UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Mutaciones bialélicas en RIPK3 en un paciente con encefalitis herpética

Bi-allelic RIPK3 mutations in a patient with herpes simplex encephalitis

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Eduardo Javier García Reino

DIRECTORES

Rebeca Perez de Diego
Shen-Ying Zhang

Madrid

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Mutaciones bialélicas en *RIPK3* en un paciente con encefalitis herpética
Bi-allelic *RIPK3* mutations in a patient with herpes simplex encephalitis

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Eduardo Javier Garcia Reino

DIRECTOR

Rebeca Perez de Diego
Shen-Ying Zhang

Acknowledgements

I want to thank my friends and my family back home for their encouragement throughout these years. In particular, I would like to thank my parents for their unconditional love and support.

I am forever grateful to my boyfriend Matt, for cheering me up, showing me endless patience, and being so understanding along the way.

I want to warmly acknowledge all the beautiful people I have met during these years in New York, and that have been a part of this journey. Brandon, Miri, Ed, Dena, Carla, Lindsey, Andrew, Eli, etc.

I also want to express my gratitude to all the members of the Casanova lab. Dominick, Tanya, Jackie, Yelena, and Mark for their assistance and consideration; the past and present trainees (Ruben, Scott, Franck, Noe, Simon, Jill, Janet, Serkan, Andras, etc.) for sharing their knowledge and expertise; and the seniors for their advice and help. I am very appreciative of the personal relationships that I got from this experience, and I only have good words for every one of them.

I am thrilled to have shared this experience with the HSE group, Mary, Jie, Zhiyong, Fabien, Anat, Hye-Kyung, Daxing, Yoon, and especially Paul. It has been a joy to share this experience with him and to discuss our projects together, he has become a true friend, and I am sure our paths will cross again in the future.

I want to express my gratitude to Jose Ramon Regueiro, who accepted to be my academic tutor and has shown nothing but kindness towards me; and Maria Angeles Vicente, for her excellent work as the coordinator of my Ph.D. program.

This work was conducted under the mentorship of Rebeca Perez de Diego, Jean-Laurent Casanova, and Shen-Ying Zhang. All three of them have had a tremendous impact on my life and have shaped me as a scientist.

My longstanding gratitude goes to Rebeca Perez de Diego for her warmth, for giving me my first opportunity in science, and for introducing me to the field of primary immunodeficiencies. I feel very fortunate to have found her and will always look up to her.

I heartily thank Jean-Laurent Casanova for allowing me to join his lab. It has been a privilege to work and learn from a world-class scientist like him. He is an example of scientific excellence, and I wish nothing but more years of success for him and his lab.

But more than anything, I am profoundly grateful to Shen-Ying. She is the person that has made this possible and has taken care of me during these years. She took me under her wing and has always been generous with her time. I thank her for sharing her passion and her science with me and for discussing with me every experiment I performed during these years.

And to the reader, thank you. I hope you enjoy reading this thesis as much as I did writing it.

Lista de publicaciones

- **Eduardo J. Garcia Reino**, Zhiyong Liu, Hongyan Guo, Oliver Harschnitz, Paul Bastard, Osefame Ewaleifoh, Mary L. Hasek, Kerry Dobbs, Jie Chien, Yoon S. Lee, Lazaro Lorenzo, Claire Gay, Deal-Louis Stephan, Rebeca Perez de Diego, Laurent Abel, Stephane Chabrier, Luigi D. Notarangelo, Gregory A. Smith, Lorenz Studer, Edward S. Mocarski, Jean-Laurent Casanova, Shen-Ying Zhang. "*Bi-allelic RIPK3 mutations in a patient with Herpes Simplex Encephalitis"*. **In preparation**

- Paul Bastard, Jeremy Manry, Jérémie Rosain, Yoann Seeleuthner, Omar AbuZaitun, Lazaro Lorenzo, Jie Chen, Mary Hasek, Nicholas Hernandez, Benedetta Bigio, Peng Zhang, Romain Levy, Shai Shrot, **Eduardo J. Garcia Reino,** Mélodie Aubart, Vivien Beziat, Zhi Li, Sandra Pellegrini, Isabelle Meyts, Flore Rozenberg, Bertrand Boisson, Aurélie Cobat, Jacinta Bustamente, Qian Zhang, Emmanuelle Jouangy, Laurent Abel, Raz Somech, Jean-Laurent Casanova, Shen-Ying Zhang. "*Herpes simplex encephalitis in a patient with a distinctive form of inherited IFNAR1 deficiency".* **Journal of Clinical Investigation (In Press)**

- Hye Kyung Lim, Sarah X.L. Huang, Jie Chen, Gaspard Kerner, Olivier Gilliaux, Paul Bastard, Kerry Dobbs, Nicholas Hernandez, Nicolas Goudin, Mary L. Hasek, **Eduardo Javier García Reino**, Fabien G. Lafaille, Lazaro Lorenzo, Priya Luthra, Tatiana Kochetkov, Benedetta Bigio, Soraya Boucherit, Flore Rozenberg, Catherine Vedrinne, Michael D. Keller, Yuval Itan, Adolfo García-Sastre, Marie Celard, Jordan S. Orange, Michael J. Ciancanelli, Isabelle Meyts, Qian Zhang, Laurent Abel, Luigi D. Notarangelo, Hans-Willem Snoeck, Jean-Laurent Casanova, Shen-Ying Zhang. "*Severe influenza pneumonitis in children with inherited TLR3 deficiency*." **Journal of Experimental Medicine** (2019): jem-20181621.

- Zhang SY, Clark NE, Freije CA, Pauwels E, Taggart AJ, Okada S, Mandel H, Garcia P, Ciancanelli MJ, Biran A, Lafaille FG, Tsumura M, Cobat A, Luo J, Volpi S, Zimmer B, Sakata S, Dinis A, Ohara O, **Garcia Reino EJ**, Dobbs K, Hasek M, Holloway SP, McCammon K, Hussong SA, DeRosa N, Van Skike CE, Katolik A, Lorenzo L, Hyodo M, Faria E, Halwani R, Fukuhara R, Smith GA, Galvan V, Damha MJ, Al-Muhsen S, Itan Y, Boeke JD, Notarangelo LD, Studer L, Kobayashi M, Diogo L, Fairbrother WG, Abel L, Rosenberg BR, Hart PJ, Etzioni A, Casanova JL. *"Inborn Errors of RNA Lariat Metabolism in Humans with Brainstem Viral Infection."* **Cell** 172.5 (2018): 952-965.

Abstract

Life-threatening herpes simplex virus encephalitis (HSE) is the most common form of sporadic viral encephalitis worldwide. A diverse collection of genetic etiologies can predispose to this condition: inborn errors of the TLR3 responsive pathway impair central nervous system (CNS) cortical neuron- and oligodendrocyte-intrinsic immunity to herpes simplex virus type 1 (HSV-1) and underlie forebrain HSE; heterozygous *SNORA31* variants have also been associated with forebrain HSE; last, genetic defects of RNA lariat metabolism, due to mutations in *DBR1*, can trigger brainstem HSE. Still, the vast majority of HSE patients enrolled in our cohort lack a genetic diagnose. Here, we report autosomal recessive RIPK3 deficiency in a patient with recurrent forebrain HSE. The patient is compound heterozygous for one nonsense and one frameshift *RIPK3* mutations that lead to impaired protein expression and function of RIPK3 via distinct mechanisms. We show that the patient's fibroblasts do not phosphorylate MLKL upon stimulation via TNFR1 or TLR3, while the production of IFN-β or -λ was normal upon TLR3 activation. We further demonstrate that the TLR3-induced RIPK3-dependent necroptosis signaling cascade is selectively impaired in fibroblasts from previously described patients with TLR3 pathway deficiencies. Taken together, our findings suggest that RIPK3 deficiency predisposes to HSE, due to disruption of the TLR3-RIPK3-mediated necroptosis pathway. This novel genetic etiology expands our understanding of the TLR3 circuit as a non-redundant mechanism of antiviral immunity of the CNS in humans.

Abstract en español

La encefalitis herpética (HSE) es la forma más común de encefalitis esporádica en todo el mundo. La predisposición a esta condición está causada por un grupo heterogéneo de etiologías genéticas: Mutaciones en la vía del TLR3 dañan a la inmunidad intrínseca de las neuronas y los oligodendrocitos del Sistema Nervioso Central (SNC) frente al virus del herpes simple tipo 1 (VHS-1) y son responsables de ciertos casos de encefalitis herpética en el lóbulo frontal. Del mismo modo, mutaciones heterocigóticas en SNORA31 también ha sido asociadas con la encefalitis herpética frontal; por último, defectos genéticos del metabolismo de los lazos de ARN, causados por mutaciones en DBR1, pueden causar encefalitis herpética en el tallo cerebral. Sin embargo, la mayor parte de los pacientes de nuestra cohorte de pacientes con encefalitis herpética no tiene una etiología genética identificada. Aquí describimos un paciente con encefalitis herpética con deficiencia en RIPK3 autosómica recesiva. El paciente es heterocigoto compuesto por una mutación *nonsense* y una mutación *frameshift* que conducen a la pérdida de expresión y función de la proteína mediante distintos mecanismos. Nuestros resultados muestran que los fibroblastos del paciente no fosforilan MLKL tras la estimulación de la vía del TLR3 y la del TNFR1. Sin embargo, la producción de interferones β y λ es normal tras la activación del TLR3. También demostramos que la vía inducida por el TLR3 y mediada por RIPK3 está selectivamente dañada en fibroblastos de pacientes previamente descritos con mutaciones en la ruta del TLR3. Esto sugiere que deficiencia en RIPK3 predispone a la encefalitis herpética y que lo hace de forma dependiente del TLR3. Esta nueva etiología genética expande nuestro conocimiento sobre el papel del circuito gobernado por el TLR3 como un mecanismo no redundante en la inmunidad antiviral del SNC en humanos.

Contents

1. INTRODUCTION

1.1 Herpes Simplex Viruses

Herpesviruses are a large family of double-stranded DNA viruses that can infect a broad spectrum of species of a minimum of two animal phyla, the Chordata (mammals, birds, fishes, reptiles, and amphibians) and also the Mollusca (oysters) (Pellet and Roizman 2007). Additionally, these viruses can enter and replicate in a wide selection of cell types within the same host, suggesting that, throughout evolution, these viruses have developed effective tactics to enter and manipulate different spaces where they can replicate. The family Herpesviridae is split into three subfamilies (Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae) based on their biological properties and genome sequences (Davison et al. 2009). The Alphaherpesvirinae subfamily contains five documented genera containing a total of 37 distinctive species, of which three virus species typically infect humans: herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV).

One characteristic of human herpesviruses is that, following primary infection, the viruses can remain silent in cells they populate. At intervals, they can reactivate and transport to a portal of egress from the body where they will disseminate to other individuals. This property has made HSV-1 and HSV-2 great model systems for the study of the latent infection (Whitley et al., 2009). These observations were initially made at the beginning of the 20th century, when Cushing (Cushing et al. 1905) realized that, following the sectioning of the connection between trigeminal ganglion (TG) and the brain, typical herpetic lesions appeared along the sites innervated by the TG. These studies were meticulously documented patient by patient and led to the irrefutable conclusion that the reactivation originated from sensory ganglia. The connection between primary infection and reactivation was nevertheless controversial until Burnet and Williams (Burnet and Williams, 1939) proposed that, following primary infection, the virus enters into a latent state, occasionally reactivating and causing recurrent lesions. We currently know that HSVs codify a

minimum of 84 proteins and several long noncoding RNAs and micro RNAs in infected cells (Roizman et al., 2006). The virus is transported retrogradely to sensory or autonomic neurons (peripheral ganglia innervating that site). Once it establishes latency in peripheral ganglia, the viral DNA is maintained within the cells as an episome; the virus expresses a prominent noncoding intron called latency-associated transcript (LAT), a group of RNAs and a group of miRNAs (Fuller et al., 1989; Gross et al., 2003; Hill et al., 1972; Hill et al., 1973). When the virus reactivates, it replicates within the neuron and then travels through anterograde transport to a portal of entry into the body.

Exposure of the mucosal surfaces of a seronegative individual to infected secretions from a seropositive individual may end in primary infection. Seeding of innervating ganglia follows, and new latency is established as a consequence of this sequence of events. Depending on many factors, the virus might reactivate with the appearance of symptomatic lesions.

The transmission of HSVs is very common, as measured by the seroprevalence of HSV-1 and HSV-2 (Feierbach et al., 2007). In developed countries, around 20% of teenagers to 60-80% of adults by the age of fifty are infected with HSV-1. In contrast, in developing countries, HSV-1 is acquired much earlier in life, approaching 50% by early adulthood and 90% by midlife.

Our knowledge of HSV-2 infections, in many respects, is significantly more advanced than it is for HSV-1 due to its propensity for sexual transmission with psychosocial implications, attendant morbidity, and mortality in the infected newborn, and the higher risk of acquisition of HIV infection by infected individuals (Hook et al.,2006; Mark et al., 2008).

Globally, the acquisition of HSV-2 begins with the onset of sexual intercourse, and like HSV-1, it occurs earlier in life within the developing world than in developed societies. From the mid-1990s

to 2005, the seroprevalence of HSV-2 declined from about 24% to 19% (Fleming et al., 1997; Xu et al., 2006). Still, seroprevalence rises overall to near 30% by midlife, varying to some extent from nation to nation. It is worth mentioning that these data do not account for HSV-1 infections of the genital tract due to our lack of serologic or biologic markers to define their contribution to genital infections. The infection burden is high worldwide; however, most infected individuals are unaware that they are infected, and plenty experience asymptomatic recurrences and shed the virus.

Although the prevalence of HSV infections is very high, most infected individuals are either asymptomatic or develop mild symptoms. However, these infections can lead to life-threatening conditions in some patients. Therefore, a deep understanding of the interactions between herpes simplex viruses and humans is necessary to prevent and treat those cases.

1.2 Clinical manifestations of Herpes Simplex virus in humans

HSVs have coevolved with the species they infect for millions of years: HSV-1 for around 6 million years and HSV-2 for 1.6 million years approximately. Humans and HSVs have developed a prosperous equilibrium, where the viruses can establish latency and remain inside its host for the rest of its lifespan. This balance between humans and virus involves host recognition of the virus infection and efforts to clear it, while the virus changes to escape the host immune responses. Through this evolutionary chess game, the HSVs and the human host have developed a durable coexistence. However, disease may result if the viral load is too high or if the host fails to provide a proper immune response.

1.2.1 Herpes labialis

Herpes labialis, also known as cold sores, is a type of infection that affects the lips primarily (Opstelten et al., 2008). Patients with herpes labialis usually suffer from burning pain within the infected area, followed by small blisters or sores. The primary infection may include fever, pharyngitis, and inflammation of the lymph nodes (Stoopler et al., 2014). Testing is rare, and diagnosis usually relies on symptoms. The rash usually lasts around ten days, but the virus remains dormant in the trigeminal ganglion. The virus can reactivate periodically and create new sores in the mouth or the lips. HSV-1 causes the vast majority of the cases of oral herpes. However, cold sores caused by HSV-2 have also been reported (Opstelten et al., 2008). The infection is transmitted among individuals by non-sexual physical contact. Several agents can trigger the activation of the latent virus, including sunlight exposure, fever, mental stress, or menstruation. Physical contact with the genitalia can result in genital herpes.

Prevention includes avoiding kissing or using personal items of an infected person. A zinc oxide, anesthetic, or antiviral creams can decrease the duration of symptoms and the frequency of outbreaks (Rahimi et al., 2012). About 0.2% of the population suffers from herpes labialis outbreaks in any given year. After the first episode, one-third of people develop subsequent outbreaks. Onset occurs in children and young adults, but most of the affected individuals produce antibodies against the virus, and the frequency of outbreaks tends to decrease over the years.

1.2.2 Herpes gingivostomatitis

Herpes gingivostomatitis (HGS) is an inflammation of the oral mucosa and gingiva caused by a herpes simplex infection (Kolokotronis et al., 2006). It is of greater severity than herpes labialis, which is often how the infection presents in individuals presenting with this condition. Similar to herpes labialis, HGS is induced predominantly by HSV-1 and affects mainly children and young adults. The disease manifests as copious vesicles, which rupture speedily to make uncomfortable ulcerations screened by yellow-grey membranes. Well-known symptoms observed in these include sub-mandibular lymphadenitis, halitosis, and refusal to drink. (Kolokotronis et al., 2006).

1.2.3 Herpes genitalis

Herpes genitalis is an infection of the genitals caused by herpes simplex viruses (Gupta et al., 2007). Most infected individuals do not present any symptoms and therefore are not aware of the infection. (Gupta et al., 2007). The most common symptom of this condition is the appearance of blisters, which occasionally lead to the formation of painful ulcers. Other symptoms like aching, fever or swollen lymph nodes are observed in a smaller fraction of patients. Onset occurs around four days after infection, and symptoms can last for a month. Once infected, further outbreaks can happen, but they are generally milder.

Herpes genitalis is a sexually transmitted disease typically spread by direct contact with the skin surface or secretions of someone infected. HSV-2 is commonly believed to be the most common and almost only cause of herpes genitalis. However, in the past decade, the USA, Canada, and several European countries have seen an increase in genital HSV-1 infections, which now

account for at least half of the first episodes of the cases. (Roberts et al., 2003; Scoular et al., 2002; Manavi et al., 2004; Coyle et al., 2003). This increase may result from a delay in the acquisition of oral HSV-1 infection early in life in developing countries, therefore remaining susceptible to genital HSV-1 infection at the initiation of sexual activity (Xu et al., 2006). HSV-2 infection has also been linked to a higher risk of sexually acquired HIV (Freeman et al., 2006). The mucosal disruption caused by genital ulcers facilitates HIV acquisition by providing an immediate gate (Corey et al., 2004). Besides, HSV-2 reactivation results in mucosal infiltration with activated CD4-bearing lymphocytes, which HIV-1 targets for attachment (Koelle, 1994).

1.2.4 Herpes gladiatorum

Herpes gladiatorum, also referred to as herpes rugbiorum, “mat herpes” or “wrestler herpes” due to its association with contact sports, is an infection of the skin that can affect the head, extremities, and trunk (Likness et al., 2011). Symptoms include rash with blisters on the neck, chest, face, stomach, and legs; and less often, lymphadenopathy, fever, sore throat, and headache. (CDC, 1990).

1.2.5 Herpes esophagitis

Herpes esophagitis is an infection of the esophagus caused by HSVs. The disease is most frequent in immunocompromised patients (Mcbane et al., 1991), but immunocompetent individuals have nonetheless been reported (Kato et al., 2005). Symptoms include pain with eating trouble swallowing and, on rare occasions, upper gastrointestinal bleeding (Takeno et al., 2002). Herpes esophagitis is usually diagnosed by inference based on symptoms. However, the obtention of biopsies through endoscopy can is more accurate. Pathologists may find inclusion bodies by microscopy and immunochemical staining (Bennett et al., 2009). Treatment consists of antiviral drugs such as valacyclovir or acyclovir. Patients who cannot swallow because of the pain, individuals with other systemic manifestations of herpes, and severely immunocompromised individuals are treated with intravenous acyclovir.

1.2.6 Herpes whitlow

A herpetic whitlow is an unpleasant infection on a finger or thumb caused by HSVs. In some instances, disease happens on the nail cuticle or the toes (Clark, D, 2003). Healthcare workers represent a sizable fraction of infected individuals as they come in contact with the virus, primarily dental workers and medical workers exposed to oral secretions (Avitzur et al., 2002; Lewis et al., 2004). It is also observed in children with HSV-1 oral infection with a tendency to suck their thumbs before seroconversion (autoinoculation) (Clark, D, 2003) and in young adults following contact with infected genitalia (Wu et al., 2007).

1.2.7 Herpes keratinitis

Herpes keratinitis is a type of inflammation caused by HSV infection of the cornea. HSV infects the epithelial cells around the eye, and retrograde transport of the virus allows it to reach the cornea (Carr et al., 2001). Primary infection typically presents as puffiness of the conjunctiva and eyelids, accompanied by tiny white itchy lesions on the corneal surface. The severity of the lesions ranges from minor disruption of the epithelium to more severe consequences like the formation of dendritic ulcers (Kaye et al.,2006). Other symptoms include pain inside the eye, dryness, and sinusitis, occurring in only one eye in most cases. These primary infections usually resolve spontaneously in a couple of weeks. Oral and topical antivirals can boost healing.

Recurrent infections can be more severe, with infected epithelial cells showing larger dendritic ulceration, and lesions forming white plaques. (Kaye et al.,2006). Mild inflammation may occur in the underlying stroma of the iris. Eye dryness and conjunctivitis may also occur. Persistent infection can trigger a massive immune response in the eye which can potentially lead to the destruction of the corneal stroma, resulting in loss of vision (Faaroq et al., 2012).

1.2.8 Herpes simplex encephalitis

1.2.8.1 Pathology

Herpes simplex encephalitis (HSE; OMIM ID 613002) is a virus infection-triggered high inflammation of the central nervous system (Gnann et al., 2017). The mechanism by which the virus reactivates and reaches the CNS is not fully understood. Reactivation of HSV has been proposed to occur in peripheral ganglia with axonal transport to the temporal lobe or directly in the brain. Four main routes of infection have been suggested:

1) Primary infection of the oropharynx with CNS invasion via the trigeminal or olfactory tracts, which is the most widely accepted (Jennische et al., 2015; Menasria et al., 2015).
2) Primary infection with CNS infection resulting from hematogenous spread.
3) CNS invasion after an episode of recurrent orofacial HSV-1 infection, representing peripheral viral reactivation with the subsequent axonal spread. (Whitley et al., 1982)
4) CNS infection without defined peripheral HSV-1 infection, possibly representing the reactivation of latent virus within the CNS (Baringer et al., 1994).

HSE is characterized by necrotizing encephalitis involving -in most cases- the temporal lobe, the frontal lobe, and/or the insular cortex of the cerebral hemispheres (Taylor et al., 2005). Necrosis occurs in all cell types, including neurons, astrocytes, and oligodendrocytes. This life-threatening condition usually starts with infection in only one temporal lobe. Still, bilateral involvement is often seen in autopsy studies, indicating that the increase in viral load eventually leads to the invasion of the contralateral hemisphere. CNS pathology in these patients is produced by a combination of cytolytic viral replication and immune-mediated mechanisms. Type I interferons are required for survival in mice models of HSE (Wang et al., 2012). In addition, patients with HSE show detectable levels of cytokines and soluble cytokine receptors (including IL- 6, interferon-gamma, TNF-alpha, IL-2 receptor, and soluble CD8 antigen) in serum and CSF at various stages of the illness. The existence of these inflammatory markers indicates that during acute HSV, the robust immune response might be contributing to the pathological effects (Aurelius et al., 1994).

1.2.8.2 Clinical manifestations

Patients typically present with fever, headache, focal neurologic findings (including cranial nerve deficits, hemiparesis, dysphasia, aphasia, or ataxia), altered mentation, and reduced level of consciousness of short duration (less than one week). Behavioral changes (especially in adults: elevated mood, excessive animation, decreased need for sleep, inflated self-esteem, and hypersexuality, and Kluver-Bucy syndrome) and occasional hallucinations have also been reported. (Riera-Mestre., 2009; Domingues et al., 1997). Seizures are also observed in about one-half of cases (Singh et al., 2016). Interestingly, HSE is not accompanied by the dissemination of the virus to other organs, and patients with HSE are not particularly susceptible to other HSV-1 diseases of the skin or mucosa.

1.2.8.3 Epidemiology

Herpes simplex virus type 1 (HSV-1) encephalitis (HSE) is the most common cause of sporadic viral encephalitis in Western countries, occurring at a rate of 1-2 per 500,000 individuals per year (Hjalmarsson et al., 2007; Kohl et al., 1998; Mailles et al., 2007; Whitley et al., 2002). HSE occurs worldwide and has no seasonal variation; prevalence among males and females is the same. HSE can affect patients at almost any age, but there are 2 peaks of incidence, the first at age 6 months to 3 years, corresponding mainly to primary infection, and the second at age >50 years, probably reflecting viral reactivation (De Tiege et al., 2003 Whitley et al., 2005; Abel et al., 2010). About one-third of HSE cases are related to primary HSV infection (more commonly in children and adolescents), while the remaining are attributed to HSV reactivation. Clinical features do not help to discriminate between HSE caused by one or the other. The introduction of acyclovir treatment has considerably reduced HSE mortality, although neurologic sequelae are common, especially in young children (Hjalmarsson et al., 2007; McGrath et al., 1997, Raschilas et al., 2002). Studies evaluating the clinical outcome of children with HSE treated with acyclovir have

shown that although the overall mortality rate was low, a substantial proportion (35%-62%) had significant neurological sequelae (Elbers et al., 2007; Hsich et al., 2007).

1.2.8.4 Diagnosis

A lumbar puncture (LP) must be performed in all individuals with suspected HSE unless there is a contraindication (e.g., elevated intracranial pressure). Standard cerebrospinal fluid (CSF) abnormalities include increased protein (50–200 mg/ dL or more; median about 80 mg/dL), CSF leukocytosis (5– 500 cells/mm3 or more, median about 70 cells/mm3) with predominance of lymphocytes (60–98%, median about 80%). CSF pleocytosis with >5 WBC/mm3 is observed in the vast majority of the cases, although HSE cases without CSF leukocytes have been described (Lopez Roa et al., 2013; Rawal et al., 2015; Razavi et al., 2001). However, the absence of CSF leukocytes should not eliminate HSE from diagnostic consideration when the clinical phenotype is otherwise indicative of this being the case. Red blood cells in CSF occurs more often in HSE patients with substantial tissue necrosis. Glucose values are usually average, although cases of hypoglycorrhachia have been described (Davis et al., 2004). The demonstration of HSV DNA in CSF using a polymerase chain reaction (PCR) assay is the most reliable evidence for HSE (Aurelius et al., 1991; Lakeman et al., 1995; Steiner et al., 2010). In HSE caused by HSV-1, the sensitivity of PCR is estimated to be 98% with specificity 94– 99%. The SimplexaTM HSV-1 and 2 Direct kit (Focus Diagnostics) is FDA-approved for the detection of HSV DNA in CSF (Binnicker et al., 2014).

PCR is generally positive after 24 hours of symptom onset and remains positive during the first week of acyclovir therapy. Negative CSF PCR has been reported in HSE patients who were tested very early in their clinical course (Adler et al., 2011; Weil et al., 2002). In those cases, if the clinical manifestations strongly suggest that it is HSE, acyclovir therapy should not be discontinued, and

the patient should undergo a second lumbar puncture; when the patient is retested the PCR normally comes positive. False-negative PCR results may also occur as a result of a suboptimal assay or due to the presence of inhibitory substances in CSF, such as blood. False-positive results are rare but occasionally occur (as with any PCR-based assay), probably because of cross-contamination. Due to its high efficacy, the PCR has substituted brain biopsies. However, brain biopsy may still be required in exceptional cases when the clinical and radiographic evidence indicate HSE, but the PCR is nondiagnostic. HSV can be identified in brain tissue by PCR, viral culture, in situ hybridization, or immunohistochemical staining. Assays for the detection of HSV antigens and anti-HSV antibodies in CSF have been described but have been replaced by PCR. These tests can be helpful as an alternative to brain biopsy in those cases where HSE is heavily suspected, but the PCR is repeatedly negative (Bhullar et al., 2016; Denes et al., 2010).

Magnetic resonance imaging (MRI) is the preferred imaging technique for patients with this condition (McCabe et al., 2003; Mekan et al., 2005). However, antiviral therapy should be initiated as soon as possible, while imaging studies are performed. After a median duration of neurologic symptoms of 3 days, 95% of patients had an abnormal MRI, although scans performed very early in the course of the disease may occasionally be normal (Tyler et al., 2004). The MRI typically shows abnormalities in the temporal lobe or neighboring areas early in the course of infection and can normally distinguish HSE from a cerebrovascular injury, tumor, or brain abscess. HSE usually affects only one lobe, but can be bihemispheric, especially later on in the course of the disease. Compared with MRI, computed tomography (CT) scanning is less sensitive for detecting the early stages of HSE. However, CT may be useful in the emergency department for assessing mass effect and excluding other possible diagnoses (e.g., intracranial hemorrhage) (Stahl et al., 2012). The MRI pattern of HSV-2 encephalitis is more heterogeneous than HSV-1 encephalitis (Singh et al., 2016). Dynamic single-photon emission computed tomography (SPECT) scanning may have utility in the diagnosis of HSE, but experience is currently limited (Kataoka et al., 2007).

1.2.8.5 Treatment

Medical management of HSE includes intensive supportive care, seizure control (if necessary), and early initiation of antiviral therapy with intravenous acyclovir (Sellner et al., 2012; Tunkel et al., 2008). Early diagnosis is essential, but clinicians should not wait for confirmatory laboratory testing before starting intravenous acyclovir therapy in patients with clinically suspected HSE (Steiner et al., 2010; Hughes et al., 2012). Neurological intervention has been beneficial in some patients with life-threatening increases in intracranial pressure (Jouan et al., 2015; Maraite et al., 2010). Following the completion of antiviral treatment, survivors will often benefit from physical therapy and occupational therapy.

The efficacy of acyclovir for the treatment of HSE was proven in a series of randomized, controlled clinical trials conducted in the 1980s that demonstrated a reduction in mortality from about 70% (without antiviral therapy) to about 25% (Skoldenberg et al., 1984; Whitley et al., 1986). Acyclovir is the only antiviral drug that is FDA-approved for the treatment of HSE. In early clinical trials, acyclovir was studied at doses of 10 mg/kg administered every 8 hours (30 mg/kg/ day) for 10 days. However, due to suboptimal outcomes even in patients receiving appropriate acyclovir therapy, many experts recommend the administration of higher doses (36–45 mg/kg/day) for longer durations (14–21 days), even though these conditions have not been rigorously examined in controlled clinical trials. These recommendations are based on the observation that neonates with persistently positive CSF for HSV after 10 days of acyclovir therapy had higher rates of morbidity and mortality (Kimberlin et al., 1996). If the PCR is negative, but the clinical manifestations strongly suggest HSE (compatible MRI findings, CSF pleocytosis >5 cells/mm3, abnormal EEG, or seizures), acyclovir therapy should not be discontinued.

In theory, corticosteroids could suppress the inflammatory responses in the CNS during HSE and reduce immune-mediated pathology. In a mouse model of HSE, combination therapy with acyclovir plus glucocorticosteroids significantly reduced the frequency of long-term MRI abnormalities (Meyding-Lamade et al., 2003). The main concern is that immunosuppressive corticosteroid therapy could enhance viral replication and increase the risk of dissemination; however, no increase in intracranial viral load or extent of brain involvement has been seen in animal models (Thompson et al., 2000; Meyding-Lamade et al., 2003). Evidence is not yet sufficient to endorse the use of concomitant therapy with corticosteroids in HSE as a standard practice (Ramos-Estebanez et al., 2008).

1.2.8.6 Post infection complications

Herpes simplex encephalitis, and possibly other viral encephalitides, can trigger antibodies against the NMDAR and other neuronal cell-surface proteins; such antibodies are responsible for the relapsing neurologic symptoms that arise weeks after the onset of HSE. (Armangue et al., 2015; Linnoila et al., 2016) This delayed complication affects approximately 20% of patients with HSE and is manifested predominantly as choreoathetosis in children and as psychiatric and behavioral alterations in adults (Armangue et al., 2015; Hacohen et al., 2014) Immunotherapy with glucocorticoids, plasma exchange, intravenous immune globulin, or rituximab is somewhat effective during relapse and does not appear to confer a predisposition to reactivation of the herpes simplex virus (Nosadini et al., 2017). The rapid increase in the number of syndromes and autoantibodies identified over the past 10 years suggests that other autoimmune encephalitides have yet to be discovered (Dalmau et al., 2017; Dalmau et al., 2018). It is still unclear whether the molecular mechanism that leads to herpes simplex encephalitis is directly related to the episode of autoimmune encephalitis or whether it is the viral infection itself what triggers an autoimmune response via a different mechanism.

1.2.9 Genetics of HSE

1.2.9.1 Lines of evidence

The idea that HSE could be not only an infectious disease but also a genetic condition is a cornerstone of the genetic theory of infectious diseases (Casanova et al., 2012; Casanova, 2015a; Casanova, 2015b). The field of human genetics of infectious diseases started to gain weight in the early 1950s with the birth of the modern cellular and molecular biology. Clinical geneticists started to describe primary immunodeficiencies (PIDs). PIDs were then considered rare, Mendelian diseases with complete penetrance caused by numerous recurrent, and opportunistic infections that presented with evident immunological abnormalities (Casanova & Abel, 2007). The first three PIDs that described a selective predisposition to a single infectious agent were epidermodysplasia verruciformis (a predisposition to oncogenic human papillomavirus infection), membrane attack complement defects (a predisposition to Neisseria), and X-linked lymphoproliferative disease (a predisposition to Epstein-Barr virus) (Casanova et al. 2013; Orth et al., 2008; Picard et al., 2006). These studies paved the way for the discovery, in the 1990s and beyond, of mutations underlying selective predisposition to various other infections, including mycobacterial disease, pneumococcal disease, chronic mucocutaneous candidiasis, and HSE (Jouanguy et al., 1996, Newport et al., 1996).

HSE is one of the most remarkable examples of an isolated pediatric condition shown to result from a monogenic inborn error of immunity. Patients with myeloid and lymphoid PIDs, including children with no T cells, are not particularly susceptible to HSE. The disease is sporadic in most cases, with very few multiplex kindreds reported. However, a high frequency of parental consanguinity (12%) observed in a French survey (Abel et al., 2010) suggested that HSE could indeed occur due to single-gene inborn errors of immunity displaying incomplete clinical penetrance.

Last but not least, the beginning of the 21st century came with a few reports of rare cases of patients with complex clinical phenotypes that included HSE in which an inborn error of immunity had been identified. *NEMO* and *STAT1* mutations leading to, respectively, impaired IFN-γ and IFN-α/β production and abolished IFN-γ and IFN-α/β responses were found in two children with syndromic HSE who also suffered from mycobacterial infections (Dupuis et al., 2003; Puel et al., 2006).

1.2.9.2 Patients with known genetic etiologies: the base camp

The first genetic etiology of HSE to be identified was autosomal recessive UNC-93B deficiency, resulting in an impairment of cellular responses to the four intracellular Toll-like receptors (TLRs), including TLR3 (Casrouge et al., 2006). Involvement of the TLR3 pathway was then suspected, because IRAK-4- and MyD88-deficient patients, whose cells do not respond to TLR7–9 agonists, are not prone to HSE (Casanova et al., 2011; Ku et al., 2007; Picard et al., 2003; Picard et al., 2010; von Bernuth et al., 2008). TLR3 was formally implicated in the disease when autosomal dominant and autosomal recessive TLR3 deficiencies were discovered in other patients with HSE (Zhang et al., 2007; Guo et al., 2011; Lim et al., 2014).

Soon after, other molecules downstream TLR3 were described as genetic etiologies of HSE: *TRIF*, *TRAF3*, *TBK1*, and *IRF3* mutations were found in some HSE patients (Sancho-Shimizu et al., 2011; Perez de Diego et al., 2010; Herman et al., 2012, Andersen et al., 2015). These seminal studies provided the first explanation for the molecular pathogenesis of forebrain HSE in a sizable fraction of patients: HSV-1 produces viral double-stranded RNA (dsRNA) during its replication, this dsRNA is recognized by TLR3 inducing the activation of the IRF3 and NF-κB pathways via TRIF, leading to the production of IFN-α/β and IFN-λ and other cytokines. TLR3, UNC-93B, TRIF, TRAF3, TBK1, and NEMO deficiencies are associated with impaired IFN-α/β

and IFN-λ production, particularly during HSV-1 infection; while STAT1 deficiencies are associated with impaired IFN-α, -β, and -λ responses (Figure 1).

Besides, a critical role of neuron and oligodendrocyte cell-intrinsic TLR3-mediated immunity in the defense against HSV-1 infection of the central nervous system has been further proposed (Lafaille et al., 2012). In contrast, iPSC-derived trigeminal neurons are naturally permissive to HSV-1 infection, regardless of their TLR3 genotype (Zimmer et al., 2018).

More recently, forward genetic studies performed in our cohort of HSE patients lead to the identification of novel and more striking genetic etiologies by means of genetic homogeneity:

AD mutations in *SNORA31* have been associated with forebrain HSE, although no evidence suggests that it is linked to the TLR3 pathway (Lafaille et al., 2019) (Figure 1). SnoRNA31 directs the isomerization of uridine residues to pseudouridine in position 218 of the 18S ribosomal RNA (rRNA) and position 3713 of the 28S rRNA (Kiss et al., 2004). Studies with human pluripotent stem cell (hPSC)-derived cortical neurons showed that snoRNA31 is produced and functional in human cortical neurons that acts as a restriction factor for HSV-1.

Likewise, inborn errors of RNA lariat metabolism due to mutations in *DBR1* have been shown to cause brainstem HSE in a TLR3-independent manner, disrupting cell-intrinsic and tissue-specific immunity to HSV-1 and other viruses (Zhang et al., 2018) (Figure 1). The underlying molecular mechanism remains elusive. The accumulation of RNA lariats could impair host cell recognition of the virus, thereby damaging cell-intrinsic mechanisms against viral infection. Alternatively, DBR1 could regulate the processing of host or viral RNA lariats (Han et al., 2017; Murray et al., 2014; Galvis et al., 2017)

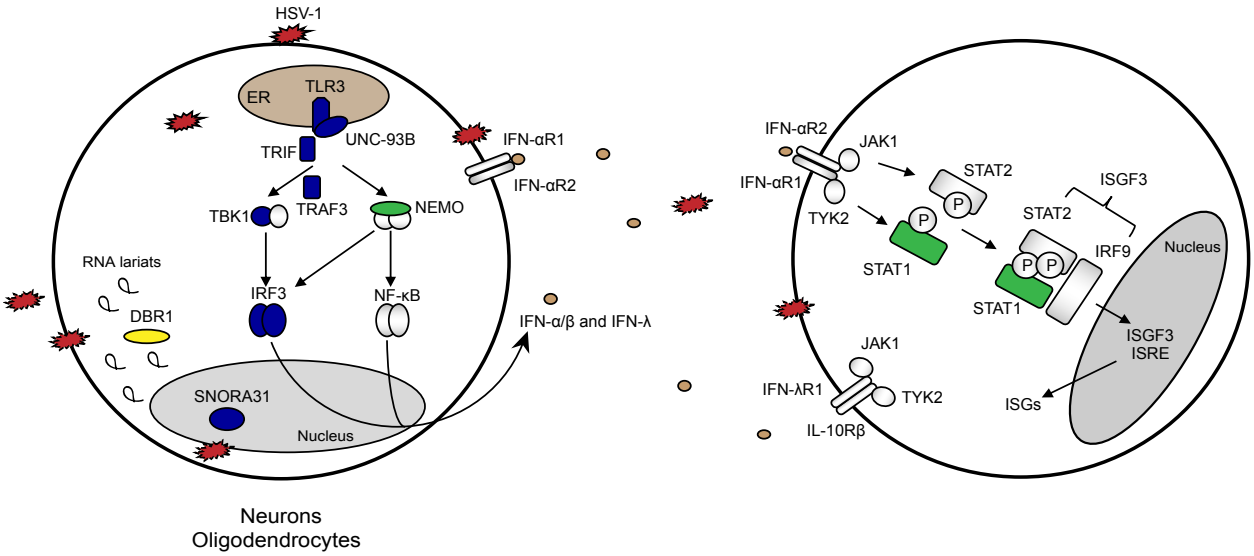

Figure 1. Schematic representation of the known genetic etiologies of HSE. In dark blue, described genetic etiologies underlying forebrain HSE (UNC93B, TLR3, TRIF, TRAF3, TBK1, IRF3 and SNORA31). In yellow, the only known genetic etiology to underlie brainstem HSE (DBR1). In green, genetic etiologies of "syndromic HSE" (NEMO and STAT1).

1.2.9.3 Patients without known genetic etiologies: the challenge

The advent of next-generation sequencing (NGS) technologies has clearly facilitated the discovery of new disease-causing variants in patients with all types of PIDs (Meyts et al., 2016), including HSE. Still, only a small fraction of the patients in our HSE cohort carry mutations in the aforementioned genes, probably due to the complex genetic architecture of the disease (i.e low clinical penetrance). We hypothesize that other single-gene inborn errors of immunity are yet to be discovered.

We currently have a cohort of ~400 patients (and we are happy to recruit as many as possible) and multiple strategies are being pursued to analyze the whole-exome sequencing data of the patients to further understand the molecular mechanism(s) that underlie the genetic predisposition of the disease. These approaches include both forward and reverse genetic strategies.

For the purpose of this thesis, we searched for novel HSE-causing candidate genes under an autosomal recessive (AR) mode of inheritance, following an unbiased genome-wide approach, and identified compound heterozygous *RIPK3* mutations in one patient.

1.3 Receptor Interacting Protein Kinase 3 (RIPK3)

1.3.1 Regulation of necroptosis

RIPK3 was first described in 1999 independently by two groups (Yu et al., 1999; Sun et al., 1999) as an apoptosis-inducing molecule that belonged to the RIP family of kinases. Back then, the different types of programmed cell death were poorly understood and it would take almost a decade until RIPK3 was rebranded as the key regulator of the necroptotic pathway (Declercq et al., 2009). Traditionally, pathologists had discriminated only two types of cell death: apoptosis and necrosis, and they were differentiated based on their morphological properties. Apoptosis was defined by cell shrinking, the appearance of membrane blebs called apoptotic bodies, and condensation of chromatin; while necrosis was associated with cell and organelle swelling and limited chromatin condensation. Apoptosis was believed to be the only form of regulated cell death while necrosis was seen as the result of a trauma or injury. In the late nineties and early 2000s, studies on TNFR1, FAS, caspases and RIPK proteins started to suggest that other forms of cell death could share morphological similarities to necrosis but still be regulated by cellular programs (Vercammen et al., 1997; Vercammen et al., 1998; Holler et al., 2000; Degterev et al., 2005); and necroptosis started to appeared as a caspase-8 independent regulated form of cell death

morphologically similar to necrosis. The role of RIPK1 and RIPK3 in necroptosis was highlighted at the end of the decade with the identification of RIPK1 as the target of a group of small molecules called necrostatins in 2008 (Degterev et al., 2008), and the redefinition of RIPK3 as the key regulator of this new type of cell death in 2009 (Cho et al., 2009; Zhang et al., 2009; He et al., 2009). RIPK3 has been intensively studied ever since due to its key role in necroptosis (Newtown et al., 2015).

1.3.2 RIPK3 signaling

1.3.2.1 TNFR1-induced RIPK3 mediated signaling

Upon ligand binding, TNFR1 recruits RIPK1 and other molecules in what is known complex I (Micheau et al., 2003). This interaction occurs between either the intracellular death domain (DD) of TNFR1 and the DD of RIPK1 or indirectly through the DD of TRADD (Ermolaeva et al., 2008; Pobezinskaya et al., 2008). cIPAs ubiquitylate RIPK1 and likely other molecules of the complex I (Varfolomeev et al., 2008; Mahoney et al., 2008) creating a scaffold for the recruitment of TAB1-TAB2/3-TAK1 and LUBAC (Cheung et al., 2004; Kanayama et al., 2004; Tokunaga et al., 2011; Ikeda et al., 2011; Gerlach et al., 2011). Linear ubiquitin chains regulated by LUBAC leads to the recruitment of the IκB kinase (IKK) complex, known as NEMO (Rahighi et al., 2009; Lo et al., 2009). Once TAK1 and NEMO are active, they signal activation of the kinases JNK, p38, and ERK as well as NF-κB transcription factors, culminating in the expression of proinflammatory genes (Newtown et al., 2012) (Figure 2). If this TNFR1-mediated cascade is compromised, RIPK1, TRADD and other molecules of the complex I move into cytoplasmic complex II (Figure 2) (Micheau et al., 2003). FADD gets recruited into the complex II through its C-terminal DD and then its N-terminal death effector domain (DED) can bind to a DED in procaspase-8 and/or its catalytically inactive homolog FLIP (Dickens et al., 2012; Schleich et al., 2012; Majkut et al., 2014). If FLIP is scarce, then autoprocessing of caspase-8 homodimers within complex II triggers apoptosis.

Complex II signaling can also be disrupted through caspase-8 inhibition. This inhibition would then lead to the activation of RIPK3 (in the cell types where RIPK3 is expressed) (Cho et al., 2009; Zhang et al., 2009; He et al., 2009), and its substrate, mixed lineage kinase-like (MLKL) (Sun et al., 2012; Zhao et al., 2012; Murphy et al., 2013; Wu et al., 2013) eventually triggering necroptosis. In this signaling cascade, RIPK3 uses RIPK1 as a cytosolic platform for the activation of necroptosis. RIPK1 and RIPK3 bind through their RIP Homotipic Interaction Motif (RHIM) domain (Sun et al., 2002) and this interaction eventually leads to the autophosphorylation of RIPK3 (Wu et al., 2014). Phosphorylated RIPK3 recruits and then phosphorylates MLKL within its C-terminal pseudokinase domain (Chen et al., 2013). MLKL is then transported to the membranes and leads to necroptotic cell death by a poorly understood mechanism that involves the disruption of the membrane through the creation of pores (Wang et al., 2014; Su et al., 2014; Cai et al., 2014).

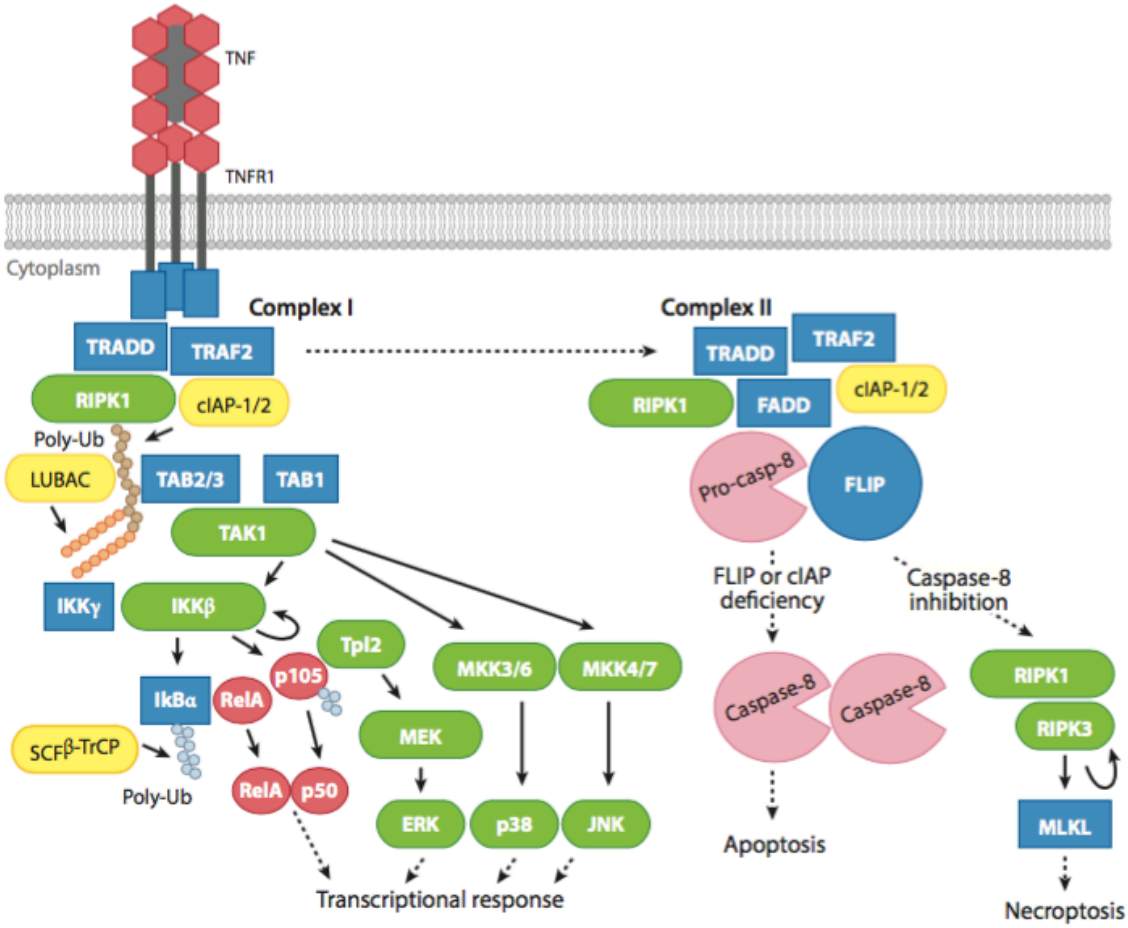

Figure 2. TNFR1-mediated RIPK3 signaling pathway (Newton, K., & Manning, G. 2016). Upon TNF binding to its receptor, complex I (a mix of adaptor proteins, protein kinases and ubiquitin kinases) is formed. Ubiquitylation and phosphorylation events lead to the activation of NF-kB and mitogen activated protein kinases, resulting in expression of prosurvival and proinflammatory genes. When complex I is internalized and shifts to form complex II, two types of cell death can occur, apoptosis and necroptosis. The type of cell death is determined by caspase-8 activity, when active, the cell die by apoptosis. If compromised, subsequent phosphorilation of RIPK3 and MLKL lead to necroptosis.

1.3.2.2 PRR-induced RIPK3 mediated signaling

Although RIPK3 has been most extensively investigated in the context of TNFR1 signaling, it can also be activated by some pathogen recognition receptors (PRRs) (TLR3, TLR4, and ZBP1) (He et al., 2011; Kaiser et al., 2013; Upton et al., 2013; Upton et al., 2010). TLR3 and TLR4 respond to double-stranded RNA and lipopolysaccharide (LPS), respectively, by recruiting the RHIM-containing adaptor protein TRIF. The RHIM in TRIF can engage the RHIM in RIPK1 (Meylan et al., 2004) or RIPK3 (Kaiser et al., 2005). Interestingly, the kinase activity of RIPK1 is required for TLR3- or TNFR1-induced necroptosis in macrophages (He et al., 2011; Kaiser et al., 2013), but is dispensable for TLR3-induced necroptosis in endothelial and fibroblastic cells (Kaiser et al., 2013). DAI, also known as ZBP1, is an intracellular PRR that recognizes double-stranded RNA. It also contains a RHIM domain and can directly activate RIPK3 (Upton et al., 2012). RIPK3 activation leads to autophosphorylation and phosphorylation of MLKL, which is translocated to the membranes of the organelles and the membrane eventually disrupting their homeostasis and leading to a necrotic-like cell death similar to the one mediated by the TNFR1.

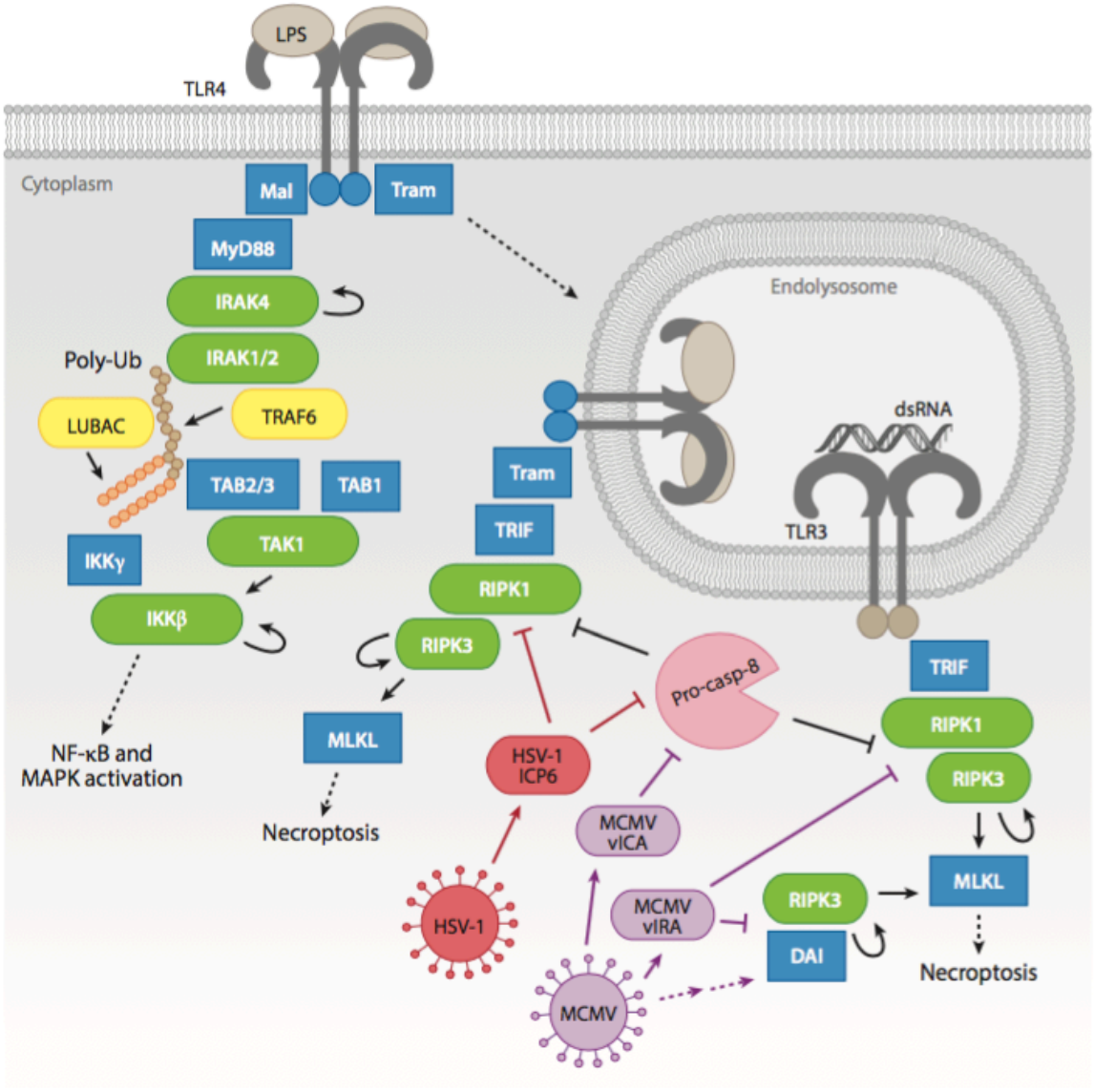

Figure 3. PRR-induced RIPK3 mediated signaling pathway (Newton, K., & Manning, G. 2016). TLR3 and TLR4 are activated by double-strand RNA (dsRNA) and lipopolysaccharide (LPS), respectively. Recruitment of the adaptor protein TRIF to TLR3 or TLR4 within endolysosomes facilitates engagement of RIPK1 and/or RIPK3, and if caspase-8 catalytic activity is impaired, then this leads to necroptosis.

1.3.3 RIPK3 in the context of viral infections

Hallmark studies using vaccinia virus raised the notion that viral-induced necroptosis contributes to host defense (Li et al., 2000). A plethora of evidence has supported this concept ever since and it is widely accepted now that RIPK3-mediated cell death can be triggered by various viral agents including influenza A virus (IAV) (Nogusa et al., 2016), human and murine cytomegalovirus (HCMV and MCMV) (Upton et al., 2010), herpes simplex viruses (HSV-1 and HSV-2) (Guo et al., 2015; Huan et al., 2015), and the aforementioned vaccinia virus (Cho et al., 2009) among others. More recent studies have also shown that, unlike the canonical function normally ascribed to RIPK3, ZIKV and WNV infections of the central nervous system are also combated by murine RIPK3 in a necroptosis-independent manner (Daniels et al., 2017; Daniels et al., 2019). Overall, these seminal studies depict RIPK3 as a pleiotropic antiviral molecule whose signaling cascade is virus-, tissue- and species-dependent.

2. MATERIALS AND METHODS

2.1 Human subjects

Informed consent was obtained in France, in accordance with local regulations and a human subjects research protocol approved by the institutional review board (IRB) of INSERM. Experiments using samples from human subjects were conducted in the United States, in accordance with local regulations and with the approval of the IRB of The Rockefeller University.

2.2 Cell culture

Primary human fibroblasts were obtained from skin biopsy specimens from controls and P1 and were cultured in DMEM medium (GIBCO BRL, Invitrogen) supplemented with 10% fetal calf serum (FCS) (GIBCO BRL, Invitrogen). To create immortalized SV40-transformed fibroblast cell lines (SV40-fibroblasts), 4 mg of a plasmid containing T antigen DNA was used to transfect about 5 millions of cells by electroporation. The cells were then placed into two fresh 75 cm2 flasks containing 12 mL of DMEM medium (GIBCO BRL, Invitrogen) supplemented with 10% FCS (GIBCO BRL, Invitrogen). SV40-fibroblasts clones appeared after about 15 days. They were then grown and passaged for experimental usage. To create Epstein-Barr virus (EBV)-transformed B cell lines (EBV-B), fresh blood samples were obtained from controls and P1, peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Paque PLUS (GE Healthcare, USA). PBMCs were then infected with EBV, and kept in culture in RPMI medium (GIBCO BRL, Invitrogen) supplemented with 10% FCS (GIBCO BRL, Invitrogen). EBV-B clones will appear in about 15 days. They could then be grown and passaged for experimental usage. HEK293T cells (ATCC) were maintained in DMEM supplemented with 10% FCS. HeLa cells (ATCC) were maintained in

DMEM supplemented with 10% FCS. HT29 cells (ATCC) were maintained in McCoy medium. Transfected cell lines were generated by transfecting pTRIP or mock vector (Luc), with X-tremegene 9, according to the manufacturer's protocol. HT-29 cells stably expressing pTRIP constructs were generated by lentiviral transduction. These cells were kept in culture under puromycin selection (10 µg/ml).

2.3 Whole-exome sequencing

Whole-exome sequencing (WES) was performed on genomic DNA extracted from whole blood from P1 by the New York Genome Center, using an Illumina HiSeq 2500 machine and an Agilent 71Mb SureSelect exome kit.

2.4 Sanger sequencing of genomic DNA

Genomic DNA Samples from controls, P1 and her parents were used as a template for the amplification of 300-600-bp regions encompassing the mutation by PCR with site-specific oligonucleotides. Amplicons were sequenced with BigDye Terminator technology on an ABI 3730 DNA sequencer. SnapGene was used for sequence analysis.

The sequences of the primers used are as follows:

R422* forward: GATTCGATGGCCCAACCTCC

R422* reverse: GCCCCCTGCAAACAGCACAG

P493fs9* forward: GACCGCTCGTTAACATATAC

P493fs9* reverse: GGCCAGACTGCCCTAGAAG

2.5 Western blots

Total cell extracts were prepared from primary fibroblasts, SV40-fibroblasts and B-EBV cells from healthy controls and P1 or HEK293T cells, HeLa cells or HT29 cells. Equal amounts of protein from each sample were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). These PVDF membranes were then probed with antibodies against RIPK3, RIPK1, MLKL, P-MLKL. Membranes were stripped and reprobed with an antibody against GAPDH (Santa-Cruz, USA), to control for protein loading. Antibody binding was detected by enhanced chemiluminescence (ECL; Amersham-Pharmacia-Biotech).

2.6 Measurement of RIPK3 transcript levels by RT-qPCR

Gene expression was assessed by measuring transcript levels. Total RNA was extracted from SV40-fibroblasts, EBV-B cells and HEK293T cells. RNA was reverse-transcribed directly, with random hexamers (Invitrogen) (for RNA from SV40-fibroblasts or EBV-B cells) or with the SuperScript III Cells-Direct kit (Life Technologies, for HEK293T cells), to determine mRNA levels for RIPK3. Reverse transcription-quantitative PCR (RT-qPCR) was performed with Applied Biosystems 2 x universal Taqman reaction mixture and Assays-on-Demand probe/primer combinations, in an ABI PRISM 7700 Sequence Detection System. GUS and GAPDH genes were used for normalization. Hs01011171_g1 (RIPK3 exons 2-3) and Hs01011177_g1 (RIPK3 exons 9-10) probes were used. Results are expressed according to the ΔCt method, as described by the manufacturer. Two independent experiments were performed, for measurement of RIPK3 transcript levels in primary fibroblasts, SV40-fibroblasts or EBV-B cells, technical duplicates were used in each experiment. For measurement of RIPK3 transcript levels in HEK293T cells, one experiment was performed using biological duplicates for each condition.

2.7 Microscopy

HELA cells were subjected to transient transfection with WT or mutant RIPK3 using X-tremeGENE9 transfection reagent (Sigma-Aldrich). After 48h of incubation, cells were fixed with 4% paraformaldehyde for 15 min at 37°C, washed 3 times with PBS, permeabilized with 0.1% Triton and blocked with 0,1% Triton containing 6% Donkey serum for 1h. The cell cultures were stained overnight with the RIPK3 primary antibody (Santa-Cruz, USA). Cells were washed 3 times with PBS and incubated with secondary antibodies: anti-mouse Alexa 633 (Invitrogen). Cells were washed 3 times with PBS and mounted in DAPI containing Prolong Gold mounting media (Thermo Fisher Scientific). Slides were examined with a confocal laser microscope (Confocal Leica SP8 gSTED). All images were acquired with APO CS2 63x/1.4 OIL objectives. Images were exported as tiff files and image analysis was performed with ImageJ software. For each independent experiment, 5-12 cells were analyzed per condition. Each field was selected from DAPI-positive staining, which was used for defining the nuclear region (NR).

2.8 Necroptosis induction

For the detection of MLKL phosphorylation, necroptosis was induced by combined treatment with PBZ (poly(I:C), 25ug/ml; BV6, 1uM; z-VAD.fmk, 20uM) or TBZ (TNF-a, 1000 units/ml, BV6, 1uM; z-VAD.fmk, 20uM) for four hours. Cell lysates were collected and Western blotting was performed.

2.9 Cell viability

Cells (5,000 cells/well) were seeded into Corning 96-well tissue culture plates. 16–24 h post-seeding. Cells were treated with the indicated reagents, and solvent, DMSO, was kept constant for all experiments. 30 ng/ml TNF, 5 uM BV6, and/or 25uM -VAD-fmk were used. Cell viability was assessed by measuring the intracellular levels of ATP using the Cell Titer-Glo luminescent cell viability assay kit (Promega) according to the manufacturer's instructions.

2.10 TLR3 agonists

We used a synthetic analog of dsRNA, polyinosine-polycytidylic acid (poly(I:C)), as a nonspecific agonist of TLR3 and MDA5/RIG-I. SV40-fibroblast cells were activated in 24-well plates, at a density of 100,000 cells/well, for 24 hours with poly(I:C) at 1, 5 and 25 μg/mL. Cells were stimulated with 25μg/mL of poly(I:C) in the presence of Lipofectamine 2000 to activate MDA5/RIG-I signaling. After 24h, supernatants were collected for cytokine determination by ELISA.

2.11 ELISAs

Levels of IFN-a, -β, -λ and IL-6 production were assessed by ELISA after 24 h of cell stimulation. Separate ELISAs were performed for each of IFN-a (eBioscience, USA), IFN-β (PBL, USA), IFN-λ (R&D Biosystems, USA), and IL-6 (eBioscience, the Netherlands), according to the kit manufacturer's instructions.

3. RESULTS

3.1 A case of recurrent HSE and suspected post-herpetic autoimmune encephalitis

We studied a patient (P1) born in France to non-consanguineous parents and presented at 6 months of age with herpes simplex encephalitis. P1 was previously healthy and had received all recommended vaccinations. She presented with fever, a brief episode of loss of consciousness with hypotonia and an episode of right hemi-body seizure lasting 10 minutes. Lumbar puncture showed elevated protein content (0.45g/L) and elevated nucleated cells (53/mm^3), mostly lymphocytes. PCR for herpes simplex virus (HSV1/2) was positive and CSF interferon levels were elevated (50U.I.). Electroencephalogram showed a left centro-temporal slowness, without paroxystic elements. Brain MRI showed a slight hypersignal in the left hemisphere (Figure 4). She was treated for 10 days with intravenous acyclovir and discharged after a good clinical evolution. A year later, she was readmitted to the hospital for another episode of HSE. She presented with elevated fever, seizures and abnormal level of consciousness. Lumbar puncture showed elevated protein content (0.57g/L) and elevated white blood cells (45/mm^3). PCR for herpes simplex virus (HSV) was again positive. A CT scan showed bilateral frontal hypodense regions, predominantly in the right hemisphere with hemorrhagic foci. She was treated with intravenous acyclovir and admitted to the intensive care unit (ICU). During her hospitalization, she presents with persistent seizures, hypotonia and dysmetria. She was discharged after 3 weeks of acyclovir treatment, with neurological sequelae.

A week later, she was again hospitalized for an episode of encephalitis strongly suggestive of auto-immune encephalitis. She presented with altered levels of consciousness, dysphagia and abnormal ventilation. Lumbar puncture showed elevated protein levels (1,32g/L), 10 white blood cells but negative PCR for HSV1/2. Brain CT scanner showed diffuse hypodense regions in both frontal lobes, both parietal lobes, right occipital lobe and basal ganglia. EEG was suggestive of a

reactive coma. She was treated with high dose intravenous steroids and acyclovir. She later developed movements disorders with dyskinesia and decreased level of consciousness. After 2 weeks of treatment, a control MRI was performed showing T2 hypersignal predominantly in the white matter. She was discharged from the hospital with severe neurological consequences.

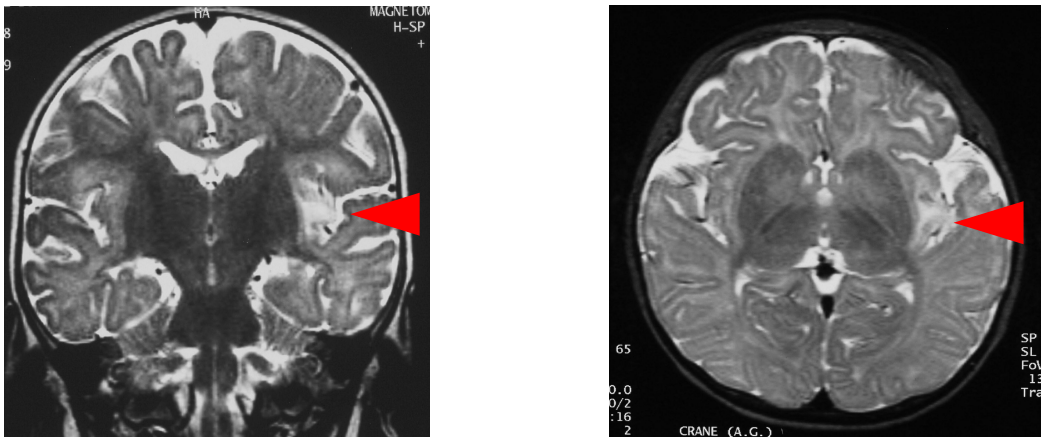

Figure 4. MRI images of the brain of the patient, showing lesions affection the right frontotemporal lobe. On the left, coronal MRI. On the right, axial MRI. T2 hypersignal is shown in red.

3.2 Identification of bi-allelic RIPK3 mutations in the proband

We tested our recurrent hypothesis that a single gene inborn error of immunity could underlie the clinical phenotype of the patient. To test this hypothesis, we perform Whole-exome sequencing (WES) on genomic DNA extracted from whole blood of P1. We then applied a filtering criteria according to an autosomal recessive mode of inheritance and found 6 genes harboring homozygous or more than one heterozygous nonsynonymous mutations (figure 5 and tables 1 and 2), among which we identified two heterozygous mutations p.Arg422* (R422*) and p.Pro493Thrfs*9 (P493fs9*) in *RIPK3* encoding Receptor Interacting Protein Kinase 3 (RIPK3).
RIPK3 is a likely candidate to underlie HSE from a biologically point of view. First, RIPK3 is able to signal downstream TLR3 via direct interaction with TRIF (He et al., 2011; Kaiser et al., 2013), which are both genetic etiologies of HSE. Second, RIPK3 is targeted by HSV-1 through interaction of the RHIM domain of RIPK3 with the RHIM domain of the viral protein ICP6 (Guo et al., 2015; Huan et al., 2015) . Last, RIPK3 has been shown to play an important role in viral restriction in the central nervous system (Daniels et al., 2017; Daniels et al., 2019). In fact, soon after we started this study of the role of RIPK3 in HSE, Katherine B. Ragan and Jason W. Upton made very similar observations to ours, and they independently predicted that mutations in RIPK3 could underlie HSE (Ragan & Upton; 2017).

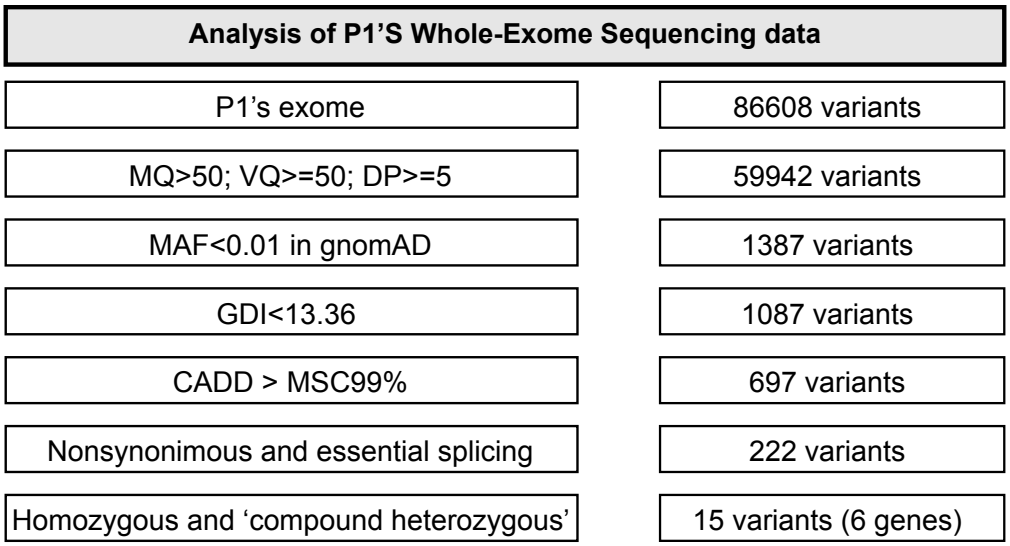

Figure 5. Analysis of P1's Whole-Exome sequencing. Filtering criteria applied to the Whole-Exome sequencing data of the patient under a potential autosomal recessive mode of inheritance. MQ, Mapping quality; VQ, variant quality; DP, combined depth across samples; MAF, minor alleles frequency; GDI, gene damage index, CADD, combined annotation dependent depletion; MSC, mutation significance cutoff.

Gene	Function	Annotation	Zygosis	Allele frequency	CADD
CDC27	missense	p.Trp644Arg	het	0.002817	29.4
CDC27	missense	p.Tyr641Cys	het	0.00702138	28
PABPC1	missense	p.Arg481Cys	het	0.00380623	27.6
PABPC1	missense	p.Arg475Gln	het	0.00189407	23.8
PABPC1	missense	p.Ile454Thr	het	7.32E-04	20.8
PABPC1	missense	p.Phe409Leu	het	4.68E-04	23.1
PABPC1	missense	p.Tyr408Phe	het	8.76E-04	22.5
PABPC1	stop-gained	p.Glu345*	het	0.00150455	40
PCDHA1	missense	p.Asn449His	hom	0.00427296	23.8
RIPK3	**indel-frameshift**	**p.Pro493fs**	**het**	**0.00207218**	**24.2**
RIPK3	**stop-gained**	**p.Arg422***	**het**	**0.00105122**	**35**
ZNF208	missense	p.Asp22Asn	het	0.00267752	14.52
ZNF208	missense	p.Thr5Ile	het	3.34E-05	11.56
ZNF785	missense	p.Val193Gly	het	0.00753272	21.5
ZNF785	missense	p.Glu119Lys	het	0.00751832	8.72

Table 1. Homozygous and 'compound heterozygous' rare nonsynonymous variations found in P1 after filtering Whole-Exome Sequencing data.

Gene description	Function	Clinical significance
Cell Division Cycle 27 (*CDC27*)	Subunit of the anaphase-promoting complex (APC)	Unknown
Polyadenylate-binding protein 3 (*PABPC3*)	Might bind RNA homopolymers	Unknown
Protocadherin-alpha 1 (*PCDHA1*)	Mediate cell-cell interaction	Unknown
Receptor-Interacting Protein Kinase 3 (*RIPK3*)	**Necroptosis; neuroinflammation**	**This study**
Zinc Finger Protein 208 (*ZNF208*)	Unknown	Unknown
Zinc Finger Protein 785 (*ZNF785*)	Unknown	Unknown

Table 2. Characteristics of the genes that remained in the exome of P1 after filtering Whole-Exome Sequencing data.

Sanger sequencing of the patient and her parents confirmed both mutations in the patient, and revealed that the R422* variant was inherited from the mother and that the P493fs9* was inherited from the father (figure 6A and 6B), corroborating the true compound heterozygosity nature of the mutations. R422* and P493fs9* yield a minor allele frequency of 0.001568 and 0.002555, respectively, as reported in gnomAD (http://gnomad.broadinstitute.org/) (figure. 6C and table 3), where we found only one homozygous carrier for each variant out of the 140,312 sequenced individuals. The number of homozygous carriers of these variants in the general population is, therefore, compatible with the incidence and the penetrance of the disease. No other homozygous or compound heterozygous carrier of rare nonsynonymous variants was found in our in-house database (>6000 patients). Last, both variants have combined annotation-dependent depletion (CADD) (http://cadd.gs.washington.edu/) scores well above the mutation significance cutoff (MSC) of *RIPK3* (http://pec630.rockefeller.edu:8080/MSC/) (figure 6C). probably because both of them are predicted to generate aberrant forms of the protein (figure 6D) Both combined, R422* and P493fs mutations may lead to AR RIPK3 deficiency underlying HSE.

Figure 6

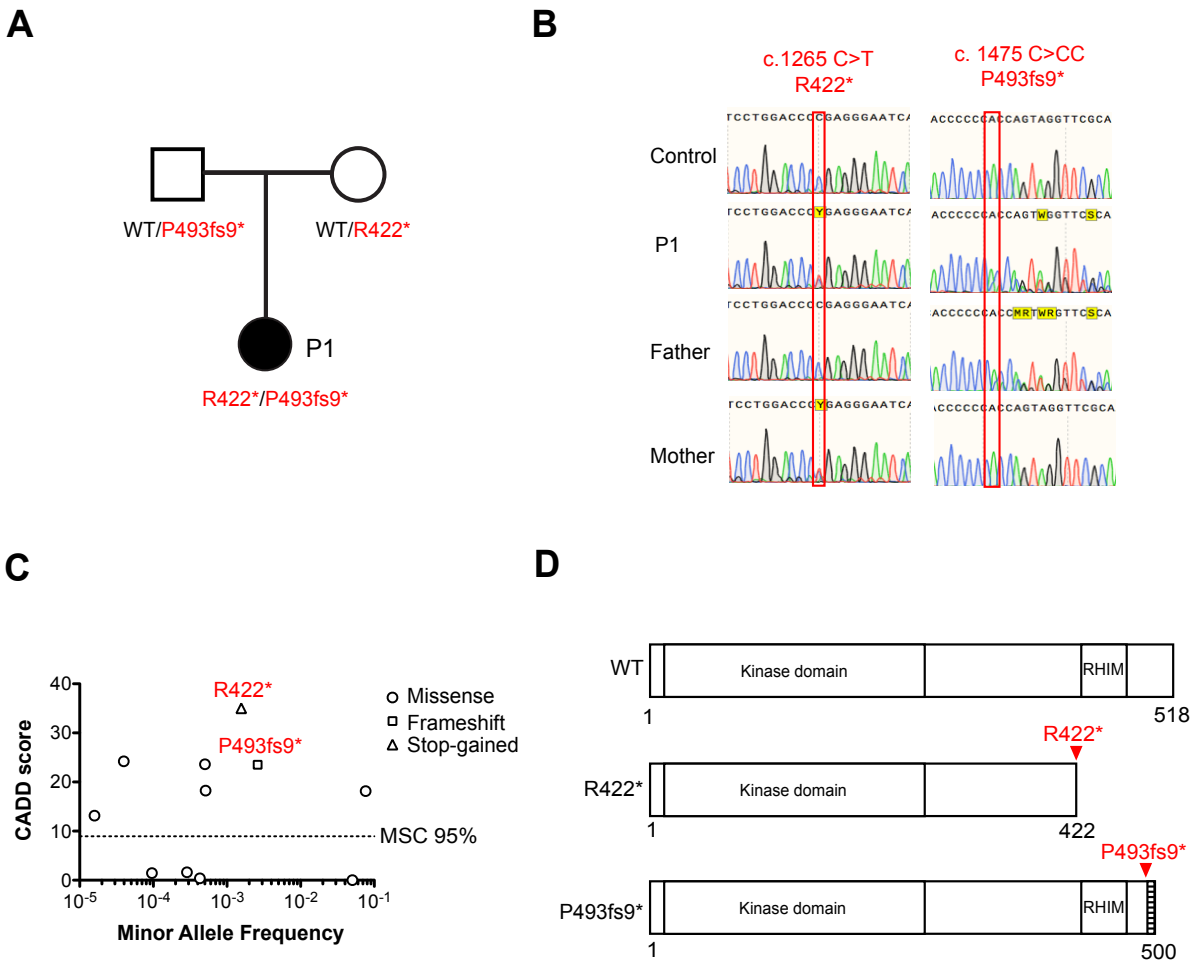

Figure 6. Compound heterozygous *RIPK3* mutations in a French girl from a non-consanguineous family. (A) Familial segregation of *RIPK3* mutations in a nonconsanguineous French family. (B) Electropherogram showing the mutations in exons 8 and 9 in *RIPK3* in P1 and her parents, respectively. (C) Representation of all homozygous nonsynonymous *RIPK3* variants reported in gnomAD. The minor allele frequency and CADD score of each variant is shown. CADD MSC of RIPK3: the 95% confidence interval mutational significant cutoff CADD score of RIPK3. (D) Schematic representation of RIPK3 protein structure and the impact of the mutations.

3.3 Investigation of the alleles through plasmid mediated overexpression

We studied the expression and function of the R422* and P493fs9* RIPK3 mutants through plasmid-mediated overexpression in HEK293T cells, which have very low endogenous level RIPK3 expression; HeLa cells; and RIPK3 knock-out (KO) HT29 cells. We first investigated the expression of the mutant alleles by transiently transfecting RIPK3 constructs in HEK293T cells. Compared with *RIPK3* WT, both mutants resulted in normal mRNA production as assessed by RT-qPCR using two different probes (figure 7A). At the protein level, the mutants are predicted to codify proteins of a smaller molecular size: R422* would translate into a 46 kDa protein, and P493fs9* would translate into a 56 kDa protein, while the WT protein size is 57 kDa. When we analyzed the expression of the mutant alleles in HEK293T cells overexpressing the RIPK3 plasmids, we observed that the encoded proteins were indeed truncated forms of RIPK3 (figure 7B). In the case of R422*, the introduction of a premature stop codon led to the expression of a protein of ~46 kDa. In addition, R422* also showed an impaired autophosphorylation and cleavage, as the levels of expression of the N-terminus cleaved fragment are significantly lower when compare to the WT. On the other hand, the introduction of a frameshift mutation in the P493fs9* codified a protein of ~56 kDa, which is slightly smaller than the WT protein. Therefore, neither of the mutant constructs lead to reinitiation of translation (figure 7B). We then examined the ability of the mutant alleles to induce NF-κB activity upon transient transfection in HEK293T cells, and we found that R422* but not P493fs9* was loss of function (figure C-E). In contrast, neither the expression nor the function was affected when we overexpressed RIPK3 constructs containing the common variants found in public databases (figure 8).

Figure 7

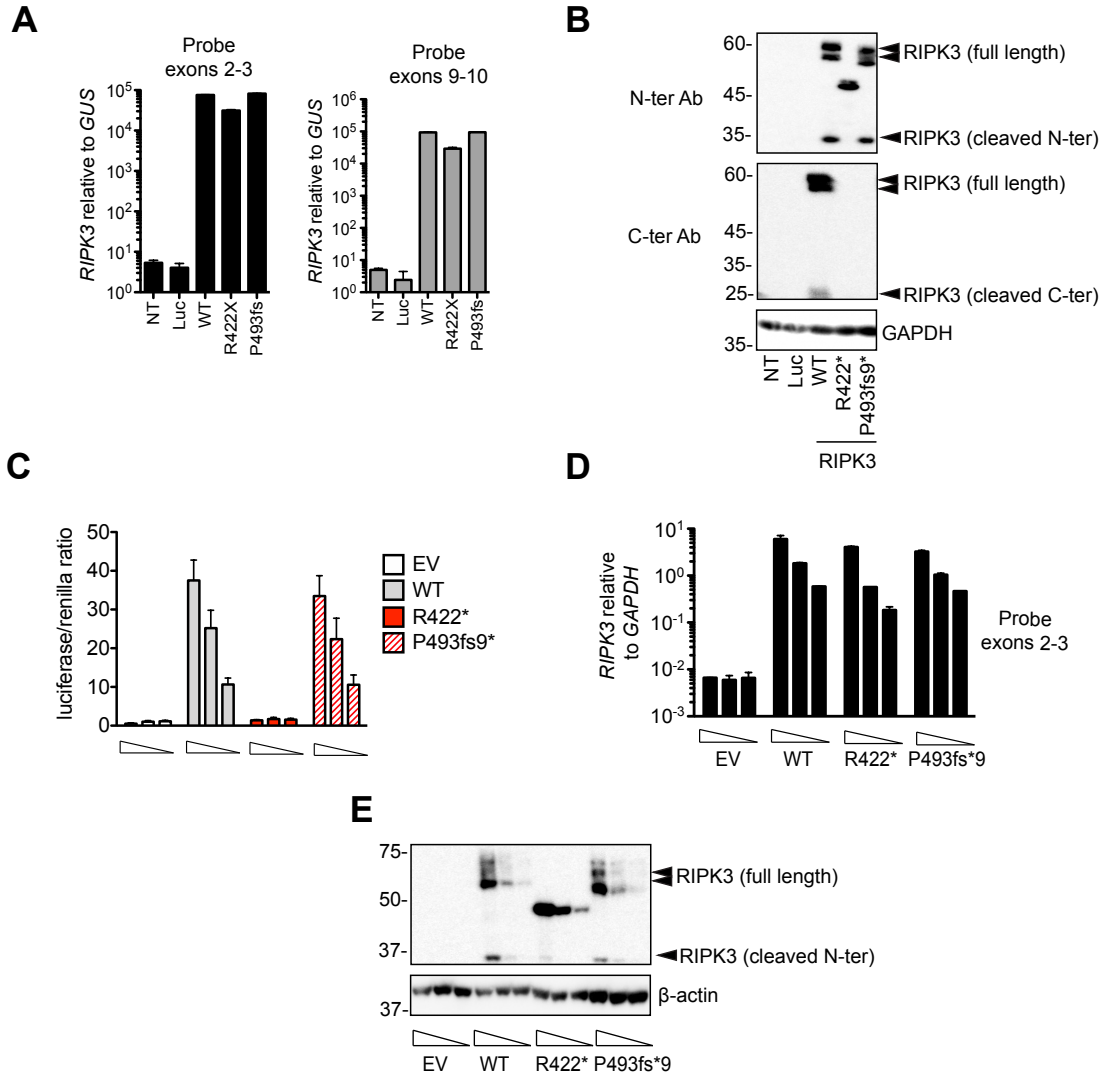

Figure 7. The mutant alleles encode truncated forms of the protein but only R422* is loss of function in terms of its NF-κB activation activity. (A) Wild-type (WT) and mutant *RIPK3* mRNA levels in transfected HEK293T cells. Levels of WT, R422* or P493fs9* were assessed by RT-qPCR using a probe that binds exons 2-3 (left) and exons 9-10 (right) of RIPK3 cDNA. (B) Wild-type (WT) and mutant RIPK3 protein levels in transfected HEK293T cells assessed by western blotting. RIPK3 protein was detected using a monoclonal antibody directed against the N terminus of RIPK3 and an monoclonal antibody directed against the C terminus of RIPK3. Results are representative of at least three independent experiments. (C) RIPK3 dependent NF-kB activity. HEK293T cells were cotransfected with NF-κB luciferase reporter plasmid (100 ng) and Renilla luciferase reporter plasmid (10 ng) with equal amounts (50ng, 25ng, and 12.5ng) of pTRIP EV, pTRIP RIPK3 WT and mutants, respectively. Cells were harvested at 24h post-transfection for Firefly and Renilla luciferase activity assay using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase values were corrected for transfection efficiency with Renilla activity. (D) Wild-type (WT) and mutant RIPK3 dose-dependent mRNA levels in transfected HEK293T cells. Levels of WT, R422* or P493fs9* were assessed by RT-PCR. (E) Wild-type (WT) and mutant RIPK3 dose-dependent protein levels in transfected HEK293T cells. Levels of WT, R422* or P493fs9* were assessed by western blotting.

Figure 8

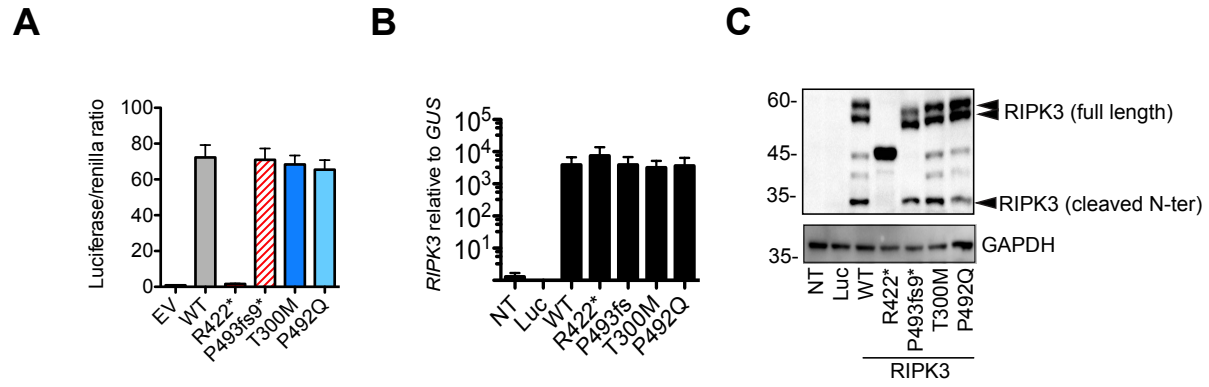

Figure 8. Overexpression of constructs containing common RIPK3 variants show normal function and expression. (A) RIPK3 dependent NF-κB activity. HEK293T cells were cotransfected with NF-κB luciferase reporter plasmid (100 ng) and Renilla luciferase reporter plasmid (10 ng) with equal amounts (50ng) of pTRIP EV and RIPK3 constructs, respectively. Cells were harvested at 24h post-transfection for Firefly and Renilla luciferase activity assay using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase values were corrected for transfection efficiency with Renilla activity. (B) *RIPK3* mRNA levels in transfected HEK293T cells as assessed by RT-qPCR using a probe that binds exons 2-3. (C) RIPK3 protein levels in transfected HEK293T cells assessed by western blotting. RIPK3 protein was detected using a monoclonal antibody directed against the N terminus of RIPK3.

We interrogated the subcellular localization of the mutant alleles. To do that, we transfected HeLa cells with RIPK3 constructs and analyzed their localization by confocal microscopy. Similar to their WT counterpart, the mutants were detected in the cytoplasm (figure 9A). Because the mutants localized normally in the cytoplasm, we decided to study the capability of these alleles to interact with RIPK1, RIPK3 WT, with themselves, and with each other. Only P493fs9* was able to bind to their homolog monomers, although unable to bind to R422*. P493fs9* also maintained the ability to the RIPK3 WT, as well as with RIPK1 WT (Fig. 9B and 9C), which is consistent with the fact that this construct contains an intact RIP Homotypic Interaction Motif (RHIM) domain, which is required for this interaction as well as the interaction of RIPK3 with the other RHIM-containing proteins (RIPK1, TRIF, and ZBP1 in vertebrates and ICP6, ICP10 and M45 in herpesviruses) (Upton et al., 2017).

Figure 9

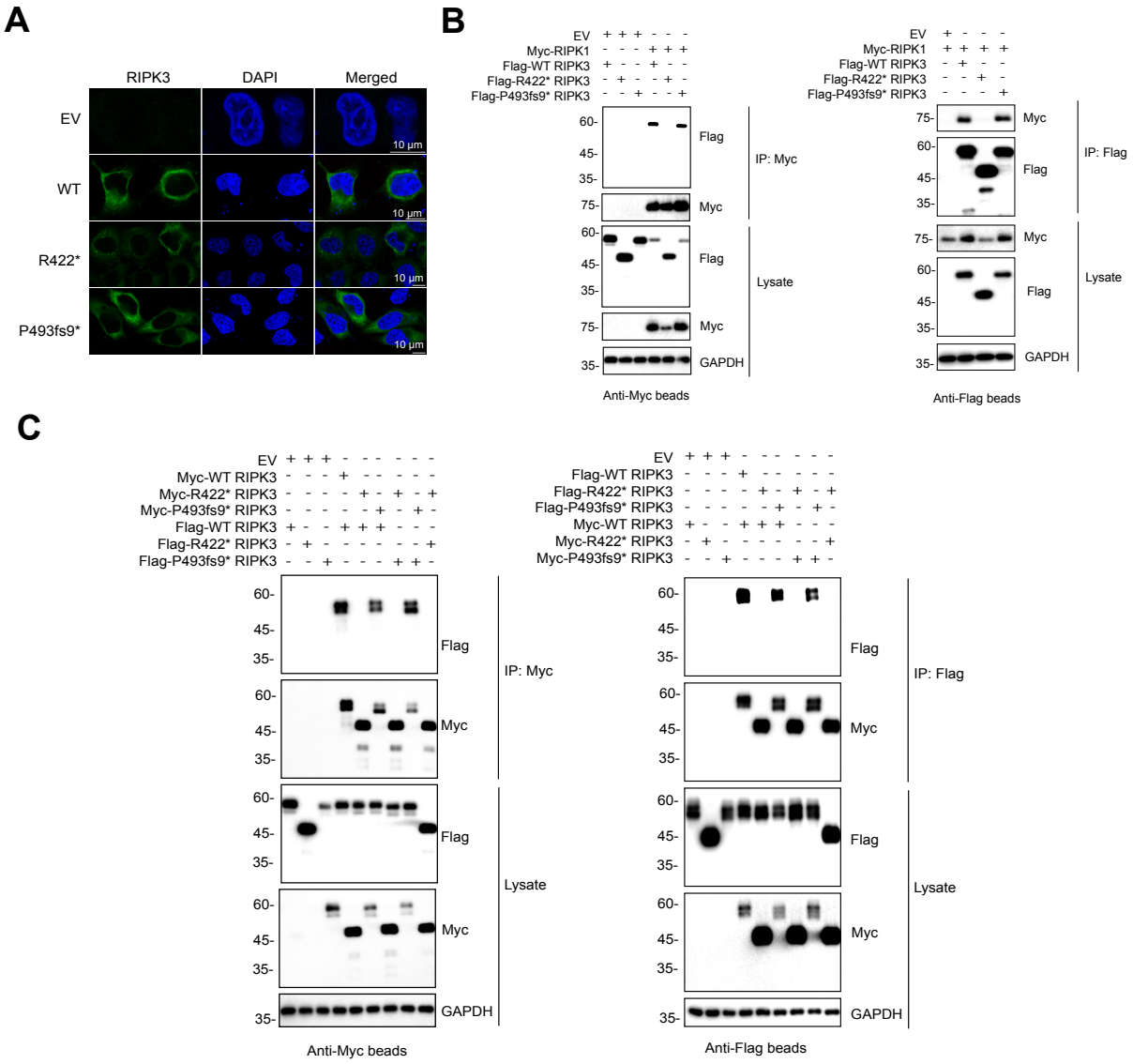

Figure 9. The mutant alleles are normally expressed in the cytoplasm but only P493fs9* can interact with other RHIM-containing constructs. (A) Wild-type (WT) and mutant RIPK3 subcellular localization in upon construct transfection in HeLa. Subcellular localization was obtained by confocal microscopy. (B) Flag-tagged RIPK3 WT and mutant constructs were co-expressed with Myc-tagged RIPK1 WT in HEK293T cells, and whole cell lysates were immunoprecipitated with anti-Flag and anti-Myc beads, followed by immunoblotting with anti-flag or anti-Myc antibodies. Results are representative of three independent experiments. (C) Flag-tagged RIPK3 WT and mutant constructs were co-expressed with Myc-tagged RIPK3 WT and mutant constructs in HEK293T cells, and whole cell lysates were immunoprecipitated with anti-Flag and anti-Myc beads, followed by immunoblotting with anti-flag or anti-Myc antibodies. Results are representative of two independent experiments.

Next, we investigated the expression and function of the mutants after retroviral transduction in HT29 RIPK3 knock out cells. Similar to the results obtained in HEK293T cells, both mutants encoded aberrant proteins. However, in this system P493fs9* showed lower levels of protein expression (Fig. 10A), although normal levels of mRNA expression (Figure 10B), suggesting that this mutant construct could be less stable than the WT protein.

Finally, we investigated the ability of the mutants to induce the phosphorylation of MLKL, a main substrate of RIPK3, and the effector molecule of the necroptotic pathway (Sun et al., 2012). HT29 RIPK3-/- cells transduced with RIPK3 WT, and mutant constructs were stimulated with combinations of the caspase inhibitor z-VAD, the smac-mimetic BV6, and either poly(I:C) (PBZ) or TNF-α (TBZ), as previously described (Guo et al., 2015). We observed that R422* but not P493fs9* was loss of function in terms of MLKL phosphorylation (p-MLKL) both via TLR3 (figure 10C) and via TNFR1 (figure 10D). Consistent with these results, cell viability of HT29 RIPK3-/- cells transduced with WT and P493fs9*, but not with R422*, was severely impaired upon PBZ and TBZ stimulation (Figures 10E and 10F).

Figure 10

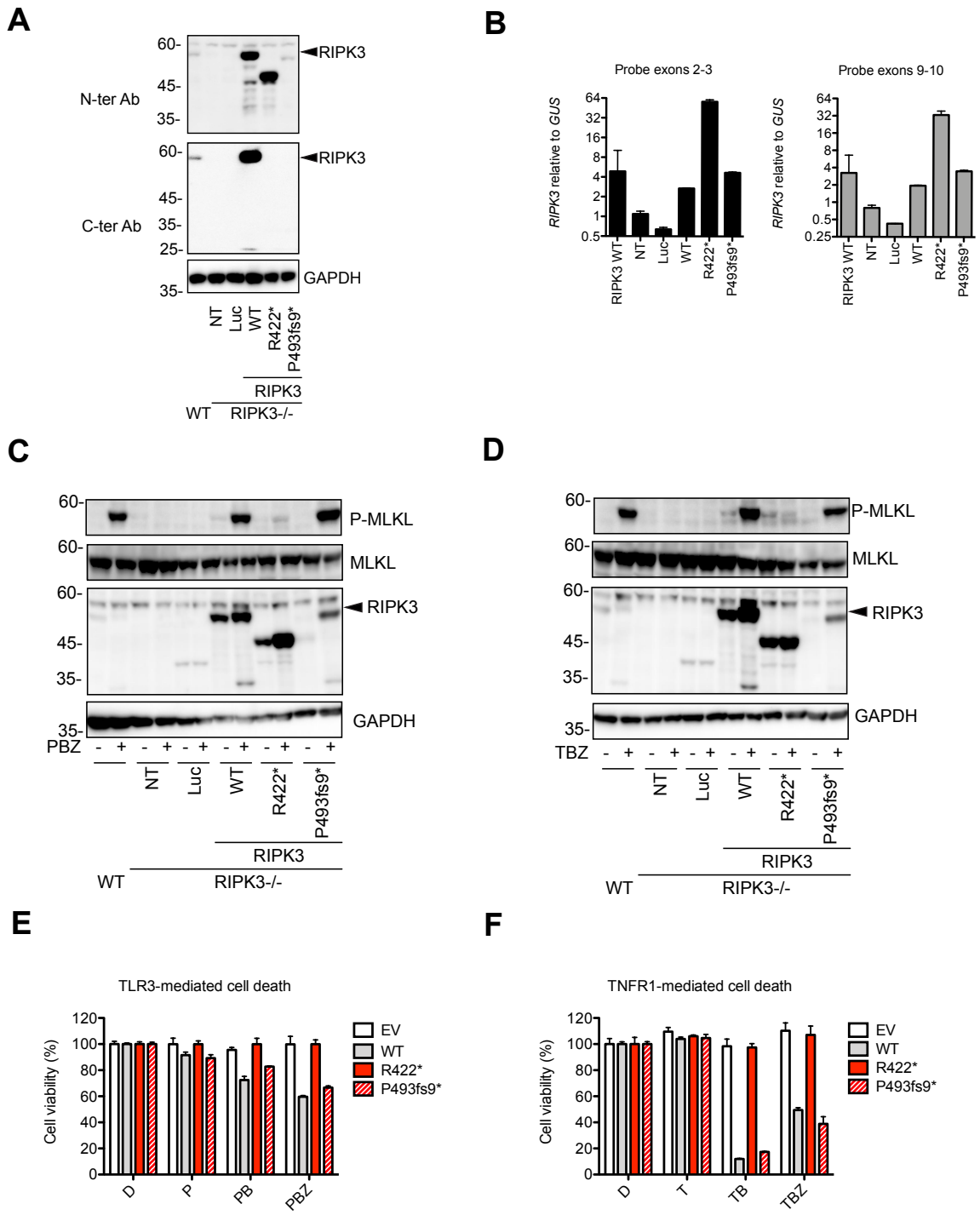
A
N-ter Ab
C-ter Ab
RIPK3
GAPDH
NT
Luc
WT
R422*
P493fs9*
RIPK3
WT
RIPK3-/-
B
Probe exons 2-3
Probe exons 9-10
RIPK3 relative to GUS
C
D
P-MLKL
MLKL
PBZ
TBZ
E
TLR3-mediated cell death
Cell viability (%)
EV
WT
R422*
P493fs9*
D
P
PB
PBZ
F
TNFR1-mediated cell death
T
TB
TBZ

Figure 10. RIPK3 overexpression in HT29 RIPK3-/- cells. (A) Wild-type (WT) and mutant RIPK3 protein levels in retro-transduced HT29 RIPK3-/- cells. Levels of WT, R422* or P493fs9* were assessed by western blotting. Results are representative of at least 3 independent experiments. (C) MLKL phosphorylation after PBZ stimulation in HT29 RIPK3-/- stably transfected with RIPK3 WT and mutant constructs. Results are representative of two independent experiments. (D) MLKL phosphorylation after TBZ stimulation in HT29 RIPK3-/- stably transfected with RIPK3 WT and mutant constructs. Results are representative of three independent experiments. (E) Cell viability assay was performed upon PBZ stimulation in HT29 RIPK3-/- transduced with RIPK3 WT and mutant constructs. (F) Cell viability assay was performed upon TBZ stimulation in HT29 RIPK3-/- transduced with RIPK3 WT and mutant constructs. (A, C and D) are representative of at least three independent experiments. (E and F) are mean values of three independent experiments. Error bars represent SEM.

3.4 Study of mRNA and protein RIPK3 expression in the patient cells

We next interrogated the impact of the mutations on the RIPK3 mRNA and protein expression in cells from the patient. The overall amounts of RIPK3 mRNA from B-EBV cells, SV40-fibroblasts, and primary fibroblasts from P1 were still within the range of controls (figures 11A-C), probably because of the interindividual variability of RIPK3 mRNA observed in these cell types. Nevertheless, TOPO-cloning of cDNA generated from gDNA of the patient fibroblasts revealed that *RIPK3* mRNA containing R422* mutant represented ~2.75% of the total amount of transcripts and P493fs9* the ~97.25% left (figure 11D). R422* might, therefore, lead to nonsense mRNA decay.

In contrast, RIPK3 protein expression was undetectable in the patient B-EBVs, SV40-fibroblasts and primary fibroblasts with two different antibodies (figures 11E-G). These data, in combination with the results obtained in overexpression, suggest that the total amount of RIPK3 protein expression in the patient cells might be severely reduced due to (i) nonsense mRNA decay of R422* and (ii) protein instability of P493fs9*.

Figure 11

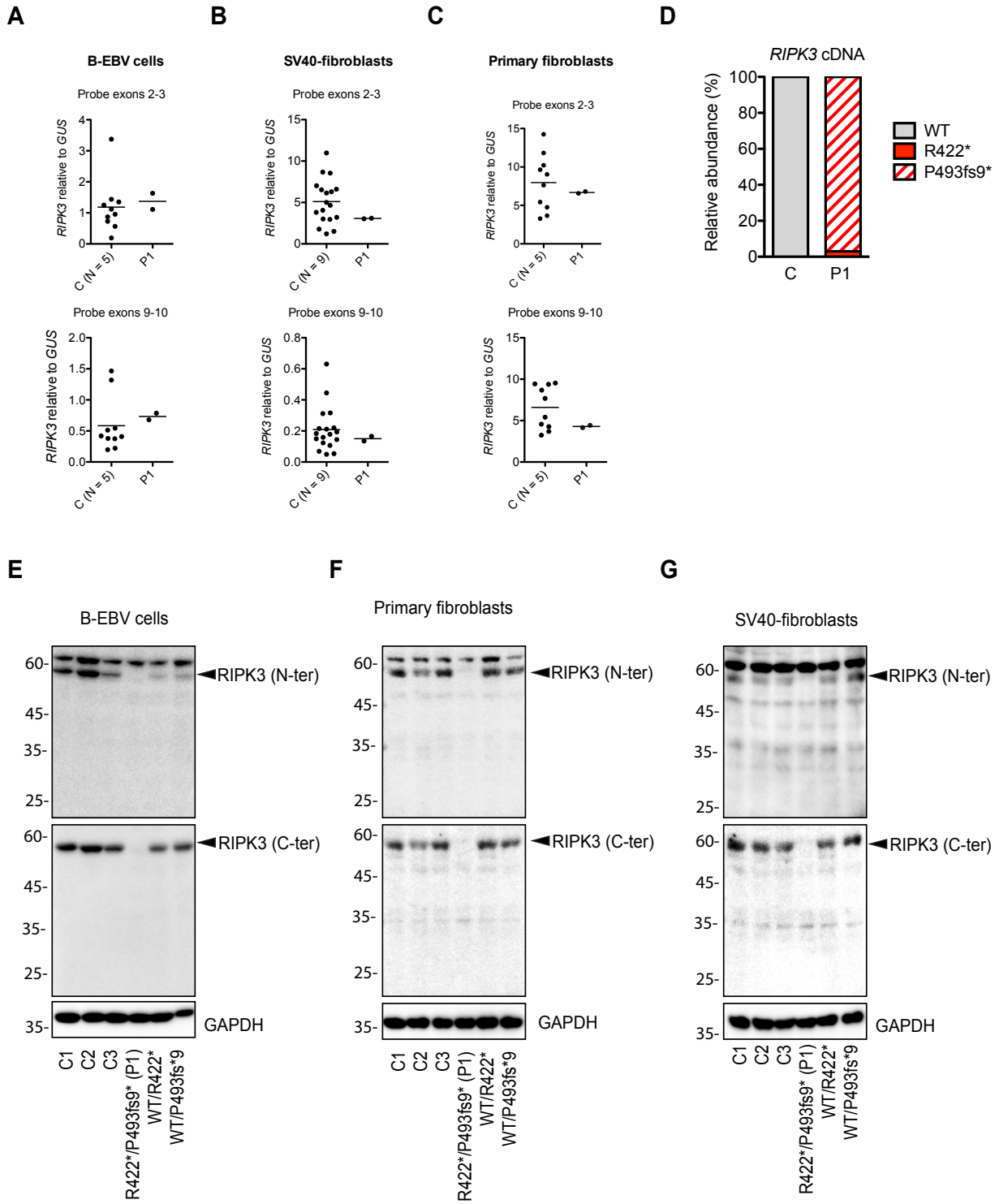
A
B
C
D
B-EBV cells
SV40-fibroblasts
Primary fibroblasts
Probe exons 2-3
Probe exons 9-10
RIPK3 relative to GUS
C (N = 5)
C (N = 9)
P1
RIPK3 cDNA
Relative abundance (%)
WT
R422*
P493fs9*
E
F
G
RIPK3 (N-ter)
RIPK3 (C-ter)
GAPDH
C1
C2
C3
R422*/P493fs9* (P1)
WT/R422*
WT/P493fs*9

Figure 11. The RIPK3 mutant alleles are loss of expression at endogenous levels by different mechanisms. (A) RIPK3 mRNA levels assessed by RT-qPCR in immortalized B-EBVs from controls and P1. (B) RIPK3 mRNA levels assessed by RT-qPCR in immortalized SV40-fibroblasts from controls and P1. (C) RIPK3 mRNA levels assessed by RT-qPCR in primary fibroblasts from controls and P1.(A-C) Results represent the average of two experiments with two biological duplicates. (D) Relative abundance (%) of RIPK3 cDNA generated from mRNA extracted from patient's immortalized SV40-fibroblasts assessed by TOPO-TA cloning. (E) RIPK3 protein levels in immortalized B-EBVs from controls and P1.. (F) RIPK3 protein levels in primary fibroblasts from controls and P1 assessed by western blotting. (G) RIPK3 protein levels in SV40-fibroblasts from controls and P1 assessed by western blotting. (E-G) RIPK3 protein was detected using a monoclonal antibody directed against the N terminus of RIPK3 and a monoclonal antibody directed against the C terminus of RIPK3. Results are representative of at least three independent experiments.

3.5 MLKL phosphorylation is broadly impaired in P1 and selectively impaired via TLR3 in other HSE patients with mutations in the TLR3 pathway

In spite of having certain pleiotropy as a restriction factor during viral infection (Daniels et al., 2017; Daniels et al., 2019), RIPK3 is well-known for its ability to phosphorylate MLKL and, in a broader sense, regulate necroptosis (Newton & Manning., 2016). In order to decipher whether the patient mutations would impair RIPK3-mediated signaling, we decided to investigate whether MLKL could be phosphorylated in immortalized SV40-fibroblasts from the patient either via TLR3 or TNFR1. To this end, SV40-fibroblasts from controls and from the patient were stimulated with PBZ and TBZ (figure 12A) using HT29 cells as a positive control (figure 12B), similar to the conditions used to test the ability of the alleles to induce necroptosis. The phosphorylation of MLKL was detected in control fibroblasts upon PBZ and TBZ stimulation, but not in the patient SV40-fibroblasts, suggesting that the mutants disrupt RIPK3 mediated signaling at endogenous levels. To further confirm our hypothesis, we decided to rescue this phenotype by transiently transfecting RIPK3 WT plasmid into the patient's SV40-fibroblasts. Upon transfection of the RIPK3 WT plasmid, levels of MLKL phosphorylation were detectable after stimulation with both PBZ and TBZ stimulation (Figures 12C and 12D) indicating that the abrogated expression and function of the *RIPK3* mutants carried by the patient were indeed accountable for the impaired

RIPK3-mediated signaling observed in the patient cells. Interestingly, levels of full-length RIPK3 protein expression in transfected patient cells decreased after stimulation, probably because of the cleavage that follows the activation of the protein. Taken together, these results suggest that the compound heterozygous *RIPK3* mutations of the proband indeed lead to autosomal recessive RIPK3 deficiency, at least in SV40-fibroblasts.

Since other HSE patients carry mutations in the TLR3 responsive pathway, and RIPK3 can signal downstream TLR3, we contemplated the possibility that the inborn errors of the TLR3 pathway previously described in other HSE patients could present with disrupted RIPK3-mediated signaling. To test this hypothesis, we interrogated dermal fibroblasts from patients with autosomal recessive (AR) complete TLR3, TRIF, and UNC93B deficiency and autosomal dominant (AD) partial TBK1 deficiency in terms of MLKL phosphorylation upon TBZ and PBZ stimulation. Consistent with the literature, TLR3-, TRIF- and UNC93B-deficient fibroblasts were unable to phosphorylate MLKL via TLR3 (figure 12D) but were able to phosphorylate MLKL via TNFR1 (figure 12E), showing a selective impairment on its ability to induced necroptosis, restricted to TLR3 but otherwise normal. In contrast, fibroblasts from a HSE patient with AD TBK1 deficiency were able to phosphorylate MLKL normally via both TLR3 and TNFR1 (Figures 12D and E). These data suggest that either TBK1 is, at least to some extent, redundant in the mediation of necroptosis in fibroblasts, or that the partial defect of the mutant allele us not sufficient to impair the activation of RIPK3 and consequent phosphorylation of MLKL.

Overall, our dataset indicates that, in addition to the decreased TLR3-mediated production of interferons and activation of the NF-κB pathway, patients with mutations in the TLR3 pathway upstream RIPK3 (i.e. UNC93B, TLR3, and TRIF) also present with an abrogated signaling in another branch of the TLR3 circuit that is mediated by RIPK3, which might account, at least to some extent, for the HSE phenotype observed in those patients.

Figure 12

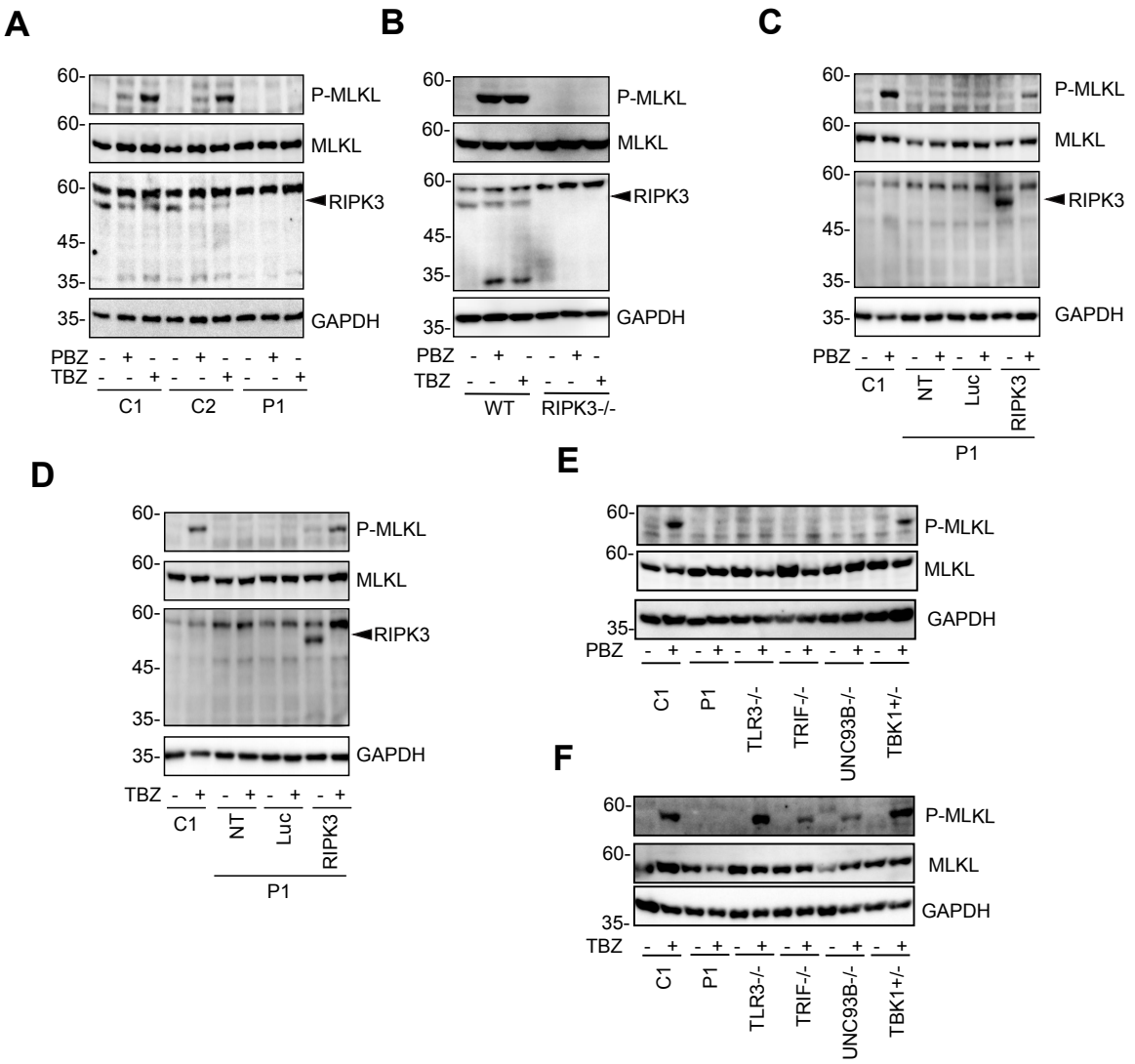

Figure 12. RIPK3-dependent signaling is broadly impaired in P1 and selectively impaired in other HSE patients via TLR3. (A) SV40-fibroblasts from two healthy controls and P1 were stimulated with either PBZ or TBZ. (B) HT29 WT and HT29 RIPK3-/- cells were stimulated with either PBZ or TBZ. (C) Complementation of MLKL phosphorylation via TLR3 in the patient SV40-fibroblasts. SV40-fibroblasts from a control and P1 either left untransfected or transiently transfected with a mock vector (Luc) or RIPK3 WT, were stimulated with PBZ. (D) Complementation of MLKL phosphorylation via TNFR1 in the patient SV40-fibroblasts. SV40-fibroblasts from a control and P1, either left untransfected or transiently transfected with a mock vector (Luc) or RIPK3 WT, were stimulated with TBZ. (A-D) Cell lysates were prepared and Western blotting was performed with antibodies against p-MLKL, total MLKL, RIPK3 and GAPDH. (E) MLKL phosphorylation via TLR3 in HSE patients with mutations in the TLR3 responsive pathway. SV40-fibroblasts from a control, P1, and HSE patients with AR TLR3, TRIF, and UNC93B deficiency and AD TBK1 deficiency were stimulated with PBZ. (E) MLKL phosphorylation via TNFR1 in HSE patients with mutations in the TLR3 responsive pathway. SV40-fibroblasts from a control, P1, and HSE patients with AR TLR3, TRIF, and UNC93B deficiency and AD TBK1 deficiency were stimulated with TBZ. (E-F) Cell lysates were prepared and Western blotting was performed with antibodies against p-MLKL, total MLKL, and GAPDH. (A-F) Western blotting results are representative of at least 3 independent experiments.

3.6 Human RIPK3-deficient fibroblasts show normal levels of cell viability and interferon production.

We next tested whether the impaired RIPK3-mediated signaling that leads to a lack of MLKL phosphorylation would result in decreased necroptotic signaling. We stimulated SV40-fibroblasts from controls, the patient, and patients with AR TLR3 and TRIF deficiency with combinations of Poly(I:C), TNF-α, BV6 and z-VAD and analyzed the viability of these cells. Although the cell viability was decreased in controls and patient cells upon stimulation with Poly(I:C) or TNF-α in combination with BV6, which is considered a marker of apoptotic cell death, PBZ and TBZ stimulations barely decrease the cell viability in these cells (Figures 13A and 13B). These results are consistent with previous reports that indicate that the levels of expression of RIPK3 in human fibroblasts are not sufficient to cross the threshold of MLKL phosphorylation that would lead to necroptotic cell death (Omoto et al., 2015).

Last, since other patients with mutations in the TLR3 responsive pathway usually show an impaired or abolished TLR3-dependent production of interferons and other cytokines, we tested whether RIPK3-deficient fibroblasts could produce interferons and other cytokines via TLR3. Interestingly, TLR3-mediated production of both interferon-λ and interferon-β as well as the production of IL-6 was normal in the patient fibroblasts (figures 13C-E), indicating that RIPK3 is redundant, at least in fibroblasts, for the TLR3-mediated production of interferons and other cytokines.

Figure 13

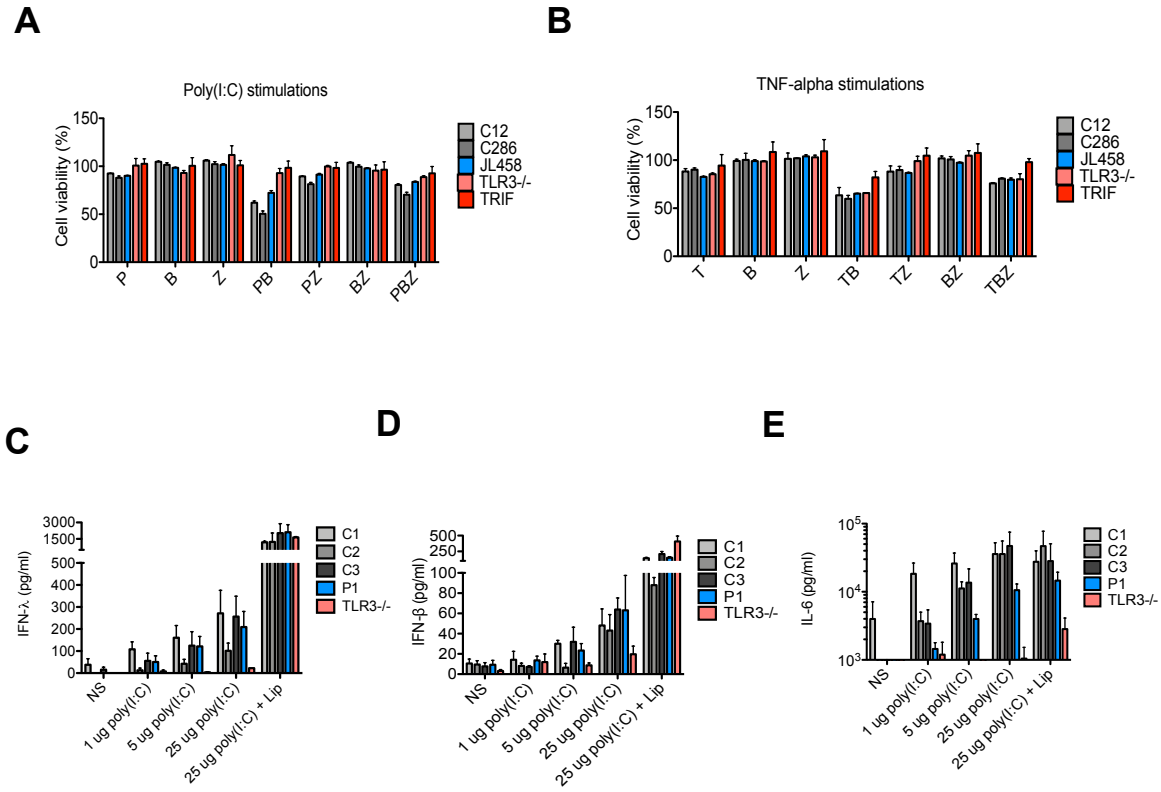

Figure 13. Human RIPK3-deficient fibroblasts show normal levels of cell viability and TLR3-mediated interferon production. (A) Susceptibility of SV40-fibroblasts to TLR3-mediated cell death. SV40-fibroblasts from three different controls, P1, and HSE patients with AR TLR3, and TRIF deficiency were stimulated with combinations of Poly(I:C), IAP antagonist/SMAC mimetic, BV6 (*S*; 5M), and/or caspase inhibitor, Z-VAD-fmk (*V*; 25M), alone or in the combinations shown. Viability was determined by measuring intracellular ATP as percentage of vehicle (0.1% DMSO) of each genotype. (B) Susceptibility of SV40-fibroblasts to TNFR1-mediated cell death. SV40-fibroblasts from three different controls, P1, and HSE patients with AR TLR3, and TRIF deficiency were stimulated with combinations of TNF-α, IAP antagonist/SMAC mimetic, BV6 (*S*; 5M), and/or caspase inhibitor, Z-VAD-fmk (*V*; 25M), alone or in the combinations shown. Viability was determined by measuring intracellular ATP as percentage of vehicle (0.1% DMSO) of each genotype. **(C)** SV40-fibroblasts from three healthy controls, P1 and a TLR3-/- HSE patient were stimulated with different doses of poly(I:C). Poly(I:C) in combination with lipofectamine served as positive control for TLR3-independent engagement of poly(I:C). Supernatants were collected 24h after stimulation and IFN-λ ELISA was performed. (D) SV40-fibroblasts from three healthy controls, P1 and a TLR3-/- HSE patient were stimulated with different doses of poly(I:C). Poly(I:C) in combination with lipofectamine served as positive control for TLR3-independent cytokine production. Supernatants were collected 24h after stimulation and IFN-β ELISA was performed. (E) SV40-fibroblasts from three healthy controls, P1 and a TLR3-/- HSE patient were stimulated with different doses of poly(I:C). Poly(I:C) in combination with lipofectamine served as positive control for TLR3-independent cytokine production. Supernatants were collected 24h after stimulation and IL-6 ELISA was performed. (A-B) Mean values from two independent experiments with biological duplicates. Error bars represent SEM. (C-E) Mean values from three independent experiments with biological duplicates. Error bars represent SEM.

4. DISCUSSION

We report here the first case of human RIPK3 deficiency. This study provides experimental evidence of a causal relationship of a human *RIPK3* genotype and a recessive phenotype of herpes simplex encephalitis.

The mutant alleles carried by the patient are rare in the general population and predicted to be deleterious *in silico.* Our results on plasmid overexpression of the mutant constructs showed that the variants are indeed deleterious. At the expression level, both mutants codify aberrant proteins of a smaller molecular size, with P493fs9* showing lower levels of expression upon retrotransduction. At the functional level, the R422* mutant showed a lack of activity in all the assays tested. These results are consistent with the literature, as R422*, but not P493fs9*, lacks the RHIM domain, which is required for the binding of upstream proteins (through RHIM-RHIM interactions) and consequently, ligand-dependent activation of RIPK3 through both auto- and transphosphorylation. We also confirmed that autosomal recessive RIPK3 deficiency at the population level is compatible with the incidence of the disease, as none of the common variants found in public databases seem to affect the expression or the function of RIPK3.

RIPK3 protein expression was abolished in the patient in all the cell types tested. Probably due to the combinatory loss of R422* mRNA and P493fs9* protein. As such, RIPK3 signaling was impaired in the patient SV40-fibroblasts in both a TNFR1- and TLR3-dependent manner. These phenotypes can be rescued by RIPK3 overexpression, proving that the proband is indeed RIPK3-deficient. Remarkably, we also showed for the first time that other patients with mutations in the TLR3 pathway (TLR3, TRIF, and UNC-93B) share an impaired RIPK3-mediated signaling, but in contrast to what we observed in our proband, this phenotype is only observed upon TLR3 stimulation but not TNFR1 stimulation. These results combined, highlight the TLR3-RIPK3 axis

as the only pathway that is impaired in all these patients. Interestingly, TBK1+/- fibroblasts from a previously described HSE patient are able to signal through RIPK3 in a TLR3-dependent manner. Autosomal dominant TBK1 deficiency was described in two HSE patients and was originally thought to underlie this condition because of its involvement in the TLR3-IFN axis. However, a recent study using cells from these patients has shown that TBK1 is needed for early autophagy upon HSV-1 infection, which contrasts with other molecules of the TLR3 pathway such as TRIF, which was used in that study as a control (Ahmad et al., 2018).

These new studies challenge the idea that mutations in the TLR3 pathway underlie HSE due to an impaired production of interferons. Our new model proposes that both IFNs and TLR3-RIPK3 signaling are non-redundant mechanisms in humans to control HSV-1 in the brain; however, it might not be the direct activation of interferons by TLR3 what makes them essential (Figure 14). That would explain why TBK1+/- cells from HSE patients show a pattern of cellular phenotypes that differ from those observed in the rest of the patients.

This shift in the paradigm that used to explain the molecular pathogenesis of HSE opens the door to new lines of research. RIPK3 crucial's role in necroptosis suggests that necroptosis, similar to apoptosis, could be a non-redundant type of cell death in humans *in natura*. Necroptosis has been shown to restrict viral infection in several cell and mice models, and multiple viruses, including HSV-1, have been shown to both induce and target RIPK3 activity. However, to the date, there is no proof of principle that necroptosis is, as a biological mechanism, essential in humans. Although it is tempting to speculate whether necroptosis act as a non-redundant mechanism of antiviral immunity in the human forebrain, we remain cautious in our claims, as we do not have evidence that it is the dysregulation of the necroptotic signaling rather than another branch of the RIPK3 cascade what is predisposing to HSE. Future studies in other patients (e.g., a patient with

mutations in MLKL or other RIPK3-associated molecules) may help elucidate the pathogenesis of the disease.

Admittedly, with only one patient, we cannot draw firm conclusions about the spectrum of clinical manifestations that can be caused by other pathogens that were not encountered by our proband (such as CMV, ZIKV, or WNV). However, the report of this patient has tremendous medical implications. Although mice models of Ripk3 deficiency leading to viral encephalitis have been described (Daniels et al., 2017; Daniels et al., 2019), this *experiment of nature* provides the first evidence that human RIPK3 is protective from viral encephalitis *in vivo*. Recent studies have suggested the use of RIPK3 inhibitors for the treatment of ischemia-reperfusion injury or heart failure (Zhang et al., 2016). Our findings suggest that caution is required when considering this approach for disease treatment.

Lastly, it is still unclear whether the mutations in RIPK3 are also responsible for the episode of post-herpetic autoimmune encephalitis. Some groups have connected RIPK3 to the NMDA receptor through the phosphorylation of CaMKII by RIPK3 (Wang et al., 2014; Bayer et al., 2001; Zhang et al., 2016), which in turn, phosphorylates one of the subunits of this receptor, regulating its conformation . This is an intriguing question that deserves to be addressed in future research.

Figure 14

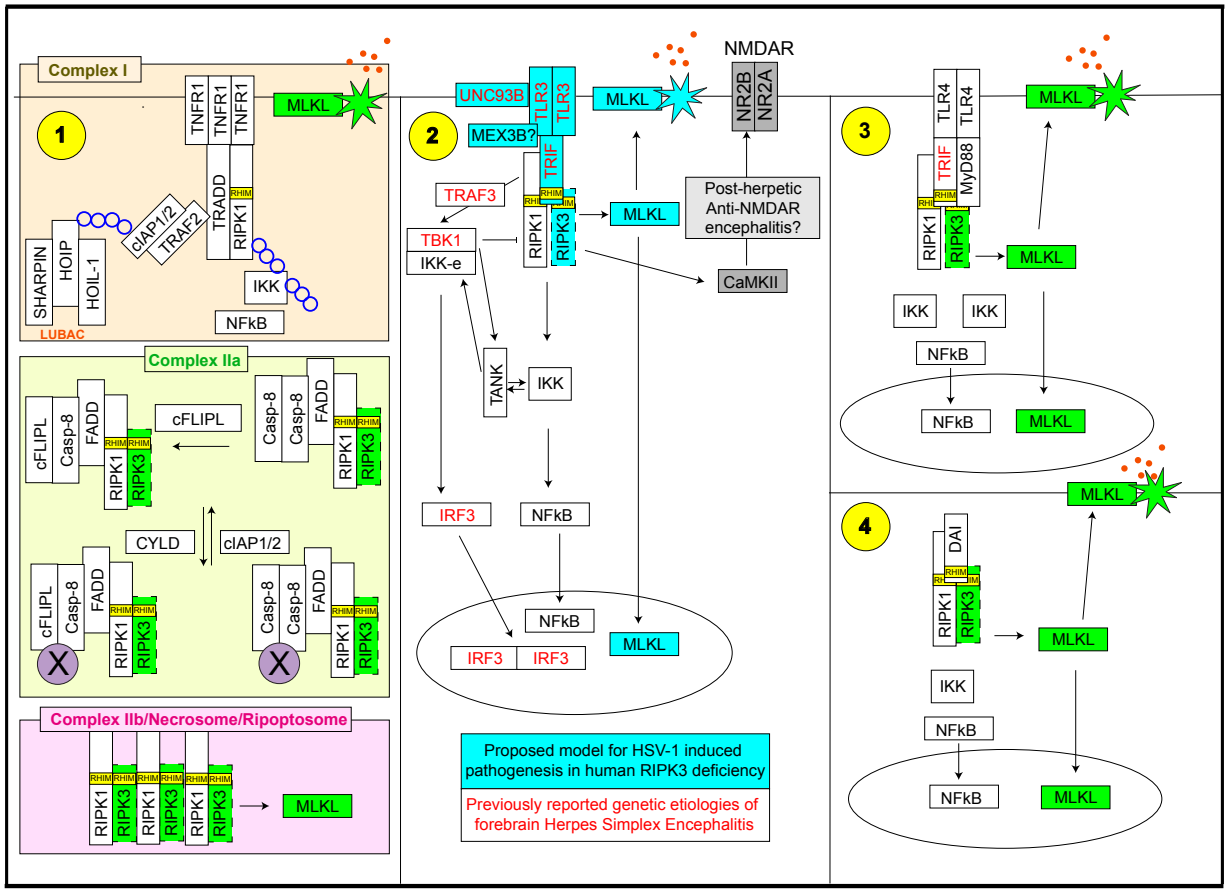

Figure 14. RIPK3 signaling pathways and proposed role for its implication in the pathogenesis of HSE.

5. REFERENCES

1. Abel, Laurent, et al. "Age-dependent Mendelian predisposition to herpes simplex virus type 1 encephalitis in childhood." The Journal of pediatrics 157.4 (2010): 623-629.

2. Adler AC, Kadimi S, Apaloo C, Marcu C. Herpes simplex en- cephalitis with two false-negative cerebrospinal fluid PCR tests and review of negative PCR results in the clinical setting. Case Rep Neurol. 2011;3:172–8.

3. Ahmad, Liyana, et al. "Human TANK-binding kinase 1 is required for early autophagy induction upon herpes simplex virus 1 infection." *Journal of Allergy and Clinical Immunology* 143.2 (2019): 765-769.

4. Andersen, L. L. et al. Functional IRF3 deficiency in a patient with herpes simplex encephalitis. J Exp Med 212, 1371-1379, doi:10.1084/jem.20142274 (2015).

5. Armangue T, Moris G, Cantarín- Extremera V, et al. Autoimmune post- herpes simplex encephalitis of adults and teenagers. Neurology 2015;85:1736- 43.

6. Aurelius E, Andersson B, Forsgren M, Skoldenberg B, Strannegard O. Cytokines and other markers of intrathecal immune response in patients with herpes simplex encephalitis. J Infect Dis. 1994;170:678–81.

7. Aurelius E, Johansson B, Skoldenberg B, Staland A, Forsgren M. Rapid diagnosis of herpes simplex encephalitis by nested poly- merase chain reaction assay of cerebrospinal fluid. Lancet. 1991;337:189–92.

8. Avitzur, Y., and J. Amir. "Herpetic whitlow infection in a general pediatrician-an occupational hazard." Infection 30.4 (2002): 234-236.

9. Baringer JR, Pisani P. Herpes simplex virus genomes in human nervous system tissue analyzed by polymerase chain reaction. Ann Neurol. 1994;36:823–9.

10. Bayer, K-Ulrich, et al. "Interaction with the NMDA receptor locks CaMKII in an active conformation." *Nature* 411.6839 (2001): 801-805.

11. Bennett Jr, William E., and Phillip I. Tarr. "Enteric infections and diagnostic testing." Current opinion in gastroenterology 25.1 (2009): 1-7.

12. Bhullar SS, Chandak NH, Baheti NN, et al. Diagnosis of herpes simplex encephalitis by ELISA using antipeptide antibodies against type-common epitopes of glycoprotein B of herpes sim- plex viruses. J Immunoass Immunochem. 2016;37:217–27.

13. Binnicker MJ, Espy MJ, Irish CL. Rapid and direct detection of herpes simplex virus in cerebrospinal fluid by use of a commercial real-time PCR assay. J Clin Microbiol. 2014;52:4361–2.

14. Burnet FM, Williams SW. 1939. Herpes simplex: new point of view. Med. J. Aust. 1:637–40

15. Cai Z, Jitkaew S, Zhao J, Chiang HC, Choksi S, et al. 2014. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat. Cell Biol. 16:55–65

16. Carr, Daniel JJ, Peter Härle, and Bryan M. Gebhardt. "The immune response to ocular herpes simplex virus type 1 infection." Experimental biology and medicine 226.5 (2001): 353-366

17. Casanova JL, Abel L. 2007. Primary immunodeficiencies: a field in its infancy. Science 317:617–19

18. Casanova JL, Abel L, Quintana-Murci L. 2011. Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. Annu. Rev. Immunol. 29:447–91

19. Casanova, Jean-Laurent, and Laurent Abel. "The genetic theory of infectious diseases: a brief history and selected illustrations." Annual review of genomics and human genetics 14 (2013): 215-243.

20. Casanova, Jean-Laurent. "Human genetic basis of interindividual variability in the course of infection." Proceedings of the National Academy of Sciences 112.51 (2015): E7118-E7127

21. Casanova, Jean-Laurent. "Severe infectious diseases of childhood as monogenic inborn errors of immunity." Proceedings of the National Academy of Sciences 112.51 (2015): E7128-E7137.

22. Casrouge, A. et al. Herpes simplex virus encephalitis in human UNC-93B deficiency. Science 314, 308-312 (2006).

23. Centers for Disease Control (CDC). "Herpes gladiatorum at a high school wrestling camp--Minnesota." MMWR. Morbidity and mortality weekly report 39.5 (1990): 69.

24. Chen W, Zhou Z, Li L, Zhong CQ, Zheng X, et al. 2013. Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. J. Biol. Chem. 288:16247–61

25. Cheung PC, Nebreda AR, Cohen P. 2004. TAB3, a new binding partner of the protein kinase TAK1. Biochem. J. 378:27–34

26. Cho, YoungSik, et al. "Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation." Cell 137.6 (2009): 1112-1123

27. Chow FC, Glaser CA, Sheriff H, et al. Use of clinical and neuro- imaging characteristics to distinguish temporal lobe herpes sim- plex encephalitis from its mimics. Clin Infect Dis. 2015;60:1377– 83.

28. Clark, Dwayne C. "Common acute hand infections." American family physician 68.11 (2003): 2167-2176.

29. Corey L, Wald A, Celum CL, Quinn TC. The Effects of Herpes Simplex Virus-2 on HIV-1 Acquisition and Transmission: A Review of Two Overlapping Epidemics. J Acquir Immune Defic Syndr 2004; 35: 435–45.

30. Coyle PV, O'Neill HJ, Wyatt DE, McCaughey C, Quah S, McBride MO. Emergence of herpes simplex type 1 as the main cause of recurrent genital ulcerative disease in women in Northern Ireland. J Clin Virol 2003; 27: 22–29.

31. Cushing H. 1905. Surgical aspects of major neuralgia of trigeminal nerve: report of 20 cases of operation upon the Gasserian ganglion with anatomic and physiologic notes on the consequences of its removal. JAMA 44:773–79, 860–65, 920–29, 1002–8

32. Dalmau J, Geis C, Graus F. Autoantibodies to synaptic receptors and neuronal cell surface proteins in autoimmune dis- eases of the central nervous system. Physiol Rev 2017;97:839-87.

33. Dalmau, Josep, and Francesc Graus. "Antibody-mediated encephalitis." New England Journal of Medicine 378.9 (2018): 840-851.

34. Daniels, B. P. et al. RIPK3 Restricts Viral Pathogenesis via Cell Death-Independent Neuroinflammation. Cell 169, 301-313 e311, doi:10.1016/j.cell.2017.03.011 (2017).

35. Daniels, B. P. et al. The Nucleotide Sensor ZBP1 and Kinase RIPK3 Induce the Enzyme IRG1 to Promote an Antiviral Metabolic State in Neurons. Immunity 50, 64-76 e64, doi:10.1016/j.immuni.2018.11.017 (2019).

36. Davis R, Jeffery K, Atkins BL. Hypoglycorrhachia in herpes sim- plex encephalitis. Clin Infect Dis. 2004;38:1506–7.

37. De Tiege X, Heron B, Lebon P, Ponsot G, Rozenberg F. Limits of early diagnosis of herpes simplex encephalitis in children: a retrospective study of 38 cases. Clin Infect Dis 2003;36:1335-9.

38. Declercq, Wim, Tom Vanden Berghe, and Peter Vandenabeele. "RIP kinases at the crossroads of cell death and survival." Cell 138.2 (2009): 229-232

39. Degterev A, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol. 2005;1:112–19.

40. Degterev, Alexei, et al. "Identification of RIP1 kinase as a specific cellular target of necrostatins." Nature chemical biology 4.5 (2008): 313

41. Denes E, Labach C, Durox H, et al. Intrathecal synthesis of specific antibodies as a marker of herpes simplex encephalitis in patients with negative PCR. Swiss Med Wkly. 2010;140: w13107.

42. Dickens LS, Boyd RS, Jukes-Jones R, Hughes MA, Robinson GL, et al. 2012. A death effector domain chain DISC model reveals a crucial role for caspase-8 chain assembly in mediating apoptotic cell death. Mol. Cell 47:291–305

43. Domingues RB, Tsanaclis AM, Pannuti CS, Mayo MS, Lakeman FD. Evaluation of the range of clinical presentations of herpes simplex encephalitis by using polymerase chain reaction assay of cerebrospinal fluid samples. Clin Infect Dis. 1997;25:86–91.

44. Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, et al. 2003. Impaired response to interferon-α/β and lethal viral disease in human STAT1 deficiency. Nat. Genet. 33:388–91

45. Elbers JM, Bitnun A, Richardson SE, Ford-Jones EL, Tellier R, Wald RM, et al. A 12-year prospective study of childhood herpes simplex encephalitis: is there a broader spectrum of disease? Pediatrics 2007;119:e399- 407.

46. Ermolaeva MA, Michallet MC, Papadopoulou N, Utermohlen O, Kranidioti K, et al. 2008. Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat. Immunol. 9:1037–46

47. Fuller AO, Santos RE, Spear PG. 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J. Virol. 63:3435–43

48. Gross SP. 2003. Dynactin: coordinating motors with opposite inclinations. Curr. Biol. 13:R320–22

49. Farooq, Asim V., and Deepak Shukla. "Herpes simplex epithelial and stromal keratitis: an epidemiologic update." Survey of ophthalmology 57.5 (2012): 448-462.

50. Feierbach B, Bisher M, Goodhouse J, Enquist LW. 2007. In vitro analysis of transneuronal spread of an alphaherpesvirus infection in peripheral nervous system neurons. J. Virol. 81:6846–57

51. Fleming DT, McQuillan GM, Johnson RE, Nahmias AJ, Aral SO, et al. 1997. Herpes simplex virus type 2 in the United States, 1976–1994. N. Engl. J. Med. 337:1105–11

52. Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. AIDS 2006; 20: 73–83.

53. Galvis AE, Fisher HE, Fan H, Camerini D (2017) Conformational changes in the 5′ end of the HIV-1 genome dependent on the debranching enzyme DBR1 during early stages of infection. J Virol. https://doi.org/10.1128/JVI.01377-17

54. Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, et al. 2011. Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471:591–96

55. Gnann, J. W., Jr. & Whitley, R. J. Herpes Simplex Encephalitis: an Update. Current infectious disease reports 19, 13, doi:10.1007/s11908-017-0568-7 (2017).

56. Guo, Y. et al. Herpes simplex virus encephalitis in a patient with complete TLR3 deficiency: TLR3 is otherwise redundant in protective immunity. J Exp Med 208, 2083-2098, doi:jem.20101568

57. Guo, H., et al. Herpes simplex virus suppresses necroptosis in human cells. Cell Host Microbe 17, 243-251 (2015).

58. Gupta, Rachna, Terri Warren, and Anna Wald. "Genital herpes." The Lancet 370.9605 (2007): 2127-2137.

59. Han B, Park HK, Ching T, Panneerselvam J, Wang H, Shen Y, Zhang J, Li L, Che R, Garmire L, Fei P (2017) Human DBR1 modulates the recycling of snRNPs to afect alternative RNA splicing and contributes to the suppression of cancer development. Oncogene. https://doi.org/10.1038/onc.2017.150

60. Hacohen Y, Deiva K, Pettingill P, et al. N-methyl-D-aspartate receptor antibodies in post-herpes simplex virus encephalitis neurological relapse. Mov Disord 2014;29: 90-6.

61. He, Sudan, et al. "Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-α." Cell 137.6 (2009): 1100-1111

62. He S, Liang Y, Shao F, Wang X. 2011. Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. PNAS 108:20054–59

63. Herman, M. et al. Heterozygous TBK1 mutations impair TLR3 immunity and underlie herpes simplex encephalitis of childhood. J Exp Med, doi:jem.20111316 [pii]
64. 10.1084/jem.20111316.

65. Hjalmarsson A, Blomqvist P, Skoldenberg B. Herpes simplex encephalitis in Sweden, 1990-2001: incidence, morbidity, and mortality. Clin In- fect Dis 2007;45:875-80.

66. Hill TJ, Field HJ. 1973. The interaction of herpes simplex virus with cultures of peripheral nervous tissue: an electron microscopic study. J. Gen. Virol. 21:123–33

67. Hill, T. J., H. J. Field, and A. P. C. Roome. "Intra-axonal location of herpes simplex virus particles." Journal of General Virology 15.3 (1972): 253-255

68. Hsieh WB, Chiu NC, Hu KC, Ho CS, Huang FY. Outcome of herpes simplex encephalitis in children. J Microbiol Immunol Infect 2007;40:34-8.

69. Holler, Nils, et al. "Fas triggers an alternative, caspase-8–independent cell death pathway using the kinase RIP as effector molecule." Nature immunology 1.6 (2000): 489-495

70. Hook LM, Lubinski JM, Jiang M, Pangburn MK, Friedman HM. 2006. Herpes simplex virus type 1 and 2 glycoprotein C prevents complement-mediated neutralization induced by natural immunoglobulin M antibody. J. Virol. 80(8):4038–46

71. Huang, Z., et al. RIP1/RIP3 binding to HSV-1 ICP6 initiates necroptosis to restrict virus propagation in mice. Cell Host Microbe 17, 229-242 (2015).

72. Hughes PS, Jackson AC. Delays in initiation of acyclovir therapy in herpes simplex encephalitis. Can J Neurol Sci. 2012;39:644–8.

73. Ikeda F, Deribe YL, Skanland SS, Stieglitz B, Grabbe C, et al. 2011. SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. Nature 471:637–41

74. Jarrin I, Sellier P, Lopes A, et al. Etiologies and management of aseptic meningitis in patients admitted to an internal medicine department. Medicine. 2016;95, e2372.

75. Jennische E, Eriksson CE, Lange S, Trybala E, Bergstrom T. The anterior commissure is a pathway for contralateral spread of her- pes simplex virus type 1 after olfactory tract infection. J Neurovirol. 2015;21:129–47.

76. Jouan Y, Grammatico-Guillon L, Espitalier F, Cazals X, Francois P, Guillon A. Long-term outcome of severe herpes simplex encephalitis: a population-based observational study. Crit Care. 2015;19:345.

77. Jouanguy, Emmanuelle, et al. "Interferon-γ–receptor deficiency in an infant with fatal bacille Calmette–Guérin infection." New England Journal of Medicine 335.26 (1996): 1956-1962.

78. Kaiser WJ, Offermann MK. 2005. Apoptosis induced by the Toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. J. Immunol. 174:4942–52

79. Kaiser, William J., et al. "Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL." Journal of Biological Chemistry 288.43 (2013): 31268-31279

80. Kataoka H, Inoue M, Shinkai T, Ueno S. Early dynamic SPECT imaging in acute viral encephalitis. J Neuroimaging. 2007;17: 304–10.

81. Kato, S., et al. "Herpes simplex esophagitis in the immunocompetent host." Diseases of the Esophagus 18.5 (2005): 340-344.

82. Kaye, Stephen, and Anshoo Choudhary. "Herpes simplex keratitis." Progress in retinal and eye research 25.4 (2006): 355-380.

83. Kim YS, Jung KH, Lee ST, et al. Prognostic value of initial standard EEG and MRI in patients with herpes simplex encephalitis. J Clin Neurol. 2016.

84. Kimberlin DW, Lakeman FD, Arvin AM, et al. Application of the polymerase chain reaction to the diagnosis and management of neonatal herpes simplex virus disease. National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group. J Infect Dis. 1996;174:1162–7.

85. Kiss AM (2004) Human box H/ACA pseudouridylation guide RNA machinery. Mol Cell Biol 24:11

86. Koelle DM, Abbo H, Peck A, Ziegweld K, Corey L. Direct recovery of herpes simplex virus (HSV)—specific T lymphocyte clones from recurrent genital HSV-2 lesions. J Infect Dis 1994; 169: 956–61.

87. Feigin, Ralph D., and James D. Cherry. Textbook of pediatric infectious diseases. No. 616.9-053.2. WB Saunders, 1998.

88. Kolokotronis, A., and S. Doumas. "Herpes simplex virus infection, with particular reference to the progression and complications of primary herpetic gingivostomatitis." Clinical microbiology and infection 12.3 (2006): 202-211.

89. Ku, Cheng-Lung, et al. "Selective predisposition to bacterial infections in IRAK-4–deficient children: IRAK-4–dependent TLRs are otherwise redundant in protective immunity." The Journal of experimental medicine 204.10 (2007): 2407-2422

90. Lafaille, F. G. et al. Impaired intrinsic immunity to HSV-1 in human iPSC-derived TLR3-deficient CNS cells. Nature, doi:nature11583 [pii] 10.1038/nature11583.

91. Lafaille, F et al., Human SNORA31 variations impair cortical neuron-intrinsic immunity to HSV-1 and underlie herpes simplex encephalitis. Nat Med 25, 1873–1884 (2019).

92. Lakeman FD, Whitley RJ. Diagnosis of herpes simplex encepha- litis: application of polymerase chain reaction to cerebrospinal fluid from brain-biopsied patients and correlation with disease. National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group. J Infect Dis. 1995;171: 857–63.

93. Lewis, M. A. O. "Herpes simplex virus: an occupational hazard in dentistry." International dental journal 54.2 (2004): 103-111.

94. Li, Ming, and Amer A. Beg. "Induction of necrotic-like cell death by tumor necrosis factor alpha and caspase inhibitors: novel mechanism for killing virus-infected cells." Journal of virology 74.16 (2000): 7470-7477.

95. Likness, Lincoln P. "Common dermatologic infections in athletes and return-to-play guidelines." The Journal of the American Osteopathic Association 111.6 (2011): 373-379.

96. Lim, H. K. et al. TLR3 deficiency in herpes simplex encephalitis: high allelic heterogeneity and recurrence risk. Neurology 83, 1888-1897, doi:10.1212/WNL.0000000000000999 (2014).

97. Linnoila JJ, Binnicker MJ, Majed M, Klein CJ, McKeon A. CSF herpes virus and autoantibody profiles in the evaluation of encephalitis. Neurol Neuroimmunol Neuroinflamm 2016;3(4):e245.

98. Lo YC, Lin SC, Rospigliosi CC, Conze DB, Wu CJ, et al. 2009. Structural basis for recognition of diubiquitins by NEMO. Mol. Cell 33:602–15

99. Lopez Roa P, Alonso R, de Egea V, Usubillaga R, Munoz P, Bouza E. PCR for detection of herpes simplex virus in cerebrospinal fluid: alternative acceptance criteria for diagnostic workup. J Clin Microbiol. 2013;51:2880–3.

100. Mahoney DJ, Cheung HH, Mrad RL, Plenchette S, Simard C, et al. 2008. Both cIAP1 and cIAP2 regulate TNFα-mediated NF-κB activation. PNAS 105:11778–83

101. Mailles A, Stahl JP. Infectious encephalitis in France in 2007: a national prospective study. Clin Infect Dis 2009;49:1838-47.

102. Majkut J, Sgobba M, Holohan C, Crawford N, Logan AE, et al. 2014. Differential affinity of FLIP and procaspase 8 for FADD's DED binding surfaces regulates DISC assembly. Nat. Commun. 5:3350

103. Manavi K, McMillan A, Ogilvie M. Herpes simplex virus type 1 remains the principal cause of initial anogenital herpes in Edinburgh, Scotland. Sex Transm Dis 2004; 31: 322–24.

104. Maraite N, Mataigne F, Pieri V, Dang T, Diederich NJ. Early decompressive hemicraniectomy in fulminant herpes simplex encephalitis. Bull Soc Sci Med Grand Duche Luxemb. 2010:279–82.

105. Mark KE, Wald A, Magaret AS, Selke S, Olin L, et al. 2008. Rapidly cleared episodes of herpes simplex virus reactivation in immunocompetent adults. J. Infect. Dis. 198(8):1141–49

106. McBane, Robert D., and John B. Gross. "Herpes esophagitis: clinical syndrome, endoscopic appearance, and diagnosis in 23 patients." Gastrointestinal endoscopy 37.6 (1991): 600-603.

107. McCabe K, Tyler K, Tanabe J. Diffusion-weighted MRI abnor- malities as a clue to the diagnosis of herpes simplex encephalitis. Neurology. 2003;61:1015–6.

108. McGrathN, Anderson NE,CroxsonMC,PowellKF.Herpessimplexen- cephalitis treated with acyclovir: diagnosis and long-term outcome. J Neurol Neurosurg Psychiatry 1997;63:321-6.

109. Mekan SF, Wasay M, Khelaeni B, Saeed Z, Hassan A, Sheerani M. Herpes simplex encephalitis: analysis of 68 cases from a ter- tiary care hospital in Karachi, Pakistan. JPMA J Pak Med Assoc. 2005;55:146–8.

110. Menasria R, Canivet C, Piret J, Boivin G. Infiltration pattern of blood monocytes into the central nervous system during experimen- tal herpes simplex virus encephalitis. PLoS One. 2015;10, e0145773.

111. Meyding-Lamade UK, Oberlinner C, Rau PR, et al. Experimental herpes simplex virus encephalitis: a combination therapy of acyclovir and glucocorticoids reduces long-term magnetic resonance imaging abnormalities. J Neurovirol. 2003;9:118–25.

112. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, et al. 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-κB activation. Nat. Immunol. 5:503–7

113. Meyts, Isabelle, et al. "Exome and genome sequencing for inborn errors of immunity." Journal of Allergy and Clinical Immunology 138.4 (2016): 957-969.

114. Micheau O, Tschopp J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–90

115. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, et al. 2013. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. Immunity 39:443–53

116. Murray JL, Sheng J, Rubin DH (2014) A role for H/ACA and C/D small nucleolar RNAs in viral replication. Mol Biotechnol 56:429–437. https://doi.org/10.1007/s12033-013-9730-0

117. Newport MJ, Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, et al. 1996. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N. Engl. J. Med. 335:1941–49

118. Newton K, Dixit VM. 2012. Signaling in innate immunity and inflammation. Cold Spring Harb. Perspect. Biol. 4:a006049

119. Newton, K. RIPK1 and RIPK3: critical regulators of inflammation and cell death. Trends Cell Biol 25, 347-353, doi:10.1016/j.tcb.2015.01.001 (2015).

120. Newton, K., & Manning, G. (2016). Necroptosis and inflammation. Annual review of biochemistry, 85, 743-763.

121. Nogusa, S. et al. RIPK3 Activates Parallel Pathways of MLKL-Driven Necroptosis and FADD-Mediated Apoptosis to Protect against Influenza A Virus. Cell Host Microbe 20, 13-24, doi:10.1016/j.chom.2016.05.011 (2016).

122. Nosadini M, Mohammad SS, Corazza F, et al. Herpes simplex virus-induced anti-N-methyl-D-aspartate receptor encephalitis: a systematic literature review with analysis of 43 cases. Dev Med Child Neurol 2017;59:796-805.

123. Omoto, S., et al. Suppression of RIP3-dependent necroptosis by human cytomegalovirus. *Journal of biological chemistry* **290**, 11635-11648 (2015).

124. Opstelten, Wim, Arie Knuistingh Neven, and Just Eekhof. "Treatment and prevention of herpes labialis." Canadian Family Physician 54.12 (2008): 1683-1687.

125. Orth G. 2008. Host defenses against human papillomaviruses: lessons from epidermodysplasia verruciformis. Curr. Top. Microbiol. Immunol. 321:59–83

126. Perez de Diego, R. et al. Human TRAF3 Adaptor Molecule Deficiency Leads to Impaired Toll-like Receptor 3 Response and Susceptibility to Herpes Simplex Encephalitis. Immunity 33, 400-411, doi:S1074-7613(10)00319-5 [pii]

127. Picard C, Puel A, Bonnet M, Ku CL, Bustamante J, et al. 2003. Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science 299:2076–79

128. Picard C, von Bernuth H, Ghandil P, Chrabieh M, Levy O, et al. 2010. Clinical features and outcome of patients with IRAK-4 and MyD88 deficiency. Medicine 89:403–25

129. Picard, Capucine, Jean-Laurent Casanova, and Laurent Abel. "Mendelian traits that confer predisposition or resistance to specific infections in humans." Current opinion in immunology 18.4 (2006): 383-390

130. Pobezinskaya YL, Kim YS, Choksi S, Morgan MJ, Li T, et al. 2008. The function of TRADD in signal- ing through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. Nat. Immunol. 9:1047–54

131. Prichard MN, Kern ER, Hartline CB, Lanier ER, Quenelle DC. CMX001 potentiates the efficacy of acyclovir in herpes simplex virus infections. Antimicrob Agents Chemother. 2011;55:4728– 34.

132. Puel, A. et al. The NEMO Mutation Creating the Most-Upstream Premature Stop Codon Is Hypomorphic Because of a Reinitiation of Translation. Am J Hum Genet 78, 691-701 (2006).

133. Ragan, Katherine B., and Jason W. Upton. "Host response: Neurons loosen the gRIP of death." Nature microbiology 2.7 (2017): 1-2

134. RahighiS,IkedaF,KawasakiM,AkutsuM,SuzukiN,etal.2009.Specificrecognitionoflin earubiquitin chains by NEMO is important for NF-κB activation. Cell 136:1098–109

135. Rahimi, Hanieh, et al. "Effectiveness of antiviral agents for the prevention of recurrent herpes labialis: a systematic review and meta-analysis." Oral surgery, oral medicine, oral pathology and oral radiology 113.5 (2012): 618-627.

136. Ramos-Estebanez C, Lizarraga KJ, Merenda A. A systematic re- view on the role of adjunctive corticosteroids in herpes simplex virus encephalitis: is timing critical for safety and efficacy? Antivir Ther. 2014;19:133–9.

137. RaschilasF,WolffM,DelatourF,ChaffautC,DeBrouckerT,ChevretS, et al. Outcome of and prognostic factors for herpes simplex encephalitis in adult patients: results of a multicenter study. Clin Infect Dis 2002;35: 254-60.

138. Rawal G, Yadav S, Wani UR, Ambastha AK. HSV encephalitis with normal CSF—a case report with review of literature. J Clin Diagn Res. 2015;9:Od06–7.

139. Razavi B, Razavi M. Herpes simplex encephalitis—an atypical case. Infection. 2001;29:357–8.

140. Riera-Mestre A, Gubieras L, Martinez-Yelamos S, Cabellos C, Fernandez-Viladrich P. Adult herpes simplex encephalitis: fifteen years' experience. Enferm Infecc Microbiol Clin. 2009;27:143–7.

141. Roberts CM, Pfister JR, Spear SJ. Increasing proportion of herpes simplex virus type 1 as a cause of genital herpes infection in college students. Sex Transm Dis 2003; 30: 797–800.

142. Roizman B, Knipe D, Whitley R. 2006. Herpes simplex virus. In Fields Virology, ed. D Knipe, P Howley, D Griffin, R Lamb, M Martin, S Straus, pp. 2501–601. Philadelphia: Lippincott-Williams & Wilkins. 5th ed.

143. Sancho-Shimizu, V. et al. Herpes simplex encephalitis in children with autosomal recessive and dominant TRIF deficiency. J Clin Invest 121, 4889-4902, doi:59259

144. Schleich K, Warnken U, Fricker N, Ozturk S, Richter P, et al. 2012. Stoichiometry of the CD95 death- inducing signaling complex: experimental and modeling evidence for a death effector domain chain model. Mol. Cell 47:306–19

145. Scoular A, Norrie J, Gillespie G, Mir N, Carman WF. Longitudinal study of genital infection by herpes simplex virus type 1 in Western Scotland over 15 years. BMJ 2002; 324: 1366–67.

146. Sellner J, Trinka E. Seizures and epilepsy in herpes simplex virus encephalitis: current concepts and future directions of pathogenesis and management. J Neurol. 2012;259:2019–30.

147. Singh TD, Fugate JE, Hocker S, Wijdicks EF, Aksamit Jr AJ, Rabinstein AA. Predictors of outcome in HSV encephalitis. J Neurol. 2016;263:277–89.

148. Stahl JP, Mailles A, De Broucker T. Herpes simplex encephalitis and management of acyclovir in encephalitis patients in France. Epidemiol Infect. 2012;140:372–81.

149. Steiner I, Budka H, Chaudhuri A, et al. Viral meningoencephalitis: a review of diagnostic methods and guidelines for management. Eur J Neurol. 2010;17:999–e57.

150. Skoldenberg B, Forsgren M, Alestig K, et al. Acyclovir versus vidarabine in herpes simplex encephalitis. Randomised multicentre study in consecutive Swedish patients. Lancet. 1984;2:707–11.

151. Stoopler, Eric T., and Thomas P. Sollecito. "Oral mucosal diseases: evaluation and management." Medical Clinics 98.6 (2014): 1323-1352.

152. Studahl M, Bergstrom T, Hagberg L. Acute viral encephalitis in adults—a prospective study. Scand J Infect Dis. 1998;30:215–20.

153. Su L, Quade B, Wang H, Sun L, Wang X, Rizo J. 2014. A plug release mechanism for membrane permeation by MLKL. Structure 22:1489–500

154. Sun, Xiaoqing, et al. "RIP3, a novel apoptosis-inducing kinase." Journal of Biological Chemistry 274.24 (1999): 16871-16875

155. Sun X, Yin J, Starovasnik MA, Fairbrother WJ, Dixit VM. 2002. Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J. Biol. Chem. 277:9505–11

156. Sun, L., et al. Mixed Lineage Kinase Domain-like Protein Mediates Necrosis Signaling Downstream of RIP3 Kinase. Cell 148, 213-227 (2012).

157. Takeno, M., et al. "Herpes esophagitis presenting upper gastrointestinal bleeding: report of a case." Nihon Shokakibyo Gakkai zasshi= The Japanese journal of gastro-enterology 99.8 (2002): 935.

158. Taylor SW, Smith RM, Pari G, Wobeser W, Rossiter JP, Jackson AC. Herpes simplex encephalitis. Can J Neurol Sci. 2005;32:246– 7.

159. Thompson KA, Blessing WW, Wesselingh SL. Herpes simplex replication and dissemination is not increased by corticosteroid treatment in a rat model of focal Herpes encephalitis. J Neurovirol. 2000;6:25–32.

160. Townend BS, Hanson JA, Sturm JW, Whyte S. Stroke or encephalitis? Emerg Med Australas. 2005;17:401–4.

161. Tokunaga F, Nakagawa T, Nakahara M, Saeki Y, Taniguchi M, et al. 2011. SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex. Nature 471:633–36

162. Tunkel AR, Glaser CA, Bloch KC, et al. The management of encephalitis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis. 2008;47:303–27.

163. Tyler KL. Update on herpes simplex encephalitis. Rev Neurol Dis. 2004;1:169–78.

164. Upton, Jason W., William J. Kaiser, and Edward S. Mocarski. "Virus inhibition of RIP3-dependent necrosis." Cell host & microbe 7.4 (2010): 302-313

165. Upton, Jason W., William J. Kaiser, and Edward S. Mocarski. "DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA." Cell host & microbe 11.3 (2012): 290-297

166. Upton, J. W., Shubina, M. & Balachandran, S. RIPK3-driven cell death during virus infections. Immunol Rev 277, 90-101, doi:10.1111/imr.12539 (2017).

167. Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, et al. 2008. c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor α (TNFα)-induced NF-κB activation. J. Biol. Chem. 283:24295–99

168. Vercammen, Dominique, et al. "Tumour necrosis factor-induced necrosis versus anti-Fas-induced apoptosis in L929 cells." Cytokine 9.11 (1997): 801-80

169. von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, et al. 2008. Pyogenic bacterial infections in humans with MyD88 deficiency. Science 321:691–96

170. Wang JP, Bowen GN, Zhou S, et al. Role of specific innate immune responses in herpes simplex virus infection of the central nervous system. J Virol. 2012;86:2273–81.

171. Wang H, Sun L, Su L, Rizo J, Liu L, et al. 2014. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. Mol. Cell 54:133–46

172. Wang, Ning, et al. "Active calcium/calmodulin-dependent protein kinase II (CaMKII) regulates NMDA receptor mediated postischemic long-term potentiation (i-LTP) by promoting the interaction between CaMKII and NMDA receptors in ischemia." *Neural plasticity* 2014 (2014).

173. Weil AA, Glaser CA, Amad Z, Forghani B. Patients with suspected herpes simplex encephalitis: rethinking an initial nega- tive polymerase chain reaction result. Clin Infect Dis. 2002;34: 1154–7.

174. Whitley R, Lakeman AD, Nahmias A, Roizman B. Dna restriction-enzyme analysis of herpes simplex virus isolates obtained from patients with encephalitis. N Engl J Med. 1982;307: 1060–2.

175. Whitley RJ, Alford CA, Hirsch MS, et al. Vidarabine versus acy- clovir therapy in herpes simplex encephalitis. N Engl J Med. 1986;314:144–9.

176. Whitley RJ, Gnann JW. Viral encephalitis: familiar infections and emerging pathogens. Lancet 2002;359:507-13.

177. Whitley RJ, Kimberlin DW. Herpes simplex encephalitis: children and adolescents. Semin Pediatr Infect Dis 2005;16:17-23.

178. Whitley RJ, Roizman B. 2009. Herpes simplex viruses. In Clinical Virology, ed. RJ Whitley, D Richman, F Hayden, pp. 409–36. Washington, DC: ASM Publ. 3rd ed.

179. Wu, Ines B., and Robert A. Schwartz. "Herpetic whitlow." Cutis 79.3 (2007): 193-6.

180. Wu J, Huang Z, Ren J, Zhang Z, He P, et al. 2013. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res. 23:994–1006

181. Wu XN, Yang ZH, Wang XK, Zhang Y, Wan H, et al. 2014. Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. Cell Death Differ. 21:1709–20

182. Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, Lee FK, et al. 2006. Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. JAMA 296(8):964–73

183. Yu, Pei Wen, et al. "Identification of RIP3, a RIP-like kinase that activates apoptosis and NFκB." Current biology 9.10 (1999): 539-S3

184. Zhang, Duan-Wu, et al. "RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis." Science 325.5938 (2009): 332-336

185. Zhang, S. Y. et al. TLR3 deficiency in patients with herpes simplex encephalitis. Science 317, 1522-1527 (2007).

186. Zhang, S. Y. et al. Inborn Errors of RNA Lariat Metabolism in Humans with Brainstem Viral Infection. Cell 172, 952-965 e918, doi:10.1016/j.cell.2018.02.019 (2018).

187. Zhang, T., et al. CaMKII is a RIP3 substrate mediating ischemia-and oxidative stress–induced myocardial necroptosis. *Nature medicine* **22**, 175 (2016).

188. Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, et al. 2012. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. PNAS 109:5322–27

189. B. Zimmer, et al., Human iPSC-derived trigeminal neurons lack constitutive TLR3-dependent immunity that protects cortical neurons from HSV-1 infection. Proc. Natl. Acad. Sci. U.S.A. 115, E8775–E8782 (2018