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Trondheim, June 2011

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Intracellulær transport av Toll-liknende reseptor 4 etter reseptoraktivering og Gramnegative bakteriers evne til å unngå reseptordeteksjon

I de tidligste fasene av en bakteriell infeksjon spiller det medfødte immunforsvaret en essensiell rolle. Ved hjelp av sine sensorer/reseptorer som gjenkjenner mønstre felles for mange ulike bakterier er det i stand til å sette i gang en kraftig immunrespons som fører til begrensning av vekst og fjerning av bakteriene, samt å utløse det adaptive immunforsvaret. En familie av slike reseptorer har fått navnet Toll-liknede reseptorer, og gjenkjenner både bakterier, virus og skadelige stoffer basert på deres molekylære strukturer.

En av de mest studerte Toll-likende reseptorerene er TLR4 som sammen med MD-2 gjenkjenner et viktig overflatemolekyl fra Gramnegative bakterier kalt lipopolysakkarid (LPS). Gramnegative bakterier er en av to hovedklasser bakterier og denne klassen rommer flere sykdomsfremkallende bakterier slik som bakterien som forårsaket svartedauden, *Yersinia pestis*; bakterien som forårsaker hjernehinnebetennelse, *Neisseria meningitidis*; i tillegg til mage/tarmbakterier som *Escherichia coli*.

Denne avhandlingen tar for seg de prosessene som skjer etter at en infeksjon av Gramnegative bakterier har ført til aktivering av TLR4. I immunceller som spiser bakterier ligger TLR4/MD-2-reseptorer klare på overflaten for å oppdage eventuelle bakterielle inntrengere. I tillegg finner man TLR4 i et intracellulært depot, som man tidligere trodde var en del av en eksportvei til celleoverflata etter nysyntese av TLR4. Våre resultater viser imidlertid at dette depotet mer sannsynlig er det intracellulære kammeret, ERC, og at det fungerer mer som et lager hvor TLR4 ligger klar til å transporteres ut til innkapslete bakterier i intracellulære rom kalt fagosom. Vi fant at denne transporten er kontrollert av GTPase-proteinet Rab11a, og at Rab11a med dette også kontrollerer det spesifikke signalsporet fra TLR4 i fagosom som fører til produksjon av type I interferon, IFN-β.

Effektive forsvarsmekanismer i det medfødte immunsystemet, slik som TLR4-mediert anti-bakteriell motstand, kan føre til et seleksjonspress for bakterier som er i stand til å unngå deteksjon, og dermed kan formere seg fritt i vertsorganismen. Det ser ut som om *Y. pestis* gjennom evolusjon har utviklet en egenskap til å modifisere LPS etter hvilken vertsorganisme som fungerer som bærer. I mennesker lager den en form for LPS som ikke lar seg detektere effektivt av TLR4. Vi viser at denne evnen kommer som et resultat av tap av genet *lpxL*, og argumenterer for at dette tapet var et sentralt trinn da *Y. pestis* evolverte til en svært virulent patogen fra den mindre farlige Y. pseudotuberculosis. Til sist viser vi at den intracellulære inflammasomreseptoren NLRP12 gir beskyttelse mot *Y. pestis* ved å aktivere caspase-1, med påfølgende aktivering av cytokinene IL-1β, og IL-18.

Samlet sett gir resultatene ny kunnskap om flere sentrale komponenter i det medfødte immunforsvaret, og øker vår forståelse av hvordan immunforsvaret fungerer under infeksjoner.

Bakgrunnen for forskningsprosjektet var studier som viste at *Y. pestis* var i stand til å unngå aktivering av TLR4 ved å uttrykke en antagonistisk LPS i pattedyr, samt upubliserte forskningsresultater som viste en klar intracellulær kolokalisering av Rab11a og TLR4 i monocytter.

Sentrale metoder har vært konfokalmikroskopi, Western blot, ulike former for PCR, knock down av genuttrykk ved hjelp av siRNA, ELISA, kloningsteknikker, Luciferase assay, standard cellebiologiske teknikker som celleisolering og kultivering, i tillegg til en nyutviklet kvantifiseringsmetode basert på bilder fra konfokalmikroskop.

Kandidat: Marie Hjelmseth Aune

Veiledere: Terje Espevik og Egil Lien

Institutt: Institutt for kreftforskning og molekylær medisin

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My family must be thanked for their unconditional love and support, I love you all. Gunhild, Vebjørn, Lea, Vidar and Mariann, Gerd and Harald; you have given me reasons to consider other aspects of life than work, and usually at least pretended to understand what I do and why I love my job when it involves scary bacteria. Turid and Erik, Ivar and Naz, and Reidun, thank you for including me as a member of your family.

To my best friend and husband Trond Erik. Thank you for being my rock, for being there when I need someone to bounce ideas of from and for making it possible for me to get to know the best scientist and explorer of us all, our beautiful daughter Minda. I love you both so much.

ABSTRACT

In the early phases of an infection the innate immune system plays a vital role. By the use of pattern recognition receptors it is able to recognize pathogen associated molecular patterns shared by many microbes. The activated receptors mount a powerful responses leading to removal and growth limitation of the intruder followed by activation of the adaptive immune defense. A family of such receptors is the Toll-like receptors (TLRs), which recognizes bacteria, virus and noxious agents based on molecular structure.

One of the best characterized TLRs is TLR4, which together with MD-2 recognize Gram-negative bacteria via the bacterial outer membrane molecule lipopolysaccharide (LPS). The Gram-negatives include several pathogenic bacteria like *Yersinia pestis* (the causative agent of plague), *Neisseria meningitidis* (the causative agent of meningitis) and the enteric pathogen *Escherichia coli*.

This thesis investigates certain processes of the immune defenses upon activation of TLR4 by Gram-negative bacteria. TLR4 is found on the plasma membrane of phagocytes, on endosomes and in an area near Golgi. We describe a novel intracellular trafficking route of TLR4 towards phagosomes, regulated by Rab11a and resulting in an activation of the MyD88 independent signaling pathway and subsequent production of IFN-β.

Effective defense mechanisms may lead to a selective pressure favoring bacteria enabled to evade immune detection. *Y. pestis* expresses a slightly different LPS dependent on its host temperature and in humans this leads to an antagonistic, tetra-acylated LPS which is a poor inducer of TLR4 activation. Our results indicate that this is a consequence of the absence of the acyltransferase LpxL; a loss which we argue hallmarks the evolution of *Y. pestis* into a highly virulent organism from the less virulent *Y. pseudotuberculosis*. Finally we report that NLRP12 participates in the defense against *Y. pestis* through activation of inflammasomes, shedding light over an NLR with unclear function in innate immunity.

Collectively this work provides new knowledge about central receptors and signaling pathways of innate immunity, and increases our understanding of immune defense during infections.

ABBREVIATIONS

3D	Three dimensional
AIM2	Absent in melanoma, gene #2
AMP	Antimicrobial proteins and peptides
AP-1	Activating protein-1
ATP	Adenosine triphosphate
Blr	Group B, leucine rich
CD-14	Cluster of differentiation 14
CpG	Deoxycytidyl-deoxyguanosine
DNA	Deoxyribonucleic acid
ds	Double stranded
ERC	Endocytic recycling compartment
FIP	Rab11-family interacting proteins
FRAP	Fluorescence recovery after bleaching
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatases
HEK	Human epithelial kidney
IFN	Interferon
IFNR	Interferon receptor
IL	Interleukin
IPAF	ICE-protease-activating factor
IRAK	Interleukin-1 receptor associated kinase
IRF	IFN regulatory factor
JNK	Jun N-terminal kinase
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen.activated protein kinase

Mal	MyD88-adaptor-like
MD-2	Myeloid differentiation factor 2
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	NACHT nucleotide-binding domain
NF-κB	Nuclear Factor- κB
NIK	NF-κB-inducing kinase
NK cells	Natural kiler cells
NLR	Nucleotide-binding domain leucine-rich repeat–containing receptors
NOD	Nucleotide oligomerization domain
PAMP	Pathogen-associated molecular pattern
PRR	Pathogen-recognition receptor
Rab	Ras related in brain
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RIG-1	Retinoic acid inducible gene-1
RIP-1	Receptor-interacting protein-1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
ss	Single stranded
TAB	TAK1 binding protein
TAG	TRAM adaptor with GOLD domain
TAK1	TGFβ-activated kinase 1
TIR	Toll and Interleukin-1 receptor
TIRAP	TIR domain-containing adapter protein/ Mal
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAM	Toll-receptor adapter molecule
TRAF	TNFR-associated factor

TRIF	Toll-receptor-associated activator of interferon
TS3	Type III secretion systems
IFN-1	Type I interferons
UPEC	Uropathogenic *E. coli*
wt	Wild type

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1 INTRODUCTION

1.1 Innate immunity

The human immune system consists of two subgroups, the innate and the adaptive immune system, and includes mechanisms for removing microbes, allergenic and toxic substances. While the adaptive immune system is a work-in-progress that evolves in response to its encounters, the innate immune system is congenital and includes physical barriers (e.g. skin), soluble proteins (e.g. complement proteins), small molecules (e.g. reactive oxygen species) and receptors (e.g. Toll-like receptors). Molecules of the innate immune system are expressed ubiquitously on several different cell types and confer quick and efficient response to challenges in a first line of defence. Infection by microorganisms activates the inflammatory response through pattern recognition receptors (PRRs), which include Toll-like receptors, RIG-I-like receptors, NOD-like receptors, AIM2 and C-type lectin receptors (Takeuchi and Akira, 2010, Hornung et al., 2009). These receptors recognize microorganisms from host cells using structural patterns shared by many microbes called pathogen associated molecular patterns (PAMPs). Because the patterns are shared PRRs can offer protection against several different intruders using relatively few components. In addition to recognize non-self, PRRs are also able to recognize damaged or altered self, like uric acid crystals in gout (Liu-Bryan et al., 2005). Cells of the innate immune system include mast cells, phagocytes (macrophages, neutrophils and dendritic cells), basophils, eosinophils and natural killer cells. Upon bacterial infections, the microbes will be recognized by PRRs of the local macrophages and mast cells which upon activation produce inflammatory mediators like chemokines and cytokines (Medzhitov, 2008). However, the response mechanism between the cell types differ as the mast cells are poor in their ability to internalize LPS and bacteria, hence incapable of instigating signalling pathways that require intracellular initiation (Dietrich et al., 2010). Macrophages and dendritic cells are able to present antigens from the invader and initiate an adaptive immune response. Inflammatory mediators promote inflammation, in which leukocytes and plasma proteins are recruited to the infected area for elimination of the intruder, followed by repair of tissue damaged by the non-specific responses of the inflammation (Medzhitov, 2008).

1.1.1 Phagocytosis

Phagocytosis is the internalization of particles larger than 0.5 μm by phagocytes like macrophages, neutrophils, and dendritic cells. The existence of this process has been known for about 100 years, and in 1908 Ilya Metchnikoff was awarded the Nobel Prize for his work on phagocytosis (Tauber, 2003). This highly conserved mechanism is now recognized as vital for clearance of pathogens and maintenance of tissue homeostasis by removing apoptotic cells and cell debris.

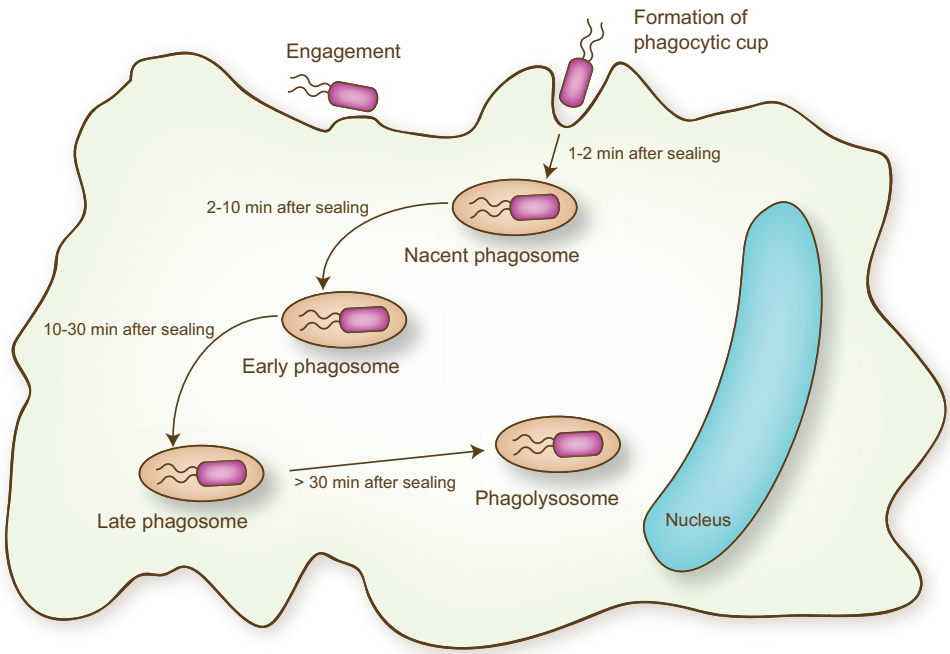

Figure 1.1: The six stages of phagocytosis as described by Steinberg and Grinstein (2008). Phagosome formation consists of engagement, formation of the phagocytic cup and formation of the nacent phagosome. Phagosome maturation involves the three last stages; early phagosome, late phagosome and phagolysosome. Elapsed time between the different stages will depend on cell type and nature of phagosomal content.

Upon engulfment of the particle, a membrane bound structure called the phagosome is formed by actin cytoskeletal rearrangement (Groves et al., 2008). The bacteria will then proceed through the endolysosomal degradation pathway, of which six stages may be identified (Figure 1.1): Particle engagement, formation of the phagocytic cup, nascent phagosome, early phagosome, late phagosome and phagolysosome (Steinberg and Grinstein, 2008). In the last three stages the phagosome will undergo a strict series of fusion and fission events with endocytic compartments, called phagosomal maturation, to gain biocidal and degenerative abilities (Flannagan et al., 2009). Early phagosomes fuse preferentially with early endosomes and gain many of the early endosomal properties, before they acquire an increasing inclination to fuse with later endocytic structures (Desjardins et al., 1997). Phagolysosomes are formed by the fusion of late phagosomes with lysosomes and marks the endpoint of phagosomal maturation (Steinberg and Grinstein, 2008). At this time the phagolysosome has its complete arsenal of antimicrobial properties (Flannagan et al., 2009):

Decrease in internal pH: By recruitment of ATP-hydrolysis-driven proton pumps called V-ATPases the phagosome experiences a drop in pH from about 5.4 to about 4.5 (Trombetta et al., 2003), resulting in a hostile growth environment for the microbe,

activating pH dependent proteases, sequestering nutrients and enabling fusion with lysosomes (Haas, 2007).

Generation of reactive oxygen species (ROS): Upon activation by phagocytosed microbes or certain molecules like bioactive lipids and antibodies, phagocytes may go through a respiratory burst where NADPH oxidase complex produce superoxide via expenditure of oxygen. This feature is microbiocidal, usually extremely rapid, and leads to the generation of ROS like hydrogen peroxide (Robinson, 2009).

Antimicrobial proteins and peptides (AMP): AMPs are evolutionary conserved defense molecules found in many different species. They display an extensive diversity and are found on skin and mucosal tissue as well as in phagocytes. In humans they have been divided into cathelicidins and defencins and while their exact antimicrobial mechanism is unknown, it is thought to involve membrane disruption through electrostatic interactions with the negatively charged bacterial membrane (Guani-Guerra et al., 2010).

As the phagocytes are removing foreign components, they become instrumental in both innate and adaptive immune responses through the activation of pattern recognition receptors and presentation of antigens to lymphocytes.

1.1.2 Toll-like receptors

Toll-like receptors (TLRs) are membrane bound PRRs that have been extensively studied over the last thirteen years. The name originates from the protein Toll in the fruit fly *Drosophila melanogaster* which is involved in both embryonic development and clearance of fungal infections (Anderson et al., 1985, Lemaitre et al., 1996). A homolog of the Toll protein was later found to control immune response in humans (Medzhitov et al., 1997), and the homology gave rise the name of the receptor family. Today, 10 of these type I trans membrane receptors have been discovered in humans providing the host with means to discriminate between self and non-self (McGettrick and O'Neill, 2010). The TLR family members respond when exposed to a diverse set of ligands. Binding of any of these ligands to their respective receptor triggers signaling cascades that ultimately lead to the induction of type I interferons (IFN-1), inflammatory cytokines and chemokines (Medzhitov et al., 1997, Kawai et al., 2001, Kawai and Akira, 2010). Responsible for the actual binding of PAMPs are the extracellular leucine-rich repeats (LRRs) which give the receptor its falcate shape (Matsushima et al., 2007). Prior to activation TLR9 constitutes a homodimer, following ligand binding it undergoes conformational changes to bring the receptors cytoplasmic toll and Interleukin-1 receptor (TIR) domains in a position that enables signaling (Latz et al., 2007). Other TLR family members are thought to be activated much in the same way (O'Neill and Bowie, 2007). The signal is transmitted through the cascade via a small group of adapters including myeloid differentiation factor 88 (MyD88), TIR domain-containing adapter protein (TIRAP/ Mal), Toll-receptor-associated activator of interferon (TRIF) and Toll-receptor adapter molecule (Tram) (Kawai and Akira, 2006). All TLRs and members of the IL-1R/TLR superfamily contain a cytoplasmic TIR domain. This domain is essential for signaling and is also present on adapters of the TLR signaling cascade of which two

differential pathways are recognized, the MyD88 dependent pathway and the MyD88 independent pathway (Doyle and O'Neill, 2006).

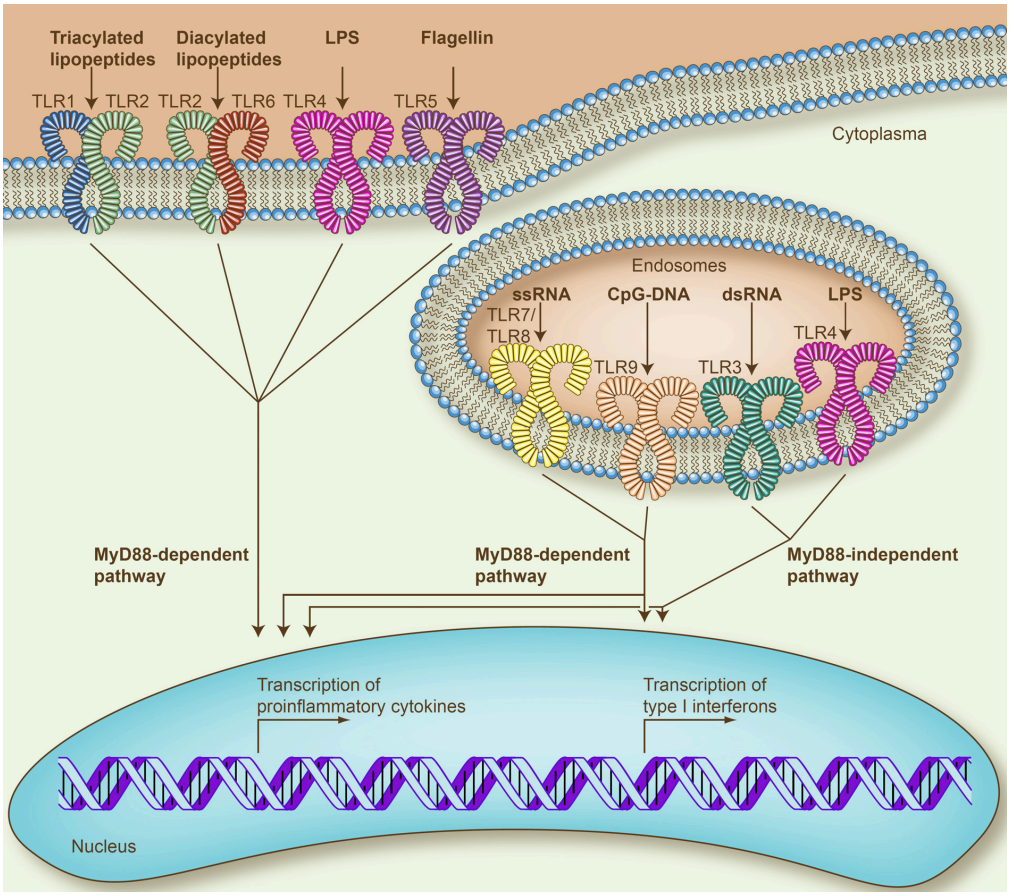

Figure 1.2: Human TLRs: Their ligands and signalling pathways. TLRs form homodimers or heterodimers in response to several different PAMPs and activate transcription of proinflammatory cytokines or interferons through the MyD88-dependent or MyD88-independent signalling pathway.

TLRs are either cell-surface receptors or intracellular receptors. The reason for this expression pattern may be the difference in ligand accessibility. Some ligands are found on the surface of microbes (lipid structures and flagellin), and can be detected more easily by surface receptors (TLR1, 2, 4, 5 and 6). Ligands derived from the genome (nucleic acids) require phagosomal degradation to become exposed and are therefore detected in intracellular compartments by TLR3, 7, 8 or 9 (McGettrick and O'Neill, 2010). Figure 1.2 shows the distribution of TLRs, their ligands and signalling pathways. Expression of TLRs is not a feature unique to immune cells, and non immune cells express functional TLRs that are able induce an immune response (Cario and Podolsky, 2000, Sha et al., 2004). For the epithelial cell line m-IC_{cl2} TLR4 is expressed solely in intracellular compartments and their response is attenuated compared to that of an immune cell (Hornef et al., 2002). This might be a result of the need for non-immune

cells to have some responsiveness to high concentrations of a ligand, but as their position cause constant exposure of minute ligand concentrations, full responsiveness is disadvantageous (McGettrick and O'Neill, 2010). The role of TLRs in maturation of phagosomes has been comprehensively debated. Several reports indicated deficiencies in microbial clearance by MyD88 knock out murine macrophages (de Veer et al., 2003, Henneke et al., 2002, Liu et al., 2004). This was followed by an article showing that activation of TLRs upon bacterial exposure could initiate an accelerated phagosomal maturation process in the same cells (Blander and Medzhitov, 2004). Conflicting to these results, a subsequent study demonstrated no effect of TLR2 or TLR4 activation on phagosomal maturation in wild type (wt) murine macrophages (Yates and Russell, 2005). The discrepancy was suggested to be a result of reduced phagocytic ability in the MyD88 knock outs because of differential gene expression due to the knock out itself. This explanation was repudiated by Blander and Medzhitov (Blander and Medzhitov, 2006), but may be explained by the utilization of different experimental systems, and the issue remains unresolved. Interestingly, TLR4 signalling has been shown to accelerate internalization of bacteria in human primary cells substantiating the connection between phagocytic ability and TLRs (Jain et al., 2008).

1.1.2.1 Signalling downstream of Toll-like receptors

All TLRs are able to activate the transcription factor Nuclear Factor-κB (NF-κB) by initiating phosphorylation and subsequent ubiquitination and degradation of the inhibitor of NF-κB (IκB). This activation may proceed through the adapters MyD88 in the MyD88 dependent pathway or TRIF in the MyD88 independent pathway (Kawai and Akira, 2006). The MyD88-dependent pathway can be initiated by all TLRs, except TLR3, and activates NF-κB through IRAK-1, IRAK-4, TRAF6 and the TAK1/TAB1/TAB2 complex (Doyle and O'Neill, 2006). TLR3 and TLR4 can also signal through the MyD88 independent pathway via the TRIF adapter that for TLR4 also requires TRAM (Yamamoto et al., 2002, Fitzgerald et al., 2003). This pathway is able to drive expression of genes downstream of both the NF-κB and IFN-β promoters (Yamamoto et al., 2002). NF-κB is activated through direct TRIF/TRAF6 interaction or via RIP1. The transcription factor responsible for IFN-β transcription, Interferon regulatory factor 3 (IRF-3), is activated by phosphorylation through TBK1 (Doyle and O'Neill, 2006). Additional transcription factors activated by TLRs are the activating protein 1 (AP-1), via TAK1 and mitogen-activated protein kinases (MAPK), JNK and p38, and other members of the IRF family (Carmody and Chen, 2007). Figure 1.4 shows the key steps in the activation of NF-κB and IRF-3 by the MyD88-dependent and independent pathways by example of TLR4. For a more comprehensive illustration of signaling cascades downstream of TLRs see review from Kawai and Akira (2010). Upon activation, the transcription factors in question translocate to the nucleus and promoter regions of inflammatory genes for transcriptional activation and expression of cytokines and interferons like interleukins, tumor necrosis factor (TNF), RANTES, type I and II interferons (Medzhitov et al., 1997, Genin et al., 2000, Takeuchi et al., 2000). Activation of genes regulated by NF-κB results in expression of proinflammatory cytokines while genes regulated by interferon

regulatory factors (IRFs) results in expression of interferons (Sheedy and O'Neill, 2007). Some cytokines and interferons of particular interest for this work are listed in table 1.1.

Table 1.1: Functions in host defense for some cytokines and interferons of particular interest for this work.

Name	Type	Function in microbial host defense
TNF	Cytokine	Almost all PRRs induce production of TNF. Among the many functions TNF is able to stimulate, the best known is its ability to induce apoptosis in many tumor cell-lines. It can also supply antiapoptotic and proinflammatory signals via NF-κB, providing an example of its often dualistic behavior. TNF's role in bacterial infections depends on species and circumstance, as granuloma formation in mycobacteria infections is TNF dependent and TNF deficient mice show either increased or decreased pathogen resilience depending on the infecting species[1].
IFN-β	Type I interferon	IFN-β is secreted after activation by either TLRs or cytosolic receptors upon viral or certain bacterial infections. It induces autocrine or paracrine activation of IFN-1 receptor, leading to expression of hundreds of genes to create an "antiviral state"[2]. In bacterial infections it may also have adverse effects[3].
IFN-γ	Type II interferon	IFN-γ is secreted primarily by NK cells and T lymphocytes upon exposure to cytokines secreted by myeloid cells[4]. This type II interferon is important in defense against several intracellular bacteria[5,6,7]. In monocytes, it enhances the expression of MHC class II[8], prompting amplified antigen presentation and activation of adaptive immune responses.
IL-1β	Cytokine	IL-1β is expressed upon activation of NF-κB and MAPKs, and can induce pleiotropic effects like fever, angiogenesis and leukocyte extravasation via the IL-1 receptor[9].
IL-18	Cytokine	IL-18 is expressed upon exposure to various PAMPs, including TLR agonists[10]. It induces the production of IFN-γ in synergy with IL-2, IL-12 or IL-15 through the receptor IL-18Rα and coreceptor IL-18Rβ[12]. It has a role in induction of proinflammatory cytokine and chemokine production, accumulation of neutrophils to lung and liver during bacteremia, attracting dendritic cells to virus infected tissue, and enhancing expression of adhesion molecules[12].

[1](Wajant et al., 2003), [2](Stetson and Medzhitov, 2006), [3](Shahangian et al., 2009), [4](Zhang et al., 2008), [5](Flynn et al., 1993), [6](Bitsaktsis et al., 2004), [7](Rottenberg et al., 2002), [8](Virelizier et al., 1984), [9](Franchi et al., 2010), [10](Arend et al., 2008), [11](Dinarello, 2009), [12](Iannello et al., 2009).

IFN-1 has conventionally been recognized mainly for their antiviral activity, but their role in bacterial infections is now also appearing (van den Broek et al., 1995, Pitha, 2004, Mancuso et al., 2007). Reports show that IFN-1 signaling is important in clearing bacterial infections such as group B streptococci, *Streptococcus pneumoniae*, *E. coli, Legionella pneumophila* and *Salmonella typhimurium* (Mancuso et al., 2007, Plumlee et al., 2009, Kim et al., 2005, Freudenberg et al., 2002). On the other hand, IFN deficiencies are beneficial against bacteria like *Listeria monocytogenes* and *Chlamydia muridarum*, (Qiu et al., 2008, Auerbuch et al., 2004, O'Connell et al., 2004, Carrero et al., 2006). It is not entirely obvious how to explain the differences in IFN-1 sensitivity among bacterial

species. It is possible that IFN-1 induced immune responses like induced cell death in macrophages in conjunction with IFN-β induced inhibition of neutrophil attraction (O'Connell et al., 2004, Qiu et al., 2008, Dietrich et al., 2010), creates an "antiviral" state more suited to deal with viral infections. The IFN-1 induced production of IFN-γ, NO and TNF is beneficial in dealing with a bacterial intruder (Mancuso et al., 2007), and might dominate in the induction of more of an "antibacterial" response. By some means certain bacterial strains may therefore be able to wrongfully induce an antiviral state, possibly as part of an evasion scheme. In line with this thought is how the IFN-1 response induced during influenza infections renders the host susceptible to a secondary infection by *Streptococcus pneumoniae* (Shahangian et al., 2009). This might point toward a model in which high amounts of IFN-1 relative to the level of other cytokines can direct the response towards being "antiviral" (Dietrich et al., 2010), explaining the detrimental effects of administering poly I:C during an *L. monocytogenes* infection or IFN-1 during *M. tuberculosis* infections (O'Connell et al., 2004, Manca et al., 2001).

1.1.2.2 Intracellular trafficking of Toll-like receptors

The localization of TLRs is important for several reasons, i.e. how internalization of surface TLRs may desensitize the cell and prevent overstimulation (McGettrick and O'Neill, 2010). Endosomal trafficking of TLR4 after exposure to lipopolysaccharide (LPS) leads to receptor ubiquitination and progression to lysosomal degradation suggesting exactly such a role (Husebye et al., 2006). Internalization of the receptors is also pivotal for proper signaling, as blocking of *Staphylococcus aureus* phagocytosis using cytochalasin D prevent TLR2 signaling (Ip et al., 2008), and inhibition of LPS uptake abolished IRF-3 activation from the MyD88-independent pathway leaving the MyD88-dependent pathway undisturbed (Kagan et al., 2008). A probable reason for this internalization requirement in TRAM/TRIF mediated TLR4 signaling is the need for TRAF3 proximity. This adaptor is not found on the plasma membrane, but by forcing its presence to the plasma membrane TLR2 induced IRF-3 activation is rendered possible indicating regulation by trafficking (Kagan et al., 2008). Indeed, compartmentalization appears to often play a role in signaling trough availability of adaptors or negative regulators, and one example of a negative regulator is the splice variant of TRAM named TAG. It displaces TRIF from TRAM in late endosomes hence negatively regulating the MyD88-independent pathway and providing another example of internalization dependent desensitizing (Palsson-McDermott et al., 2009). Trafficking also contributes in allowing for appropriate receptor-ligand interactions. TLR1, 2 and 6 are known to accumulate to phagosomes regardless of their content (Ozinsky et al., 2000), while FRAP experiments demonstrated a rapid movement of TLR4 between the plasma membrane and intracellular compartments (Latz et al., 2002). This kind of intracellular translocation of surface receptors may provide a surveying mechanism in which all phagosomes and endosomes are scanned for their contents. The intracellular TLR7 and 9 translocate to endosomes upon cell activation regardless of ligand identity, providing additional examples of endosome content sampling (Kim et al., 2008). Having receptors for nucleic acids are essential when it comes to detection of viral infections, but also

hazardous as inappropriate self-recognition may occur under given circumstances leading to autoimmune diseases (Kawai and Akira, 2010). Confining genome sensing TLRs to endosomal structures where access of intrinsic ligands can be limited and xonotic ligands can be amplified may be important to prevent such a contingency. However, how this discrimination should be obtained remains inadequately described (Barton and Kagan, 2009). To at all be able to interact with their ligands TLRs need to be a part of the endosomal/phagosomal pathway after synthesis. The chaperone protein gp96 is involved in trafficking of TLR2, 4, 5, 7 and 9 to their respective sites for ligand assembly (Randow and Seed, 2001, McGettrick and O'Neill, 2010). Finally, trafficking also plays a role in the bridge between innate and adaptive immunity as antigens against the TLR4 receptor complex were presented by HLA class II to T helper cells (Husebye et al., 2006).

1.1.2.3 Toll-like receptor 4

TLR4 is activated by lipopolysaccharide (LPS) from Gram-negative bacteria (Poltorak et al., 1998). In addition to LPS TLR4 recognizes various proteins from viruses and toxins (Kawai and Akira, 2010). TLR4 forms a receptor complex with the soluble protein myeloid differentiation factor 2 (MD-2), which is required for LPS responsiveness (Shimazu et al., 1999, Nagai et al., 2002). The complex receives LPS from the protein cluster of differentiation 14 (CD14) in concert with the soluble lipid transferase LPS-binding protein (LBP) (Guha and Mackman, 2001). Based on experiments using murine TLR4 and the LPS antagonist eritoran, TLR4 and MD-2 were predicted to form a dimer by MD-2 binding the concave region of the receptor upon LPS delivery (Kim et al., 2007). Later investigations confirmed LPS to indeed bridge human MD-2 and TLR4 as seen in figure 1.3 (Park et al., 2009).

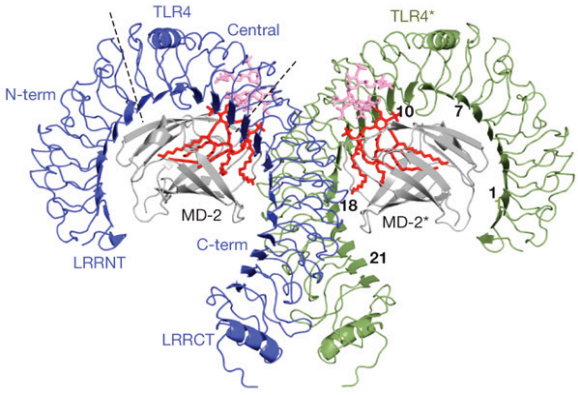

Figure 1.3: Crystal structure of TLR4 in complex with MD-2 and lipid A. TLR4 is displayed as the signalling homodimer (blue and green). One MD-2 molecule is found associated with the concave face of each TLR4 molecule, and five of the six acyl chains of Lipid A (red) is buried in a hydrophobic pocket of MD-2 leaving the sixth acyl chain to interact with TLR4 (Park et al., 2009). Printed with permission.

TLR4 is expressed in a variety of cells, both of immune and non-immune origin (Backhed and Hornef, 2003). Its distribution in resting cells includes the plasma membrane, endosomes and a Golgi-near area (Latz et al., 2002, Husebye et al., 2006). Following LPS activation the receptor is internalized in a dynamin and clathrin dependent manner to endosomes where it colocalizes with TLR4. TLR4 is further ubiquitinated and sent to lysosomes for degradation (Husebye et al., 2006). TLR4 seems to be exceptional among TLRs in that it is able to signal through both the MyD88-dependent and independent pathway (Doyle and O'Neill, 2006). NF-κB activation by the MyD88-dependent pathway was for several years recognized to ensue earlier than activation by the MyD88-independent pathway (Kawai et al., 1999). This was later explained by the pathways being initiated at either the plasma membrane or on endosomes (Kagan et al., 2008), illustrated in figure 1.4.

LPS is highly immunostimulatory causing a dual role of TLR4 in microbial infections as the gain of a strong immunological response for clearance of the intruder can be overshadowed by the potential destructivity of a septic shock. Gram-negative bacteria are able to cause sepsis by activating cells to a degree that result in an uncontrolled release of proinflammatory cytokines, activation of coagulation and microvascular dysfunction as a result of endothelial activation, potentially causing multiorgan failure and death (Waage et al., 1989, Schouten et al., 2008). It is the hydrophobic lipid A moiety of LPS in the outer monolayer of the bacteria that is a ligand for the TLR4/MD-2 complex (Lien et al., 2000, Raetz et al., 2007). Lipid A from *E. coli* is made up from two phosphorylated glucosamines acylated by six lipid chains, where five chains interact with MD-2, leaving the last chain to interact with TLR4 (Park et al., 2009), as seen in figure 1.3.

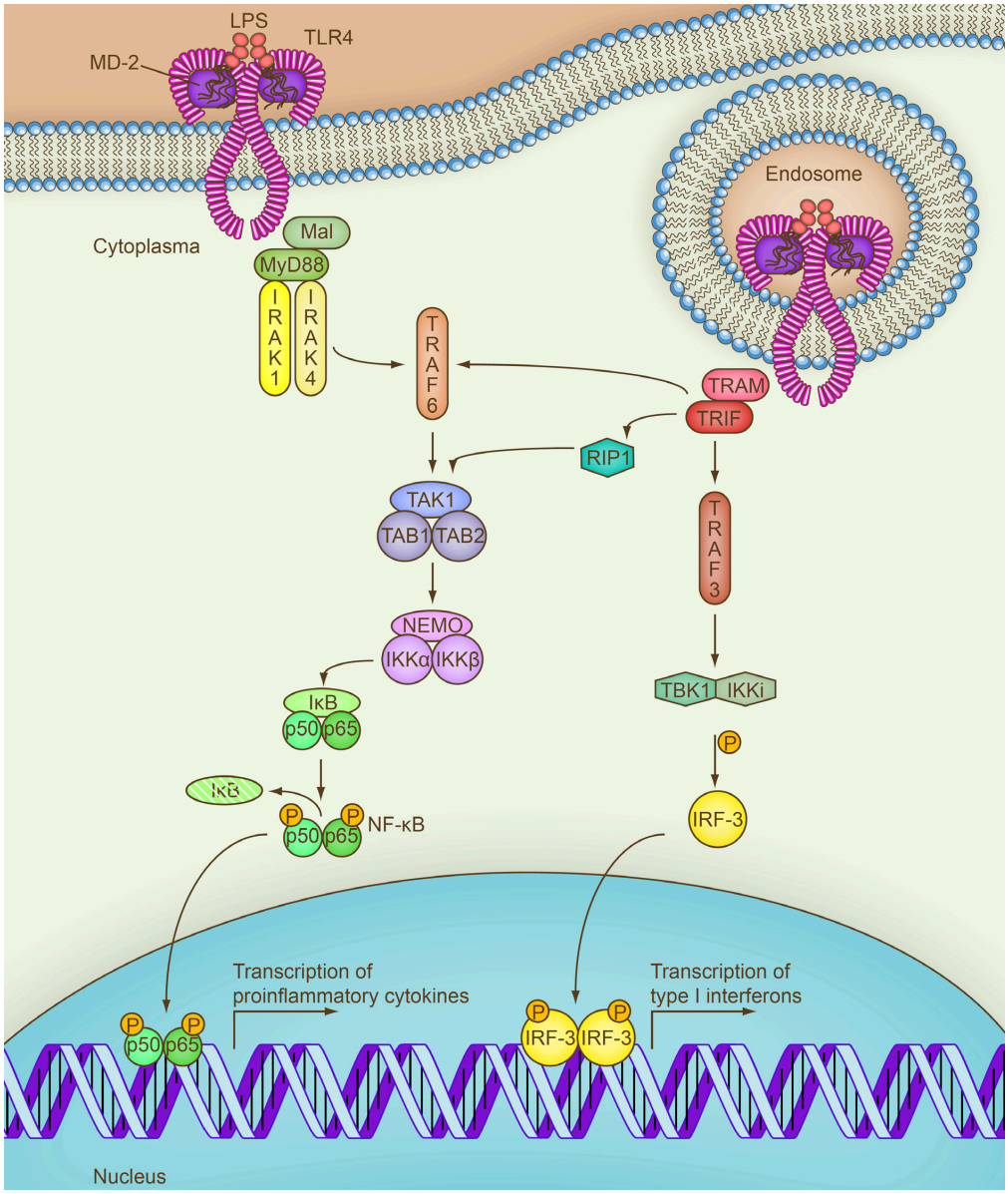

Figure 1.4: **Key steps in the activation of NF-κB and IRF-3 downstream of TLR4.** The TLR4/MD-2 receptor complex is activated by LPS binding and signals from the plasma membrane through the MyD88-dependent pathway and from endosomes/phagosomes via the MyD88-independent pathway. The two pathways end up with phosphorylation and nuclear translocation of the transcription factors NF-κB (here with typical subunits p50 and p65) and IRF-3, followed by transcription of genes for proinflammatory cytokines and IFN-1, respectively. Both the MyD88-dependent and the MyD88-independent pathways are able to activate TRAF6, which further activates the TAK1 complex leading to phosphorylation and degradation of IκB for reversal of its inhibitory function and release of NF-κB. TAK1 can also be activated by the MyD88-independent pathway through RIP1. The MyD88-independent pathway activates TRAF3, which activates the kinase TBK1 and finally phosphorylate IRF-3.

1.2 NLRP12 and the inflammasomes

Activation of TLRs leads to the expression of several cytokines, including IL-1β (Medzhitov et al., 1997). Although several TLR ligands are reported to induce IL-18 production it is also constitutively expressed, producing a ready made pool prior to inflammatory excitants (Arend et al., 2008). The two interleukins are unique in that they are expressed as inactive pro forms that require proteolytic cleavage by caspase-1 in order to be secreted as active cytokines (Cerretti et al., 1992, Gu et al., 1997). This activation step is mediated by molecular complexes called inflammasomes (Martinon et al., 2002). Central in this complex is a family of innate immune receptors; the nucleotide-binding domain leucine-rich repeat–containing receptors (NLRs). As the name indicates they contain LRRs, believed to function in autoregulation and ligand recognition, and a NACHT nucleotide-binding domain (NBD) that enables ribonucleotide interaction and may facilitate activation via oligomerization (Stutz et al., 2009). Although the NLRs are mostly known as important components of inflammasomes, they can also work independently of these structures (Shaw et al., 2010). NLRs and other inflammasome associated receptors are known to recognize a diverse assembly of signals including; fragments of bacterial cell walls (NOD1 and 2), bacterial and viral PAMPs and environmental irritants (NLRP3), lethal toxin from *Bacillus anthracis* (NLRP1), type III and IV secretion systems from Gram-negative bacteria (IPAF) and double stranded DNA (AIM2) (Schroder and Tschopp, 2010). NLRs assemble different inflammasomes by unknown mechanisms that include one or more NLRs, typically in association with the adaptor ASC. Activation of NF-κB via PRRs can also induce an increase of NLRP3 transcription which in turn serves to directly activate inflammasomes (Bauernfeind et al., 2009). A common denominator of inflammasomes is pro-caspase-1, which is cleaved by autocatalysis upon activation and further reassembled into its active form before proceeding to convert cytokines like IL-1β and IL-18 into their active forms (Guarda and So, 2010). Figure 1.5 shows the inflammasomes' role in inducing an IL-1β and IL-18 release. IL-1β and IL-18 activate receptors that signal via MyD88 and in consequence leads to activation of several inflammatory responses by the inflammasome (Stutz et al., 2009). After detection by inflammasome sensors several mechanisms is employed to battle the intruder. One important defense response is the caspase-1 dependent and proinflammatory programmed cell death named pyroptosis (Boise and Collins, 2001, Cookson and Brennan, 2001, Bortoluci and Medzhitov, 2010). The process was first observed for *Shigella flexneri* and thought to be apoptosis (Zychlinsky et al., 1992). However it was later discovered that the process was caspase-1 dependent rather than dependent on the specific marker for apoptosis; caspase-3 (Chen et al., 1996, Hilbi et al., 1998). The ingested bacteria from a pyroptotic cell are released to the extracellular milieu, thereby exposing them to uptake and successive killing by neutrophils (Miao et al., 2010). Other methods of killing due to IL-1β and IL-18 release include recruitment of immune cells and amplification of cytokine production leading to an inflammatory state (Toda et al., 2002, Iannello et al., 2009, Canetti et al., 2003).

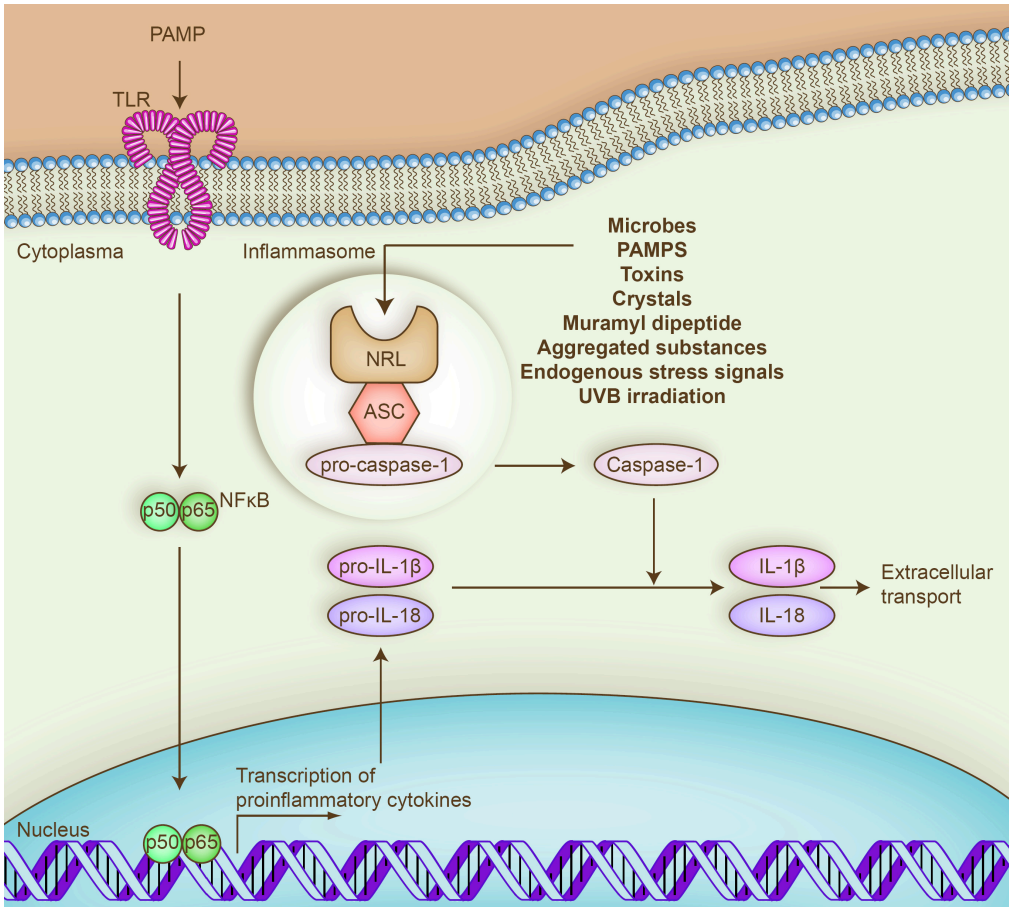

Figure 1.5: Proteolytic activation of IL-1β and IL-18 by the inflammasome. PAMPs will activate TLRs and result in the expression of pro-IL-1β and pro-IL-18. PRRs can also directly activate NLRP3 inflammasomes. Proteolytic activation of caspase-1 and subsequent proteolytic activation of IL-1β and IL-18 by the inflammasomes are initiated by a multitude of stimuli. Known inflammasomes consists of one or more NLRs and pro-caspase-1. ASC is not always obligate, but is usually needed for full IL-1β secretion.

NLR12, also known as Nalp12, Monarch-1 and Pypaf-7, is found only on immune cells where it forms an inflammasome together with the adaptor ASC (Wang et al., 2002). The protein has an N-terminal pyrin-domain, which is essential for NLRP12-mediated activation of IL-1β, a central NBD-domain and a LRR-domain, which is subject to differential splicing (Wang et al., 2002, Williams et al., 2003). Exposure to TLR ligands and examples of both Gram positive and negative bacteria in either the THP-1 cell-line or human leucocytes resulted in reduced expression of the receptor, prompting the hypothesis that it has a function as a negative regulator of inflammatory responses downstream of TLRs (Williams et al., 2005). NLRP12 has been suggested to be a negative regulator of NF-κB activation through interactions leading to proteasomal degradation of IRAK1 and NF-κB-inducing kinase (NIK) (Lich and Ting, 2007). Certain

mutations in *NLRP12* are associated with hereditary periodic fever (Jeru et al., 2008, Borghini et al., 2010). In a mouse model of dermatitis NLRP12 plays a role by promoting accumulation of neutrophils to inflamed tissue and enhance migration of dendritic cells to lymph nodes (Arthur et al., 2010). Additionally, single nucleotide polymorphisms are weakly linked with human dermatitis (Macaluso et al., 2007). While co-expression of NLRP12 and ASC results in synergistic caspase-1 activation and successive IL-1β secretion (Wang et al., 2002), its exact role in caspase-1 activation induced by pathogens remains unsolved.

1.3 Rab proteins

The process of intracellular trafficking in eukaryotes is exceedingly intricate and requires strict control mechanisms. In this effort the Ras related in brain (Rab) protein family is absolutely central as they control most aspects of intracellular trafficking including vesicle budding, uncoating, motility and fusion (Stenmark, 2009). The family has at least 60 members of which more than 15 has been localized to the Golgi (Goud and Gleeson, 2010). Each Rab shows a distinct pattern of localization, providing a convenient tool in studies of intracellular trafficking (Pfeffer, 2005). Rab5 is perhaps the best studied Rab protein and resides on early endosomes where it controls endocytosis and endosome fusion with clathrin-coated vesicles and phagosomal maturation (Stenmark, 2009). Rab10 is found in a perinuclear region where the localization is resistant to treatment with the Golgi disrupting agent Brefeldin A (Chen et al., 1993) and Rab7 is found on late endosomes about to fuse with lysosomes where the protein is needed for degradation of the phagolysosomal content (Meresse et al., 1995, Feng et al., 1995). Transport of secretory vesicles to the plasma membrane are mediated by Rab3 A, B, C and D in addition to Rab26, Rab27 A and B and Rab37 (Fukuda, 2008). Rab proteins are small guanosine triphosphatases (GTPases) working as molecular switches, usually obtaining an on state in its GTP-from, and an off state when bound to GDP (Zahraoui et al., 1989). They are reversibly associated with cellular membranes and interact with coat complexes, motor proteins and tethering factors like soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs) (Stenmark, 2009). To gain a specific selection of cargo into vesicles, different coat complexes recognize the cargo identity and interact with Rab proteins via sorting adaptors to gain increased affinity for their cargo (Carroll et al., 2001, Stenmark, 2009). In vesicle motility, Rab GTPases are known to indirectly recruit motor proteins for transport along both actin filaments and in either direction along the microtubule (Echard et al., 1998, Bielli et al., 2001, Deacon and Gelfand, 2001). Rab proteins recruit tethering factors like SNAREs, small vesicle membrane proteins that intermingle with SNAREs on other vesicles, and thereby aiding in vesicle docking and fusion (Armstrong, 2000). Tethering factors also often contain proteins that facilitates GDP to GTP exchange in the Rab (Guanine nucleotide exchange factors) resulting in a positive feedback loop that maintains local Rab activation (Stenmark, 2009). The prominent position intracellular trafficking has in essential cell functions leads Rab proteins to be implicated in many diseases, ranging from hereditary diseases and cancer through to microbial infections (Stenmark, 2009). Interestingly, a

recent study reveals a role for Rab10 in recruiting TLR4 to the plasma membrane in murine cells, providing an example of Rab-mediated trafficking of TLR4 (Wang et al., 2010).

1.3.1 Rab11a

Rab11a, together with the isoforms Rab11b and Rab25, make up the Rab11 subfamily. Rab11a is found in most tissues where it controls the endosomal recycling pathway (Schwartz et al., 2007). First isolated in bovine brain membrane (Kikuchi et al., 1988), homologs were later found in several species including humans (Drivas et al., 1991). It has been implicated in recruiting membrane to the forming phagosome in macrophages (Cox et al., 2000). Rab11a localizes to post-Golgi membranes, especially the endocytic recycling compartment (ERC)/ pericentriolar recycling endosomes, co-localizing with the transferrin receptor and regulating the recycling of transferrin back to the exterior of the cell (Ullrich et al., 1996). Crystal structure of Rab11a with one of its effector proteins FIP2, seen in figure 1.6, reveals a complex of two Rab11a molecules bridged by a FIP2 homodimer (Jagoe et al., 2006).

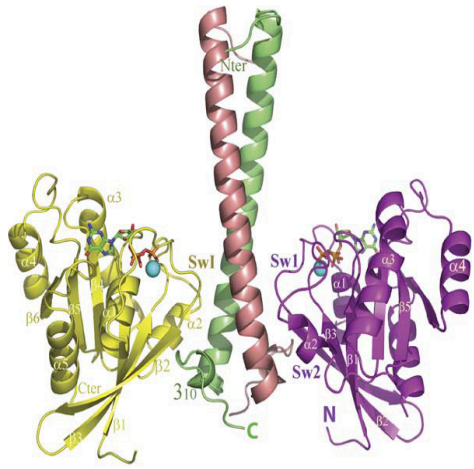

Figure 1.6: Structure of Rab11a and effector protein. Crystal structure of Rab11a molecules (yellow and purple), in complex with FIP2 (pink and green) shows two FIP2 molecules bridging two molecules of Rab11a (Jagoe et al., 2006). Printed with permission.

To achieve its different functions, Rab11a associates with many effector proteins, including the following (Horgan and McCaffrey, 2009):

Rab11-family interacting proteins (FIPs): This is a five member family of evolutionary conserved proteins containing a C-terminal Rab11-binding domain. FIPs control recycling of several molecules such as transferrin, GLUT4 transporter and the chemokine receptor CXCR2 to the plasma membrane, delivery of membrane during cell division and links Rab11 to motor proteins (Horgan and McCaffrey, 2009).

Rab11BP: Rab11BP (bovine) is also known as Rabphilin-11 (rat) and associates with the active GTP-form of Rab11a at both perinuclear regions and along microtubules (Mammoto et al., 1999). This cytosolic protein was identified as important for the Rab11a-mediated recycling of the transferrin receptor (Zeng et al., 1999).

Myosin Vb: Myosin Vb is an actin filament motor protein, involved in several cellular processes. It interacts with all members of the Rab11 family and is essential for plasma membrane recycling systems (Lapierre et al., 2001).

Phosphatidylinositol 4-kinase *β*: Phosphoinositides are phospholipids important in regulating key features of the cell including organization of the cytoskeleton and vesicle trafficking (Simonsen et al., 2001). Phosphatidylinositol 4-kinase β is found to colocalize with markers of the Golgi complex, where it serves as a docking factor to Golgi for the GTP-bound Rab11a and regulates transport from the complex to the plasma membrane (de Graaf et al., 2004).

1.4 Bacterial strategies for evasion of innate immunity

Vertebrate immune systems have developed elaborate mechanisms to fight off bacterial assaults. This evolutionary pressure has in turn resulted in bacteria gaining more and more inventive methods to counter the effects of an immune attack, some of which are summarized in figure 1.7. We now have knowledge about numerous evasion tactics, revealing targeted processes in the immune system and suggesting new drug targets for use in treatment of pathological bacterial infections (Finlay and McFadden, 2006, Roy and Mocarski, 2007).

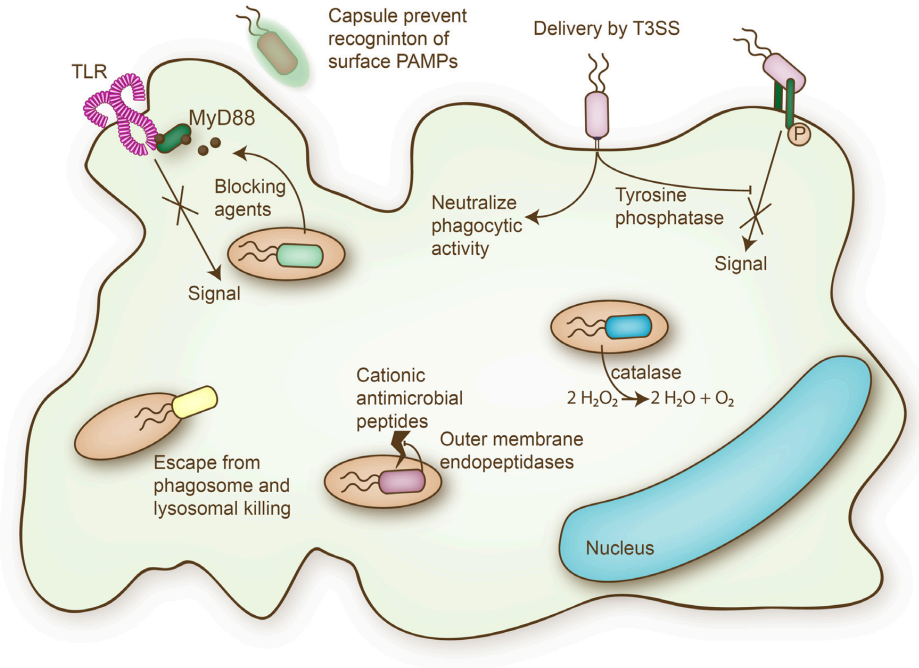

Figure 1.7: Some mechanisms of bacterial evasion employed by immune cells. Bacteria are able to evade the innate immune system by several mechanisms including modification of surface, escape from the phagolysosomal pathway, expression of enzymes to neutralize ROS and AMPs, subversion of phaogcytes and immunosupression.

Modification of surface
A fairly obvious way to avoid detection by the pattern recognition based innate immune system is for the bacterium to modify its surface. A popular approach is to simply express a more or less inert capsule in which to hide the different surface molecules. *Streptococcus agalactiae* (group B) is a pathogen that mainly cause disease in infants or if present as a secondary disease in adults. By the use of a polysaccharide capsule it is able to prevent recognition of its surface lipoprotein Blr by macrophage scavenger receptor A, hence avoiding phagocytosis (Areschoug et al., 2008).

Escape from phagolysosomal degradation pathway
Francisella tularensis is a Gram-negative bacterium and the agent of tularemia, a widespread zoonosis able to cause serious pneumonic disease in humans. It is phagocytosed by macrophages, but the phagosomes show reduced ability to mature before the phagosomal membrane disrupts allowing *F. tularensis* entry to the cytoplasm for further replication and intracellular occupancy (Clemens et al., 2004).

Expression of enzymes to neutralize reactive oxygen species (ROS)
To enable an intracellular life cycle the bacteria must avoid the biocidal effects of ROS. *Salmonella typhimurium* is an intracellular pathogen causing gastroenteritis in humans. To obtain its intracellular state it must employ several evasion mechanisms including suppression of bacterial killing by ROS. *S. typhimurium* has five hydrogen peroxide degrading enzymes which all facilitate intracellular survival (Hebrard et al., 2009).

Resistance towards antimicrobial peptides
Upon infection a bacteria can increase its resistance toward antimicrobial peptides by several mechanisms including surface remodelling, neutralizing or promoting active efflux of the peptides (Nizet, 2006). *Staphylococcus aureus* is a Gram-positive bacterium often associated with skin disease. The outer membrane endopeptidase staphylokinase produced by *S. aureus*, has several roles in immune evasion, one being the ability to inactivate the bactericidal α-defensins from neutrophils (Jin et al., 2004).

Subversion of phagocytes
Phagocytes are important in host defence, and naturally a key target in evasion manoeuvres. Type III secretion systems (T3S) are found in several different Gram-negative bacteria and the apparatus enables injection of bacterial effector proteins into host cells to manipulate their function (Coburn et al., 2007). *Yersinia pseudotuberculosis* usually causes fever and abdominal pain in humans. It can deliver the effector YopE to the host cell through its T3S, thereby providing antiphagocytic functions (Black and Bliska, 2000).

Immunosupression
Not all evasion mechanisms aim at hindering detection of the invading bacteria, some focus on blocking the response following detection. Uropathogenic *E. coli* (UPEC) is responsible for most urinary tract infections in otherwise healthy individuals. Secreted

TcpC protein from UPEC directly inhibits MyD88, drastically reducing innate immune activation (Cirl et al., 2008)

1.4.1 *Yersinia pestis* and evasion of Toll-like receptor 4

Yersinia pestis is the causative agent of the widespread zoonotic disease plague and the infectious agent of the medieval Black Death (Raoult et al., 2000, Haensch et al., 2010). From 1987 to 2001 36876 cases of plague was reported, resulting in 2847 deaths, with mortality reaching 100 % in untreated cases of the more rare primary pulmonary form (Prentice and Rahalison, 2007). The organism is dependent on a life cycle that alternates between flea and mammals (usually rodents), as the flea fetus and larvae are protected from persistent colonization. The flea is infected after a blood meal where bacteria may proceed to multiply and colonize the flea gut, preventing feeding and promoting regurgitating of bacteria as the hungry animal tries to feed, effectively spreading the disease to its prey (Perry and Fetherston, 1997). In humans the disease is often presented by a single tense swelling (bubo) near the bite area accompanied by malaise, dizziness, high fever, and enlarged lymph nodes (Anisimov and Amoako, 2006). If untreated it proceeds to secondary pneumonic plague that may be the cause of person-to-person contagion by aerosolized droplets after coughing, resulting in the very serious and deadly primary pneumonic plague (Prentice and Rahalison, 2007). *Y. pestis* evolved in China from its close relative *Y. pseudotuberculosis* and spread to other continents causing three main pandemic waves (Achtman et al., 1999, Achtman et al., 2004, Morelli et al., 2010). While *Y. pestis* is a highly virulent pathogen, *Y. pseudotuberculosis* only causes a self-limiting gastroenteritis. Important acquired virulence genes in *Y. pestis* are found on the plasmids pMT1 (enables extensive colonization of flea gut, and expression of a capsule-like antigen) (Hinnebusch et al., 2002, Du et al., 2002), and pPCP1 (promotes dissemination from infection site) (Sodeinde et al., 1992). Also important for virulence is the *Yersinia* Type III secretion system for delivery of virulence effector proteins, which is shared by all pathogenic *Yersinia* species (Prentice and Rahalison, 2007). Whole genome comparison between *Y. pestis* and *Y. pseudotuberculosis* is seen in figure 1.8 and reveals acquisition of some new genetic material (32 chromosomal, and 2 genes on plasmids) in *Y. pestis*, however, the striking difference is a major loss of genes (317 lost, and 149 inactivated) (Chain et al., 2004).

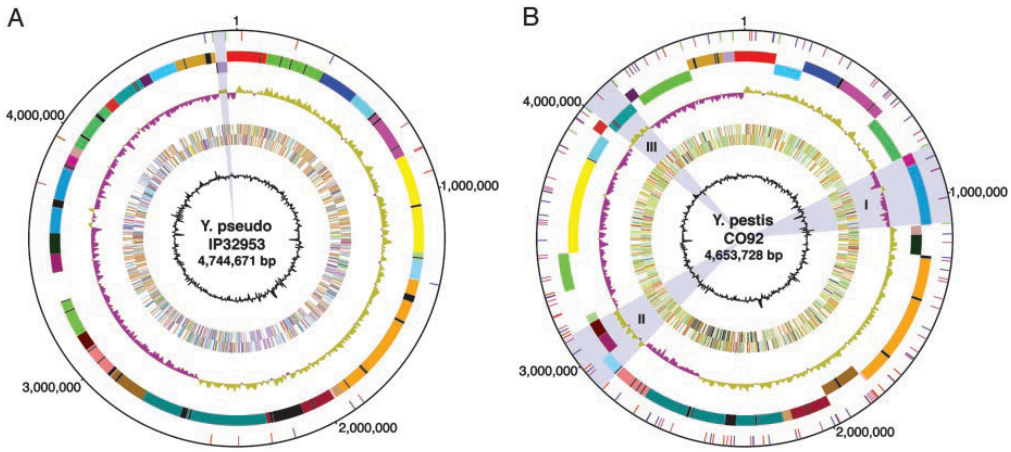

Figure 1.8: Whole genome comparison of *Y. pestis* and *Y. pseudotuberculosis*. Genome map of *Y. pseudotuberculosis* IP32953 (A) and *Y. pestis* CO92 (B) shows great genetic similarity (75 % more than 97 % identical) between the organisms and a complex genetic rearrangement. Circles 5 and 6 from the centre show homologous regions in colour code, black segments represent genome specific regions. The orientation of each region is indicated by strand (circle 5; - strand, circle 6; + strand) (Chain et al., 2004). Printed in accordance with PNAS guidelines for reprint.

Y. pestis expresses different forms of LPS dependent on the temperatures it experiences during its life cycle. At flea temperature (26°C) the lipid A is mainly hexa-acylated, resembling the potent immunostimulatory *E. coli* LPS, at mammalian temperature (37°C) a shift occurs towards a tetra-acylated lipid A population (Kawahara et al., 2002, Knirel et al., 2005, Rebeil et al., 2004). The tetra-acylated lipid A results in an antagonistic LPS that inhibits TLR4 activation in contrast to the potent hexa-acylated lipid A-LPS (Kawahara et al., 2002, Golenbock et al., 1991). A recombinant *Y. pestis* strain expressing the acyl transferase LpxL from *E. coli* yielded hexa-acylated LPS also at mammalian temperature allowing TLR4 activation and promoting clearance of the infection (Montminy et al., 2006). This corroborated the existing hypothesis that production of low stimulatory LPS-lipidA is an evolved evasion strategy of *Y. pestis* (Dixon and Darveau, 2005). Having a bacterial strain that differ in the ability to express either an immunostimulatory or non-stimulatory LPS offers an excellent molecular tool for studying innate immunity, TLR4 activation, down stream signalling events and their effects on pathogenicity.

2 AIMS OF THE STUDY

Despite the fact that new research articles involving TLR4 are published almost daily, key features regarding this receptor still remains to be elucidated. The aims of this study were to gather new knowledge about the intracellular trafficking of TLR4 upon challenge of various bacteria, and to examine the way Gram-negative bacteria are able to evade immune detection using *Y. pestis* as a model organism. Because of the central role TLR4 has in so many aspects of human health and disease, novel findings in this area have the potential to be of great importance in understanding the host responses during infections with Gram-negative bacteria in general. Another currently dynamic science field is inflammation and in particular the role of inflammasomes in pathology. We were especially interested in inflammasome function in *Y. pestis* infections on the basis of the immune evasive nature of this bacterium.

Specifically, we wanted to:

- Establish a method for quantification of fluorescence intensity in marked subcellular compartments based on confocal images (Imaris-method) and use this to measure the trafficking of TLR4 to phagosomes.
- Extend the application of the Imaris-method to include investigation of phagosomal trafficking of other interesting intracellular components like Rab11a, Tram, IRF-3, p65 and MyD88 together with trafficking of Rab11a and TLR4 to ERC.
- Ascertain whether an intracellular trafficking route of TLR4 as a result of phagosomal maturation of Gram negative bacteria could be described; how TLR4 was recruited, where it was recruited from and the implications this trafficking would have on TLR4 induced signalling.
- Investigate the relationship between TLR4 and Rab11a, such as physical interaction, the role of Rab11a in TLR4 mediated signaling and trafficking of TLR4.
- Determine if intracellular trafficking of TLR4 differed in monocytes upon infection by *Y. pestis* expressing either immunostimulatory or non-stimulatory LPS.
- Examine the inability of *Y. pestis* to hexa-acylate LPS at mammalian temperatures as an evolved evasion strategy by comparing the pathogens LPS acylation pattern and immune stimulatory abilities with that of its close relative *Y. pseudotuberculosis.*
- Evaluate the roles of inflammasomes and NLRs such as NLRP12 in host defense against *Y. pestis* strains.

3 LIST OF PAPERS

Paper I:

Husebye, H., M. H. Aune, J. Stenvik, E. Samstad, F. Skjeldal, O. Halaas, N. J. Nilsen, H. Stenmark, E. Latz, E. Lien, T. E. Mollnes, O. Bakke and T. Espevik (2010). "The Rab11a GTPase Controls Toll-like Receptor 4-Induced Activation of Interferon Regulatory Factor-3 on Phagosomes." Immunity

Paper II:

Vladimer, G., S. M. Paquette, D. Weng, V. Rathinam, M. H. Aune, J. Burbage, J. Conlon, C. Sweet, K. Stamper, A. Beardsley, R. J. Cotter, Q. Liu, G. Reed, J. C. Mecsas, Y. Iwakura, J. Bertin, J. D. Goguen, K. A. Fitzgerald, E. Lien. Central role of NLRP12 in resistance to *Yersinia pestis* producing a *Y.pseudotuberculosis*-like LPS. *Submitted*.

4 SUMMARY OF RESULTS AND DISCUSSION

4.1 Confocal image analysis of cellular compartments

At an early stage of this work it became clear that a way of quantifying TLR4 in individual phagosomes would be greatly beneficial. By utilizing the functions of the imaging software Imaris from Bitplane, a method for quantification of structures visualized in a confocal microscope was established. It relies on creating pseudolayers that covers the structures in question during the analysis work after image capture, as seen in figure 4.1. In contrast to the visualized structures in a confocal image, a structure covered by a pseudolayer allows for the recognition of the pseudostructure as an individual object. This enables the structures to be manipulated, i.e. segmented based on proximity to other structures like an *E. coli* cell to retrieve phagosomes or deleted if they do not fit the pre-defined criteria for that particular experiment. The software provides various parameters from the actual information in the image covered by the pseudolayer, and by that also the observed structure without loss of information from the image. A particular useful parameter for this study has been “sum of voxel intensity”. This is simply a sum of all the intensity values of the voxels (3D pixels) that make up a defined pseudostructure, and has been used as a measure of relative amounts of i.e. TLR4 in phagosomes. To obtain valid results, images must be captured in the dynamic range of the microscope. Using an 8-bit microscope the intensity of the signal emitted from the observed pixel making up any structure will range from 0 (no light emitted) to 255 (fully saturated). Before starting the image acquisition, several test images of the sample must be captured to ascertain if the microscope settings are suitable to stay within this range. The goal is to allow for as much light as possible to be detected but simultaneously not allow structures to become over saturated. When using a confocal microscope for quantification purposes, minimizing bleaching is essential and care should be taken to reduce laser output, number of scans in a z-stack, zoom, and scan time. The strength of the method lies in its versatility, as it can be used to quantify voxels or pixels in almost any structure visualised in a three dimensional confocal image. As you have an image of the quantified structures you are also able to exclude cells that might be unsuitable for the analysis, like apoptotic cells. The method, however, is very time consuming, and all images that are to be compared must be captured on the same day, restricting the number of samples per experiment and the number of observations per group.

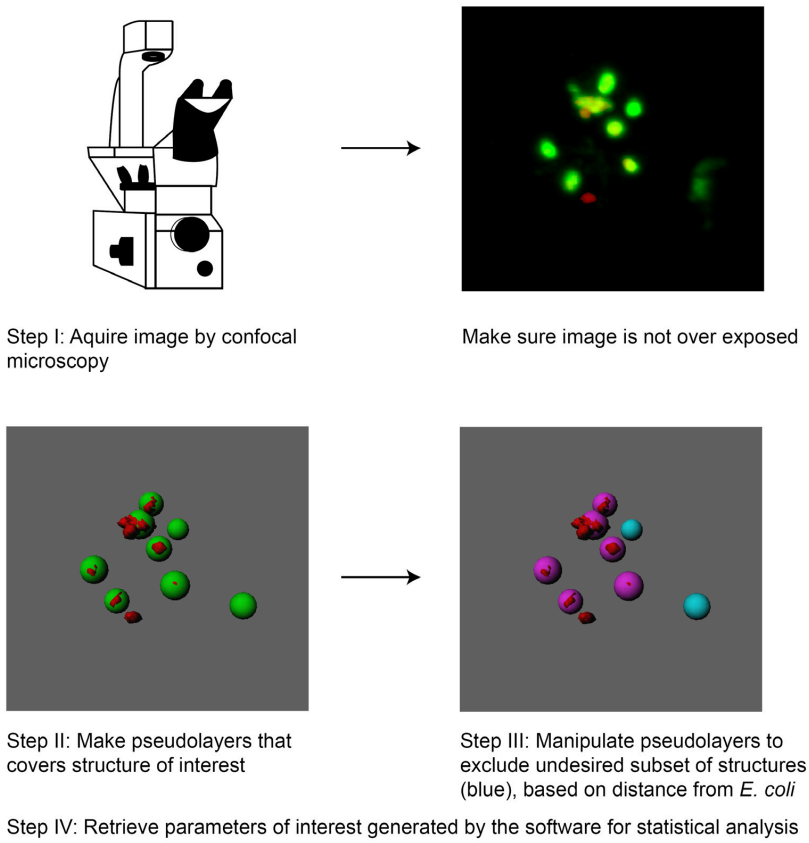

Figure 4.1: Confocal image analysis example. Step I: Acquire 3D images by confocal microscopy making sure the images are within the dynamic range of the microscope and minimizing bleaching of the sample. Example image shows phagocytosed *E. coli* (red) and fluorescently marked TLR4 (green). Step II: Make pseudolayers that cover the structure of interest. *E. coli* and TLR4 are kept red and green, respectively. Step III: Pseudolayers are manipulated to retrieve the desired subset of structures. In this example the figure shows discrimination of TLR4 structures not containing *E. coli* (blue) from TLR4 structures containing *E. coli* /phagosomes (magenta). Step IV: The software produces the parameters of each subset of structures using the information in the actual confocal image that is covered by the pseudolayers to be used for statistical analysis.

4.2 A novel trafficking route of TLR4 to phagosomes

In article I a novel intracellular trafficking route of TLR4 to phagosomes is described. It was previously assumed that TLR4 on endosomes were attained from the plasma membrane as it was co-internalized with the particle (Kagan et al., 2008). An intracellular pool of TLR4 was described, but as it is close to Golgi, and partially overlaps with Golgi markers this was thought to be part of a post transcriptional trafficking route

to the plasma membrane (Hornef et al., 2002, Latz et al., 2002). By carefully examining the position of the TLR4 pool in relation to different markers of the Golgi in three dimensions, it became clear that the overlap with Golgi was minimal. Based on the co-localization with Rab11a in this compartment we argue that the compartment in fact is the ERC, found inside the tube-like structure of Golgi (Ullrich et al., 1996, Saraste and Goud, 2007, Jones et al., 2006). TLR4 on phagosomes was quantified and interestingly the amount of TLR4 increased during phagosomal maturation, leading to the conclusion that TLR4 was recruited to the phagosome also after internalization. Rab11a was present on phagosomes displaying opposite kinetics of TLR4 during phagosome maturation, which may reflect a drop in fission/fusion ratio. The amount of TLR4 on phagosomes increases at the same time as the amount of TLR4 and Rab11a in the ERC decreases, suggesting that TLR4 is recruited to phagosomes from this compartment. Knock down of Rab11a resulted in less TLR4 recruited to phagosomes, less TLR4 in the ERC but not a reduction in over all TLR4 present in the cell. Based on experiments with cells expressing a dominant negative GDP-bound version of Rab11a (Rab11aSN^{GFP}) causing TLR4 to redistribute into endosomes close to the plasma membrane, it is likely that knock down of Rab11a lead to a similar redistribution. The constitutively active GTP variant of Rab11a (Rab11aQL^{GFP}) sequestered TLR4 in Rab11aQL^{GFP} positive vesicles, further corroborating the function of Rab11a in TLR4 trafficking. Because Rab11a is described in literature to regulate the continuous recycling of the transferrin receptor back to the plasma membrane (Ullrich et al., 1996), we propose a Rab11a dependent, constitutive transport of TLR4 from ERC to phagosomes. Our study show how the bulk of TLR4 in monocytes is found in the ERC rather than on the plasma membrane, and that dynamin is not required for TLR4 trafficking to phagosomes. Both observations are in line with our proposed hypothesis. Furthermore, as LPS induced, MyD88-independent signaling was selectively reduced in Rab11a knock-down cells; we also propose a role for Rab11a in the transport of TLR4 to endosomes. By the use of inhibitors the TLR4 recruitment to phagosomes was shown to be dependent on actin (cytochalasin D) and resistant to disruption of Golgi by brefeldin A. Taken together the results indicate an unanticipated intracellular trafficking route of TLR4 from ERC to phagosomes along actin filaments regulated by the GTPase Rab11a.

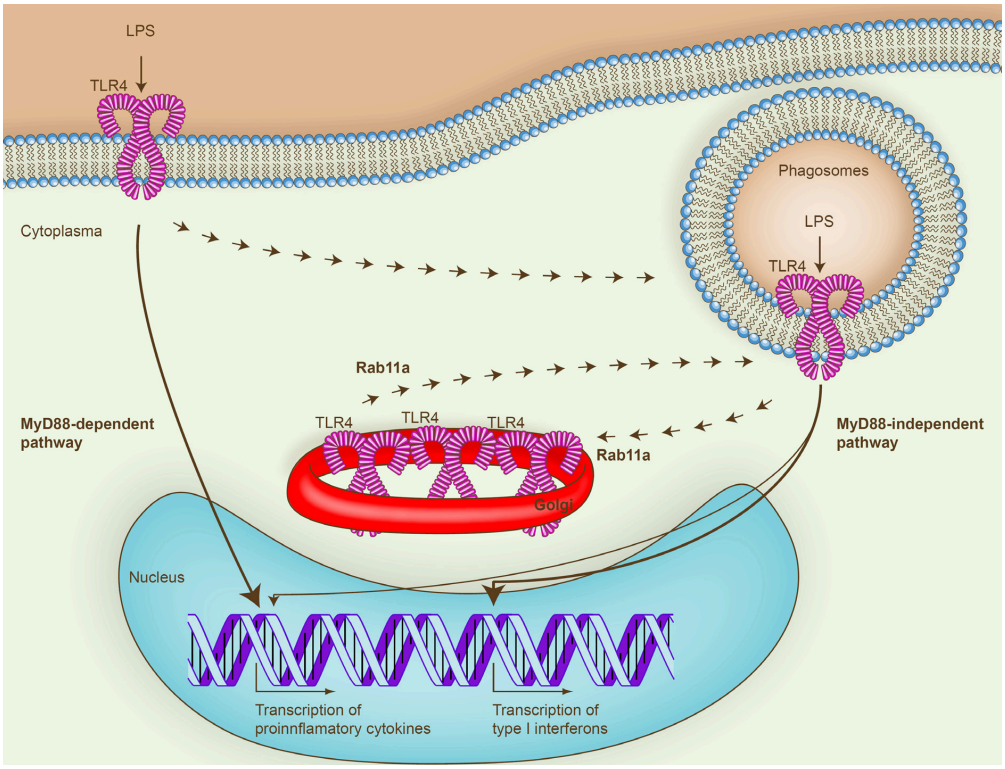

Figure 4.2: Trafficking of TLR4 to the phagosome. TLR4 on the plasma membrane is internalized during phagosome formation. ERC is found inside the tube-like structure of Golgi (Red) and transport of TLR4 (small arrows) from this compartment to phagosomes is controlled by Rab11a. Transcription of proinflammatory cytokines is mediated by the MyD88-dependent pathway (unbroken line) with a smaller contribution form the MyD88-independent pathway (thin, unbroken line). The MyD88-independent pathway (unbroken line) is initiated from phagosomes and governs the transcription of IFN-1s.

4.3 Consequences of the ERC-to-phagosome trafficking route on TLR4 signalling

Tram, IRF-3 and MyD88 were detected in phagosomes, prompting the deduction that this compartment is a site of signalling. *Y. pestis* was cultivated at different temperatures to provoke differential acylation of lipid A and used to show how TLR4 accumulation towards bacteria expressing the antagonistic LPS in contrast to agonistic LPS was reduced. The result points towards a dependency for stimulating LPS in TLR4 recruitment to phagosomes. This is in accordance with our results showing that the Gram positive *S. aureus* was unable to recruit TLR4. Restricting TLR4 transport to phagosomes by knock down of Rab11a lead to decreased amounts of Tram in this compartment, but left the amounts of MyD88 unchanged. Blocking phagocytosis by the actin filament inhibitor cytochalasin D resulted in an asymmetric impairment of the MyD88 independent pathway. This was manifested by a marked inhibition of IFN-β

transcripts upon activation by *E. coli*, but not TNF transcripts. In consequence, a strong IFN-β response is dependent on phagocytosis of the bacterium and is in line with previous work demonstrating initiation of the MyD88 independent pathway only from endosomes (Kagan et al., 2008). LPS alone is also able to induce IFN-β, but as the *E. coli* particle is much more potent in this respect, phagocytosis of the whole bacterium is probably of greater biological significance. Further investigations into the ability of Rab11a to control differential activation of the MyD88 independent signalling pathway downstream of TLR4 were performed and showed that Rab11a controls phosphorylation and nuclear translocation of IRF-3, expression from the IFR3 promoter and transcription of IFN-β. At the same time only minimal or no effects of Rab11a knock down was registered on IκB degradation, nuclear translocation of the NF-κB subunit p65, expression from the NF-κB promoter and transcription of TNF. The transport of TRAM to phagosomes was also shown to be dependent on Rab11a, perhaps indirectly by providing the phagosomes with TLR4 as a starting point for a signalling platform. The results do not exclude the possibility of a parallel signalling mechanism including initial activation of TLR4 on the plasma membrane through the MyD88 dependent pathway before the same receptor molecules initiate MyD88 independent signalling from phagosomes. As a result, we suggest that Rab11a regulates IFN-1 expression upon TLR4 activation by recruiting TLR4 to phagosomes, and initiating MyD88 independent signalling.

4.4 Evolution of *Y. pestis* virulence

In article II the effect on *Y. pestis* virulence after spontaneously losing one of its acyltransferase genes, *lpxL*, was examined. Compared to its close relative *Y. pseudotuberculosis* the *Y. pestis* genome is missing a section of genes containing among others *lpxL* (Chain et al., 2004). An essential virulence factor of *Y. pestis* is its exhibition of a mainly tetra-acylated LPS population at mammalian temperatures (37 °C), which is a poor TLR4 inducer and a likely consequence of the lack of LpxL (Montminy et al., 2006). Modification of the bacteria by expressing *Y. pseudotuberculosis lpxL* (also known as *htrB*) in *Y. pestis* (*Y. pestis*-pYtbLpxL) enables the latter to produce an LPS population that includes hexa-acylated lipid A at mammalian temperatures, similar to that of *Y. pseudotuberculosis*. The transgenic bacterium was cleared from mice upon infection and displayed LPS with increased ability to induce TNF, IL-8 and IL-1β compared to that of the wt. Clearance of the infection in mice was TLR4 dependent, and reflected in a major decrease of spleen bacterial load, stressing the importance of non-stimulatory LPS for evasion purposes. The ability to induce IL-1β release was dependent on the H132 amino acid in the proposed active site of *E. coli* LpxL, as a mutation (H132A) led to less IL-1β release and a LPS population skewed toward tetra-acylated lipid A. On the other hand, *Y. pestis* expressing *E. coli lpxL* does not differ in key phenotypes like growth, membrane stability, Pla activity and type III secretion activity (Montminy et al., 2006), excluding them as contributors for the increased immune response. It did, however, lead to a presumably better clearance of the infection of *Y. pestis*-pEcLpxL compared to *Y. pestis*-pYtbLpxL. This statement is based on how some experiments resulted in one dead wt

mouse following *Y. pestis*-pYtbLpxL exposure, while this was not seen when *Y. pestis* was expressing *E. coli lpxL*. A plausible reason for this may be that infection with the *E. coli-lpxL* expressing *Y. pestis* was able to induce an increased TLR4-mediated clearance caused by a higher proportion of hexa-acylated LPS. The expression of *lpxL* from *Y. entrocolitica* in *Y. pestis* resulted in a strain with similar LPS to that of *Y. entrocolitica* both in chemical structure and immune stimulatory abilities. Finally, an adequate IL-1β release required live bacteria, suggesting that other factors than LPS is necessary for inflammasome activation. Taken together this provides additional evidence to support the hypothesis that loss of *lpxL* was a key step in the evolution of *Y. pestis* virulence, substantiating the essential role of TLR4 in the early phases of an infections by Gram-negative bacteria.

4.5 The role of NLRP12 in resistance to infections

While examining the effect expression of *lpxL* in *Y. pestis* had on virulence a possible link to inflammasomes became evident when experiments indicated an important role of IL-1R in resistance of *Y. pestis* expressing *E. coli lpxL*. Results showing that MyD88 was important in resistance against wt *Y. pestis* supported the inflammasome hypothesis and prompted experiments with NLR-knock out macrophages. Upon infection with wt *Y. pestis* and *Y. pestis*-pYtbLpxL of NLRP12 deficient macrophages we observed reduced IL-1β and IL-18 secretion. Cleavage of IL-1β after *Y. pestis* or *Y. pestis*-pYtbLpxL infection in cells lacking inflammasome agents or TLR4 was reduced. NLRP12 expression increased in macrophages after infection with both wt and modified *Y. pestis*, this upregulation might be a part of a mechanism for increasing the activity of the inflammasome, as it has been implied for NLRP3 (Bauernfeind et al., 2009). ASC and Caspase-1 were also participating in controlling IL-1b/IL-18 release following infection with the *Y. pestis* strains, emphasizing the involvement of inflammasomes. NLRP12 likely does not participate in signaling via other NLRs like NLRP3, AIM2 and NLRC4, as known activators of signaling via these receptors gave a normal IL-1b release upon exposure of NLRP12 knock out macrophages. IL-1β and IL-18 secretion was almost abolished after infection of neither *Y. pestis* strains in TLR4 deficient macrophages, indicating a critical role of the receptor in production of these cytokines. There are species dependent differences in the TLR4/MD-2 complex' ability to recognize tetra-acylated LPS in which mice are better equipped than its human counterpart (Lien et al., 2000, Poltorak et al., 2000, Akashi et al., 2001, Hajjar et al., 2002, Montminy et al., 2006, Meng et al., 2010); hence the role of TLR4 in a human setting may prove less important in infections by strains with hypo-acylated LPS. To test the effects of NLRP12 in resistance against *Y. pestis* infections in vivo, experiments using NLRP12 knock out mice were undertaken. The studies showed that infection with *Y. pestis*-pYtbLpxL, which in wt mice is cleared quickly, caused a mortality rate of 80 % in NLRP12 deficient animals. Survival in wt mice coincided with significant increase of IL-1β and IL-18 detected in spleens and sera, an increase not detected in NLRP12 knock out animals. When comparing infections in IL-1R, IL-1β and IL-18R knock out mice it became clear that IL-18 signalling had the most profound effect on resistance against *Y. pestis* with hexa-acylated LPS. Further

experiments with IL-18R knock out mice revealed a decrease of both IL-18 and IL-1β in spleens when infecting with *Y. pestis*-pYtbLpxL compared to wt mice, which may indicate a positive feed-back loop for the production of the cytokines. Inflammation is a prerequisite for a full and efficient clearance of an intrusive agent such as bacteria. Examination of histological samples showed recruitment of inflammatory cells to bacterial foci in livers of mice infected with modified *Y. pestis*, while wt *Y. pestis* seemed to have evaded detection as no signs of inflammation was identified in conjunction with the fully virulent strain. In liver samples from NLRP12 or IL-18R deficient mice, only some or no signs of inflammation were seen around *Y. pestis*-pYtbLpxL, indicating that the immune response seen in wt animals was hampered by the absence of these genes. The same trend was observed in post-infection spleens, where a significantly higher bacterial load was seen for bacteria with the non-stimulating LPS compared to the genetically engineered strain. Knocking out either NLRP12 or IL-18R lead to a significant increase in bacterial load compared to wt mice, but the difference between the two bacterial strains was not apparent in these mouse strains. IL-18 is a known link between innate and adaptive immunity because it induces IFN-γ production (Yang et al., 1999), leading us to explore the role of IFN-γ in infection with the *Y. pestis*-pYtbLpxL strain. Using knock out mice for the IFN-γ receptor, IFN-γ was shown to play a particular important role in survival a *Y. pesits*-pYtbLpxL infection. Targeted deletion of either NLRP12 or IL-18R resulted in a significant drop in IFN-γ concentrations in spleens as a result of *Y. pesits*-pYtbLpxL infection, almost to the level of uninfected samples. Together these observations point toward a central role of IFN-γ in resistance against the transgenic *Y. pestis* initiated through signalling via NLRP12 and IL-18R. In summary our work shows that NLRP12 has a role in resistance against *Y. pestis*. This supports the view that NLRP12 functions as a classical participant in an inflammasome leading to processing of IL-1β and IL-18. NLRP12 activation also, leads to the secretion of IFN-γ and consequently a likely initiation of an adaptive immune response. We propose the existence of redundant inflammasomes, as NLRP12 phenotype in mice was not as dramatic as seen for IL-18R, and we note that a cooperation between NLRP3 and NLRC4 has been proposed for optimal resistance to Salmonella infection (Broz et al., 2010). The actual factor for activating NLRP12 is still unknown, but to achieve full release of IFN-γ, signalling via NLRP12 and IL-18R seem important. Bacterial growth in tissues of NLRP12 knock out animals was not controlled, whereas some sign of inflammation such as recruitment of inflammatory cells to infected livers was detected. Thus, NLRP12 may not be the main initiator of phagocyte attraction, but rather induce other antibacterial defenses.

4.6 The role of IFN-1 in infections by Gram negative pathogens and possible links between the activation of TLR4 and NLRP12

A major part of this thesis concerns IFN-1, from the production of IFN-β after intracellular TLR4 activation in paper I to a study of how a model infection of a Gram

negative pathogen in an *in vivo* system is influenced by IFN-1 in paper II. It is therefore only natural to ask if IFN-1 induced responses are of major significance in the defense against infections by *Y. pestis* and other Gram negative bacteria. As earlier described the role of IFN-1 in bacterial infections is somewhat unclear, as IFN-1 may cause adverse effects in some bacterial infections. A possible explanation for why IFN-1 sometimes gives undesirable effects may be that its beneficial role is best suited for less severe infections and that the IFN-1 effects become increasingly detrimental as the infection becomes more severe (Kelly-Scumpia et al., 2010). Reasons why IFN-1 is of importance in defence against bacterial infections are demonstrated by IFN-1 induced inhibition of *in vivo* bacterial growth, production of proinflammatory cytokines and activation of NK or DC cells (Ishihara et al., 2005, Buss et al., 2010, Mancuso et al., 2007, Decker et al., 2005). The capacity of bacteria to induce IFN-1 makes it improbable that IFN-1 mainly confers a disadvantage to the host in the defence against bacterial infections. Our experiments showed an LPS dependency in the production of IFN-β, indicating IFN-β production as a mean for the host to direct its efforts towards responses suitable for fighting the intruder. However, because several Gram positive bacteria also induce IFN-β production with both beneficial and detrimental effects on the host (Auerbuch et al., 2004, O'Connell et al., 2004, Mancuso et al., 2007), the IFN-β secretion alone is not specific for Gram negative bacteria. The balance between proinflammatory and IFN-1 responses may communicate to the host whether the intruder is of bacterial or viral origin (Dietrich et al., 2010), pointing towards a necessity of looking at the whole picture of the complexity of cytokines and interferons when the host mounts its most specific immune response. Using IFN-1 receptor knock out mice, only a small contribution towards the survival in *Y. pestis KIM1001-pEcLpxL* infections (80% survival) was demonstrated compared to that of IFN-γ receptor knock out (10% survival). This indicates that type II IFN signalling axis is much more important for resistance against *Y. pestis* expressing hexa-acylated LPS than IFN-1 signalling. Since IFN-1 signalling is known to induce IFN-γ production (Freudenberg et al., 2002, Mancuso et al., 2007), and we show a central role for IFN-γ in *Y. pestis* clearance, some of the sensitivity of the IFN-1 receptor knock out could be explained by the subsequent decreased amounts of IFN-γ as a result of lack of IFN-1 signalling. However as the IFN-αβR x IFN-γR double knock out is more deadly than IFN-γ receptor knock out IFN-αβ dependent mechanisms must also exist.

A currently undetermined question is if the activation of TLR4 could be necessary for NLRP12 activation. LPS signaling is required for the formation of pyroptosome upon NLRP3 activation, but not AIM2 activation (Bauernfeind et al., 2009). As our results show, the expression of NLRP12 is induced upon *Y. pestis* infection. The fact that more NLRP12 is induced in the LpxL expressing clone may point towards a model where priming of cells as a result of TLR4 signaling is important, if perhaps not necessary, for NLRP12 activation. It is also tempting to wonder whether NLRP12 is likely to accumulate on phagosomes, as we have demonstrated for TLR4, Rab11a and other signaling proteins. The most plausible answer is not entirely obvious as neither the

activating mechanism, nor the activating agent is known for NLRP12. This NLR is, however, known to colocalize with ASC in punctuate structures necessary for NF-κB and caspase-1 activation when coexpressed (Wang et al., 2002), indicating the presence of an ASC containing NLRP12 inflammasome. In macrophages infected with *S. typhimurium*, ASC forms a single focus that does not overlap with phagosomes (Broz et al., 2010). This might indicate the formation of an inflammasome separate from any phagosomal structures. On the other hand ASC and the inflammasome receptor AIM2 form complexes with bacterial nucleic acids (Jones et al., 2010) and phagocytosis and lysosomal degradation of *S. aureus* is required for IL-1β secretion (Shimada et al., 2010). This opens for the possibility of inflammasome formation in connection with late phagosomal structures. Thus, further studies may find NLRP12 to be associated with phagosomes.

5 CONCLUSIONS

In this study it has been shown that Rab11a controls a previously unknown trafficking route of TLR4 from the ERC to phagosomes along actin filaments. A thorough examination of the consequences this has on down stream signalling events has revealed that Rab11a differentially controls expression of IFN-β. Furthermore, we argue that the loss of the acyl transferase LpxL was a hallmark in the evolution of *Y. pestis* towards becoming a highly virulent pathogen. We suggest that NLRP12 participates in the defence against *Y. pestis* with or without stimulatory LPS via activation of inflammasomes by a currently unknown mechanism. This study sheds light on the function of a NLR with a previously unclear function in the innate immunity. Collectively this work adds to the understanding of bacterial evasion and detection via one of the most studied innate immune receptors, TLR4.

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Paper I

Immunity
Article

The Rab11a GTPase Controls Toll-like Receptor 4-Induced Activation of Interferon Regulatory Factor-3 on Phagosomes

Harald Husebye,[1,9] Marie Hjelmseth Aune,[1,9] Jørgen Stenvik,[1,9] Eivind Samstad,[1] Frode Skjeldal,[3] Øyvind Halaas,[1] Nadra J. Nilsen,[1] Harald Stenmark,[1,4] Eicke Latz,[1,5] Egil Lien,[1,6] Tom Eirik Mollnes,[2] Oddmund Bakke,[3,7] and Terje Espevik[1,8,*]

[1]Norwegian University of Science and Technology, Department of Cancer Research and Molecular Medicine, N-7489 Trondheim, Norway
[2]Institute of Immunology, Rikshospitalet University Hospital, University of Oslo, N-0027 Oslo, Norway
[3]Department of Molecular Biosciences, Centre for Immune Regulation, University of Oslo, N-0316 Oslo, Norway
[4]Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway
[5]Institute of Innate Immunity, Biomedical Center, University of Bonn, Sigmund-Freud-Str. 25, 53127 Bonn, Germany
[6]Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01605, USA
[7]The Gade Institute, University of Bergen, 5021 Bergen, Norway
[8]St. Olavs Hospital, N-7489 Trondheim, Norway
[9]These authors contributed equally to this work
*Correspondence: terje.espevik@ntnu.no
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SUMMARY

Toll-like receptor 4 (TLR4) is indispensable for recognition of Gram-negative bacteria. We described a trafficking pathway for TLR4 from the endocytic recycling compartment (ERC) to *E. coli* phagosomes. We found a prominent colocalization between TLR4 and the small GTPase Rab11a in the ERC, and Rab11a was involved in the recruitment of TLR4 to phagosomes in a process requiring TLR4 signaling. Also, Toll-receptor-associated molecule (TRAM) and interferon regulatory factor-3 (IRF3) localized to *E. coli* phagosomes and internalization of *E. coli* was required for a robust interferon-β induction. Suppression of Rab11a reduced TLR4 in the ERC and on phagosomes leading to inhibition of the IRF3 signaling pathway induced by *E. coli*, whereas activation of the transcription factor NF-κB was unaffected. Moreover, Rab11a silencing reduced the amount of TRAM on phagosomes. Thus, Rab11a is an important regulator of TLR4 and TRAM transport to *E. coli* phagosomes thereby controlling IRF3 activation from this compartment.

INTRODUCTION

Toll-like receptor 4 (TLR4) has an essential role in host defense against Gram-negative bacteria (Montminy et al., 2006; Poltorak et al., 1998). The signaling receptor for lipopolysaccharide (LPS) is TLR4 in complex with myeloid differentiation factor 2 (MD-2), which receives LPS from CD14 (Gioannini et al., 2004). The TLR4 signaling cascade is mediated through the Toll and Interleukin-1 receptor (TIR)-adaptors myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP, also known as MyD88 adaptor-like protein), Toll-receptor-associated activator of interferon (TRIF), Toll-receptor-associated molecule (TRAM), and Sterile α- and armadillo-motif containing protein (SARM) (O'Neill and Bowie, 2007). TLR4 signaling proceeds through a MyD88-dependent and MyD88-independent pathway. The MyD88-dependent pathway rapidly activates NF-κB and mainly takes place at the plasma membrane (Kagan and Medzhitov, 2006; Latz et al., 2002), whereas the MyD88-indenpendent pathway activates interferon regulatory factor-3 (IRF3) and occurs at early endosomes (Halaas et al., 2007; Kagan et al., 2008). Systemic infections with Gram-negative bacteria may lead to septic shock, multiorgan failure, and death (Waage et al., 1989). Therefore, fine-tuning of the TLR4 response is of essential importance in regulating inflammatory reactions against Gram-negative bacteria.

Macrophages and neutrophils eliminate invading pathogens and foreign particles by first ingesting them into a plasma membrane-derived intracellular vacuole termed the phagosome. The formation of the phagosome and phagocytosis per se is a receptor-mediated and actin-dependent process. The resulting phagosome undergoes a series of fusion and fission events through a sequence that resembles the endocytic pathway, referred to as phagosomal maturation (Flannagan et al., 2009). Although soluble LPS is a primary inducer of host responses against Gram-negative bacteria, LPS on the bacterial surface contributes to both phagocytosis and TLR4 signaling. This is demonstrated by recent data showing that MD-2 acts as an opsonin for Gram-negative bacteria (Jain et al., 2008; Tissières et al., 2008). Having MD-2 on the surface of Gram-negative bacteria is likely to result in binding to and aggregation of TLR4 on the envelope that forms during phagocytosis. A consequence of TLR4 aggregation is potent signaling events that can be demonstrated as high tumor necrosis factor (TNF) production in human PBMC (Latz et al., 2002).

Rab proteins are small guanosine triphosphatases (GTPases) that have key roles in membrane transport and fusion (Jordens et al., 2005). Rab4 is a main regulator of exocytosis and rapid

recycling of membrane receptors whereas Rab5 and Rab7 control early endocytic transport and late endocytic membrane traffic, respectively. Thus, both Rab5 and Rab7 have essential functions in phagosome maturation (Kinchen and Ravichandran, 2008). The Rab11 subfamily consists of three isoforms—Rab11a, Rab11b, and Rab25. Rab11a and Rab11b are ubiquitously expressed, whereas Rab25 expression is restricted to epithelial cells (Goldenring et al., 1993; Lapierre et al., 2003). Rab11 proteins regulate the "slow" recycling of the transferrin receptor to the plasma membrane and Rab11 is highly concentrated in the perinuclear endocytic recycling compartment (ERC) (Ullrich et al., 1996). Like all GTPases of the Ras family, Rab11 functions as a molecular switch. Rabs associate with membranes in the guanosine diphosphate (GDP)-bound form and switch to a GTP-bound conformation in a reaction catalyzed by a GEF (guanine-nucleotide-exchange factor). In the active GTP-bound state, they interact with downstream effector proteins that coordinate vesicle formation, vesicle and organelle motility, and the tethering of vesicles to their target compartment (Zerial and McBride, 2001). Rab11 binds to Rab11 family interacting protein 2 (FIP2) that recruits the actin motor MyoVb that mediates transports of cargo along actin filaments (Jordens et al., 2005; Wei et al., 2009). Of interest, Rab11 has been reported to be associated with phagosomes (Cox et al., 2000) and to be involved in the intracellular transport of pro-TNF from the Golgi complex to the phagocytic cup (Murray et al., 2005). TLR2 is recruited to macrophage phagosomes containing zymosan particles and *S. aureus* (Ip et al., 2008; Nilsen et al., 2004) as well as to immunoglobulin-G (IgG)-coated erythrocytes (Ozinsky et al., 2000). Also, TLR4 accumulates at the phagocytic cup that develops around *E. coli* in HEK293 cells overexpressing TLR4, CD14, and MD-2 (Espevik et al., 2003). However, no information exists on possible functions of Rab11 in cytokine responses and whether Rab11 controls trafficking of TLR4 to phagosomes containing Gram-negative bacteria.

We found that TLR4 strongly colocalized with Rab11a, in particular in the ERC. Our data demonstrate that Rab11a controls trafficking of TLR4 in and out of ERC and that Rab11a is involved in transport of TLR4 to the *E. coli* phagosome. Furthermore, we have documented that phagocytosis of *E. coli* is required for interferon-β (IFN-β) production and that Rab11a is a specific regulator of TLR4-induced IRF3 activation and IFN-β production.

RESULTS

Perinuclear TLR4 Is Located in the Rab11a$^+$ ERC

Earlier reports suggest that the large perinuclear pool of TLR4 is localized to the Golgi apparatus (Hornef et al., 2002; Latz et al., 2002; Uronen-Hansson et al., 2004). However, because the Rab11-positive perinuclear endocytic recycling compartment (ERC) is in close vicinity to the Golgi apparatus (Jones et al., 2006; Saraste and Goud, 2007), we wanted to carefully analyze in 3D the spatial localization of TLR4 in relation to markers for Golgi and ERC. HEK293 cells stably expressing TLR4 fused to yellow fluorescent protein (TLR4^{YFP}) were transfected with a trans-medial Golgi marker β-1,4-galactosyl transferase fused to cyan fluorescent protein (β-1,4-GT^{CFP}) and subjected to optical sectioning. As can be seen in Figure 1A, there is only a partial overlap between TLR4 and β-1,4-GT^{CFP} that identifies the trans-medial Golgi as a ring structure (Misaki et al., 2007). The highest TLR4 intensity was found inside the center of the Golgi ring, which is clearly visualized in both planar and orthographic projections (Figure 1A). We next examined whether the high amounts of TLR4 expression inside the Golgi ring represented the Rab11a-positive ERC. HEK293-TLR4^{YFP} cells were labeled with Rab11a antibodies and examined by confocal microscopy. The results demonstrate that there is a strong colocalization of TLR4 and endogenous Rab11a (Figure 1B) suggesting that TLR4 accumulates in the ERC. Similar data were also obtained when Rab11a^{CFP} was expressed in the HEK293-TLR4^{YFP} cells (Figure 1C; Figure S1A available online). Transfection of a dominant-negative GDP-bound version of Rab11a (Rab11aSN^{GFP}) into HEK293-TLR4^{Cherry} caused a redistribution of TLR4 from ERC to endosomes close to the plasma membrane, whereas the constitutively active GTP variant of Rab11a (Rab11aQL^{GFP}) sequestered TLR4 in numerous vesicles that were positive for Rab11aQL^{GFP} (Figures 1D and 1E). Thus, Rab11a has an essential role in localization of TLR4 to the ERC. In addition to the marked accumulation in ERC, Rab11a was also expressed on tubular structures throughout the cell and on the limiting membrane of enlarged endosomes where it frequently colocalized with TLR4 (Figure S1A). Three-dimensional reconstruction of optical sections revealed that TLR4 and Rab11a were colocalized inside the *cis*-Golgi ring in HEK293-TLR4^{YFP} cells (Figure 1C; Figure S1B). Because TLR4^{YFP} is overexpressed in HEK293 cells, we wanted to confirm the above data in human monocytes. Relatively low amounts of TLR4 are found on the plasma membrane of human monocytes, whereas considerable amounts of TLR4 are detected in intracellular compartments (Latz et al., 2002). By using antibodies against a trans-medial Golgi marker and TLR4, we found that TLR4 was concentrated inside the Golgi ring also in monocytes (Figure 2A). Furthermore, TLR4 and Rab11a displayed a marked colocalization in human monocytes, particularly in the perinuclear ERC (Figure 2B). Rab11 has been reported to interact with G protein-coupled receptors (Parent et al., 2009). Therefore, we examined whether TLR4 and Rab11a could physically interact. Coimmunoprecipitation experiments were carried out in HEK293 cells stably expressing HA-tagged TLR4 and cotransfected with Flag-tagged Rab11a; however, no association between TLR4 and Rab11a was observed (Figure S2). These data demonstrate that TLR4 accumulates in the Ra11a$^+$ ERC that is located inside the Golgi ring.

TLR4 and Rab11a Accumulate around Phagocytosed *E. coli* but Not around *S. aureus*

Because TLR4 and Rab11a showed prominent colocalization, we wanted to investigate the role for Rab11a in trafficking of TLR4 to phagosomes containing *E. coli*. Human monocytes were incubated with pHrodo (a pH-sensitive, rhodamine-based dye)-labeled *E. coli* or *S. aureus* bioparticles before they were fixed and stained for TLR4. A strong accumulation of TLR4 around *E. coli* phagosomes was observed (Figure 3A). In early phases of phagosome formation, TLR4 seemed to be recruited to the phagocytic cup from intracellular vesicles whereas later in the phagocytosis process, TLR4 was found around the *E. coli* particle (Figures S3A and S3B). Recruitment of TLR4 to

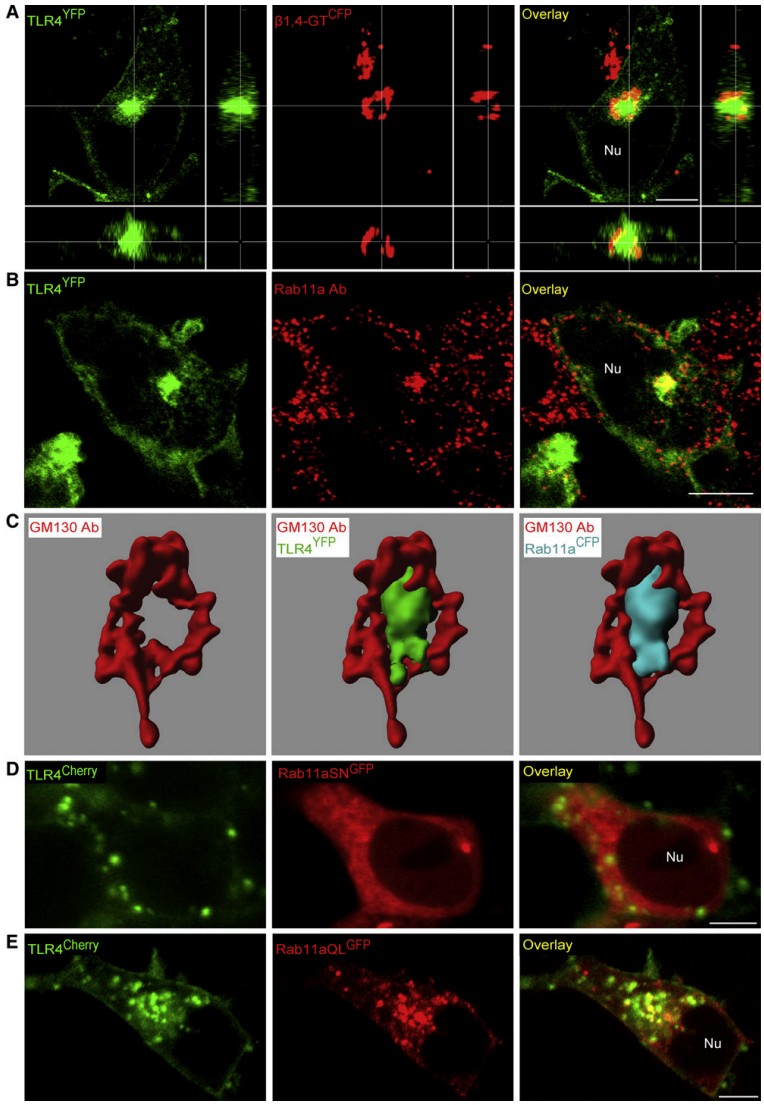

Figure 1. The Rab11a-Positive ERC Contains High Amounts of TLR4 in HEK293 TLR4^{YFP} Cells

Orthographic and planar projection data were obtained via confocal microscopy on fixed cells. Overlapping regions appear as yellow in the overlay panels.
(A) Orthographic projections of HEK293-TLR4^{YFP} cells coexpressing the trans-medial Golgi marker β-1,4 galactosyl transferase linked to CFP (β-1,4 GT^{CFP}).
(B) Planar sections of HEK293-TLR4^{YFP} cells stained for endogenous Rab11a via the Rab11a antibody (ab3612).
(C) 3D modeling of the cis-Golgi (GM130), TLR4^{YFP}, and Rab11a^{CFP} in HEK293-TLR4^{YFP} cells cotransfected with Rab11a^{CFP}, MD-2, and CD14.
(D and E) HEK-TLR4^{Cherry} cells cotransfected with dominant-negative GDP-bound Rab11aSN^{GFP} (D) or constitutive active GTP-bound Rab11aQL^{GFP} (E) together with MD-2 and CD14.
Scale bars represent 10 μm. Data are representative of four independent experiments. See also Figure S1.

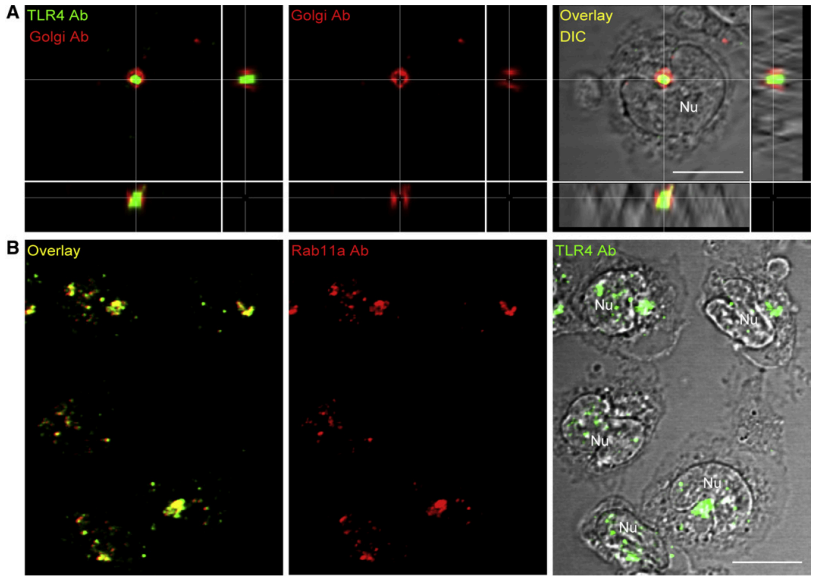

Figure 2. The Rab11a-Positive ERC Contains High Amounts of TLR4 in Human Monocytes

Orthographic and planar projection data were obtained with confocal microscopy on fixed cells. Overlapping regions appear as yellow in the overlay panels.

(A) Orthographic projections of a human monocyte costained for TLR4 and trans-medial Golgi (Golgin-97).

(B) Human monocytes costained for TLR4 and Rab11a.

Scale bars represent 10 μm. Data are representative of three independent experiments. See also Figure S2.

E. coli phagosomes was also apparent when live *E. coli*^{YFP} was used (data not shown). No accumulation of TLR4 was observed around *S. aureus* phagosomes (Figure 3B), suggesting that this phenomenon is specific for Gram-negative bacteria. We next investigated whether Rab11a was recruited to phagosomes. As seen for TLR4, Rab11a showed a clear accumulation around *E. coli*- but not *S. aureus*-containing phagosomes in human monocytes (Figures 3C and 3D).

Phagocytosis of *E. coli* Reduces TLR4 and Rab11a in the ERC

The effect of a challenge with *E. coli* on the TLR4 pool in ERC was investigated. In order to obtain a nonbiased and accurate measurement of TLR4 intensities, optical sections of monocytes were obtained and the amounts of TLR4 in the ERC were estimated by measuring the sum of voxel intensity of each ERC via 3D image analysis. The median TLR4 voxel intensity in the ERC was estimated in monocytes with and without added bacteria for 15 min (Figure 4A). A significant reduction of TLR4 in the ERC was observed when *E. coli* was added. Also, the voxel intensity of Rab11a in the ERC was reduced after addition of *E. coli* for 15 min (Figure 4B). These data suggest that addition of *E. coli* is emptying the ERC for both TLR4 and Rab11a.

TLR4 and Rab11a on Phagosomes Are Affected by *E. coli* Incubation Time

Next, we monitored the amount of TLR4 and Rab11a on phagosomes at different maturation states. In a time kinetic study, monocytes were exposed to *E. coli* for 15 min, for 30 min, and for 30 min followed by an additional 30 min in medium ("pulse-chase"). The median TLR4 voxel intensity on *E. coli* phagosomes was measured by 3D image analysis for each of the three time points (Figure 4C). This revealed that the amount of TLR4 on phagosomes increased as a function of *E. coli* incubation time. Worth noting is that the 30+30 min pulse-chase resulted in significant increase of TLR4 on phagosomes compared to the 30 min exposure (Figure 4C). This result suggests that TLR4 is recruited to the maturing phagosome by mechanisms that involve trafficking of TLR4 mainly from intracellular compartments. We also wanted to compare the kinetics of TLR4 and Rab11a accumulation on *E. coli* phagosomes. In contrast to TLR4, the amount of Rab11a was highest around *E. coli* after 15 min followed by a gradual decrease on maturing phagosomes (Figure 4D). The dynamics of Rab11a recruitment to *E. coli* was further studied by confocal live cell imaging of immortalized murine macrophages stably expressing Rab11a^{GFP} (Figures S4A–S4C and Movie S1). Time series images of an *E. coli* entering a macrophage revealed that Rab11a^{GFP} rapidly trafficked by small vesicles and tubules that seemed to fuse with the developing phagosome (Figure S4A). After 35 min of *E. coli* incubation, a considerable amount of Rab11a^{GFP} was observed on the phagosome and processes resembling both fusion and fission events of Rab11a^{GFP}-positive vesicles on phagosomes were observed (Figures S4B and S4C). The observed reduction in the Rab11a on maturing phagosomes (Figure 4D) may thus be

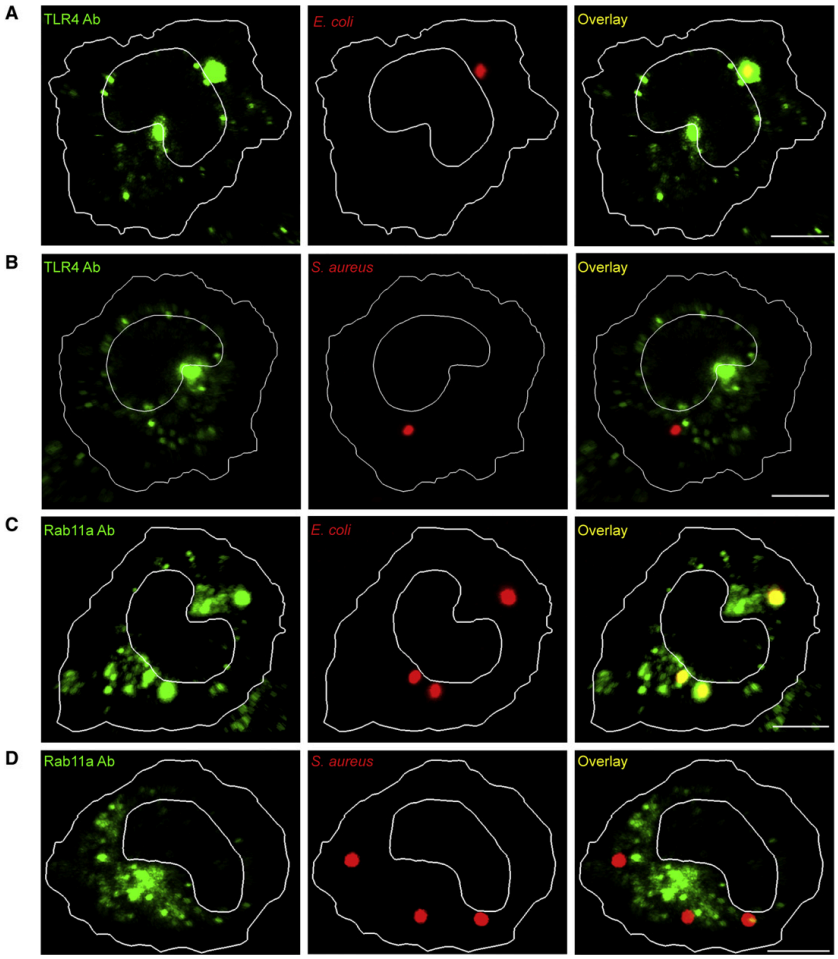

Figure 3. TLR4 and Rab11a Accumulate around Phagocytosed *E. coli*, but Not around *S. aureus*

Human monocytes were incubated with *E. coli* or *S. aureus* particles (5.0×10^6/ml) for 15 min and fixed and stained for TLR4 or Rab11a. Overlapping regions appear as yellow in the overlay panels.

(A) TLR4 accumulates toward the *E. coli* phagosome. TLR4 (green), *E. coli* (red), and overlay (right).

(B) TLR4 does not accumulate toward the *S. aureus* phagosome. TLR4 (green), *S. aureus* (red), and overlay (right).

(C) Rab11a accumulates toward the *E. coli* phagosome. Rab11a (green), *E. coli* (red), and overlay (right).

(D) Rab11a does not accumulate toward the *S. aureus* phagosome. Rab11a (green), *S. aureus* (red), and overlay (right).

Scale bars represent 10 μm. Data are representative of three independent experiments. See also Figure S3.

due to a time-dependent increase in the fission-fusion ratio of Rab11a vesicles associated with *E. coli* phagosomes.

Accumulation of TLR4 around Phagosomes Requires TLR4 Signaling and Results in Strong IFN-β Induction

We next wanted to see whether Gram-negative bacteria other than *E. coli* induced mobilization of TLR4 to the phagosomes, and whether this phenomenon required an LPS type that activates TLR4. *Y. pestis* grown at 37°C produces a tetraacylated LPS that poorly stimulates TLR4 whereas *Y. pestis* grown at 26°C produces a hexaacylated LPS with high TLR4-stimulating properties (Montminy et al., 2006). The two *Y. pestis* variants were labeled with CFP, heat-killed bacteria were added to monocytes, and the median voxel intensity of TLR4 around *Y. pestis* phagosomes was quantified with 3D image analysis. As can be seen from Figure 5A, *Y. pestis* grown at 37°C was

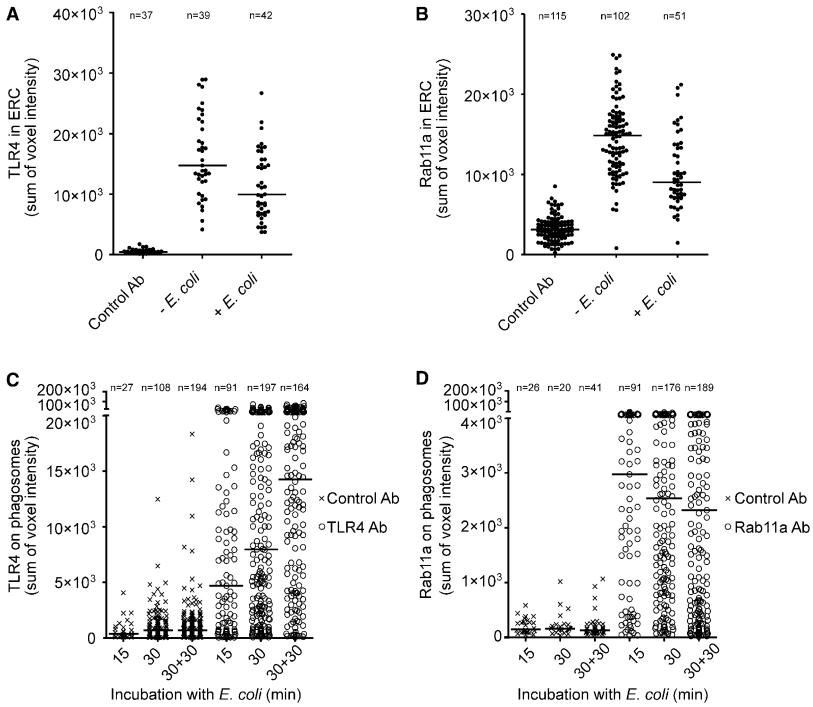

Figure 4. Addition of *E. coli* to Human Monocytes Results in Intracellular Redistribution of TLR4 and Rab11a

Human monocytes were incubated with *E. coli* particels (2×10^6/ml), fixed, and immunostained for TLR4 or Rab11a.

(A) TLR4 voxel intensity in the ERC in cells incubated with or without *E. coli* for 15 min was measured ($p = 0.001$).

(B) Rab11a voxel intensity in the ERC in cells incubated with or without *E. coli* for 15 min ($p < 0.0001$).

(C) TLR4 voxel intensity on *E. coli* phagosomes as a function of incubation time ($p < 0.0001$ for TLR4 from 15 min to 30+30 min).

(D) Rab11a voxel intensity on *E. coli* phagosomes as a function of incubation time ($p = 0.046$ for Rab11a from 15 min to 30+30 min).

Bars in plots represent the median. n = number of observations. Data are representative of three independent experiments. See also Figure S4 and Movie S1.

a poor inducer of TLR4 recruitment to the phagosomes, whereas TLR4 was strongly recruited to phagososmes with *Y. pestis* grown at 26°C. This result suggests that phagosomal TLR4 accumulation requires Gram-negative bacteria that produce a TLR4-stimulating LPS.

A likely consequence of TLR4 accumulation at the phagosomes is that TLR4 signaling occurs from this compartment. In order to initiate TLR4 signaling from the phagosomes, adaptor molecules must also be recruited. TRAM interacts with TRIF and is required for IRF3 activation. MyD88 is the universal adaptor for TLRs that activates NF-κB, c-Jun kinase, and p38 mitogen-activated protein kinase (O'Neill and Bowie, 2007). Therefore, we wanted to examine whether the adaptor molecules TRAM and MyD88 were recruited to phagosomes containing *E. coli*. We found that both TRAM and MyD88 strongly accumulated around phagosomes (Figure 5B; Figures S5A, S5B, and S5E). In line with the TRAM observation, we also detected prominent recruitment of IRF3 to *E. coli* phagosomes (Figure 5C; Figure S5C). These data suggest that *E. coli* initiates powerful signaling from this compartment.

Having described that TLR4 accumulation on phagosomes is associated with TRAM, MyD88, and IRF3 recruitments, we next wanted to address the involvement of actin filaments, Golgi, and dynamin GTPase in the recruitment of TLR4 to phagosomes. Inhibition of actin polymerization with cytochalasin D (cyto D) reduced the number of internalized bacteria by approximately 65% (Figure S6C) as well as the recruitment of TLR4 to *E. coli* phagosomes (Figure 5D). In contrast, brefeldin A, which inhibits ADP-ribosylation factor 1 (Arf 1) leading to retraction of Golgi membranes back into the ER, did not influence the amount of TLR4 associated with *E. coli* phagosomes (Figure 5E). It has previously been reported that TLR4 is internalized from the plasma membrane by a dynamin-dependent process (Husebye et al., 2006; Kagan et al., 2008). Pulse chase experiments with *E. coli* showed that TLR4 was almost doubled during the 30+30 min chase experiment (Figure 4C), so we added the dynamin inhibitor Dynasore (Kagan et al., 2008) during the last 30 min of the pulse-chase to see whether this treatment inhibited TLR4 recruitment to phagosomes. Quantitative analyses of phagosomal TLR4 revealed no inhibitory effect of Dynasore. In fact,

Dynasore treatment weakly increased the TLR4 amounts (Figure 5F). Dynasore treatment inhibited the LPS-induced IFN-β mRNA by approximately 70%, whereas the LPS-induced TNF response was inhibited by only 30% (Figure S5F). The *E. coli*-induced IFN-β and TNF responses were only weakly inhibited (15%–18%) when Dynasore was added with the last 30 min chase (Figure S5F). These results suggest that dynamin is not involved in TLR4 transport to phagosomes and that the plasma membrane is not the main source of phagosomal TLR4.

Because of the pronounced accumulation of TLR4, TRAM, and IRF3 to *E. coli* phagosomes, we wanted to address the involvement of phagocytosis in cytokine production. In the first set of experiments, we compared the potency of *E. coli* and LPS from *E. coli* in inducing IFN-β and TNF mRNA. We found that the maximum IFN-β response induced by *E. coli* was around 700% higher compared to the maximum IFN-β response induced by LPS. In contrast, the TNF mRNA was only around 60% higher for *E. coli* compared to LPS (Figures 6A and 6B). The robust IFN-β response obtained with *E. coli* seemed to be specific for Gram-negative bacteria as shown by the fact that *S. aureus* did not induce detectable IFN-β under the same experimental conditions (data not shown). Furthermore, the potent IFN-β induction required LPS in the bacteria because a *N. meningitidis* mutant without LPS induced minimal IFN-β production compared to the wild-type *N. meningitides* (Figure S6A). Thus, *E. coli* is far more potent in inducing IFN-β than LPS, whereas a considerable lesser difference between *E. coli* and LPS is seen for the TNF response. Because opsonization of bacteria increases phagocytosis (Johnston et al., 1969), we used serum-opsonized and nonopsonized *E. coli* and compared the induction of IFN-β and TNF. We observed that opsonization of *E. coli* increased the IFN-β production by 300%, whereas opsonization increased the TNF response by only 20%. The fact that opsonization clearly increases the number of internalized *E. coli* in monocytes was verified by flow cytometry (data not shown). Another way to inhibit phagocytosis is to use cyto D. We found that 2 μM cyto D inhibited *E. coli*-induced IFN-β by 53% whereas the TNF response was reduced by only 17%. According to the dose response curve in Figure 6A, a 10-fold (90%) reduction in *E. coli* concentration (from 10^7/ml to 10^6/ml) corresponded to a 50%–60% reduction in the IFN-β mRNA. If we assume a linear relationship between added *E. coli* to monocytes and internalized *E. coli*, a 90% reduction in phagocytosed *E. coli* would result in a 50%–60% inhibition of IFN-β mRNA. Because cyto D treatment inhibited the IFN-β response and *E. coli* uptake by 53% and 65%, respectively, it is likely that the reduction in IFN-β mRNA after cyto D treatment is an effect mainly resulting from reduced uptake, but also from a contribution from failure to deliver TLR4 to the phagosome membrane. Addition of cyto D had minimal effect on LPS-induced IFN-β and TNF (Figures 6C and 6D). Together these data clearly demonstrate a selective and important role of *E. coli* phagocytosis in the IFN-β response.

Suppression of Rab11a Results in Emptying of TLR4 from the ERC and Reduction of TLR4 around Phagosomes

To investigate the role of Rab11a in trafficking of TLR4 from the ERC to phagosomes, we performed experiments where we specifically silenced Rab11a. Consequently, human monocytes and HEK293-TLR4^{YFP} cells were treated with Rab11a siRNA or nonsilencing control RNA (NS RNA). The silencing was verified by immunoblotting. The Rab11a amount was reduced by approximately 90% in HEK293-TLR4^{YFP} cells and 60% in monocytes when quantified with a Kodak Imager (Figure 6E). Suppression of Rab11a minimally affected the Rab7a in monocytes and HEK293-TLR4^{YFP} cells (Figure 6E). Also, Rab11a siRNA treatment reduced the mRNA for Rab11a by approximately 60% in monocytes, whereas the Rab11b mRNA was unaffected (Figure S6C). After establishing the conditions for proper Rab11a silencing, we examined whether the intensity of TLR4 in ERC was affected by this treatment. We observed a significant reduction in TLR4 voxel intensity in the ERC in monocytes when the Rab11a was reduced (Figure S6E). This observation was also verified in HEK293-TLR4^{YFP} cells where the intense accumulation of TLR4 in the ERC disappeared completely in cells treated with Rab11a siRNA (Figure 6F). Additional experiments via flow cytometry showed that Rab11a suppression only minimally affected the total amount of TLR4 in HEK293-TLR4^{YFP} cells (data not shown). Furthermore, immunoblotting of TLR4 in monocytes did not reveal a reduction in total TLR4 amounts in Rab11a-silenced cells (data not shown). We did not observe a reduction in the number of phagocytosed bacteria in monocytes treated with Rab11a siRNA (Figure S6D). The amount of phagocytosed bacteria in Rab11a-silenced monocytes was quantified both by manual counting from the 3D data obtained from the confocal microscope and by flow cytometry (data not shown).

We next addressed the role of Rab11a in trafficking of TLR4 to phagosomes. The amount of TLR4 around *E. coli* was determined in monocytes treated with Rab11a siRNA and control oligo. The result from this experiment revealed an approximately 50% reduction in TLR4 voxel intensity around *E. coli* in Rab11a-silenced cells compared to the control siRNA-treated cells (Figure 6G). The reduced amount of TLR4 on phagosomes was observed at both 15 min and 30 min after addition of *E. coli*. In addition, we observed that recruitment of TRAM to *E. coli* phagosomes was reduced by approximately 50% in monocytes treated with Rab11a siRNA (Figure 6H). In parallel experiments we did not observe reduction in recruitment of MyD88 to *E. coli* phagosomes in monocytes treated with Rab11a siRNA (Figure S6G). Taken together, our results show that Rab11a controls trafficking of both TLR4 and TRAM to the *E. coli* phagosome most probably by mechanisms affecting the transport of TLR4 and TRAM vesicles in and out of the ERC.

Suppression of Rab11a Selectively Inhibits *E. coli*-Induced IRF3 Activation and IFN-β Production

TLR4 activates MyD88- and TRAM-TRIF-dependent signaling pathways from the plasma membrane and the endosome, respectively. Therefore, we wanted to address whether Rab11a differentially affected these two pathways in monocytes and in HEK293-TLR4 cells. We first examined the effect of Rab11a siRNA treatment on IRF3 phosporylation in monocytes stimulated with *E. coli* and LPS. As can be seen from Figures 7A and 7B, Rab11a silencing resulted in an almost complete inhibition of IRF3 phosphorylation induced by both *E. coli* and LPS. We next explored whether activation of NF-κB was affected by Rab11a siRNA treatment. NF-κB activation was measured as

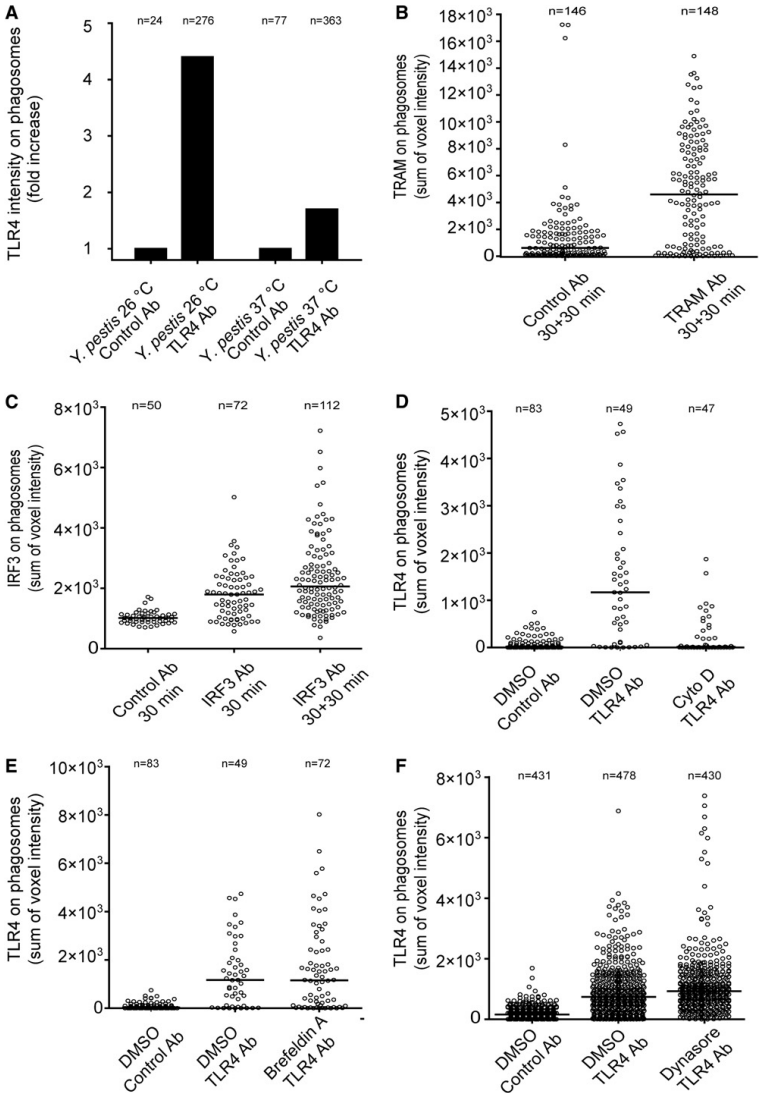

Figure 5. Recruitment of TLR4 to *E. coli* Phagosomes Requires TLR4 Signaling and Intact Actin Filaments, but Not Dynamin

Human monocytes incubated with bacterial particles, fixed, and immunostained for TLR4, TRAM, and MyD88; with normal rabbit IgG as control antibody.

(A) TLR4 recruitment to the phagosome requires TLR4 signaling. Fold increase in phagosomal TLR4 voxel intensity in cells immunostained for TLR4 versus cells stained with control antibody. Cells were incubated 30 min with heat-killed *Y. pestis* (2×10^6/ml) cultured at 26°C or 37°C.

(B) TRAM voxel intensity on *E. coli* phagosomes ($p < 0.0001$).

(C) IRF3 voxel intensity on *E. coli* phagosomes. An increase in IRF3 was observed from 30 min to 30+30 min ($p < 0.0028$).

(D) TLR4 recruitment to *E. coli* phagosomes requires intact actin filaments. Cells were pretreated with 2 μM cytochalasin D (cyto D) or vehicle (DMSO) for 30 min and incubated with *E. coli* for 30 min in the presence of inhibitor or vehicle ($p < 0.0001$).

(E) TLR4 recruitment to *E. coli* phagosomes occurs independent of Golgi. The cells were pretreated with 5 μg/ml Brefeldin A or vehicle (DMSO) and incubated 30 min with *E. coli* in the presence of inhibitor or vehicle.

degradation of IκB-α after addition of *E. coli.* Suppression of Rab11a did not affect IκB-α degradation either in monocytes (Figure 7C) or in HEK293-TLR4^{YFP} cells expressing MD-2 and CD14 (Figure 7D). This result suggests that NF-κB activation is not controlled by Rab11a. The role of Rab11a in IRF3 activation was confirmed via IRF3 and NF-κB reporter assays. The Gal4-IRF3 fusion protein and the endothelial adhesion molecule-luciferase (ELAM-luc) reporter were used to measure IRF3 and NF-κB activation, respectively, in HEK293-TLR4^{Cherry} cells expressing MD-2 and CD14. Rab11a knockdown gave a marked inhibition of *E. coli*- and LPS-induced signaling to IRF3, whereas NF-κB activation was unaffected (Figures 7E and 7F). Additional support for the selectivity of Rab11a in controlling *E. coli*-induced signaling to IRF3 was obtained by measuring nuclear translocation of IRF3 and NF-κB subunit p65 in monocytes. Suppressing the Rab11a led to a significant inhibition of IRF3 that translocated to the nucleus in response to *E. coli* (Figure 7G). In contrast, p65 translocation was not inhibited by the siRNA treatment (Figure 7H). In fact, Rab11a suppression resulted in a weak increase in p65 translocation after 30 min with *E. coli*. In line with these findings, treatment of monocytes with Rab11a siRNA led to a 65% inhibition of *E. coli*-induced IFN-β mRNA production at both 30 and 60 min of stimulation, whereas the TNF mRNA amounts were unaffected (Figures 7I and 7J). When *E. coli* and LPS responses were compared, it became apparent that Rab11a siRNA treatment selectively inhibited LPS-induced IFN-β production that was comparable to *E. coli* (Figure S7).

In summary, our results demonstrate that Rab11a has an essential function in localizing TLR4 to phagosomes and controlling IRF3 activation and IFN-β production in response to *E. coli* and also LPS.

DISCUSSION

Phagocytosis and concomitant induction of proinflammatory cytokines are two instrumental events of an innate immune response against bacteria. In this study we describe an unexpected route for TLR4 recruitment to the *E. coli* phagosome by recycling mechanisms involving Rab11a. This conclusion is based on our observations that TLR4 is strongly expressed in the Rab11a-positive ERC that is localized inside the Golgi ring. Furthermore, TLR4 and Rab11a were recruited to *E. coli* phagosomes and suppression of Rab11a significantly inhibited the amount of TLR4 and TRAM around the phagosomes as well as signaling to IRF3 and IFN-β induction. The rapid accumulation of TLR4 was not observed in response to Gram-positive bacteria *S. aureus* or against *Y. pestis* with a nonstimulating LPS, suggesting that recruitment of TLR4 to *E. coli* requires TLR4 signaling.

Phagocytosis of *E. coli* and recruitment of TLR4, TRAM, and IRF3 to the phagosome implicate a strong type I IFN response from the vacuole. Indeed, our data have demonstrated that phagocytosis of *E. coli* is required for a robust IFN-β response, whereas phagocytosis is minimally involved in the TNF response. Recently, Ip and coworkers (Ip et al., 2010) reported that phagocytosis is necessary for MyD88-dependent TNF production induced by Gram-positive bacteria although this is not the case for Gram-negative bacteria. They also showed that phagosome acidification was needed to initiate the TNF production induced by *S. aureus*. We found that lysosomal inhibitors like Bafilomycin A1 and NH_4Cl inhibited neither IRF3 phosporylation nor the IFN-β response induced by *E. coli* (data not shown), suggesting that LPS in the bacterium is already accessible for TLR4 sensing without the need for enzymatic processing in the phagosome. The IRF3 pathway can be activated by LPS in the absence of phagosomes; however, because *E. coli* is much more potent in inducing IFN-β compared to LPS, it is likely that internalization of the bacteria is the prevailing pathway for IFN-β production during *E. coli* infections.

Our data have demonstrated that Rab11a mediates trafficking of TLR4 from the ERC to *E. coli* phagosomes. We suggest that TLR4 recycling to phagosomes from the ERC may occur constitutively without the need for TLR4 signaling. This would be in accord with the fact that Rab11 regulates the constitutive recycling of transferrin receptors through the ERC (Ullrich et al., 1996). When TLR4 encounters a phagosome with *E. coli*, TLR4 is activated for signaling, which may lead to formation of stable receptor complexes and development of signaling platforms resulting in TLR4 accumulation. This statement is supported by our observations that TRAM, MyD88, and IRF3 are all present on *E. coli* phagosomes. Moreover, as the maturation proceeds, TLR4 may then be internalized into the lumen of intermediate and late phagosomes by mechanisms similar to the described sorting of the LPS receptor complex to late endosomes and lysosomes (Husebye et al., 2006; Kobayashi et al., 2006).

Kagan and coworkers have suggested that TLR4 is recruited to the endosome from the plasma membrane and that TRAM-TRIF displaces MyD88 on the endosome as the TLR4 signaling switches from a MyD88- to a TRAM-TRIF-dependent pathway. Our data have suggested a different model where Rab11a mediates recruitment of TLR4 from the ERC to the *E. coli* phagosome. Our model is supported by data showing that TLR4 is strongly expressed in the ERC compared to the plasma membrane, silencing of Rab11a results in a marked reduction of TLR4 in the ERC and also around *E. coli* vacuoles, and the amount of TLR4 on phagosomes increases during phagosomal maturation. Furthermore, inhibition of dynamin with Dynasore inhibits internalization of TLR4 (Kagan et al., 2008); however, Dynasore did not inhibit TLR4 recruitment to *E. coli* phagosomes. Thus, our data suggest that TLR4 is recruited from the ERC to phagosomes allowing for TRIF signaling to occur. This is further supported by our observations showing that Rab11a controls TRAM recruitment to phagosomes. Despite the evidence for our model that the ERC is the major source for phagosomal TLR4, it cannot be excluded that also a sequential MyD88- and

(F) Inhibition of dynamin does not cause a reduction in phagosomal TLR4. Monocytes were incubated for 2 hr under serum-free conditions and treated for 30 min with opsonized *E. coli*. Subsequently the cells were washed two times and incubated for 30 min with 80 μM Dynasore or vehicle (DMSO) in serum-free medium ($p = 0.0002$ for DMSO and Dynasore treatments).

Monocytes were incubated with *E. coli* particles ($3.0–8.0 \times 10^6$/ml) (B–F).

Bars in plots represent the median. n = number of observations. Data are representative of three independent experiments. See also Figure S5.

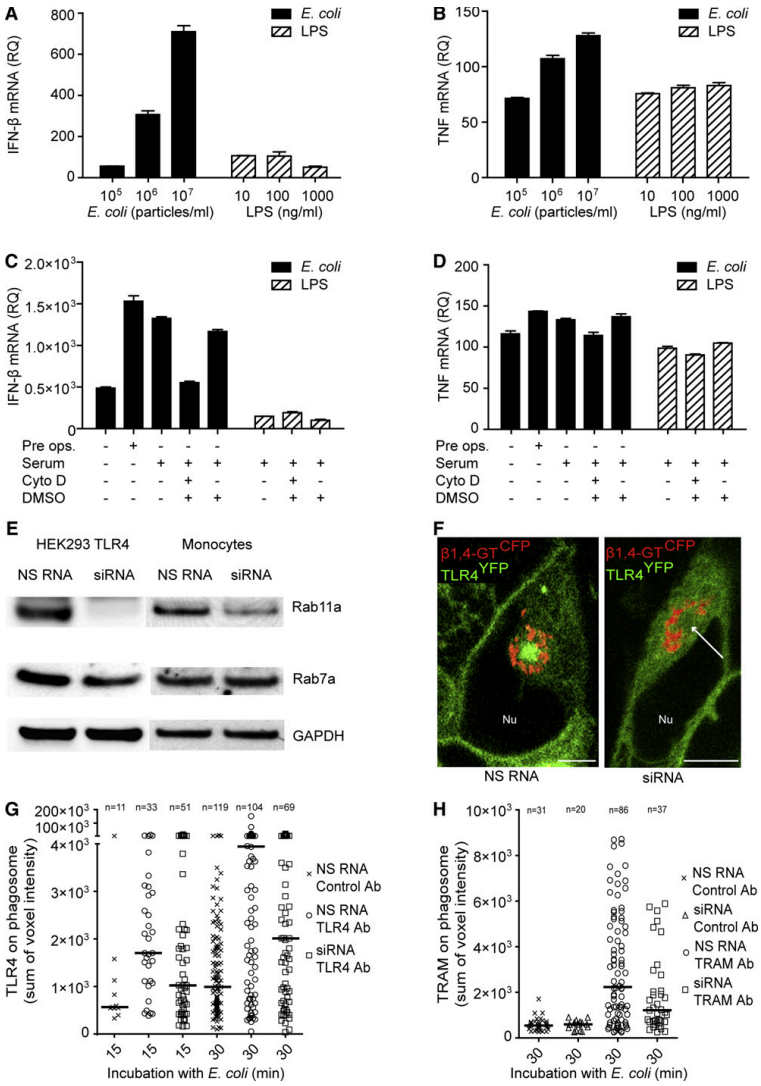

Figure 6. *E. coli*-Induced IFN-β Signaling Requires Phagocytosis, and TLR4 Recruitment to Phagosomes Is Rab11a Dependent

(A and B) Induction of IFN-β mRNA (A) and TNF mRNA (B) in monocytes stimulated for 60 min with different concentrations of *E. coli* particles or LPS. The amounts of IFN-β and TNF mRNAs were determined by QPCR and is presented as mean relative to nonstimulated monocytes and standard deviations.

(C and D) Induction of IFN-β (C) and TNF mRNAs (D) in monocytes stimulated with *E. coli* particles (8.0×10^6/ml) or LPS (100 ng/ml) for 60 min. The impact of phagocytosis was examined by incubation with or without human A+ serum, with or without preopsonization as well as the phagocytosis inhibitor Cyto D or vehicle (DMSO). The amounts of IFN-β and TNF mRNAs were determined by QPCR and are presented as mean relative to nonstimulated monocytes and standard deviations.

(E) HEK293-TLR4^{YFP} cells or human monocytes were treated with nonsilencing RNA oligo (NS RNA) or Rab11a siRNA, cellular lysates were made, and immunoblotting performed with Rab11a antibody (ab3612) or Rab7a antibody. GAPDH was used for control of equal loading.

(F) Confocal image of TLR4^{YFP} (green) in HEK293-TLR4^{YFP} cells coexpressing β-1,4 GT^{CFP} (red) treated with NS RNA (left) or Rab11a siRNA (right). The arrow indicates the center of the Golgi ring.

(G) TLR4 on *E. coli* phagosomes in monocytes treated with NS RNA or Rab11a siRNA and stimulated with *E. coli* particles (3×10^6/ml), $p = 0.05$ and $p = 0.03$ for TLR4 amounts in NS RNA compared to Rab11a siRNA-treated cells at 15 min and 30 min, respectively.

TRIF-dependent signaling pathway may operate as cell surface TLR4 is internalized during the very early stages of *E. coli* phagocytosis.

The fact that Rab11a is such an important regulator of TLR4 trafficking and IRF3 signaling from *E. coli* phagosome may also be expanded to include LPS-induced IRF3 signaling from endosomes. By comparing *E. coli* particles and LPS, we found that Rab11a silencing also selectively reduced LPS-induced TRAM-TRIF signaling, suggesting that Rab11a also can regulate transport of TLR4 to endosomes.

In summary, we have uncovered an unexpected transport pathway of TLR4 toward the internalized *E. coli* vacuole that involves ERC and Rab11a. This route of TLR4 transport results in the production of IFN-β that takes place from the *E. coli* phagosome. We have found that phagocytosis of Gram-negative bacteria is essential for type I IFN production, which may serve as an important link between the innate and adaptive immune systems in providing host defense against Gram-negative infections.

EXPERIMENTAL PROCEDURES

Reagents and Bacteria

E. coli and *S. aureus* pHrodo Bioparticles (Invitrogen) were used. LPS (0111:B4) from *E. coli* (Invivogen) was Cy5 labeled as previously described (Latz et al., 2002). Cytochalasin D and Brefeldin A1 were purchased from Sigma. DRAQ5 was from Biostatus Limited. Plasmids for bacterial expression of CFP was electroporated into *Y. pestis* KIM5 strains and cultured at 26°C or 37°C to gain bacteria expressing hexaacylated or tetraacylated LPS, respectively (Montminy et al., 2006). Alexa Fluor labeling of Golgin-97 was performed according to the manufacturer's protocol (Invitrogen). The following antibodies were used: mouse-anti-Golgin-97 from Invitrogen; mouse-anti-GAPDH (ab9484) and rabbit-anti-Rab11a (ab3612) from AbCam; rabbit-anti-phospho-IRF3 (Ser396), rabbit-anti-total IRF3, and rabbit-anti IκB-α from Cell Signaling; rabbit-anti-TLR4 (H-80), rabbit-anti-Rab7 (H-50), goat-anti-Rab11a (K-15), rabbit-anti-NF-κB p65 (A) antibody, rabbit-anti-IRF3 (FL-425), rabbit-anti-MyD88 (HFL-296), rabbit-anti-TRAM (TICAM-2) (H-85), normal rabbit IgG, and goat IgG from Santa Cruz Biotech. Alexa Fluor labeled secondary antibodies were from Invitrogen. The following mammalian expression vectors were used: pcDNA3.1 (Invitrogen), huCD14, and Elam-luc (Latz et al., 2002); and huMD-2 in pEF-BOS was kindly provided by K. Miyake (University of Tokyo). Rab11aS25N^{GFP} and Rab11aQ70L in pEGFP (Clontech) were kindly provided by M. Zerial (Max Planck Institute of Molecular Cell Biology and Genetics). pGEM-Rab11a was kindly provided by T.G. Iversen (The Norweigan Radium Hospital, University of Oslo). The Gal4-IRF3 reporter assay was kindly provided by K. Fitzgerald (UMass Med School, Worcester, MA) and T. Maniatis (Harvard University, Cambridge, MA). The ECFP-Rab11a vector was made by inserting the human Rab11a cDNA from pGEM-Rab11a into the pECFP$_{C1}$ vector (Clontech).

Cells

Human monocytes were isolated from buffycoat as previously described (Husebye et al., 2006). Use of human monocytes from blood donors was approved by the Regional Committees from Medical and Health Research Ethics at NTNU. The monocytes were maintained in RPMI1640 (GIBCO) supplemented with 10% or 25% of pooled human A$^+$ serum (The Blood Bank, St Olavs Hospital, Trondheim, Norway). 25% A$^+$ serum was used if the incubation time exceeded 24 hr. The HEK293 TLR4^{Cherry} cells were made as previously described (Latz et al., 2002). The HEK293 TLR4^{YFP} and HEK293 TLR4^{Cherry} cells were cultured in DMEM with 10% fetal calf serum (FCS) and 0.5 mg/ml G418 and transfected with GeneJuice transfection reagent (Novagen).

Luciferase Assays

NF-κB activation was determined by the ELAM-luc NF-κB luciferase reporter assay as described (Latz et al., 2002) and IRF3 activation determined with the Gal4-IRF3 assay as described (Fitzgerald et al., 2003). The Renilla luciferase pRL-TK vector (Promega) was cotransfected for normalization.

siRNA Treatment

Monocytes or HEK293 cells were seeded on 35 mm glass-bottom γ-irradiated tissue cell dishes (MatTek Corporation) or 6-well plates (NUNC) at 10 × 10^6 cells per dish/well containing medium free of antibiotics, before transfection with siRNA via Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's recommendations. The AllStars Negative Control (QIAGEN) was used as a nonsilencing control RNA and the Hs_RAB11A_5 HP Validated siRNA (QIAGEN) was used to target Rab11a. The cells were treated for 20–72 hr with 20 nM siRNA and, when stated, transfected with indicated plasmid DNA for 24 hr.

Gene Expression Analysis

Gene expression analysis was carried out via QPCR as described in Supplemental Experimental Procedures.

Immunoblotting

Immunoblotting was performed on HEK293 cells expressing TLR4^{YFP} and transfected with MD-2 and CD14 or monocytes as previously described (Husebye et al., 2006). The blots were developed with the SuperSignal West Femto (Thermo Scientific) and visualized with the Image Estimation 2000R (Kodak). For quantification the Kodak 1D Image Analysis software was used.

Confocal Laser Scanning Microscopy

Images of live cells were captured at 37°C with a Zeiss LSM 510 META scanning unit, a heating stage, and a 1.4 NA × 63 objective. For intracellular staining, the cells were fixed with 2% paraformaldehyde in PBS, put 15 min on ice, permeabilized with PEM buffer (80 mM K-Pipes [pH 6.8], 5 mM EGTA, 1 mM $MgCl_2$, 0.05% saponin) for 15 min on ice, quenched of free aldehyde groups in 50 mM NH_4Cl with 0.05% saponin for 5 min, and blocked in PBS with 20% human serum and 0.05% saponin (monocytes) or in PBS with 10% FCS and 0.05% saponin (HEK293 cells) for 20 min. The cells were incubated with primary antibody 2 μg/ml (polyclonal antibodies) or 10 μg/ml (monoclonal antibodies) in PBS with 1% human serum and 0.05% saponin (monocytes) overnight at 4°C or PBS with 0.05% saponin (HEK293 cells) for 60 min at room temperature. Alexa Fluor-labeled secondary antibodies (Invitrogen) were incubated 15 min at room temperature after three washes in PBS with 0.05% saponin.

Quantification of Relative Amounts of Fluorescently Labeled Proteins in Intracellular Compartments

3D data were captured with identical settings and avoiding saturation of voxels (3D pixels) intensities. The ImarisXT software (Bitplane) was used to surface render the imaged structures giving one surface for each ERC. The pHrodo or DRAQ5 fluorescence was used to spot or surface render the volume of individual phagosomes or nuclei, respectively. The software produced a numerical value of the relative amount of TLR4, Rab11a, IRF3, or p65 as a sum of voxel intensities from the original image in each compartment. The values for voxel intensities did not follow a Gaussian distribution, and therefore we used median as a measure of average intensities and the nonparametric Mann-Whitney test to evaluate statistical significance.

(H) TRAM on *E. coli* phagosomes in monocytes treated with NS RNA or Rab11a siRNA stimulated with *E. coli* particles (3 × 10^6/ml), p = 0.02 for TRAM amounts in NS RNA- compared to siRNA-treated cells.

Bars in plots represent the median. n = number observations. Data are representative of four independent experiments. Scale bars represent 5 μm. See also Figure S6.

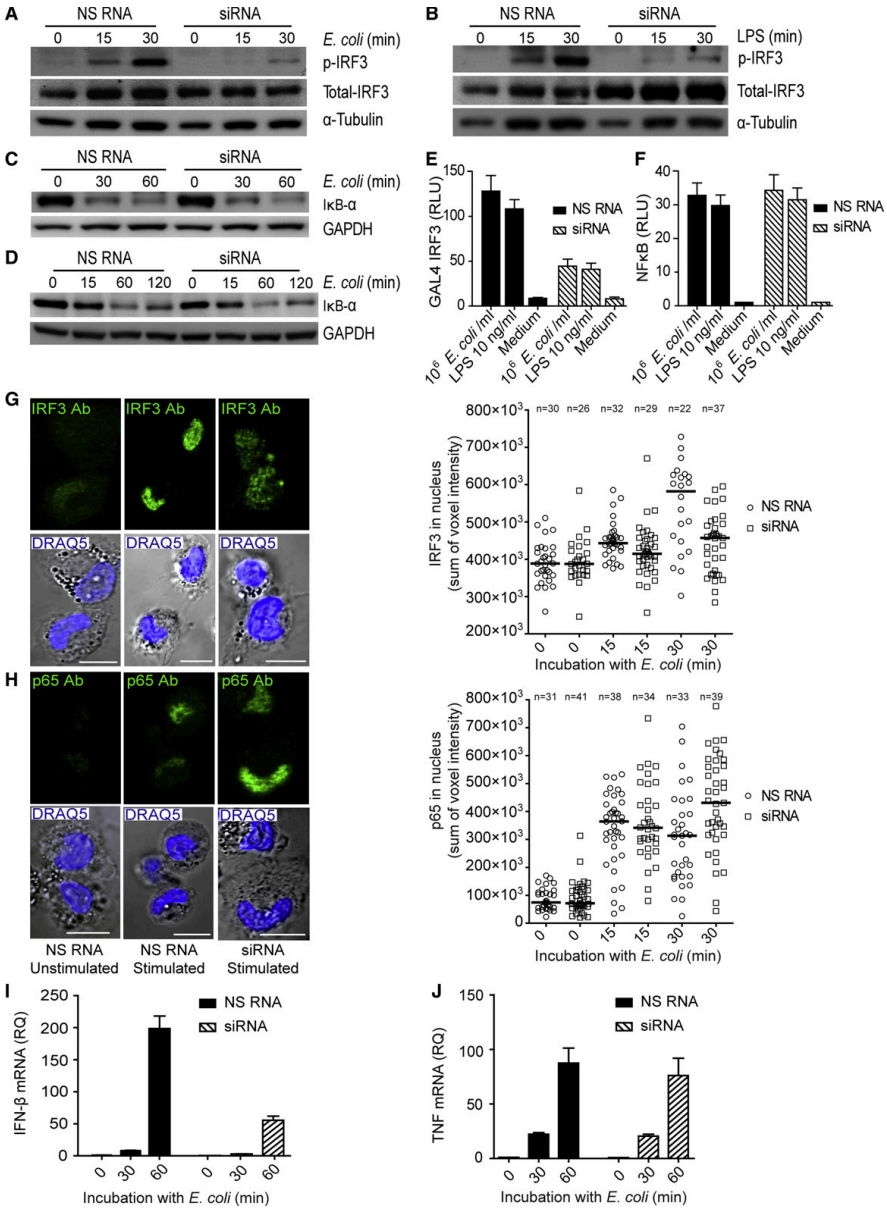

Figure 7. Silencing of Rab11a Selectively Affects TLR4-Mediated IRF3 Activation and IFN-β Induction

(A and B) Human monocytes were treated with NS RNA or siRNA (48 hr) and stimulated with *E. coli* particles (A) or LPS (B) as indicated. Immunoblotting was performed with IRF3 antibodies recognizing phosphorylated-IRF3 (Ser 396) and total-IRF3. α-Tubulin was used as a control of equal loading.

(C and D) IκB-α degradation in siRNA-treated monocytes (C) and HEK293TLR4^{YFP} cells (D) stimulated with *E. coli* particles (1.0×10^7/ml) as indicated, with GAPDH as loading control.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one movie and can be found with this article online at doi:10.1016/j.immuni.2010.09.010.

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(E and F) HEK293 TLR4^{Cherry} cells treated with siRNA or NS RNA for 72 hr, plated, and transfected with MD-2, CD14, and the Gal4-IRF3-luciferase reporter (E) or the NF-κB-luciferase reporter (F). The cells were incubated with *E. coli* or LPS for 9 hr. Gal4-IRF3 activation was normalized toward Renilla Luciferase. Activation is presented as mean relative luciferase units (RLU) to Gal4DBD signals and standard deviations.
(G and H) Human monocytes treated with NS RNA or siRNA and stimulated with *E. coli* (3.0×10^6/ml) as indicated, fixed, and immunostained for total IRF3 (G) or total p65 (H). Quantification of nuclear IRF3 (G). Silencing of Rab11a resulted in decreased nuclear IRF3 translocation compared to NS RNA-treated cells (15 min; $p = 0.0145$, 30 min; $p = 0.0009$). Quantification of nuclear p65 (H). Silencing of Rab11a resulted in increased nuclear p65 translocation at 30 min ($p = 0.0031$). Bars represent the median. n = number of observations. Scale bars represent 10 μm.
(I and J) Human monocytes treated with NS RNA or siRNA for 20 hr were stimulated with *E. coli* particles (3.0×10^6/ml) as indicated. Total RNA was isolated and the level of IFN-β and TNF mRNA quantified by QPCR shown as mean relative to reference sample (NS RNA, 0 min) and standard deviations. Data are representative of three independent experiments. See also Figure S7.

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Supplemental Information

The Rab11a GTPase Selectively Controls Toll-like Receptor 4-Induced Interferon Regulatory Factor-3 Activation by Regulating Trafficking to Phagosomes

Harald Husebye, Marie Hjelmseth Aune, Jørgen Stenvik, Eivind Samstad, Frode Skjeldal, Øyvind Halaas, Nadra J. Nilsen, Harald Stenmark, Eicke Latz, Egil Lien, Tom Eirik Mollnes, Oddmund Bakke, and Terje Espevik

Inventory of Supplemental Information:

- Supplemental data for Figure 1: Figure S1.
  A: Co-localization of TLR4 and Rab11a. Figure 1 B focus on the ERC. Figure S1 A focuses on enlarged Rab11a endosomes.
  B: Dataset used to make a 3-D model of the TLR4 and Rab11a in relation to Golgi, as seen in Figure 1 C.

- Supplemental data for Figure 2: Figure S2.
  Rab11a is shown to not physically interact with TLR4 despite the prominent co-localization of Rab11a and TLR4 seen in figures; Figure 1B, 1C,1E and 2B.

- Supplemental data for Figure 3: Figure S3.
  A: TLR4 accumulation towards *E. coli* on the forming phagocytic cup. (Figure 3A shows a more mature phagosome).
  B: TLR4 accumulation towards *E. coli* seen as a halo around the phagosome when the level of exposure is reduced compared to that shown in Figure 3 A.

- Supplemental data for Figure 4: Figure S4.
  A: Rab11a association with *E. coli* in very early phagocytic events. (Figure S4 A shows point of entry for *E. coli.* Figure 4 D shows later time points).
  B: Rab11a vesicle fusion with *E. coli* phagosome used to explain the kinetics of Rab11a on phagosomes in Figure 4 D.
  C: Rab11a vesicle fission from *E. coli* phagosome used to explain the kinetics of Rab11a on phagosomes in Figure 4 D.

- Supplemental data for Figure 4: Movie S1.
  Movie used to make still image representation of Rab11a association with *E. coli* in very early phagocytic events in Figure S4 A. (Movie S1 shows point of entry for *E. coli.* Figure 4 D shows later time points).

- Supplemental data for Figure 5: Figure S5.
  A, B and C: TRAM, MyD88 and IRF3 accumulation towards the *E. coli* phagosome. Representative images for the data used to generate Figure 5B, C and E.
  D: MyD88 distribution in resting cells.
  E: MyD88 recruitment to *E. coli* phagosomes at 30 min and 30+30 min.
  F: Effect of Dynasore on *E. coli* and LPS induced IFN-β and TNF mRNA.

- Supplemental data for Figure 6: Figure S6.
  A and B: Effect of LPS expression by *N. meningitides* strain on induction of IFN-β and TNF mRNA. Relates to Figures 5A, 6A and 6B.
  C: Uptake of *E. coli* by cyto D treated monocytes. Treatment protocol for cyto D inhibition is equivalent to that used in Figure 6 C and D.
  D: Rab11a and Rab11b mRNA levels after Rab11a siRNA treatment. Treatment protocol equivalent to that used in Figure 6E, G and H.
  E: Uptake of *E. coli* by Rab11a siRNA treated vs. NS RNA treated monocytes. Treatment protocol is equivalent to that used in Figure 6E, G and H.
  F: TLR4 levels in ERC in Rab11a siRNA treated vs. NS RNA treated monocytes. Relates to Figure 6G.
  G: MyD88 and TRAM recruitment to *E. coli* phagosomes in monocytes treated with Rab11a siRNA. Relates to Figure 6H.

- Supplemental data for Figure 7: Figure S7.
  A and B: IFN-β or TNF mRNA induction in human monocytes treated with NS RNA or Rab11a siRNA following 120 min *E. coli* or LPS treatment. Relates to Figure 7I and 7J.

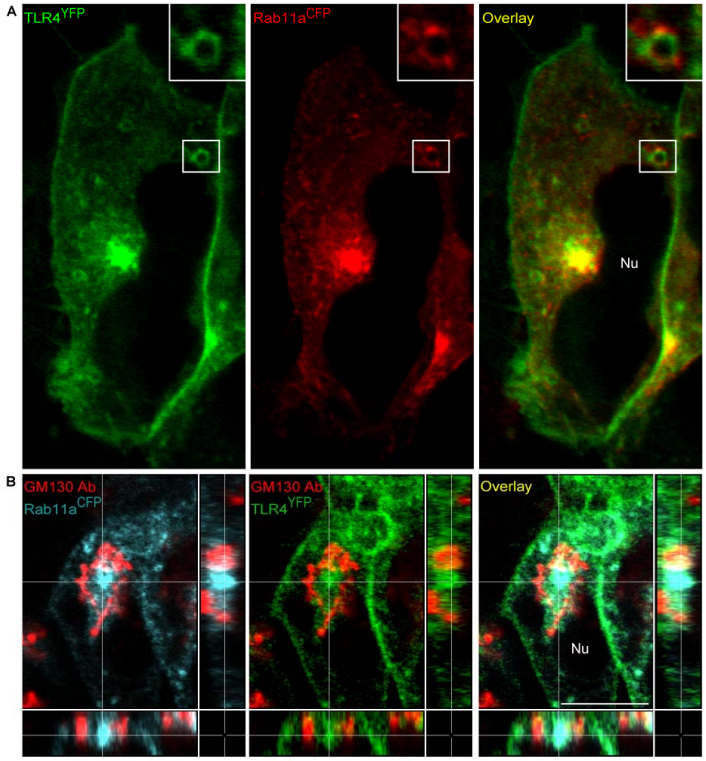

Figure S1. TLR4 and Rab11a show extensive co-localization in many cellular compartments. (**A**) Confocal image of live HEK293-TLR4^{YFP} cells expressing Rab11a^{CFP}, MD-2 and CD14. The boxed area shows co-localization of TLR4 and Rab11a on a single endosome. (**B**) Orthographic projection raw data used for 3D modeling of the cis-*Golgi* (GM130), TLR4^{YFP} and Rab11a^{CFP} in fixed HEK293-TLR4^{YFP} cells co-expressing plasmids encoding Rab11a^{CFP}, MD-2 and CD14 shown in the main article (Fig. 1C). Overlapping regions appear as yellow in the overlay panels. Nu indicates nucleus. Scalebar 10 µm.

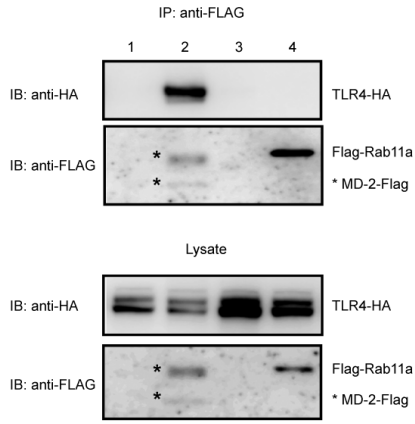

Figure S2. Rab11a does not physically interact with TLR4. HEK293 cells expressing TLR4-HA were co-transfected with pFlag-CMV2 and CD14 (lane 1 and 2) or Flag-Rab11a and CD14 (Lane 4) for 24 hours. MD-2-Flag was included as a positive control for TLR4 association (Lane 2) and non-transfected cells (Lane 3) as negative control. Cellular lysates were made and Flag-Rab11a or MD-2-Flag pulled down using Flag (M2)-conjugated agarose. The immuno-complexes were eluted using Flag-peptide. TLR4-HA was detected using a HA-antibody, MD-2-Flag and Flag-Rab11a was detected using a Flag antibody. As seen in the upper panel (Lane 4), Flag-Rab11a does not associate with TLR4-HA (Lane 4). Under the same conditions MD-2-Flag showed a strong association with the upper band of TLR4-HA (Lane 2, upper panel). Data are representative of three independent experiments.

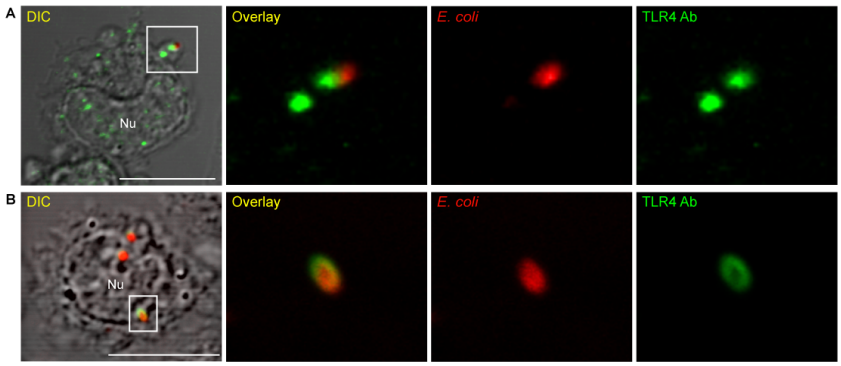

Figure S3. Accumulation of TLR4 towards *E. coli*. Human monocytes were incubated with *E. coli* particles (2 x 10^6/ml), fixed and immunostained for TLR4. (A) After 5 min. TLR4 (green) is recruited towards *E. coli* (red) in very early phagocytic events such as the formation of the phagocytic cup. (B) After 30 min. TLR4 (green) is shown to predominantly reside on the phagosomal membrane around the *E. coli* particle (red) by presenting the shape of a halo. Nu indicates nucleus. Scalebars: 10 µm.

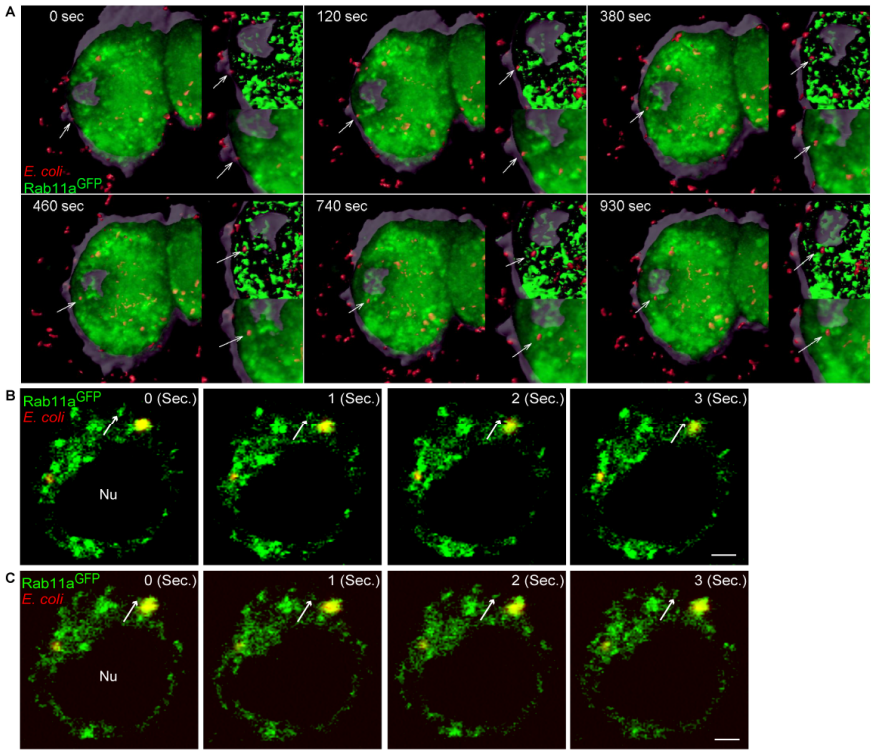

Figure S4. Rab11a is associated with *E. coli* in very early phagocytic events and the *E. coli* containing phagosome undergoes fusion and fission events with Rab11a vesicles. Live murine B6 macrophages with retrovirally transduced Rab11a^{GFP} were incubated with pHrodo *E. coli* particles (2 x 10^6/ml) and images captured using a spinning disc confocal microscope. Single frames are shown with the first time point set to 0 sec. (A) Arrow follows a single *E. coli* particle (red) before and after phagocytosis. The cells surface is indicated in grey and is processed and rendered 3D in Imaris. Images show that Rab11a (green) is recruited towards the *E. coli* particle at point of entry to the cell, and that additional Rab11a is accumulated in very early maturation events. (B) Arrow follows a Rab11a^{GFP} (green) vesicle as it fuses with the *E. coli* (red) containing phagosome. (C) Arrow follows a Rab11a^{GFP} (green) vesicle as it separates and moves away from the *E. coli* (red) phagosome. Nu indicates nucleus. Scalebars: 2 µm.

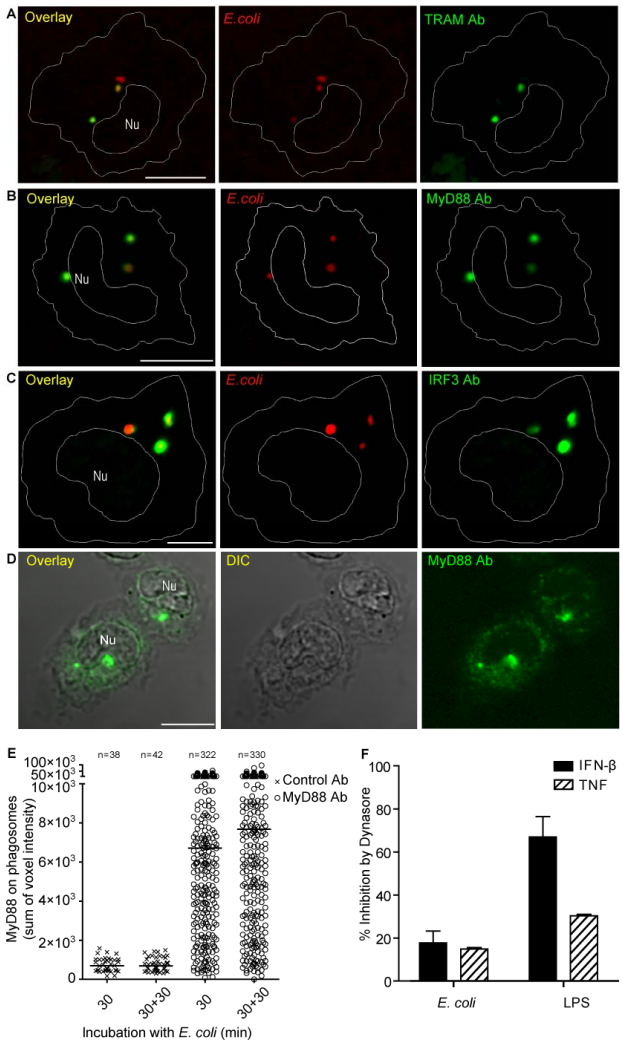

Figure S5. MyD88, TRAM and IRF3 accumulate around phagocytosed *E. coli.*
Human monocytes incubated with *E. coli* pHrodo bioparticles (2 x 10^6/ml) for 30 min and immuno-stained for TRAM, MyD88 or IRF3 following fixation. (A) *E. coli* treated monocytes showing TRAM (green) recruitment to *E. coli* phagosomes (red). (B) *E. coli* treated monocytes showing MyD88 (green) recruitment to *E. coli* phagosomes (red). (C) *E. coli* treated monocytes showing IRF3 (green) on recruitment to *E. coli* phagosomes (red). (D) MyD88 in unstimulated monocytes. (E) MyD88 on *E. coli* phagosomes. Monocytes incubated with *E. coli* pHrodo bioparticles as indicated. The moderate increase in MyD88 on phagosomes from 30 min to 30+30 min was not significant (p = 0.07). (F) Effect of Dynasore on *E. coli* and LPS induced IFN-β and TNF mRNA. Monocytes were serum starved for 2 hrs before *E. coli* (8.0 x 10^6/ml) or LPS treatment (1 ng/ml). The cells were treated for 30 min with opsonised *E. coli* and subsequently washed two times before an additional 30 min incubation with 80 µM Dynasore or vehicle (DMSO) in medium supplemented with 1% A^+

serum. In parallel, monocytes were pre-treated with 80 µM Dynasore or vehicle (DMSO) in medium supplemented with 1% A^+ serum for 5 min before addition of LPS for 1 hr. Scalebars: 10 µm.

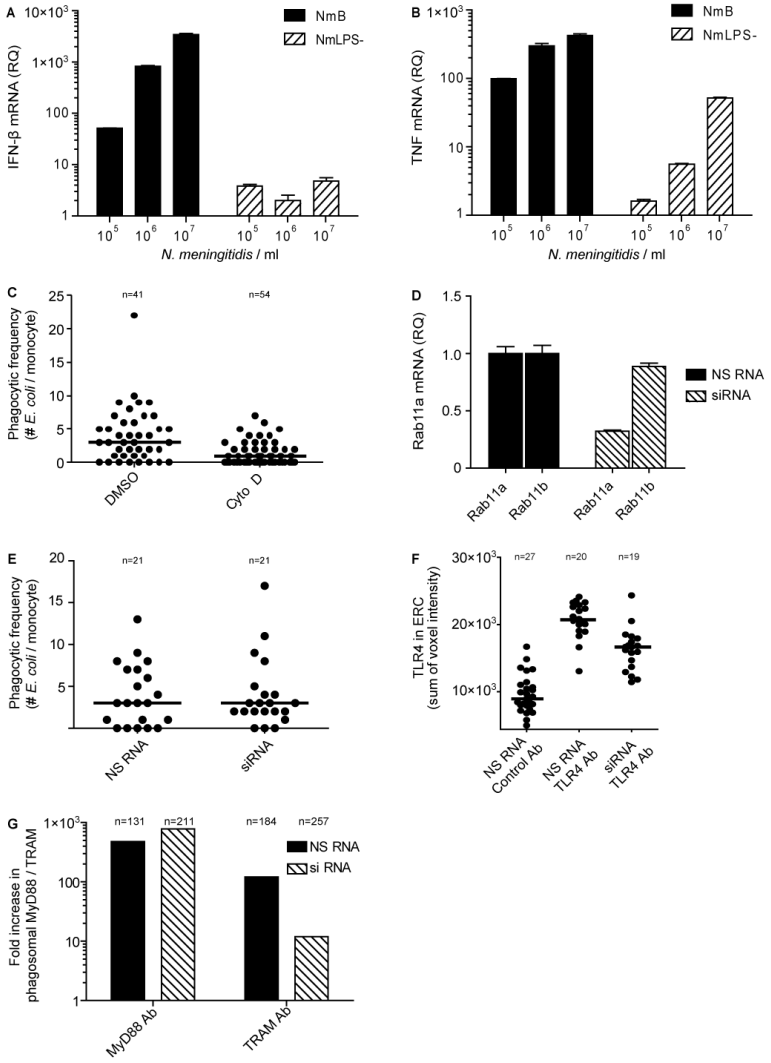

Figure S6. TLR4 specificity of IFN-β induction and Rab11a siRNA specificity. Induction of IFN-β mRNA (A) and TNF mRNA (B) in monocytes stimulated with different concentrations of wild type (NmB) or LPS deficient (NmLPS-) heat killed *N. meningitidis* bacteria. Total RNA was isolated 60 min after stimulation and the level of IFN-β and TNF mRNA determined by QPCR presented as mean and s.d. relative to non-stimulated monocytes. (C) Plot of phagocytic frequency (number of *E. coli* /cell) obtained by analysis of 3D-confocal data from monocytes incubated with DMSO or 2 µM Cytochalasin D (Cyto. D). The cells were pretreated for 15 min with inhibitor or vehicle and incubated with *E. coli* (3 x

10^6/ml) for 30 min prior fixation. (D) Rab11a knock down does not affect the level of Rab11 isotype Rab11b. Human monocytes were treated with NS RNA or Rab11a siRNA for 40 hrs. Rab11a and Rab11b mRNA were quantified by QPCR and shown relative to reference sample (NS RNA, 0 min) and standard deviations. (E) Plot of phagocytic frequency (number of *E. coli* /cell) obtained from Rab11a siRNA and NS RNA treated cells (40 hrs). The monocytes were incubated with *E. coli* (2 x 10^6/ml) prior fixation. The data is representative for six independent experiments. (F) Plot of TLR4 voxel intensity in the ERC of human monocytes 30 min following *E. coli*. Silencing of Rab11a resulted in a significant decrease of TLR4 in ERC compared to the NS RNA treated cells ($p < 0.0001$). (G) Fold increase in median levels of MyD88 and TRAM on *E. coli* phagosomes in monocytes treated with NS RNA or Rab11a siRNA (40 hrs). Fold increase in median levels is relative to normal rabbit antibody control. Monocytes were stimulated with 3 x 10^6/ml *E. coli* particles. Under the same conditions the silencing of Rab11a resulted in a significant decrease in TRAM levels on phagosomes at 30 min ($p = 0.0002$) while the MyD88 recruitment was not impaired ($p = 0.48$) compared to NS RNA treated cells. Bars in plots represent the median. n = number observations. Data above are representative of three or more independent experiments.

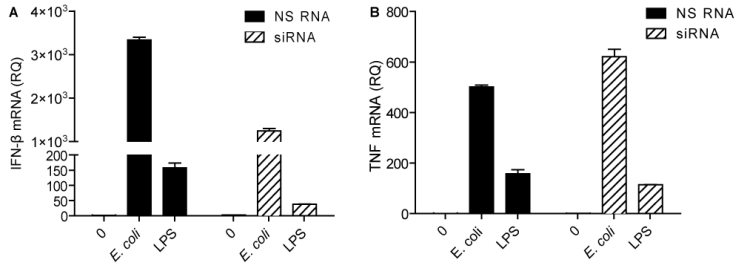

Figure S7. Silencing of Rab11a selectively affects TLR4 induced IRF3 activation and IFN-β induction (A,B) Human monocytes treated with NS RNA or siRNA for 20 hours and stimulated with *E. coli* particles (C) or LPS (100ng/ml) (D). Total RNA was isolated from monocytes before and after 120 min of stimulation. The levels of IFN-β and TNF mRNA were quantified by QPCR and presented as mean and standard deviations relative to reference sample (NS RNA, 0 min).

Movie S1. Trafficking of Rab11a during the initial phases of phagocytosis. Immortalized murine macrophages expressing $Rab11a^{GFP}$ were placed in heating chamber at 37°C on a spinning disk microscope. Images were acquired and *E. coli* particles were added so that the initial process of phagocytosis was captured. The cell surface is indicated in grey and is processed and rendered 3D in Imaris. Three 4D timelapse movies were put together to demonstrate the internalization of *E. coli* and the recruitment of $Rab11a^{GFP}$ to the phagocytosed particle (indicated by white arrows). A single image montage of the movie was prepared to better follow the internalization and recruitment (Figure S4, white arrows). Movie available online.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Reagents and bacterial strains.

The following antibodies were used for Western blotting: Mouse-anti Flag (M2) and mouse-anti-HA (HA-7) (Sigma). The following antibodies were used for immunofluorescence: Rabbit-anti-TLR4 (H80) Rabbit-anti-NFκB p65 (A), rabbit-anti-total IRF3 (FL-425), rabbit-anti-MyD88 (HFL-296) and rabbit-anti- TICAM-2 (H-85) all from Santa Cruz Biotech. *N. meningitidis* H44/76*lpxA*- (denoted NmLPS- in this study) is a viable encapsulated isogenic mutant of H44/76 that completely lacks LPS due to insertional inactivation of the *lpxA* gene which is essential for the first committed biosynthesis step of LPS (Steeghs et al., 1998). The LPS-deficient *N. meningitidis* strain (H44/76*lpxA*-) was created by L. Steeghs and P. van der Ley, National Institute of Public Health and Environment, the Netherlands and donated to the National Institute of Public Health, Oslo, for research purpose. *E. coli* DH5-α strain carrying YFP (BRL Life Technologies).

Cloning of Flag-Rab11a. The Flag-Rab11a vector was made by inserting the human Rab11a cDNA from pGEM-Rab11a into the pFLAG CMV2 vector (Sigma).

Cells and cell lines: Human monocytes were isolated as described in the main article. HEK293 cell lines stably expressing human TLR4-HA was purchased from Invivogen and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10 μg/ml of Blasticidin. Transfection of plasmids was performed as described in the main article. For confocal imaging, the cells were seeded on 35 mm glass bottom γ-irradiated tissue. An immortalized B6 murine macrophage cell line expressing $Rab11a^{GFP}$ was made using J2 recombinant retrovirus and $Rab11a^{GFP}$ cloned into the lentiviral plasmid FugW as described previously described (Bauernfeind et al., 2009).

Co-immunopreciptiation and Western bloting: Immunoprecipitation was performed using Flag (M2) - conjugated agarose or HA (HA-7) conjugated agarose from Sigma, according to manufacturers instructions. Cellular lysates were made from HEK293 cells expressing TLR4-HA transfected with MD-2, CD14 and Flag-Rab11a or pFLAG-CMV2. The immuno-complexes were eluted from the Flag-or HA -agarose using the Flag (M2) peptide or the HA (HA-7) peptide, respectively. Western blotting was preformed as described in the main article.

Gene expression analysis. Total RNA from monocytes were isolated with the RNeasy Mini Kit, including DNAse digestion, using the QIAcube robotic work station (Qiagen). cDNA was made with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, ABI), according to the recommended procedure. Realtime PCR (QPCR) was performed with the StepOnePlus^{TM} Realtime PCR System, TaqMan® Gene Expression Assays, and TaqMan® Universal Master Mix (ABI) with 20µl reaction volume in duplicate wells. The PCR-efficiencies were determined by series dilution of monocyte cDNA in control cDNA made from yeast tRNA (Sigma), thus ensuring a constant cDNA background level in the standard curve. The efficiencies of the TaqMan® Gene Expression Assays for IFN-β (Hs01077958_s1), TNF (Hs00174128_m1), Rab11a (Hs00900539_m1), Rab11b (Hs00188448_m1), and GAPDH (Hs99999905_m1) ranged from 89-103 percent. Efficiency-correction was used in the analysis using the StepOne^{TM} Software 2.1 (ABI). No-RT controls were negative. The level of GAPDH mRNA was used for normalization.

Live cell imaging. In some experiments live cell image acquisition was performed on an Andor Revolution XD spinning disc microscope. The spinning disc unit CSU22 was synchronized with an iXon^{EM+} 885 EMCCD camera. The spinning disc data was 3D rendered and processed in ImarisSuite (Bitplane). Fluorescent average intensity of *E. coli* and Rab11a^{GFP} was masked to be reconstructed in 3D and iso-surfaced. The 4D supplementary video with 3 pictures in one movie was created in Adobe Premiere Pro and compressed with AVI jpeg mode in ImageJ.

SUPPLEMENTARY REFERENCES

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