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The discovery of the PYRIN domain's role in IFI16-TLR4 interaction unveils a new category of endogenous inflammatory molecules

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Contents

1. Summary

The interferon inducible protein IFI16 is associated to a wide range of biological functions, such as cell cycle regulation, tumor suppression, apoptosis, DNA damage signaling, virus sensing, and virus restriction. In addition, a number of inflammatory diseases have been associated with abnormal IFI16 expression and release in the extracellular space. The current hypothesis is that overexpression of the IFI16 protein occurs in tissue compartments where it is not physiologically expressed during inflammation. As a result, IFI16 is released into the extracellular space, where it may function as a damage-associated molecular pattern (DAMP) that communicates with the Toll-like receptor 4 (TLR4) to initiate inflammation either on its own or in response to interactions with external molecules, including bacterial lipopolysaccharide (LPS).However, the precise molecular mechanisms underlying the extracellular function of IFI16 as DAMP remain to be elucidated. Here, human macrophages were used as target to define the proinflammatory activity of a panel of IFI16 recombinant domains or truncated proteins, along with the recombinant domains of other PYHIN family members. Moreover, surface plasmon resonance (SPR) experiments, 3D structure prediction and site-directed mutagenesis were used to identify the IFI16 moiety responsible for TLR4 binding and signaling. Collectively, our data provide compelling evidence that the proinflammatory activity of the IFI16 protein specifically lies within its N-terminal region and that the IFI16-PYRIN domain is involved in TLR4/MD2 binding and activation. Moreover, we expand previous knowledge by showing that the proinflammatory activity of the PYRIN domain is conserved among the PYHIN family members,and that specific amino acids located within the IFI16-PYRIN domain, and conserved across the family, are critically involved in their TLR4-mediated proinflammatory activity. These data strengthen the notion that specific PYRIN domains, including the one in the IFI16 N-terminus, behave as DAMP, and can be envisaged as targets of new drug candidates to be exploited for dampening the inflammatory response in different pathological settings.

2. Introduction

2.1 Innate immunity and the role of interferon

Maintaining homeostasis upon changes in the external and internal environments is a critical challenge for living organisms. Controlling variations in nutrition and water supply, physical stress, temperature changes, physiological stress, infections, and cancers are among them. The varieties of life and biological systems that can best deal with these obstacles have been chosen during billions of years of evolution. The removal of microorganisms and aberrant or damaged cellular material is a difficulty that all organisms undergo (Paludan et al., 2021). In addressing these challenges, the mechanisms for recognizing microbial structures play a crucial role.

These mechanisms can be divided into two broad categories. First, there are hard-wired responses encoded by genes in the host's germ line, which comprehend pattern recognition receptors (PRRs) that bind unique pathogen-associated molecular patterns (PAMPs) shared by many microbes as well as toxins that are not present in the mammalian host. Included in PRRs are Toll-like receptors (TLRs), RIG-I-like receptors (RIG-Is), nucleotide-binding domain and leucine-rich repeat- containing receptors (NLRs), cyclic GMP-AMP (cGAMP) synthase, AIM2-like receptors (ALRs), and stimulator of interferon genes (STING) (Taguchi and Mukai, 2019). Secondly, there are responses encoded by gene elements that somatically rearrange to assemble antigen-binding molecules with exquisite specificity for individual unique for each microbe, constituting innate immune response. Because the innate system's recognition molecules are widely expressed on a vast number of cells, this system is ready to respond quickly once an invading pathogen or toxin is met, and so comprises the primary host response (Hillion et al., 2020). The second set of responses constitutes the adaptive immune response (not treated in this elaborate) (Chaplin, 2010).In the innate immunity, when PRRs are activated, they initiate intracellular signaling cascades that result in the transcriptional production of proinflammatory cytokines, antiviral proteins and interferons (IFNs), that all work cooperatively to eliminate pathogens and infected cells. Concerning Interferons, these polypeptides, when released by infected cells, play three distinctive roles. They first generate cell-intrinsic antimicrobial states in infected and adjacent cells, limiting the transmission of infectious agents, notably viral infections. Second, they regulate innate immune responses by promoting antigen presentation and natural killer cell capabilities while suppressing pro-inflammatory pathways and cytokine generation (Ivashkiv and Donlin, 2014). Third, they stimulate the adaptive immune system, increasing the formation of antigen-specific T and B cell responses as well as immunological memory [(Katze et al., 2002) ;(Toshchakov et al., 2002)]. The IFN family consists of two main types of related cytokines: type I IFNs and type II IFNs. There are several type I IFNs all of which have significant structural similarity. These include IFN-α (which may be further classified into 13 distinct subtypes), IFN-β, IFN-δ, IFN-ε,IFN-κ, IFN-τ and IFN-ω1,2,3. In contrast, IFN-γ is the sole type II IFN. IFN-γ is a distinct cytokine from type I IFNs, but it was first categorized as an IFN because of its capacity to 'interfere' with viral infections, which is compatible with the original description of IFNs (Platanias, 2005). Type I and II IFNs bind to different cell-surface receptors found on the majority of cell types and promote gene expression via pathways involving the protein tyrosine kinases JAKs and STATs (signal transducers and activators of transcription) (Figure 1).The binding of type I IFNs to their heterodimeric receptor IFNAR activates the receptor- associated protein tyrosine kinases JAK1 and TYK2, leading to activation of STAT1 and STAT2 and their interaction with the IFN transcription factor IRF9, resulting in the formation of the heterotrimeric complex IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to interferon- sensitive response elements (ISREs) in DNA (with the consensus sequence TTTCNNTTTC) andactivates ISGs, including genes producing antiviral proteins like Mx1 and OAS, as well as

transcription factors like interferon-regulatory factors (IRFs). In the other hand, type II IFNs (i.e. IFN-γ) binds to its receptor called interferon gamma receptor 1 and 2(IFNGR1 and IFNGR2), it activates JAK1 and JAK2, as well as STAT1 homodimers. STAT1 binds to a specific DNA region known as a gamma-activated site (GAS; consensus sequence TTCNNNGGA) and directly activates a subset of ISGs, including chemokines like CXCL10 and transcription factors like IRFs. Given their unique basic signaling pathways type I and type II IFN signatures should be easily identifiable, revealing which IFNs are driving gene expression and, by extension, disease etiology. (Barrat et al., 2019). According to research anomaly-expressed IFN-Is and/or type I IFN-inducible gene signatures have been connected to pathophysiology, clinical symptoms, and disease activity in individuals with autoimmune disease (Jiang et al., 2020).

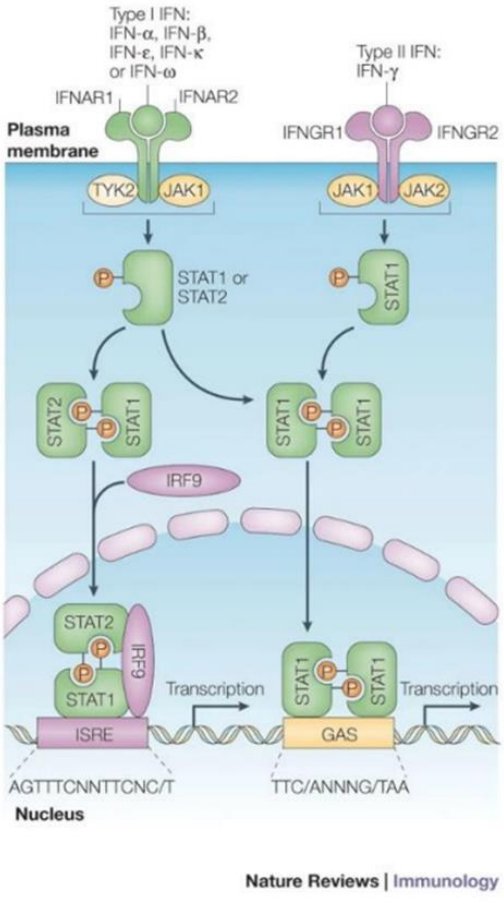


Figure 1. Signal transduction pathways ofinterferons (IFNs) on target cells. Distinct types of interferons bind to distinct receptors. Type II interferon receptors are tetramers, whereas types I are dimers [Adapted from Platanias 2005]

2.2 Toll-like receptors: a focus on the TLR4/MD2 pathway

The understanding of the ways the innate immune system uses to identify microbial components and its crucial function in the host's defense against infection has advanced quickly during the last ten years. The discovery of the Toll-like receptors (TLRs) in the mid-1990s contradicted the early theory that the innate immune system recognized microbes in an indiscriminate manner. Instead, it became clear that the innate immune system functions selectively, depending on germline- encoded pattern-recognition receptors (PRRs), including TLRs, which have evolved to identify pathogen-associated molecular patterns (PAMPs). In detail, TLRs are type I transmembrane proteins containing ectodomains with leucine-rich repeats—that facilitate PAMPs recognition— transmembrane domains, and an intracellular Toll- interleukin 1 (IL-1) receptor domain (TIR) that serves as a platform for the recruitment of downstream signaling molecules (Balka and De Nardo, 2019). Humans and mice contain 10 and 12 functional TLRs, respectively, with TLR1-TLR9 being preserved in both species. Following the identification of the TIR domain-containing adaptor protein MyD88, TLR signaling pathways were thoroughly investigated. The discovery of further TIR domain-containing adaptors later on has demonstrated that different TLRs attract different adaptor molecules, eliciting immune responses that are specifically shaped on the microbe causing the infection. Toll-like receptor 4 (TLR4), in particular, was discovered as the long-sought receptor that reacts to bacterial lipopolysaccharide (LPS), a component of gram-negative bacteria's outer membrane that may induce septic shock (Kawai and Akira, 2010).Since then, various studies have identified a large number of other TLR4 agonist, both pathogen-derived as well as endogenous, such as HSP60, beta-amyloid, α-synuclein, fibrinogen, HMGB1, opioids and IFI16 [(Tian et al., 2013); (Apetoh et al., 2007); (Zhang et al., 2020); (Hughes et al., 2020); (Leavy, 2013); (Iannucci et al., 2020); (Long et al., 2022)]. Considering LPS as the paradigmatic ligand of TLR4, the pathway shows multistep process involving different proteins (Figure 2). Human TLR4 alone, however, is not capable of sensing the presence of LPS (Akashi et al., 2000). The lipopolysaccharide-binding protein (LBP) was the first recognized actor in the recognition process [(Schumann et al., 1990); (Tobias et al., 1986)]. Extracellular LBP makes direct connections with the bacterial outer membrane (or LPS micelles) and changes the outer membrane, allowing the protein CD14 to extract a single molecule of LPS (Gioannini et al., 2004). CD14 is a free soluble extracellular protein or a glycosylphosphatidylinositol (GPI)-anchored protein found in plasma membrane nanodomains rich in cholesterol and sphingolipids known as rafts, which are thought to be TLR4 activation sites. TLR4-TLR4 dimerization happens at this stage as CD14 transfers LPS to Myeloid differentiation factor 2 (MD2), a key mediating protein (Akashi et al., 2000). Furthermore, contact with the lipid A moiety of LPS causes structural modifications in MD2, generating hydrophilic connections between MD2 and TLR4, further stabilizing the newly formed complex. Indeed, a crystallographic analysis of the human TLR4/MD-2 complex with LPS bound revealed that five of the six acyl chains of LPS are buried in the hydrophobic pocket of MD-2, while the sixth interacts with TLR4 of another TLR4/MD-2 complex, and that dimerization of the TLR4/MD-2 complexes is strengthened by ionic bonds between the phosphate group of lipid A and the neighboring TLR4 molecule (Ciesielska et al., 2021). Upon TLR4-TLR4 dimerization, TIR domain containing adaptor protein (TIRAP) detects the TLR4 cytosolic Toll/IL-1R (TIR) domain of TRL4 C-term (Horng et al., 2001). TIRAP is a phosphoinositide-binding protein which contains a TIR domain and leads to the assembly of a supramolecular organizing center named myddosome (Kagan et al., 2014). TIRAP functions as a sorting adaptor that recruits MyD88 to TLR4 through its ability to interact with

phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), a component of the cellular membrane (Kagan and Medzhitov, 2006). From a molecular point of view, the TIR domain of TIRAP interacts with the TIR domain of TLR4 forming a homodimer interaction (Valkov et al., 2011). This interaction is facilitated by tyrosine phosphorylation of TIRAP by several tyrosine kinases, including Bruton's tyrosine kinase (BTK) (Rajpoot et al., 2021). The homo- and heterotypic TIR–TIR domain interactions play a pivotal role in the assembly of the TLR signalosomes and in initiation of the signaling pathway, in fact they are central also in the binding between TIRAP and MyD88 (Ohnishi et al., 2009). Specifically, the adapter protein myeloid differentiation primary response protein 88 (MyD88) is a modular protein. Along with a TIR domain, it contains a death domain (DD) by which it recruits the IRAKs to the receptor complex via homotypic DD interactions. Activation of the IRAKs involves auto- and cross- phosphorylation, followed by K63-ubiquitination mediated by Pellino-3 (Gay et al., 2011). At this stage, the complex formed by TIRAP, MyD88, and multiple IL-1 receptor-associated kinases (IRAKs) are the proteins is called myddosome. The assembled myddosome recruits E3 ubiquitin ligase TRAF6, which triggers a signaling cascade involving TAK1 kinase and leading, through the phosphorylation and activation, among different protein, IκB kinases α/β (IKKα/β), to nuclear translocation of the NF-κB transcription factor. In addition, downstream of TRAF6 and TAK1, MAP kinases are phosphorylated to activate transcription factors AP-1 and CREB which lead to the expression of pro-inflammatory cytokine (such as TNF and IL6) and anti-inflammatory (such as IL10) (Ciesielska et al., 2021). The MyD88-dependent signaling described until now, is followed by internalization of TLR4. During endocytosis, PtdIns(4,5)P2 concentrations on the invaginating membrane drop precipitously, thereby releasing the TIRAP-MyD88 complex from the invaginating membrane, which will ultimately become an early endosome. Concurrently, CD14 facilitates the internalization of LPS-TLR4/MD2 from plasma membranes into endosomes (Zanoni et al., 2011). This pathway is entirely subject to CD14 and MD2 (Tan et al., 2015) and is not dependent on TLR4-TIR domains, even though CD14 may increase LPS endocytosis in the absence of TLR4 (Dunzendorfer et al., 2004). Thus, the Loss of the TIRAP-MyD88 complex allows the TRAM-TRIF complex to engage the TIR domain of TLR4 on early endosomes and induce the second phase of signaling from an intracellular location. Now, the so called Triffosome is formed and TLR4 is found in the endosome, with the adaptor molecules translocating chain- associated membrane protein (TRAM) and TIR-domain-containing adapter-inducing interferon (TRIF) (Yamamoto et al., 2003). By analogy with TIRAP, TRAM may also function as a sorting adaptor to recruit TRIF to TLR4. TRAM is targeted to the plasma membrane by myristylation, and the present model of TRAM activation is that it recruits TRIF to the plasma membrane, where TLR4 is located. Moreover, TRAM contains a bipartite sorting signal that controls its trafficking between the plasma membrane and endosomes. The formed triffosome recruits TRAF3, which catalyzes its own K63-linked polyubiquitination. This leads to the activation of the TBK1 and the non-canonical IKK, IKKε, which in turn phosphorylates IRF3 (Dhillon et al., 2019). Finally, this lead to the expression of genes encoding type I interferon and CCL5/RANTES. In summary, TLR4 triggered by LPS promotes two sequential signaling pathways depending on receptor redistribution: the MyD88-dependent signaling is induced by TLR4 localized to the plasma membrane, whereas the TRIF-dependent signaling is triggered by TLR4 internalized in endosomes. The two pathways result in coordinated synthesis of pro- and anti-inflammatory mediators, contribute to NLRP3 inflammasome activation, control cell metabolism, trigger adaptive immunological responses, and other cell type-specific reactions.

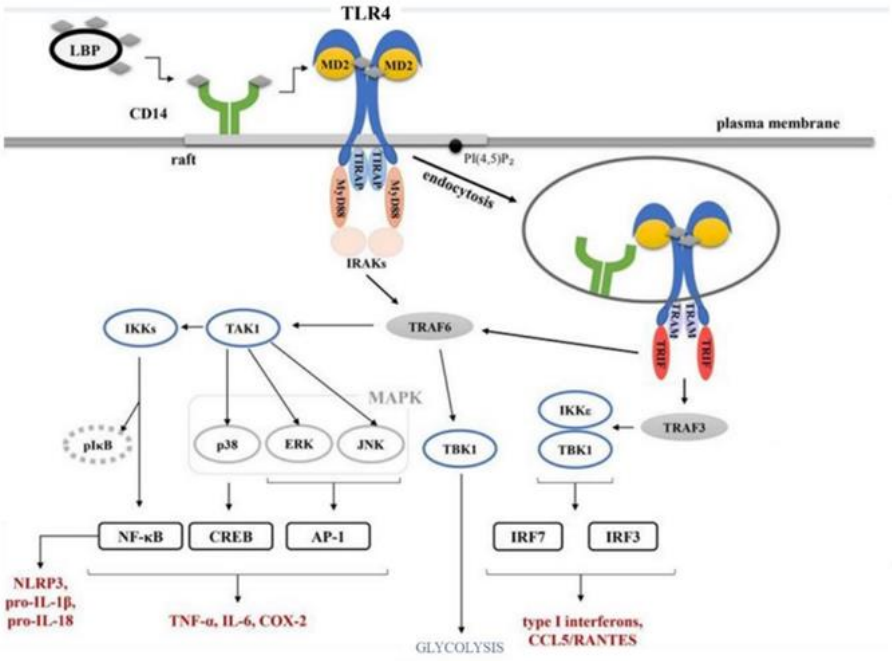


Figure 2. Paradigmatic LPS-induced TLR4 signaling pathway that promote inflammation. TLR4 stimulates the MyD88-signaling pathway at the plasma membrane, triggering the TRIF-dependent cascade after CD14-dependent endocytosis. TLR4 also contributes to the activation of the cytosolic NLRP3 inflammasome via activated NF-B. [Adapted from Ciesielska et al., 2021].

2.3 The HIN200/PYHIN gene family

Interferons, previous described, have the property to regulate around 10% of the genes in the human genome (Schoggins, 2019), and most of them are indeed classified as IFN-stimulated genes. The HIN200/PYHIN (PYHIN hereinafter) gene family comprises most of them. Since the discovery of the first murine PYHIN gene in 1982 (Ifi202, coding for the p202 protein) in chromosome 1) (Samanta et al., 1982), a family of at least 13 genes in mice and four in humans have been cloned and described (Ludlow et al., 2005). The human PYHIN family members are: IFN inducible protein 16 (IFI16), myeloid cell nuclear differentiation antigen (MNDA), Absent in melanoma 2 (AIM2), and interferon-inducible protein X (IFIX); sometimes the PYRIN domain only protein 3 (POP3) has been also considered as a variant of the PYHIN family, which resulted from a HIN domain deletion (Khare et al., 2014) (Figure 3). In mouse, the most characterized proteins are p202, p203, p204, p205, p206, p 207 , Aim2/p210, and Mndal, plus several predicted proteins [(Fan et al., 2022); (Cridland et al., 2012); (Landolfo et al., 1998); (Schattgen and Fitzgerald, 2011)]. PYHIN family is historically also named HIN200 because of the presence of either one or two consecutive 200-amino acid DNA binding HIN (for hematopoietic expression, interferon-inducible nature, and

nuclear localization) domains (Ludlow et al., 2005). Although the overall structure of HIN domains is highly conserved, their superposition reveals significant flexibility in the loops in oligonucleotide/oligosaccharide binding (OB) folds [(Shaw and Liu, 2014); (Theobald et al., 2003)]. These HIN domains exhibit different surface charges, indicating distinct DNA-binding surfaces (Fan et al., 2022). Furthermore, the structural superposition of the HIN dsDNA complex reveals two distinctly different DNA-binding modes in the PYHIN family. To bind to dsDNA, AIM2 HIN, IFI16 HINB, p204 HINA, and HINB adopt the linker joining two OB folds and the surrounding residues, whereas IFI16 HINA and p202 HINA use an opposing surface created by the loops of two OB folds that exhibited as well as a greater affinity for single- stranded DNA (ssDNA), the possibility to wrap, stretch, and form oligomers with ssDNA [(Fan et al., 2022); (Yan et al., 2008)]. With the exception of murine p202, the N-terminus of the PYHIN proteins contains a PYRIN domain. PYRIN has been identified in more than 20 human proteins with putative functions in apoptotic and inflammatory signaling pathways and it belongs to the Death Domain Fold (DDF) superfamily [(Liepinsh et al., 2003); (Liu et al., 2003); (Steward et al., 2009); (Bürckstümmer et al., 2009)]. DDF is a highly conserved protein interaction domain spanning approximately 90 amino acid residues (Stehlik, 2007) consisting of six anti-parallel alpha helices arranged in a Greek key structure (Steward et al., 2009). PYRIN is implicated in homo-oligomerization interaction (Yi, 2017); (Lum et al., 2019) and it is frequently involved in inflammation and immune responses (Piao et al., 2019). Especially, the engagement of PYRIN during stress stimuli leads to the assembly of an inflammasome, and the subsequent activation of caspase-1 and release of IL-1β and IL-18. An important step in this process is the recruitment of ASC to PYRIN. Via its N-terminal PYRIN domain, ASC enters a PYRIN-PYRIN homotypic interaction with PYRIN, which induces its oligomerization of micrometer-sized assemblies (Schnappauf et al., 2019). The PYHIN proteins have been demonstrated to localize to the nucleus and the cytoplasm. IFI16, IFIX, MNDA and p204 contain either a monopartite or bipartite nuclear localization sequence (NLS) and are predominantly found in the nucleus (Ludlow et al., 2005). Following activation, these proteins can be delocalized from the nucleus to the cytoplasm. AIM2 and p202, on the other hand, lack a NLS and are mostly, if not only, found in the cytoplasm. Because of their tissue-specific inducibility by IFN treatment, the PYHIN proteins have been linked to the regulation of growth and cell differentiation. The IFI16, p202, and p204 nuclear phosphoproteins are rather well defined in terms of their function in IFN action: these proteins have been shown to contribute in cell cycle progression suppression, differentiation regulation, and cell survival. In general, PYHIN proteins are assumed to operate as scaffolds for the assembly of large protein complexes involved in transcriptional control (Ludlow et al., 2005). Finally, due to their ability to act as a new class of pattern recognition receptors (PRRs), some PYHIN proteins, including AIM2, IFI16 and p204, have been recently grouped as AIM2-like receptors (ALRs). PRRs act as sensors, detecting signals of infection or tissue damage in the extracellular and intracellular compartments. These PRRs, include the Toll-like receptors (TLRs), the retinoic acid inducible gene-like receptors (RLRs), the nucleotide oligomerization domain-like receptors (NLRs), and the AIM2-like receptors (ALRs) [(Kawai and Akira, 2009); (Unterholzner et al., 2010); (Gray et al., 2016); (Caneparo et al., 2018)].

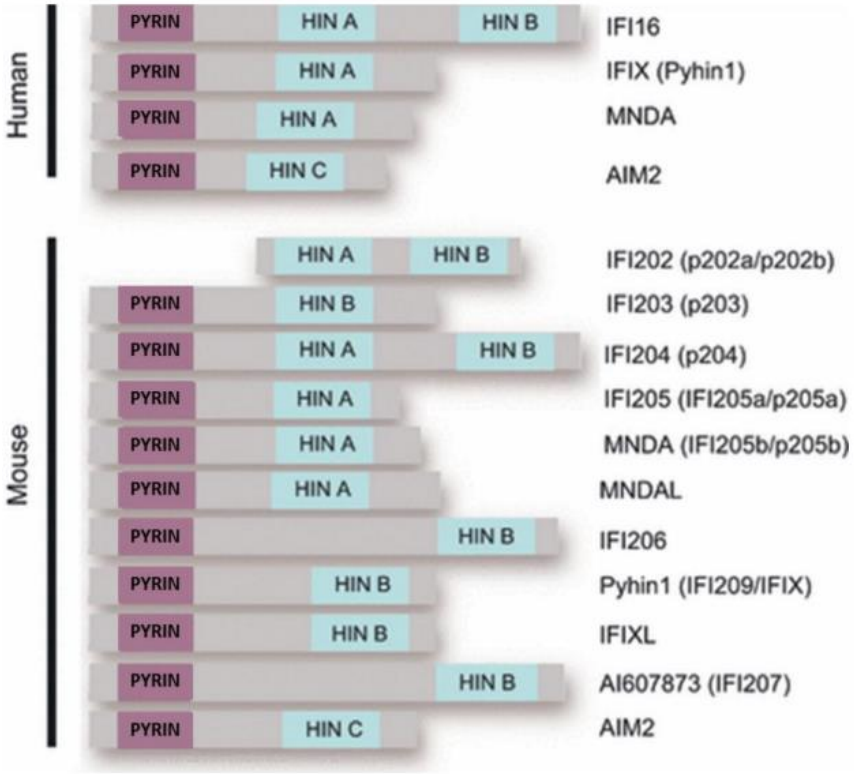


Figure 3. The human and murine PYHIN protein families. The PYHIN proteins consist of an N-terminal PYRIN domain and one or more HIN-200 domains which can be one of 3 subtypes (HINA, HINB, or HINC) based on their sequence [adapted form Schattgen and Fitzgerald, 2011].

2.4 The human interferon-inducible protein 16

The human interferon-inducible protein 16 (IFI16) was discovered in 1994, coded by a gene that was constitutively expressed in human lymphoid cell lines and inducible in myeloid cell lines following IFN-γ treatment or differentiation stimuli (Trapani et al., 1994). IFI16, like the other members of the PYHIN family, has a PYRIN domain at its N-terminus, which is well-known for promoting homotypic protein interactions (Figure 4). The presence of the PYRIN was originally associated with a role of the protein in apoptosis, since it may regulate the activity of specific nuclear transcription factors that are involved in cell death commitment. Characteristically, IFI16 also contains the HINA and HINB domains, which are separated by a 116 amino-

acid serine- threonine-proline (S/T/P) rich spacer (Figure 4). The length of this spacer is controlled by alternative mRNA splicing, which results in three IFI16 isoforms called A, B, and C, respectively. In human fibroblasts, epithelial cells, keratinocytes, macrophages, and T cells, the B isoform is the most prevalent (Jakobsen and Paludan, 2014). IFI16 is expressed also in other cells, such as in CD34+ myeloid precursor cells and is seen in high concentrations in monocyte progenitors, peripheral blood monocytes, and throughout lymphoid development (Dawson et al., 1998). Furthermore, immunohistochemical analysis of normal human tissues indicated that IFI16 is expressed in a highly restricted pattern in selected cells within certain organs [(Gariglio et al., 2002); (Wei et al., 2003)]. Indeed, IFI16 has been found in epithelial cells of the skin, gastrointestinal system, urogenital tract, and breast glands and ducts. Its expression was shown to be prominent in stratified squamous epithelia, especially in basal cells in proliferative compartments, although it gradually diminishes in a more differentiated supra-basal compartment. Connective tissue staining was limited to scattered fibroblasts in the underlying dermis. Furthermore, IFI16 was found in all vascular endothelial cells from both blood and lymph channels. IFI16 is an important player involved in innate immunity along with inhibition of cell cycle progression and in the regulation of apoptosis [(Caposio et al., 2007); (Li et al., 2021)]. Overexpression of IFI16 can lead to reduced cell proliferation and arrest in cell cycle progression at the G1-S phase transition. The interaction of IFI16 with p53 and pRb appears to cause IFI16-mediated growth arrest [(Aglipay et al., 2003); (Liao et al., 2011)]. Indeed, prostate cancer cell lines harboring functioning p53 and pRb were much more susceptible to IFI16's antiproliferative activities (Xin et al., 2003). In medullary thyroid cancer cells, IFI16 has also been identified as an important growth-specific effector of the cell extrinsic growth-inhibitory pathway of Ras/Raf signaling (Kim et al., 2005). Finally, IFI16 expression has been discovered to be altered in a variety of human cancers [(Azzimonti et al., 2004); (Fujiuchi et al., 2004); (Xin et al., 2003)]. In vitro experiments, including chemotaxis, matrigel invasion, tube morphogenesis, and cell cycle progression, have shown that IFI16 overexpression inhibits tube morphogenesis and proliferation of human endothelial cells (Raffaella et al., 2004). Overall, these findings suggest that IFI16 plays a role in the control of cell proliferation, differentiation, and angiogenesis. As mentioned before, IFI16 has a pivotal role in innate immunity and inflammation. Not only IFN-γ, but also other pro- inflammatory cytokines like IL-1b and TNFa, can considerably increase IFI16 expression (Mondini et al., 2007). Anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-13, and IL-17), on the other hand failed to stimulate IFI16 expression. The physiologic nuclear localization of IFI16 is regulated by a bipartite nuclear localization sequence (NLS) located at the protein's N-terminus (Li et al., 2012). In contrast, stress stimuli like UVB irradiation of human keratinocytes have been demonstrated that can promote the IFI16 translocation from nucleus to cytosol and eventually in extracellular space [(Cao et al., 2016); (Costa et al., 2011)]. IFI16 is peculiar due to the fact that it can shuttle between the cytoplasm and the nucleus and detect DNA from a variety of viruses, including dsDNA from herpes simplex virus type 1 (HSV-1), Kaposi sarcoma-associated herpesvirus (KSHV), and human cytomegalovirus (HCMV) (Dell'Oste et al., 2015), and ssDNA or dsDNA from bacteria, [(Li et al., 2012); (Veeranki and Choubey, 2012)]. Overall, IFI16 is found mostly in the nucleus in many cells, functioning as a DNA sensor to identify pathogenic DNA and subsequently activating cytoplasmic inflammasome and innate signaling pathways to initiate an immunological response against pathogen invasion. Out of all the many IFI16 functions that have been previously described, one that has recently been discovered by our group is that IFI16 behaves as a DAMP triggering inflammation through Toll-like receptor 4 (TLR4) activation (Iannucci et al., 2020).

As mentioned before, IFI16 can translocate in the extracellular environment following stress stimuli (i.e., UVB irradiation or bacterial/viral infection) or upon tissue inflammation. Here, alone or upon binding to subtoxic concentrations of strong TLR4-activating lipopolysaccharide (LPS) variants, IFI16 can bind TLR4 and induce inflammation (Iannucci et al., 2020). This opens a new perspective in the study of extracellular IFI16, its potential role in autoimmune and autoinflammatory diseases, and how to exploit it as a therapeutic target.

Figure 4. Schematic illustration of IFI16 protein structure. Residues 1-83 constitute the N-terminalPYRIN domain (dotted box). The solid-shaded boxes denote the HINA (residues 192-393) and theHINB (residues 515-710) domains. Diagonal stripped bars indicate the S/T/P-rich seven amino acidrepeat motifs between the two HIN domains, resulting from alternative splicing of the gene. Verticalbars indicate the nuclear localization sequence (NLS). The conserved MFHATVAT motifs are also shown. Numbers located below each diagram represent amino acid positions (Adaptation from Dell'Oste et al., 2015).

2.6 IFI16 as a DAMP in autoinflammatory/autoimmune diseases

One of the essential properties of the immune system, leading to immunological responses against pathogens, is the ability to discriminate between "self" and "non-self". However, the innate immune response can be engaged even when there is no infection, as introduced in 1994 by Polly Matzinger with the so-called "danger theory" which claimed that danger signals from stressed or damaged cells can trigger immune responses (Matzinger, 1994). This notion led to the identification of a variety of endogenous molecules generated after tissue injury, which were identified as damage-associated molecular patterns (DAMPs) (Land, 2003). DAMPs are ordinarily sequestered intracellularly and hence remain unrecognized by the immune system under normal physiological settings. However, dying cells following cellular stress or damage might release these molecules into the extracellular environment, independent of pathogen infection, resulting in sterile inflammation (Chen and Nuñez, 2010). Like pathogens, DAMPs may in turn activate both non-immune cells, such as epithelial cells, endothelial cells, and fibroblasts, as well as innate immune cells, such as neutrophils, macrophages, and dendritic cells (DCs). Once activated, these cells produce a variety of cytokines and chemokines, which attract inflammatory cells and trigger adaptive immune responses (Gong et al., 2020). Furthermore, certain DAMPs can directly activate adaptive immune cells. Although sterile inflammation is necessary for tissue repair and regeneration, unresolved chronic inflammation is harmful to the host and can result in metabolic disorders, neurological diseases, autoimmune diseases, and cancer. Numerous novel DAMPs have been found in distinct damage conditions throughout the last 20 years and their number is still increasing. Furthermore, DAMPs differ widely depending on the kind of cell (epithelial or mesenchymal) and wounded tissue. They can

arise from extracellular matrix degradation processes during tissue injury and include hyaluronan, biglycan and tenascin C, among the others, or from intracellular compartments, as in the case of high-mobility group box 1 (HMGB1), histones, S100 proteins, heat-shock proteins (HSPs), oxidized phospholipids (oxPAPC,) and plasma proteins (e.g., fibrinogen, Gc-globulin, serum amyloid A or SAA) [(Tsung et al., 2005); (Schaefer, 2014); (Smiley et al., 2001); (Zhou and Binder, 2014); (Sokolove et al., 2010); (Sohn et al., 2012); (Ye and Sun, 2015); (Zanoni et al., 2017)]. Along with the discovery of new DAMPs, numerous related receptors have been also discovered. As a major broad distinction, released nucleic acids can activate transmembrane TLR3, TLR7, TLR8 and TLR9, whereas released intracellular proteins can activate mostly TLR2 and TLR4. Among these, TLR4 is of special importance since it can be bound and activated by a large number of DAMPs. As a consequence, several therapeutic approaches are being developed to inhibit TLR4 activation in a variety of diseases [(Garcia et al., 2020); (Romerio and Peri, 2020)]. Tenascin-C, an extracellular matrix glycoprotein linked with tissue damage and fibrotic activity (Ummarino, 2016), has been shown to stimulate the creation of pro-inflammatory cytokines in macrophages via its fibrinogen-like globe (FBG), and TLR4 neutralizing antibodies, genetic deletion of TLR4, or production of a dominant negative MyD88 mutant, totally abrogate Tenascin-C proinflammatory activity (Midwood, 2009). Subsequential analyses have also identified tenascin-C specific sites that directly and cooperatively interact with TLR4 through its fibrinogen-like globe domain and (Zuliani-Alvarez et al., 2017). Levels of Tenascin-C and the delivered persistence state of inflammation are elevated in Systemic sclerosis (SSc) skin and in circulation (sera) making it a possible biomarker of skin and lung fibrosis in SSc and related diseases [((Bhattacharyya et al., 2022); (Bhattacharyya et al., 2016)]. HMGB1 proinflammatory activity was also demonstrated in necrotic wild-type (WT) or HMGB1-knockout (HMGB1-KO) cells, demonstrating that TLR4 activation was not caused by bacterial contaminants in the recombinant protein. Surface plasmon resonance (SPR) analysis revealed that the HMGB1-TLR4-MD2 interaction is initiated by HMGB1-TLR4 binding via the HMGB1 A-box domain (high affinity and slow off-rate), and then the HMGB1 B-box domain binds to MD2 (low affinity but extremely slow off-rate) once in proximity (He et al., 2018). Despite this, HMGB-1 is a pleiotropic DAMP because it binds to and activate a variety of receptors, including TLR2 and a non-PRR, namely the receptor for advanced glycation end products (RAGE, (Yang et al., 2020). The evidence that HMGB1 can bind also a non-PRR (unlike PAMPs), suggested that it may be detected by a variety of receptors to trigger inflammation. DAMPs can stimulate the innate immune response because, following an injury, they contribute to support tissue repair and regeneration [(Pandolfi et al., 2016); (Vénéreau et al., 2015)]. However, the lack of inflammation control may lead to autoimmune disease. For instance, a wide range of endogenous TLRactivators, including heat shock proteins, HMGB1, and Tenascin-C, has been observed in the synovia of rheumatoid arthritis (RA) patients but not in those from patients with normal joints or in non-inflamed synovia from osteoarthritis (OA) patients [(Baillet et al., 2010); (Goldstein et al., 2007); (Midwood et al., 2009)]. Moreover, again high levels of HMGB1 and Tenascin-C circulate in the serum of septic patients [(Andersson and Tracey, 2003); (Li and Lu, 2021)], and high serum concentrations of DNA-containing immune complexes are associated with systemic lupus erythematosus (SLE) (Tian et al., 2007). Endogenous TLR activators are often associated with disease activity. In in vivo experimental inflammatory disease models, where high levels of circulating DAMPs were not found, the administration of exogenous DAMPs was sufficient to initiate inflammation. As an example, intra-articular injection of the TLR4 activators tenascin-C has been shown to induce joint inflammation in wild type but not

in TLR4-null mice [(Gondokaryono et al., 2007); (Midwood et al., 2009)]. Interestingly, blocking DAMP activity using neutralizing antibodies, small compounds, or genetic deletion can improve disease in vivo, indicating that DAMP play a critical role in promoting chronic inflammation and pointing to DAMP inhibition as a necessary therapeutic intervention.

3. Aim of the study

IFI16 expression is aberrantly increased in damaged tissues of individuals with autoimmune/autoinflammatory illnesses due to abnormal response to type I IFNs and/or other proinflammatory/stress stimuli (Mondini et al., 2007); IFI16 then delocalizes from nucleus to cytoplasm and eventually isreleased into the extracellular space (Dell'Oste et al., 2014)**.** The released IFI16 protein leads to abreakdown in tolerance to self-antigens thus favoring the generation of specific anti-IFI16autoantibodies [(Caneparo et al., 2013); (Alunno et al., 2015); **(**Baer et al., 2015**)**; (McMahan et al., 2016)] moreover, the freely circulating IFI16 may act as a DAMP, alone or in combinationwith bacterial LPS, by binding the TLR4/MD2 complex (Iannucci et al., 2020). Finally, through the NF-kB pathway IFI16 can induce the production and the release of proinflammatorycytokine, thus amplifying the injury of target cells (Bawadekar et al., 2015b)**.** Altogether, these results clearly point out the role of extracellular IFI16 as a DAMP. The goals of the study are:

1) Mechanistically dissect the interaction between IFI16 and TLR4, using a panel of antibodies directed against either the N- or the C-terminal region of IFI16, along with a panel of IFI16 recombinant domains or truncated proteins spanning the whole IFI16 isoform from the N-terminus to the C-terminus;
2) Evaluate whether the pro-inflammatory activity of IFI16 could be extended to other PYHIN family members;
3) Identify the amino acids potentially involved in IFI16-TLR4/MD2 binding and proinflammatory through 3D structure prediction and site-specific mutagenesis;
4) By using human keratinocytes as cellular model, characterize different stress stimuli as triggers of IFI16 delocalization and extracellular release in vivo.

4. Materials and Methods

4.1 Reagents, antibodies, and recombinant proteins

LPS from Escherichia coli O111:B4 (LPS-EB), was purchased from InvivoGen. Bovine serum albumin Fraction V pH 7 (BSA) was purchased from Euroclone. The following antibodies were used: mAb anti-human TLR4 (sc-293072, Santa Cruz Biotechnologies), mAb anti-human TLR4 (sc-13593, Santa Cruz Biotechnologies), mAb anti- human TLR4 (mabg-htlr4, InvivoGen), rabbit polyclonal anti-MD2 (AHP1717T, Bio-Rad), mAb anti-β-actin (A1978, Sigma-Aldrich), rabbit IgG-HRP (A6154, Sigma-Aldrich,), mouse IgG-HRP (NA931V, GE Healthcare), streptavidin- HRP (E2886, Sigma-Aldrich,), mouse IgG- Alexa Flour 488 (A11001, Thermo Fisher Scientific), normal mouse IgG2a isotype control (sc- 3878, Santa Cruz Biotechnologies), Rabbit polyclonal anti-IFI16 N-term and C-term were produced as described previously (Gariglio et al., 2002). Briefly, N-terminus or C-terminus IFI16 cDNA from pBKS-IFI16 (kindly provided by J. Trapani, The Peter MacCallum Cancer Institute, Victoria, Australia) were cloned into a pGEX-4T-2 vector (Pharmacia, Uppsala, Sweden) to create an in-frame fusion protein with the GST coding region. The expression of N-terminus or C-terminus GST-IFI16 fusion protein in the Escherichia coli host AD202 was induced by treatment with 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 3 h. The bacterial cells were harvested by centrifugation, resuspended in cold lysis buffer (0.5 mg/ml lysozyme, 25 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.2% Triton X-100 containing 2 mM PMSF, 50 mM pepstatin A, and 50 mM leupeptin as protease inhibitors) and lysed by sonication. Fusion proteins were purified from the cleared lysate by glutathione- Sepharose affinity chromatography. Antisera against IFI16 were raised by injecting rabbits with the purified GST-IFI16 fusion proteins. The sera obtained after bleeding at 1 week after the fourth immunization were precipitated with ammonium sulfate at 45% saturation. The precipitate was then resuspended in phosphate-buffered saline (PBS) and purified on a protein A affinity column (Pharmacia) according to the specification of the supplier. IFI16 mutated in PRYRIN domain (i.e., I17A, E85A, K64G, K34A, K86A and triple mutant K64G-K34A-K86A) and Pyrin of HIN200 family (i.e., hNLRP3, mAIM2, hAIM2, mIFI203, mIFI204, hMNDA, hIFIX, hASC) were purchased from GenScript in pET30a expression vector.Human recombinant IFI16, IFI16 domains (i.e., PYRIN, HINA and), and IFI16 variants lacking the HINB domain (i.e., IFI16ΔHINB) or the PYRIN domain (i.e., IFI16ΔPYRIN), Pyrin domain of HIN200 members and Mutated IFI16 in its Pyrin domain were produced as previously described (Bawadekar et al., 2015b-6-01). Briefly, the different coding regions were amplified from the full-length human IFI16 cDNA (isoform b) and cloned in a pET30a expression vector (Novagen) containing an N-terminal histidine tag. The expression of the proteins in the ClearColi® BL21(DE3) host (to ensure no endotoxin contamination) and the lysis of the bacteria, were performed as described above. Recombinant proteins were purified from the cleared lysate by nickel-affinity purification and stored at - 80°C in endotoxin-

free vials. GST recombinant protein was expressed using pGEX-4T2 vector and purified according to standard procedures. The purity of the proteins was assessed by 12% SDS-polyacrylamide gel electrophoresis. Recombinant TLR4 protein and TLR4/MD2 complex (478-TR-050 and 3146- TM-050/CF, respectively) were purchased from R&D Systems.

4.2 Cell cultures, treatments and cells viability

Human leukemia monocytes (THP-1) and mouse macrophages (RAW264,7), both obtained from ATCC grown in RPMI 1640 Medium (Sigma- Aldrich) containing 10% of fetal bovine serum (FBS, Immunological Sciences) and 1% of penicillin/streptomycin/glutamine solution (PSG, Gibco) at 37°C and 5% CO2. Green monkie normal epithelial cells (VERO) were obtained from ATCC too but grown in DMEM (Euroclone) containing 10% of fetal bovine serum (FBS, Immunological Sciences) and 1% of penicillin/streptomycin/glutamine solution (PSG, Gibco) at 37°C and 5% CO2. human Keratinocytes (HaCaT) kindly provided by Dr. Leonie Unterholzner (Lancaster University), and HaCaT IFI16KO previously formed in our lab grown in the same condition as VERO cells. Brightfield picture for cells morphology was taken with Leica MC120 HD (DMi1) microscope and Las X leica software. UVB irradiations were performed as previously described (Costa et al., 2011). Briefly, UV irradiation was performed in PBS and provided by a UVB lamp (HD 9021; Delta Ohm S.r.l., Padova, Italy), which emits most energy within the UVB range (280–315 nm), with an emission peak at 312 nm. Irradiation intensity was monitored by a UVB irradiance meter cosine corrector with spectral range of 280–319 nm (LP 9021 RAD; Delta Ohm). Following irradiation with the required UVB dose, cells were incubated in complete medium for 16h at 37 °C in a humidified 5% CO2 atmosphere. The resulting cell culture supernatants were centrifuged to remove any cellular pellet and stored at -80°C for the following experiments. For treatments, cells were stimulated in complete medium with equimolar concentration of IFI16, PYRIN, HINA, HINB, IFI16ΔHINB, IFI16ΔPYRIN, and only Pyrin domain of human AIM2 (hAIM2), MNDA, and IFIX and mouse PYHIN proteins aim2 (mAIM2), p203, and p204 along with those of the human NLRP3 and ASC proteins. Moreover, we treat cells with recombinant IFI16 mutated in a single ammino acid for Four different amino acid (plasmid provide by GenScrip) and with a triple-mutant all in equimolar way (111nM). Additionally, cells were stimulated with hTNF (Biolegend) 50ng/ml and 100ng/ml for 24h and 48h. All treatments were carried out at 37°C and 5% CO2. For TLR4 neutralization, THP-1 cells were pretreated with CLI-095 (Thermo scientific) during treatments with recombinant protein. For treatments with anti-IFI16 antibodies, IFI16 was incubated with rabbit polyclonal anti-IFI16 N-term or C-term for 1 h at RT before treatments. After treatment with UVB and TNF the viability was measured using 5% of Alamar Blu for (biorad) 1h and LDH release assay (Roche) following kit protocol. Both assays have been measured the relative absorbance to Spark multimode microplate reader (Tecan).

4.3 Western blot and immunoprecipitation

Whole-cell extracts were prepared using RIPA lysis and extraction buffer (Thermo Fisher Scientific) with halt protease and phosphatase inhibitor (Thermo Fisher Scientific) on ice, and total protein concentration was quantified by Bradford Reagent (Sigma-Aldrich) measuring absorbance at 595 nm. Twenty µg of cell extracts, or 30 µl of cells culture supernatants (HaCaT)were separated by electrophoresis on 7.5% or 12% SDS-polyacrylamide gels (Bio-Rad), transferred to nitrocellulose membranes, blocked with 10% non-fat milk in tris-buffered saline-tween (TBST), and probed with specific primary antibodies O/N at 4°C. After being washed with TBST, membranes were incubated with specific HRP-conjugated secondary antibodies, and binding was detected by ECL (Thermo Fisher Scientific, Super Signal West Pico). Expression of β-actin or GAPDH was used as protein loading control. Co-immunoprecipitation of TLR4 with interacting proteins was performed using the Dynabeads Protein G Immunoprecipitation Kit (ThermoFisher), according to the manufacturer's instructions with minor modifications. Briefly, after lysis of treated cells, 20 µg of total cell extracts were kept as the input control, while 90 µg of total cell extracts were incubated for 1 h at RT with 2.5 µg of anti-TLR4 antibody previously conjugated with magnetic beads. The resulting complexes were then washed, eluted, denatured, and subjected to Western blotting as described above. For DNase-treated cell extracts, DNase I (Sigma Aldrich) was added at a 1:10 dilution and incubated for 15 min at RT. Images were acquired, and densitometry of the bands was performed using Quantity One software (version 4.6.9, Bio-Rad). Densitometry values were normalized using the corresponding loading controls.

4.4 Quantitative real time PCR

Quantitative real-time PCR (qRT-PCR) was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) as previously described (Albertini et al., 2018). Briefly, total RNA was extracted using TRI Reagent (Sigma-Aldrich), and 1 µg was retrotranscribed using an iScript cDNA Synthesis Kit (Bio-Rad). Reverse-transcribed cDNAs were amplified in duplicate using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), up to 40 cycles of PCR. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, or the murine actin gene, were used as housekeeping gene to normalize for variations in cDNA levels. The relative normalized expression after stimulation as compared to control was calculated as fold change = $2^{-\Delta(\Delta CT)}$ where $\Delta CT = CT_{target} - CT_{GADPH}$ and $\Delta(\Delta CT) = \Delta CT_{stimulated} - \Delta CT_{control}$. Primer sequences are summarized in Table 2.

Table 2. List of primers used for qRT-PCR (h: human)

Gene	Forward (5' to 3')	Reverse (5' to 3')
hIL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC
hTNF-α	GCCAGAGGGCTGATTAGAGA	TCAGCCTCTTCTCCTTCCTG
hIL-1β	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG
hGAPDH	AACGTGTCAGTGGTGGACCTG	AGTGGGTGTCGCTGTTGAAGT

4.5 Cytokines measurement by ELISA

Cytokines secreted in culture supernatants after treatments were analyzed using human IL-6 DuoSet ELISA and human IL-8 DuoSet ELISA (all from R&D Systems) and human TNF-α DuoSet ELISA (all from R&D Systems) according to the manufacturer's instructions. Absorbance was measured using a Spark multimode microplate reader (Tecan).

4.6 Cloning, transformation, Transfection, Monitoring IFI16-mCherry

A chimera form of IFI16 were formed by cloning using mCherry dye plasmid provided by Addgene. pET30a vector that contains IFI16(already present in laboratory) was amplified by PCR using primers present in table below (Table 3). IFI16 (300ng) obtain purified form Agarose gel (1%) and mCherry plasmid (700ng) were digested with Restriction Enzyme BamHI e XhoI for 15min at 37°C and then using T4 ligase (Invitrogen) with molar ratio vector: insert of 1:2. Size check and second round of checking digestion was done and analyzed in Agarose gel. Then, E.coli (clear coli) transformation using thermal shock (42°C and ice) is done followed by 1h of resting. The selection occurs in plate dishes with LB agar auditioned with Kanamycin (50ug/ml) as selection marker, overnight at 37°C incubation, it. After amplification of the colony, plasmid DNA was extracted and checked with digestion with Restriction Enzyme. Plasmid DNA of IFI16- mCherry chimera obtained has been used for HACAT cells transfection. Cells were transfected with specific Lipo3000 following kit protocol (thermo scientific) with lower amount of Lipo proposed and 2.5ug of DNA. Following 24h of resting cells were monitored before at FLoid™ Cell Imaging Station (Thermo Scientific) and then after cells treatment in IncuCyte (Sartoriuss) by setting acquisition plate every 30min.

Table 3. Primers for IFI16 cloning in mCherry vector

Gene	Forward (5' to 3')	Reverse (5' to 3')
IFI16-ORF	CCGCTCGAGATGGGAAAAAAATACAAG	CCGGGATCCGAAGAAAAAGTCTGGTGAAGT

4.7 Surface plasmon resonance analysis

The Biacore X100 (GE Healthcare) instrument was used for real-time binding interaction experiments. Recombinant TLR4 or TLR4/MD2 complex was covalently immobilized onto the surface of sensor CM5 (cat # BR100012, GE Healthcare) chips via amine coupling. TLR4 was diluted to a concentration of 10 µg/ml in 10 mM sodium acetate at pH 4.0, while TLR4/MD2 complex was diluted to a concentration of 20 µg/ml in the same buffer. Both proteins were injected on CM5 chips at a flow rate of 10 µl/min, upon activation of the carboxyl groups on the sensor surface with 7-min injection of a mixture of 0.2 M EDC and 0.05 M NHS. The remaining esters were blocked with a 7-min injection of ethanolamine. Taking into account the ligands (TLR4 or TLR4/MD2) and analytes (IFI16, HIN200 protein Members and IFI16 mutated) molecular weights (MW) of 70 or 90 kDa, and 90, 10 or 100 kDa 15 kDa respectively, the appropriate ligand density (RL) on the chip was calculated according to the following equation: RL = (ligand MW/analyte MW) × Rmax × (1/Sm), where Rmax is the maximum binding signal and Sm corresponds to the binding stoichiometry. The target capture level of the TLR4 or TLR4/MD2 was 596.0 or 1223.9 response units (RUs), respectively. The other flow cell was used as a reference and was immediately blocked after the activation. Increasing concentrations of IFI16, IFI16 subdomain, HIN200 family members; IFI16 mutated were flowed over the CM5 sensor chip coated with TLR4 or TLR4/MD2 at a flow rate of 30 µl/min at 25°C with an association time of 120 s and a dissociation phase of 180 s. A single regeneration step with 50 mM NaOH was performed following each analytic cycle. All the analytes tested were diluted in the HBS-EP+ buffer (GE Healthcare). Recombinant IFI16 was covalently immobilized onto the surface of sensor CM5 chips via amine coupling as done for TLR4 and TLR4/MD2 complex. IFI16 was diluted to a concentration of 25 µg/ml in 10 mM sodium acetate at pH 4.0. The target capture level of IFI16 was 1926.6 response units (RUs). A single regeneration step with 50 mM NaOH was performed following each analytic cycle. The KDs were evaluated using the BIAcore evaluation software (GE Healthcare) and the reliability of the kinetic constants calculated by assuming a 1:1 binding model supported by the quality assessment indicators values. For the binding inhibition experiments, a fixed concentration of IFI16 (500 nM) was incubated with increasing concentrations of rabbit polyclonal anti-IFI16 N-term or C-term for 1 h at RT, diluted in HBS- EP+ buffer. IFI16–antibody complexes were injected over the TLR4/MD2 sensor chip surface for 120 s and allowed to dissociate for 180s. A single regeneration step with 50 mM NaOH was performed following each analytic cycle. Data were background-subtracted using the adjacent control flow cell and buffer-alone injections. The reported RUs were calculated using the BIAcore evaluation software.

4.8 Sequence alignment, mutagenesis and protein structure docking

Sequence alignments of the Pyrin domains were performed with the program MEGAX using the ClustalW algorithm. The primary amino acid sequences were obtained from UniProtKB with the following codes: IFI16 Q16666, MNDA P41218, AIM2 O14862, IFIX Q6K0P9, IFI203 O35368, IFI204 P0DOV2, mAIM2 Q91VJ1, NLRP3 Q96P20, ASC Q9ULZ3. The genes encoding the mutated variants of IFI16 at level of the PYRIN domain (i.e., I17A, E85A, K64G, K34A, K86A and triple mutant K64G-K34A-K86A), were purchased from GenScript in pET30a expression vector. Structure prediction of IFI16 PYRIN domain was performed using Robetta software (Kim et al., 2004). Protein-protein interaction has been simulated using the Haddock web server (van Zundert et al., 2016). The binary complex between C-term of TLR4 (fist 626 amino acids) or MD2 with PYRIN, obtained through the docking approach, were ranked based on the Haddock score, which represents a binding free energy–like value. Proteins 3D structures were evaluated and figures obtained using PyMOL software (The PyMOL Molecular Graphics System, Version 2.3.4).

4.9 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The data are expressed as mean ± SD. For comparisons between two groups, means were compared using a two-tailed Student's t test. For comparisons among three groups, means were compared using one-way or two-way ANOVA followed by Dunnett's test. Differences were considered statistically significant at a P value < 0.05.

5. RESULTS

5.1 The proinflammatory activity of the IFI16 protein lies within its N-terminal region

We previously demonstrated that the IFI16 protein is a specific TLR4-ligand and that IFI16 per se or upon binding to LPS can trigger TLR4-mediated inflammation (Iannucci et al., 2020). To identify the region of IFI16 responsible for TLR4 activation, we took advantage of a panel of antibodies directed against either the N- or the C-terminal region of IFI16. Phorbol 12-myristate 13-acetate- (PMA)-differentiated human THP-1 cells (human macrophages hereinafter) were stimulated with 10 μg/ml of full-length IFI16, alone or pre-incubated for 1h at RT with increasing concentrations of anti-IFI16 antibodies. After 24h, total RNA was extracted and analyzed using qRT-PCR to assess the inflammatory reaction. As expected, and consistent with the data previously obtained (Iannucci et al., 2020), TNFα, IL8, and IL1β mRNAs were strongly upregulated when cells were treated with IFI16 alone compared to untreated cells (61-fold for TNFα, 97-fold for IL8, and 50-fold for IL1β, respectively) (Figure 5A). Interestingly, a concentration-dependent inhibition of IFI16-mediated TNFα, IL8, and IL1β upregulation was observed when the anti-N-term-IFI16 antibodies were pre-incubated with the IFI16 protein, reducing the transcriptional activation of around 85% when the highest concentration of

antibody (10 µg/ml) was used. By contrast, no significant changes were observed when anti-C-term-IFI16 antibodies were pre-incubated with the recombinant IFI16 protein when compared to the protein alone. Consistent with the transcriptional data, TNF-α and IL-8 release in cell culture supernatants was strongly increased when the cells were stimulated with IFI16, and this effect was significantly inhibited in a concentration-dependent manner when anti-N-term-IFI16 antibodies, but not anti- C-term, were used (Figure 5B). Moreover, the inhibitory activity exerted by anti-N-term-IFI16 antibodies has been further investigated by surface plasmon resonance (SPR) in order to validate the possible interference of the selected antibodies in the physical interaction between IFI16 and TLR4/MD2 receptor. Briefly, IFI16, alone or pre-incubated for 1h at RT with increasing concentrations of anti-IFI16 antibodies, flowed over a TLR4/MD2-coated chip. Consistent with the results obtained in the cellular assays, anti-N-term-IFI16 antibodies significantly inhibited IFI16 binding to the receptor in a concentration dependent manner, exhibiting approximately 75% and 45% of binding inhibition versus the control setting without antibodies when the highest or the lowest concentrations of antibodies— 500nM and 62.5nM, respectively— were used (Figure 5C). As expected, preincubation with anti-C-term-IFI16 antibodies did not impair IFI16 binding to the TLR4/MD2 receptor even at the highest concentrations used. Collectively, our findings strongly suggest that the IFI16 N-terminal region is mainly responsible for the recognition and activation of the TLR4/MD2 complex.

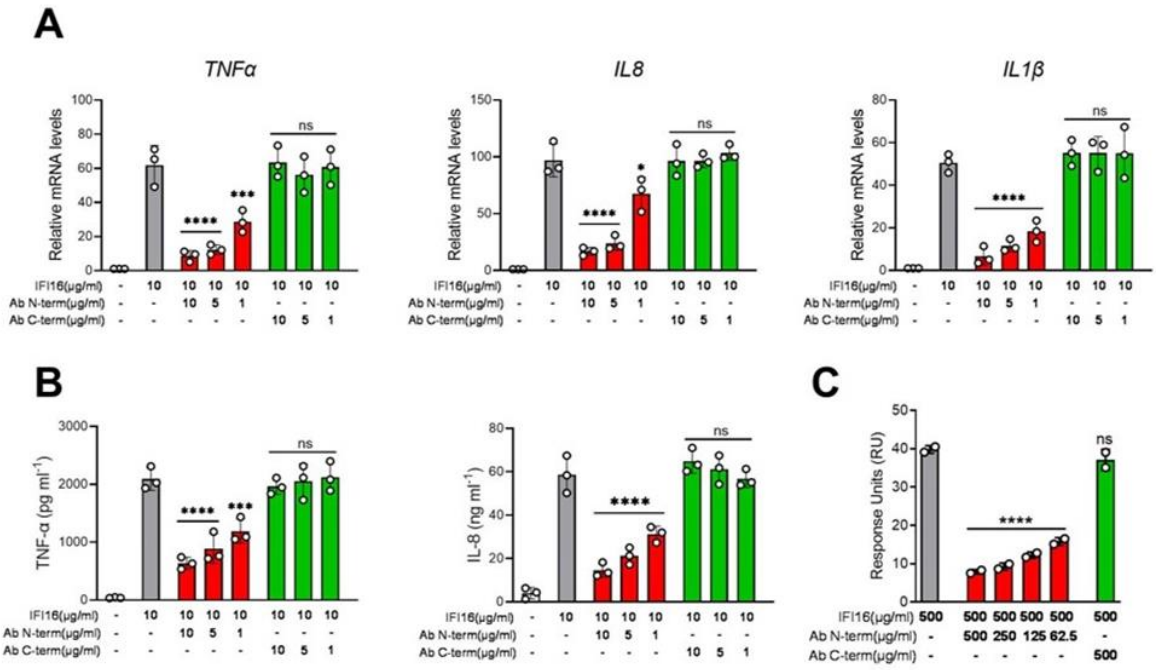


Figure 5. Antibody against the N-term region of IFI16 dampens the inflammatory response in human macrophages. (**A**)qRT-PCR analysis for TNF-α, IL-8, and IL-1β mRNA expression levels in human macrophages stimulated for 24 h with IFI16 alone (10 μg/ml) or pre-incubated for 1 h with the indicated amounts of anti-IFI16 polyclonal antibodies directed against either the N- or C-terminal region of the protein. Values are normalized to GAPDH mRNA and plotted as fold of induction over untreated cells. qRT-PCR data are presented as mean values of biological triplicates. (**B**) Proteinconcentration of TNF-α and IL-8 determined by ELISA in the culture supernatants harvested from human macrophages stimulated for 24 h as described in A. (**C**) Surface plasmon resonance (SPR) analysis of IFI16 binding to immobilized TLR4. Five-hundreds 500nM of IFI16 diluted in running buffer, alone or pre-incubated for 1h at RT with increasing concentrations (62.5 to 500 nM) of the anti-N-term-IFI16 antibody or with 500nM of anti-C-term antibody, were flowed over a TLR4/MD2-coated chip. Data are representative of three independent experiments, shown as the mean ± SD (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; two-way ANOVA followed by Dunnett's test).

5.2 The PYRIN domain of IFI16 is involved in TLR4/MD2 binding and activation

To map the IFI16 domain that is involved in TLR4/MD2 binding and activation, we generated a panel of IFI16 recombinant domains spanning the whole protein from the N-terminus to the C- terminus that comprises the PYRIN, HINA or HINB domains as well as truncated IFI16 proteins that lack either the PYRIN or HINB domains (IFI16ΔPYRIN or IFI16ΔHINB, respectively) (Figure 6A). The human macrophages were stimulated with 10 μg/ml of IFI16, or equimolar concentrations of IFI16 domains or truncated proteins, and total RNA was extracted 24h later and analyzed using qRT-PCR to assess the inflammatory reaction. Consistent with Figure 6A and our previous work (Iannucci et al, 2020), TNFα, IL8, and IL1β mRNAs were significantly upregulated when IFI16 or the IFI16ΔHINB truncated protein were used (Figure 6B). By contrast, this proinflammatory activity dropped when the IFI16ΔPYRIN protein was used (from 57- to 10-fold for TNFα, from 113- to 20-fold for IL8, and from 48- to 5-fold for IL1β respectively, compared to untreated cells). Interestingly, out of the three IFI16 domains, only the PYRIN domain retained the ability to upregulate the expression levels of TNFα, IL8, and IL1β mRNAs, although at a lower extent when compared to the full-length protein (37-fold for TNFα, 66-fold for IL8, and 38-fold for IL1β respectively, compared to untreated cell). Consistently, TNF-α and IL-8 protein levels were significantly increased in the culture supernatants of the cells treated with IFI16, PYRIN or IFI16ΔHINB but not when cells were stimulated with the IFI16ΔPYRIN truncated protein or the HINA or HINB domains, compared to untreated cells (Figure 6C). As already reported for the full-length protein (Iannucci et al, 2020), the observed proinflammatory activity was TLR4 dependent, being inhibited by the addition of the TLR4 antagonist CLI-095 (Figure 6C). Next, SPR analysis was performed to ascertain that the PYRIN domain was exclusively able to interact with the TLR4/MD2 receptor complex. To this end, increasing concentrations of the IFI16 protein, the truncated forms, or the single domains were flowed over a TLR4/MD2-coated chip. As shown in Figure 6D, we found that both IFI16ΔHINB and the PYRIN domain bound the TLR4/MD2 receptor complex with an affinity (KD=7.8*10-8 and 4.1*10-6, respectively) that was comparable to that displayed by the full-length protein (KD=1.7*10-7). Both HINA and HINB domains still retained binding affinity for the TLR4/MD2 complex but to much lower extent when compared to the full-length protein

(KD=1.6*10-4 and 3.2*10-5, respectively). As expected, the binding to TLR4/MD2 complex was completely lost when the IFI16ΔPYRIN protein was probed. The binding activity of the PYRIN domain to the TLR4 receptor was then confirmed in vivo using co-immunoprecipitation experiments. Human macrophages were treated for 1h with IFI16, the PYRIN domain, or left untreated, and after that the cell lysates were incubated with anti-TLR4 antibodies pre-adsorbed on protein G beads. Subsequently, SDS-PAGE and immunoblotting were used to detect the presence of TLR4, or the recombinant proteins in the immunoprecipitated protein complexes. As shown in Figure 6E, the protein bands corresponding to IFI16 and PYRIN domain were found when cells were treated with relatives' recombinant protein. The specificity of the interactions was confirmed by the absence of any bands in untreated cells Taken together, our findings strongly suggest that the PYRIN domain of the IFI16 protein, through its interaction with the TLR4 receptor, is necessary and sufficient to induce a proinflammatory phenotype in target cells, e.g., human macrophages, as demonstrated by the transcriptional activation and release of proinflammatory cytokines.

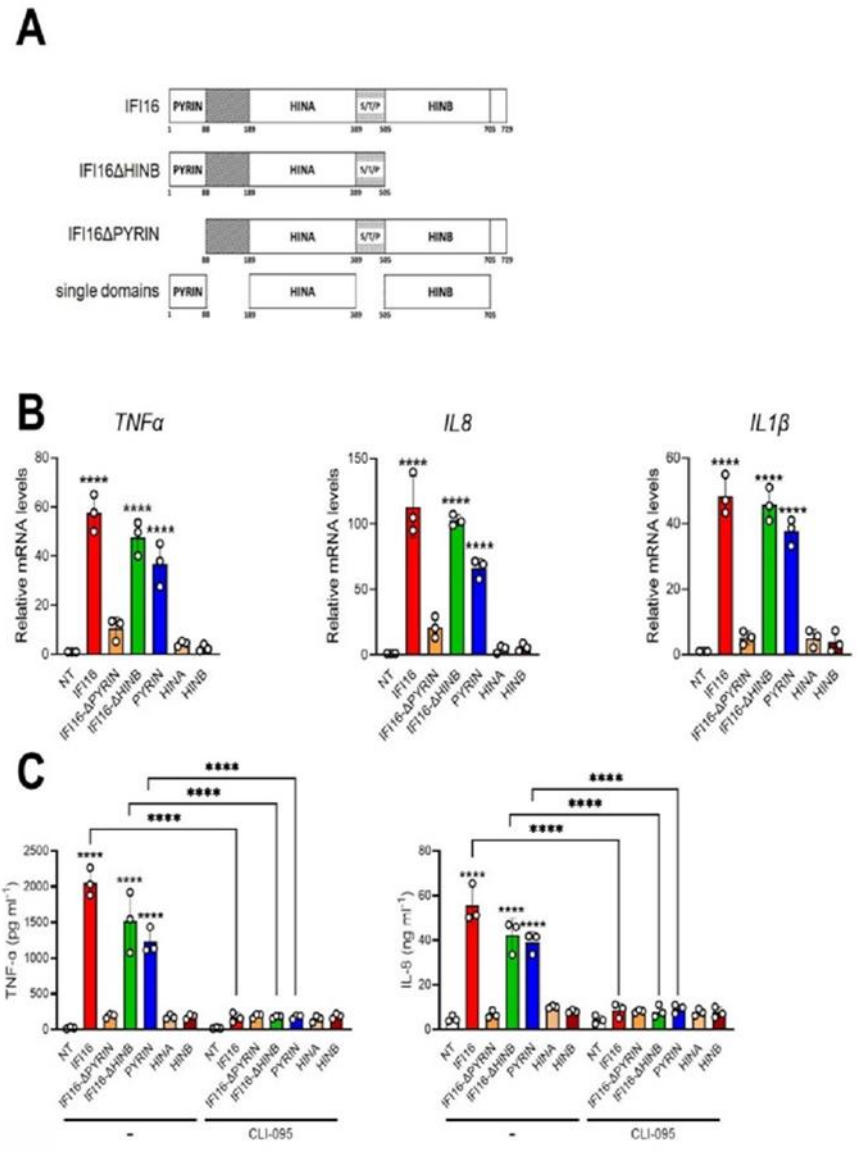
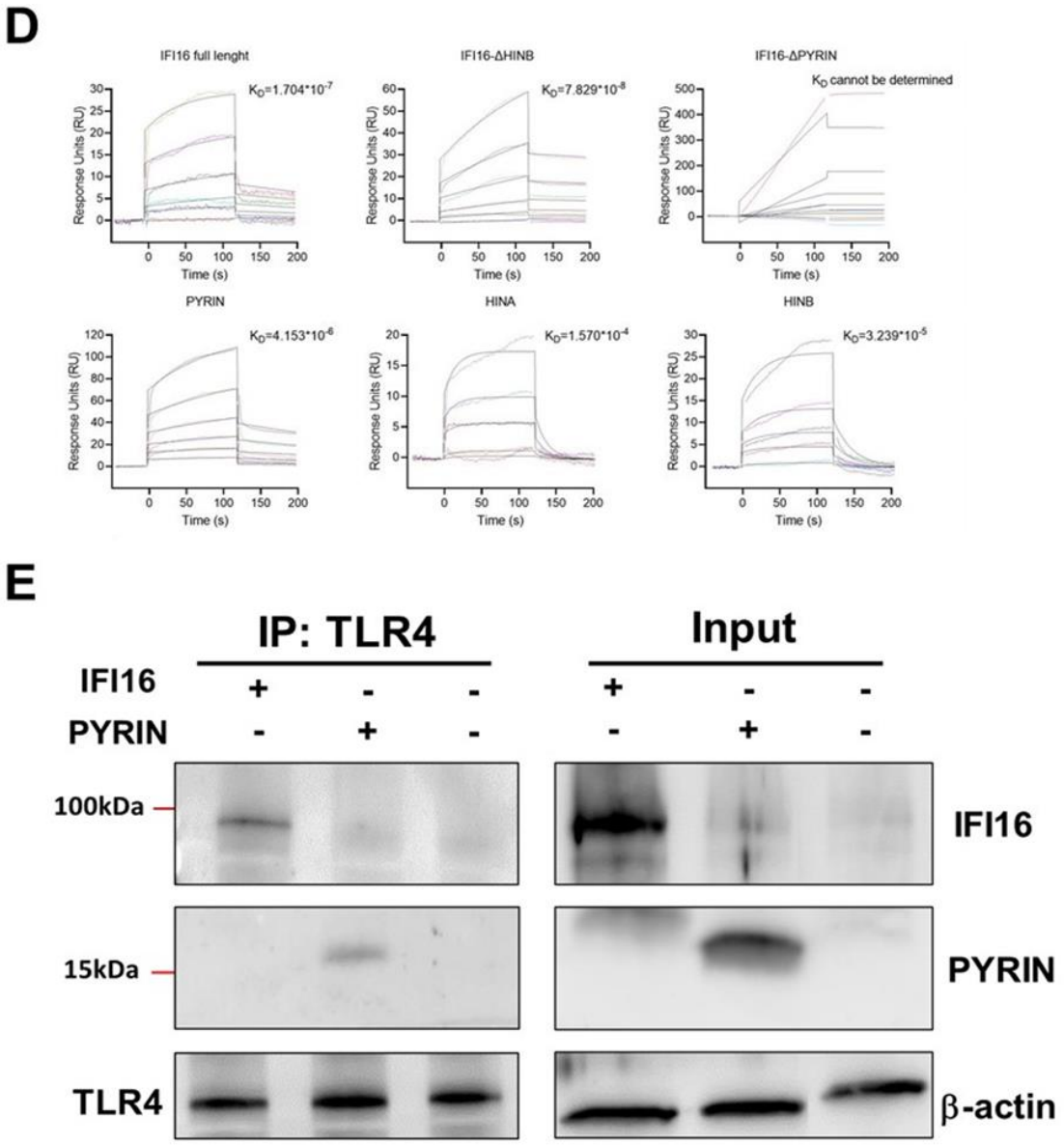


Figure 6. The PYRIN domain of IFI16 mediates TLR4 activation and pro-inflammatory activity. A)Schematic representation of the full length (IFI16), truncated forms IFI16DHINB and IFI16DPYRIN, and recombinant IFI16 single domains used in this study. Numbers represent the amino acid positions based on the NCBI Reference Sequence NP_005522. (B, C) Human macrophages were stimulated with equimolar concentrations (111nm) of IFI16 domains or truncated proteins and B) total RNA or C) culture supernatants were analyzed to assess the inflammatory reaction. Cytokines were significantly upregulated only when IFI16, PYRIN domain or the IFI16ΔHINB truncated protein were used, and similar results were obtained when TNF-α and IL-8 release was tested in ELISA. This proinflammatory activity was TLR4-dependent, being inhibited by the addition of the TLR4 antagonist CLI-095. Data are representative of three independent experiments, shown as the mean ± SD (* p<0.05, ** p<0.01, *** p<0.001; one-way ANOVA.



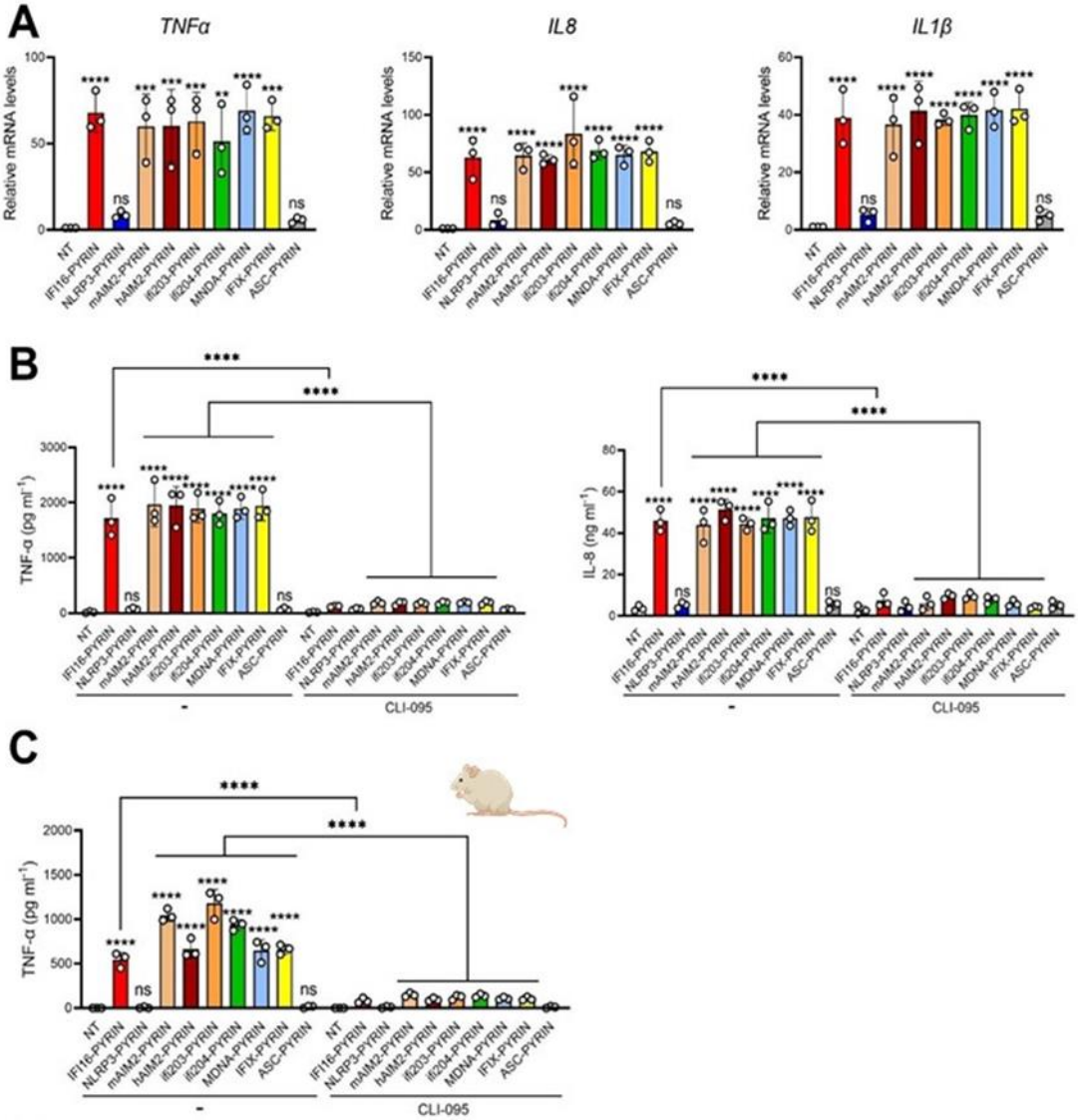
D) Surface plasmon resonance (SPR) analysis of IFI16 domains binding to immobilized TLR4/MD2. After immobilization of TLR4/MD2 on the CM5 sensor chip surface, increasing concentration of full length IFI16, PYRIN domain, IFI16ΔPYRIN,

HINA or HINB domains (20–800 nM) diluted in running buffer were injected over immobilized complex. Data are representative of three different experiments, and for each analysis the dissociation constant (KD) value is shown. E) Human macrophages were stimulated for 1h with full-length FI16 or the PYRIN domain (30ug/ml). Total cell extracts were subjected to immunoprecipitation using a TLR4 monoclonal antibody. Immunoprecipitated (left, IP:TLR4) and whole- cell lysates (right, Input) were analyzed by immunoblotting with anti-N-term-IFI16 (recognizing both flIFI16 and the PYRIN domain) or anti-TLR4 antibodies. β-actin protein expression was used for protein loading control. Data are representative of three independent experiments with similar results.

5.3 The proinflammatory activity of the PYRIN domain is conserved among the PYHIN family members

The results so far described prompted us to investigate whether the proinflammatory activity of the IFI16 PYRIN could be extended to the other members of the PYHIN family or even to other proteins that also harbor a PYRIN domain, such as the NOD-, LRR- and PYRIN domain- containing protein 3 (NLRP3), and the apoptosis-associated speck-like protein containing a CARD (ASC). To this end, we generated the recombinant PYRIN domains of other representative human AIM2 (hAIM2, hereinafter), MNDA, and IFIX and mouse PYHIN proteins aim2 (mAIM2, hereinafter), Ifi203, and Ifi204 along with those of the human NLRP3 and ASC proteins. Then, equimolar concentrations of these PYRINs were used to stimulate human macrophages for 24h and then total RNAs were extracted and analyzed by qRT-PCR for the expression of proinflammatory cytokine genes. As shown in Figure 7A, TNFα, IL8, and IL1β mRNA expression levels were significantly upregulated when the cells were stimulated with all the PYRIN domains of the PYHIN proteins (> 40-fold for all the three cytokines when compared to untreated cells under any condition), being very similar to those reported upon exposure to the IFI16-PYRIN domain, even when murine proteins were used. Notably, we didn't observe any significant upregulation of the proinflammatory cytokines when the cells were stimulated with the NLRP3-PYRIN or with the ASC-PYRIN, compared to untreated cells. Enhanced release of TNF-α and IL-8 was also shown in the culture supernatants of the cells stimulated with the PYRIN domain from the PYHIN family members but not in those of the cells exposed to the NLRP3- PYRIN or to the ASC-PYRIN (Figure 7B). Importantly, the small-molecule TLR4 antagonist CLI-095 was able to block the proinflammatory activity elicited by the PYRIN domain of the PYHIN family members confirming their function through the canonical TLR4/MD2 pathway. Noteworthy, similar results were obtained when murine macrophages (i.e., RAW264.7) were used as target cells. Indeed, as shown in Figure 7C, both human and murine PYHIN PYRIN domains were able to induce TLR4-dependent TNF-α release in cell culture supernatants of murine macrophages, while the NLRP3-PYRIN or the ASC-PYRIN did not. Next, to confirm that the proinflammatory activity exerted by the

PYRIN domain derived from the PYHIN family members but not by other PYRIN domain derived from other families, e.g. NRLP3, consistently involved the TLR4/MD2 complex, increasing concentrations of hAIM2-PYRIN (human PYHIN), Ifi203- PYRIN (murine PYHIN) or NRLP3-PYRIN (non-PYHIN) were used to run SPR analyses on a sensor chip coated with TLR4/MD2. As expected, high affinity binding between both hAIM2 and Ifi203 PYRIN domains and TLR4/MD2 complex was found with a KD comparable to that observed with IFI16-PYRIN



(KD = 3.5*10-6 for hAIM2 and KD =6.1*10-6 for Ifi203). By contrast, NLRP3-PYRIN still retained binding affinity for the TLR4/MD2 complex but to much lower extent when compared to the hAIM2 and Ifi203 PYRIN domains (KD = 4.4*10-3) (Fig 12D). Altogether, these findings clearly indicate that the PYRIN domains of the PYHIN family members specifically retain the ability to induce a TLR4-mediated proinflammatory phenotype in target cells. Remarkably, this activity is observed in both human and mouse PYHIN members, suggesting a conserved interaction mechanism between the two species.

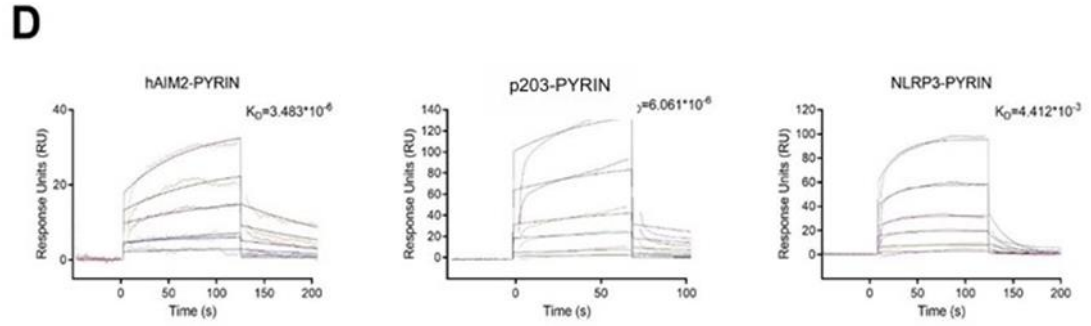


Figure 7. The proinflammatory activity of the PYRIN domain is conserved across the PYHIN family members. Equimolar concentrations of the PYRINs from different PYHIN family members, along with those from NLRP3 and ASC proteins, were then used to stimulate (A, B) human or C) mouse macrophages for 24h, with or without CLI-095 (TLR4 inhibitor, 5µM). Then, A) the relative amounts of TNF-a, IL8 and IL-1b mRNA were calculated through qRT-PCR analysis, and (B, C) TNFa and IL-8 release were measured through ELISA. Data are representative of three independent experiments, shown as the mean ± SD (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; one-way ANOVA followed by Dunnett's test). D) Surface plasmon resonance (SPR) analysis of different PYRINs' binding to immobilized TLR4. After immobilization of TLR4/MD2 on a CM5 sensor chip surface, increasing concentration of the indicated PYRIN domains (20–800 nM) diluted in running buffer were injected over immobilized complex. Data are representative of three different experiments, and for each analysis the dissociation constant (KD) value is shown.

5.4 Four amino acids located within the IFI16 PYRIN domain are critically

Having determined the TLR4-dependent proinflammatory activity of the PYHIN PYRIN domains, we chose IFI16-PYRIN as paradigmatic for the entire family to uncover the specificity of the interaction with the receptor at a structural level. To this end, we predicted its 3D structure taking advantage of Robetta server (Kim et al., 2004, Figure 8A) and used it to perform a molecular docking study with the extracellular portion of TLR4 (PDB:3FXI, Figure 8B) or with MD2 (PDB:2E56, Figure 8C) using High Ambiguity Driven protein-protein DOCKing (HADDOCK, van Zundert et al., 2016). In parallel, we performed sequences alignment of the PYHIN PYRIN domains, along with those of NLRP3 and ASC, to identify PYHIN-specific amino acids with similar chemical properties (Figure 8D). Notably, the integration of sequences alignment and molecular docking information, specifically considering i) the bindings length under 3Å, ii) the chemical nature of the amino acids involved, and iii) the characteristics of the PYHIN PYRIN domains, led to the identification of three residues in the IFI16-PYRIN that are potentially involved in the interaction with TLR4 — i.e., Lys34, Lys64, and Lys86 (Figure 8E) and one potentially involved in the interaction with MD2 —i.e., Glu85 (Figure 8F). Interestingly, the alignments between IFI16-PYRIN's projected 3D structure and those of NLRP3- and ASC-PYRIN using PyMOL software revealed that the amino acids in those positions are replaced not only by residues with different orientations but also shown an exeternal loop, that preventing the interaction with

the TLR4 receptor (Figure 9). Moreover, NLRP3- and ASC- PYRIN structures are characterized by an extended loop that could interfere with the receptor binding by steric hindrance phenomena (respectively Figure 9A and 9B). In particular, the loop moiety occupies the region that in IFI16-PYRIN is designated to interact with TLR4 through electrostatic interaction involving Lys34 and Lys64. To assess the significance of these predictions, and to confirm the contribution of these residues in activating the TLR4/MD2 receptor, a series of alanine mutations were constructed by site- directed mutagenesis of the identified polar residues, and the IFI16 mutants produced (namely, K34A, E85A, and K86A). Additionally, glycine mutagenesis was performed on the amino acid residue Lys64 (K64G mutant) to minimize the non-specific interaction that can occur with methyl group of alanine due to steric hindrance in its specific interaction background with TLR4. Finally, to validate the specificity of the interaction between PYHIN PYRIN domains and TLR4/MD2, alanine mutagenesis was also performed on a PYRIN superfamily conserved amino acid, in a region non predicted to be involved in the binding with the receptor —i.e., Ile17— resulting in the I17A mutant. All the IFI16 mutants, including the triple mutant (K34A/K64G/K86A), were then used along with the IFI16 wild-type protein to stimulate human macrophages for 24h at equimolar concentrations. Interestingly, as shown in Figure 10A a significant decrease in TNF-α production was detected in the culture supernatants of cells treated with the aforementioned mutants except for the I17A mutant. Specifically, the decrease in TNF-α release was about 95%, 75%, and 90% for the K34A, K64G, and K86A mutants, respectively, compared to the IFI16 wild- type protein, indicating their potential direct involvement in the IFI16-TLR4 binding process. Consistently, the triple mutant showed a similar decrease in TNF production (about 85%). Additionally, the E85A mutant, carrying a substitution in the amino acid implicated in MD2 binding, resulted in a significant decrease in TNF-α production (about 65%, compared to IFI16 wild-type protein) even if less pronounced compared to the other mutations targeting the amino acids implicated in TLR4 receptor binding. Next, SPR analysis was performed to establish the ability of each mutant to physically interact with the TLR4/MD2 receptor complex. To this end, increasing concentrations of the IFI16 mutants or the wild type form were flowed over the TLR4/MD2-coated chip. As shown in Figure 10B, we observed that the single mutations targeting the amino acids potentially involved in the interaction with TLR4 (K34A, K64G, and K86A) or MD2 (E85A) significantly affected the ability to bind the receptor, with consequences also in the kinetic of the interaction since the sensorgrams of the mutated variants do not reach the saturation levels representative of the steady state and the Kinetic constant measured is not statistically significative. Consistently, a similar picture was observed for the triple K34A/L64G/K86A mutant, while the I17A mutant still retained the high significantly affinity binding to the TLR4/MD2 receptor complex (KD=1.7*10-7) which was comparable to that observed with the wild type of form (KD=4.2*10-6).Taken together, these findings confirm the specificity of the interaction between PYHIN PYRIN

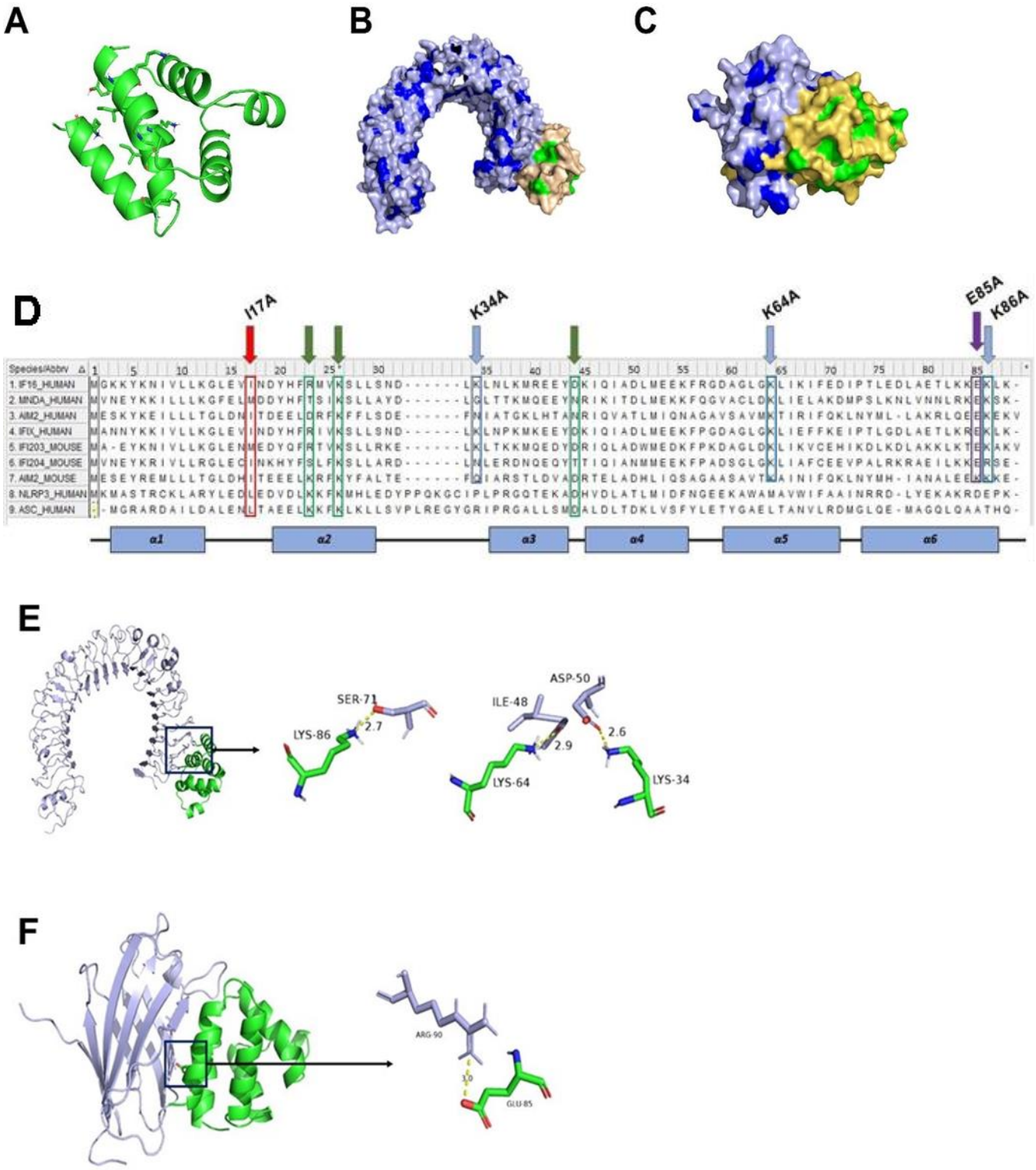


Figure 8. Structural analysis of the interaction moiety between the PYRIN domain of IFI16 and TLR4/MD2 complex. A) Predicted 3D structure of the IFI16 PYRIN domain using the Alphafold method (Jumper et al., Nature 2021). (B, C) Molecular docking analysis of the IFI16 PYRIN domain (green) and the extracellular portion of TLR4 (light blue) (PDB:3FXI, B) or with MD2 (PDB:2E56, C) using High Ambiguity Driven protein-protein DOCKing (HADDOCK, van Zundert et al., J. Mol. Biol., 2016). D) Alignment of the PYHIN PYRIN domains, along with those of NLRP3 and ASC, was performed using MEGAX and ClustalW algorithm to identify PYHIN-specific amino acids with similar chemical properties. Green arrows

identify amino acids conserved across the different proteins, whereas light blue and purple arrows identify amino acids that are conserved only across the PYHIN family members and that were mutated (see Figure 6). The red arrow identifies the aminoacid which is conserved in all the proteins and that wasmutated as control. The six α helices of the PYRINs with known structures are also indicated. (E, F) Identification of the IFI16-PYRIN residues (green) potentially involved in the interaction with TLR4 (Lys34, Lys64, and Lys86, E) or MD2 (Glu85),(F) obtained by integrating the alignment and molecular docking information.

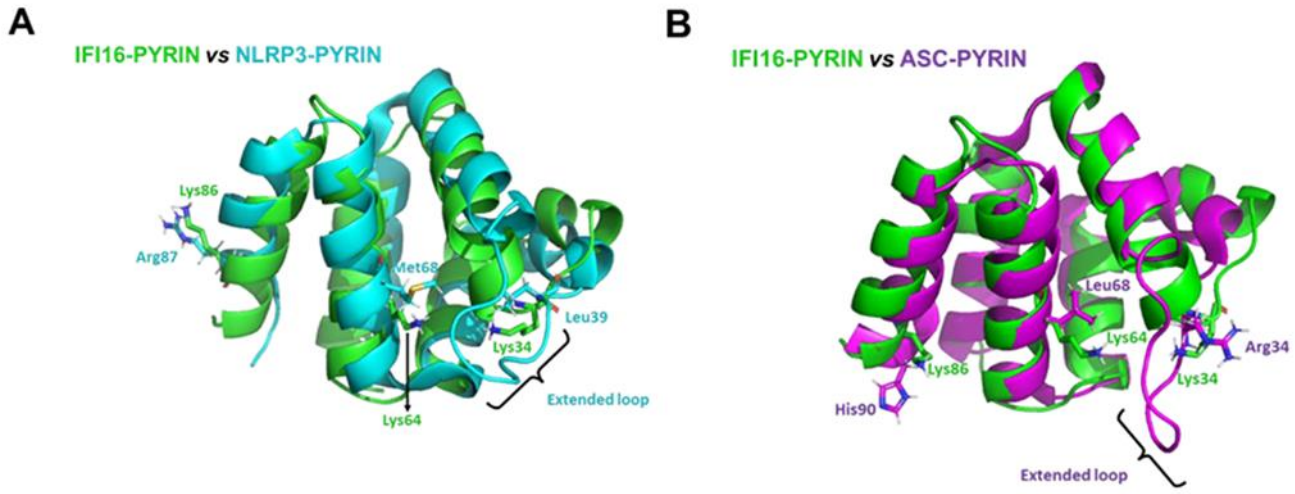


Figure 9. 3D structure alignment between the PYRIN domains of IFI16 and NLRP3 or ASC proteins. Superimposed alignments between the projected 3D structure of IFI16-PYRIN (green) and those of NLRP3-PYRIN (light blue, A) and of ASC-PYRIN (purple, B) using PyMOL software.

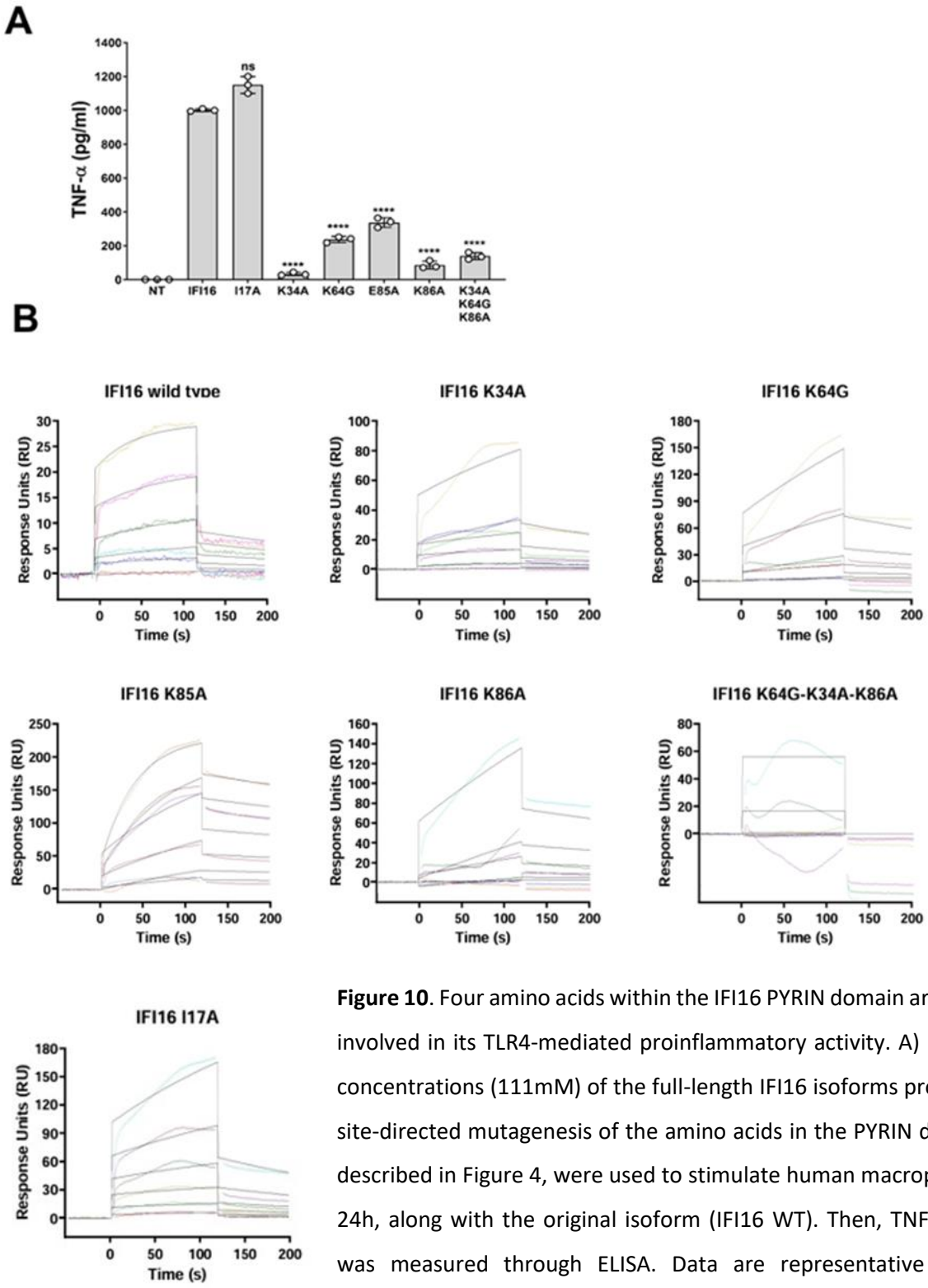


Figure 10. Four amino acids within the IFI16 PYRIN domain are critically involved in its TLR4-mediated proinflammatory activity. A) Equimolar concentrations (111mM) of the full-length IFI16 isoforms produced by site-directed mutagenesis of the amino acids in the PYRIN domain, as described in Figure 4, were used to stimulate human macrophages for 24h, along with the original isoform (IFI16 WT). Then, TNF-a release was measured through ELISA. Data are representative of three independent experiments, shown as the mean ± SD (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; one-way ANOVA followed by Dunnett's test).B) The same mutants were also used to run surface plasmon resonance (SPR) analysis to immobilized TLR4/MD2. After immobilization of TLR4/MD2 on the CM5 sensor chip surface, increasing concentration of IFI16 wt and mutants (20–800 nM) diluted in running buffer were injected over immobilized complex. Data are representative of three different experiments, and for each analysis the dissociation constant (Kd) is shown.

5.5 IFI16 is released in the extracellular milieu after stress stimuli

The results described so far prompted us to evaluate the pathogenetic mechanism underlying IFI16 release. Since we have previously observed the occurrence of antibodies against IFI16 in the sera of patients with SLE, as well as UVB-induced IFI16 release in keratinocytes from SLE patients (Costa et al., 2011), we reasoned that keratinocytes could represent a valuable model to characterize IFI16 release upon different stimuli. To this end, we took advantage of HaCaT cells, a spontaneously transformed immortal keratinocyte cell. We first confirmed that IFI16, normally restricted to the nucleus, can be induced to translocate in the extracellular milieu upon UVB- induced cell injury. Briefly, HaCaT were treated with different doses of UVB and then the presence of the protein in the culture supernatants was tested by immunoblotting. As shown in Figure 11A, IFI16 release was clearly both time- and dose-dependent, with maximum signals detected at 6hpt and upon 400-800J/m2 of UVB radiation, that were paralleled by a slight decrease of the intracellular protein. Analysis of LDH activity in the same supernatants confirmed that the extracellular presence of this soluble cytoplasmic protein as a consequence of cell death and lysis (Legrand et al., 1992) was barely detectable at 3hpt upon any irradiation doses even though start to increase at 6hpt with 200 and 400 J/m2 (~10% and ~20% of activity, respectively), when the IFI16 protein was already detectable. At higher irradiation dose (800J/m2), LDH activity increased dramatically in the extracellular medium, very likely due to cellular membrane rupture and the nonselective leakage of cytoplasmic proteins (Figure 11B, red bars). As further vitality control, we measured the cell viability and metabolic activity through the Alamar blue assay (Bonnier et al., 2015). As shown in Figure 11B (green bars), the metabolic activity decreasing compared with not treated cells was always not less than 75% regardless of the type of treatment, suggesting that IFI16 release could be associated to a partial active mechanism. To follow the localization of IFI16 after UVB irradiation, we generated a chimera form where the full-length protein was fused with a mCherry fluorescent dye. Stable IFI16-knockout human keratinocytes (HaCaT IFI16KO cells) were transfected with IFI16-mCherry plasmid and then, after 48h, cells were irradiated with 400J/m2 of UVB and inserted in the Incucyte system. In IFI16-mCherry-transfected cells the fluorescent signal clearly spread and increase in the surrounding cells after 18h of incubation, whereas in cells transfected with the empty vector (mCherry alone) the fluorescent signal was constantly restricted to transfected cells (Figure 11C). Although further controls should be generated, this experiment can be considered the first qualitative direct evidence proving the extracellular mobilization of IFI16 upon UVB treatment. Then, to assess whether acute inflammation involving the epithelial cells, as those observed in some autoimmune conditions (Pisetsky, 2023) or viral infections [(Milora et al., 2014); (Novak et al., 2021)], could be also associated to IFI16 release, HaCaT cells were subjected to TNFa treatment. As shown in Figure 12A, 24h of treatment with 100ng/ml of TNF induced a considerable release of IFI16 in the supernatants, paralleled by an important decrease of protein expression in the total extracts. A significant IFI16 release occurred also at 48hpt, whereas these conditions were associated to significant cells death as

demonstrated by the increase of LDH release (Figure 12B) used as a hallmark of cell distress (Takasu et al., 2016).

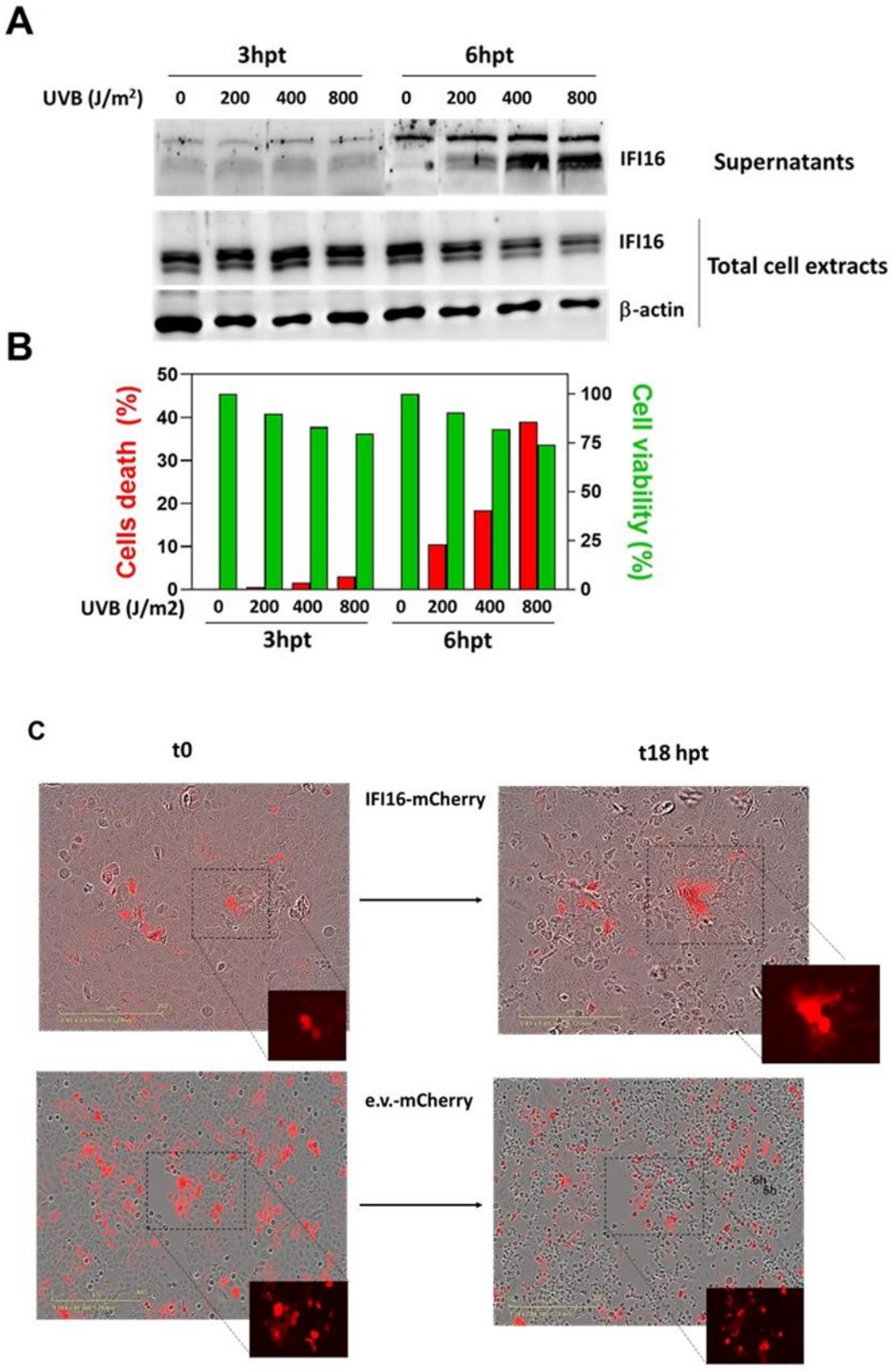


Figure 11. IFI16 release from HaCaT cells upon UVB exposure. (**A, B**) HaCaT cells were irradiated at the indicated doses of UVB (*i.e.* 200, 400, 800 J/m^2), for the indicated period of time (*i.e.* 3 and 6 hpt*)*. Then, **A**) immunoblotting with anti-IFI16 antibody were performed by using 30ul of supernatants and 20ug of total cell extracts (b-actin was used as loading control). **B**) The same supernatants were also usedto test LDH release (red bars), and cells were tested for metabolic activity through Alamar blue assay (green bars). (**C**) IFI16KO-HaCaT cells, transfected with the IFI16-mCherry construct or with empty control vector (e.v.-mCherry), were UVB irradiated (400 J/m2) and monitored in an Incucyte system every 30 minute. Representative figures taken at T0 (immediately after the UVB exposure, left panels) and 18 hours after UVB exposure (T18, right panels) with bright-field and a red fluorescent filter (for mCherry) are shown.

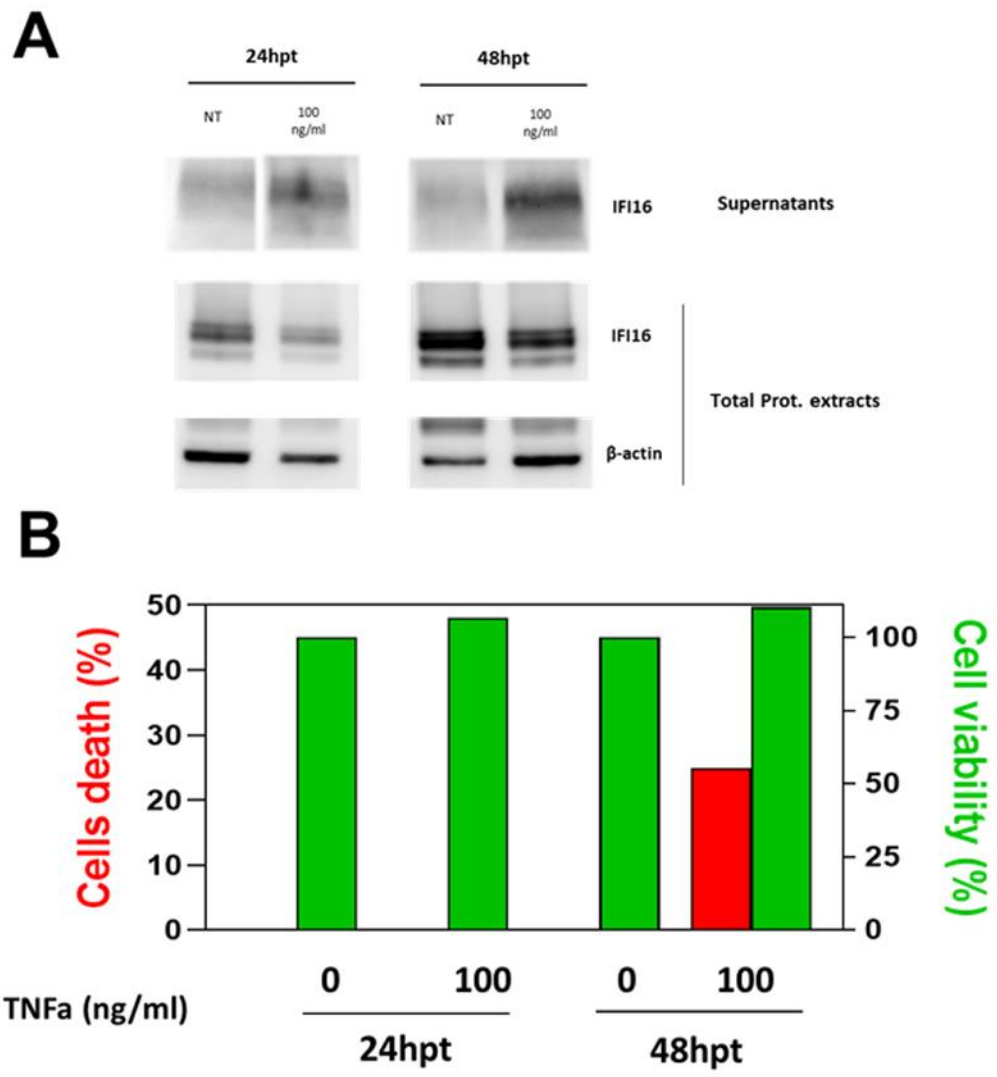


Figure 12. IFI16 release from HaCaT cells upon TNFα treatment. Cells were treated with 100 ng/ml of TNFα, for the indicated period of time (24 and 48hpt). Then, **A**) immunoblotting with anti-IFI16 antibody were performed using 30ul of supernatants and 20ug of total cell extracts (b-actin was used as loading control). **B)** The same supernatants were also used to test the LDH release (red bars) and cells were also tested for metabolic activity through Alamar blue assay (green bars).

6. Discussion

Besides sensing exogenous microbial components, in recent years TLRs have been shown to be involved in the recognition of endogenous material released during cellular injury, thereby promoting a non-microbial-induced inflammatory state known as sterile inflammation. If not resolved, sterile inflammation can in turn lead to severe acute and chronic inflammatory conditions [(Piccinini and Midwood, 2010); (Rifkin et al., 2005); (Schaefer, 2014)]. Recently, we have demonstrated that IFI16 represents a novel trigger of sterile inflammation acting through the TLR4 signaling pathway (Iannucci et al., 2020). Here, we expanded on those observations by systematically dissecting the IFI16 sequence elements to define the IFI16 and TLR4 interaction and signaling mechanism. For the first time, we showed that the proinflammatory activity of the IFI16 protein specifically lies within its N-terminal region and that the IFI16-PYRIN domain is involved in TLR4/MD2 binding and activation. In relation to this, antibodies against the N-terminal part of IFI16 i) decreased the ability to induce inflammation in human macrophages in a dose-dependent fashion, and ii) decreased the ability to bind the TLR4/MD2 complex in saturation binding experiments. This led us to perform experiments by using specific recombinant subdomain of IFI16. Among all, only the PYRIN domain was able to induce IL-1β, IL-8 and TNF-α transcriptional activation and cytokine release into the supernatants of human macrophages. Moreover, by means of in vivo co- immunoprecipitation assay and in vitro saturation binding experiments, we have provided the first evidence that the IFI16-PYRIN directly mediates the binding with TLR4/MD2 complex. Interestingly, IFI16 pro-inflammatory activity lies within its PYRIN domain, which is necessary and sufficient to induce overexpression of pro-inflammatory cytokines. The PYRIN domain is commonly composed of six α-helices tightly packed around a hydrophobic core with a highly charged surface like other members of the death domain (DD) superfamily (Jin et al., 2013). Indeed, it is widely spread along different proteins (Dorfleutner et al., 2015). There are more than 20 PYRIN-containing proteins in the human genome (Kwon et al., 2012), most of which share some residues in their PYRIN domains (Jin et al., 2013), nevertheless no one consensus protein sequence for the PYRIN domain exists across all. Our hypothesis that specific members of PYHIN family could mimic the activity of IFI16-PYRIN domain, paving the way toward the identification of a new class of DAMPs that, once extracellularly released by damaged cells, can trigger sterile inflammation through the engagement of the TLR4/MD2 receptor. In line with this hypothesis, we here provide evidence that the proinflammatory activity of the PYRIN domain is conserved among the PYHIN family members, including both the human and mouse gene families. By producing the recombinant PYRIN

domains belonging to different PYHIN proteins we confirmed that sterile inflammation in human and mouse macrophages occurred through the TLR4 pathway, in the same way as for IFI16-PYRIN. The inflammatory signature significantly decreased when we utilized a selective signaling inhibitor of TLR4 (i.e., CLI-095) which hinders both the MyD88- and the TRIF-dependent pathways by binding the cysteine 747 in the intracellular domain of TLR4 (Kawamoto et al., 2008)]. Along with inducing inflammation, we also tested the ability to bind the TLR4/MD2 complex in saturation binding experiments. Consistent with the observation with IFI16-PYRIN, human AIM2-PYRIN and mouse p203-PYRIN (chosen as representative of the PYHIN family) shared the ability to bind TLR4 receptor with high-affinity, while the NLRP3- PYRIN (as paradigm of non-PYHIN protein) did not. All in all, we propose that the PYHIN- PYRINs can be considered as a new class of DAMPs. Finally, we have also mapped and those specific amino acids located within the IFI16-PYRIN domain and partially conserved across the PHYN family but absent in the PYRIN domain from other gene families, e.g. NRLP3, that are critically involved in their TLR4-mediated proinflammatory activity of the family members. In this context we chose IFI16 as paradigmatic of the entire PYHIN family to underline the strength interaction with TLR4/MD2 receptor complex to find and inhibit them with new small drugs. Thanks to structural prediction software, we hypothesized either the molecular structure and the interaction mode between IFI16PYRIN and the extracellular domain of TLR4 receptor, revealing three amino acids of IFI16PYRIN involved in the binding with TLR4 and one amino acid with MD2. By site-directed mutagenesis, we generated mutated recombinant domains in the predicted amino acids, and we showed that by mutating them there was a significant decrease in both cytokine release and kinetics of receptor binding. To support this idea, the comparison of quaternary structures between IFI16-PYRIN and NLRP3-PYRIN or ASC-PYRIN, revealed that the amino acids mutated in IFI16 correspond to residues lying in an external loop in both NLRP3 and ASC. This observation suggests a potential explanation for why neither NLRP3 nor ASC can bind to TLR4/MD2. Remarkably, all these information allow to identify a specific region of IFI16 Pyrin responsible for the binding to TLR4/MD2 complex and pave the way for the synthesis new compounds specifically targeting this interaction as future strategy against autoinflammatory/autoimmune disease. In this regard, abnormal IFI16 expression in otherwise negative cells, or IFI16 delocalization, has been reported in a variety of inflammatory conditions, including SLE (skin, Costa et al., 2011), psoriasis (skin), [Cao et al., 2016; (Chiliveru et al., 2014); Tervaniemi et al., 2016), SSc (skin), (Mondini et al., 2006), IBD (colonic epithelium) [(Caneparo et al., 2016; Vanhove et al., 2015) and SS (salivary epithelial and inflammatory infiltrating cells, [(Alunno et al., 2015); Alunno et al., 2016; Antiochos et al., 2019)]. We discovered that high levels of circulating IFI16 (27 ng/ml) were related with overall worse clinical parameters in three RA, SS, and PsA patient cohorts. Interestingly, circulating IFI16 was discovered more often in RA patients with RF/anti-CCP- positive blood and was substantially linked with pulmonary involvement (Alunno et al., 2016). Furthermore, in SS patients, circulating IFI16 is associated with an increased prevalence of both RF and the degree of glandular infiltration, whereas in PsA patients, it correlates with heightened C-reactive protein (CRP) levels (De Andrea et al., 2020).

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