UNIVERSITY OF AMSTERDAM

Analysis of the structure function of the human IRAK-M Death Domain
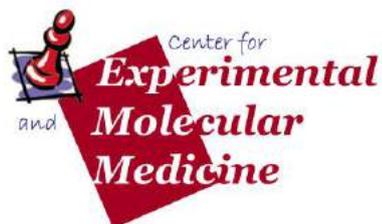
Liza Pereverzeva
6059163

Daily/Senior tutor:
C. van 't Veer, PhD
E-mail: c.vantveer@amc.uva.nl
Tel. number: +31 20 566 65928

Center for Experimental Molecular Medicine, AMC

ABSTRACT

IRAK-M is a negative regulator of the innate immunity by inhibition of the Toll-like receptor signaling. This protein is expressed in lung epithelial cells, monocytes and macrophages. IRAK-M deficient mice showed an increased inflammatory response and better outcomes of both mortality in bacterial pneumonia and host response in tumor models. Therefore, IRAK-M is believed to be a potential therapeutical target. IRAK-M contains a N-terminal Death Domain (DD), an inactive kinase domain and a C-terminal domain. As the interaction with other signaling proteins takes place through the DD, further understanding of the structural function of the human IRAK-M-DD is crucial before inhibitors against IRAK-M may be developed.

This study investigates the structure function of the human IRAK-M-DD by transfecting several cell lines with IRAK-M-DD mutants and other signaling proteins of interest. Through immunoprecipitation of WT IRAK-M and IRAK-M mutants, the complex formation of IRAK-M with MyD88/IRAK-4, IRAK-4/TRAF6 and IRAK-1/TRAF6 could be further characterized.

This research demonstrates that IRAK-M-DD residue Y105 in combination with R97 plays a pivotal role in the IL-8 production and complex formation of IRAK-M with TRAF6. Furthermore, a new schematic representation of the IRAK-M, IRAK-4 and TRAF6 complex formation is demonstrated, taking into account that TRAF6 forms a trimeric complex through its TRAF-domains and dimerizes with its RING and zinc finger domains. Lastly, our results suggest that the active kinase domains of IRAK-1 and IRAK-4 play a role in the phosphorylation of TRAF6. Therefore, this study contributes to the knowledge of the complex formation between IRAK-M and important signaling proteins in the pathways of innate immunity.

INTRODUCTION

Interleukin-1 receptor-associated kinase M (IRAK-M) is one of the IRAK proteins that are involved in pathways of innate immunity. The IRAK family (IRAK-1, -2, -M and -4) plays a part in the signaling of the cytokines interleukin-1 (IL-1) and interleukin-18 (IL-18), as well as in the the Toll-like receptor (TLR) signaling, which results in nuclear factor-κB (NF-κB) activation and transcription of inflammatory genes [1]. The interleukins act as an endogenous pyrogen and have potentiating effects on cell proliferation, differentiation, and functioning of many innate and specific immunocompetent cells. Many inflammatory diseases are mediated by IL-1, which initiates and potentiates immune and inflammatory responses [2]. Toll-like receptors are pattern-recognition receptors (PRR) that are able to recognize pathogen-associated molecular patterns (PAMPs). These PAMPs are ideal targets for the innate immunity to recognize microorganisms, because they are solely produced by microbes and not by host cells. Moreover, they are almost invariant between microorganisms of a given class and they are essential for microbial survival, hence there are virtual no 'escape mutants' that survive [3].
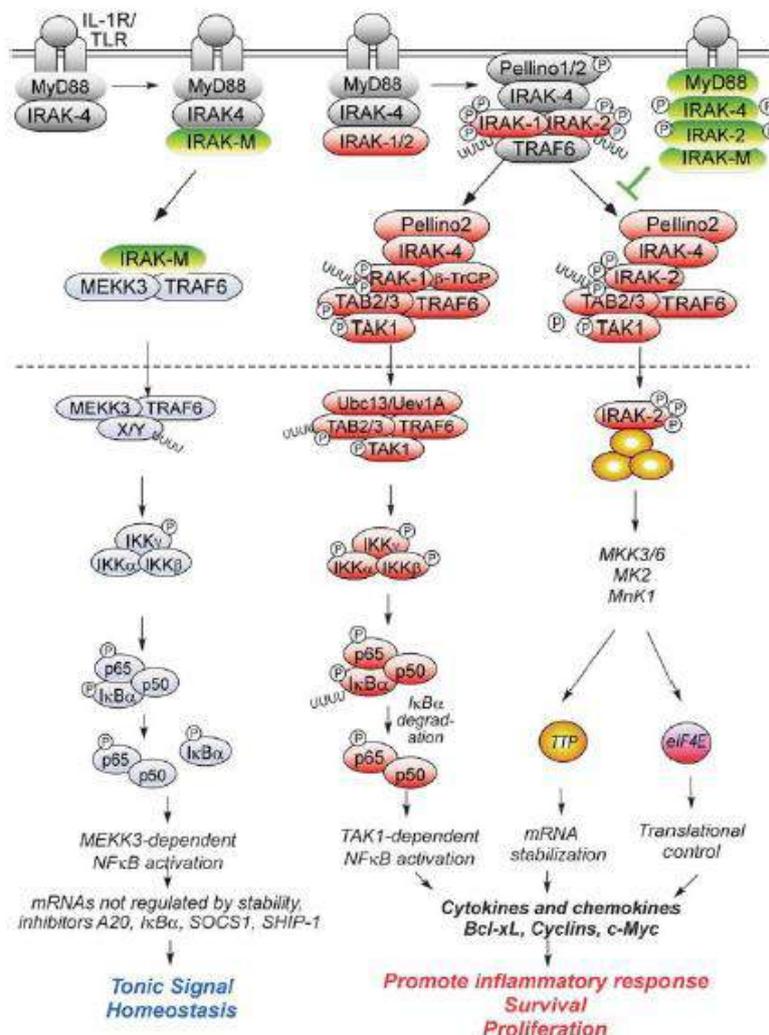

Figure 1. *Model for the regulatory roles of IRAK-1, IRAK-2, IRAK-4 and IRAK-M in TLR-IL-1R signaling. IRAK-4 interactions with IRAK-1 and IRAK-2 give rise to an inflammatory response by NFκB activation and translational control and mRNA stabilization of pro-inflammatory genes. IRAK-M creates an overall inhibitory effect on inflammatory response through the MEKK3 dependent pathway. Also, IRAK-M specifically interacts with IRAK-2, suppressing TLR-IL-1R-induced IRAK-2-mediated translation of cytokines and chemokines. Figure from Zhou et al. [4].*

The signaling pathway of the Toll and IL-1 receptor (IL-1R) families includes the adaptor protein called myeloid differentiation protein 88 (MyD88), which dimerizes by activation of the TLR/IL-1R. Subsequently, IRAK-4 and other IRAK's are recruited, to form multimeric complexes, known as myddosomes, through interaction of the death domains (DD) of these proteins. The myddosome MyD88/IRAK-4/IRAK-1 leads to auto- and hyper-phosphorylation of IRAK-1 and the binding of TNF receptor-associated factor 6 (TRAF6), which forms a complex with IRAK-1, after which IRAK-1 and TRAF6 dissociate from MyD88. This complex activates TAB2/3 and TAK-1 to eventually activate NF-κB and transcription of inflammatory genes [5]. Hyper-phosphorylation of IRAK-2 and TAB/TAK/TRAF6 activity results in specific IRAK-2 dependent mRNA stabilization and a translational control of pro-inflammatory genes [6]. As described, IRAK-1, IRAK-2 and IRAK-4 have a pro-inflammatory effect by activating NF-κB. In contrast, IRAK-M is found to be a negative regulator of TLR signaling, illustrated in IRAK-M deficient mice with an increased inflammatory response [7]. These mice turned out to have better outcomes of mortality in bacterial pneumonia [8,9], an improved host response in tumor models [10] and bone marrow transplantation [11]. In humans injected with the endotoxin LPS, the LPS tolerance phase was found to be associated with higher whole blood transcript levels of IRAK-M [12]. Therefore, inhibition of IRAK-M appears to have a therapeutic potential in infections and tumor models.

IRAK-M is found to be expressed in lung epithelial cells, monocytes and macrophages. Zhou *et al.* [4] found IRAK-M to be interacting with MyD88/IRAK-4, forming a IRAK-M myddosome to mediate TLR7-induced MEKK3-dependent second wave NF-κB activation. Although IRAK-M is able to form a myddosome (like the other IRAKs), this IRAK-M-dependent pathway only induces expression of genes that are not regulated at the post-transcriptional levels (including inhibitory molecules SOCS-1, SHIP-1, A20 and IκBα), leading to an overall inhibitory effect on inflammatory response. IRAK-M is involved in a second pathway, where it specifically inhibits the IRAK-2-mediated translation of cytokines and chemokines (Figure 2). The structure of IRAK-M is similar to the other IRAKs: it contains a N-terminal Death Domain (DD), which is crucial for binding to other IRAKs and therefore in activating NF-κB, an inactive kinase domain (KD) and an unstructured C-terminal domain (CTD) with a TRAF6 binding site [1]. Zhou *et al.* [4] explored the functional relationships between IRAK-M and the other IRAKs in a murine model, leading to the functional model of IRAK-M pictured in Figure 1. However, it is demonstrated that the IRAK-pathway differs in humans and mice, illustrating that the human TLR signaling was particularly dependent on IRAK-1, while the murine system is more dependent on IRAK-2 [13]. Therefore, further investigation of the human IRAK-M structure function is important, specifically its death domain, as it is crucial in the interaction with other proteins.
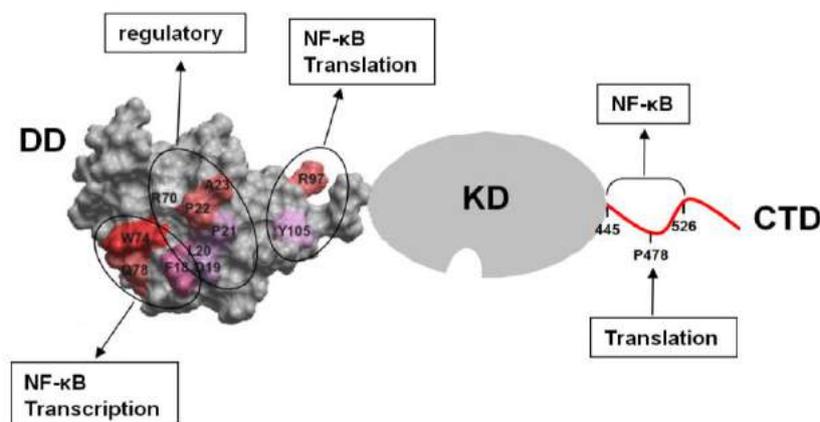

Figure 2. Schematic representation of the structure of IRAK-M and the functional residues of its death domain and C-terminus, involved in NF-κB dependent transcription and translation. Figure from Du et al. [1].

Previous research on the human IRAK-M DD by Du *et al.* [1] predicted that DD residue W74 was essential for the binding of IRAK-M to IRAK-4, preceding MEKK3 dependent NF-κB activation and expression of inhibitory genes. In addition to W74, IRAK-M DD residue R97 was identified to be involved in NF-κB activation. These two sites are predicted to be located at opposite positions on the DD and are predicted to bind on different sides of the IRAK-4 DD. These findings formed the hypothesis of an IRAK-M/IRAK-4 sandwich, in which an IRAK-4 tetramer is sandwiched by two IRAK-M tetramers and forms a complex with TRAF6. Recent research of Nollet [14] observed that the IRAK-M W74A mutant stimulated IL-8 release driven by the R97Q mutant while W74A itself had no IL-8 stimulation activity at all. When the W74 mutant was co-expressed with the R97Q/Y105A double mutant, no IL-8 expression occurred, indicating that Y105A fulfils a pivotal role in IL-8 production. The interaction of TRAF6 and IRAK-M is described by Banchaewa [15], who demonstrated that for this interaction residues W74 and R97/Y105 are essential. Furthermore, Du *et al.* [1] demonstrated that apart from its death domain, the C-terminus of IRAK-M is also of great importance, providing the TRAF6 binding site P478. A schematic representation of the IRAK-M/IRAK-4/TRAF6 complex is provided in figure 3.
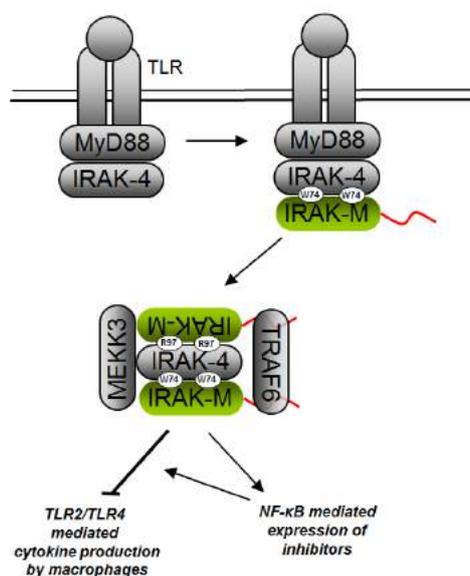

Figure 3. Schematic representation of the IRAK-M/IRAK-4 sandwich that gives rise to IRAK-M's anti-inflammatory effects, emphasizing the pivotal residues of IRAK-M, W74 on one side and R97 on the opposite side. Figure adapted from Du et al. [1].

Nollet [14] demonstrated that a stable MyD88/IRAK-4/IRAK-M complex could be found in 293T cells by immunoprecipitating IRAK-M. Surprisingly, IRAK-M was not recognized by a polyclonal antibody directed against the very C-terminal end of IRAK-M when in complex with MyD88/IRAK-4. This suggests that the C-terminus of IRAK-M, which contains two tyrosines (Y590 and Y593), becomes modified when forming a complex with MyD88/IRAK-4. However, the exact modification has not been described yet.

In this study we will further investigate the structure function of the human IRAK-M death domain and its complex formation with other regulatory proteins by immunoprecipitation of IRAK-M in several cell lines. We will investigate the formation of the IRAK-4/IRAK-M sandwich and its complex with TRAF6 by transfecting cells with IRAK-M, IRAK-4 and TRAF6 vectors. Also, we will try to understand what the role is of DD residue Y105, by generating a W74A/Y105A double mutant and determine its IL-8 stimulatory capacity when co-expressed with the R97Q mutant. Finally, we will attempt to determine the exact modification of the C-terminus when in complex with MyD88/IRAK-4, illustrating whether phosphorylation or ubiquitination occurs.

METHODS

Immunoprecipitation

Sample preparation for immunoprecipitation in 293T cells and HEK Null cells

Human embryonic kidney (HEK) 293T cells (ATCC) and HEK-Blue Null cells (Invivogen) were cultured in DMEM with 10% FCS, 2 mM L-glutamine, pen/strep (complete). For transfection and subsequent immunoprecipitation 700,000 cells were plated in 2 mL of complete DMEM without antibiotics in a 6-well plate. They were grown overnight at 37°C in a 5% CO_2 incubator, to a 70-90% confluence at the time of transfection. The plasmid DNA transfection was done in duplicate wells for each condition, diluting a total of 4 µg of DNA in 250 µL DMEM per well. 10 µL Lipofectamine® 2000 (Invitrogen) was diluted in 250 µL DMEM per well and incubated for 5 – 25 minutes at room temperature. After this, the diluted DNA was combined with diluted Lipofectamine® 2000, after which it was left to incubate for 20 minutes at room temperature. After the incubation, 500 µL of the transfection solution was added to each well containing cells and medium. The cells were incubated at 37°C in a 5% CO_2 incubator overnight.

293T and HEK Null cells were detached from the wells by pipetting and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was removed and replaced with 10 mL cold PBS, after which the samples were vortexed and centrifuged at 1500 rpm for 5 minutes at 4°C. All PBS was removed, and the pellet was resuspended in 400 µL cold Cell Lysis Buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin) (Cell Signaling), supplemented with 1:100 Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). This was incubated on ice for 10 minutes, following by sonication at 30% amplitude for 5 seconds with a 3 mm probe in a VCX 130 sonicator from Vibra-Cell™, Sonics®. Subsequently, samples were centrifuged at 14,000 rpm at 4°C for 10 minutes.

Immunoprecipitation

To 50 µL of the cell lysis supernatant, 50 µL 3x SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol and 0.03% bromophenol blue) was added and stored as lysate samples at -20°C. 300 µL of the lysate was used for the immunoprecipitation. 1,5 µg of the monoclonal anti-IRAK-M antibody 1F6 (Abnova) was added, after which the samples were vortexed and incubated on ice for 1 hour. Next, 50 µL A/G PLUS-agarose beads (Santa Cruz Biotechnology) were added after thorough vortexing. Samples were vortexed again and incubated for one hour at 4°C with rotation. Samples were washed with Cell Lysis Buffer (Cell Signaling) with 1:100 Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) by removing the supernatant, adding 300 µL of the lysisbuffer, vortexing and centrifuging at 2000 rpm for 2 minutes at room temperature. After the third time washing, all the supernatant was carefully removed. Finally, 50 µL 3xSDS sample buffer was added, after which the samples were stored at -20°C.

Cell stimulation

For one experiment we incubated HEK Null cells with an IRAK-1/4 inhibitor, a potent and selective inhibitor of IRAK-1 (IC50 = 300 nM) and IRAK-4 (IC50 = 200 nM) (Calbiochem®), with DMSO as a control. The inhibitor and DMSO were diluted 1:100 in DMEM with 10% FCS and 2 mM L-glutamine. To end up with a total dilution of 1:2500 (4 µg/mL of the inhibitor), 100 µL per well was added to the 2.5 mL medium three hours after the start of transfection of the cells. The cells were incubated overnight at 37°C in a 5% CO_2 incubator.

For preparation of the lysate and immunoprecipitation samples, IRAK-1/4 inhibitor or DMSO control were added 1:2500 to the Cell Lysisbuffer (Cell Signaling) with 1:100 Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). This lysisbuffer was used for both the I.P. incubation and washing steps.

Western Blotting

To reduce the lysate and immunoprecipitation samples, 20% β-mercaptoethanol 1:10 was added. All samples were denatured at 95°C for 5 minutes, before being separated by SDS electrophoresis on 10% polyacrylamide gels. The proteins were then transmitted to PVDF membranes, which were blocked in 5% non-fat dry milk in TBS-T (20 mM Trisma Base, 135 mM NaCl, 2% Tween, pH=7.4) for 1 hour at room temperature after the transfer. Incubation with the first antibody (1:1000) was effectuated in 1% non-fat dry milk TBS-T overnight at 4°C with rotation. Membranes were washed three times in TBS-T before being incubated with the second antibody (1:1000) for 1.5 hours at room temperature with rotation. Again, the membranes were washed three times in TBS-T, after which detection of immunoreactive bands was performed, using Lumi-Light[PLUS] Western Blotting Substrate (Roche) and an ImageQuant[TM] LAS4000 biomolecular imager (GE Healthcare Life Sciences).

IL-8 production

293T and HEK Null cells were plated in 96-well plates (50,000 cells in 100 µL) in DMEM without antibiotics (10% FCS, 2 mM L-glutamine). After overnight incubation at 37°C in a 5% CO_2 incubator, transfections were performed with 0.2 µg vector DNA in total and 0.5 µL Lipofectamine® 2000 (Invitrogen) in a final volume of 50 µL DMEM added per well in accordance with the manufacturer's instruction. After transfection overnight at 37°C in a 5% CO_2 incubator, medium was replaced with 150 µL DMEM complete (10% FCS, 2 mM L-glutamine, pen/strep). Next day, supernatants from the wells were collected in a 96-well F-bottom sample microplate. IL-8 production was measured using a Duoset ELISA kit (R&D systems), performing all incubation steps on a shaker and washing them with PBS/0.05% Tween after each step. 96-well high binding ELISA microplates were coated overnight with 25 µL/well anti-IL-8 coating antibody diluted in PBS 1:180. After blocking the coated plates with 150 µL 1% BSA/PBS for 1 hour, 50 µL of the samples (1:5 and 1:25 diluted in 1% BSA/PBS) and standard curves of purified IL-8 were loaded on the plates. After one hour incubation, plates were washed and 25 µL anti-IL-8 detection antibody (1:180) in 1% BSA/PBS was incubated for one hour. The plates were then incubated with Streptavidin Poly-HRP (1:5000) in 1%BSA/PBS for 30 minutes. Finally, TMB substrate was made by combining 5 mL TMB substrate buffer (8.2 gram NaAc, 21 gram citric acid monohydrate in 1 liter H_2O, pH=5.0), 50 µL TMB chromogen and 10 µL 3% H_2O_2, from which 25 µL was added to the wells for a coloring reaction. To stop the reaction, 12.5 µL 1 M H_2SO_4 was added. To measure the IL-8 production, iMark™ Microplate Absorbance Reader (Bio-Rad) was used.

Mutant Strand Synthesis Reaction

IRAK-M death domain mutant (DDM) W74A+Y105A was generated using the existing mutant Y105A, generated by D. Kruijswijk and C. van 't Veer. Mutant strand synthesis reaction was then used to generate IRAK-M DDM W74A. To synthesize two complimentary oligonucleotides containing the desired mutation, oligonucleotide primers #1 (5' CCA TGA TTA CGC CAA GCG CGC AAT TAA CCC TCA C 3') and #2 (5' GTG AGG GTT AAT TGC GCG CTT GGC GTA ATC ATG G 3') were used. The QuikChange® Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) provided the materials. Together with 2.5 µL 10x reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.8), 20 mM $MgSO_4$, 1% Triton® X-100, 1 mg/ml BSA), 2 µL IRAK-M DDM Y105A plasmid (20 ng/µL), 0.5 µL oligonucleotide primer #1, 0.5 µL oligonucleotide primer #2, 0.5 µL dNTP mix and 18.75 µL H_2O the sample reaction was generated. At last, 0.5 µL *PfuTurbo* DNA polymerase (2.5 U/µL) was added to extend the oligonucleotide primers. The reactions were cycled in the thermal cycler according to Table 1. After cooling down the samples to <37°C, 0.5 µL *Dpn* I restriction enzyme (10 U/µL) was added. The reaction mixtures were spinned down in a microcentrifuge for 1 minute and then incubated at 37°C for 1 hour.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	16	95°C	30 seconds
		55°C	1 minute
		68°C	7 minutes

Table 1. *PCR program Mutant Strand Synthesis Reaction*

Minipreparation

Transformation of XL1-Blue Supercompetent Cells

For each reaction, 25 µL XL1-Blue supercompetent cells were thawed on ice after being transferred to a prechilled 14-mL BD Falcon polypropylene round-bottom tube. Then, 1 µL of the *Dpn* I-treated DNA sample was added, followed by incubation on ice for 30 minutes, and subsequently a heat shock at 42°C for 45 seconds. After incubation on ice for 2 minutes, cells were supplied with 250 µL SOC Outgrowth Medium (New England BioLabs) and incubated at 37°C for 1 hour with shaking at 225 rpm. Lysogeny Broth (LB)-Blasticidin agar plates were prepared and 50 or 100 µL of the transformation reaction was plated and incubated overnight at 37°C. After incubation, colonies were transferred to 200 µL LB medium supplemented with Blasticidin S HCl (100 µg/mL) (Invitrogen™) each, and incubated at 37°C with shaking at 225 rpm for 5 hours. Subsequently, 150 µL of the suspension was transferred to 3 mL LB+blasticidin medium and incubated overnight at 37°C with shaking.

Plasmid DNA purification

Suspensions were centrifuged at 4000 rpm for 5 minutes. DNA purification was done according to the NucleoSpin® Plasmid EasyPure manual (Macherey-Nagel).

Plasmid concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific).

Sequencing

To determine if the mutation was implanted correctly, the samples were subjected to Big Dye Terminator (BDT) sequencing. Sequence reactions were prepared by adding 2 µL BDT buffer, 1 µL BDT, 1 µL PRIMER 20 µM, 1 µL sample DNA and ddH$_2$O up to the volume of 10 µL. The samples were then cycled in a thermal cycler according to Table 2.

The samples were sent to the sequence laboratory, where the sequencing was performed. Codon Code Aligner was used as software for sequence assembly and mutation detection.

Segment	Cycles	Temperature	Time
1	1	92°C	60 seconds
2	35	92°C	10 seconds
		55°C	10 seconds
		60°C	4 minutes

Table 2. *PCR program BDT sequencing*

Midipreparation

Transformation DH5α Competent cells

The same protocol was used as for the transformation of XL1-Blue Supercompetent Cells for the minipreparation. Clones 1 and 5 of the minipreparation were used for the midipreparation, as they had the highest DNA concentration.

Clones were transferred to 3 mL LB medium, supplemented with Blasticidin S HCl (100 µg/mL) (Invitrogen™) and incubated for 5 hours at 37°C with shaking at 225 rpm. The media with bacteria were then transferred to flasks with each 200 mL LB medium with Blasticidin S HCl (100 µg/mL) (Invitrogen™) and left at 37°C with shaking at 225 rpm overnight for incubation.

Endotoxin-free plasmid DNA purification
Endotoxin-free plasmid DNA purification was performed according to the NucleoBond® Xtra Midi EF user manual from Macherey-Nagel.

Plasmid concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific) and again verified by sequencing as described above.

Antibodies
An overview of used antibodies is provided in Table 3.

Antibody	Host	Directed against	Purchased from
Mouse IgG (HRP-linked)	Horse	Mouse IgG	Cell Signaling
Rabbit IgG (HRP-linked)	Goat	Rabbit IgG	Cell Signaling
HA	Rabbit, monoclonal	HA tag	Cell Signaling
IRAK-M	Rabbit, polyclonal	C-terminus	Cell Signaling
IRAK-M 1F6	Mouse, monoclonal	497 a.a. ~ 596 a.a.	Abnova
IRAK-4	Rabbit, polyclonal	Lysine 41	Cell Signaling
IRAK-1	Rabbit, polyclonal	C-terminus	Cell Signaling
TRAF6	Rabbit, monoclonal	Amino terminus	Cell Signaling
PY20	Mouse, monoclonal	Phosphotyrosine	Abcam
Ubiquitin	Rabbit, polyclonal	Keyhole limpet hemocyanin	Sigma-Aldrich
β-actin	Rabbit, polyclonal	Amino terminus	Cell Signaling

Table 3. *Overview of antibodies used in this study*

RESULTS

Modification of IRAK-M C-terminus in 293T cells

To follow up on the findings of Nollet [14] who described a modification of the C-terminus of IRAK-M, we attempted to reconstruct this modification and determine its sort. Figure 4A shows that cotransfection of 293T cells with MyD88/IRAK-4/IRAK-M generates a stable complex that can be immunoprecipitated by anti-IRAK-M. As MyD88 was HA-tagged, we were able to demonstrate by an anti-HA blot, that death domain residue W74 is a crucial binding site of IRAK-M to form a complex with MyD88 and IRAK-4. In contrast to the findings of Nollet [14], both anti-IRAK-M antibodies – the one directed against the C-terminus, as well as the monoclonal antibody 1F6 – showed binding to the immunoprecipitated IRAK-M and its death domain mutants. However, when the anti-HA blot was stripped with stripbuffer (0,1 M Glycine, 0,5 M NaCl, pH 2,7) and incubated with the antibody against the C-terminus of IRAK-M, identical results to those of Nollet [14] were observed (Figure 4B).
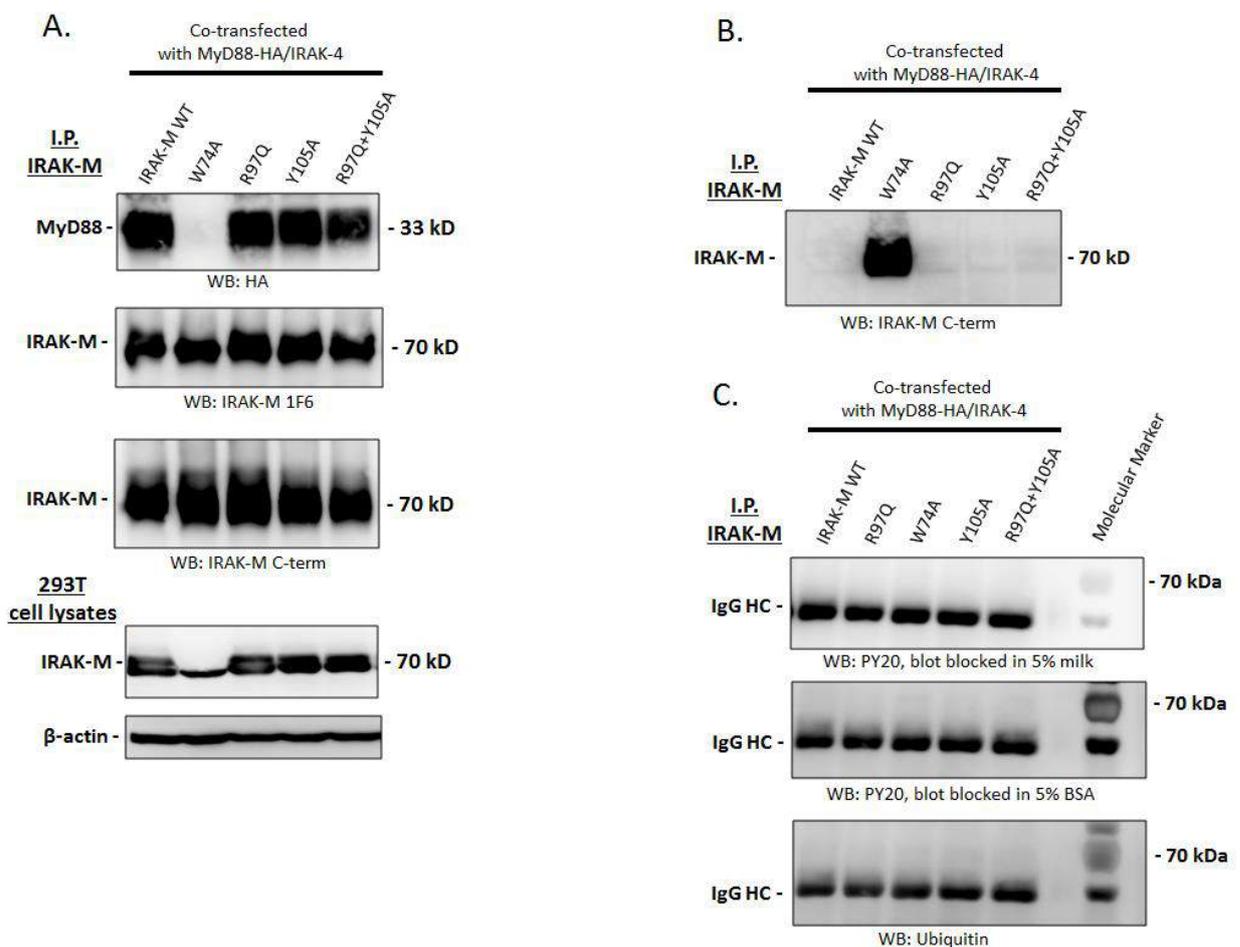

Figure 4. Modification of C-terminus of IRAK-M in MyD88/IRAK-4 complex. (A) *Cotransfection of MyD88/IRAK-4/IRAK-M WT and DD mutants in 293T cells and subsequent immunoprecipitation of IRAK-M shows a stable complex. Single mutation of W74 prevents IRAK-M to form a complex with MyD88/IRAK-4. Immunoprecipitated IRAK-M was detected both by the antibody used for precipitation (1F6), as well as a polyclonal antibody directed against the very C-terminus of IRAK-M. β-actin was used as a control for uniform loading.* ***(B)*** *The anti-HA/MyD88 blot shown in (A) was stripped and re-incubated with the polyclonal antibody directed against the C-terminus of IRAK-M. IRAK-M was not detected in immunoprecipitation samples where MyD88 had been co-immunoprecipitated.* ***(C)*** *Phosphorylation and ubiquitination were not detected at the molecular weight of IRAK-M in the I.P. samples, only the immunoglobulin G heavy chains (IgG HC).*

As the C-terminus of IRAK-M contains two tyrosines (Y590 and Y593), we hypothesized that the C-terminus becomes modified by phosphorylation of those two tyrosines. Furthermore, we hypothesize that IRAK-M could get ubiquitinated, preventing the antibody to bind the C-terminus. Therefore, we looked into possible phosphorylation or ubiquitination by incubating the blots with an anti-PY20 (directed against phosphotyrosine) and an anti-ubiquitin antibody respectively. Neither showed a reactive band at the molecular weight of IRAK-M, but only bands at the molecular weight of the heavy and light chains of the mouse antibody IgG (Figure 4B). To determine whether 5% milk was not an adequate block for the blots, the anti-PY20 blot was stripped and blocked in 5% BSA. Re-incubation with anti-PY20 did not generate different results, suggesting that in these circumstances, the C-terminus of IRAK-M does not get modified by phosphorylation or ubiquitination.

Functional analysis of IRAK-M-DD mutants after transient overexpression in 293T and HEK Null cells

Nollet [14] found that the cotransfection of the IRAK-M-DD mutants W74A and R97Q enhanced the IL-8 production, indicating that these two mutants appear to take over each other's binding site, as W74A still has an intact R97 binding site and vice versa. However, cotransfection of W74A and the double mutant R97Q/Y105A did not restore IL-8 production, demonstrating that Y105 plays an important role in IL-8 production. To further investigate the role of Y105, we generated a W74A/Y105A double mutant, to determine whether cotransfection of R97Q and W74A/Y105A would still increase IL-8 production, which would suggest that the double mutant W74A/Y105A can still bind with its R97 site.
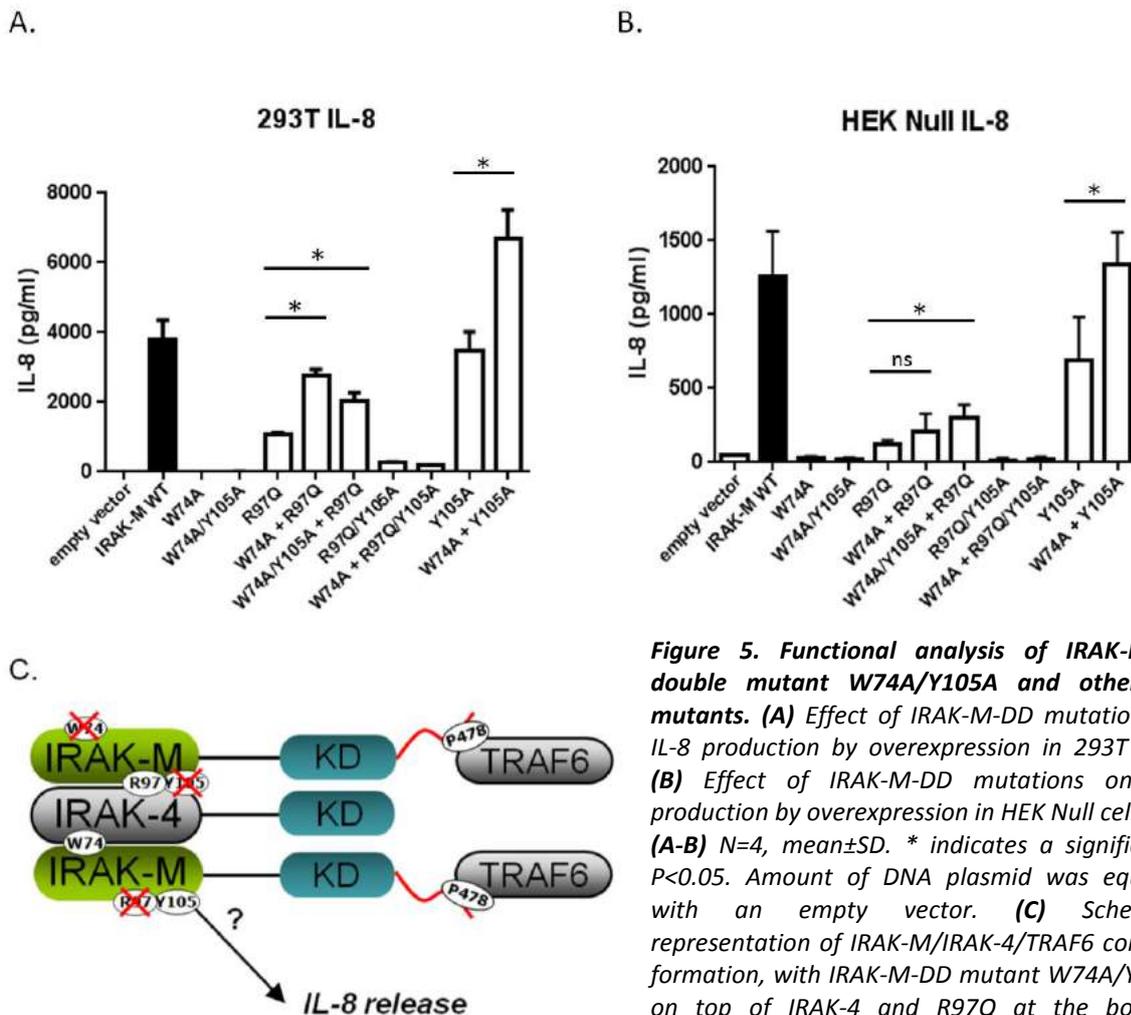

Figure 5. Functional analysis of IRAK-M-DD double mutant W74A/Y105A and other DD mutants. (A) Effect of IRAK-M-DD mutations on IL-8 production by overexpression in 293T cells. (B) Effect of IRAK-M-DD mutations on IL-8 production by overexpression in HEK Null cells. (A-B) N=4, mean±SD. * indicates a significance P<0.05. Amount of DNA plasmid was equated with an empty vector. (C) Schematic representation of IRAK-M/IRAK-4/TRAF6 complex formation, with IRAK-M-DD mutant W74A/Y105A on top of IRAK-4 and R97Q at the bottom. Combination of W74 and Y105 is predicted to be pivotal for IL-8 release, but the role of Y105 is unknown.

Both 293T and HEK Null cells were subsequently transfected with WT IRAK-M and mutants to stimulate the IL-8 production. As previously mentioned, IL-8 production was found to depend on W74 and R97, and our data confirmed this (Figure 5). Furthermore, single mutant Y105A showed no significant decrease in IL-8 production compared to IRAK-M WT in 293T cells, but in HEK Null cells the production was reduced. As expected, the W47A/Y105A double mutant did not induce IL-8 production, which could be explained by the lack of an intact W74 residue. Cotransfection of mutants W74A and R97Q resulted in significantly increased IL-8 production in 293T cells compared to R97Q alone, which is consistent with the results of Nollet [14]. While it can be deduced from Figure 5 that the W47A/Y105A double mutant still binds with its R97 site, it does not seem to need Y105 to increase IL-8 production. This is in contrast to the R97Q/Y105A mutant, that still binds with W74, but for this interaction Y105 is apparently needed to signal IL-8 production, as cotransfection of R97Q/Y105A and W74A does not restore the signal.

Cotransfection of W74A with Y105A resulted in an increased IL-8 production compared to cells transfected with IRAK-M WT. However, it should be kept in mind that the cells transfected with IRAK-M WT were co-transfected with pUNO, an empty vector to control for the total amount of DNA in each reaction. Therefore, these cells were transfected with only 50% IRAK-M WT, whereas the cotransfected cells with W74A and Y105A had a higher total level of IRAK-M with an intact R97 binding site. This again indicates that residue R97 plays a pivotal role in IL-8 production.

Potency of IRAK-M-DD mutants to form a complex with TRAF6 in HEK Null cells

Apart from the functional analysis, we also examined the role of Y105 in the complex formation between IRAK-M and TRAF6. As mentioned previously, it is predicted that IRAK-M can form a sandwich-like complex with IRAK-4, subsequently binding TRAF6. As this can lead to IL-8 production, we investigated whether the IL-8 production of IRAK-M described in the above section can be correlated to the complex formation with TRAF6. Banchaewa [15] already investigated those residues of the IRAK-M-DD that play a role in its interaction with TRAF6, demonstrating that W74 and R97/Y105 are important binding sites. Next, we explored the formation of a complex between IRAK-M and TRAF6 using the same transfection strategy as described in the above functional analysis.
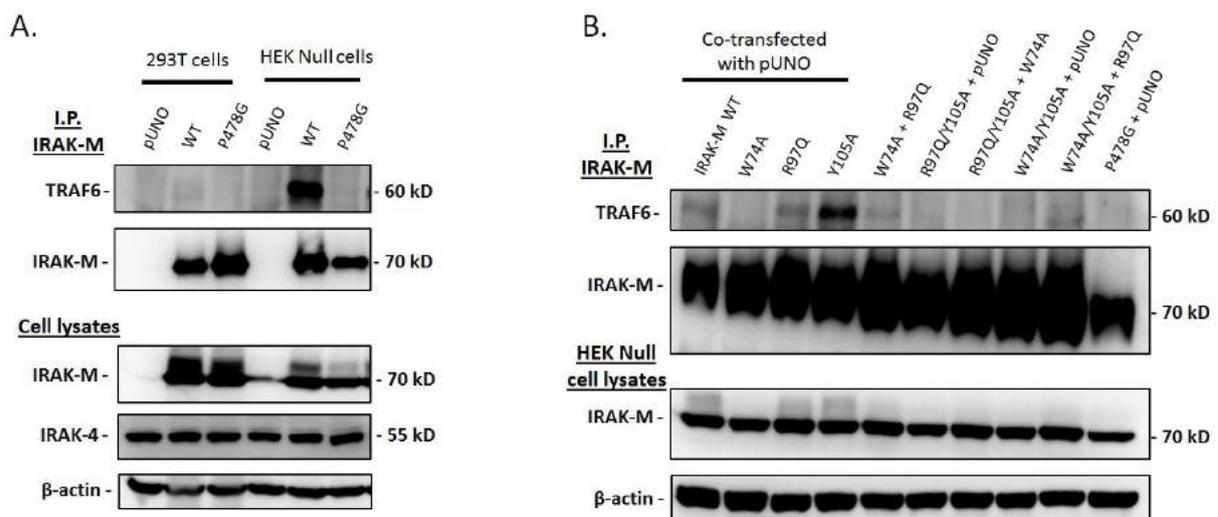

Figure 6. Co-immunoprecipitation of TRAF6 with IRAK-M after overexpression of IRAK-M-DD mutants.
(A) 293T and HEK Null cells were transfected; only HEK Null cells showed TRAF6 co-immunoprecipitation with IRAK-M WT. (B) Effect of IRAK-M-DD mutations on co-immunoprecipitation of TRAF6, which occurs with IRAK-M-DD mutants R97Q, Y105A and combined mutants W74A+R97Q and W75A/Y105A+R97Q. β-actin was used as a control for uniform loading.

First we explored whether TRAF6 forms a complex with overexpressed IRAK-M by immunoprecipitating IRAK-M in 293T and HEK Null cells. A stable complex with IRAK-M and TRAF6 was only formed in HEK Null cells (Figure 6A), which led us to proceed further investigations with those cells. Figure 6B demonstrates that, as expected, the Y105A mutant showed a strong ability to form TRAF6 complex. R97Q displayed a decreased complex formation, but still bound some TRAF6, while W74A did not bind any TRAF6. Cotransfection of R97Q with either W74A or W74A/Y105A did not lead to increased TRAF6 binding compared to R97Q alone. IRAK-M WT seems to bind TRAF6 weakly in this experiment, however it should be taken into account that immunoprecipitation of IRAK-M WT was not conducted properly, as the I.P. samples show less IRAK-M WT. Taken these results into account, the potency of the IRAK-M-DD mutants to form a complex with TRAF6 is similar to the capacity to induce IL-8 production.

Overexpression of IRAK-M /TRAF6 complex with IRAK-4 or IRAK-1 in HEK Null cells

To further investigate the possibility of an IRAK-M/IRAK-4 sandwich forming a complex with TRAF6, we tried to determine whether IRAK-M, IRAK-4 and/or TRAF6 are limiting factors for the formation of this hypothetical complex. HEK Null cells were cotransfected with IRAK-M WT and IRAK-4 and/or TRAF6, with different amounts of IRAK-M plasmid. As the total amount of DNA in each reaction remained identical, the amount of (different) plasmids differed. Figure 7A shows that transfection with only IRAK-M WT leads to more complex with TRAF6 compared to co-transfection of IRAK-M WT with an empty vector (pUNO). Fewer IRAK-M was immunoprecipitated when the reactions contained less IRAK-M WT plasmids.

Cotransfection of IRAK-4 and IRAK-M increases TRAF6 binding to IRAK-M (Figure 7A), suggesting that IRAK-4 is a limiting factor for a stable IRAK-M/TRAF6 complex formation. Furthermore, cotransfection of IRAK-M with TRAF6 alone leads to even more complex formation, indicating that with limited IRAK-4, IRAK-M may bind TRAF6 independently from IRAK-4, or that under combined overexpression of IRAK-M and TRAF6, the IRAK-4 becomes saturated and sandwiched with IRAK-M and IRAK-M-bound-TRAF6.

Surprisingly, simultaneous transfection with IRAK-M WT, IRAK-4 and TRAF6 shows no TRAF6 complex. This is despite comparable expression of IRAK-4 and TRAF6 in the lysates, and of IRAK-M in the lysates and I.P. samples (figure 7A). Inability of TRAF6 to form a stable complex with IRAK-M could be caused by deactivation of TRAF6, or possible competition with another molecule.

As can be seen in figure 7A, transfection of HEK Null cells with TRAF6 results in an extra reactive band in the lysates with a higher molecular weight. We hypothesize that overexpression of TRAF6 leads to ubiquitination, which causes an increase of the molecular weight with ~8 kD. Because Nollet [14] demonstrated that co-immunoprecipitation of IRAK-4 was not successful with antibodies directed against the kinase domain, nor against amino acids 410-460, we did not attempt to investigate IRAK-4 co-immunoprecipitation in these IRAK-M I.P. samples.

IRAK-1 is known to form complexes with IRAK-M, as well as with IRAK-4 and TRAF6 (Figure 1), making it a potential competitor which prevents TRAF6 to form a stable complex with IRAK-M (figure 7A). Therefore, we repeated the above experiment, but performed the cotransfection of HEK Null cells with IRAK-1 instead of IRAK-4. Figure 7B shows that there is no increase in TRAF6 complexes when IRAK-M WT is cotransfected with IRAK-1 instead of an empty vector, indicating that IRAK-1 is not a limiting factor in this TRAF6 complex. Cotransfection with IRAK-M and TRAF6 alone does increase IRAK-M/TRAF6 complexes, but remarkably, this effect disappears with simultaneous transfection of IRAK-1. Again, cotransfection of IRAK-M WT with a kinase, in this case IRAK-1, prevents TRAF6 to form a complex with IRAK-M. Co-immunoprecipitation of IRAK-1 with IRAK-M shows a reactive band
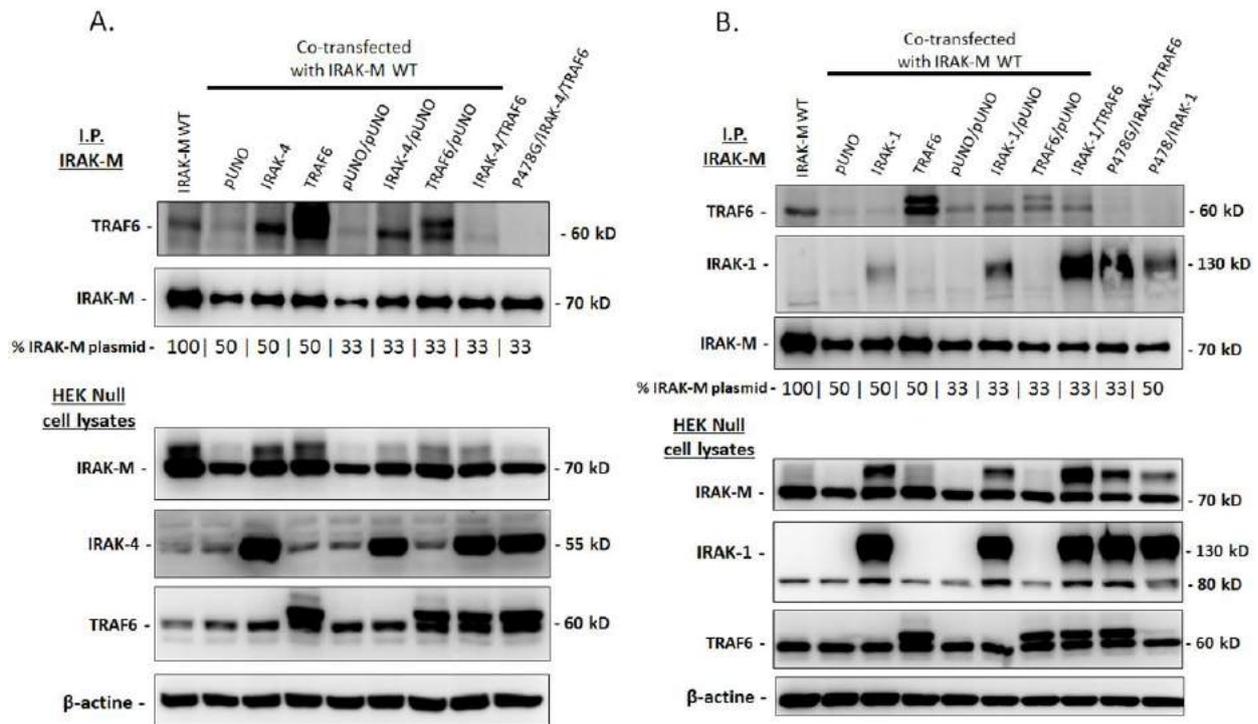
Figure 7. Immunoprecipitation of IRAK-M/TRAF6 complexes with IRAK-4 and IRAK-1 co-expression in HEK Null cells. (A) *Cotransfection of IRAK-M with TRAF6 and/or IRAK-4. Three different IRAK-M plasmid concentrations were used, i.e. 33%, 50% and 100% of the total DNA amount. More co-immunoprecipitation of TRAF6 is observed with co-transfection of IRAK-4 or TRAF6, but this effect disappears with co-transfection of IRAK-M with both IRAK-4 and TRAF6. Effect on IRAK-M, IRAK-4 and TRAF6 in lysates when overexpressed in HEK Null cells shows the difference between reactions with endogenous and overexpressed IRAK-4 and TRAF6.*
(B) *Cotransfection of three different plasmid concentrations of IRAK-M with and without TRAF6 and/or IRAK-1. Cotransfection of IRAK-M with both IRAK-1 and TRAF6 shows no increase in IRAK-M/TRAF6 complex formation. Modified IRAK-1 at 130 kD is co-immunoprecipitated with IRAK-M, and also binds to the C-terminal mutant P478G. Cotransfection of IRAK-M with both IRAK-1 and TRAF6 shows more IRAK-M/IRAK-1 complex formation. Effect on IRAK-M, IRAK-1 and TRAF6 in lysates when overexpressed in HEK Null cells shows the difference between reactions with endogenous and overexpressed IRAK-1 and TRAF6. Overexpression of IRAK-1 shows modification of both IRAK-M and IRAK-1.* **(A) and (B)** *β-actin was used as a control for uniform loading.*

at 130 kD (Figure 7B), which indicates that the active IRAK-1 is co-immunoprecipitated with IRAK-M, instead of the inactive form that has a molecular weight of 80 kD. IRAK-1 seems to co-immunoprecipitate with IRAK-M more efficiently when cotransfected with TRAF6. This does not depend on P478, the binding site of the C-terminus of IRAK-M to TRAF6. Thus, IRAK-1 appears to play a role in the formation of a complex with IRAK-M and TRAF6.

IRAK-M in the lysates shows an additional band of a higher molecular weight when cotransfected with IRAK-1. This modified IRAK-M is only seen in the lysates and not in the I.P. samples, which potentially indicates that the modified IRAK-M cannot be detected by the antibody.

Potency of IRAK-M-DD mutants to form a complex with IRAK-4 and TRAF6 in HEK Null cells

As can be deduced from figure 7A, overexpression of IRAK-4 enhances the binding of TRAF6 to IRAK-M. To investigate which IRAK-M-DD residues play a role in this complex formation, we transfected HEK Null cells with IRAK-4 and IRAK-M-DD mutants and observed which mutants did not co-immunoprecipitate TRAF6 with IRAK-M. Figure 8 illustrates that single mutation of W74A, R97Q

and P478G, and combined mutation of D19N-L20A-P21A, F18A-P21A, D19N-A23S, F18A/Q78A and R97Q/Y105A prevents IRAK-M to bind IRAK-4/TRAF6. Compared to the co-immunoprecipitation of TRAF6 to IRAK-M WT, mutant Y105A shows a comparable binding, R70Q results in a stronger binding, and F18A, P22A-A23S and Q78A lead to a reduced binding capacity.
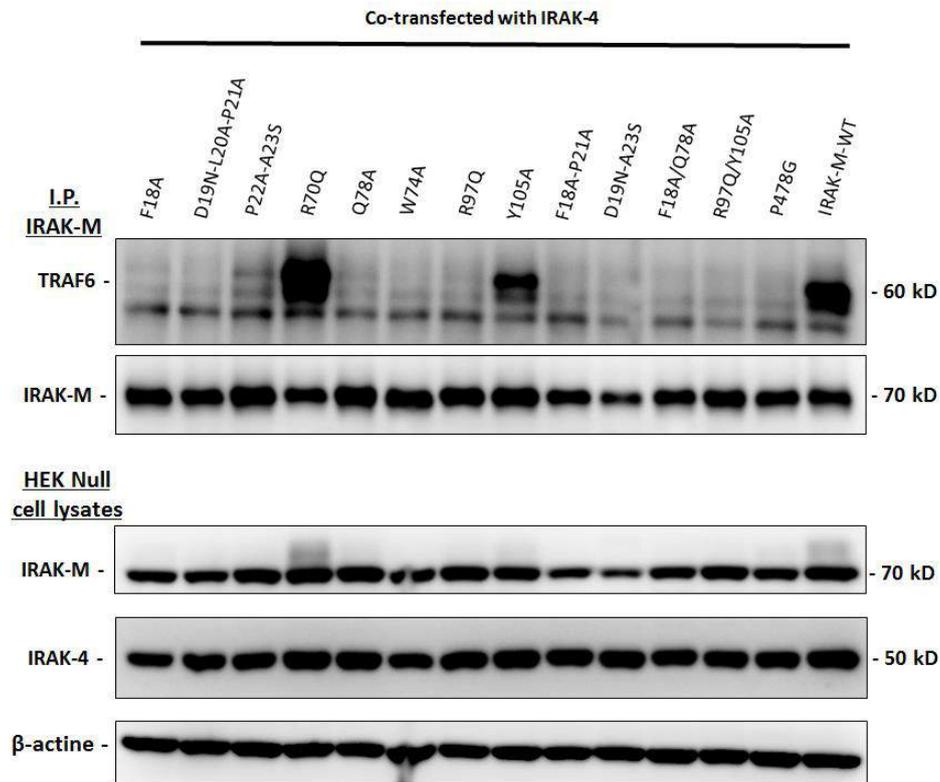

Figure 8. Co-immunoprecipitation of TRAF6 with IRAK-M-DD mutants after overexpression of IRAK-4 in HEK Null cells. *Single mutation of W74, R97 and P478 abolished the binding of TRAF6 to IRAK-M/IRAK-4. Combined mutants D19N-L20A-P21A, F18A-P21A, D19N-A23S, F18A/Q78A and R97Q/Y105A also showed a highly diminished capacity to bind IRAK-M/IRAK-4. β-actin was used as a control for uniform loading.*

The fact that the R70Q mutant tends to lead to more complex formation of TRAF6 with IRAK-M/IRAK-4 is in line with the prediction of Du *et al*. [1] that R70Q results in an increased IRAK-4 interaction through an extra hydrogen bond between Q70 in IRAK-M and R54 in IRAK-4.

Whereas mutation of P22-A23 still resulted in a partially active mutant, mutation of the whole D19-A23 stretch led to no complex formation. This D19N-A23S mutant is known to be hyperactive in NF-κB and IL-8 production in 293T cells [1], but as can be seen in figure 8, this is not due to binding with IRAK-4/TRAF6.

Effect of IRAK-1/4 inhibitor on the complex formation of TRAF6 with IRAK-M

As shown in figure 7, cotransfection of HEK Null cells with IRAK-M/IRAK-1/TRAF6 or IRAK-M/IRAK-4/TRAF6 does not lead to the formation of a stable TRAF6-complex. We hypothesized that this could be as a result of phosphorylation of TRAF6 by the kinase activity of IRAK-1 or IRAK-4. To investigate this hypothesis, we inactivated the kinase domain of IRAK-1 and -4 by incubating the cells with an IRAK-1/4 inhibitor. Figure 9 depicts that addition of the IRAK-1/4 inhibitor to HEK Null cells transfected with IRAK-M WT, TRAF6 and an empty vector, resulted in slightly more complex formation. However, when the inhibitor was added to cells transfected with IRAK-M WT, IRAK-4 and

TRAF6, the inhibitor caused a profound increase in IRAK-M/TRAF6 stable complex formation. While in previous experiments no stable complex was formed with cotransfection of IRAK-M WT, TRAF6 and IRAK-4, in this experiment some complex formation was observed, comparable to that of IRAK-M WT and TRAF6. Nevertheless, cotransfection of IRAK-4 was believed to result in more binding of TRAF6 to IRAK-M, which in this experiment again does not happen. Cotransfection of IRAK-M/TRAF6 and IRAK-1 in the presence of the IRAK-1/4 kinase inhibitor also resulted in more complex formation, which showed to be more efficient than cotransfection of IRAK-M and TRAF6 alone. These results illustrated in Figure 9 therefore suggest that inactivation of the kinase activity of IRAK-1 and IRAK-4 leads to more stable IRAK-M/TRAF6 complex formation.
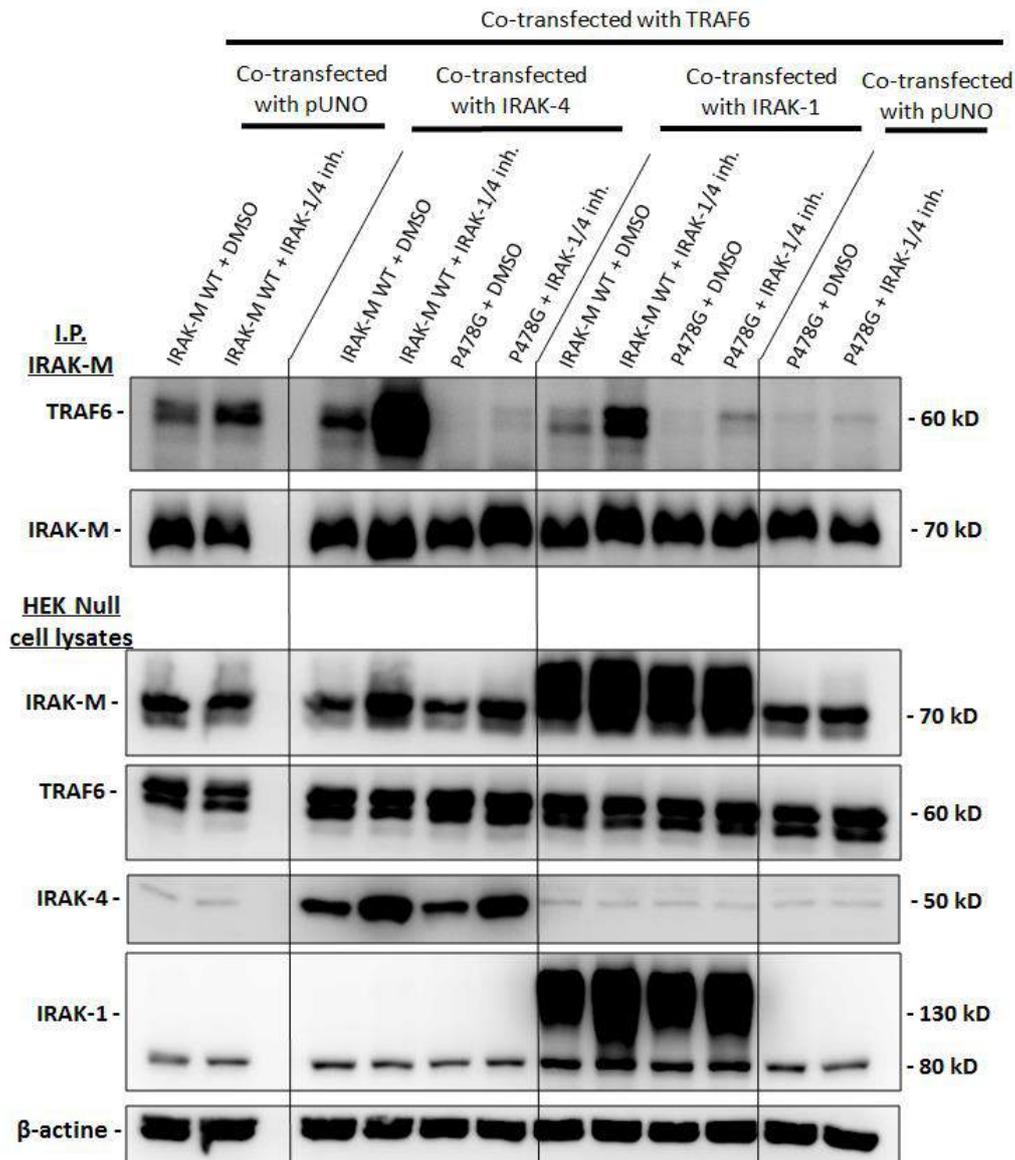

Figure 9. Stabilization of IRAK-4/IRAK-M/TRAF6 and IRAK-1/IRAK-M/TRAF6 complexes with an IRAK-1/4 inhibitor in HEK Null cells. Cotransfection of the indicated factors with 4 µg/mL of IRAK-1/4 kinase inhibitor or control DMSO added to cells 3 hours after start of the transfection. Cells were lysed after overnight transfection and immunoprecipitated with anti-IRAK-M in the presence of the inhibitor or DMSO control. In all samples, stimulation with the IRAK-1/4 inhibitor leads to an increased complex formation of IRAK-M and TRAF6, as well as more stable IRAK-1 and IRAK-4 in the lysates. β-actin was used as a control for uniform loading.

Furthermore, some TRAF6 binding to the P478G mutant was observed, which is the IRAK-M mutant that lacks its binding residue for TRAF6. As can be deduced from figure 7B, IRAK-1 is capable to bind to P478G, so it is possible that the complex formation of the P478G IRAK-M mutant with TRAF6 takes place through DD interaction with IRAK-1, and IRAK-1 binding of TRAF6.

The lysates show that overexpressed IRAK-1 and IRAK-4 in presence of the IRAK-1/4 inhibitor lead to a broader reactive band, indicating that IRAK-1 and IRAK-4 are more stable when their own kinase activity is inhibited.

Both figure 7B and 9 illustrate that IRAK-M becomes modified when overexpressed with IRAK-1, resulting in a band at a higher molecular weight. This modification of IRAK-M by IRAK-1 does not diminish in the presence of the IRAK-1/4 inhibitor (Figure 9), indicating that it is not the kinase activity of IRAK-1 that causes this modification of IRAK-M.

DISCUSSION

Here we report the structure function studies on human IRAK-M, investigating its complex formation with MyD88/IRAK-4 and TRAF6 in particular. First, we followed up on the results of Nollet [14], who described that the C-terminus of IRAK-M becomes modified when in complex with MyD88. However, our results suggest that this phenomenon only appears when the membranes of the western blots are stripped at low pH (pH 2.7) and re-incubated with the antibody against the C-terminus of IRAK-M (Figure 4). It appears that the antigenic site for the antibody only disappears after a modification and subsequent acidification. IRAK-M-DD mutant W74A does not undergo this modification, suggesting that only IRAK-M that was in complex with MyD88 and IRAK-4 is susceptible to undergo the causative modification. Our findings did not indicate that phosphorylation or ubiquitination is the cause of the modification. However, the western blots for phosphorylation and ubiquitination were lacking a positive control. To determine the exact modification, IRAK-M's C-terminus should be further checked for post-translational modifications.

Furthermore, we examined the function of IRAK-M-DD residue Y105 both in IL-8 production and complex formation with TRAF6. Du *et al.* [1] predicted that IRAK-M could bind IRAK-4 through both its W74 and R97 site, forming a sandwich with two IRAK-M molecules and one IRAK-4 molecule in the middle. Our findings confirm this hypothesis and show that combined mutation of W74A with R97Q restores the IL-8 production, as those mutants can take over each other's binding (Figure 5). Furthermore, residue Y105 also plays a pivotal role in the IL-8 production, as R97Q/Y105A is shown to have no IL-8 production, which surprisingly could not be restored by cotransfection with W74A. By constructing a new IRAK-M-DD double mutant of W74A and Y105A, we demonstrated that the reduced IL-8 production of R97Q can still be enhanced by W74A/Y105A. This leads to the hypothesis that specifically the combination of IRAK-M-DD residues W74 and Y105 plays an important role, because the R97Q/Y105A mutant can bind with its W74 site to IRAK-4, but needs Y105 to signal to IL-8 production.

Since it was demonstrated by Du *et al.* [1] that IRAK-M's TRAF6 binding motif at P478 was essential for IL-8 production, we expected to detect a correlation between the IRAK-M-DD residues playing a role in TRAF6 complex formation and in IL-8 production. The findings of IL-8 production correspond with the results of co-immunoprecipation of TRAF6. Here as well, mutants W74A, R97Q/Y105A and W74A/Y105A do not show a stable complex between IRAK-M and TRAF6 (Figure 6B). Combined mutation of R97Q/Y105A and W74A does not enhance the complex formation, neither does the combined mutation of W74A/Y105A and R97Q, as only the TRAF6 bound to the W74 site of mutant R97Q is observed. In the experiment in Figure 6B the TRAF6 signal bound to WT IRAK-M is hampered by inefficient immunoprecipitation of IRAK-M, because the Y105A mutant shows good TRAF6 binding with efficient immunoprecipitation. We think the data on the other mutants in this experiment with efficient immunoprecipitation are valid. Even though this experiment should be repeated, we do believe that the trend in these results will not change.

Our results establish that 293T and HEK Null cells behave differently upon transfection with IRAK-M in terms of IL-8 production and IRAK-M/TRAF6 complex formation. We did not succeed in creating a stable IRAK-M/TRAF6 complex in 293T cells (Figure 6A), while these cells show a higher IL-8 production compared to HEK Null cells (Figure 5). Possibly, TRAF6 becomes hyper activated in 293T cells and dissociates from a stable complex, resulting in more downstream signaling and IL-8 production. Incubation of 293T cells with the IRAK-1/4 inhibitor could disclose whether the kinase activity of IRAK-1/4 is responsible for this phenomenon.

As the formation of an IRAK-M/IRAK-4/TRAF6 complex through an IRAK-M/IRAK-4 sandwich (Figure 3) is an assumption supported by most data, we tried to test this hypothesis by investigating whether and how IRAK-M, IRAK-4 and TRAF6 are limiting factors for the IRAK-M/TRAF6 complex

formation. Our findings suggest that all three factors are limiting (Figure 7A). This strengthens the hypothesis of the IRAK-M/IRAK-4 sandwich illustrated in Figure 3, where IRAK-M sandwiches IRAK-4 by R97 and W74 interaction, bringing together TRAF6 binding sites of multiple IRAK-M molecules. However, in theory two IRAK-M molecules may also bind each other, without IRAK-4 between them. This could be tested by creating IRAK-4 knockout cells through CRISPR, and transfecting those cells with IRAK-M to investigate whether a TRAF6 complex can be generated when no IRAK-4 is present.

Because our results demonstrated that IRAK-4 is a limiting factor for TRAF6 binding to IRAK-M, we investigated which death domain residues of IRAK-M are playing a role in this complex formation. Up till now, co-immunoprecipitation of IRAK-4 with IRAK-M was unsuccessful in our hands. For this reason, we examined the involvement of the different IRAK-M-DD residues to form the IRAK-M/IRAK-4/TRAF6 complex by cotransfecting DD mutants in HEK Null cells with IRAK-4 and TRAF6. Nollet [14] investigated the IRAK-M-DD residues necessary for the myddosome formation with MyD88 and IRAK-4 through co-immunoprecipitation of MyD88. In contrast with that complex formation, we found that R97 seems to play a special role in the formation of the complex with IRAK-4 and TRAF6, as the mutation of this residue eliminated the capacity of IRAK-M to bind IRAK-4 in complex with TRAF6 (Figure 8), but not with MyD88 (Figure 4A) and [14]. Furthermore, the hypothesis stated by Du *et al.* [1] that single mutation of residue R70 is capable to form an extra hydrogen bond with R54 of IRAK-4 tetramers, was affirmed by the observation that the enforced interaction of R70Q with IRAK-4 leads to more TRAF6 binding (Figure 8). Mutation of the D19-A23 stretch inhibits the binding with TRAF6, but Du *et al.* [1] demonstrated that this mutant does not lose its capacity to generate IL-8 and NF-κB production. Du *et al.* [1] hypothesized that this stretch (in particular residues P22 and A23) is pivotal for interactive lateral binding between IRAK-M molecules in tetramers. To investigate this hypothesis, one specific residue that is predicted to play a role only in binding with IRAK-M itself and not in formation of a myddosome should be determined, and then mutated. According to Du *et al.* [1] (Supplemental Figures S3A) this could be L53, K60, Q64, or G65.

Combining our assumption that the IRAK-M-DD residue Y105 plays a crucial role in the complex formation with TRAF6 and our knowledge of the structure function of TRAF6, we created a new schematic representation of the IRAK-M/IRAK-4/TRAF6 complex. TRAF6 contains a N-terminal RING and zinc finger (ZF) domain, followed by a C-terminal TRAF domain. Three TRAF domains are capable of forming a trimeric complex. Furthermore, the N-terminal region of TRAF6 is dimeric, with the RING and ZF domains arranged linearly in a rigid, golfclub-like conformation. For its activity which leads to polyubiquitination and NF-κB activation, dimerization of TRAF6 through its N-terminal is critical [16]. Therefore, we hypothesize that the complex of IRAK-M, IRAK-4 and TRAF6 does not only facilitate a structure for the formation of trimeric TRAF6, but also for the dimerization of TRAF6. A schematic representation of this predicted complex is shown in figure 10.

Surprisingly, triple transfection with IRAK-M, IRAK-4 and TRAF6 did not lead to co-immunoprecipitation of TRAF6, despite the fact that the lysate samples showed that the amount of TRAF6 was identical for all samples (Figure 7A and 9). This leads to the conclusion that overexpression of the whole IRAK-M/IRAK-4/TRAF6 complex results in an absence of a stable TRAF6 complex. We thought of several possible explanations for this phenomenon. First, competition with another protein, in particular IRAK-1, could be a reason for TRAF6 not binding IRAK-M/IRAK-4. Furthermore, we hypothesized that TRAF6 may be deactivated by phosphorylation or methylation.

As IRAK-1 contains three potential TRAF6-binding sites [17], we repeated the experiment of cotransfecting cells with the IRAK-M/TRAF6 complex, replacing IRAK-4 with IRAK-1. At first, cotransfection of IRAK-M with IRAK-1 did not increase the IRAK-M/TRAF6 complex formation (Figure 7B). Furthermore, no TRAF6 complex was formed with overexpression of the C-terminal mutant P478G with IRAK-1 and TRAF6, indicating that in this experiment, IRAK-1 bound to IRAK-M does not bind TRAF6 through its three TRAF6 binding motives.
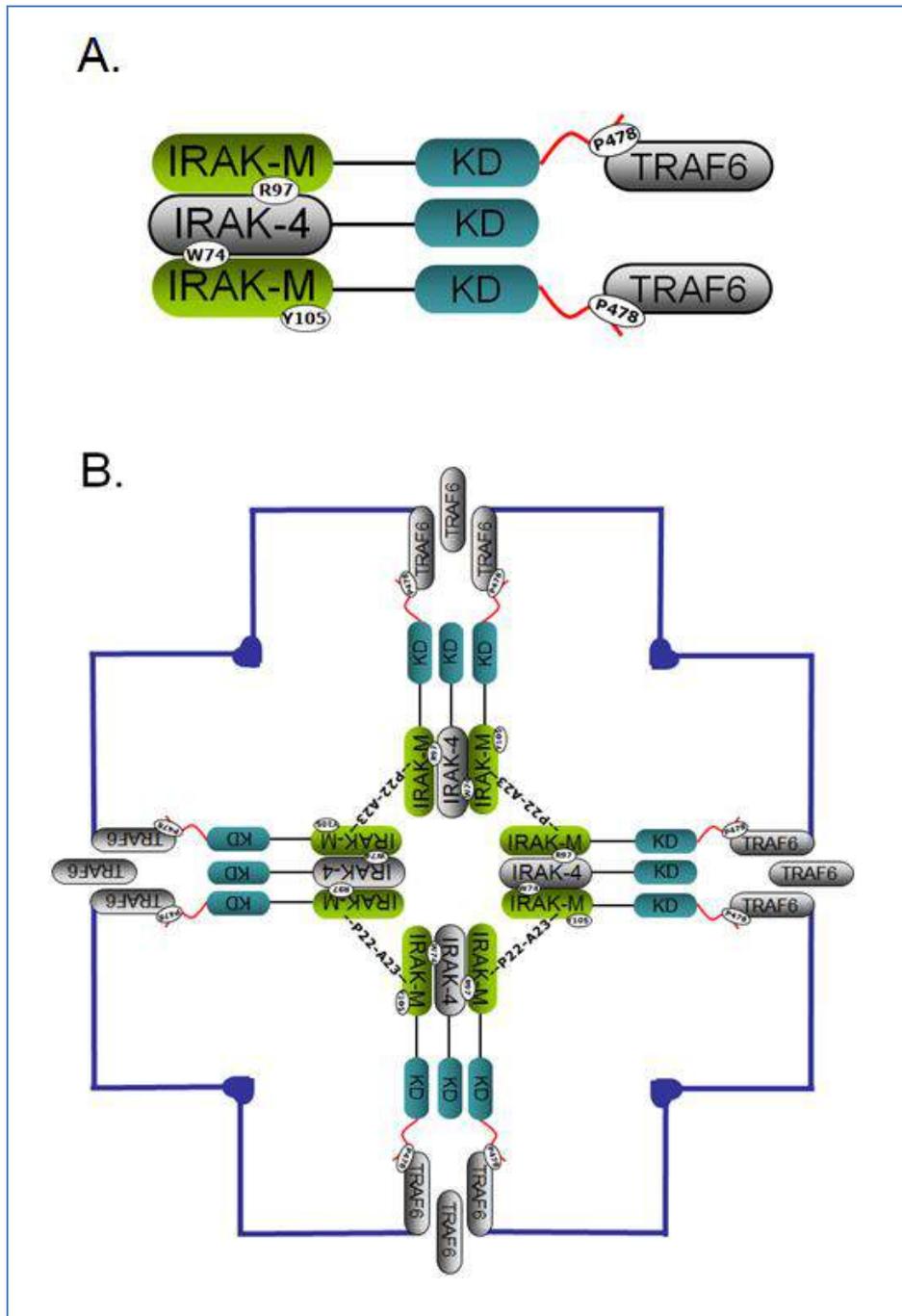
Figure 10. Schematic representation of IRAK-M/IRAK-4/TRAF6 complex formation. (A) *Schematic representation of the sandwich formation, with IRAK-M-DD residues W74, R97 and Y105 playing a pivotal role, and C-terminal residue P478 providing the binding motif for TRAF6.* ***(B)*** *TRAF6 forms a trimeric complex through its TRAF-domain (gray), and dimerizes through its RING and ZF domains (blue). The four TRAF6 molecules that are illustrated in the trimeric complexes, but are not showed to dimerize, potentially connect this complex with other identical complexes through dimerization of their RING and ZF domains, or with each other. IRAK-M-DD residues P22 and A23 are predicted to play a role in the interactive lateral binding of IRAK-M.*

Interestingly, cotransfection of IRAK-M, IRAK-1 and TRAF6 again resulted in the absence of the TRAF6 complex, indicating that overexpression of specifically the whole complex generates the condition for an unstable TRAF6 complex (Figure 7B and 9).

Cotransfection with IRAK-1 showed some other interesting results: cotransfection of IRAK-M, IRAK-1 and TRAF6 did not lead to co-immunoprecipitation of TRAF6, but it did result in more IRAK-1 binding to IRAK-M. TRAF6 seemed to have enhanced this binding, but TRAF6 did not stay in the complex itself. Moreover, this expanded binding of IRAK-M and IRAK-1 is not dependent on the P478 binding site of IRAK-M.

Furthermore, cotransfection or IRAK-M with IRAK-1 results in reciprocal modification of both molecules, illustrated as reactive bands on the western blots at a higher molecular weight than expected. It is has been established that IRAK-1 undergoes modification when activated, increasing the molecular weight from 80 kD to 115 kD and above [18]. Interestingly, only this modified form of IRAK-1 is co-immunoprecipitated with IRAK-M (Figure 7B). IRAK-1 dependent modification of IRAK-M results in an increase of the molecular weight with approximately 8 kD, which is most likely caused by ubiquitination, as the ubiquitin molecule weighs 8.5 kD. In this case, an antibody against ubiquitin should recognize the modified IRAK-M. The monoclonal 1F6 antibody does not seem to bind the modified IRAK-M, as these molecules do not appear in the western blots of the I.P. samples (Figure 7B and 9). In the future, supernatants of the I.P. samples could be tested with other antibodies against IRAK-M to determine whether this modified IRAK-M is still present after the precipitation.

Deactivation of TRAF6 can be generated by phosphorylation [19]. It is established that TRAF6 has two threonine residues at positions 463 and 486 that can be phosphorylated, resulting in an altered electric charge. This makes them repel one another and prevents them from forming a trimeric complex. Jiao *et al.* [19] demonstrated that TRAF6 can be phosphorylated by MST4, making it a potential regulator of TRAF6's deactivation. IRAK-1 and IRAK-4 are also able to generate phosphorylation through their active kinase domains. Incubation of the cells with an IRAK-1/4 inhibitor that specifically inhibits the kinase activity of IRAK-1 and IRAK-4, resulted in an enormous increase in TRAF6 complex formation. In both overexpression of the IRAK-M/IRAK-4/TRAF6 and IRAK-M/IRAK-1/TRAF6 complexes, the inhibitor stabilized the complexes, especially for cotransfection with IRAK-4 (Figure 9). In combination with the inhibitor, some binding of TRAF6 to the IRAK-M C-terminal mutant P478G was observed, when co-transfected with IRAK-1. This indicates that in the presence of the IRAK-1/4 inhibitor, TRAF6 is associated to IRAK-M bound IRAK-1.

Despite these results of a stable complex between IRAK-M and TRAF6 when overexpressed with either IRAK-4 or IRAK-1, some reserves should be taken to conclude that the kinase activity of IRAK-1/4 causes the deactivation of TRAF6. As it is established that MST4 plays a crucial role in phosphorylation of TRAF6 [19], the possibility exists that IRAK-1/4 activity somehow activates MST4 and thereby deactivates TRAF6. Specific inhibition of MST4 in cells transfected with the IRAK-M/IRAK-4/TRAF6 and IRAK-M/IRAK-1/TRAF6 complexes would provide this information. Furthermore, immunoprecipitation of TRAF6 and incubation with an anti-phosphothreonine antibody could confirm that phosphorylation of TRAF6 causes the reduced complex formation with IRAK-M/IRAK-4 and IRAK-M/IRAK-1. Because deactivation of TRAF6 can also be regulated by arginine methylation through PRMT1 [20], overexpression of the IRAK-M/IRAK-4/TRAF6 could lead to accumulation of PRMT1 and consequently deactivation of TRAF6. To investigate this possibility, stimulation with a methyltransferase inhibitor could be considered.

In conclusion, our studies on the structure function of IRAK-M contribute to the existing literature, demonstrating that IRAK-M-DD residue Y105 plays a role in combination with R97. Furthermore, we provided a new schematic representation of the IRAK-M, IRAK-4 and TRAF6 complex formation through dimerization of TRAF6. Lastly, our results suggest that active kinase domains of IRAK-1 and IRAK-4 play a role in the phosphorylation of TRAF6. Our research presents further understanding of IRAK-M as a negative regulator of the innate immunity, in order to help the development of inhibitors targeted against IRAK-M.

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20.   Tikhanovich I, Kuravi S, Artigues A, et al. **Dynamic Arginine Methylation of Tumor Necrosis Factor (TNF) Receptor-associated Factor 6 Regulates Toll-like Receptor Signaling.** J Biol Chem. 2015 Sep 4;290(36):22236-49.

ATTACHMENT 1

Explanatory list of symbols and abbreviations	
*	Significance P<0.05
a.a.	Amino acids
BDT	Big dye terminator
BSA	Bovine serum albumin
CTD	C-terminal domain
DD	Death domain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
HA	Hemagglutinin
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
IκBα	Inhibitor of κBα
IL-1	Interleukin-1
IL-8	Interleukin-8
IL-18	Interleukin-18
IL-1R	Interleukin-1 receptor
I.P.	Immunoprecipitation
IRAK-1	Interleukin-1 receptor-associated kinase 1
IRAK-2	Interleukin-1 receptor-associated kinase 2
IRAK-4	Interleukin-1 receptor-associated kinase 4
IRAK-M	Interleukin-1 receptor-associated kinase M
KD	Kinase domain
kD	Kilodalton
LB	Lysogeny broth
LPS	Lipopolysaccharide
MEKK3	Mitogen-activated protein kinase kinase kinase 3
MyD88	Myeloid differentiation protein 88
NF-κB	Nuclear factor-κB
ns	not significant
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRMT1	Protein arginine-N-methyltransferase 1
PRR	Pattern-recognition receptor
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
SHIP-1	SH2 (Src homology 2)-containing inositol phosphatase-1
SOCS-1	Suppressor of cytokine signaling 1
TAB 2 and 3	TAK1-binding proteins 2 and 3
TAK1	TGFβ-activated kinase 1
TBS-T	Tris-buffered saline and tween
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TRAF6	TNF receptor-associated factor 6
WT	Wild type

ATTACHMENT 2

Learning goals and participation
At the Center of Experimental Molecular Medicine (CEMM), C. van 't Veer (daily/senior tutor) and his research group have been working on IRAK-M for several years now, focusing on the structure function of its death domain. Before the start of my internship, two other students worked on IRAK-M under supervision of C. van 't Veer. My role was to continue their work by participating in the ongoing research on IRAK-M by C. van 't Veer and his analyst D. Kruijswijk.

During the weekly meetings on Monday morning I would present my results from the previous week to C. van 't Veer and D. Kruijswijk, after which we would discuss what the next step in my research could be. During the week I would perform the experiments, in the beginning together with D. Kruijswijk, as I had not been working in a laboratory before, but after some time I could perform the experiments myself. Because the preparation of a DNA plasmid was only done once during my internship, this was mostly performed by D. Kruijswijk, with me observing her work and participating where possible.

As this was my first internship in a laboratory, my main goal was to obtain basic knowledge of laboratory work and to learn to interpret scientific writing about basic research. After approximately 4 weeks I was taught to do immunoprecipitation, western blotting and ELISA individually. By reading articles about the innate immune system, IRAK-M and other proteins mentioned in my thesis, I widened my knowledge about these subjects significantly.

During my internship I attended the weekly research meetings of the Infectious Disease group, where PhD students, post-docs and staff members shared recent results from their research, followed by a group discussion. During one of those meetings, I presented my own research. Once in two weeks someone discussed a published article during the journal club. Furthermore, I attended a CEMMinar, where three PhD students from the CEMM presented their research. In December 2015 I went to the annual symposium of CEMM, the CEMMposium.

ATTACHMENT 3

PROJECTBESCHRIJVING WETENSCHAPPELIJKE STAGE / PROJECT PLAN RESEARCH INTERNSHIP

Naam / name student: Liza Pereverzeva	Student nummer: 6059163
Project titel / project title: Analysis of the structure function of the human IRAK-M Death Domain	

Research line (250 words): Provide a short description of the research line in which the current project will be embedded. Possibly add some references to publications from the hosting group.

The Center for Experimental and Molecular Medicine (CEMM) is a laboratory within the AMC which is fully devoted to interdisciplinary fundamental and clinical research of a variety of common diseases (cardiovascular and thrombotic disease, host defense, infectious disease, cancer, and others). Within the research group of the Infectious Diseases, Dr. C. van 't Veer analyses mechanisms that underlie the activation of the innate immune system, in particular Toll-like receptor (TLR) mediated inflammation. The Interleukine-1 receptor-associated kinase (IRAK) protein family is crucially involved in signalling initiated by the cytokines IL-1 and IL-18 and in TLR activation. Previous research shows that one member of the IRAK-family, IRAK-M, is a negative regulator of TLR signalling, expressed in lung epithelial cells, monocytes and macrophages. Therefore, IRAK-M seems to be potential therapeutic target. The research group of Dr. C. van 't Veer investigates the structure function, binding properties and (anti-)inflammatory signalling paths of human IRAK-M (Du et al., 2014). Recently, Edgar Nollet, a student of Biomedical Sciences, investigated the structure function of human IRAK-M and its binding to IRAK-4 and TNF receptor-associated factor 6 (TRAF6). My study will continue the analysis on IRAK-M, following up on the previous results of this research group.

Du J, Nicolaes GA, Kruijswijk D, Versloot M, Van der Poll T, & Van 't Veer C. (2014). The structure function of the death domain of human IRAK-M. Cell Communication and Signaling, 12(1), 77.

Background and research questions (500 words): Provide the background of the research that will be carried out by the student. Summarize the current body of knowledge on the subject as a background to the aim of the project.

Interleukine-1 receptor-associated kinase M (IRAK-M) is a part of the IRAK protein family, which is involved in pathways of innate immunity. The IRAK family plays part in IL-1 and IL-18 signaling, as well as the Toll-like receptor (TLR) signaling. Whereas IRAK-1, IRAK-2 and IRAK-4, the other members of the IRAK family, have a pro-inflammatory effect by activating NF-κB, IRAK-M is found to be a negative regulator of TLR signaling. This was demonstrated in IRAK-M deficient mice, which turned out to have better outcomes of mortality in secondary Pseudomonas aeruginosa pneumonia, an improved host response in tumor models and bone marrow transplantation. Therefore, inhibition of IRAK-M seems to have therapeutic potential.

IRAK-M is found to be expressed in lung epithelial cells, monocytes and macrophages. By activation of TLRs, they cause adaptor protein MyD88 to dimerize, leading to the recruitment of IRAK-4 and subsequently other IRAKs. Together with MyD88 these IRAKs then form multimeric complexes known as myddosomes, which induce expression of inflammatory genes dependent on the specific IRAK assembled on the myddosome. Zhou et al. (2013) found IRAK-M to be interacting with MyD88/IRAK-4 to form IRAK-M myddosome to mediate TLR7-induced MEKK3-dependent second wave NF-κB activation. This IRAK-M-dependent pathway only induces expression of genes that are not regulated

at the post-transcriptional levels (including inhibitory molecules SOCS-1, SHIP-1, A20 and IκBα), exerting an overall inhibitory effect on inflammatory response. 293T cells were found to create a good environment to form a stable MyD88/IRAK-4/IRAK-M complex, where immunoprecipitation could successfully be performed. By immunoprecipitating IRAK-M and labelling it with both an antibody against 1F6 and the C-terminus, it was observed that IRAK-M which had been in complex with MyD88/IRAK-4 was no longer recognized by an antibody directed to the very C-terminal end of IRAK-M which contains two tyrosines (Y590 and Y593).

The structure of IRAK-M is similar to the other IRAKs: it contains a N-terminal Death Domain (DD), which is crucial for binding to other IRAKs and therefore in activating NF-κB, an inactive kinase domain (KD) and an unstructured C-terminal domain (CTD) with a TNF receptor-associated factor 6 (TRAF6) binding site. IRAK-M DD residue W74 was shown to be essential for the binding of IRAK-M to IRAK-4, preceding MEKK3 dependent NF-κB activation and expression of inhibitory genes. Furthermore, it was demonstrated by Du et al. (2014) that in human IRAK-M both the DD and the CTD are of great importance for NF-κB activation, induced by IRAK-M overexpressed in 293T cells. In addition to W74, IRAK-M DD residue R97 was identified to be involved in NF-κB activation. These two sites are predicted to be located at opposite positions on the DD and are predicted to bind on different sides of the IRAK-4 DD. These findings formed the hypothesis of a IRAK-M/IRAK-4 sandwich, in which an IRAK-4 tetramer is sandwiched by two IRAK-M tetramers. It has been found that Y105 fulfils a pivotal role in IL-8 production, as no IL-8 production was to be found with Y105 mutants, but it is unknown what its exact role is.

In this research we try to determine whether the C-terminus of IRAK-M gets modified when in complex with IRAK-4 and MyD88, and if so, what the exact modification is. Furthermore, we will further investigate the formation of the IRAK-4/IRAK-M sandwich, determining whether IRAK-M needs to bind IRAK-4 to form a stable complex with TRAF6 or if IRAK-M can interact with itself. Also, we will try to understand what the role of Y105 is.

Research questions and/or hypotheses (150 words)

Does the C-terminus of IRAK-M modify when forming a complex with MyD88 and IRAK-4 and if so, what is the exact modification? We expect to find similar results as previous studies, where IRAK-M could not be detected with an antibody against the C-terminus of IRAK-M when IRAK-M forms a complex with MyD88 and IRAK-4. We expect that this is possibly due to phosphorylation of tyrosines (Y590 and Y593) in the C-terminus.
Can IRAK-M interact with itself? Does IRAK-M need IRAK-4 to form a stable complex with TRAF-6? What is the role of Y105? We expect that IRAK-M sandwiches IRAK-4 and does not bind itself, in order to form a stable complex with TRAF6. Furthermore, we expect Y105 interaction is needed on both IRAK-M molecules present in the sandwich.

Research design (500 words): Provide the set-up of the research and the methods. Describe the outcome measures and statistical analyses.

The analysis of the structure function of the human IRAK-M will take place in the laboratory of the Centre for Experimental and Molecular Medicine in the Academic Medical Centre of Amsterdam.

To determine whether the C-terminus of human IRAK-M gets modified when forming a complex with MyD88 and IRAK-4, we will perform immunoprecipitation with IRAK-M. We will transfect HEK Null- and 293T-cells with MyD88, IRAK-4, IRAK-M wild type, and several mutated types of the IRAK-M death domain (mutated at residue W74A, R97Q, Y105A and R97Q+Y105A). With western blotting of the cell lysates and immunoprecipitation samples with antibodies against IRAK-M (1F6 and C-

terminus) and MyD88 (HA-tag), we will try to determine whether the C-terminus is modified when IRAK-M binds to IRAK-4/MyD88. If so, we will assess whether this is due to phosphorylation of the two tyrosines in the C-terminus by western blotting with anti-phosphotyrosine antibodies and IRAK-4/IRAK-1 kinase inhibitors. Furthermore, it is possible to use peptide mass fingerprinting, which will yield the modifications that occur on IRAK-M and potentially IRAK-4. C-terminal sequencing of IRAK-M could also be used to identify the modification.

The interaction of IRAK-M with TRAF6 was observed without TLR stimulation or MyD88 overexpression, so the question is whether this IRAK-M/TRAF6 interaction needs IRAK-4. To determine this, we will use siRNA to eliminate IRAK-4, observing whether it will be possible for IRAK-M to form a complex with TRAF6.

Edgar Nollet observed that the IRAK-M W74A mutant stimulated IL-8 release driven by the R97Q mutant while W74A itself had no IL-8 stimulation activity at all. When the W74 mutant was co-expressed with the R97Q/Y105A double mutant, no IL-8 expression occurred indicating that Y105A is involved in a functional interaction. To assess whether Y105 interaction is needed on both IRAK-M molecules present in the sandwich we will make the W74/Y105 mutant and determine its IL-8 stimulatory capacity when co-expressed with the R97Q mutant.

Workplan and Internship specific learning goals (500 words)

Week 1:
Introduction in laboratory activities. Observing my tutors perform cell culturing, sample preparation for immunoprecipitation, immunoprecipitation and western blotting. First time pipetting individually with supervision of my tutors.
Doing research on current literature of IRAK-M.

Week 2 - 4:
Performing cell culturing, sample preparation for immunoprecipitation, immunoprecipitation and western blotting myself, with help and supervision of my tutors.

From week 5 on:
Performing cell culturing, sample preparation for immunoprecipitation, immunoprecipitation and western blotting individually.
Start writing Introduction and Methods for my paper.

Week 5 – 12:
Based on the results obtained in week 1 – 4 we will decide which following parts of laboratory research I will perform. These will all have the goal to create more knowledge of the structure function of IRAK-M. If these will contain new laboratory tests, I will first observe my tutors performing them, after which I will be able to perform them myself.

Week 13 – 16:
- Completing last laboratory test
- Writing Results, Discussion and Abstract
- Prepare presentation
- Present my research during the weekly meeting of the Infection-research-group of the Center for Experimental and Molecular Medicine in the AMC.

Which specific facilities are needed for this internship research and what has been arranged (250 words)

For this research, access to the laboratory of the Center for Experimental Molecular Medicine in the AMC is required. I will be working with 293T cells, HEK Null cells to perform immunoprecipitation mouse monoclonal antibody 1F6 against IRAK-M (Abnova). For this, vectors for overexpressing MyD88, IRAK-4 and IRAK-M, and mutants of IRAK-M DD will be used. For Western Blotting, antibodies against IRAK-M (Cell Signaling and Abnova) and MyD88 (Cell Signaling), and anti-mouse and anti-rabbit antibodies (Cell Signaling) will be required. All these facilities will be present in de CEMM lab, or will be ordered.

Ethics Review Approval: If for the research activities that the student will be perfoming approval of any Ethics Review Board (or related) is required this approval should have been obtained at the time of submission of the current proposal, otherwise the proposal can not be approved by the director.

For these research activities an approval to work with Genetically Modified Organisms (GMO) is required. This approval is submitted for the research group (GMO licence number 03-110). I will be registered to work in ML-I and ML-II labs, by signing a letter to declare adherence to the official regulations and AMC rulers with respect to working with genetically modified organisms.

Professional development (250 words): How does this internship fit in the professional development of this student?

As this is my first internship in a laboratory, my main goal will be to obtain basic knowledge of laboratory work. In the end I hope to be able to work individually with cell culturing, (sample preparation for) immunoprecipitation and western blotting. Hopefully I can develop enough knowledge on the basics of laboratory research to think critically of mine and others research and maybe to come up with new ideas.
Furthermore, I hope to gain knowledge on the working mechanisms of IRAK-M and it's pathways.

By participating in this research I also hope that this new experience will learn whether I would like to do research in the future, and whether I would like to do this in the experimental and molecular field.