



# Functional assessment of the mutational effects of human *IRAK4* and *MyD88* genes

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## ABSTRACT

Human interleukin-1 receptor-associated kinase 4 (IRAK4) deficiency and myeloid differentiating factor 88 (MyD88) deficiency syndromes are two primary immune-deficiency disorders with innate immune defects. Although new genetic variations of *IRAK4* and *MyD88* have recently been deposited in the single nucleotide polymorphism (SNP) database, the clinical significance of these variants has not yet been established. Therefore, it is important to establish methods for assessing the association of each gene variation with human diseases. Because cell-based assays, western blotting and an NF- $\kappa$ B reporter gene assay, showed no difference in protein expression and NF- $\kappa$ B activity between R12C and wild-type IRAK4, we examined protein–protein interactions of purified recombinant IRAK4 and MyD88 proteins by analytical gel filtration and NMR titration. We found that the variant of IRAK4, R12C, as well as R20W, located in the death domain of IRAK4 and regarded as a SNP, caused a loss of interaction with MyD88. Our studies suggest that not only the loss of protein expression but also the defect of Myddosome formation could cause IRAK4 and MyD88 deficiency syndromes. Moreover a combination of *in vitro* functional assays is effective for confirming the pathogenicity of mutants found in IRAK4 and MyD88-deficiency patients.

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## 1. Introduction

Interleukin-1 receptor-associated kinase (IRAK) 4 is the one of the essential molecules of the Toll/interleukin-1 receptor signaling pathway (Suzuki et al., 2002). In this pathway, ligand-induced hetero- or homodimerization of receptors recruits the Toll/interleukin-1 receptor homology domain (TIR domain) containing adaptor oligomers. One of these adaptors, MyD88, then binds IRAK4 (Burns et al., 2003). Recently, defects in the innate

**Abbreviations:** DD, death domain; HEK, human embryonic kidney; ID, internal domain; IRAK, Interleukin-1 receptor-associated kinase; MyD88, myeloid differentiating factor 88; Mal, MyD88 adaptor-like; NMR, nuclear magnetic resonance; ELISA, enzyme-linked immunosorbent assay; TIR domain, Toll/interleukin-1 receptor homology domain; WT, wild type; SNP, single nucleotide polymorphism; IRAK4-DD, death domain of IRAK4; IRAK4-DD+ID, death domain and internal domain of IRAK4; MyD88-DD, death domain of MyD88; MyD88-DD+ID, death domain and internal domain of MyD88; MyD88-TIR, TIR domain of MyD88; Mal-TIR, TIR domain of Mal; TRAF, TNF receptor associated factor.

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immune system have been shown to cause newly categorized human primary immune-deficiency syndromes (Al-Herz et al., 2011) such as human IRAK4 deficiency (Picard et al., 2003).

In affected IRAK4 deficient patients, invasive infections such as bacterial meningitis, sepsis, arthritis, or osteomyelitis are caused by *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Picard et al., 2010). Human MyD88 deficiency (von Bernuth et al., 2008) has remarkably similar clinical features to human IRAK4 deficiency. Interestingly, the life-threatening infections in IRAK4 or MyD88 deficient patients first occur during early infancy, but their frequency and severity reduce after the teenage years (Picard et al., 2011). Therefore, it is necessary for them to be diagnosed quickly.

IRAK4 and MyD88 proteins both consist of two major functional domains. In IRAK4, the death domain (DD) interacts with MyD88, while the kinase domain phosphorylates downstream signaling factors such as IRAK1, IRAK2, and subsequently causes activation of TNF Receptor Associated Factor 6 (TRAF6). In MyD88, both the DD and TIR domains interact in homotypic binding to similar domain structures. The domain–domain interactions are critical for these signaling pathways. IRAK4 and MyD88 form a hetero-oligomeric signaling complex via a shared DD, so-called Myddosome (Motshwene et al., 2009). Appropriate Myddosome

formation can induce activation of the downstream signaling pathway, which eventually leads to the activation of NF- $\kappa$ B and activator protein 1 (AP-1).

Most previously identified causative mutations of human IRAK4 deficiency are nonsense or frame shift mutations that create early stop codons (Cardenes et al., 2006; Davidson et al., 2006; Enders et al., 2004; Krause et al., 2009; Ku et al., 2007; Medvedev et al., 2003; Picard et al., 2010; Takada et al., 2006; Yoshikawa et al., 2010), however, three missense mutations (M1V, R12C, and G298D) have been reported (Bouma et al., 2009; de Beaucoudrey et al., 2008; Hoarau et al., 2007). In human MyD88 deficiency, one nonsense mutation (E53X) and three missense mutations (E52del, L93P, and R196C) were reported as causative mutations (Conway et al., 2010; von Bernuth et al., 2008). Recently, new gene variations of *IRAK4* and *MyD88* have been deposited in the single nucleotide polymorphism (SNP) database following next-generation DNA sequencing, but the significance of these variants has not been evaluated. It is therefore important to establish methods to determine the association of gene variations with human diseases. For example, about MyD88, previous attempts have used western blotting, reporter gene assays, immunoprecipitation, and size exclusion chromatography of recombinant proteins to show that the SNPs MyD88 S34Y and R98C were loss-of-function variants (George et al., 2011), while another study used immunofluorescence to determine that S34Y fails to interact with IRAK4 (Nagpal et al., 2011).

Methods to detect the impaired responses to the Toll/interleukin-1 receptor agonists, such as enzyme-linked immunosorbent assay (ELISA) and flow-cytometry, are useful for rapid screening of innate immune deficiency syndromes (Davidson et al., 2006; Ohnishi et al., 2012a; Takada et al., 2006; von Bernuth et al., 2006). However, no *in vitro* method to assess the pathogenicity of novel variants of human *IRAK4*, *MyD88* and the other possible signaling components has been established. Therefore, when novel gene variants are found in that possible cases of IRAK4 or MyD88 deficiency syndromes, it is difficult to analyze the pathogenetic significance of these variants. In this study, we used a cell-based assay as well as *in vitro* protein-interaction analyses to show that IRAK4 R12C and R20W caused a loss of interaction with MyD88. This suggested that not only the loss of full-length IRAK4 and MyD88 protein expression but also the loss of Myddosome formation could cause IRAK4 and MyD88 deficiency syndromes.

## 2. Materials and methods

### 2.1. Cell culture

Human embryonic kidney (HEK) 293T cells were cultured in high glucose-containing DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 2.2. Vector preparations

cDNA encoding full-length IRAK4 (amino acid residues 1–460) or the DD and the internal domain (ID) of IRAK4 (IRAK4-DD+ID, amino acid residues 1–150) were tagged at the N terminus with a FLAG-epitope and cloned into the plasmid vector pcDNA3.1+ (Invitrogen). M1V was tagged at the C terminus because of a substitution of the start codon, and wild type (WT) tagged at the C terminus was prepared as a reference. Similarly, cDNA encoding full-length MyD88 (amino acid residues 1–296) or the TIR domain of MyD88

(MyD88-TIR, amino acid residues 148–296) tagged at the N terminus with a myc-epitope were cloned into the plasmid vector pcDNA3.1+ (Nada et al., 2012; Ohnishi et al., 2009). IRAK4 mutants and SNPs taken from dbSNP135 of the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/snp>) were generated using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Fitchburg, WI). The pUNO hIL1R1(mb) vector (InvivoGen, San Diego, CA) was purchased and cDNA encoding IL-1RAcP and IL-18RAcP were cloned into the plasmid vector pcDNA3.1+. The pGL4.32[luc2P/NF- $\kappa$ B-RE/Hygro] vector, used as an NF- $\kappa$ B luciferase reporter vector, and the pGL4.70[hRluc] vector, used as an internal control Renilla luciferase reporter vector, were purchased from Promega.

### 2.3. Western blot analysis

To detect protein expression, HEK293T cells were seeded on six-well plates at a density of  $2 \times 10^5$ /ml and transfected with 1  $\mu$ g of expression plasmids of FLAG-tagged IRAK4 full length, FLAG-tagged IRAK4-DD, and myc-tagged MyD88 full length using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h incubation, cells were harvested, washed with PBS, and lysed using CytoBuster Protein Extraction Reagent (Novagen, Darmstadt, Germany) containing a protease inhibitor mix (Roche Applied Science, Indianapolis, IN). All extracts were adjusted to contain equal amounts of total cellular proteins, as determined using the Bradford method. Supernatants and whole cell lysates were separated by electrophoresis on SDS polyacrylamide gels and transferred to nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen). Membranes were blocked for 1 h in 5% BSA in TBST (pH 8.0, 10 mM Tris buffer containing 0.15 M NaCl and 0.1% Tween 20), then incubated at room temperature for 2 h with an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), anti-myc antibody (Invitrogen), or anti- $\beta$ -actin antibody (Sigma-Aldrich) followed by incubation with anti-mouse IgG HRP conjugate (Promega) at room temperature for 30 min. Detection was performed using the ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) and LightCapture system AE6970CP (ATTO, Tokyo, Japan).

### 2.4. NF- $\kappa$ B reporter gene activity

For the functional assessment of IRAK4, HEK293T cells, HEK293-hTLR1/2 cells (InvivoGen), HEK293-hTLR4-MD2-CD14 cells (InvivoGen) and HEK293-hTLR5 cells (InvivoGen) were transfected with NF- $\kappa$ B luciferase reporter vector, Renilla luciferase reporter vector, pcDNA3.1+ empty vector or pcDNA3.1+ FLAG-IRAK4 WT using Lipofectamine 2000. After transfection, cells were incubated for 24 h then stimulated with recombinant IL-1 $\beta$  (10 ng/ml) prepared as previously described (Wang et al., 2010), Pam3CSK4 (10 ng/ml, InvivoGen), LPS (10 ng/ml, Sigma-Aldrich), and recFLA-ST (10 ng/ml, InvivoGen) for 6 h. In a similar way, HEK293T cells were transfected as described above and with IL-18RAcP, and stimulated with recombinant IL-18 (50 ng/ml) prepared as previously described (Kato et al., 2003; Li et al., 2003) for 6 h. Luciferase reporter gene activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega). Similarly, HEK293T cells were transfected with pUNO-hIL1R1 vector, pcDNA3.1+ IL-1RAcP vector, NF- $\kappa$ B luciferase reporter vector, Renilla luciferase reporter vector, pcDNA3.1+ empty vector or pcDNA3.1+ FLAG-IRAK4 WT or variants, as described above. After transfection, cells were incubated for 24 h and luciferase reporter gene activities were analyzed.

For the functional assessment of MyD88, HEK293T cells were transfected with NF- $\kappa$ B luciferase reporter vector, Renilla luciferase reporter vector, and different amounts of pcDNA3.1+ myc-MyD88

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