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FIP2 promotes *E. coli*-induced IFN-β production and phagocytosis, through its interaction with TRAM

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Abstract

The Toll-like receptor 4 (TLR4) senses LPS from Gram-negative bacteria and triggers two distinct signaling pathways by the use of distinct pairs of signaling adaptors. Following activation at the plasma membrane the adaptors pair TIRAP and MyD88 are immediately recruited, leading to induction of pro-inflammatory cytokines. However, TLR4 located on the phagosomal membrane induces the type I interferons such as IFN-β, through the adaptor pair TRAM and TRIF. The small GTPase Rab11a is involved in the TLR4-induced IFN- β production, delivering TLR4 and TRAM to *E. coli* phagosomes. Here we report that the Rab11a effector protein, Rab11-FIP2, binds TRAM promoting its delivery to forming *E. coli* phagosomes and that both Rab11-FIP2 and TRAM are involved in phagocytosis of *E. coli* in human macrophages. These results show that FIP2 and TRAM are effectors involved in the *E. coli*-induced IFN- β induction and phagocytosis, which may point at new strategies for treatment Gram-negative induced inflammation that in severe cases may result in the development of sepsis.

Table of Contents

Abbreviations

BSA	Bovine serum albumin
CD	Cluster of differentiation
Cdc42	Cell division control protein 42
cDNA	Complementary DNA
DAMPs	Damage associated molecular patterns
DCs	Dendridic cells
DD domain	Death domain
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ERC	Endocytic recycling compartment
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FcγRs	Fc gamma receptors
FSC	Forward scatter
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HEK	Human Embrionic Kidney
IFN	Interferferon
IL	Interleukin
IRAKs	IL-1 receptor-associated kinases
IRFs	IFN regulatory factors
IκBα	NF-κB inhibitor, alpha
KD	Knock down
LBP	LPS binding protein
LDS	Lithium dodecyl sulfate
LPS	Lipopolysaccharide

LRR	Leucine rich repeats (LRR)
MAPKs	Mitogen-activated protein kinases
MD2	Myeloid differentiation factor 2
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene88
NF-κB	Nuclear factor-κB
NLRs	NOD (nucleotide-binding oligomerization domain)likereceptors
NS RNA	Non-silencing RNA oligo
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PMA	Phorbol 12-myrstate 13-acetate
PRRs	Pattern-recognition receptors
PtdIns(3,4,5)P_3	Phosphatidylinositol-3,4,5-trisphosphate
PtdIns(4,5)P_2 (PIP_2)	Phosphatidylinositol-4,5-bisphosphate
q-PCR	Quantitative Polymerase chain reaction
Rab GTPase	Ras-related in brain
Rab11-FIPs	Rab11 family interaction protein
Rac 1	Ras-related C3 botulinum toxin substrate 1
RBD	Rab11-binding domain
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
S. aureus	*Staphylococcus aureus*
SD	Standars deviation
siRNA	Silencing RNA
SSC	Side scatter
TBK1	TANK binding kinase 1
TBP	TATA-Box binding Protein
TBS	Tris Buffered Saline
TIR domain	Toll-Interleukin-1 receptor domain

TIRAP	TIR-containing adaptor protein
TLRs	Toll-like receptors
TMED7	Transmembrane emp24 domain-containing protein 7
TNF	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-containing adaptor-inducing
WT	Wild type
YFP	Yellow fluorescent protein

1 INTRODUCTION

1.1 The innate immune system

Human beings are colonized by a large variety of microbes which exist in close association with their hosts.

The consequence of such colonization can affect the host's health and depends on the microbial adaptation strategy: if the microbes provide a range of benefits to the host the effect can be positive (e.g. intestinal microbiota), but if microbes are harmful to the host they are called pathogens and can cause infectious diseases [1]. A third group of microbes are called opportunistic pathogens, which are not dangerous for healthy people but can affect individuals who are weakened or immunosuppressed, thus many factors can contribute to infection.

Mammals have evolved different defense mechanisms that are able to counteract microbial infections and these are traditionally dividend in to two types of defense, innate and adaptive immunity [1].

Innate immunity is the first line of defense against invading microbes and its response is immediate. As postulated by Janeway, already 27 years ago, the innate immune system is regulated by germline-encoded pattern-recognition receptors (PRRs) which recognize conserved and invariant features of microorganisms [2], by sensing pathogen associated molecular patterns (PAMPs). PAMPs are molecules with essential roles in microbial physiology, such as components of the cell walls (lipopolysaccharides, lipoproteins, lipotheicoic acid, etc.) proteins or nucleic acids [1]. Moreover, it has been shown that PRRs can also recognize endogenous molecules released from cells of damaged tissues, known as damage associated molecular patterns (DAMPs) [3].

Several classes of PRR families have been identified [4] and divided into two main classes: transmembrane proteins, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) expressed at the cell surface and/or in endosomal compartments; and cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) [5]. Another feature of the innate immunity is the inflammasome, a multi protein complex triggered by NLRs or ALRs (AIM2-like receptors)

which assemble in the cytosol upon a PAMP or DAMP stimulus and activate pro-inflammatory caspases 1 (canonical inflammasome) and 11 (non-canonical inflammasome). Activation of these proteases by auto-proteolytic cleavage leads to the generation of biologically active IL-1β and IL-18 from their respective precursors and to the death of the cell by pyroptosis [6].

PRRs are expressed in both immune cells (e.g. macrophages, dendritic cells and B- and T-cells) as well as non-immune cells, such as fibroblasts and epithelial cells [3, 7]. Innate immune cells directly kill pathogens through phagocytosis or inducing the production of cytokines, chemokines and type I interferons (IFN-α or IFN-β), which lead to elimination of the pathogens [1, 8-10] and development of the long-lasting pathogen-specific adaptive immune responses. Adaptive immune recognition is mediated by B- and T-cells, which leads to pathogen-specific immunity, thanks to somatic rearrangement of antigen receptors [11], a process that provide an extremely diverse set of receptors. Even though the innate immunity is not as specific as the adaptive immunity, it can discriminate between self and non-self and if this discrimination is not perfect, it can lead to the development of autoimmune diseases [12]. Moreover, the adaptive immune response leads to an antigen-specific activation of the cells belonging to the innate immunity system. This activation is more efficient than direct activation and therefore a constant interaction between innate and adaptive immunity is often required for a complete pathogen clearance [1].

1.2 Phagocytosis

Phagocytosis is a very complex process that contributes to host defense [13] and plays a crucial role in immune system. Phagocytosis was first observed by Elie Metchnikoff in 1882 and for this he was awarded the Nobel Prize in Physiology/Medicine in 1908.

Phagocytosis can be defined as the process by which cells ingest particles larger than 0.5 µm, inside their plasma-membrane [13]. This process appeared very early during evolution, since unicellular organisms use it as source of nourishment [14].

By contrast, in Metazoans, phagocytosis is mainly involved in development, tissue homeostasis maintenance, and immunity [15].

Thus, cells can be divided into professional phagocytes such as neutrophils, macrophages, DCs and osteoclasts, and non-professional or facultative phagocytes such as epithelial cells, which are capable to engulf particles only during certain, often pathological, conditions [13]. Phagocytes belong to the innate arm of immunity and are able to recognize both altered self-particles, such as apoptotic cells and non-self particles such as pathogens and potential pathogenic microbes, discerning from commensals [13]. Moreover, some phagocytes, macrophages and DCs in particular, use phagocytosis to internalize pathogens for degradation in phagosomes and loading of antigens for antigen presentation on MHC I and II molecules [16], making phagocytosis a bridging process between the innate and adaptive immunity [17]. In order to recognize their targets, immune cells possess a unique repertoire of receptors, which are sensitive to particular and definite molecules [14].

Interaction between the phagocytic cells and pathogens can occur directly through PRR, which recognize PAMPs, or indirectly, through opsonisation. Opsonins are host factors, such as immunoglobulin G (IgG) or components of the complement cascade that bind to the surface of the pathogen, leading to a recognition by phagocytic receptors such as CD11b (also known as complement receptor 3, CR3) and Fcγ receptors (FcγRs) respectively [18]. Once activated, these receptors undergo clustering that triggers downstream signaling transduction which culminates with the engulfment of the pathogen [19]. Even though signal transduction is not completely understood, remodeling of actin filaments plays a central role. This is controlled by small GTPases proteins belonging to the Rho family such as Rac1 and Cdc42, that control lamellipodia and filopodia, respectively [19]. Moreover, also phosphoinositides such as phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] and phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3] are important for actin remodeling and phagocytosis [20]. Thus, the recruitment of actin, Rac1, Cdc42 and molecules such as Myosin X [20] and the Wiskott-Aldrich syndrome protein (WASP) [18] lead to the formation of the phagocytic cup and pseudopod-like structures and regulate the engulfment of the pathogen [14]. Thereby, the formed phagosome undergoes a series of fusion and fission events involving cytosolic organelles, which lead to phagosome maturation [13].

It's still unclear whether a complete fusion between membranes or the so called 'kiss and run' model is involved or whether a combination of both occurs [20].

During these events the phagosomal membrane fuses in sequence with early endosomes, late endosomes and eventually with lysosomes [21], which lead to the phagolysosome formation, through a Rab7a-dependent process [20] (Figure 1).

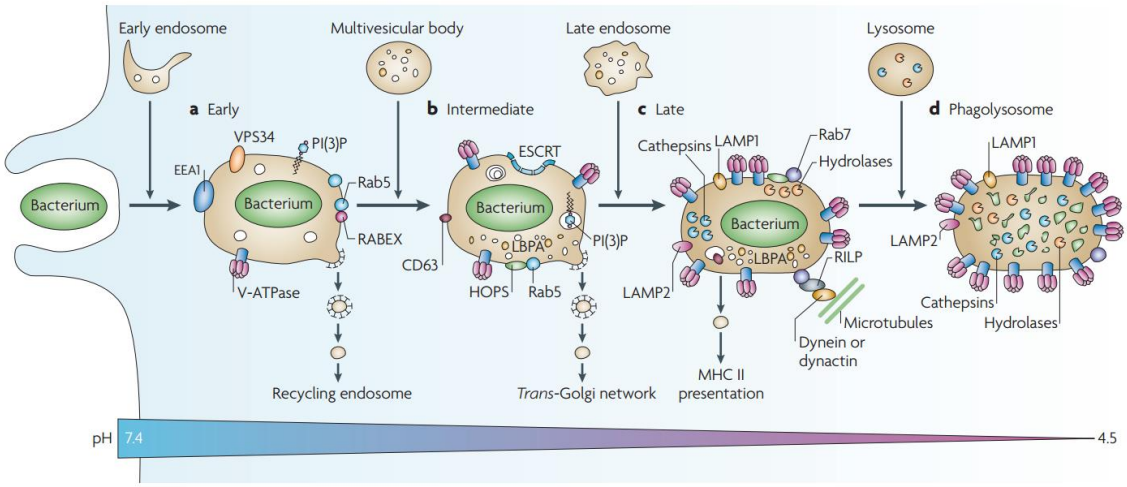


Figure 1. Stages of phagosomal maturation. Early (a), intermediate (b) and late (c) phagosomes, that culminate with the formation of phagolysosomes (d).
Figure taken from Flannagan et al., 2009 [20].

Furthermore, during the maturation events, the phagolysosome acquires different microbicidal characteristics, such as lumen acidification, synthesis of reactive oxygen species (ROS), reactive nitrogen species (RNS) and synthesis of antimicrobial proteins and peptides [20]. Lumen acidification, with a pH around 4.5, prevents microbial growth [22], impairing pathogen metabolism and activating many hydrolytic enzymes. ROS are generated by NOX2 NADPH oxidase, whereas RNS are generated by inducible nitric oxide synthase (iNOS) and both of them act to cause an oxidative damage in pathogen DNA, proteins and lipids [20]. Antimicrobial proteins and peptides are able to impair growth or the integrity of the microorganism, for example by limiting the presence of nutrients inside the phagosome [20].

Nevertheless, several pathogens have evolved diverse strategies in order to survive host defense, eluding one of these features [23-25] and some of them can even replicate

inside phagocytes [20]. However, when phagocytes can successfully kill the pathogens, phagolysosome resorption occurs and the products digestion are either moved into the cytoplasm (if they are beneficial to the host cell) or exported out of the cell by exocytosis [14].

1.3 Toll-like receptors (TLRs)

Toll-like receptors family is the best characterized among PRRs and is able to sense invading pathogens at the cell surface and in intracellular compartments upon internalization [8], or molecules released from damaged cells [3]. The TLRs were discovered almost 20 years ago and led to increased focus on innate immunity, seen at that time as the less important and interesting part of the immune system [26]. In 2011 the key investigators, Hoffman and Beutler, were awarded the Nobel Prize in Physiology/Medicine. The first human TLR discovered was TLR4, homologue to the Toll receptor in the fruitfly *Drosophila melanogaster* a decade earlier [27]. In the fly, the Toll protein was known to regulate the dorsoventral polarity during embryonic development, but in 1996 Toll was shown to be also involved in immunity towards fungal infections in the fly [28]. Thus, since 1997, 10 different functional TLRs in humans and 13 in mice have been identified [4, 8].

All TLRs are type I membrane glycoproteins and consist of an extracellular domain rich in leucine repeats (LRR), a transmembrane domain and a cytoplasmic domain containing the Toll-Interleukin-1 receptor domain (TIR) [29]. The extracellular LRR domain (or ectodomain) folds into a characteristic solenoid structure and consist of 16–28 tandem repeats of the LRR motif [30, 31]. It is involved in the recognition of ligands such as the lipids LPS (lipopolysaccharide) and LTA (lipoteichoic acid), proteins flagellin and porin, sugar zymosan, nucleic acids CpG-containing DNA and single or double stranded RNA and derivatives of proteins or peptides [8].

By contrast, the TIR domain, which got this name because of its homology with the cytoplasmic region of the IL-1 receptor [29, 32], consists of approximate 150 amino acids. This domain is critical for the downstream signal transduction.

Several TIR-domain-containing adaptors have been identified: MyD88 (myeloid differentiation primary response gene88), TIRAP [TIR-containing adaptor protein, also known as MAL (MyD88-adaptor-like)], TRIF [TIR-containing adaptor-inducing interferon-β, also known as TICAM1 (TIR-domain containing adaptor molecule 1)], TRAM (TRIF-related adaptor molecule, also known as TICAM2) and SARM1 (sterile-α- and armadillo-motif-containing protein 1). Moreover, recently, a protein termed BCAP (B cell adaptor for PI3K; also known as PIK3AP1) has been proposed as a sixth TIR adaptor molecule [33, 34]. These adaptors, in turn, can activate MAPKs (mitogen-activated protein kinases) and transcription factors, such as NF-κB (nuclear factor-κB) through the MyD88-dependent pathway, or IRFs (IFN regulatory factors) in order to induce the type I interferons (IFNs) production through the TRAM-TRIF-dependent pathway (Figure 2). In addition, the different TLRs show a different localization within the cell, depending on the type of PAMAPs/DAMPs they recognize. Thus, TLR1, TLR2, TLR5 and TLR6 are expressed on the plasma membrane, whereas TLR3, TLR7, TLR8 and TLR9 are expressed on the endosomal or endolysosomal membranes. TLR4 is expressed on both locations and since it has a unique signaling among TLRs that will be separately discussed below.

Exposition to their specific ligands induces TLRs dimerization: TLRs on the cell surface can homo- or heterodimerize. TLR5 can homodimerize [35], whereas TLR2 heterodmerize with TLR1 or TLR6 when it binds triacyl or diacyl lipopeptides, respectively [36, 37]. On the contrary, almost all the members of the endosomal TLR subfamily are synthesized as stable preformed dimers [38, 39]. TLR3 is the exception, since it is a monomer when inactive [30].

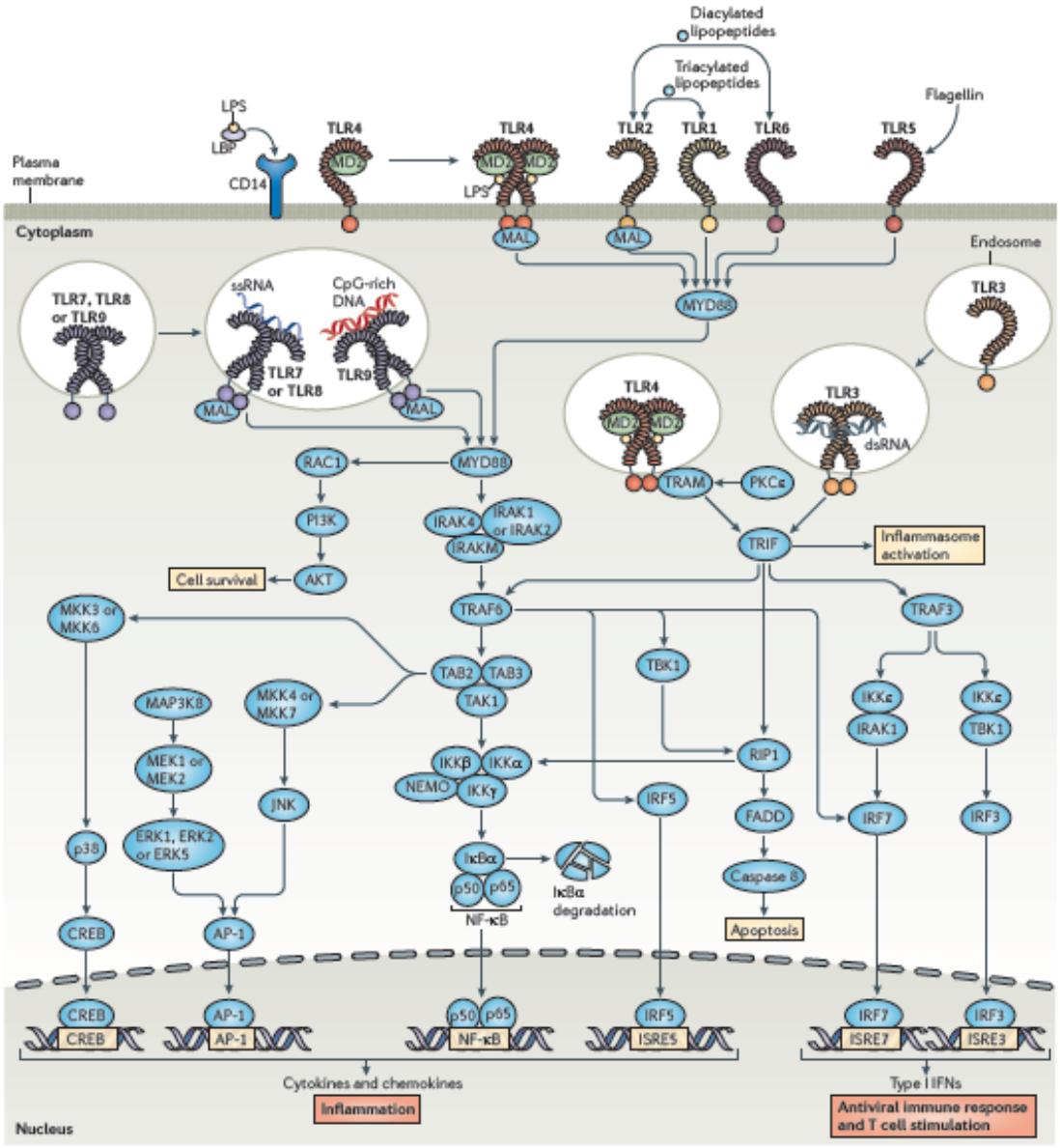


Figure 2. Summary of the TLRs family signaling pathways and TLR localization with respective ligands. Upon stimulation, MyD88- and TRAM-TRIF-dependent pathways can be activated. Figure taken from Gay et al., 2016 [30].

In addition, endosomal TLRs needs a proteolytic cleavage of their ectodomain before efficient binding with their ligands [40, 41]. This process might be necessary in order to give an additional protection from self-nucleic acids, avoiding autoimmune responses.

1.4 Toll-like receptor 4 (TLR4)

As mentioned above, TLR4 was the first human TLR to be discovered, showing that it had a homology with the cytosolic domain of the Toll receptor found in *Drosophila* [28]. TLR4 has an extracellular domain and an intracellular domain, made of 608 and 187 amino acidic residues, respectively. It binds to several types of PAMPs and DAMPs, leading to both infectious and non-infectious inflammatory diseases, such as ischemia/reperfusion injury and neurodegenerative diseases [42]. The first TLR4 ligand to be discovered, and by far the most studied is LPS (also known as endotoxin). LPS is the major component of the outer membrane of Gram-negative bacteria [43, 44]. LPS comprises three parts: O antigen (or O polysaccharide), core oligosaccharide and Lipid A, responsible for most of the toxicity of Gram-negative bacteria. This endotoxin is a strong immunogenic molecule and a high concentration in the blood can lead to septic shock [45].

For a TLR4 ligand-induced activation, a physical association between the extracellular domain of TLR4 and myeloid differentiation factor 2 (MD2) is necessary [46-49]. Moreover LPS needs to bind LBP (LPS binding protein). LBP, a plasma protein which binds the lipid A moiety of LPS, also contributes to the cytokines release in response to LPS [46, 47]. MD2 is a small secreted glycoprotein and it is critical for the LPS to bind TLR4 [48, 49]. After LPS binding, two TLR4/MD2 complexes dimerize, leading to conformational changes of the TLR4 homodimer which allows binding of the TIR-adaptor proteins [30]. Another accessory molecule which enhances LPS sensing is CD14 (cluster of differentiation 14). CD14 is a glycosylphosphatidylinositol (GPI)-linked protein, found on the surface of many TLR4 expressing cells and called mCD14 (membrane-CD14) [50]. A soluble form of CD14 (sCD14) is circulating in the blood and might enhance the cells response to LPS [51]. Its role is to transfer LPS-LBS complex to the TLR4-MD-2 heterodimer [52-54].

TLR4 is the most studied in the TLR family. It engages four TIR domain–containing adaptors: MyD88, TIRAP, TRIF and TRAM [32] and can induce both MyD88-dependent signaling pathways and MyD88-independent production of type I IFNs through

recruitment of TRAM and TRIF signaling adapters (TRAM-TRIF-dependent pathway) [55]. It can signal from two different location of the cell. The MyD88-dependent pathway starts from the plasma membrane, whereas the TRAM-TRIF-dependent pathway is activated from the phagosomal membrane [56, 57]. TLR2 was also shown to be able to recruit the TRAM-TRIF adaptors, however the TRAM-TRIF-mediated responses induced by TLR2 ligands, are weaker compared to TLR4 ligands [58].

1.4.1 TLR4 trafficking

As all TLRs, TLR4 undergoes a highly regulated trafficking through different cellular compartments in order to sustain the signal transduction and regulation. Proper processing in the ER (endoplasmatic reticulum) requires the chaperone molecules, heat shock protein HSP90β1 (also known as endoplasmin, GRP94, GP96 and TRA1) and the protein associated with TLR4 PRAT4A (also known as CNPY3) [59-61]. Moreover, in the ER, the association of TLR4 with MD2 occurs and this step has been demonstrated to be critical for a correct glycosylation, transport to the plasma membrane and a proper response to LPS [53, 62, 63]. TLR4 delivery to the plasma membrane might also require the COPII (coat protein complex II) adaptor protein called TMED7 (transmembrane emp24 domain-containing protein 7) [64]. Furthermore several small Rab GTPase (Ras-related in brain) proteins are involved in TLR4 trafficking. Rab10 controls the rate of TLR4 trafficking from the Golgi to the plasma membrane in response to LPS [65], whereas, Rab11a is responsible for TLR4 trafficking from the endocytic recycling compartment (ERC) to the phagosomal membrane [57]. Rab7b is a negative regulator of the TLR4 signaling pathway since it promotes the movement of TLR4 into late endosomes for its degradation [66].

1.4.2 The TLR4-activated MyD88-dependent pathway

After LPS binding and TLR4/MD-2 tetramerization the β2 integrin CD11b, which interact with CD14, leads to the activation of the GTPase ARF6 (ADP ribosylation factor 6) and the resulting induction of PtdIns(4,5)P_2 (PIP_2) synthesis at the plasma membrane, by the

kinase PI5K (Phosphatiidylinositol 4-phosphate 5-kinase) [67]. PIP_2 enrichment at the cell surface mediates TIRAP recruitment, which has a PIP_2 binding domain and can bind TLR4 more easily trough a TIR-TIR interaction. By another TIR-TIR interaction TIRAP, acting as a "sorting adaptor" can recruit MyD88 to PIP_2-rich plasma-membrane subdomains and facilitates the interaction between TLR4 and MyD88, which can be defined as the "signaling adaptor" [67]. When MyD88 is recruited, MyD88-dipendent pathway can start to signal. Thus, MyD88 triggers, through its DD domain (death domain), IRAK4 (IL-1 receptor-associated kinase 4) which consequently activates IRAK1, inducing its kinase-activity (akira2004 37). IRAK-1 can be functionally substituted by IRAK-2 and thus these kinases might have a redundant role in this pathway [68].

Moreover, in vitro studies showed that the MyD88 DD domain forms, in the presence of IRAK4 a heterocomplex called the Myddosome [69]. The Myddosome can be composed of six to eight molecules of MyD88 and four molecules of IRAK4. Whereas a variant Myddosome containing IRAK2 consists of three layers with six MyD88 DDs, four IRAK4 DDs and four IRAK2 DDs, facilitating their interaction and thus their phosphorylation [70]. It seems that the Myddosome might exist even in physiological conditions [71].

The activated IRAKs proteins can associate with the E3 ubiquitin ligase TRAF6 (TNF receptor-associated factor 6). The polyubiquitinated TRAF6 activates the preformed complex composed of the protein kinase TAK1 (transforming growth factor-β-activated kinase 1) and TABs (TAK1-binding proteins) TAB1 and TAB2 or TAB3. This complex can activate the canonical IKK complex (inhibitor of nuclear factor kappa-B kinase complex) or MAPKs (Mitogen-activated protein kinase) [29]. IKK complex (consisting of the two catalytic subunits IKK-α and IKK-β and a regulatory subunit IKK-γ) phosphorylates IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), leading to its polyubiquitylation and proteasome-mediated degradation. Since IκBα acts as an inhibitor of the transcription factor NF-*κ*B, after its degradation, NF-*κ*B can be released and translocated to the nucleus, inducing the transcription of inflammatory genes [29]. On the other hand, MAPKs such as ERK (extracellular-signal-regulated kinase), JNK (c-jun N-terminalkinase) and p38 lead to the activation of a second inflammatory transcription factor, AP-1 (activator protein 1) [72].

1.4.3 TLR4 internalization and the TRAM-TRIF-dependent pathway

The TRAM-TRIF-dependent pathway signals from the phagosomal membrane [56].
According to the model proposed by Kagan and coworkers, after the immediate MyD88-dependent signal has started, the invagination of the plasma membrane due to the phagocytic process occurs and PIP_2 concentration goes dramatically down, leading to the release of the TIRAP-MyD88 complex and allowing the TRAM-TRIF complex to bind to the TIR domain of TLR4 on intracellular compartment. This second phase of TLR4-induced signaling leads to the production of type I IFNs [56].
However, another model has been subsequently proposed by Husebye and coworkers who showed that even though in a very early stage of the phagocytic process the TRAM-TRIF-dependent signaling pathway may start with the internalization of TLR4 from the plasma membrane, the major source for phagosomal TLR4 is the ERC [57]. They also demonstrated, as mentioned above, that TLR4 is delivered from the ERC to the phagosomal membrane by the small GTPase Rab11a containing vesicles, promoting the type I IFNs production (Figure 3). Moreover, studies using Dynasore, a dynamin inhibitor, showed that TLR4 internalization is a dynamin-dependent process [56, 73]. It was also demonstrated that TLR4 uptake depends on CD14 by the tyrosine kinase Syk/PLCγ2 regulation [74] and that the involvement of CD11b is required for a proper LPS response [75-77]. Once TLR4 has been engulfed together with its ligand, TRIF is recruited from the cytoplasm by TRAM, which acts as a bridging adaptor, like TIRAP in the MyD88-dependent pathway [56]. TRIF has a critical role in this pathway, shown in TRIF-deficient cells which lose the ability to produce IFN-β [78, 79]. TRIF interacts with RIP1 (receptor-interacting protein 1) through its C-terminal part [80] and with TRAF6 (TNF receptor associated factor 6) through its TRAF6-binding motif located in its N-terminal part [81]. Both RIP1 and TRAF6 lead to the activation of the late inflammatory response [82]. TRIF can also recruit TRAF3 (TNF receptor associated factor 3) which consequently activate IKKε (also known as IKKi) [83, 84] and the IKK-related kinase TBK1 (TANK binding kinase 1). Therefore the formed complex of kinases IKKε-TBK1 phosphorylates IRF3, which dimerizes and translocate into the nucleus.

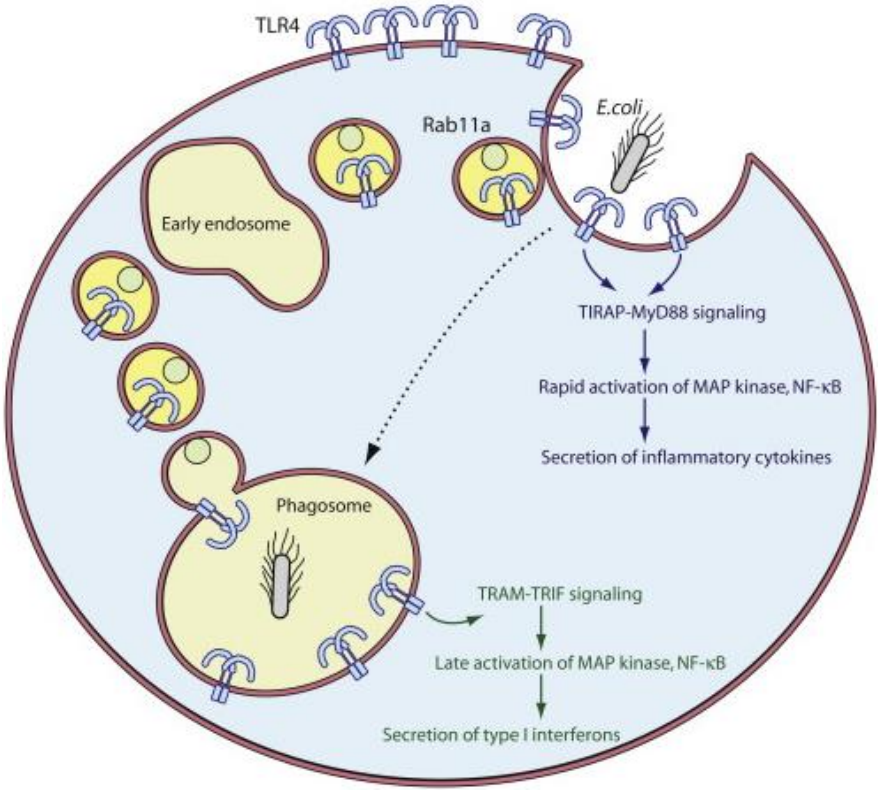


Figure 3. Schematic representation of the two signaling pathways activated by TLR4 and how it is delivered to the phagosomal membrane by Rab11a positive vesicles.
Figure taken from Kagan, based on Husebye et al., 2010 [57].

Once activated IRF3, along with the co-activators p300 and CBP [cAMPresponsive-element-binding protein (CREB)-binding protein] [29], binds to the ISREs (IFN-stimulated response elements) and activates the transcription of the type I IFNs genes and the chemokine CCL5 (RANTES) [85].

The type I IFNs, IFN-α and IFN-β, make an important link between the innate and adaptive immune response against pathogen microbes. The type I IFNs have mainly been described as anti-viral cytockines, preventing virus replication [86]. However it is now demonstrated that they are produced also in response to bacterial infections, even though their role seems to be controversial, being sometimes beneficial and sometimes detrimental [87, 88]. Indeed they were shown to be involved in suppression of innate cell recruitment and pro-inflammatory response, upon *S. aureus* infections [89, 90], but

on the other hand they are crucial for the host resistance to different bacteria, like group B streptococci, pneumococci and *E. coli* [91].

Furthermore, it has been showed that the autophagy-related 16-like 1 factor (Atg16L1) negatively regulated the TRIF-dependent signaling pathway suggesting that autophagy might be involved in innate immune regulation [92].

1.4.4 The TLR4 adaptor protein TRAM

TRAM is a TIR-containing adaptor protein used by TLR4 [93] and its role is to function as a bridging molecule between TLR4 and TRIF [56].
Moreover, it has been shown that upon LPS stimulation, Rab11a plays a crucial role to deliver TRAM from the Golgi to the ERC and subsequently to the endo/phagosomes [49]. TRAM is a protein made of 235 amino acids, with a myristoylated domain and a phosphoinositide-binding domain in its N-terminus, and the TIR domain in its C-terminus. The myristoylated domain is necessary for its localization to the plasma membrane, the trans-Golgi-network (TGN) and endosomes and it has been demonstrated to be fundamental for the activation of IRF 3 [56, 94].
Inside TRAM N-terminus, a protein kinase C-ε (PKCε) phosphorylation acceptor site has been found, responsible for TRAM phosphorylation (at S16) and its consequent dissociation from the plasma membrane upon LPS stimulation [95].
By contrast, the TIR domain is crucial for the interaction with TRIF and TLR4 TIR domains [32].
Huai and coworkers showed that upon TLR4 activation TRAM undergoes a tyrosine phosphorylation, at Y167 position. This tyrosine is located in the TIR domain and is required for the TLR4-induced IRF3 activation and IFN-β secretion. Furthermore they showed that a protein tyrosine phosphatases (PTP) called PTPN4 (PTP non-receptor type 4) is a negative regulator of the TRAM-TRIF pathway, attenuating TRAM Y167 phosphorylation and thus its cytoplasm translocation and inhibiting the TRAM–TRIF interaction [96].

Moreover, two TRAM motifs seem to be crucial for the TRAM-TRIF signaling pathway activation: the E87/E88/D89 motif, which regulates the bond of TRAM to TRIF [97] and the D91/E92 motif, which is critical for IFN-β activation [98].

An alternative splice variant of TRAM, called TAG (TRAM adaptor with Gold domain), a protein which contains a GOLD domain instead of the myristoilated domain has been shown to sequester TRIF from TRAM, acting as a negative regulator of the TRAM-TRIF signaling cascade and this disruption is TMED7-mediated [99, 100].

1.5 The role of Rab11-FIP2 as effector protein of the Rab11 family

Rab proteins are small GTPases anchored to the lipid bilayers of intracellular compartments, trough C-terminal prenylation of their cysteine residues [101], and they are known to be involved in transport regulation systems [102].

Rab11 subfamily comprises three isoforms: Rab11a, Rab11b and Rab25 [103], which localize to the ERC or to the apical recycling endosomes in polarized cells, in order to regulate endosomal trafficking through these compartments [104-106].

Rab11a has been shown to have a crucial role in both TLR4 and TRAM trafficking, upon LPS stimulation [49, 57].

Rab11a (like all the Rab proteins) needs effector proteins in order to work properly and over the last decades, several proteomic studies led to the identification of a highly conserved effector protein family, known as the Rab11-FIPs (Rab11 famyly interacting proteins) which act downstream of Rab11 [103].

Among them, Rab11-FIP2 (also known as FIP2), belonging to class I FIPs, is a 512 residue protein characterized by a conserved 20 amino acid RBD (Rab11-binding domain) and a α-helical coiled-coil structure, both located at its C-terminus and a phospholipid binding C2-domain close to its N-terminus [104].

FIP2 has a role in cellular physiological processes such as cell division, but it is mainly involved in recycling and delivery systems.

Indeed FIP2 was shown to be involved in receptor-mediated endocytosis [107], recycling of the chemokine receptor CXCR2 (CXC chemokine receptor 2) [108] and the water channel protein AQP2 (aquaporin-2) [109].

Moreover, it is has also a role in trafficking of GLUT4 (Glucose transporter type 4) [110] and the RSV (respiratory syncytial virus) [111].

Studies made on crystal structure of Rab11a-FIP2 complex, demonstrated that two FIP2 molecules form a parallel coiled-coil homodimer with two symmetrical RBD domains, interacting with two Rab11-GTP molecules [101, 112, 113] forming thereby a heterotetrameric complex (Figure 4).

Moreover it's known that FIP2 can also interact with Myosin5B, which is an actin-based motor protein and it has been showed that the tripartite association of Rab11a with both FIP2 and Myosin5B is crucial for movement regulation of Rab11a-containing recycling vesicles which can thus slide along the cytoskeleton [114, 115].

A **B**

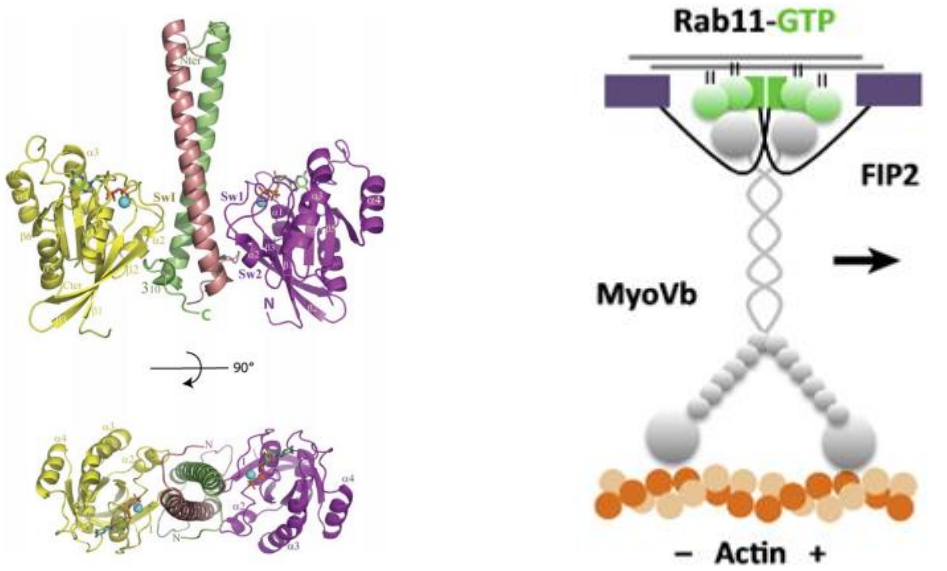


Figure 4. Ribbon representation of Rab11a-FIP2 complex. Rab11 molecules are yellow and magenta, while FIP2 is colored dark pink and green (A). Figure taken from Jagoe et al*.,* 2006 [101].
Rab11-FIP2-Myo5b tripartite complex and its interaction with actin filaments (B). Figure taken from Welz et al. 2014 [115].

2 AIMS OF THE THESIS

Since Rab11a has the critical role to recruit TLR4 and TRAM to *E. coli* phagosomes and induce IFN-β mRNA, one of the aims of this thesis was to investigate whether the Rab11 effector protein FIP2 might have a role in the *E. coli*-induced TRAM-TRIF-dependent IFN-β production.

Secondly, a potential interaction between TRAM, FIP2 and Rab11a was addressed. Moreover, since both FIP2 and TRAM accumulate at the *E. coli* phagocytic cup (along with F-actin), as well as at the early *E. coli* phagosome, their roles in phagocytosis were addressed. MyD88, already shown to be involved in *E. coli* phagocytosis and phagosome maturation in mice was included as a control.

Since FIP2 is also known to control recycling of endocytosed receptors to the cell surface, the role of FIP2 in the control of surface levels of CD11b (CR3), CD16 (FcγRIII), CD14 and TLR4 (receptors involved in *E. coli* response and internalization), was investigated.

3 MATERIALS AND METHODS

3.1 Cell cultures and their maintenance

Two clones of the human monocyte-like cells THP-1 were used: wild type (WT) (ATCC® TIB-202™) and a 50% FIP2 knock down clone, F2, made with TALEN® technology (source) and called F2 (Patanè, F., Skjesol, A., Husebye, H. et al.) THP-1 cells were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium (ATCC® #30-2001™) supplemented with 10% heat inactivated fetal calf serum (FCS), 0.05 mM β-mercaptoethanol (Sigma) and 100 nM penicillin/streptomycin (Life Technologies) and incubated at 37ºC in 5% CO_2 atmosphere. Cultures were maintained by splitting cells 2-3 times a week, to keep the density between $0{,}2x10^5$ and 10^6 cells/ml. Immortalized murine black 6 macrophages (B6 macrophages): WT and the B6 knock out macrophages, $MyD88^{-/-}$ and $TRAM^{-/-}$, were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat inactivated FCS, 10 µg/ml ciprofloxacin (CellGro®) and 700 µM L-glutamin (Sigma) and incubated at 37ºC and 8% CO_2. The cell cultures were maintained by splitting cells at 70-80% of confluence using 3 ml of Trypsin-EDTA (Lonza) in PBS (Phosphate-buffered saline) to detach cells. Human monocytes were isolated from buffycoat by Lymphoprep gradient and adherence as previously described [73]. The use of human monocytes from blood donors has been approved by the Regional Committee for Medical and Health Research Ethics at NTNU. Monocytes were maintained in RPMI1640 (Sigma) supplemented with 10% of pooled human serum (The Blood Bank, St Olavs Hospital, Trondheim, Norway). Human Embrionic Kidney (HEK 293T) cells were cultured in DMEM supplemented with 10% FCS and 100 nM penicillin/streptomycin (Life Technologies) and incubated at 37ºC in 8% CO_2. The cells were cultured and maintained by splitting cells when the confluency reached 70-80%, using 3 ml of Trypsin-EDTA (Lonza).

3.2 Differentiation and siRNA treatment

THP-1 cells were seeded in 6-well plates (Nunc™), at a density of $0.4x10^6$ per well and differentiated into macrophages using 40 ng/ml PMA (phorbol 12-myrstate 13-acetate) in antibiotic-free media for 72 hours and rested for 48 hours in PMA-free and antibiotic-free medium. When needed, the cells were stimulated 96 hours after siRNA transfection. HEK293T were seeded in 6-well plates (Nunc™) with a density of $0.4x10^6$ cells/well. Both THP-1 and HEK293T cells were transfected 24h after seeding using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. The final siRNA concentration was 16nM in all experiments. Media was changed to fresh antibiotic-free 48 hours after transfection and the cells were stimulated 96 hours after transfection. PBMCs were seeded in 6-well plates (Nunc™) with a density of $6.0x10^6$ cells per well, before being differentiated into macrophages using 50 ng/ml recombinant human M-CSF (R&D Systems) in RPMI1640 (Sigma) with 10% human serum, 700 µM L-glutamin (Sigma) and 20 µg/ml Gensumycin (Sanofi-Aventis). Media was changed on day 3 and on day 5. On day 6 and day 8, the cells were transfected with 32nM siRNA using the Lipofectamine® 3000 Transfection Reagent (Invitrogen), according with the manufacturer's instructions. Media was changed to fresh antibiotic-free media at least 2 hours before the second siRNA transfection and the cells were stimulated on day 10. The AllStars Negative Control (QIAGEN) was used as a non-silencing RNA oligo and the Hs_Rab11FIP2_5, Hs_MyD88_5 and Hs_TICAM2_2 Validated siRNA (QIAGEN) were used to target the FIP2, MyD88 and TRAM mRNA, respectively.

3.3 Cell stimulation

B6 macrophages were seeded in 6-well plates (Nunc™) with a concentration of $0.2x10^6$ cells/well (WT and $TRAM^{-/-}$) or $0.3x10^6$ cells/well ($MyD88^{-/-}$). Cells were then stimulated the day after seeding. PMA-differentiated THP-1 cells, primary human macrophages or B6 macrophages were stimulated in a final volume of 1ml/well with pHrodo® Red *E. coli* BioParticles® (ThermoFisher Scientific) or pHrodo® Red *S. aureus* BioParticles® (ThermoFisher Scientific), using 25, 50 or 100 µl (corresponding to 18,7 37,5 and 5

bioparticles per cell, respectively). *E. coli* K12 LPS Ultrapure (InvivoGen) (100ng/ml) stimulation were done in some experiments as well. All stimulations were done at different time points.

LPS and bioparticles were pre-incubated in 10% of mouse serum for of B6 macrophage stimulation or 10-12% of A^+ human serum, for opsonization. After stimulation, cells were washed in PBS and treated either with 500µl of QIAzol® (Qiagen) Lysis Reagent and frozen at -80ºC, for total RNA extraction; or with 500µl of Accutase® (Sigma-Aldrich) cell detachment solution for flow cytometry analysis.

3.4 RNA extraction and quantitative real-time PCR

The QIAzol®-treated lysates were slowly thawed on ice and left at room temperature for 5 min. After addition of 100µl of chloroform and vigorous shaking, the tubes were centrifugated at 11600g for 15 min at 4ºC. The top layer was aspirated and moved into a new tube, where 0,5X volume of absolute ethanol was added. The lowest phenol-ethanol layer was kept and stored at -80 ºC for protein isolation. Purification on RNeasy® Mini Kit (Qiagen®) spin columns as well as DNase digestion step (QIAGEN), were performed, according to the manufacturer's protocol. RNA quantity and purity was checked using Nanodrop ND-1000 (Thermo Scientific) spectrophotometer. 450 ng of extracted RNA were added to a mixture containing reverse transcriptase and converted into cDNA using the Maxima Fist Strand cDNA Synthesis Kit (ThermoFisher Scientific), according to the manufacturer's instructions. cDNA synthesis was performed in a thermocycler, following three steps: 10 min at 25ºC, 30 min at 50ºC and 5min at 85ºC. 5 µl of diluted (1:10) cDNA were added to the reaction mixture containing PerfeCTa qPCR Fast Mix (Quanta Bioscience), TaqMan probe (Applied Biosystems) and Nuclease-free water, with a final volume of 20 µl, according with the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific). TaqMan probes used were: TBP, TATA-Box binding Protein, (Hs00427620_m1) as endogenous control, RAB11-FIP2 (Hs00202593_m1), TICAM2 (Hs04189225_m1), MYD88 (Hs01573837_g1), IFNB1 (Hs01077958_s1) and TNF (Hs00174128_m1). The level of TBP mRNA was used for normalization and results

presented as relative expression compared to the control-treated sample. Relative gene expression was evaluated using StepOnePlus™ software, performing the quantitative comparative (ΔCt) program. and it was calculated using the Pfaffl's mathematical model [116].

3.5 Cell-surface receptors analysis

After differentiation, plates were put on ice and cells were washed with cold PBS and treated with 500µl of Accutase® (Sigma) for 10-15 min. Then they were harvested by gentle scraping, transferred into FACS tubes and washed with PBS and PBS containing 0,1% BSA (bovine serum albumin), removing supernatants by centrifugation. Cells were then surface-stained with different PE-labeled primary antibodies, targeting different receptors, for 30 minutes on ice in the dark. After a wash in PBS and a second one using PBS+ 0,1% BSA, cells were resuspended in 200 µl of PBS.

Antibodies used were: mouse IgG_{2B} PE-conjugated Antibody (R&D Systems) as Isotype control; anti-human CD14 (MϕP9) PE-conjugated, anti-human CD11b (D12) PE-conjugated and anti-human CD16 (3G8) PE-conjugated from Bioscience and anti-human CD284 (TLR4) (HTA125) PE-conjugated from eBioscience.

Live cells were gated setting a living cell gate based on forward scatter/side scatter (FSC/SSC) plot. Fluorescence intensity was measured by BD LRSII flow cytometer using FACS Diva software (BD Biosciences). Data were exported and analyzed using FlowJo software v10.0.5 (Trees Star).

3.6 Phagocytic Assay

This assay measures the phagocytic efficiency of macrophages after stimulation with pHrodo®-conjugated bioparticles (more details in cell stimulation section). Indeed, since pHrodo dye conjugates are pH sensitive, they are non-fluorescent outside the cell, where pH is neutral, but fluoresce brightly red in phagosomes, where the pH is strongly reduced. Thus there will be a positive correlation between high fluorescence intensity values and phagosomal acidification, necessary for an efficient phagocytosis [14, 20].

After differentiation and stimulation, plates were put on ice and cells were washed with cold PBS and treated with 500µl of Accutase® (Sigma-Aldrich) for 10-15 min. Then they were gently scraped, transferred into FACS tubes, washed with PBS and Flow wash (PBS containing 2% FCS) and resuspended with 200 µl of the same buffer.

In some experiments cells were also stained with a viability dye for 15 min on ice in the dark. Fluorescence intensity was measured by BD LRSII flow cytometer using FACS Diva software (BD Biosciences), after gating single live cells in FSC/SSC plots, as well as viability dye staining negative cells. Data were exported and analyzed using FlowJo software v10.0.5 (Trees Star).

3.7 Expression vectors and DNA transfection

Human $TRAM^{YFP}$ from K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA, USA), $RAB11a^{FLAG}$ [57], $TRAM^{FLAG}$ from Mariia Yurchenko (Norwegian University of Science and Technology, Trondheim, Norway), $FIP2^{GFP}$ from Mary McCaffrey (Cork University College, Cork, Ireland) and $Rab11a^{CFP}$ (Klein et al., 2015) were used for transfections. pDUO-hMD-2/CD14 (Invivogen) was co-expressed with $TLR4^{Cherry}$ [57] to ensure TLR4 dimer formation.

Empty EGFP -N1, empty EYFP-N1 and empty C-terminal-DYKDDDDK vectors (Clontech) were used for control GFP, YFP or FLAG protein expression, respectively. For endo-free plasmids preparations Endofree plasmid Maxi kit (QIAGEN) was used. One day after silencing, HEK 293T cells were transfected using 0.2-0.3 ug of vectors/well. Transfections were performed using GeneJuice® Transfection Reagent (Novegen), according with the manufacturer's instructions. The day after, media was changed and replaced with antibiotic-free and PMA-free media. Cells were lysed 48 hours after transfection, in order to perform immuno-precipitation.

3.8 Immunoprecipitation

HEK293T cells expressing FLAG-tagged and/or YFP-tagged proteins, were lysed using 1X lysis buffer (150 mM NaCl, 50 mM TrisHCl pH 8.0, 1 mM EDTA, 1% NP40), supplemented

with EDTA-free Complete Mini protease Inhibitor Cocktail Tablets (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche), 50 mM NaF, 2 mM $NaVO_3$ (Sigma) and 2,5U/ml Benzonase® Nuclease (Novagen®). Immunoprecipitations were carried out using anti-FLAG (M2) antibody conjugated to agarose beads (Sigma) which was incubated with lysates on rotation overnight. After washes using the same lysis buffer, agarose pellets were resuspended in 1X NuPAGE LDS (*lithium* dodecyl sulfate) sample buffer (Novex) and heated at 70 ºC for 7-8 min, before removing agarose beads sitting on the bottom of the tubes and adding 25 mM DTT (Dithiothreitol). Samples were then heated again at 70 ºC for 7-8 min and analyzed by Western-blot (Immunoblott).

3.9 Protein isolation

Proteins were isolated from the lower phenol-ethanol layer saved from QIAzol® lysates, according with the manufacturer's instructions. The protein pellet was dissolved in 50-200 µl of a buffer made of 4% SDS (Sigma), 4M urea, EDTA-free Complete Mini protease Inhibitor Cocktail Tablets (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche), 1X NuPAGE® LDS sample buffer (Novex) and 25 mM DTT. Samples were then heated at 95ºC for 5 min and analyzed by Western blot.

3.10 Immunoblotting

Heated samples were run in pre-cast protein gels NuPAGE™ Novex™ (ThermoFisher Scientific), with 1X MES buffer (ThermoFischer Scientific) and were transferred on nitrocellulose membranes, using the iBlot®2 Gel Transfer Device (Invitrogen). Membranes were washed in TBS-T (Tris Buffered Saline + 0,1% Tween) and incubated with blocking buffer (TBS-T containing 5% milk/BSA) for 45 min at room temperature on a shaker . Afterwards, membranes were incubated with primary antibodies in TBS-T containing 1% milk/BSA, at 4ºC, overnight or for 2-3-days, depending on the antibody.
The following primary antibodies were used: anti–FLAG M2, from Sigma; anti -GFP from Clontech; anti-phospho IRF3 (S396) (4D4G), anti-phospho TBK1 (S172), anti-total TBK1/NAK (D1B4), anti-phospho IκBα (I4D4) and anti-phospho p38 MAPK (T180/Y182)

from Cell Signaling; anti-phospho IRF3 (S386) (ab76493) and anti β-tubulin (ab15568) from Abcam and anti-total IRF3 (FL-425) from Santa Cruz.

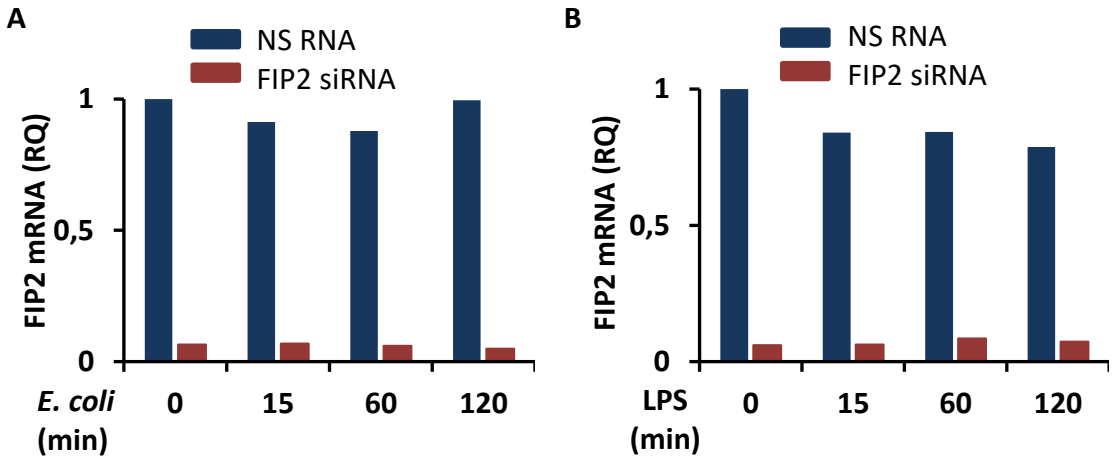
Membranes were then washed three times in TBS-T and incubated with secondary antibodies (HRP-conjugated, from DAKO Denmark A/S) for one hour at room temperature in TBS-T containing 1% milk/BSA and developed with SuperSignal™ West Femto Substrate (ThermoFisher Scientific), capturing the specific signal using the LI-COR Odissey® detection system. Images were analyzed by Odyssey Image Analysis software.

4 RESULTS

4.1 FIP2 is involved in *E.coli* induced IFN-β signaling downstream of TLR4

To investigate the role of FIP2 in *E. coli* induced IFN-β signaling downstream of TLR4, differentiated THP-1 cells were treated with a non-silencing RNA oligo (NS RNA) or FIP2 siRNA. Following silencing, the cells were stimulated with pHrodo *E. coli* particles or ultrapure LPS for 15, 60 and 120 minutes. FIP2 silencing was verified by realtime quantitative PCR (q-PCR), showing a knockdown level (KD) of approximately 95% for the investigated time points (Figures 5A and 5B, upper panels).

As shown in Figure 5A and 5B (middle and lower panels), FIP2 silencing markedly impaired both *E. coli* and LPS stimulated IFN-β induction after 60 and 120 minutes of stimulation, whereas TNF (pro-inflammatory cytokine) induction was largely unaffected. Furthermore, the effect of FIP2 depletion was stronger on *E.coli* stimulated IFN-β than LPS stimulated IFN-β, especially after 120 min of stimulation showing a 72% and 45% reduction, respectively.



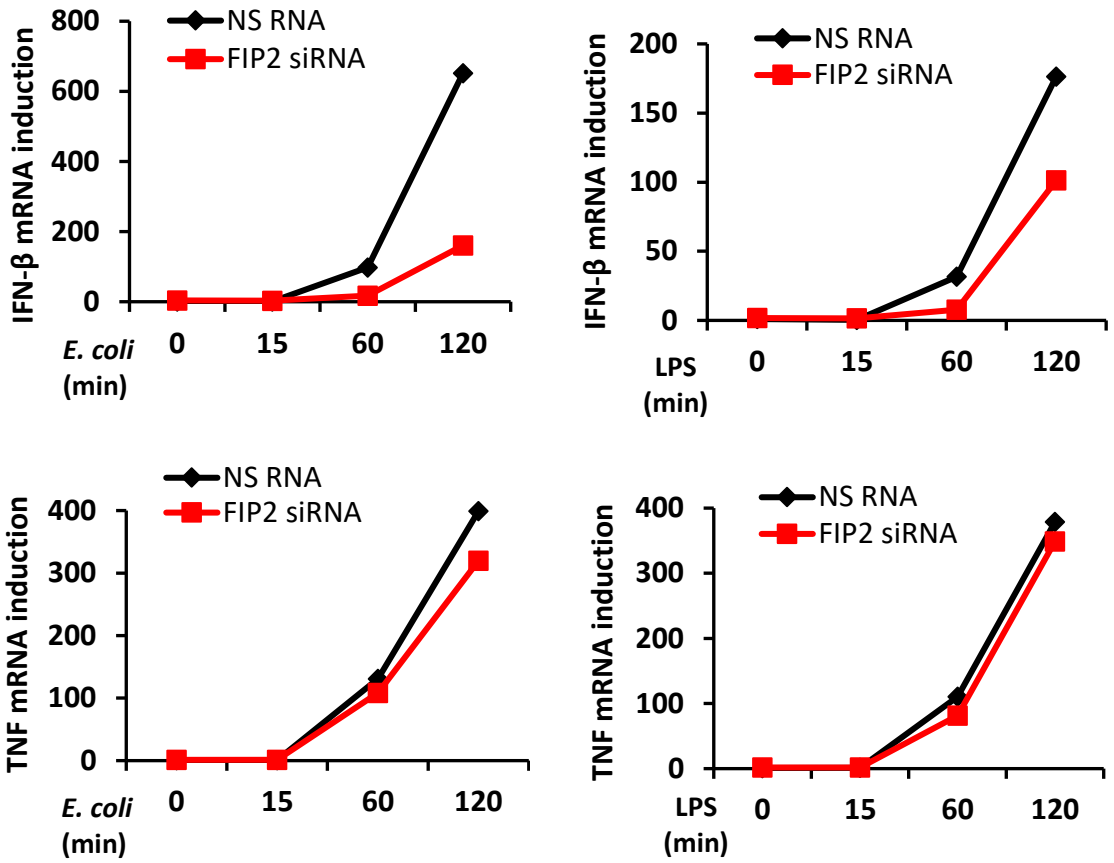
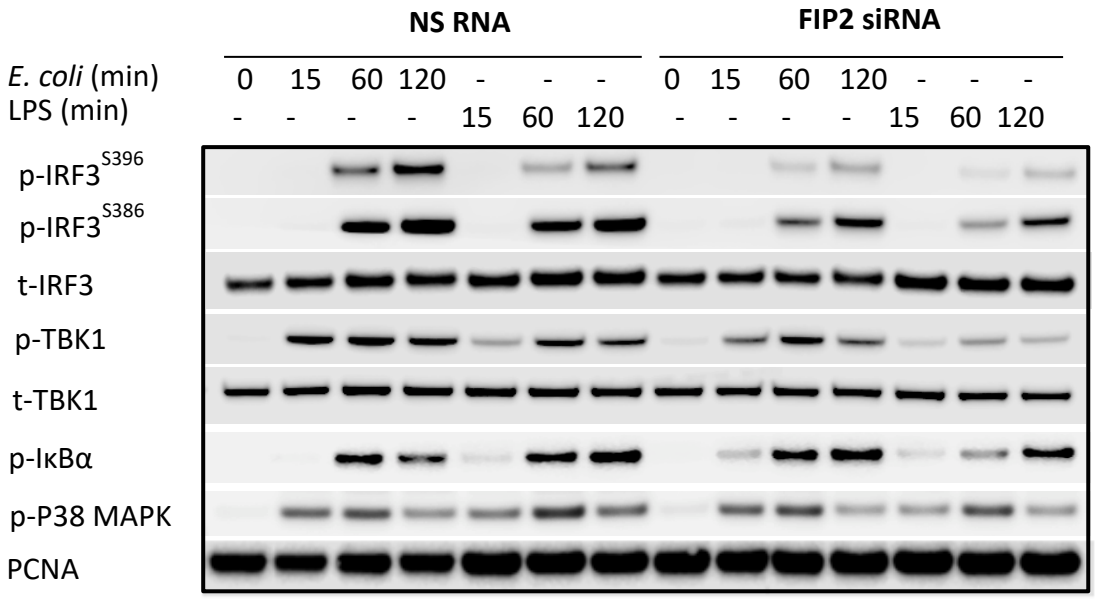


Figure 5. Relative quantification of FIP2 (upper panels), IFN-β (middle panels) and TNF (lower panels) mRNA by q-PCR in differentiated THP-1 cells, treated with a nonsilencing RNA oligo (NS RNA) or FIP2 siRNA and stimulated with *E. coli* particles ($7.5x10^6$/ml) (A) or LPS (100 ng/ml) (B). TBP was used as endongenous control for normalization. Results show one representative of three independent experiments.

To verify these results, proteins from the same biological samples were isolated and immunoblotting performed, addressing phosphorylation of TBK1 at S172 and IRF3 at S386 and S396 known to be important for the LPS-induced TRAM-TRIF-dependent signaling pathway giving IFN-β [117, 118]. Silencing of FIP2 gave a markedly reduced phosphorylation of TBK1 at S172 and IRF3 at S386 and S396 following stimulation with *E. coli* (Figures 6A and 6B). Phosphorylation of IκBα and p38 MAPK, known to be involved in the MyD88-dependent signaling pathway, were also addressed and showed no marked reduction. In contrast to *E. coli* stimulated cells, following LPS stimulation FIP2 silencing also reduced the phosphorylation of IκBα and p38 MAPK (Figure 6A and 6C).

A


NS RNA
FIP2 siRNA
E. coli (min)
LPS (min)
0 15 60 120 - - -
- - - - 15 60 120
p-IRF3S396
p-IRF3S386
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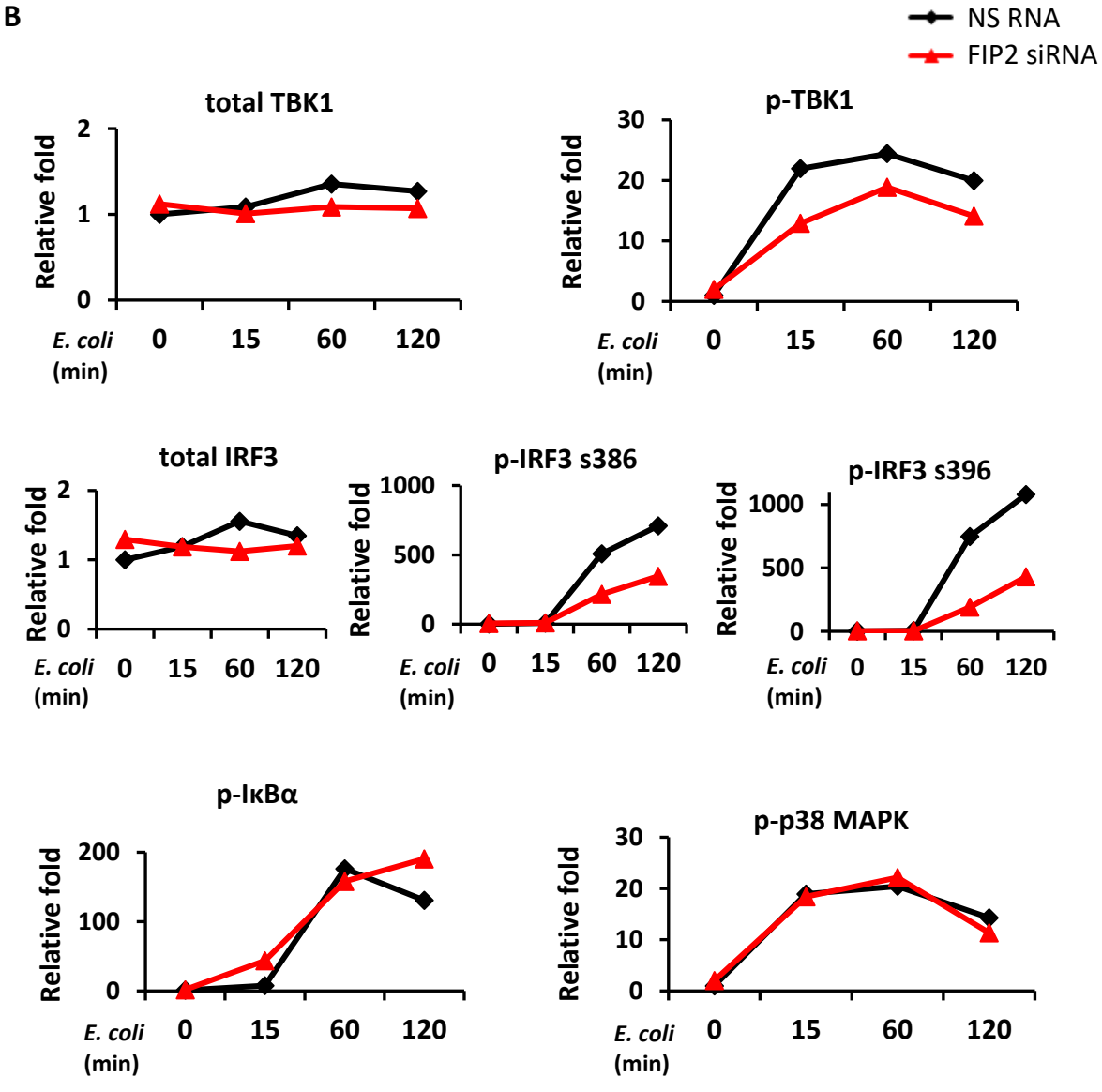
p-TBK1
t-TBK1
p-IκBα
p-P38 MAPK
PCNA

B


NS RNA
FIP2 siRNA
total TBK1
p-TBK1
total IRF3
p-IRF3 s386
p-IRF3 s396
p-IκBα
p-p38 MAPK
Relative fold
E. coli (min)
0 15 60 120

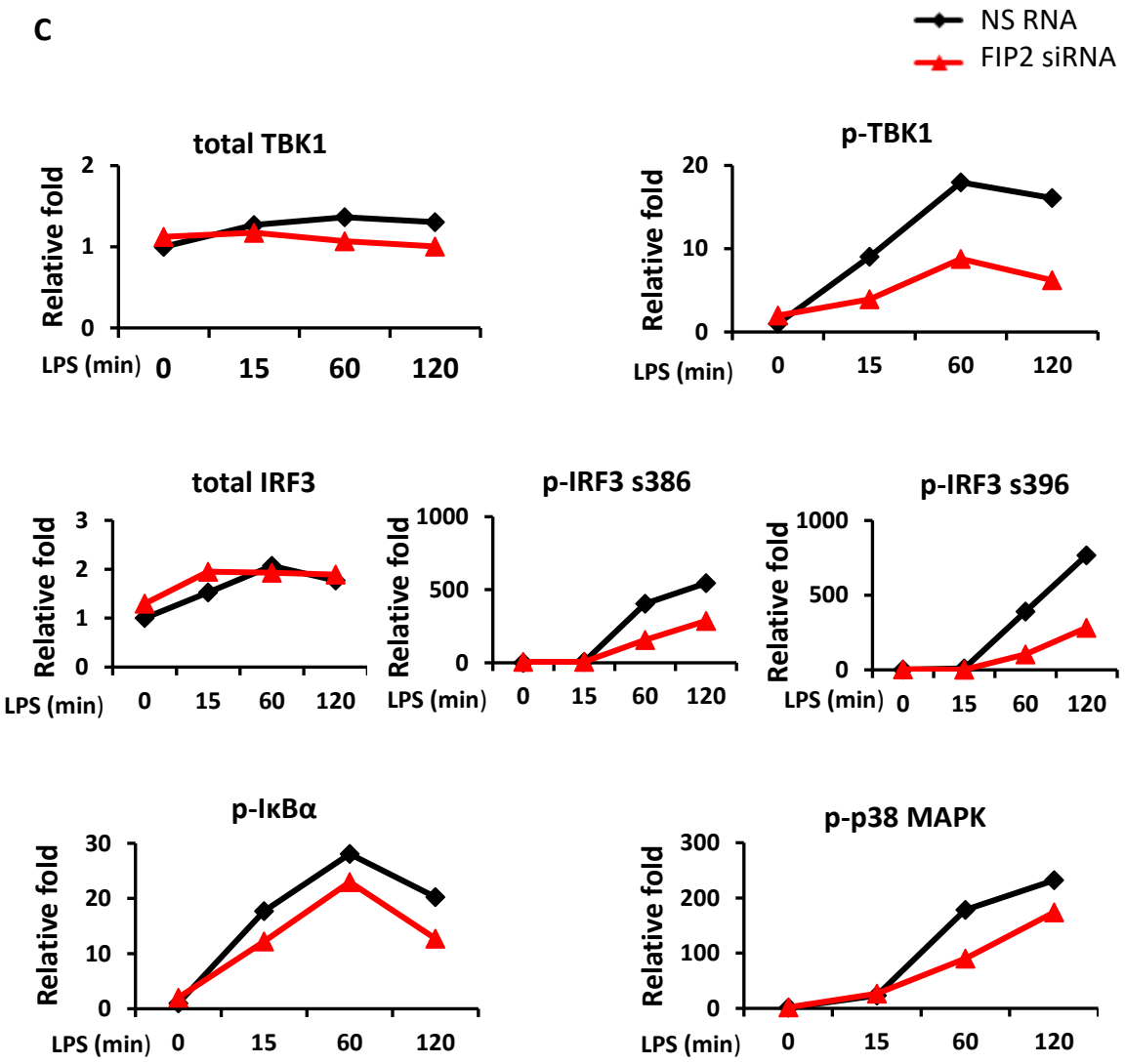


Figure 6. Immunoblot from THP1 cells treated with NS RNA or FIP2 siRNA and stimulated with *E. coli* particles ($7.5x10^6$/ml) or LPS (100 ng/ml) (A). Graphs show quantification of protein levels normalized with PCNA (B-C).
Results show one representative of three independent experiments.

In primary human macrophages only the activation of $IRF3^{S386}$ was addressed, since for still unknown reasons S396 is not detected in these cells [119]. Immunoblotting confirmed previous results, showing that $IRF3^{S386}$ phosphorylation was strongly impaired after FIP2 silencing (Figure 7A and 7B).

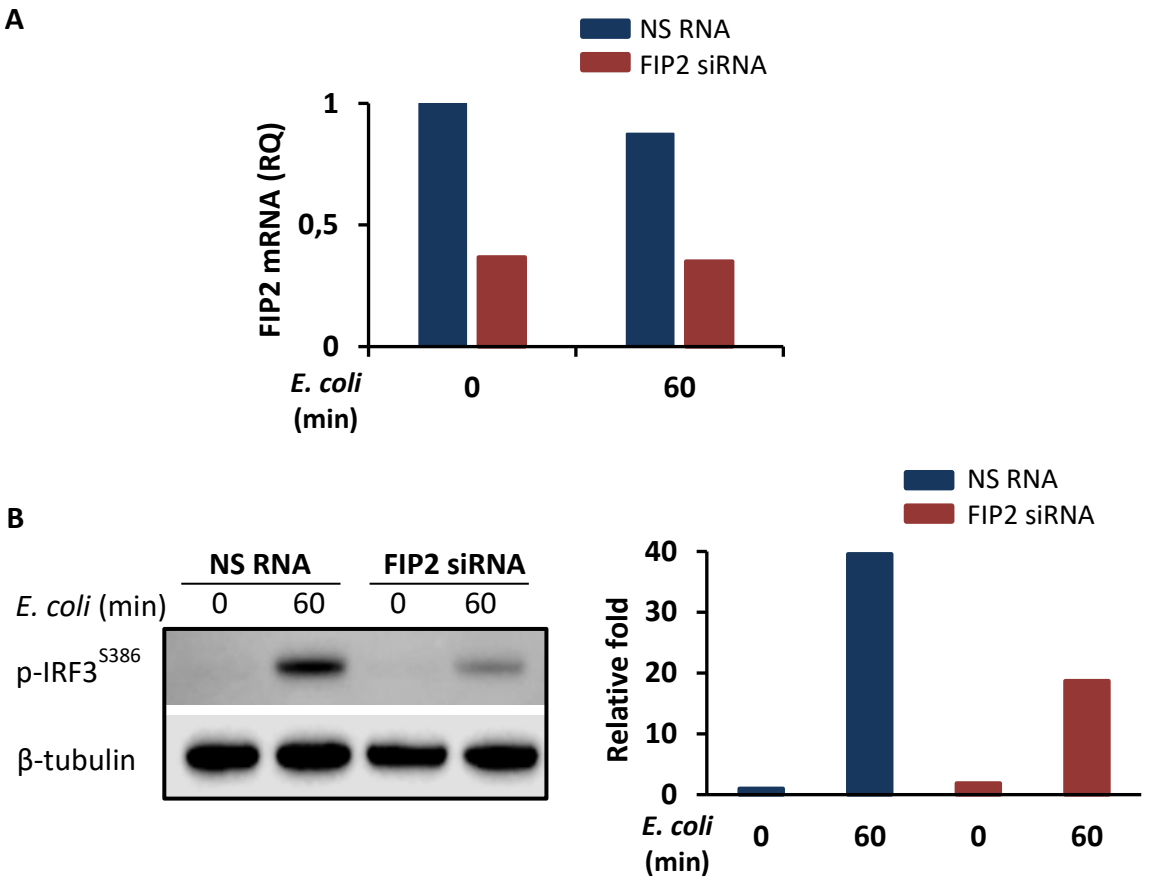


Figure 7. Relative quantification of FIP2 mRNA by q-PCR in primary human macrophages treated with NS RNA or Rab11-FIP2 siRNA and stimulated with *E .coli* particles ($3x10^7$/ml). TBP was used as endongenous control for normalization (A). Immunoblot of protein obtained from the same samples as in A (B, left panel). Graph shows the level of p-IRF3^{S386} normalized with β-tubulin (B, right panel). Results show one representative of three independent experiments.

In summary, these data suggest a crucial role for FIP2 in the TRAM-TRIF-dependent signaling upon stimulation with both E. *coli* and LPS. For LPS induced response, FIP2 also affect MyD88-dependent signaling, giving lower levels of IκBα and markedly lower levels of p38MAPK phosphorylation upon silencing.

4.2 FIP2 and TRAM form a complex with Rab11a

To investigate whether FIP2 can bind TRAM and whether Rab11a is also involved in this interaction, HEK293T cells were transfected with plasmids encoding the respective tag-proteins and immunoprecipitations performed. Results showed that FIP2^{GFP} could immunoprecipitate with TRAM^{FLAG}, with or without Rab11a^{CFP} overexpression; whereas

Rab11a^{CFP} could not immunoprecipitate with TRAM^{FLAG} when FIP2^{GFP} was not overexpressed (Figure 8A). Moreover, TRAM^{YFP} could immunoprecipitate with Rab11a^{FLAG} only when FIP2 was expressed and the interaction did not occur when FIP2 was silenced (Figure 8B).

These results suggest that Rab11a, FIP2 and TRAM all bind each other, forming a protein complex where FIP2 has the crucial role as a bridging molecule.

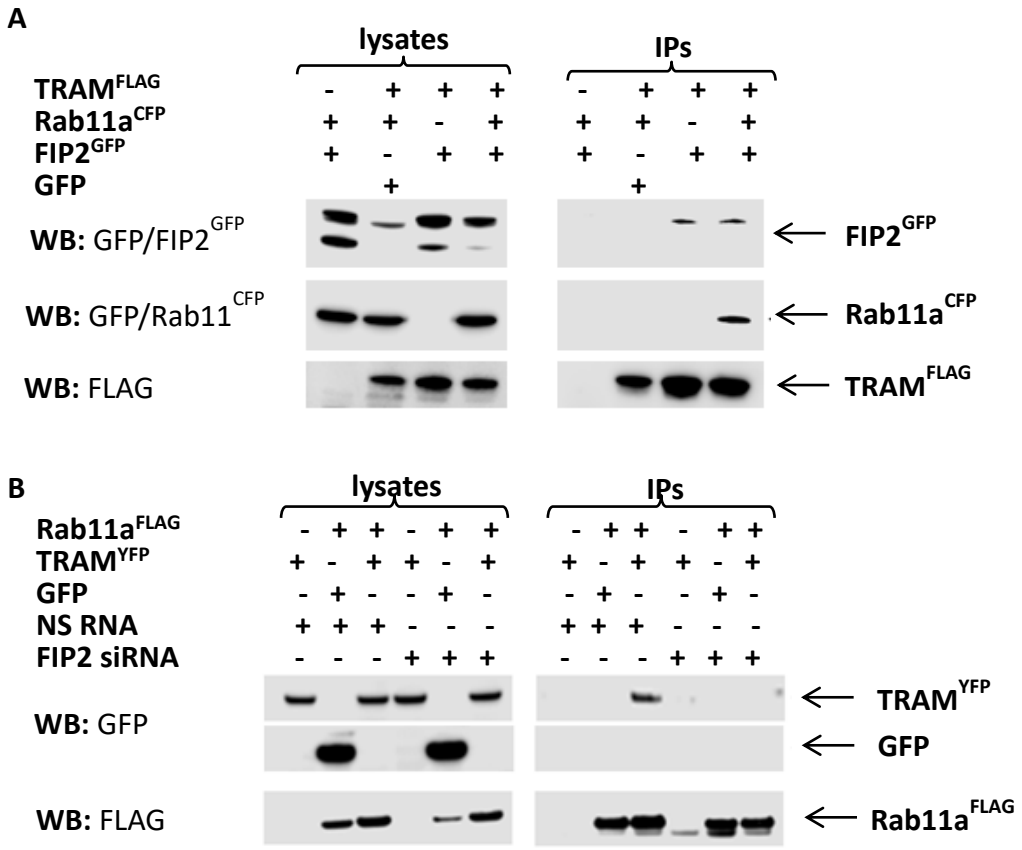
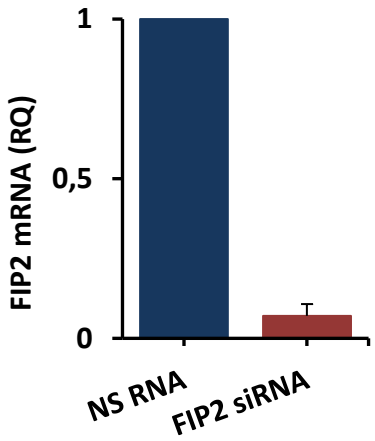


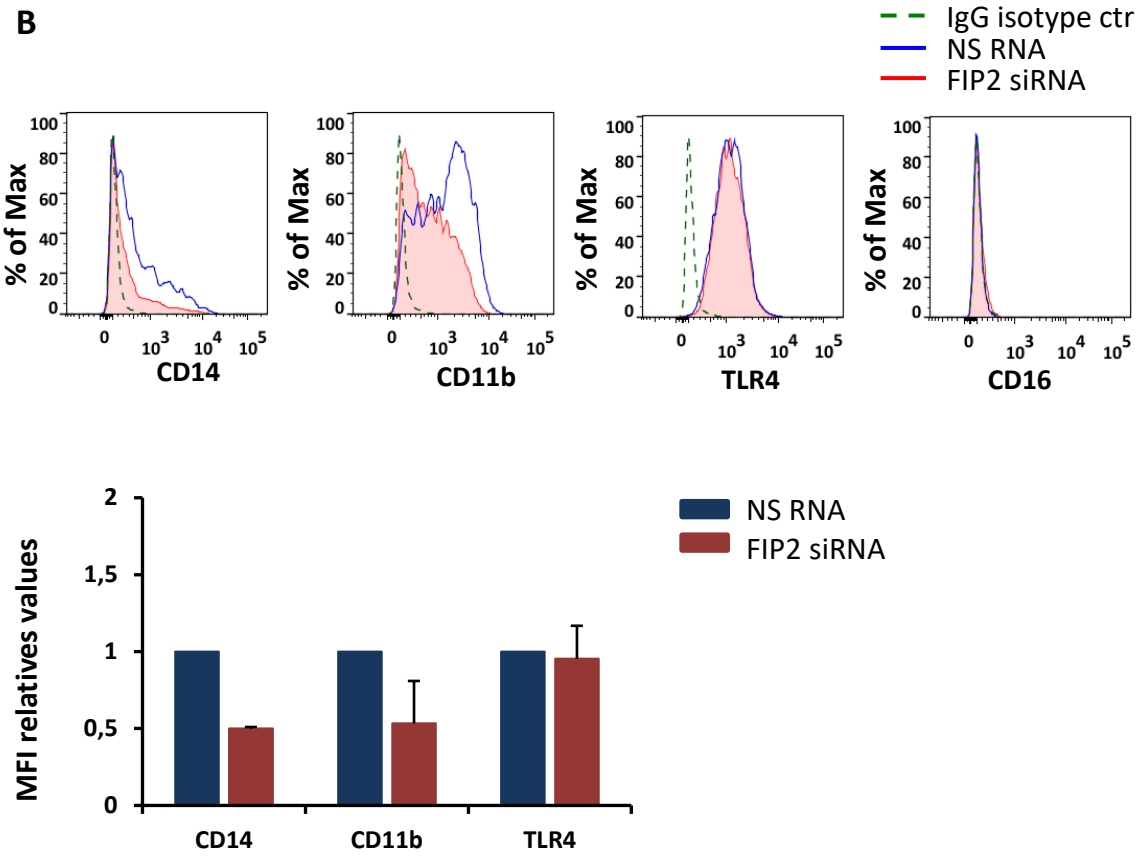
Figure 8. Immunoprecipitations (IPs) of FLAG-tagged versions of TRAM or Rab11a. HEK293T cells with co-expression of indicated proteins, without FIP2 silencing (A). HEK293T cells with co-expression of indicated proteins, treated with NS RNA or FIP2 siRNA (B). FLAG-antibody (M2) conjugated agarose beads were used carrying out the IPs, and FLAG- and GFP-antibodies used for detection. Results show one representative of three experiments.

4.3 FIP2 contributes to set the repertoire of surface receptors involved in *E. coli* uptake in THP-1 cells but not in primary human macrophages

To determine whether FIP2 is involved in the regulation of cell-surface receptors involved in *E. coli* response and phagocytosis, such as TLR4, CD14, CD11b, and CD16 [51, 74-77, 120] differentiated THP-1 cells and primary human macrophages were treated with NS RNA or FIP2 siRNA. FIP2 silencing was verified by realtime q-PCR, showing a knockdown level of approximately 95% in THP-1 cells and 65% in primary human macrophages (Figures 9A and 10A). Differentiated THP-1 cells, WT and F2 were also analyzed for this purpose. Single live cells were gated by setting FSC/SSC and FSC-A/FSC-H plots using flow cytometry. The surface level of CD14 and CD11b, were halved upon FIP2 silencing, while no difference was observed in TLR4 levels (Figure 9B). CD16 could not be detected on the surface of the THP-1 cells by any treatment. Similar results were obtained in F2 THP-1 cells (50% KD Talen clone) compared to WT, were the level of CD14 and CD11b were reduced by approximately 80% and 50%, respectively and still no differences were observed in levels of TLR4 (Figure 9C).

A




B
IgG isotype ctr
NS RNA
FIP2 siRNA
% of Max
CD14
CD11b
TLR4
CD16
MFI relatives values
NS RNA
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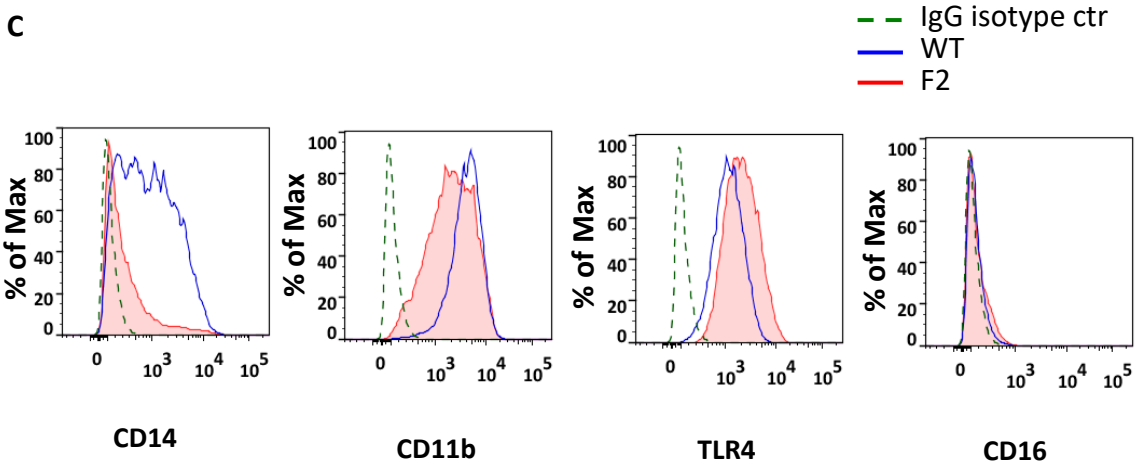
CD14
CD11b
TLR4


C
IgG isotype ctr
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CD11b
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CD16

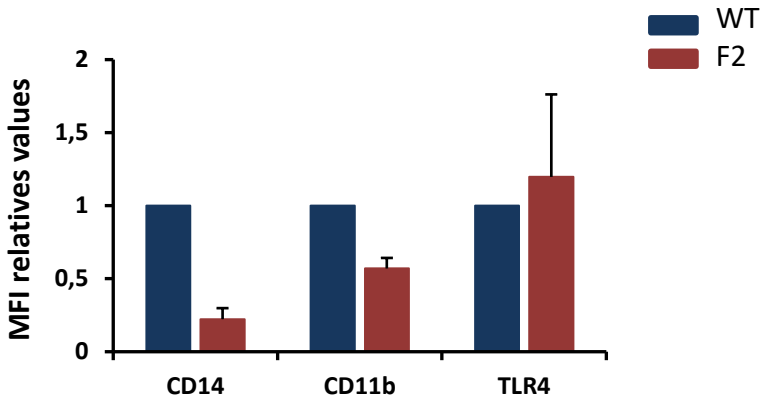
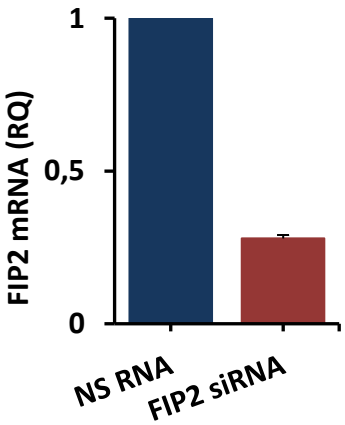


Figure 9. Relative quantification of FIP2 mRNA by q-PCR in THP-1 cells treated with NS RNA or FIP2 siRNA. TBP was used as endongenous control for normalization. Values represent means of two independent experiments ± SD (A). Expression and MFI relative values of four different cell-surface receptors in differentiated THP-1 cells treated with NS RNA or FIP2 siRNA (B) and differentiated THP-1 cells WT and F2 (C). Histograms show a representative of three experiments. Graphs represent means of three independent experiments ± SD. SD=standars deviation. MFI=median fluorescence intensity.

On the contrary, primary human macrophages showed a similar surface expression of these receptors compared to their control cells upon FIP2 silencing. Indeed the level of CD14, CD11b and TLR4 were even slightly increased, whereas the level of CD16 (which is now detected) was decreased to a non-marked extent (Figure 10B).

A



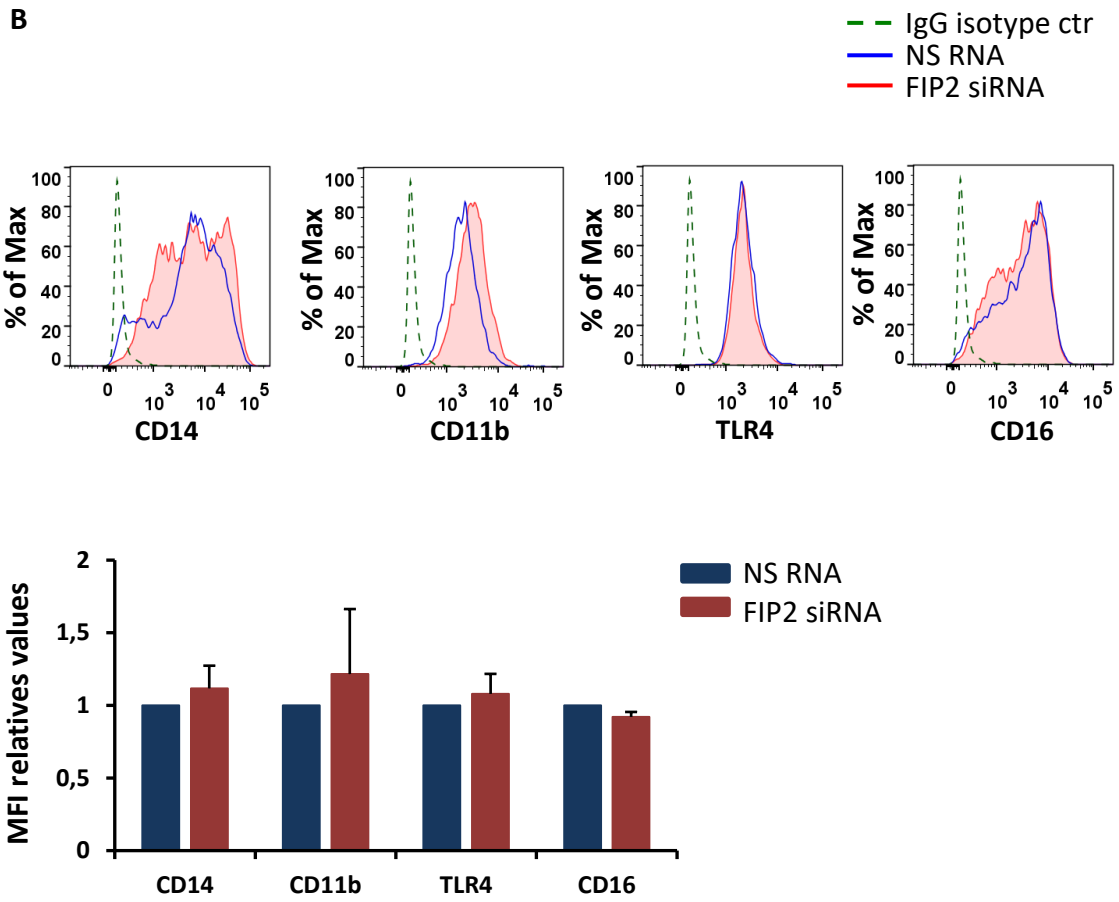


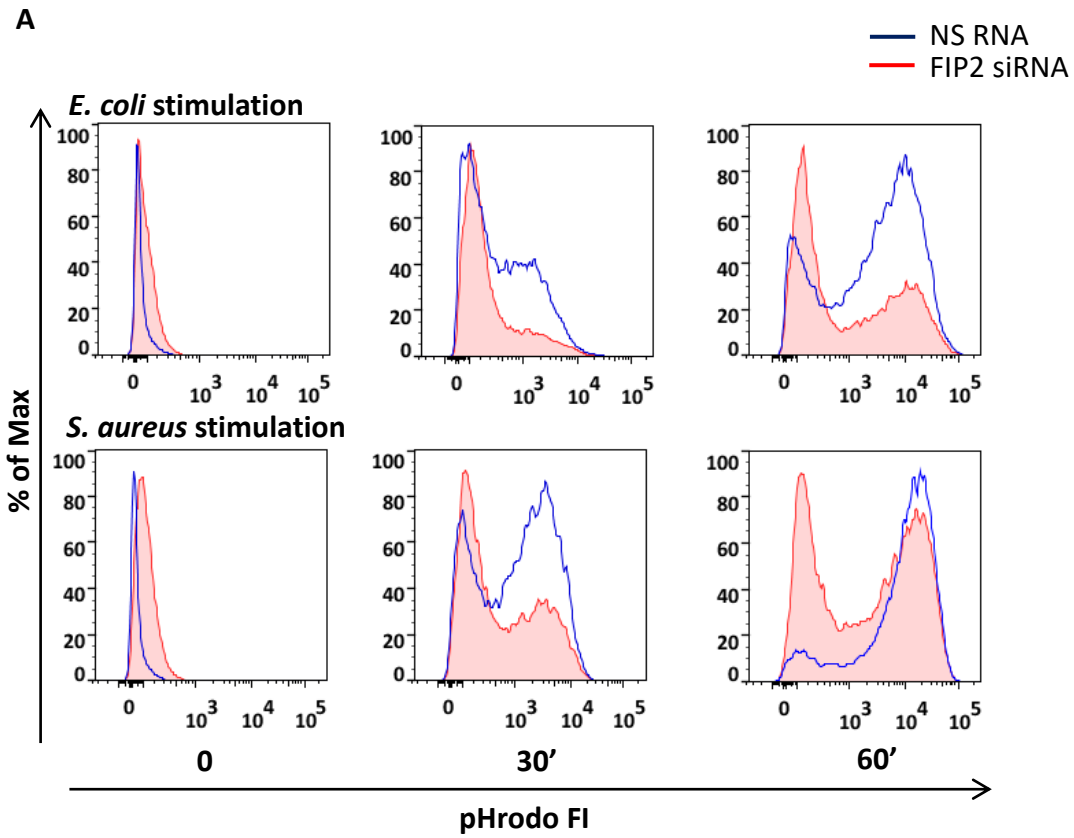
Figure 10. Relative quantification of FIP2 mRNA by q-PCR in primary human macrophages treated with NS RNA or FIP2 siRNA. TBP was used as endongenous control for normalization. Values represent means of two independent experiments ± SD (A).
Expression and MFI relative values of four different cell-surface receptors in primary human macrophages treated with NS RNA or FIP2 siRNA (B). Histograms show one representative of two independent experiments. Graphs represent means of two independent experiments ± SD.

Taken together these data suggest that FIP2 controls the surface levels of CD14 and CD11b in THP1 cells, both reported to be involved in *E. coli* phagocytosis. Whereas FIP2 behaves differently in primary human macrophages, where a relevant decrease of the receptors investigated is not observed upon silencing.

4.4 FIP2 is involved in phagocytosis of *E. coli* in human macrophages

To investigate a possible role of FIP2 in *E. coli* phagocytosis it was silenced in differentiated THP-1 cells and phagocytosis was compared to cells treated with NS RNA. The cells were stimulated for 30 and 60 minutes with pHrodo *E. coli* particles ($1.5x10^7$/ml) and harvested, before phagocytosis was monitored using flow-cytometry. FIP2 KD levels monitored by realtime q-PCR were in compliance with the ones shown in the section above (Figure 9A) and for this reason they are not shown. The cells were gated (as explained above) and the percentage of pHrodo-positive cells and MFI relative values registered.

Upon FIP2 silencing, *E. coli* phagocytosis was strongly altered: approximately -40% at 30 minutes and -90% at 60 minutes (Figures 11A, upper panels and 11B, left panel). Also primary human macrophages following siRNA treatment (KD levels by realtime q-PCR comparable with the ones shown in Figure 10A) and upon *E. coli* particles ($3x10^7$particles/ml) stimulation for 60 minutes, showed a decreased phagocytosis by around 90% (Figure 11C).



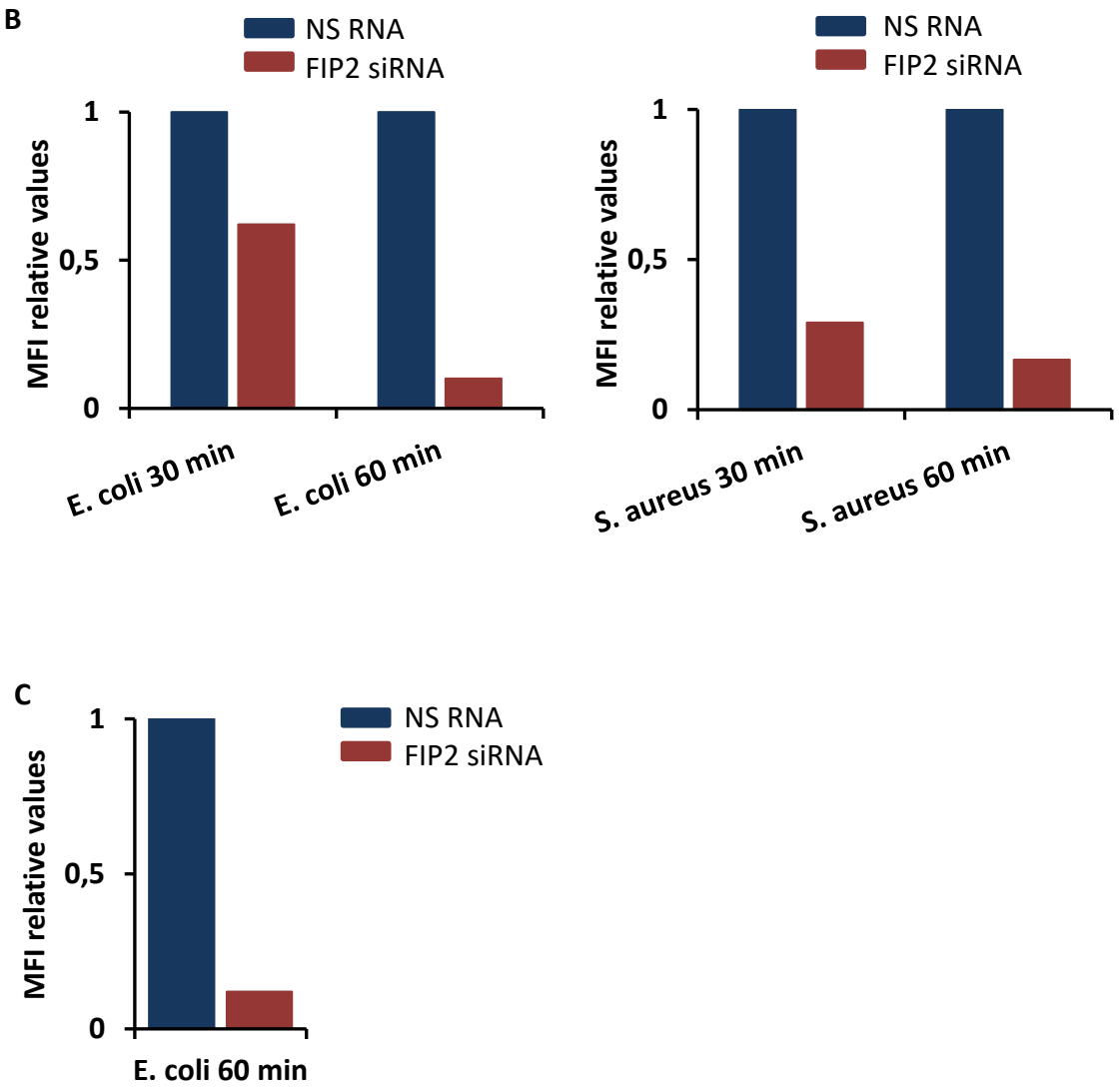


Figure 11. pHrodo fluorescence levels (A) and MFI relative values (B) in differentiated THP-1 cells treated with NS RNA or FIP2 siRNA and stimulated with *E. coli* or *S. auraeus* particles ($1.5x10^7$/ml) for 30 and 60 minutes. Results show a representative of two independent experiments. MFI relative values in primary human macrophages treated with NS RNA or FIP2 siRNA and stimulated with *E. coli* particles ($3x10^7$/ml) for 60 minutes (C). Results show one representative of two independent experiments.

Moreover, as a confirmation of these data, WT and F2 THP-1 cells were analyzed as above and stained with a viability dye (Figure 12). After *E. coli* particles ($1.5x10^7$/ml) stimulation, results showed that phagocytosis was impaired in F2 THP-1 cells compared to WT cells, to even a stronger extent compared to siRNA treatment: -90% and -95% after 30 and 60 minutes of stimulation, respectively (Figures 13A, upper panels and 13B, left panel). To address FIP2 dependence on phagocytosis of Gram-positive bacteria, *S.*

aureus was added to THP-1 cells. Following pHrodo *S. aureus* particles stimulation ($1.5x10^7$/ml), results showed that also in this case phagocytosis was strongly impaired, both at 30 and 60 minutes (Figures 11A-13A, lower panels and 11B-13B, right panels).

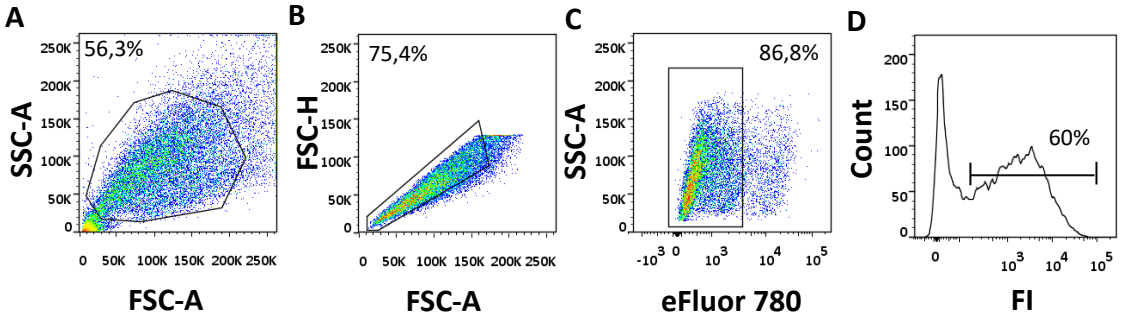
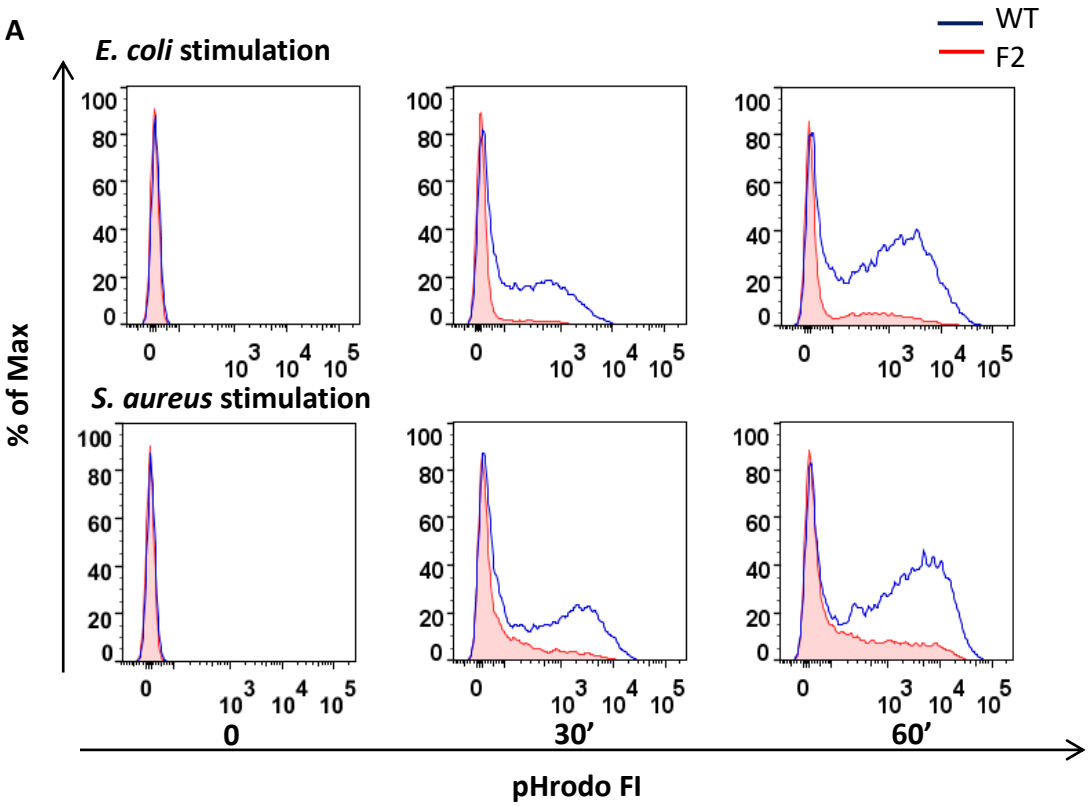


Figure 12. Typical gating strategy used for single live cells analysis. FSC-A versus SSC-A plot shows cells population (A). FSC-A versus FSC-H plot shows single cells (B). Viability Dye eFluor 780 versus SSC-A plot shows single live cells (C). Histogram shows an example of positive cells population in differentiated WT THP1 cells (D). FI=Fluorescence intensity. This strategy was also used in further flow-cytometry experiments.



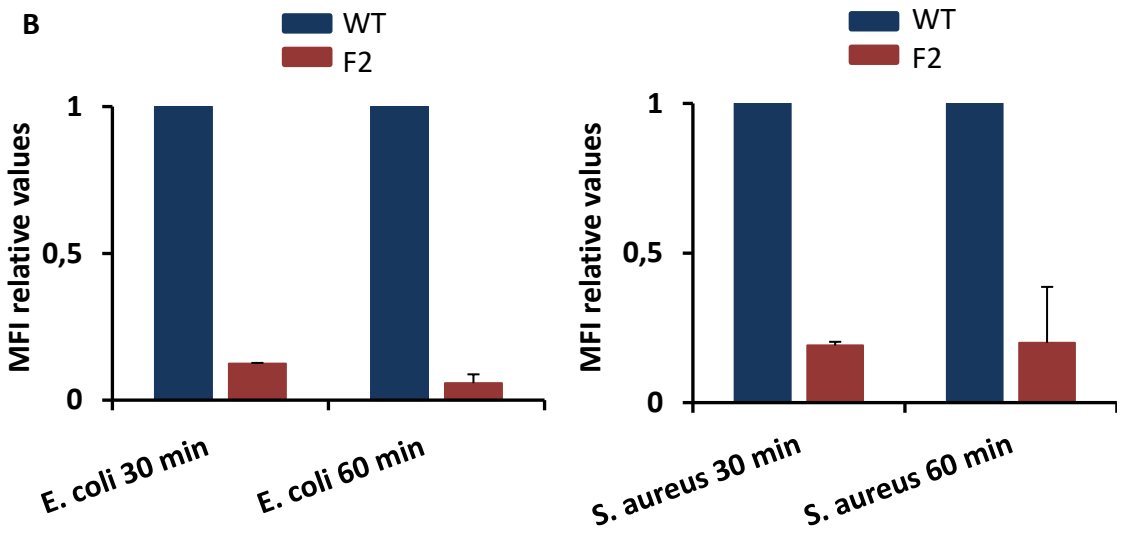


Figure 13. pHrodo fluorescence levels (A) and MFI relative values (B) in differentiated THP-1 cells WT and F2 stimulated with *E. coli* or *S. aureus* particles (1.5 x 10^7/ml) for 30 and 60 minutes. Histograms show a representative of two independent experiments. Graphs represent means of two independent experiments ± SD.

These data suggest that FIP2 is essential for a proper *E. coli* phagocytosis; however it is not a Gram-negative bacteria-related molecule, since it has also a crucial role in phagocytosis of the Gram-positive bacterium *S. aureus*.

4.5 TRAM, but not MyD88, is involved in *E. coli* phagocytosis in human macrophages

The involvement of the two adaptor proteins recruited by TLR4, TRAM and MyD88 in *E. coli* phagocytosis was also investigated.

Differentiated THP-1 cells and primary human macrophages treated with NS RNA, TRAM siRNA or MyD88 siRNA, were stimulated with pHrodo *E. coli* particles (1.5x10^7/ml and 3x10^7/ml, respectively) for 30 and/or 60 minutes. The silencing was verified by realtime q-PCR, showing TRAM and MyD88 KD levels of approximately 90% and 80%, respectively in THP-1 cells and of 80% and 45%, respectively in primary human macrophages (Figures 14A and 14B).

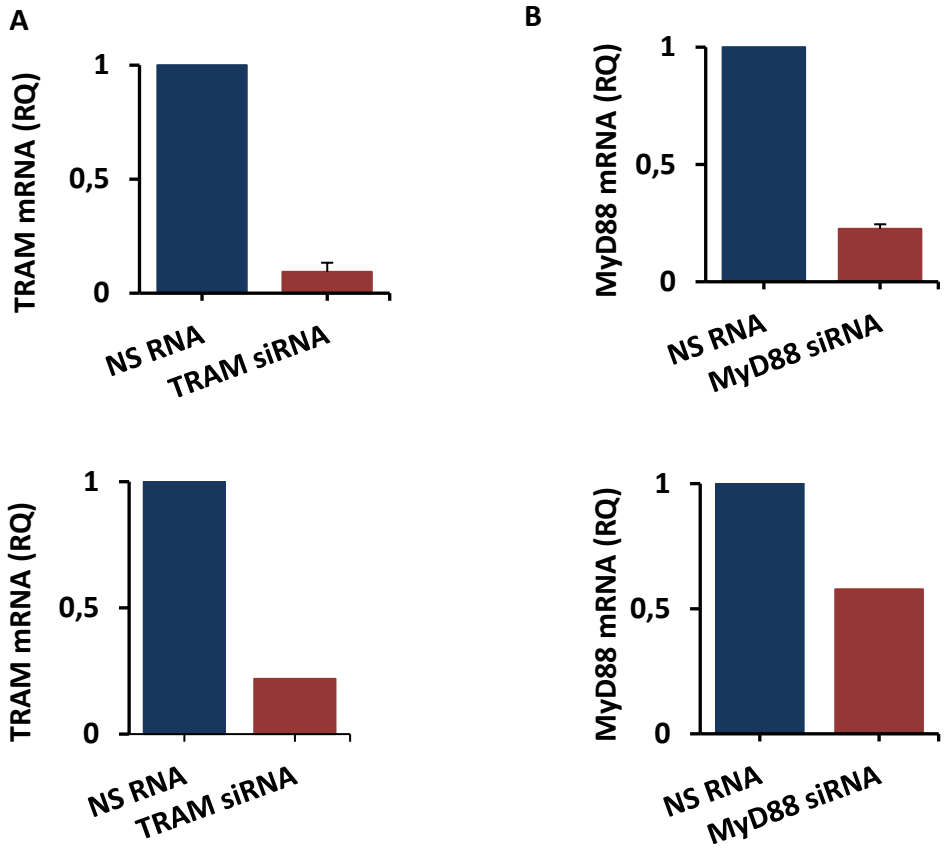
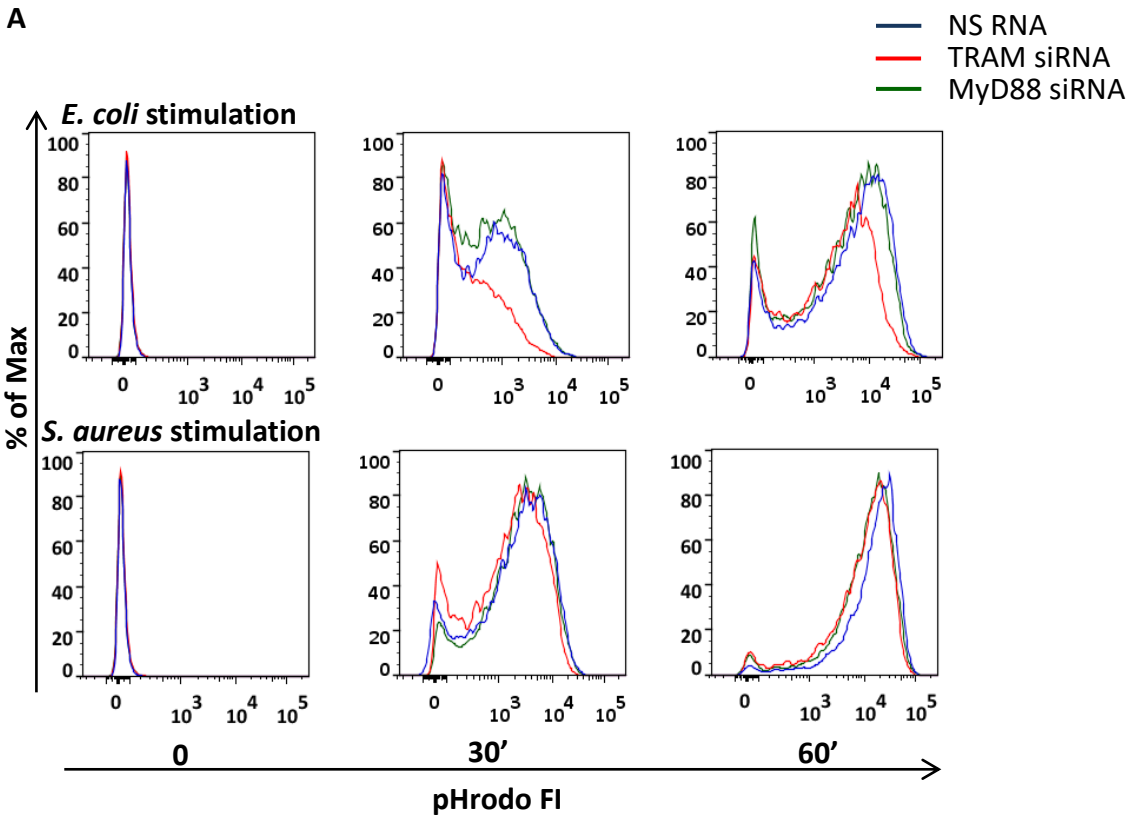


Figure 14. Relative quantification of TRAM (A) and MyD88 (B) mRNA by q-PCR in THP-1 cells (upper panels) and primary human macrophages (lower panels) treated with NS RNA, TRAM siRNA or MyD88 siRNA. TBP was used as endongenous control for normalization. THP-1 cells values represent means of two independent experiments ± SD. Primary human macrophages values show one representative of two independent experiments.

Results demonstrated that in TRAM depleted cells, *E. coli* phagocytosis was markedly impaired: -60% in THP-1 cells at both investigated time points and -50% in primary human macrophages after 60 minutes of stimulation; whereas MyD88 depleted THP-1 cells or primary human macrophages did not show any marked decrease of the phagocytic process after 60 minutes and even a slight increase after 30 minutes of stimulation (Figures 15A, upper panels, 15B, left panel and 15C). On the contrary, upon stimulation with pHrodo *S.aureus* particles ($1,5x10^7$/ml), THP-1 TRAM depleted cells showed just a weak reduction of phagocytosis after 30 minutes (-28%) and a not strong decrease at 60 minutes (-20%) of stimulation; whereas MyD88 depleted cells still did not show any marked phagocytic decrease (Figures 15A, lower panel and 15B, right panel).


A
NS RNA
TRAM siRNA
MyD88 siRNA
E. coli stimulation
S. aureus stimulation
% of Max
0
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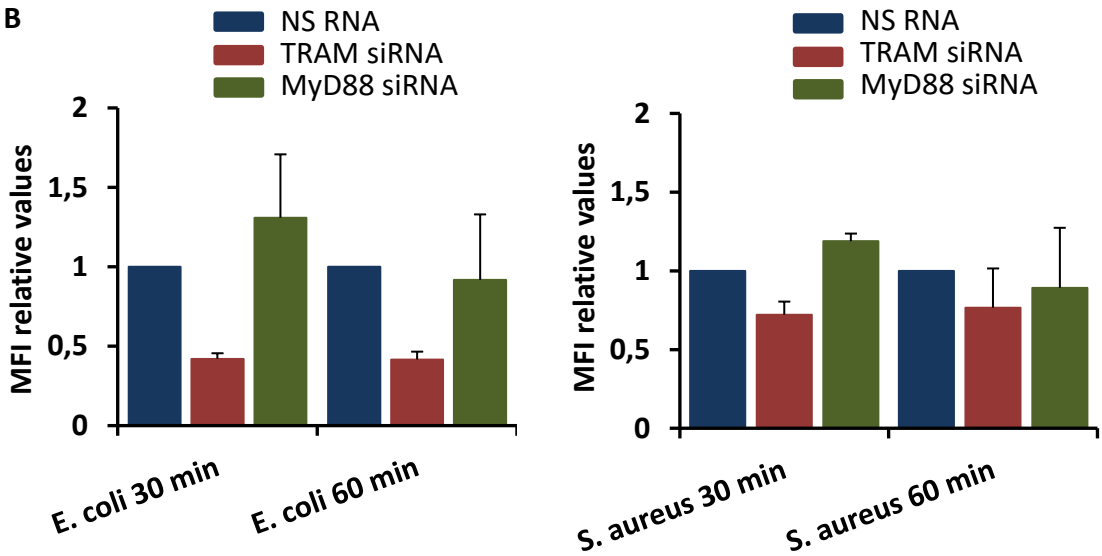
pHrodo FI


B
NS RNA
TRAM siRNA
MyD88 siRNA
MFI relative values
E. coli 30 min
E. coli 60 min
S. aureus 30 min
S. aureus 60 min

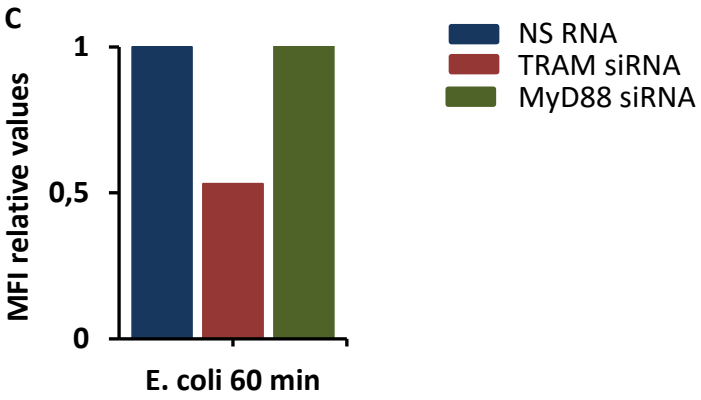


Figure 15. pHrodo fluorescence levels (A) and MFI relative values in differentiated THP-1 treated with NS RNA, TRAM siRNA or MyD88 siRNA and stimulated with *E .coli* or *S. aureus* particles (1.5 x 10^7/ml) for 30 or 60 minutes (B). MFI relative values in primary human macrophages treated with NS RNA, TRAM siRNA or MyD88 siRNA and stimulated with *E .coli* particles (3x10^7/ml) for 60 minutes (C). Histograms show one representative of three independent experiments. Graphs regarding THP-1 cells represent means of three independent experiments ± SD. Graphs regarding primary human macrophages show one representative of two independent experiments.

Furthermore, immortalized murine macrophages TRAM$^{-/-}$ and MyD88$^{-/-}$ were analyzed upon *E. coli* and *S. aureus* particles (7.5x10^6/ml) stimulation, for 30 and 60 minutes and compared to WT cells. TRAM$^{-/-}$ cells showed a decreased *E. coli* phagocytosis, even to a stronger extent (approximately -80% at both time points) compared to human macrophages; whereas *S .aureus* phagocytosis was impaired at 30 minutes (-60%) and just slightly altered at 60 minutes (-25%) (Figures 16A and 16B). In contrast, in MyD88$^{-/-}$ cells, both *E. coli* and *S. aureus* phagocytosis were strongly impaired at both analyzed time points (between -70% and -90%) (Figures 16A and 16B).

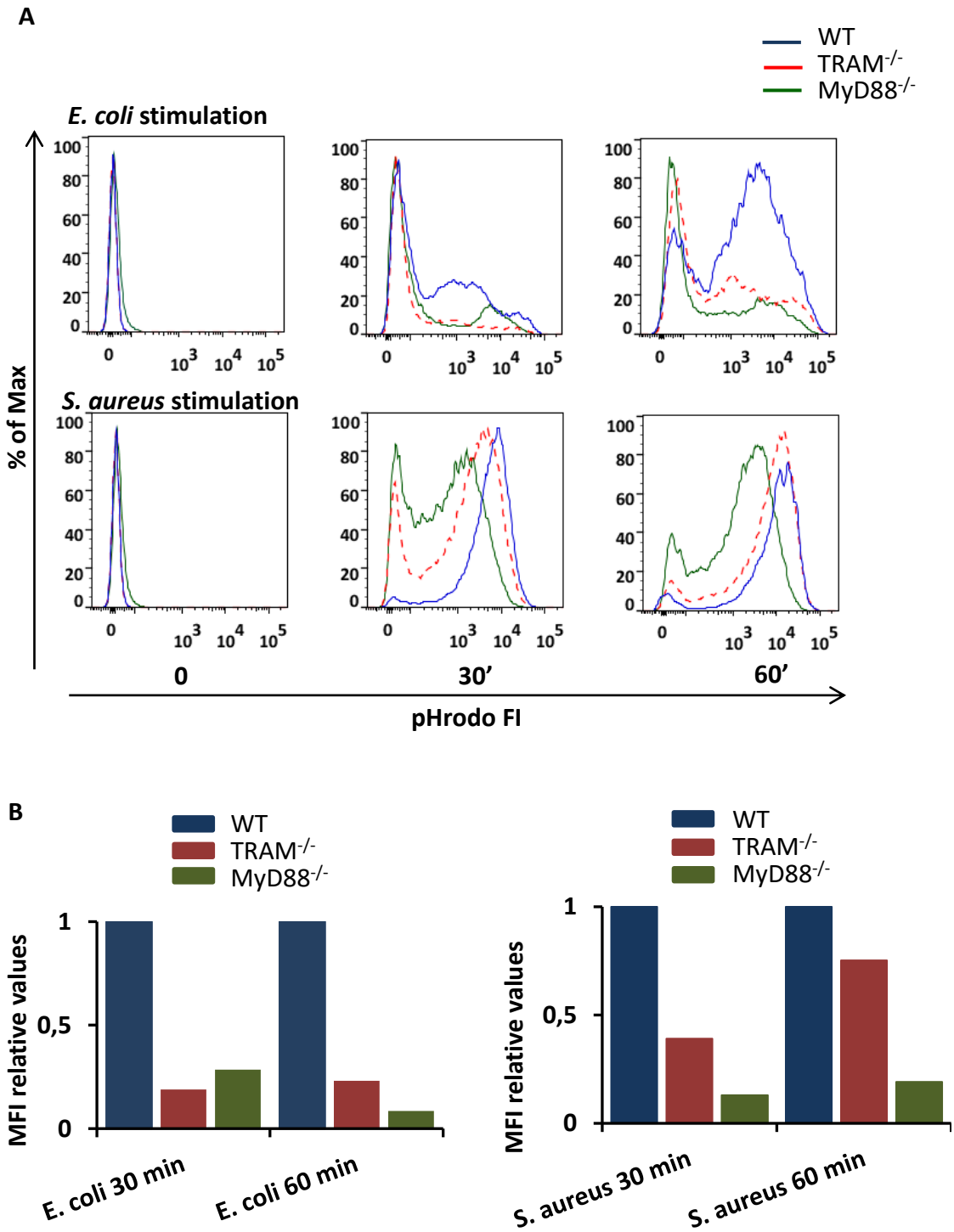


Figure 16. pHrodo fluorescence levels (A) and MFI relative values (B) in immortalized murine macrophages WT, TRAM$^{-/-}$ and MyD88$^{-/-}$, stimulated with *E .coli* or *S. aureus* particles (7.5x10^7/ml) for 30 and 60 minutes. Results show one representative of three experiments.

All these data demonstrate that TRAM is involved in *E. coli* phagocytosis, both in human and murine macrophages; whereas MyD88 is not involved in this process in human macrophages. Moreover, the involvement of TRAM is largely TLR4-dependent, at least

in human cells, since when this molecule is depleted, phagocytosis is strongly much more impaired if subsequent to *E. coli* rather than *S. aureus* response.

5 DISCUSSION

The innate immune system as first line of defense, plays a crucial role in recognition and response against microbial pathogens, by sensing them through PRRs and starting inflammation [4]. Innate immunity is the mayor driver of acute inflammation, and if over stimulated, overproduction of pro-inflammatory cytokines (cytokine storm) may result, leading to detrimental conditions for the host [5, 8, 120, 121]. Since inflammation is a required response to protect the organism, a proper balance between inflammatory and anti-inflammatory responses is crucial for the maintenance of the host's health [45]. Indeed, if this balance is not properly regulated, bacterial infections can lead to sepsis, defined as a severe and debilitating syndrome with high mortality [45, 122-124]. Sepsis is due to a PPR-mediated dysregulation of the immune system, which can be followed by organ dysfunction, alterations of coagulation and unresponsive hypotension, leading to septic shock [125, 126]. Gram-negative bacteria are responsible for about 60% of cases [126] and LPS (also called endotoxin) on their cell wall, is considered the major cause of this syndrome. TLR4 is the sensor of LPS [43] and also the best studied and characterized among PRRs. When TLR4 recognizes LPS from Gram-negative bacteria it triggers a unique signaling cascade. It immediately activates, from the plasma membrane, the MyD88-dependent pathway with the consequent NF-κB nucleus translocation and the pro-inflammatory cytokines production [29].

Following endocytosis TLR4 is internalized along with its ligand and appears at the endosomal membrane [73] where it triggers the TRAM-TRIF-dependent pathway, leading to the late pro-inflammatory response and IRF3 activation, responsible for the IFN-β induction [56]. According to Husebye and coworkers, *E.coli* phagocytosis is necessary for the IFN-β response and the small GTPase Rab11a is responsible for the delivery of most TLR4 and TRAM to the phagosomal membrane to initiate IFN-β signaling downstream of TLR4 [57].

The production of type I interferons (IFNs), like IFN-β, in response towards bacterial infections can be both beneficial and detrimental, making the role still controversial and not completely understood [87, 88].

High type I IFN production resulting from a Gram-negative infection, can suppress the adaptive immune response and make the host susceptible to secondary infections, increasing the risk of septic shock [127]. It is thus crucial to understand how the IFN response is mediated by TLR4, as well identification of the effectors involved, this to find new strategies that prevent the development of Gram-negative sepsis. For this purpose FIP2, a Rab11 binding protein able to recruit the actin motor Myosin5B, allowing Rab11 coated vesicles to move along the actin cytoskeleton [114] was studied. FIP2 plays a crucial role in regulating TLR4-mediated IFN-β signaling in macrophages, by delivering TRAM and TLR4 to *E. coli* phagosomes [128]. In addition, FIP2 seems to control phagocytosis of *E. coli.* Furthermore, both *E. coli* phagocytosis and *E.coli* induced IFN-β signaling can be blocked if actin polymerization is inhibited by Cytochalasin D [57].

The finding that FIP2 controls E. *coli* and LPS stimulated induction of IFN-β mRNA and that FIP2 did not affect the induction of TNF was reproduced demonstrating that FIP2 depleted cells showed a markedly impaired induction of IFN-β.
Moreover it was shown that FIP2 was required for both *E. coli* and LPS stimulated phosphorylation of TBK1 at S172, and IRF3 at S386 and S396 in differentiated THP1 cells. On the contrary, FIP2 did not seem to affect *E. coli*-induced phosphorylation of IκBα and p38 MAPK and in contrast FIP2 had a slight effect on LPS stimulated phosphorylation of IκB-α and a marked effect on phosphorylation of p38 MAPK.

To investigate how FIP2 might more or less selectively affect IFN-β induction downstream of TLR4, an interaction study of FIP2, TRAM and Rab11a was carried out in HEK293T cells expressing TLR4, MD-2 and CD14. This showed that FIP2 and TRAM can bind each other and that they exist in a complex together with Rab11a. Interestingly FIP2 plays the crucial role of bridging molecule of TRAM and Rab11a as demonstrated by FIP2 overexpression and silencing of FIP2, and giving a rationale how the Rab11a dependent delivery of TRAM via FIP2 can be transported to the *E. coli* phagosome.
Moreover, since FIP2 was found on the plasma membrane of human macrophages [129], at the E.coli binding site (or phagocytic cup), as well as on *E. coli* early phagosomes, the role of FIP2 in the regulation of cell-surface receptors involved in *E. coli* recognition and internalization, like CD14 and CD11b [74, 75-77], was investigated.

In FIP2 depleted THP1 cells, the level of surface CD14 and CD11b were strongly reduced. In contrast, TLR4 surface levels were not consistently altered. Nevertheless this was not observed in primary human macrophages, where FIP2 depletion did not decrease the cell-surface levels of these investigated receptors.

Furthermore, given the location of FIP2 and TRAM to the phagocytic cup, a role of these molecules in *E.coli* phagocytosis was addressed. Phagocytosis is a key process of innate immunity by which phagocytes like macrophages, engulf and kill pathogens by three steps: particle internalization, phagosomal maturation and resolution [14, 20]. Interestingly, here it was demonstrated that FIP2 was involved in *E. coli* phagocytosis not only in THP-1 cells but also in primary macrophages. Indeed, as already mentioned, in primary human macrophages, FIP2 is not involved in the regulation of CD14 and CD11b surface levels, demonstrating that its role is not to promote the expression of *E. coli* recognition-related receptors, but instead it is actively involved in the phagocytic process. In addition, THP1 is a cell line and, thus, does not necessarily represent a normal macrophage. Furthermore, FIP2 is not an exclusively Gram-negative-dependent molecule, since it is also involved in phagocytosis of the Gram-positive bacterium *S. aureus*.

TRAM seems to be involved in *E.coli* phagocytosis in both murine and human macrophages. Earlier studies have shown MyD88 to control *E. coli* phagocytosis and phagosome maturation in murine bone marrow-derived macrophages and -activation of p38 MAPK to be involved [130]. Moreover, another study carried out in RAW264.7 cells showed that LPS pretreatment stimulated phagocytosis capability of these cells through a MyD88-independent activation of the GTPases Rac1 and Cdc42 [131]. Specific inhibition of Cdc42/Rac or p38 MAP kinase, but not PI3K, reduced LPS-induced phagocytosis of *E.coli*. In contrast to the murine, the results shown in this thesis demonstrate that there is no strong involvement of MyD88 in phagocytosis of *E. coli* neither in THP-1 cells nor in primary human macrophages. Nevertheless, as already mentioned, a decrease of the LPS-induced p38 MAPK levels was observed in THP1-cells upon FIP2 silencing and might open new investigations. Moreover, it would be

interesting to figure out whether a LPS-induced activation of Rac1 and Cdc42 also occurs in human macrophages.

6 CONCLUSION AND FUTURE PERSPECTIVES

In summary this thesis mainly focused on FIP2, a protein shown to play a critical role in human macrophages both in *E.coli*-induced IFN-β production and in *E.coli* phagocytosis, through its interaction with TRAM. This is the first time a role of the TLR4 adaptor protein TRAM in phagocytosis has been shown.

These findings thus identify new players involved in the TLR4-mediated response against Gram-negative bacteria and may enable to find new therapeutic targets available in the fighting against severe bacterial infections.

However some points need to be clarified, such as FIP2 and TRAM involvement in other stages of bacterial processing following phagocytosis, like for example bacterial killing.

Another crucial point would be to identify the motif in FIP2 which physically interacts with TRAM and *vice versa* the motif in TRAM which binds FIP2. These identifications in particular might lead to find out a new strategy for anti-inflammatory drugs through the construction of peptides that inhibit the interaction between FIP2 and TRAM and possible reduce the IFN-β response.

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