sought to determine whether these factors were competent to modulate IL1R signaling activity. To develop a sensitive functional measurement of IL1R signaling activity, we optimized a cell-based assay using HEK-Blue IL1R cells. These cells express human IL1R and respond to IL1R agonists such as IL1 β with induction of nuclear factor κ B activity and consequent production of secreted embryonic alkaline phosphatase. Measurement of increased absorbance at 655 nm wavelength following incubation of cell supernatants with Quanti-Blue substrate provides evidence of IL1R signaling and exhibits a dose-dependent response curve (Fig 2). Furthermore, this response is competitively blocked by cotreatment with recombinant IL1RA. By titrating IL1 β we found that, of the concentrations tested, 32 pg/ml IL1 β was most sensitive to blockade by IL1RA while still retaining sufficient signal to noise to limit intra-assay variability. We further found that quenching HEK-Blue IL1R cells 2 hours after treatment using excess IL1RA further increased sensitivity of the assay.

Elevated IL1R Signaling Activity of FIRES Patient Serum

Using the assay described above, we measured the amount of IL1R signaling induced by factors present in serum from

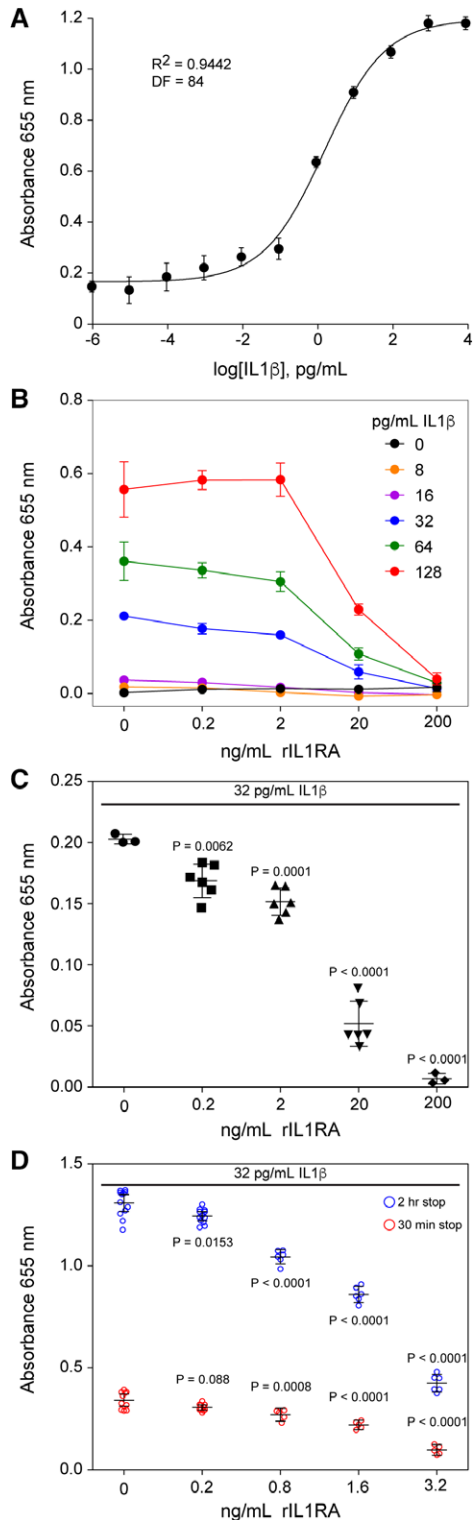


FIGURE 2: Optimization of an interleukin-1 receptor (IL1R) signaling cell-based assay. In all experiments, HEK-Blue IL1R supernatants were collected at 24 hours after stimulation and then mixed with prewarmed Quanti-Blue detection reagent for 3 hours. Background endpoint absorbance at 655 nm was subtracted from raw experimental values in panels B–D. (A) HEK-Blue IL1R cells were treated with different concentrations of IL1 β 1 ag/ml–10 ng/ml to determine the lower and upper limits of detection ($n = 8$ technical replicates). Log agonist versus response curve (nonlinear least squares fit) is shown based on a 4-parameter variable slope model (Hill equation). (B) HEK-Blue IL1R cells were treated with 0–128 pg/ml IL1 β in the presence of 0–200 ng/ml recombinant IL1R antagonist (rIL1RA) to determine the optimal IL1 β concentration that maximized the signal-to-noise ratio while also allowing for inhibition by low concentrations of IL1RA ($n = 3$ –6 technical replicates). (C) The same assay design was employed with the IL1 β concentration fixed at 32 pg/ml ($n = 3$ –6 technical replicates). (D) To maximize the detectable inhibitory effect of IL1RA, HEK-Blue IL1R cells were treated for 30 minutes or 2 hours with 32 pg/ml IL1 β and the indicated concentration of IL1RA, followed by quenching with excess IL1RA (1 μ g/ml). Supernatants were collected 24 hours after initial treatment for measurement of IL1R signaling ($n = 5$ –10 technical replicates). Multiple comparison corrected 1-way analysis of variance probability values (Dunnett) are indicated compared to the no IL1RA condition. Error bars indicate 95% confidence interval of the mean.

HCs and patients with MRE, ESES, and FIRES (Fig 3). We found that the addition of both FIRES and ESES patient serum to a final concentration of 10% (vol/vol) induced increased IL1R activity relative to that induced by HC or MRE serum (MRE did not differ from HCs). Next, we measured the impact of patient-derived serum factors on the induction of IL1R activity in response to a known concentration of exogenous IL1 β (32 pg/ml). Surprisingly, we found that FIRES and ESES patient serum synergized with exogenous IL1 β to induce even more IL1R activity than was observed when the same amount of IL1 β was added to HC or MRE serum. The ratio of exogenously induced activity measured in Figure 3B relative to the activity induced in Figure 3A is much higher in the FIRES and ESES samples. Given that the measured concentration of serum IL1 β in these patients was far below the 32 pg/ml added in the assay, this multiplicative increase in IL1R activity suggests that

other serum factors may be driving IL1R activity or that a larger reservoir of serum IL1 β remains undetected by ELISA, possibly because it is present in microvesicles, as has been previously described.²⁶ Overall, the serum IL1 β levels measured by ELISA did not correlate with the induction of IL1R activity across all subjects. Conversely, contrary to expectation, IL1RA levels weakly correlated with the amount of induced IL1R activity, suggesting the presence of serum factors that modulate the inhibitory capacity of IL1RA, perhaps by causing it to function as an IL1R agonist rather than an IL1R antagonist in this assay. These speculations were partly rejected in subsequent studies in which we confirmed that, in samples from a representative patient for each condition, the measured increase in IL1R activity could be at least partially suppressed by the addition of exogenous rIL1RA. However, again unexpectedly, the amount of IL1R signaling activity induced by serum was not completely suppressed by the addition of exogenous IL1RA in the samples derived from FIRES and ESES patients, whereas the activity was mostly suppressed in HC and MRE samples. This may mean that some portion of whatever is agonizing IL1R in the FIRES and ESES serum is not accessible to antagonism by exogenous IL1RA, as others have reported.²⁷ Of note, the HEK-IL1R cells used in this assay are also sensitive to other inflammatory cytokines such as murine IL1 α or tumor necrosis factor- α

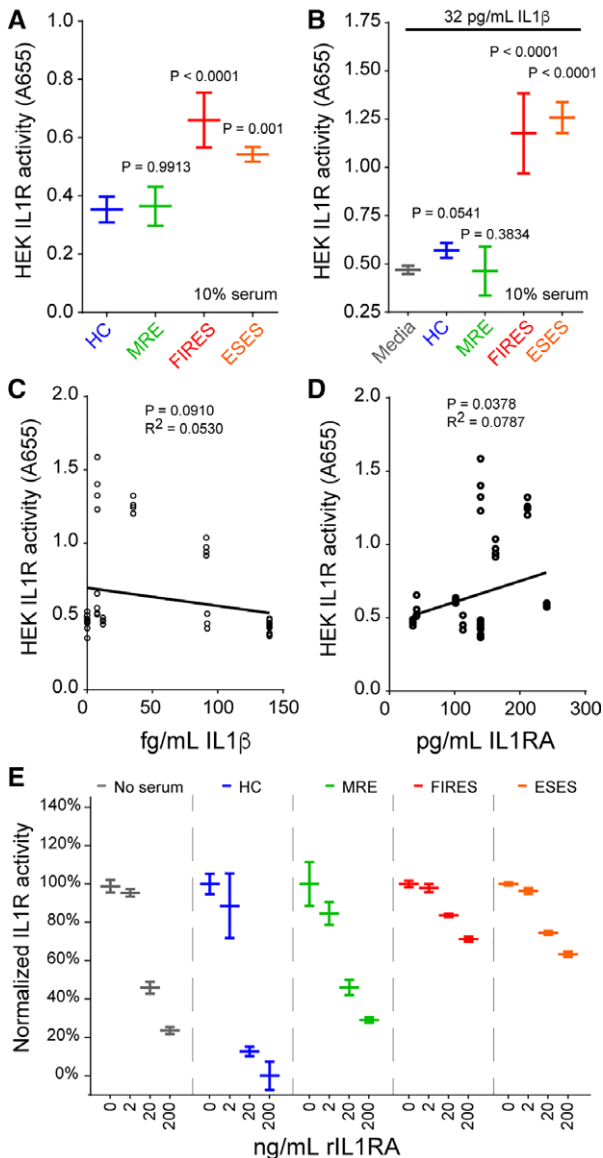


FIGURE 3: Serum levels of interleukin-1 receptor (IL1R) signaling modulators in febrile infection-related epilepsy syndrome (FIRES) and other seizure disorders. In all experiments, HEK-Blue IL1R supernatants were collected at 24 hours after stimulation and then mixed with prewarmed Quanti-Blue detection reagent for 3 hours. Background endpoint absorbance at 655 nm was subtracted from raw experimental values in all panels. (A) HEK-Blue IL1R cells were treated with 10% serum (vol/vol) from a healthy control (HC; n = 3 replicates from 1 subject), patients with medically refractory epilepsy (MRE; n = 4 replicates each from 3 patients), FIRES patients (n = 2-3 replicates each from 3 patients), and patients with electrical status epilepticus in sleep (ESES; n = 4 replicates each from 4 patient samples) for 2 hours before quenching with excess IL1R antagonist (IL1RA) as described in Figure 2. (B) IL1R activity elicited by 32 pg/ml IL1 β in the presence of 10% serum (vol/vol) from HCs (n = 4 replicates each from 4 subjects), MRE (n = 3 replicates from 1 patient), FIRES (n = 4 replicates each from 3 patients), and ESES (n = 4 replicates from 1 patient). (C) IL1R activity from the experiment in B is plotted against the concentration of IL1 β measured in the serum by enzyme-linked immunosorbent assay (ELISA). (D) IL1R activity from the experiment in B is plotted against the concentration of IL1RA measured in the serum by ELISA. (E) HEK-Blue IL1R cells were treated as in B with 10% patient serum (vol/vol) and the indicated concentration of recombinant IL1RA. The mean and 95% confidence interval are plotted based on values derived from 4 technical replicates from a single representative patient for each condition. Multiple comparison corrected 1-way analysis of variance probability values (Dunnett) are indicated in A and B compared to the HC serum condition. Probability values in C and D indicate whether the slope is significantly different from zero.

(TNF- α) when they are present at high titer. In our hands, however, the HEK-Blue IL1R cells responded to human TNF α only when present at concentrations >100 pg/ml, which was far in excess of the levels measured in patient serum (typically <1 pg/ml; data not shown). These findings suggest that, although informative, ELISA-based measures of serum IL1 β and IL1RA levels, per se, do not represent a complete biological picture of serum IL1R agonists and antagonists.

Reduced Functional Blockade of IL1R Signaling by Endogenous IL1RA in an Anakinra-Responsive FIRES Patient

It is likely that the concentration of IL1RA at the site of production in the brain interstitial space (and by extension its relevant functional activity) far exceeds the levels that we detect in CSF. IL1RA is a downstream transcriptional target of IL1R signaling and a principle component of negative feedback in this cascade. Therefore, elevated CSF IL1RA may represent either a normal acute response to terminate IL1R signaling or a chronic failure to terminate ongoing IL1R signaling. Thus, that we detected levels of endogenous IL1RA in the CSF of an anakinra-responsive FIRES patient at concentrations sufficient to cause suppression of IL1R signaling^{28,29} suggested the possibility of a dysfunctional IL1RA protein and failed termination of IL1R signaling. To test this hypothesis, we sought to determine the antagonistic activity of CSF-derived IL1RA from our FIRES index patient using the HEK-Blue IL1R cell-based assay. However, in our initial experiments using patient or NPH control CSF or artificial CSF we discovered that IL1R signaling was reduced in the presence of CSF relative to serum and that the addition of rIL1RA to cells in the presence of 50% (vol/vol) CSF or artificial CSF failed to inhibit IL1R signaling in response to 32 pg/ml IL1 β (Fig 4). Dialysis of CSF against DMEM/F12 across a 100 Da molecular weight cutoff cellulose acetate membrane restored the ability of rIL1RA to inhibit IL1 β -induced IL1R signaling. Therefore, we treated HEK-Blue IL1R Cells with 32 pg/ml IL1 β in the presence of 50% (vol/vol) dialyzed CSF that was collected from our index FIRES patient prior to initiation of anakinra therapy. Note that this CSF contained 65pM endogenous IL1RA (~1 ng/ml) as measured by ELISA but did not inhibit IL1R signaling. In contrast, addition of 65pM rIL1RA to dialyzed NPH CSF did inhibit the IL1R signaling induced by 32 pg/ml IL1 β when added at 50% (vol/vol). Moreover, treatment with 50% (vol/vol) dialyzed CSF collected from the FIRES patient after initiation of anakinra (measured at 24 ng/ml IL1RA) robustly suppressed IL1R signaling in response to 32 pg/ml IL1 β . To verify that the observed effect was not an artifact of the CSF, we repeated the stimulation of HEK-Blue IL1R cells with 50% (vol/vol) dialyzed NPH CSF in the presence

or absence of 65pM rIL1RA (added prior to dialysis) and found robust inhibition of IL1R signaling. Finally, to verify that dialysis did not introduce an unexpected agonistic effect in the FIRES CSF, we treated HEK-Blue IL1R cells with 10% (vol/vol) NPH or FIRES CSF in the absence of any other stimulus (no IL1 β) and observed no increase in IL1R signaling.

Multiple Noncoding Polymorphisms in FIRES Patient IL1RN Gene

To determine whether the observed impairment in IL1RA function was associated with a genetic mutation, we performed Sanger sequencing on overlapping 4,000 bp amplicons spanning the *IL1RN* gene of our index FIRES patient. No variants were detected within the translated portion of the coding sequences. Several novel and several previously reported variants were detected within intronic sequences, and a silent mutation was present in exon 6 (Fig 5A). Supplementary Table 2 lists each variant by row and provides the genomic location and associated risks for previously reported variants.

Marked Reduction in Expression of Intracellular Isoforms of IL1RN in FIRES Patient PBMCs

To determine whether there was aberrant expression of IL1RA that could explain the observed impairment in inhibitory function, we isolated PBMCs from HCs and our index FIRES patient. In separate experiments, we isolated protein and RNA from cell lysates and collected supernatants after 24 hours in culture. We measured secreted IL1RA in cell supernatants and intracellular IL1RA in cell lysates by ELISA. Total *IL1RN* mRNA expression was determined by RT-PCR. We did not measure a difference in the level of secreted IL1RA between our index FIRES patient and control PBMCs (see Fig 5). However, the level of intracellular IL1RA in cell lysates was significantly reduced in the FIRES patient PBMCs. This reduction corresponded to a 21-fold decrease in *IL1RN* mRNA levels in untreated FIRES PBMCs relative to controls. IL1RA is translated from 5 distinct isoforms that give rise to 2 secreted and 2 intracellular variants (isoforms 4 and 5 only differ upstream of the translational start site). Importantly, so-called intracellular forms of IL1RA are secreted in response to adenosine triphosphate and other triggers and serve as acute response molecules.³⁰⁻³² To determine whether the reduced levels of intracellular IL1RA protein were due to aberrant expression of specific *IL1RN* isoforms in the index FIRES patient, we analyzed expression of the secreted (isoform 1 and 4/5) and intracellular (isoforms 2 and 3) *IL1RN* gene products by RT-PCR. *IL1RN* isoforms 1 and 4/5 were marginally increased in FIRES PBMCs, although this difference did not reach significance relative to expression in PBMCs from HCs. In contrast, isoform 2 mRNA was present at <10% of HC levels, and isoform 3 was expressed at <5% of control.

Moreover, in control PBMCs isoforms 2 and 3 were the dominant *IL1RN* transcripts, together representing ~90 to 99% of all expressed isoforms. Thus, the profound decrease in absolute expression of *IL1RN* isoforms 2 and 3 in the FIRES patient PBMCs resulted in a proportional increase in expression of isoforms 1 and 4/5 as a percentage of total *IL1RN*

expression. For example, *IL1RN* isoform 1 represented about 2.5% of total *IL1RN* expression in HC PBMCs but represented 11.5% of *IL1RN* expression in the FIRES patient PBMCs. Likewise, *IL1RN* isoforms 4/5 represented about 1.5% of *IL1RN* expression in control PBMCs but jumped to ~15% of total *IL1RN* in the FIRES PBMCs.

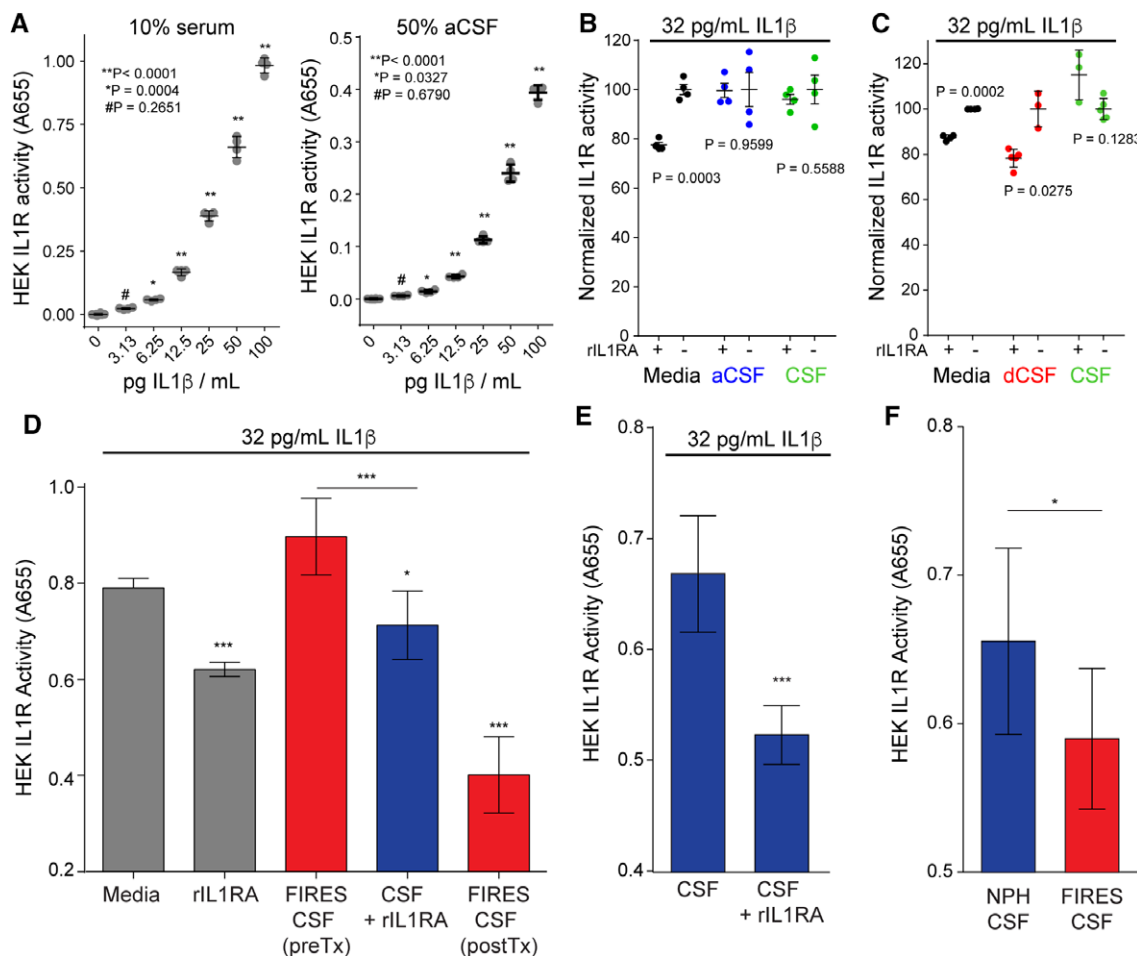


FIGURE 4: Deficient interleukin-1 receptor (IL1R) antagonism mediated by anakinra-responsive febrile infection-related epilepsy syndrome (FIRES) patient-derived IL1R antagonist (IL1RA). In all experiments, HEK-Blue IL1R supernatants were collected at 24 hours after stimulation and then mixed with prewarmed Quanti-Blue detection reagent. Absorption at 655 nm was measured by 3-hour kinetic read. Background endpoint absorbance at 655 nm was subtracted from raw experimental values in panels A–C. (A) HEK-Blue IL1R cells were treated with the indicated concentrations of IL1β in media containing 10% (vol/vol) fetal bovine serum or in media containing 50% (vol/vol) artificial cerebrospinal fluid (aCSF; n = 4 technical replicates per condition). (B) HEK-Blue IL1R cells were treated with 32 pg/ml IL1β with or without 650 pg/ml recombinant IL1RA (rIL1RA) in media or in media supplemented with 50% aCSF or 50% normal pressure hydrocephalus (NPH) CSF (n = 4 technical replicates). (C) HEK-Blue IL1R cells were treated with 32 pg/ml IL1β with or without 650 pg/ml rIL1RA in media or in media supplemented with dialyzed NPH CSF or NPH CSF (n = 3–5 technical replicates per condition). (D) HEK-Blue IL1R cells were treated with 32 pg/ml IL1β in media or in the presence of 65pM rIL1RA (gray bars) to establish dynamic range for IL1R inhibition. Cells were also stimulated with 32 pg/ml IL1β in 50% (vol/vol) dialyzed CSF collected from our FIRES index patient prior to anakinra treatment (preTx). This CSF contained 65pM endogenous IL1RA. Cells were stimulated with 32 pg/ml IL1β in 50% (vol/vol) dialyzed CSF from NPH patients supplemented with 65pM IL1RA or with 32 pg/ml IL1β in 50% (vol/vol) dialyzed CSF collected from our FIRES index patient after treatment with anakinra (postTx; 1 .3nM IL1RA measured by enzyme-linked immunosorbent assay; n = 3–5 technical replicates per condition). (E) HEK-Blue IL1R cells were treated with 32 pg/ml IL1β in the presence of 50% (vol/vol) dialyzed NPH CSF supplemented prior to dialysis with or without 65pM rIL1RA (n = 4 technical replicates per condition). (F) HEK-Blue IL1R cells were treated with 10% NPH CSF or CSF from our FIRES index patient (n = 6–8 technical replicates per condition). *p < 0.05, ***p < 0.0001. Multiple comparison corrected 1-way analysis of variance probability values (Dunnett, A–C; Holm–Sidak, D) are indicated compared to the no IL1β condition (A) or no IL1RA condition (B–D). For side by side comparisons, Student t test probability values are indicated (E, F). Error bars represent 95% confidence interval. Data are representative of 2 independent experiments.

Discussion

Our previously reported case¹⁴ highlights the importance of an anti-inflammatory response specific to the IL1 pathway in regulating neuronal excitability within the context of systemic inflammation. This patient's clinical presentation was consistent with the induction of SE by propagation of a peripheral, systemic inflammatory response into the CNS. Treatment with recombinant IL1RA terminated the patient's seizures and provided long-term neuroprotection, highlighting the

role of targeted anti-inflammatory therapy in the treatment of SE. Remarkably, the endogenous levels of IL1RA were significantly elevated in the serum and CSF of our index case prior to anakinra therapy. Furthermore, IL1RA levels were more elevated in CSF than in serum, relative to controls. This pattern was observed in 6 other FIRES patients (see Fig 1). Whereas serum levels of IL1RA did not clearly distinguish FIRES from other cases of refractory epilepsy, the CSF level of IL1RA represented a consistent biomarker for FIRES relative to ESES and MRE associated with autoimmune encephalopathy and other etiologies (see Fig 1C, Table). Although IL1RA can cross the blood-brain barrier,³³ the relative enrichment of IL1RA in CSF versus serum strongly suggests that IL1RA was being produced by CNS resident or infiltrating cells in the FIRES patients. Given previous reports showing the critical role of CNS-derived IL1 β in epileptogenesis, it is likely that the target(s) of IL1 β and by extension the target(s) of IL1RA-mediated inhibition are CNS cells that express functional IL1R, including endothelial cells, astrocytes, microglia, and neurons.

IL1 β levels are very hard to measure consistently in serum, as even levels associated with an acute inflammatory response are at or below the limit of detection in most

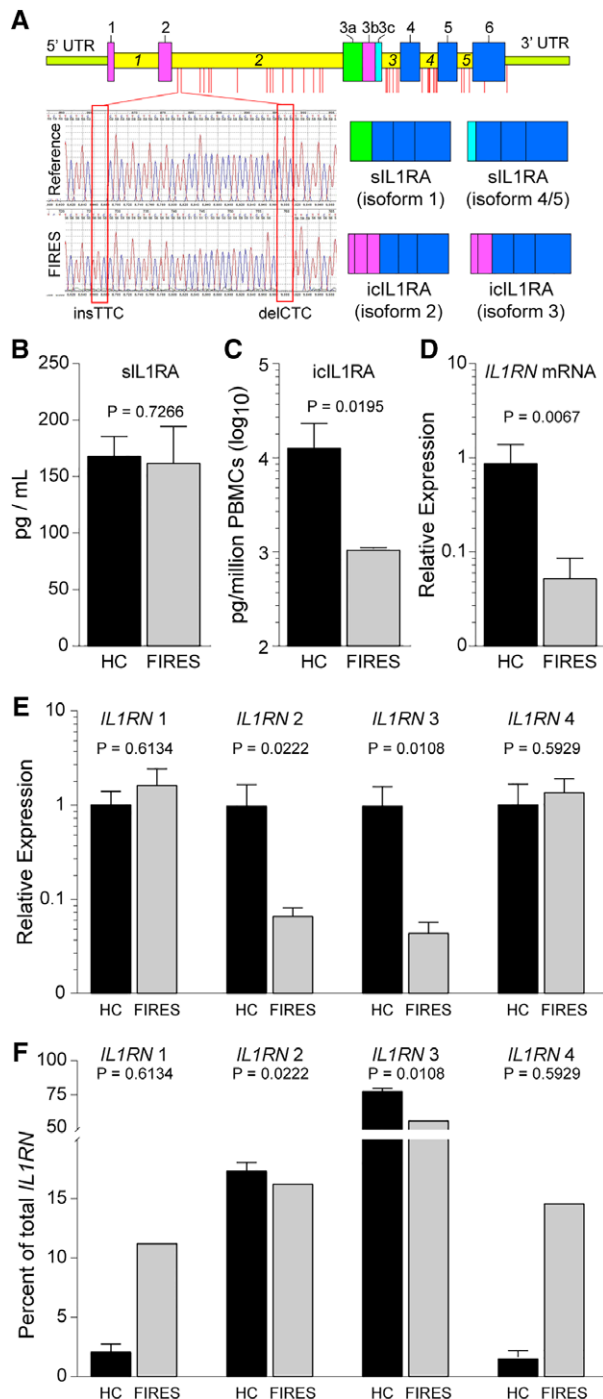


FIGURE 5: Reduced expression of intracellular interleukin-1 receptor antagonist (IL1RA) isoforms in peripheral blood mononuclear cells (PBMCs) from the index anakinra-responsive febrile infection-related epilepsy syndrome (FIRES) patient. (A) Sanger sequencing was performed on polymerase chain reaction (PCR)-amplified fragments of the *IL1RN* gene from our index FIRES patient. Exons and introns of the *IL1RN* gene are depicted along with the location of the indicated variants present in the gene. FIRES patient (bottom) and reference (top) sequences of selected regions are shown at the bottom alongside trace file images. **(B–F)** Freshly isolated PBMCs from our FIRES index patient or healthy controls (HCs) were incubated for 24 hours under resting conditions. Supernatants were collected, cells were washed, and cell pellets were lysed. **(B)** The concentration of secreted IL1RA (sIL1RA; n = 12 biological replicates) in supernatant and **(C)** the amount of intracellular IL1RA (icIL1RA; n = 4 biological replicates) in cellular lysate were measured by enzyme-linked immunosorbent assay. Graphs represent the mean of technical replicates. **(D)** In separate experiments, RNA was isolated from cell lysates and the relative expression of *IL1RN* mRNA was determined by real-time PCR (RT-PCR). **(E)** *IL1RN* isoform expression was further analyzed by RT-PCR using specific forward primers and a common reverse primer for each isoform. **(F)** Expression of each isoform was normalized to aggregate *IL1RN* expression levels, which were calculated in arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase expression. For RT-PCR experiments, values are averaged from technical triplicates representing n = 8 HCs and 2 independent samples taken 6 months apart from our index FIRES patient. Error bars indicate 95% confidence interval of the mean for HC samples and standard error of the mean for our FIRES index patient, where applicable. Student t test was used to assess significance in B–E. UTR = untranslated region.

conventional assays. Although we did detect significant increases in IL1 β in both serum and CSF in FIRES patients, the values were frequently just above detection even in high-sensitivity assays (see Fig 1D). This is a common issue for the highly labile IL1 β molecule and results in a sharply nonlinear profile for analysis of expression. In other words, levels of IL1 β in biofluids must reach a critical threshold before these changes can even be detected. However, we found that our HEK-Blue IL1R cell-based assay was more sensitive than ELISA and cytometric bead assays for detecting IL1R agonists. This assay revealed the presence of IL1R agonists even in FIRES samples that were at or below the limit of detection by ELISA (see Fig 2A). One possible explanation for this increased detection, beyond the cell-based system taking advantage of the amplification inherent to signal transduction, is the activity of IL1 α and IL1 β in microvesicular structures, such as those secreted by activated microglia.^{34,35} Such vesicular cytokines may be competent to activate IL1R³⁶ but may not be accessible to the antibodies employed in ELISA. An important caveat to our interpretation of this assay is the absence of systematic analysis of IL1 α in our study (and in most FIRES studies) and the unknown expression of IL1R isoforms on the HEK-Blue cells that may bind to factors such as IL33. These questions will need to be resolved if the cell-based assay is employed in the future as a highly sensitive detector for IL1 levels and for the functional status of patient IL1RA.

Perhaps our most salient finding is that a FIRES patient who responded to anakinra presented with high levels of endogenous IL1RA in her CSF that was ineffective at blocking IL1 signaling. Sequencing of her *IL1RN* gene revealed multiple noncoding polymorphisms and one silent exonic mutation. In addition to novel polymorphisms of unknown significance, we detected several previously identified single nucleotide polymorphisms that have been associated with risk or severity in other inflammatory diseases. Notably, rs3213448 has been associated with increased IL1RA levels,³⁷ although it is not clear whether this association is sex dependent.³⁸ Unfortunately, a polymorphism associated with decreased IL1RA expression, rs4251961,³⁹ occurs in a promoter region of the gene that was not included in our sequencing coverage. There are 5 reported splice variants of the *IL1RN* gene that produce different secreted and intracellular protein isoforms (see Fig 5A). The predominant differences between the isoforms are based on alternative splicing events at exon 3. In isoform 1, referred to as sIL1RA I, exons 1 and 2 are not translated and an upstream region of exon 3 (3a) is used as an alternative start site. Exons 1 and 2 are also not expressed in isoforms 4 and 5 (sIL1RA II), and only the final 5 residues encoded by exon 3 (3c) are translated as the start of the protein. Isoform 2, the first intracellular isoform (icIL1RA I), uses exons 1, 2, and 3B, whereas isoform

3 (icIL1RA II) uses exon 1 and 3B. All of the splice variants use exons 4, 5, and 6 (see Fig 5A). Although the sequences controlling the alternative *IL1RN* splice events are not understood, it is likely that specific intronic sequences that determine both splicing repressor/enhancer binding and splice acceptor–donor pairing are involved.⁴⁰ It is therefore notable that we detected 15 polymorphisms between exons 2 and 3 and that several of these intronic mutations are associated with possible splicing changes. Specifically, the duplication of TTC at 2:113121042 and the substitution of C to A at 2:113121134 lead to breakage of exonic splicing silencer elements, whereas the substitution of A to G at 2:113121138 creates an exonic splicing enhancer, possibly altering exon skipping and changing the complex alternative splicing of exon 3. These changes individually are not predicted by the Human Splicing Finder (HSF)⁴¹ to impact splicing, but it is not clear whether there is an effect in aggregate. The HSF algorithm does predict a potential impact of the T to A substitution at 2:113120473 and the G to C substitution at 2:113126625 via the activation of cryptic intronic acceptor sites, perhaps altering the pattern of intronic excision in such a way as to promote exon 3A translation rather than 3B. However, the ultimate significance of these mutations for IL1RA expression and function remain to be determined and are outside the scope of this study.

IL1RA loss-of-function mutations have been described previously and are characterized as a "deficiency in IL1 receptor antagonist" (DIRA). DIRA was first described in 9 pediatric patients presenting with *IL1RN* nonsense mutations or large deletions that resulted in production of a truncated, nonsecreted, or completely absent IL1RA protein. DIRA patients are generally afebrile and present perinatally with systemic sterile joint and skin inflammation. Almost all DIRA cases present with osteomyelitis and high mortality and require continuous replacement of endogenous IL1RA with anakinra.^{42,43} Depending upon the specific mutation, disease onset can present as a fatal intrauterine disease (Q119X)⁴⁴ or with onset as late as age 12 years, as reported in at least one pediatric case with a novel nonsense mutation (R26X) in *IL1RN*.⁴⁵ Several distinct *IL1RN* polymorphisms have been characterized in various inflammatory diseases,⁴⁶ but very few examples of CNS involvement are reported, although Saitoh and colleagues described an association between polymorphisms in the variable number of tandem repeat region in *IL1RN* and FIRES risk.⁴⁷ Notably, our index patient did not present with symptoms associated with global IL1RA deficiency. She had normal development and no evidence of systemic disease up until an episode of systemic inflammation that appears to have given rise to a neuroinflammatory response. It is possible that the nature of her functional deficiency did not manifest until some set of combinatorial circumstances occurred involving the precipitating infection.

TABLE. Patient and Healthy Control Demographics

Subject	Diagnosis	Blood Draws, n	CSF Draws, n	Figures	Age ^a	Sex
1	FIRES (index case)	4	3	1, 3, 5	1.5	F
2	FIRES	3	3	1	16	F
3	FIRES	1	1	1, 3	10	M
4	FIRES	5	3	1, 3	7	M
5	FIRES	0	3	1	5	M
6	FIRES	0	1	1	7	M
7	FIRES	0	2	1	5	M
8	ESES	3	1	1, 3	6	M
9	ESES	4	0	1, 3	6	M
10	ESES	1	1	1	8	M
11	MRE (LGS)	2	1	1, 3	15	M
12	MRE (EEUE)	2	1	1, 3	10	M
13	MRE (autoimmune)	1	1	1, 3	15	F
14	MRE (EEUE)	1	1	1, 3	20	F
15	Normal pressure hydrocephalus	0	2	1, 4, 5	82	M
16	Normal pressure hydrocephalus	0	2	1, 4, 5	80	M
17	Normal pressure hydrocephalus	0	1	1, 4	72	F
18	Normal pressure hydrocephalus	0	1	1	68	F
19	Healthy control	1	0	1	35	M
20	Healthy control	1	0	1	34	F
21	Healthy control	1	0	1	45	M
22	Healthy control	1	0	1, 3	19	F
23	Healthy control	1	0	1	40	M
24	Healthy control	1	0	1, 3	61	F
25	Healthy control	1	0	1	49	F
26	Healthy control	1	0	1	53	F
27	Healthy control	1	0	1	24	M
28	Healthy control	1	0	1, 3	48	M
29	Healthy control	1	0	5	49	M
30	Healthy control	1	0	5	30	F
31	Healthy control	1	0	5	27	F
32	Healthy control	1	0	5	27	M

^aAge in years at time of first sample collection.

CSF = cerebrospinal fluid; EEUE = episodic encephalopathy of uncertain etiology; ESES = electrical status epilepticus in sleep; F = female; FIRES = febrile infection-related epilepsy syndrome; LGS = Lennox–Gastaut syndrome; M = male; MRE = medically refractory epilepsy.

It is also important to note that we have only focused on one specific inflammatory pathway in this study. Others have certainly established a possible role for multiple inflammatory cytokines in FIRES pathogenesis.⁴⁸ Sakuma and colleagues showed that a number of factors were elevated in CSF in patients with acute encephalitis and refractory, repetitive partial seizures, including a nearly 200-fold increase in IL6 and large increases in CXCL10 and IL8.⁴⁹ This same study found that T-cell-associated cytokines and homeostatic chemokines were unchanged or reduced in the CSF of these patients, consistent with the general lack of improvement in response to immunotherapies such as rituximab in FIRES.³ Jun and colleagues also reported that levels of IL6 were massively increased in at least 4 patients with FIRES and provided evidence that therapeutic intervention with tocilizumab, an IL6 receptor inhibitor, terminated SE in 6 of 7 subjects.⁵⁰ These findings suggest a complex interplay between inflammatory pathways and emphasize the clear need for additional mechanistic analyses of FIRES pathogenesis.

Future studies will focus on determining the possible functional consequences of the polymorphisms we have identified. However, regardless of the underlying genetic or epigenetic cause, our findings indicate that our anakinra-responsive reference FIRES case had a functional deficit in IL1RA, providing direct mechanistic support for the therapeutic efficacy of this intervention in this specific patient. Moreover, our data indicate that at least a subset of other FIRES cases also exhibit elevated CSF levels of endogenous IL1RA, suggesting that these individuals may also have a functional deficiency in this pathway. The cell-based assay that we describe may be a useful tool for revealing such functional deficits, and we propose that screening patient-derived IL1RA for antagonistic efficacy will provide a biomarker for guiding decisions to employ anakinra in these complex seizure disorders. We predict that the variable response to anakinra anecdotally observed across FIRES treatment centers is based on the presence of functional deficiencies in only some children with this syndromic disease. Finally, our findings provide further evidence in support of a causative relationship between inflammation and seizures, especially in patients who are refractory to the standard armamentarium of antiseizure medications.

Acknowledgment

This work was supported by the NIH National Institute of Neurological Disorders and Stroke (NS064571, C.L.H.) and a Child Neurology Foundation grant (CNF#1, 93346001, E.T.P.). B.D.S.C. was supported by fellowships from the Mayo Clinic Center for Regenerative Medicine and the Center for Multiple Sclerosis and Autoimmune Neurology.

J. Sagen provided invaluable assistance with sample acquisition and regulatory fidelity.

Author Contributions

B.D.S.C., R.J.K., E.T.P., and C.L.H. contributed to the conception and design of the study. B.D.S.C., R.G.L.-C., R.F.-M., E.T.P., and C.L.H. contributed to the acquisition and analysis of the data. All authors contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

C.L.H., B.D.S.C., R.G.L.-C., and E.T.P. are coinventors on a pending provisional patent filed April 12, 2018, entitled "Methods for Guiding Therapy Decisions in Seizure Disorders," 62/656664.

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