Master's thesis

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Characterization of a Model Cell Line to Study Toll-like Receptor 9 Signaling and Trafficking

Master's thesis in Molecular Medicine
Supervisor: Lene M. Grøvdal and Nadra J. Nilsen
June 2019

NTNU
Kunnskap for en bedre verden

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Abstract

Toll-like receptors (TLRs) are a family of innate immune receptors that function to mount an immune response upon recognition of molecules associated with infection or injury. Toll-like receptor 9 (TLR9) is expressed predominantly in endosomes of plasmacytoid dendritic cells (pDCs), where it is responsible for recognition of unmethylated CpG DNA derived from virus or bacteria. Activated TLR9 initiates immune responses which are important in antiviral immunity. Upon activation, TLR9 locates to early endosomes and initiates the production of interferons (IFNs) through transcription factor IRF7. Further sorting of TLR9 to late endosomes stimulates the production of proinflammatory cytokines like TNFα and IL-12B through transcription factor NF-κB. In pDCs, TLR9 can trigger potent anti-tumor immunity, however, the receptor has also been found involved in driving tumor progression. Since pDCs are rare in the human blood, this project sought to establish and characterize a model cell system that resembles human pDCs. A THP-1 cell line with inducible expression of TLR9 mCherry was characterized and optimized for studying TLR9 signaling and trafficking. Different differentiation protocols were applied to the THP-1 TLR9 mCherry cells before cells were induced to express TLR9 and stimulated with CpG. PMA differentiation of these cells failed to produce a potent IFNB1 response in response to CpG. In contrast, GM-CSF and IL-4-differentiated and undifferentiated THP-1 TLR9 mCherry cells mimicked pDC responses and induced marked levels of IFNB1, as well as TNFα, in response to CpG. Previous findings in HEK293 cells indicated a role for the GTPase Rab39a in TLR9 signaling. Undifferentiated THP-1 TLR9 mCherry cells were used as a model cell line for studying how Rab39a silencing might affect TLR9 signaling. siRNA experiments targeting Rab39a revealed an increased tendency of IFNB1 and TNFα mRNA levels in response to CpG in undifferentiated THP-1 TLR9 mCherry cells. Combined, this project provides a novel model system to study TLR9 signaling and trafficking and suggests that Rab39a might be involved in regulation of signaling from TLR9.

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NTNU – Trondheim
Norwegian University of
Science and Technology

CEMIR
Centre of Molecular Inflammation Research

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Abbreviations

AEP	Asparagine endopeptidase
AP-1	Activator protein 1
AP-3	Adaptor protein 3
cDNA	complementary DNA
COPII	Coat protein complex II
CpG	Cytosine-guanosine
C_T	Threshold cycle
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
ER	Endoplasmic reticulum
FCS	Fetal calf serum
GOI	Gene of interest
GTP	Guanosine triphosphate
Gp96	Glycoprotein 96
HEK	Human embryonic kidney
IFN	Interferon
IκB	Inhibitor of kappa B
IKK	IκB kinase
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IKKε	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
kDa	Kilodalton
LAMP1	Lysosomal-associated membrane protein 1
LF3000	Lipofectamine 3000
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen-activated protein kinase
mRFP1	Monomeric red fluorescent protein 1
MyD88	Myeloid differentiation primary-response protein 88
NEMO	NF-kappa B essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK	Natural killer
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
rhGM-CSF	Recombinant human granulocyte macrophage-colony stimulating factor

rhIL-4	Recombinant human interleukin-4
RIP1	Receptor interacting protein 1
RT	Reverse transcriptase
SD	Standard deviation
siRNA	Small interfering RNA
TAB	TGF-β activated kinase 1 binding protein
TAK1	Transforming growth factor-β (TGF-β)-activated kinase 1
TBK1	TANK binding kinase 1
TfR	Transferrin receptor
TGF-β	Transforming growth factor-β
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-domain containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRAF	Tumor-necrosis factor (TNF)-receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain containing adapter-inducing interferon-β
Unc93B1	Uncoordinated 93 homologue B1

1 Introduction

The immune system is a complex and dynamic network of molecules, cells, and organs that serve to defend the body against a broad spectrum of potentially harmful pathogens. The immune system is commonly divided into two branches: the innate immune system and the adaptive immune system. The innate system consists of many different cell types, like macrophages and dendritic cells, as well as soluble components and physical and chemical barriers. It is the first line of defense and the players of the innate immune system respond fast and recognize a large variety of conserved structures on pathogens. They are also responsible for directing the adaptive immune system, consisting of B- and T-lymphocytes, through activating signals called cytokines. B and T-lymphocytes have high antigen specificity generated through several steps of clonal selection and proliferation and they provide long-term immunity against previous pathogens combated by the adaptive immune system. Key components of the innate immune system are pattern recognition receptors (PRRs) which recognize conserved structures of microorganisms (Reviewed in[1,2]).

1.1 Signaling pattern recognition receptors (PRRs)

Pattern recognition receptors (PRRs) are germline encoded receptor proteins expressed by cells of the innate immune system. These receptors recognize conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), which are not found in mammals[1]. PAMPs can include whole molecules or more commonly, parts of molecules such as cell wall carbohydrates of bacteria or unmethylated cytosine-guanosine (CpG) motifs in foreign DNA sequences (Reviewed in[1,3,4]). PRRs can be expressed both on the plasma membrane and intracellularly in a variety of innate immune cells[5,6]. Stimulation of PRRs through recognition of PAMPs triggers an inflammatory response involving production of cytokines which results in direct elimination of microbes[7,8], and initiation and activation of the adaptive immune system[9]. Some PRRs are also capable of detecting damage-associated molecular patterns (DAMPs) which are antigens associated with dying or dead cells[1]. PRRs are classified into five families, however, in this thesis it will only be focused on Toll-like receptors (TLRs), and further Toll-Like receptor 9 (TLR9).

1.2 Toll-like Receptors

Toll-like receptors (TLRs) are a group of transmembrane glycoproteins that serve as the first line of defense by detection of PAMPs and the initiation of an immune response. These receptors are found expressed on cells of the innate immune system, including monocytes, macrophages and dendritic cells (DCs) (Reviewed in[10]). The first TLR was found in *Drosophila* where it was shown to play a crucial role in the development of embryogenesis[11]. However, further investigations revealed that the receptor was also involved in the innate immune systems' combating of fungal infections[12]. To date, 10 TLRs have been discovered in humans, whereas 13 TLRs have been identified in mice (Reviewed in[10,13-15]). In humans, TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the plasma membrane and can sense components of bacteria and fungi. TLR2 creates heterodimers with TLR1 (TLR2/1) and TLR6 (TLR2/6) to recognize triacylated lipopeptides and diacylated lipopeptides respectively. Lipopeptides are components of the bacterial membrane[16]. TLR4 binds lipopolysaccharide (LPS), an endotoxin found on the surface of gram negative bacteria, while TLR5 recognizes flagellin which is a component of flagella, an apparatus of locomotion utilized by many bacteria (Reviewed in[10]). TLR3, TLR7, TLR8, and TLR9 are expressed in intracellular compartments like endosomes and are responsible for the detection of potentially harmful endolysosomal contents (Reviewed in[17]). These endosomal TLRs recognize structures of nucleic acids. TLR3 senses viral double-stranded RNA, TLR7, and TLR8 recognize viral single-stranded RNA, and TLR9 recognize viral and bacterial unmethylated CpG DNA. (Reviewed in[10]). The different TLRs are characterized by ligand specificity, pattern of expression and subcellular localization[18].

1.2.1 TLR signaling

TLRs are characterized by a common structure consisting of several numbers of leucine-rich repeats (LRRs) in their ectodomain, responsible for recognition of PAMPs and a Toll/interleukin-1 receptor (TIR) homology endodomain responsible for downstream signaling (Reviewed in[10,19]). The extracellular domain contains 19-25 tandem copies of LRRs[19], while the TIR-domain has a conserved region of approximately 200 amino acids and consists of three boxes highly necessary for proper downstream signaling[19,20] (Figure 1.1).

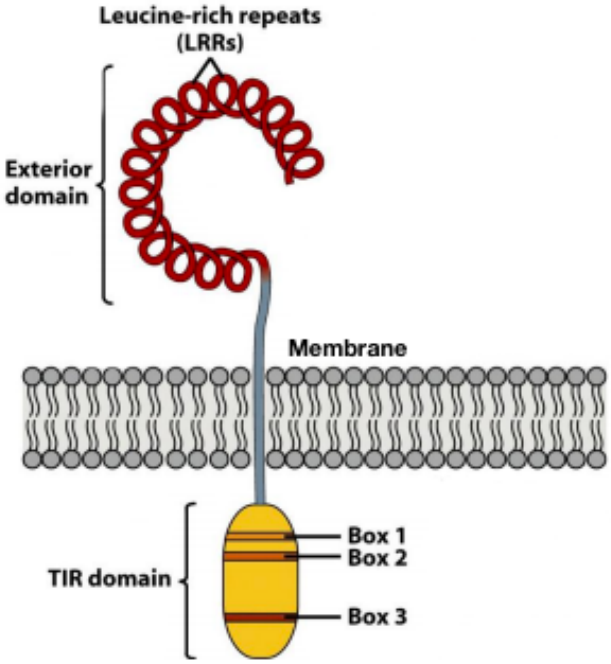


Figure 1.1 Schematic structure of Toll-like receptors. The ectodomain contains leucine-rich repeats (LRRs) responsible for ligand binding. The endodomain, known as the Toll/IL-1R (TIR) domain is liable of signal transduction and consists of three homologous regions (Box 1, 2 and 3). Modified from[21].

Recognition of ligands triggers the formation of TLR dimers. TLR2 forms heterodimers with TLR1 and TLR6 while the other TLRs make up homodimers to transduce signals to the interior of the cell[15]. Dimerization of TLRs brings the cytoplasmic TIR-domains close together which triggers downstream signal transduction[19,22]. Mutations in the TIR-domain of both TLR4 and TLR2 have been shown to disrupt signaling from these receptors in mice[20]. After ligand binding and dimerization, the TIR-domain will function as a scaffold and recruit adaptor proteins necessary for downstream signaling[19,23]. Adaptor proteins recruited by TLRs include myeloid differentiation primary-response protein 88 (MyD88), TIR-domain containing adapter-inducing interferon-β (TRIF), TIR-domain containing adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM). These adaptor proteins act as links between the ligand-bound TLRs and different serine/threonine kinases[23]. MyD88, utilized by all TLRs except TLR3, cooperate via downstream signaling molecules to activate nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and mitogen-activated protein kinases (MAPKs) which further promotes the production of proinflammatory cytokines, such as TNFα. TIRAP, employed by TLR2 and TLR4, operates as a sorting adaptor to recruit MyD88 to these receptors. TRIF, utilized by TLR4 and TLR3, promotes expression of type I interferons (IFNs) through interferon regulatory factors (IRFs) and proinflammatory cytokines through NF-κB.

TRAM functions as a sorting adaptor to recruit TRIF to TLR4 (Reviewed in[13]). Signaling from TLRs can be split into the MyD88-dependent pathway and the TRIF-dependent pathway.

1.2.1.1 The MyD88-dependent pathway

All TLRs, except TLR3, use the adaptor protein MyD88 for activation of NF-κB and MAPKs and subsequently the production of proinflammatory cytokines. MyD88 will recruit and activate different IL-1R-associated kinases (IRAKs)[13]. Activated IRAK interacts with tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) which is an E3 ligase. TRAF6 mediates a polyubiquitin chain on TGF-β activated kinase 1 binding proteins (TABs), which serve as regulatory regions of the transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) complex, and NF-kappa B essential modulator (NEMO), the regulatory component of the IKB kinase (IKK) complex. Activated TAK1 will phosphorylate and activate inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), a second component of the IKK complex. Fully activated IKK complex mediates phosphorylation and degradation of the inhibitor of NF-κB, IκB, leaving NF-κB free to access the nucleus and activate target genes[13,24,25] (Figure 1.2).

As mentioned above, MyD88 also initiates signaling through MAPKs. Activated TAK1 will provide an additional phosphorylation on MAPKs, leading to induction of the MAPK pathway. This pathway will eventually lead to activation of the transcription factor activator protein 1 (AP-1) which promotes gene transcription of target cytokines within the cell nucleus[26] (Figure 1.2).

The MyD88-dependent pathway usually stimulates to the production of proinflammatory cytokines, however, TLR7 and TLR9 expressed in endosomes in pDCs, are capable of using MyD88 to induce production of type I IFNs[23]. This pathway involves activation of the transcription factor IRF7 most likely mediated through IRAKs and TRAFs[27-29] (Figure 1.2).

1.2.1.2 The TRIF-dependent pathway

The adaptor proteins TRIF and TRAM are utilized by TLR4, whereas solely TRIF is used by TLR3 to induce the TRIF-dependent pathway leading to activation of both IRF3 and NF-κB. Activation of IRF3 promotes the production of type I IFNs while activation of NF-κB stimulates the production of inflammatory cytokines. TRIF activates NF-κB through a similar mechanism as described in the MyD88 dependent pathway. TRIF creates a signaling complex

consisting of TRAF6 and kinase receptor interacting protein 1 (RIP1) which will activate TAK1 which further activates both NF-κB and MAPK pathways (Reviewed in[13]).

The TRIF-dependent pathway also results in the activation of transcription factor IRF3. TRIF induces the formation of a signaling complex composed of the noncanonical IKKs, TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε), and TRAF3. This activated signaling complex phosphorylates and activates IRF3, enabling it to translocate to the nucleus and promote the production of type I IFNs[13,30]. However, IRF3 is presumably dependent on IRF7 to induce a robust IFN response[28] (Figure 1.2).

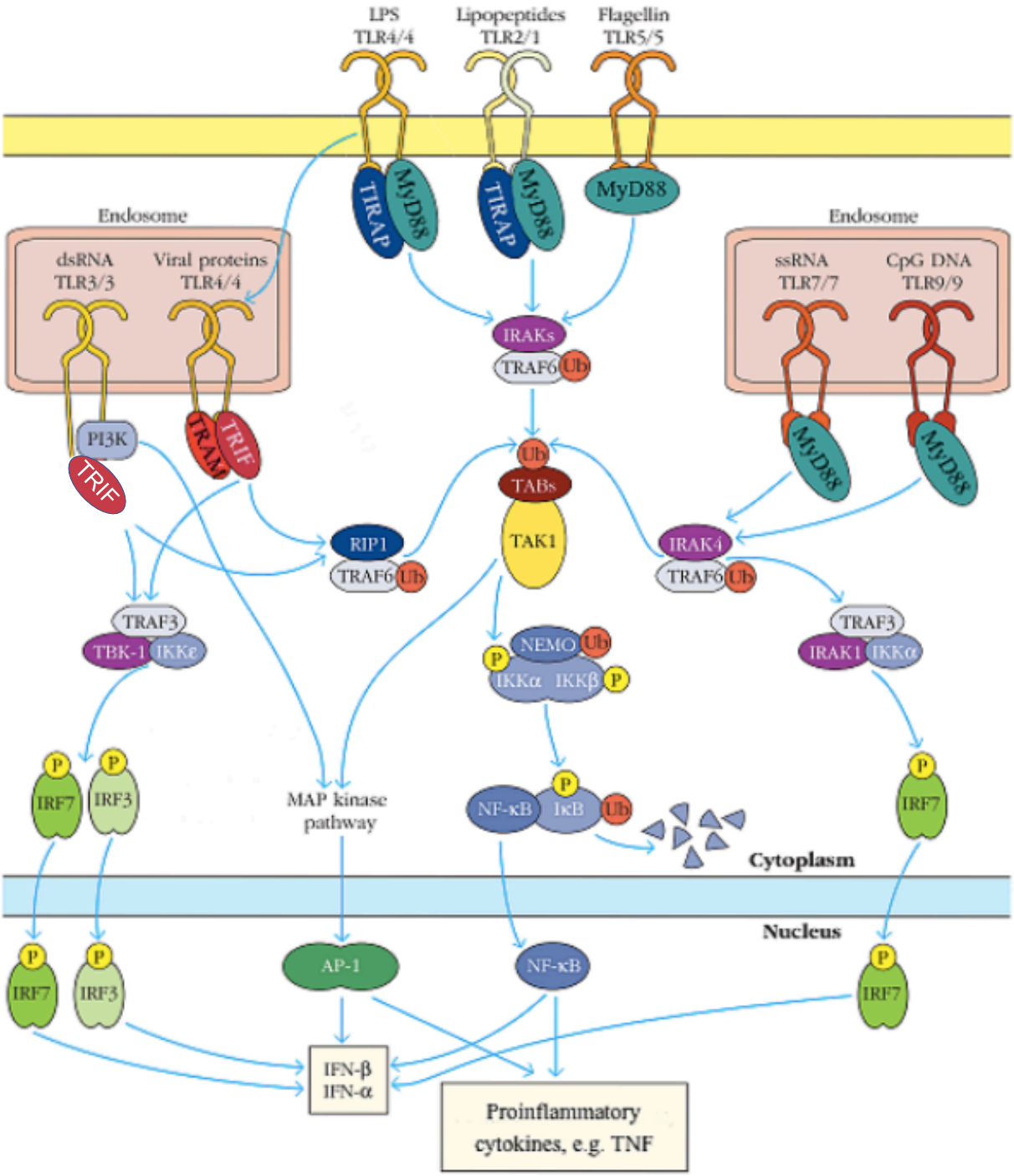


Figure 1.2 Overview of signaling cascades initiated downstream of the different TLRs. TLR1, 2, 4, and 5 are found on the plasma membrane, while TLR3, 7 and 9 are located intracellularly to endosomal compartments. Ligand recognition promotes the recruitment of distinct adaptor proteins like MyD88, TIRAP, TRIF and TRAM. These adaptor proteins initiate downstream signaling cascades leading to the production of type I interferons (IFNβ and IFNα) and proinflammatory cytokines like TNF. See the text for further details. Modified from[31].

1.2.2 TLR localization and trafficking

The different TLRs reside within different cellular compartments and can be split into two groups: surface bound TLRs which comprise TLR1, 2, 4, 5 and 6 and intracellular TLRs

including TLR3, 7, 8 and 9. Localization and trafficking of TLRs are particularly important for correct ligand sensing and activation. The intracellular TLRs reside in the endoplasmic reticulum (ER) before they are transported to endosomes upon ligand internalization. TLR4 is a well-studied surface-bound receptor that is activated by LPS. Ligand-binding at the cell surface initiates the MyD88-dependent signaling pathway before the receptor is internalized into endosomal compartments from where it initiates the TRIF-dependent pathway (Reviewed in[32]). Certain Rab proteins were found to be involved in intracellular trafficking of TLR4[33,34]. Rab proteins are monomeric GTPases involved in vesicle transport[35]. These proteins can be found both on transported vesicles and on target membranes, where they function as selective markers highly important for identification and transportation of membranes and vesicles[36]. In this project, trafficking and signaling from TLR9 will be emphasized in closer detail.

1.3 Toll-like receptor 9

Toll-like receptor 9 (TLR9) is one of the intracellular TLRs and is found expressed in pDCs and B-lymphocytes[37]. In pDCs, this receptor is unique in its ability to produce large amounts of type I IFNs in response to viral infections[17]. Additionally, TLR9 in pDCs can trigger potent anti-tumor immunity and is a promising target in cancer immunotherapy[38]. Despite these propitious features of TLR9, the receptor has also been found involved in the pathogenesis of autoimmune diseases and cancer development[38,39]. TLR9 is found overexpressed in several different cancer cells, for instance, lung cancer cells, breast- and ovarian cancer cells and glioma cancer cells[40-42]. Hence, elucidating how trafficking and signaling from TLR9 are regulated within the cell, might contribute to understanding how this receptor is involved in human disease.

1.3.1 TLR9 recognizes endocytosed CpG DNA

TLR9 is localized intracellularly to endosomal compartments and is liable of the detection of nucleic acids derived from bacteria and viruses (Reviewed in[43]). Unlike mammalian DNA, bacterial DNA contains non-methylated CpG-motifs which is the ligand recognized by TLR9[6,44-47]. Foreign CpG DNA is endocytosed by the cells in a clathrin-dependent manner[48], generating intracellular clathrin-coated vesicles[49]. Fission of clathrin-coated vesicles from the plasma membrane is mediated by dynamin[50]. Different classes of CpG-motifs are identified, including CpG class A (CpG-A) which is multimeric and CpG class B (CpG-B) which is

monomeric[51]. These CpG-motifs are TLR9 agonists composed of synthetic oligodeoxynucleotides (ODNs) frequently used in research to activate TLR9. CpG-A consists of ODNs with a central palindromic phosphodiester (PO) backbone containing CpG motifs. The 3' and 5' end consists of phosphorothioate (PS) modified poly G motifs. CpG-A is particularly efficient in activating natural killer (NK) cells and induce the production of IFN-α from precursors of pDCs[45,52,53]. On the other hand, CpG-B is characterized by a full PS backbone containing CpG motifs. CpG-B stimulates the production of proinflammatory cytokines and activates B-cells[45,54] (Figure 1.3).

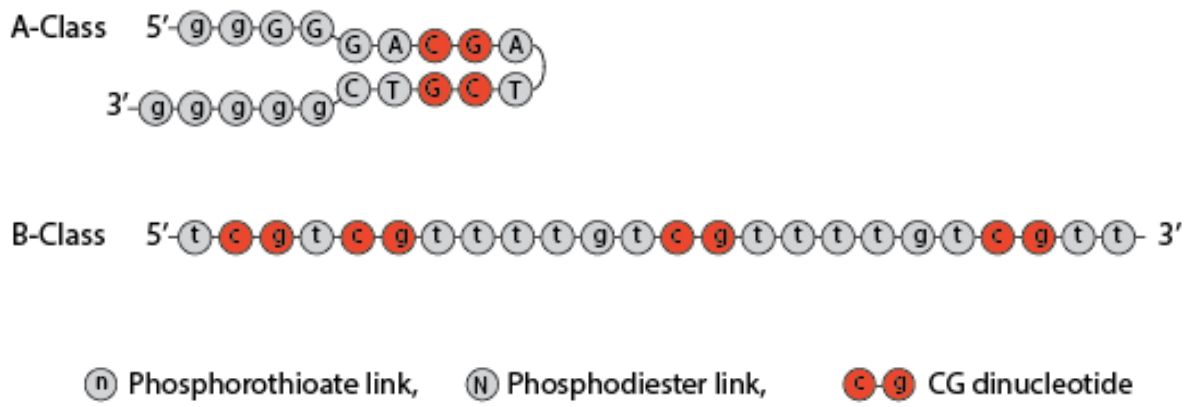


Figure 1.3 Structure of CpG class A and CpG class B. CpG-A ODN is characterized by a central palindromic PO backbone with CpG motifs and PS-modified poly G motifs at the 3' and 5' end. CpG-B ODN is characterized by a full PS backbone with CpG motifs. Adapted from[55].

In human pDCs, multimeric CpG-A was found colocalized with the early endosome marker transferrin receptor (TfR) where it stimulates the production of type I IFNs, whereas monomeric CpG-B were colocalizing with the late endosome marker lysosomal-associated membrane protein 1 (LAMP1), promoting pDC maturation[56].

1.3.2 Localization and trafficking of TLR9

TLR9 encounters its ligand in endosomal compartments. Before stimulation of TLR9, the receptor is located in the endoplasmic reticulum (ER)[8]. Upon internalization of CpG DNA, mediated by clathrin-dependent endocytic, TLR9 is trafficked from the ER to endolysosomal compartments containing CpG DNA[48]. The protein, uncoordinated 93 homolog B1 (UNC93B1) interacts with TLRs, including TLR9, in the ER, and is responsible for regulating the exit of nucleotide-sensing TLRs from the ER[57,58]. Mice having a missense mutation in the *Unc93b1* gene are incapable of signaling through TLR3, TLR7 and TLR9[58], suggesting that this physical interaction is highly necessary to ensure correct trafficking and signaling from these receptors[57].

UNC93B1 has also been detected in endolysosomes, indicating that the direct interaction between UNC93B1 and TLR9 is maintained from the ER to endolysosomal compartments[59]. Other proteins located in the ER, like PRAT4A and glycoprotein 96 (gp96), as well as adaptor protein 3 (AP-3), have also been found involved in intracellular trafficking of TLR9[51,60,61].

1.3.2.1 Trafficking route of TLR9

The trafficking route of TLR9 from the ER to CpG DNA-containing endosomes and lysosomes is not fully elucidated and has been highly debated. Latz et al. reported a direct route for the trafficking of TLR9 from the ER to endosomes, bypassing the regular trafficking route through the Golgi Apparatus[48]. A possible mechanism for TLR9 trafficking involved fusing of the ER membrane and the plasma membrane, thereby providing additional membrane used to create early endosomes. In this way, some TLR9 will become part of the plasma membrane which later will be used to create CpG DNA-containing endosomes[48]. However, this model has been controversial and more recent research proposed another trafficking route for TLR9[62].

This route suggests that TLR9 is recruited from the ER via the Golgi Apparatus from where it is distributed to endosomal compartments[62]. One study shows that UNC93B1, which interacts with TLR9, is present in cytoplasmic coat protein complex II (COPII) vesicles[59]. These vesicles are responsible for the transportation of proteins between the ER and Golgi[63]. The findings from this study support a mechanism where UNC93B1, together with TLR9, is distributed to endosomal compartments via the secretory pathway[59].

1.3.3 Activation and signaling from TLR9

TLR9 activation takes place in endosomal compartments and requires an acidic environment (Reviewed in[54]). Prior to ligand stimulation, TLR9 appears as pre-formed homodimers[64]. After trafficking from the ER to endolysosomal compartments, TLR9 is proteolytically cleaved into a C-terminal fragment of approximately 80 kDa. This event is highly necessary for TLR9 to get activated and initiate appropriate signaling pathways[65]. The site of cleavage occurs within the ectodomain of the receptor, particularly between LRR14 and LRR15[66]. The transmembrane- and the cytoplasmic domain remains unchanged[65]. Presumably, several proteases are involved in the processing of TLR9, including cathepsin K and B and asparagine endopeptidase[65-70]. Both full length and cleaved TLR9 can bind foreign, unmethylated CpG DNA. However, when

stimulated with ligand, only processed TLR9 can transduce signals via recruitment of MyD88. This mechanism restricts TLR9 activation and signaling to endolysosomal compartments[65].

Another important aspect of TLR9 activation is the fact that when TLR9 binds CpG DNA, the ectodomain undergoes a conformational change. This conformational change makes the TIR-domains come closer together which again enables recruitment of MyD88. With that, TLR9 occurs as a preformed homodimer, which upon activation by CpG DNA in endosomes will undergo a conformational change and a cleavage event, both targeting the ectodomain. These changes in TLR9 trigger recruitment of MyD88 which eventually will initiate signaling cascades[64].

Fully activated TLR9 initiate production of type I IFNs and proinflammatory cytokines. The distinct groups of cytokines are produced from different endosomal maturation states. Signaling from early endosomes typically activates IRF7 and further type I IFN production[71] while signaling from late endosomes activates NF-κB and subsequently the production of proinflammatory cytokines, like TNFα[51] (Figure 1.4). Production of type I IFNs is necessary to initiate an immune response and the IRF7-signaling pathway is thought to have an anti-tumorigenic effect on TLRs. The NF-κB signaling pathway is associated with driving carcinogenesis because of its capacity to activate proinflammatory genes (Reviewed in[38]).

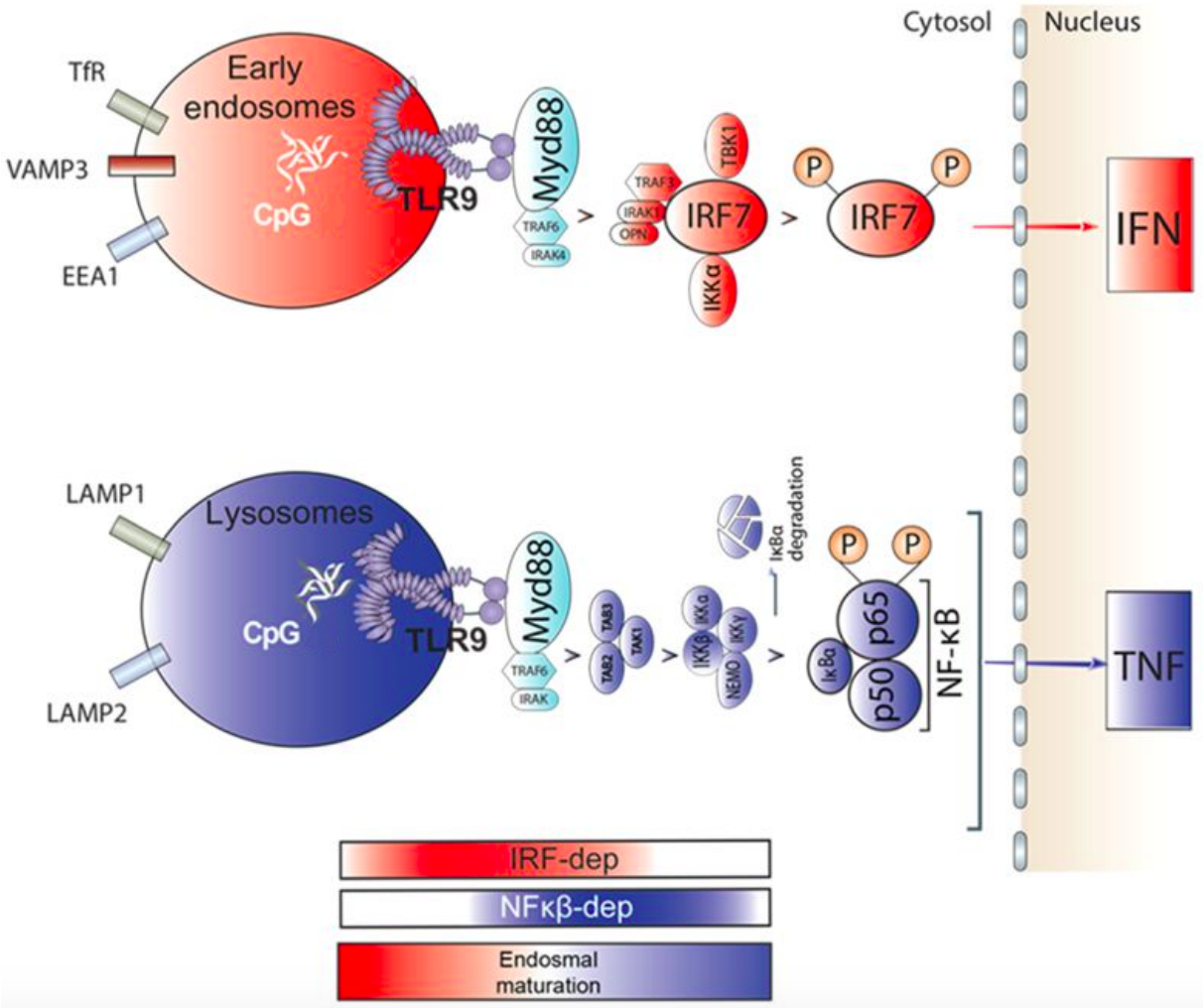


Figure 1.4 Schematic illustration of the bimodal regulation of TLR9 signaling from different endosomal maturation states in human pDCs. CpG recognized by TLR9 in early endosomes activates the IRF7 pathway leading to type I IFN production. CpG recognized by TLR9 in lysosomes activates the NF-κB pathway leading to TNF production. Modified from[72]. Creative Commons license: http://creativecommons.org/licenses/by/4.0/

2 Aim of the study

The endosome specific signaling from TLR9 has both advantageous and disadvantageous properties in human disease. Hence understanding the intracellular sorting and signaling from TLR9 is highly relevant. Much of the previous work studying TLR9 has been based on murine cells or non-immune cells. Since TLR9 is predominantly expressed in pDCs, which are rare in the human blood, this project aimed to characterize and establish a model system closer related to pDCs to study TLR9 signaling and trafficking. The cell line used in this project is a THP-1 cell line where the expression of TLR9 can be artificially induced by the antibiotic, Doxycycline.

A preliminary screening in HEK293 cells overexpressing TLR9 showed disrupted CpG-induced secretion of IL-8 in cells treated with siRNA targeting Rab39a. Since Rab proteins are involved in vesicle transportation it was hypothesized that Rab39a might play a role in the regulation of TLR9 trafficking and signaling. In this project, it will be investigated if Rab39a is involved in the regulation of TLR9 trafficking and signaling. Rab39a is a poorly characterized Rab GTPase localized in late endosomes/lysosomes and the Golgi (Reviewed in[73]). This protein has not been described as a regulator of TLR trafficking but has been shown to be important in other cellular mechanisms, for instance linking caspase-1 to IL-1B secretion functioning as a trafficking adaptor[74], as well as being a regulator of phagosomal acidification[75] and neuron cell differentiation[76].

Topics to be addressed:

1. Establish a model system for studying TLR9 signaling and trafficking.
2. Investigate if knock-down of Rab39a by siRNA transfection affects TLR9 signaling in response to CpG.

3 Materials and methods

3.1 Cell lines and cell culture

The experiments done in this project were mainly performed in THP-1 cells with inducible expression of TLR9 mCherry, termed THP-1 TLR9 mCherry cells. Wild type THP-1 cells were used as a control cell line to study cytokine induction in response to TLR4 ligand LPS, and human embryonic kidney (HEK) 293XL cells with overexpression of TLR9 were used to check CpG ODNs.

The different cell lines used in this project were grown in a 75 cm^2 cell culture flask and incubated in 5% CO_2 at 37° C. A cell concentration of 200 000-800 000 cells/ml was maintained for the two THP-1 cell lines, while HEK293XL cells were split when the cell culture flask was ≥ 80% confluent. The cells were counted (cells/ml) on the cell counter machine Z2 Coulter® Particle Count and Size Analyzer using profile C, which counts particles between 10-19 μm.

3.1.1 Reagents

Table 3.1 Reagents used in cell culture. Name, distributor and catalog number of reagents used in cell culture

Name	Distributor	Cat. Number
6 Well Cell Culture Cluster	Costar	3516
β-mercaptoethanol	Sigma-Aldrich/Merck	60-24-2
Corning 75 cm^2 Cell Culture Flask	Corning	431464U
Dulbecco´s Modified Eagle Medium (DMEM)	Lonza/BioWhittaker	12-604F
Fetal calf serum (FCS)	Gibco	10270
L-glutamine	Sigma-Aldrich/Merck	G7513
Penicillin	Sigma-Aldrich/Merck	P0781
Puromycin	Sigma-Aldrich/Merck	58-58-2
RPMI 1640 medium	Gibco	A10491-01
Streptomycin	Sigma-Aldrich/Merck	P0781
Trypsin/EDTA	Lonza/BioWhittaker	BE17-161E

3.1.2 Wild type THP-1 cells

The THP-1 cell line (ATCC) originates from the blood of a boy diagnosed with acute monocytic leukemia. These cells are suspension cells carrying monocytic markers providing a monocyte-like morphology[77]. Wild type THP-1 cells were included as a control cell line to evaluate responses to TLR4 ligand LPS in wild type THP-1 cells compared to THP-1 TLR9 mCherry cells.

THP-1 cells were cultured and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), β-mercaptoethanol (0,05 mM), L-glutamine (100 µg/ml), penicillin (100 units/ml) and streptomycin (0,1 mg/ml) (Further referred to as THP-1 medium). When seeding THP-1 cells for experiments, 400 000 cells/well were seeded in THP-1 medium (2 ml) in 6-well plates.

3.1.3 THP-1 TLR9 mCherry cells

THP-1 TLR9 mCherry cells were obtained and made by Dr. Lene M. Grøvdal. This cell line was characterized and optimized to serve as a model system for studying trafficking and signaling from TLR9 and examine the role of Rab39a regarding this. The cell line was made using gateway technology to incorporate the *TLR9* gene into lentivirus, which was further introduced into THP-1 cells. These cells have the same morphology as wild type THP-1 cells, but they can induce TLR9 expression upon stimulation with the antibiotic Doxycycline. Doxycycline is a derivate of tetracyclines and works in a tet-on system. In the presence of Doxycycline, it will bind to and trigger a conformational change in a transcription activator thus enabling it to bind to the promoter region upstream of the *TLR9* gene[78] (Figure 3.1). In this way, Doxycycline is responsible for TLR9 expression in THP-1 TLR9 mCherry cells.

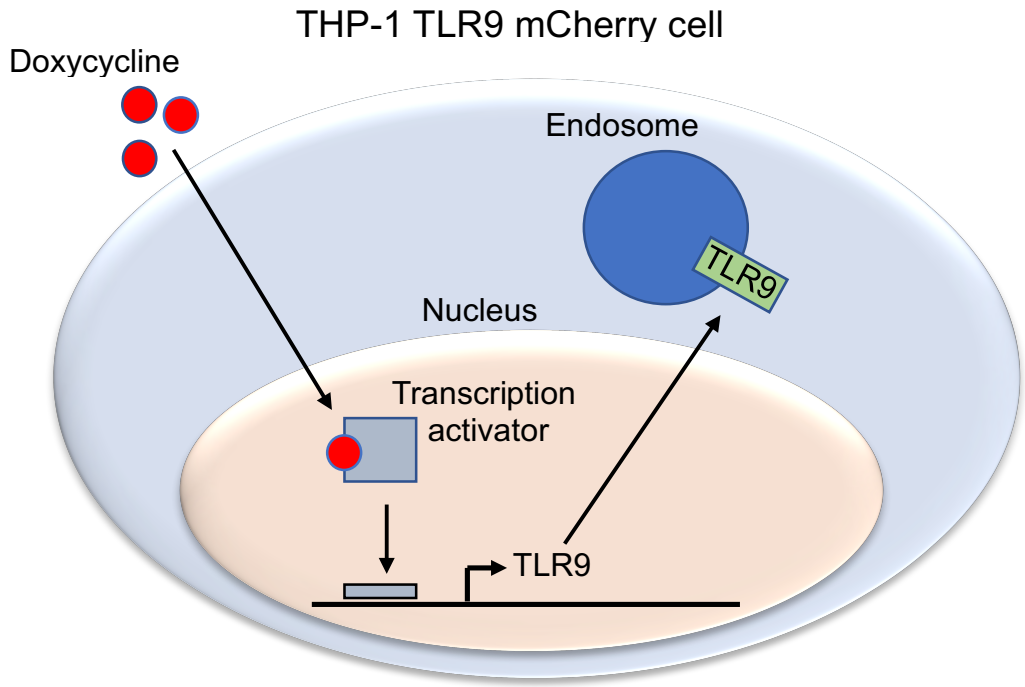


Figure 3.1 Doxycycline regulates the expression of TLR9 in THP-1 TLR9 mCherry cells. Doxycycline binds and triggers a conformational change in a transcription activator. This enables the transcription activator to attach to the promoter region of the downstream *TLR9* gene. TLR9 protein is produced and expressed in endosomes.

Additionally, the C-terminal end of TLR9 in these cells are artificially tagged with mCherry which is a monomeric red fluorescent protein derived from monomeric red fluorescent protein 1 (mRFP1)[79].

THP-1 TLR9 mCherry cells were cultured and maintained in THP-1 medium with puromycin (0,25 μg/ml). Puromycin is a selection marker for TLR9 mCherry. Puromycin resistance is encoded from the same plasmid as TLR9 mCherry, hence the cells expressing the plasmid will also be resistant to puromycin. The cells not carrying the plasmid will neither show resistance against puromycin and will consequently die. When seeding THP-1 TLR9 mCherry cells for experiments, 400 000 cells/well were seeded in regular THP-1 medium (2 ml) in 6-well plates.

3.1.4 HEK293XL/TLR9 cells

Human embryonic kidney (HEK) 293 cells were originally made by transforming the cells using sheared fragments of adenovirus type 5 DNA[80]. This cell line is particularly propitious in molecular biology because the insertion of plasmid vectors containing artificially incorporated genes can hijack the cells' normal protein synthesis, forcing the cell to produce the inserted plasmid gene[81].

HEK293XL cells overexpressing TLR9 (HEK293XL/TLR9) (Invivogen) were included in experiments to evaluate and control the function of CpG-ligands used in this project. HEK293XL cells are adherent cells and were grown and cultured in DMEM with the following additives: 10% FCS, L-glutamine (100 μg/ml), penicillin (100 units/ml) and streptomycin (0,1 mg/ml). When splitting cells, they were detached using trypsin/EDTA. For experiments, 150 000 cells/well were seeded in medium (2 ml) in 6-well plates.

3.2 Differentiation of THP-1 cells

Differentiation of cell lines is particularly convenient since isolation and obtainment of primary tissue cells, like macrophages and pDCs, can be quite comprehensive and the cells can be difficult to grow and culture *ex vivo*[82]. A differentiation process is generally characterized by a loss of cell proliferation[83].

In this project, THP-1 TLR9 mCherry and wild type THP-1 cells were differentiated with phorbol 12-myristate 13-acetate (PMA), recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) together with recombinant human interleukin-4 (rhIL-4) or left

undifferentiated. PMA differentiation is frequently used in research to study other TLRs and it was therefore interesting to try this differentiation protocol when studying TLR9. Differentiation with GM-CSF and IL-4 was conducted as an alternative protocol to obtain an IFNB1 response which turned out to be an issue in PMA-differentiated cells. Undifferentiated THP-1 TLR9 mCherry cells are shown in figure 3.2.

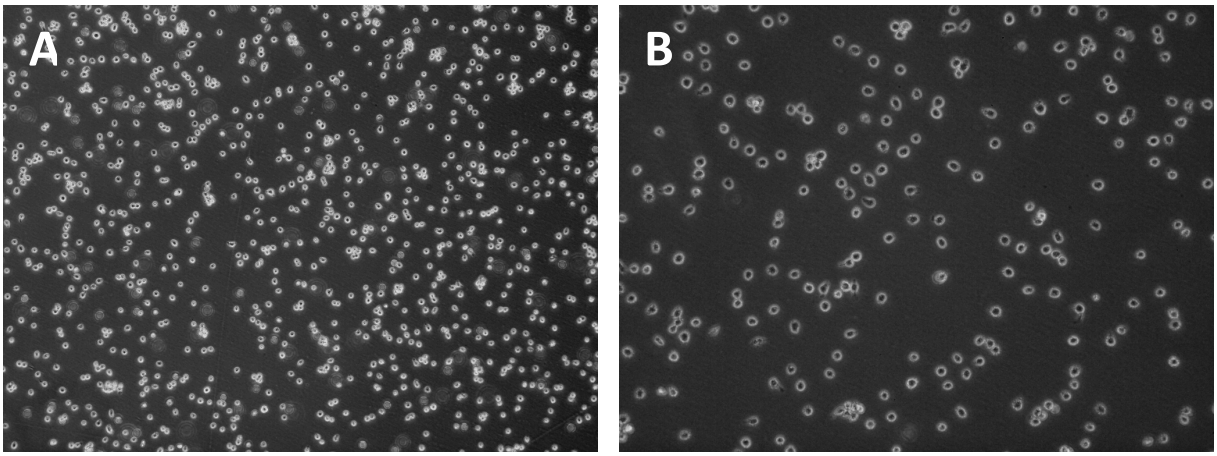


Figure 3.2 Undifferentiated THP-1 TLR9 mCherry cells. Undifferentiated THP-1 TLR9 mCherry cells imaged with A) 10X objective and B) 20X objective.

3.2.1 Reagents

Table 3.2 Reagents used in differentiation of cells. Name, distributor and catalog number of reagents used for differentiation of cells.

Name	Distributor	Cat. Number
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich/Merck	P8139
rhGM-CSF	R&D Systems	215-GM
rhIL-4	PEPRO TECH	200-04

3.2.2 Differentiation with PMA

In order to provide a macrophage-like morphology, wild type THP-1 and THP-1 TLR9 mCherry cells were seeded with phorbol 12-myristate 13-acetate (PMA) (40 ng/ml or 60 ng/ml) for 72 hours followed by 48 hours rest time in PMA-free medium before stimulation. Differentiation with PMA abolishes cell proliferation almost completely and makes the cells adherent[83]. THP-1 TLR9 mCherry cells differentiated with PMA are shown in figure 3.3.

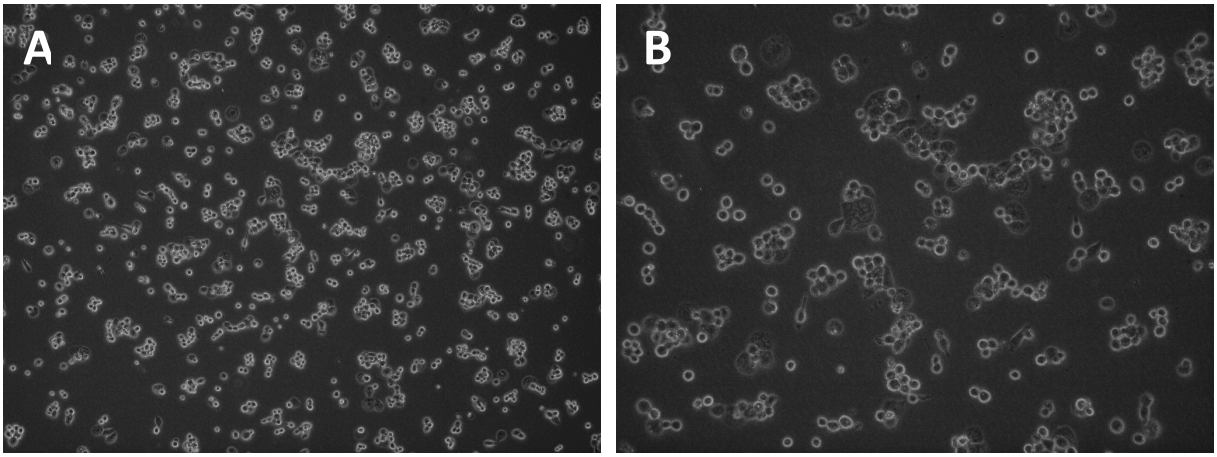


Figure 3.3 PMA-differentiated THP-1 TLR9 mCherry cells. THP-1 TLR9 mCherry cells differentiated with PMA (40 ng/ml) for 3 days imaged with A) 10X objective and B) 20X objective.

3.2.3 Differentiation with rhIL-4 and rhGM-CSF

An alternative differentiation protocol was applied involving recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and recombinant human interleukin-4 (rhIL-4). This differentiation procedure provided an immature DC-like morphology characterized by a ruffled cytoplasm with small spikes[84]. THP-1 TLR9 mCherry cells were seeded with rhIL-4 (200 ng/ml) and rhGM-CS (100 ng/ml) for a total of 5 days prior to stimulation. New medium with the same concentration of freshly added rhIL-4 and rhGM-CSF was given to the cells on day 3. When differentiating with rhIL-4 and rhGM-CSF, the cells did not become adherent, which resulted in the need for centrifugation at 200g for 5 min before medium change and lysis. THP-1 TLR9 mCherry cells differentiated with rhIL-4 and rhGM-CSF are shown in figure 3.4.

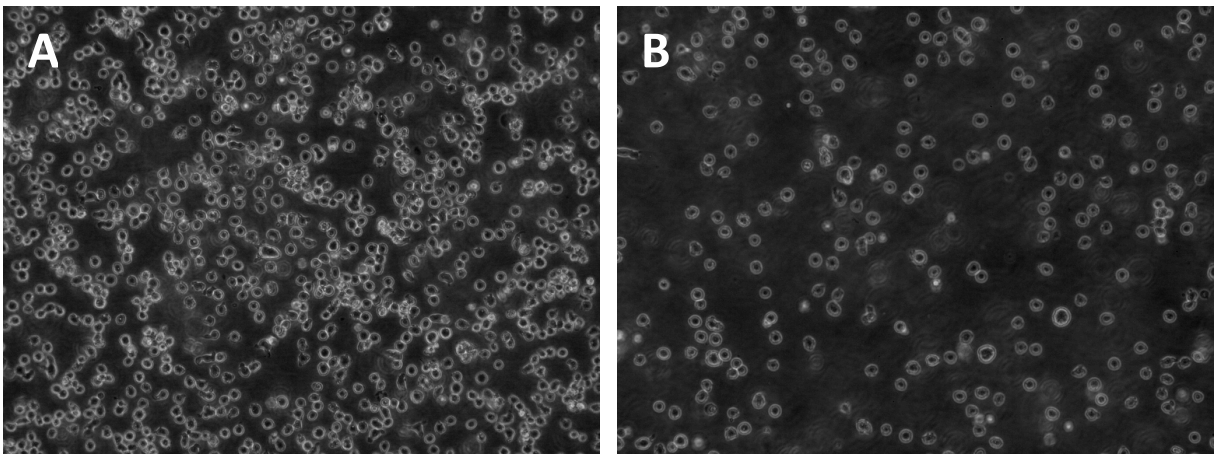


Figure 3.4 GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells. THP-1 TLR9 mCherry cells differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for 5 days imaged with A) 10X objective and B) 20X objective.

3.3 Transient transfection with small interfering RNA (siRNA)

Transfection is a technique used to deliver for instance nucleic acids like siRNAs to the interior of a cell. siRNA transfection was performed in order to silence the expression of Rab39a so that the function of this protein could be examined in the context of TLR9 signaling. siRNAs are small non-coding RNAs which together with RNA-induced silencing complex (RISC) will bind and degrade complementary mRNA molecules within the cell[85].

Rab39a siRNA oligos and AllStar negative control siRNA were transfected into the cells using two different transfection reagents: Lipofectamine RNAiMAX or Lipofectamine 3000, which embraces and delivers siRNA oligos to the cells. Opti-Mem was used to dissolve siRNA oligo and transfection reagent, making it easier to form liposomes containing siRNA.

RPMI 1640 medium supplemented with 10% FCS, L-glutamine (100 μg/ml) and β-mercaptoethanol (0,05 mM) was used when seeding cells for siRNA transfection experiments. Antibiotics were excluded to reduce cellular stress when cells were treated with siRNA. THP-1 TLR9 mCherry cells were spun down at 100g for 7 minutes, dissolved in antibiotic-free medium (10 ml) and counted on Z2 Coulter® Particle Count and Size Analyzer. 400 000 cells/well were seeded in antibiotic-free medium (2 ml) in 6-well plates. Differentiated cells were treated with siRNA 24 hours after seeding while undifferentiated cells were exposed to siRNA right after seeding.

3.3.1 Reagents

Table 3.3 Reagents used in siRNA transfection. Name, distributor and catalog number of reagents used in siRNA transfection experiments.

Name	Distributor	Cat. Number
AllStar Negative control siRNA	Qiagen	S103650318
β-mercaptoethanol	Sigma-Aldrich/Merck	60-24-2
Fetal calf serum (FCS)	Gibco	10270-106
L-glutamine	Sigma-Aldrich/Merck	G7513
Lipofectamine® 3000	Invitrogen	L3000-015
Lipofectamine® RNAiMAX	Invitrogen	13778-150
Opti-Mem®	Gibco	11058-021
Rab39a #5 siRNA	Qiagen	SI02663276
Rab39a #6 siRNA	Qiagen	SI02663283
Rab39a #7 siRNA	Qiagen	SI04439918
RPMI 1640 medium	Gibco	A10491-01

3.3.2 siRNA transfection using Lipofectamine RNAiMAX

siRNA transfection with Lipofectamine RNAiMAX was performed using forward transfection technique (Figure 3.5). Opti-Mem was preheated to 37° C before two separate mixes were made for each sample. Mix1 contained Opti-Mem (240 µl) and RNAiMAX (5 µl), and mix2 contained Opti-Mem (240 µl) and siRNA oligo or AllStar negative control siRNA (2 µl). Mix1 was vortexed and incubated for 5 min at room temperature before the two mixes were carefully blended and incubated at room temperature for 20 min. The final solution was added to cells dropwise giving a final concentration of 16 nM of siRNA in each well (Figure 3.5). The cells were treated with siRNA for 24 hours before the medium was changed and further treatments were performed.

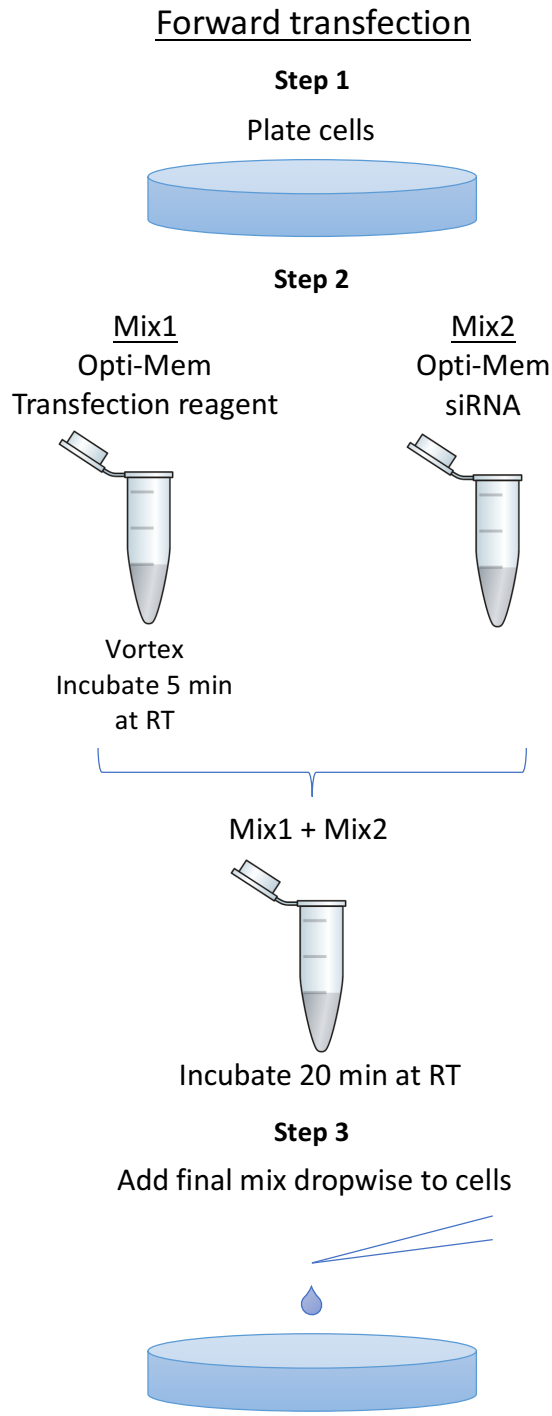
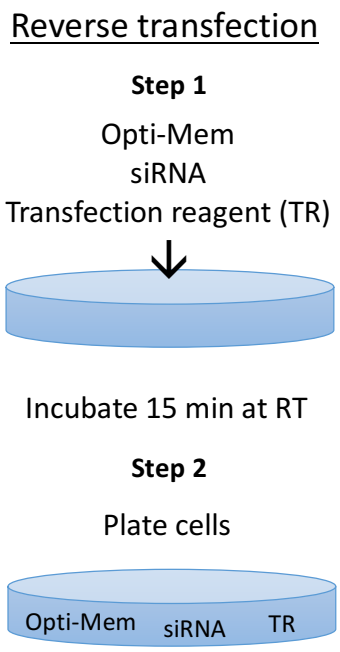


Figure 3.5 Forward transfection technique for siRNA transfection. Cells are plated (Step 1) before two separate mixes containing Opti-Mem + transfection reagent (Mix1) and Opti-Mem + siRNA (Mix2) are prepared. Mix1 is vortexed quickly and incubated for 5 min at room temperature before mix1 and mix2 are carefully combined and incubated for 20 min at room temperature (Step 2). The final mix is added to cells dropwise (Step 3).

3.3.3 siRNA transfection using Lipofectamine 3000 (LF3000)

Transfection using Lipofectamine 3000 (LF3000) was performed with forward transfection technique, in the same way as described in section 3.3.2. However, Mix2 contained Opti-Mem (240 µl) and siRNA oligo or AllStar negative control siRNA (4 µl) giving a final concentration of 32 nM of siRNA when the final mix was added to wells. The cells were treated with siRNA for 48 hours before the medium was changes and further treatments were carried out.



Transfection using LF3000 was also performed with reverse transfection technique (Figure 3.6) in combination with the LF3000 manufacturer's protocol. Before cells were seeded in wells, Opti-Mem (250 µl) was added to wells so that it covered the whole bottom. siRNA oligo or AllStar negative control siRNA (4 µl) was added to respective wells before LF3000 (7.5 µl) was added to all wells. Plates with Opti-Mem, siRNA, and LF3000 were incubated at room temperature for 15 minutes. Cell suspension (2 ml) was carefully added to wells giving a final concentration of 36 nM of siRNA in each well (Figure 3.6). The cells were exposed to siRNA for 48 hours before the medium was changed and further treatments were carried out.

Figure 3.6 Reverse transfection technique for siRNA transfection. Opti-Mem is added so that it covers the whole plate bottom before the addition of siRNA and transfection reagent (TR). The plate is incubated for 15 min at room temperature (Step 1). Cells are carefully added to plates containing Opti-Mem, siRNA and transfection reagent (TR) (Step 2).

3.4 Induction of TLR9 expression

THP-1 TLR9 mCherry cells were exposed to Doxycycline (Echelon Bioscience, B-0801) to induce the expression of TLR9. After differentiation, the cells were given fresh medium and treated with Doxycycline (1 µg/ml) for 48 or 24 hours before further stimulations were performed.

3.5 Stimulation of cells

Pathogenic unmethylated CpG DNA is the natural ligand recognized by TLR9. Synthetic CpG ODNs were used to stimulate and activate TLR9. Cells were treated with two different CpG ODNs, particularly CpG-A represented by 2216 and CpG-B represented by 2006 (Table 3.4).

In some experiments, the cells were transfected with CpG ODN using Lipofectamine LTX reagent which made liposomes capsuling the CpG ODNs. LTX was included to deliver CpG to the cells.

Table 3.4 Sequences of CpG ODNs. CpG ODN class A and B are represented by 2216 and 2006. In sequence: capital letters indicate phosphodiester bases while lower case letters indicate phosphorothioate bases. The palindromic region in CpG-A 2216 is underlined[55].

CpG Class	Name	Sequence
CpG-A	2216	5'-ggGGGACGA:TCGTCgggggg-3'
CpG-B	2006	5'-tcgtcgttttgtcgttttgtcgtt-3

Lipopolysaccharide (LPS) is the ligand recognized by TLR4[86]. Stimulation with LPS was included to activate TLR4 to investigate whether THP-1 TLR9 mCherry cells responded in the same way as wild type THP-1 cells to this ligand. Stimulation of cells was done in 1 ml medium.

3.5.1 Reagents

Table 3.5 Reagents used in stimulation of cells. Name, distributor and catalog number of ligands and reagents used in stimulation of cells.

Name	Distributor	Cat. Number
CpG ODN 2006	Biomers	00202305-1
CpG ODN 2006	TIB MOLBIOL	1611821
CpG ODN 2006	TIB MOLBIOL	1712649
CpG ODN 2216	TIB MOLBIOL	10668-3237
Fetal calf serum (FCS)	Gibco	10270-106
Lipofectamine™ LTX Reagent	Invitrogen	15338-100
RPMI 1640 medium	Gibco	A10491-01
Ultrapure K12 LPS from *E.coli*	Invivogen	tlrl-peklps

3.5.2 Stimulation with CpG ODNs

Stimulated with CpG ODNs were executed after induction of TLR9. Fresh medium was given to the cells before they were stimulated with CpG. The cells were stimulated with CpG ODNs at desired concentrations ranging from 0,5-10 μM for 2 or 3 hours.

Cells were stimulated with CpG by pre-diluting CpG-stock in RPMI 1640 which was further added to cells, or CpG-stock was added directly to cells at desired concentrations. In certain experiments, CpG was complexed with Lipofectamine LTX to transfect the cells and deliver CpG intracellularly. A stimulation solution was prepared by adding LTX (0,375 ul) to RPMI 1640. CpG was added and the solution was incubated at room temperature for 30 min before

100 µl was added dropwise to the cells. The amount of RPMI 1640 and CpG depended on the final concentration wanted in wells. In unstimulated samples, the amount of CpG was replaced by RPMI 1640.

3.5.3 Stimulation with Lipopolysaccharide (LPS)

Prior to LPS stimulation, the cells were given fresh THP-1 medium. K12 LPS was prepared by a short vortex for 45 sec, followed by sonication at room temperature for 1 min and a second vortex for 45 sec. K12 LPS (1 mg/ml) was diluted 1:1000 in RPMI 1640 with 10% FCS. The LPS dilution was heated in 37° C for 5 min before 100 µl was added to each well giving a final concentration of 100 ng/ml of LPS. Cells were stimulated with LPS for 2 hours.

3.6 Inhibition of endocytosis

Dynasore is an inhibitor of dynamin[87] which is a protein responsible for fission of clathrin-coated vesicles formed during endocytosis[50]. This inhibitor was used to investigate whether signaling from TLR9 was dynamin-dependent in THP-1 TLR9 mCherry cells. Cells were given fresh THP-1 medium and Dynasore (Sigma-Aldrich/Merck, 304448-55-3) was diluted 1:310 in RPMI 1640. 100 µl was added to each well giving a final concentration of 100 µM of Dynasore for 30 min prior to stimulation with CpG. The cells were exposed to Dynasore for a total of 3,5 hours with CpG stimulation for the last 3 hours.

3.7 Lysis of cells for qRT-PCR

Following stimulation, cells were washed once in Dulbecco's Phosphate Buffered Saline (PBS, Sigma, D8537-500ML) (1 ml) and lysed with Qiasol (Qiagen, 79306) (750 µl). Cell lysates were transferred to Eppendorf tubes and stored in -80° C for further analysis.

3.8 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) procedure

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a method used to amplify and quantify mRNA expression of a certain gene of interest. In this project, qRT-PCR was used to examine the mRNA expression of cytokines induced by TLRs in response to different stimuli and treatments. Following lysis of cells, RNA was extracted and then

converted to cDNA mediated by the enzyme reverse transcriptase. Subsequently, qRT-PCR was performed.

3.8.1 The principle of qRT-PCR

qRT-PCR involves three main steps. First, cDNA is heated to 95°C to denature the two strands. Second, the samples are cooled down to 60°C and the primers hybridize to specific nucleotides of the available cDNA strands and third, DNA polymerase will elongate the primers, creating a copy of the particular gene of interest. These three steps equal one cycle which is repeated 40 times. In this project, a TaqMan probe was used to measure the amount of amplified product. This is a short oligonucleotide sequence with a fluorophore attached to the 5' end, and a quencher attached to the 3' end of the probe. The TaqMan probe will hybridize to cDNA template in between the forward and reverse primers, and as long as the probe is intact, the quencher will inhibit signaling from the fluorophore. When DNA polymerase starts extending the primers, it will eventually reach and degrade the TaqMan probe, releasing the fluorophore from the probe and enable fluorescence signaling (Figure 3.7). The intensity of the fluorescence detected is proportional to the amount of product in the sample[88]. After some cycles, when the fluorescence from the product is higher than the background fluorescence, the reaction reaches a certain threshold-value. The number of cycles it takes to reach above this threshold-value is termed the threshold cycle (C_T) value. The C_T-value is used to calculate the absolute amount of RNA in a sample[89].

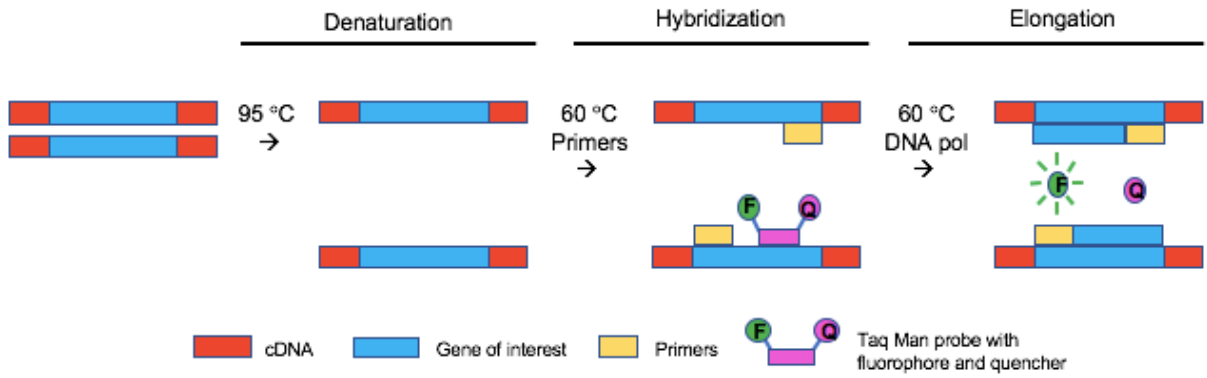


Figure 3.7 Principle of TaqMan qRT-PCR. One cycle in the qRT-PCR reaction involves denaturation of cDNA, hybridization of primers and TaqMan probe and elongation by DNA polymerase. As the reaction proceeds, DNA polymerase will degrade the TaqMan probe and the fluorophore is released from the quencher enabling fluorescence signaling.

3.8.2 Reagents

Table 3.6 Reagents used in qRT-PCR procedure. Name, distributor and catalog number of reagents used in qRT-PCR procedure.

Procedure	Name	Distributor	Cat. Number
RNA extraction	Chloroform EMSURE®	Sigma-Aldrich/Merck	67-66-3
	RNeasy mini kit	Qiagen	74106
cDNA synthesis	5X Reaction mix	Thermo Scientific	R1362
	Maxima enzyme mix	Thermo Scientific	K1642
	Water, nuclease-free	Thermo Scientific	R0581
qRT-PCR	PerfeCTa® qPCR FastMix® UNG, ROX	Quanta Bioscience	84079
	Water, nuclease-free	Thermo Scientific	R0581
	IFNB1 TaqMan probe	Thermo Fisher	HS01077958_s1
	IL-12B TaqMan probe	Thermo Fisher	HS01011518_m1
	Rab39a TaqMan probe	Thermo Fisher	HS00380029_m1
	TBP TaqMan probe	Thermo Fisher	HS00427620_m1
	TNFα TaqMan probe	Thermo Fisher	HS00174128_m1

3.8.3 qRT-PCR procedure

3.8.3.1 RNA extraction and measurement

Cell lysates were thawed on ice and chloroform (150 µl) was added to each sample. The samples were mixed by vigorous shaking for 15 sec and then centrifuged at 11600xg for 15 min at 4° C. The transparent supernatant (200 µl) was recovered into a new 1.5 ml Eppendorf tube. RNA was extracted using the Qiagen RNeasy mini kit with on-column DNase digest according to the manufacturer's instructions (Appendix I).

RNA concentrations were measured using NanoDrop® Spectrophotometer ND-1000 at 260 nm. Pure RNA has a 260/280-ratio of ~2,0 and a 260/230-ratio between 1,8 and 2,2. The concentration of RNA was adjusted to 50 ng/µl or to the lowest RNA concentration measured and stored in -80°.

3.8.3.2 Complementary DNA (cDNA) synthesis

In order to perform a qRT-PCR, RNA has to be converted into complementary DNA (cDNA). This process is carried out by the enzyme Reverse Transcriptase (RT), which generates single-stranded DNA from a template of RNA. RNA sample, nuclease-free H_2O and 5X Reaction mix were thawed on ice for ~20 min and RNA samples were vortexed and spun down. A master

mix was made according to table 3.7. The total volume of master mix and the total volume of reaction mix for cDNA synthesis are also listed in table 3.7.

Table 3.7 Contents of master mix and total volume of reaction mix for cDNA synthesis. Contents of master mix and total volume of reaction mix for cDNA synthesis for both high (50 ng/µl) and low (<50 ng/µl) RNA concentrations. The amounts in the table equal to one sample.

	50 ng/µl RNA	**< 50 ng/µl RNA**
5X Reaction mix	4 µl	4 µl
Maxima enzyme mix containing reverse transcriptase (RT) enzyme	2 µl	2 µl
Nuclease-free H_2O	5 µl	0 µl
Total volume master mix	11 µl	6 µl
RNA sample	9 µl	14 µl
Total volume reaction mix	20 µl	20 µl

Master mix (11/6 µl) and RNA samples (9/14 µl) was added to respective tubes in an 8-tube strip (0,2 ml) (Thermo Fisher, N8010580). Two controls for the qRT-PCR procedure were also included: one control to verify the master mix containing nuclease-free H_2O instead of RNA sample and one control without RT enzyme to check if DNA was present in the sample. In the latter control, the amount of enzyme mix was replaced by nuclease-free H_2O. All samples were vortexed and spun down before cDNA synthesis. The instrument, C1000™ Thermal Cycler, was used to perform cDNA synthesis which was run at the following thermocycler program: 10 min 25° C, 30 min 50° C, 5 min 85° C, ∞ 4°. If necessary, cDNA was stored in -20° C before qRT-PCR.

3.8.3.3 qRT-PCR

TaqMan probes, cDNA samples, nuclease-free H_2O and PerfeCTa® qPCR FastMix® UNG were thawed on ice for ~30 min. cDNA was vortexed and spun down before each sample was diluted 1:10 in nuclease-free H_2O in an 8-tube strip. One master mix for each TaqMan probe was made according to table 3.8.

Table 3.8 Contents of master mix for qRT-PCR. The amounts in the table equal to one sample.

Reagents	**Amount**
PerfeCTa® qPCR FastMix® UNG	10 µl
TaqMan probe	1 µl
Nuclease-free H_2O	4 µl

Master mix (15 µl) was added to duplicate wells in a MicroAmp® Fast 96-Well Reaction Plate (Thermo Fisher, 4346907). Diluted cDNA sample (5 µl) was added to respective wells and the plate was covered with an optical plastic film and centrifuged at 1500g for 2 min. qRT-PCR was run in StepOnePlus^{TM} RT PCR cycler at the following program: 2 min 50° C, 10 min 95° C, (15 sec 95° C, 60 sec 60° C) X40.

3.8.4 Calculations

To analyze the data obtained from qRT-PCR, the $\Delta\Delta C_T$- method was utilized which determines the relative change in gene expression[90]. First, the difference in CT-value (ΔC_T) of the gene of interest (GOI) between a treated sample and an untreated control sample was calculated according to the following equation:

$\Delta C_{T,\,GOI} = 2\text{^}(CT_{GOI\ untreated\ control} - CT_{GOI\ treated\ sample})$

This was also done for a control gene, usually a housekeeping gene, which is expressed at the same level independent of treatment. In this project, TATA-binding protein (TBP) was used as a control gene. When ΔC_T-values were calculated for both the gene of interest and the control gene, the ΔC_T-value for the gene of interest was normalized to the ΔC_T-value for the control gene using the equation:

$\Delta C_{T,\,GOI} / \Delta C_{T,\,control\ gene}$

The normalized values were used to make a bar chart visualizing the amount of gene of interest in the samples relative to the untreated sample. Standard deviations (SD) for either technical replicates of biological replicates/triplicates were calculated and visualized in the bar chart made in GraphPad Prism 8.0.0.

3.9 Statistical analysis

Statistical analysis included Two-way ANOVA with Sidak's multiple comparisons test and was performed in GraphPad Prism 8.0.0. Significant changes are represented by a P-value lower than 0,05 and are marked by *.

4 Results

Toll-like receptors are a group of innate immune receptors responsible for the detection of invading pathogens and the initiation of an immune response[10]. TLR9 is activated in endosomes and recognizes unmethylated CpG-rich nucleic acids coming from virus or bacteria[43,44]. While TLR9 located to early endosomes leads to activation of IRF7 and production of type I IFNs, further sorting to late endosomes and lysosomes activates NF-κB which promotes the production of proinflammatory cytokines[51,56,71]. These two signaling pathways are thought to be cancer suppressing and cancer promoting respectively[38]. In research, synthetic CpG ODNs are used to stimulate and activate TLR9. In this project, two classes of CpG ODNs were used, CpG-B 2006 and CpG-A 2216.

Much of the previous work studying trafficking and signaling from TLR9 has been performed in murine model systems or non-immune cells. The need for a model system more similar to the human immune system, in particular, human pDCs, is therefore of high relevance. This can contribute to reveal how cancer promoting vs. cancer suppressing features of TLR9 is regulated. Preliminary data indicate that Rab39a is a potential regulator of TLR9 trafficking (Grøvdal, Unpublished data).

4.1 Characterization of TLR9 responses in PMA-differentiated THP-1 TLR9 mCherry cells

THP-1 cells expressing inducible TLR9 mCherry were initially used to characterize and determine if they responded to TLR9 ligands. These cells are stably transfected with plasmids containing the *TLR9* gene which is also tagged with the red fluorescent protein mCherry. Doxycycline is used to induce the expression of TLR9.

Cells were first treated with PMA for 3 days to induce differentiation. Exposure to PMA makes the monocytic THP-1 TLR9 mCherry cells adopt a macrophage-like morphology, and they become adherent[83]. The cells were then treated with Doxycycline for 48 hours to induce the expression of TLR9 prior to stimulation with CpG-B 2006 or CpG-A 2216 for 2 hours. Previous experiments in the lab showed that TLR9 expression was induced after 2 hours incubation time with Doxycycline (Cemalovic, Grøvdal, Unpublished data). Therefore, a 2 hours incubation time with CpG was considered as sufficient to induce TLR9-responses in THP-1 TLR9 mCherry cells.

4.1.1 Lipofectamine LTX-complexed CpG-B 2006 induces TNFα, but not IFNB1, expression in PMA-differentiated THP-1 TLR9 mCherry cells

To investigate if TLR9 could signal in THP-1 TLR9 mCherry cells, the expression of TNFα and IFNB1 mRNA was measured after CpG stimulation. CpG was complexed with the transfection reagent Lipofectamine LTX to properly deliver the CpG intracellularly where TLR9 is expressed. THP-1 TLR9 mCherry cells were differentiated with PMA (40 ng/ml) for 3 days before respective samples were treated with Doxycycline (1 µg/ml) for 48 hours to induce expression of TLR9. The cells were then stimulated with Lipofectamine LTX-complexed CpG-B 2006 or CpG-A 2216 (1 µM) for 2 hours. Figure 4.1 A illustrates the treatment schedule of the cells. Following stimulation, cells were lysed and assayed for TNFα and IFNB1 mRNA expression by qRT-PCR. The results showed a marked increase in mRNA levels of TNFα after treatment with Doxycycline and stimulation with CpG-B 2006 (Figure 4.1 B). However, there was no detectable increase in mRNA levels of IFNB1 in response to CpG-B 2006 (Figure 4.1 C) in these cells. CpG-A 2216 failed to induce both cytokines, indicating that THP-1 TLR9 mCherry cells respond poorly to this type of CpG (Figure 4.1 B and C) using this protocol (Figure 4.1 A). The results suggest that THP-1 TLR9 mCherry cells are capable of responding to CpG-B 2006, but fail to induce IFNB1 expression in response to the same ligand.

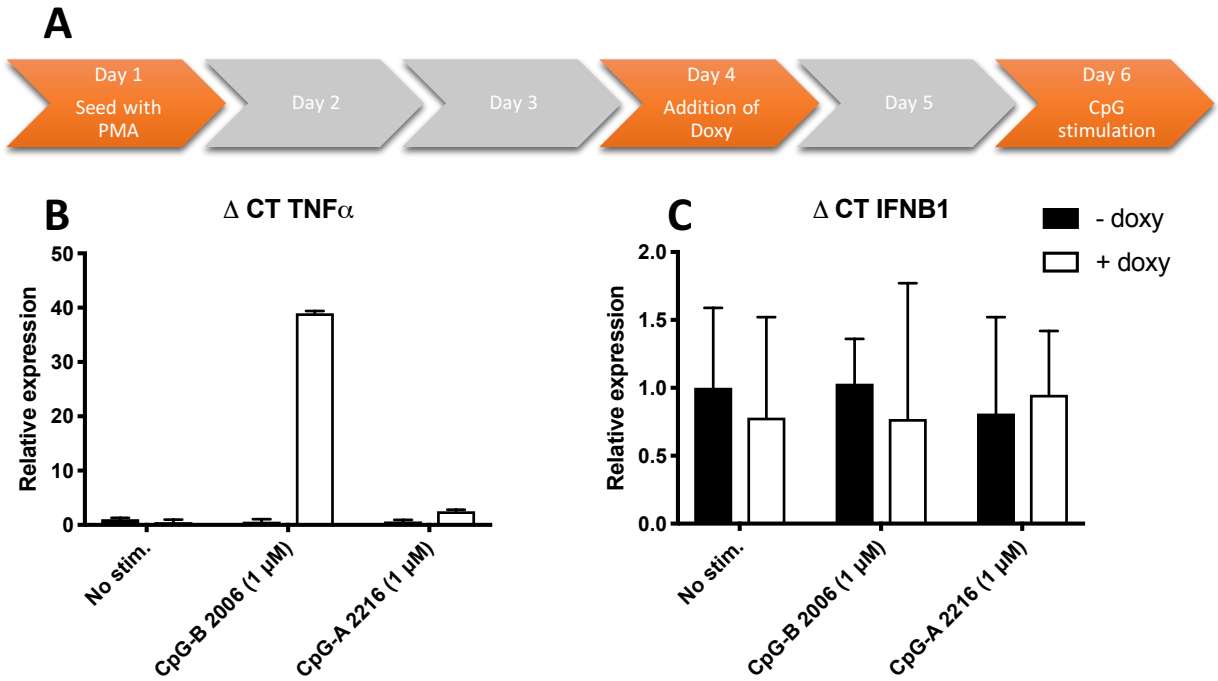


Figure 4.1 Stimulation of PMA-differentiated THP-1 TLR9 mCherry cells with Lipofectamine LTX-complexed CpG. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα and C) IFNB1 mRNA induction in PMA-differentiated THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with Lipofectamine LTX-complexed CpG-B 2006 (1 µM) or CpG-A 2216 (1 µM) for 2h. THP-1 TLR9 mCherry cells

were differentiated with PMA (40 ng/ml) for 3 days before respective samples were treated with Doxycycline (1 μg/ml) for 48h to induce expression of TLR9 prior to CpG stimulation. CpG was complexed with transfection reagent Lipofectamine LTX to deliver CpG intracellularly. Fold induction of TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

4.1.2 Lipofectamine LTX-complexed CpG-B 2006 induces TNFα, but not IFNB1, expression in undifferentiated THP-1 TLR9 mCherry cells

Undifferentiated THP-1 TLR9 mCherry cells were also tested for responsiveness to CpG since PMA differentiation could possibly affect TLR9 activation in these cells. This was done by omitting the differentiation step. THP-1 TLR9 mCherry cells were plated and treated with Doxycycline (1 μg/ml) for 48 hours prior to CpG stimulation. CpG-B 2006 and CpG-A 2216 (1 μM) were complexed with Lipofectamine LTX and added to cells for 2 hours. Figure 4.2 A illustrates the treatment schedule of the cells. Cells were subsequently lysed and assessed for TNFα and IFNB1 mRNA induction by qRT-PCR. The results showed a small increase in TNFα expression after treatment with Lipofectamine LTX-complexed CpG-B 2006 (Figure 4.2 B). However, undifferentiated cells failed to induce IFNB1 mRNA levels in response to CpG (Figure 4.2 C), in likeness with PMA-differentiated THP-1 TLR9 mCherry cells (Figure 4.1 C). The results show that undifferentiated and PMA- differentiated cells respond similarly to CpG-B 2006, but fail to induce IFNB1 in response to the same ligand.

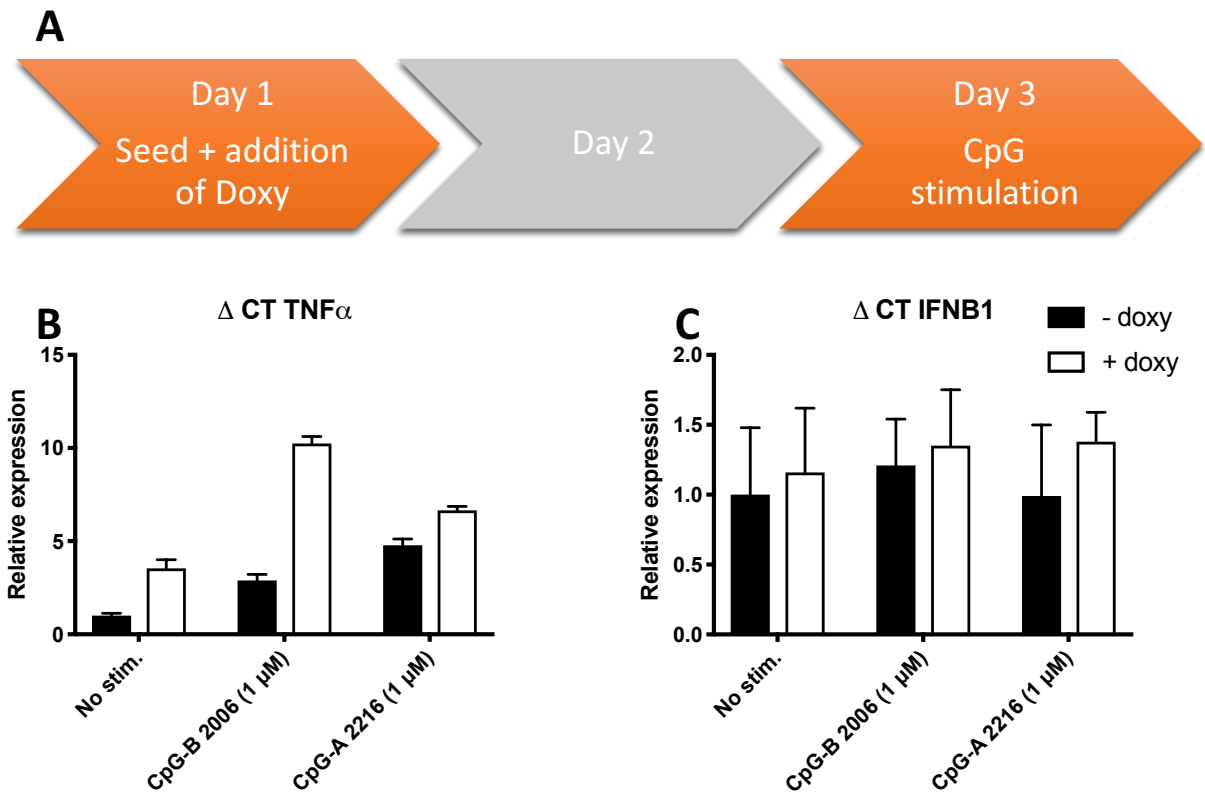


Figure 4.2 Stimulation of undifferentiated THP-1 TLR9 mCherry cells with Lipofectamine LTX-complexed CpG. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα and C) IFNB1 mRNA induction in undifferentiated THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with Lipofectamine LTX-complexed CpG-B 2006 (1 μM) or CpG-A 2216 (1 μM) for 2h. Doxycycline (1 μg/ml) was added for 48h to respective samples to induce expression of TLR9 prior to CpG stimulation. CpG was complexed with transfection reagent Lipofectamine LTX to deliver CpG intracellularly. Fold induction of TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

4.1.3 Lipofectamine LTX-complexed CpG-B 2006 induces TNFα, but not IFNB1, expression in HEK293XL/TLR9 cells

To check whether the CpG ODNs used in this project were functional, they were tested in a well-characterized system utilizing HEK293XL cells overexpressing TLR9. These cells were seeded and after 2 days stimulated with Lipofectamine LTX-complexed CpG-B 2006 (1 μM) or CpG-A 2216 (1 μM) for 2 hours. Figure 4.3 A illustrates the treatment schedule of the cells. The cells were then lysed and assayed for TNFα and IFNB1 mRNA induction by qRT-PCR. The results showed that CpG-B 2006 gave a potent induction of TNFα in these HEK293XL cells (Figure 4.3 B), while none of the CpG ODNs gave any induction of IFNB1 (Figure 4.3 C). These results confirmed that CpG-B 2006 is functional, while CpG-A 2216 is unable to trigger responses in this system. CpG-B 2006 failed to induce IFNB1expression in these cells,

which might indicate that HEK293XL cells lack signaling components needed to induce IFNB1 downstream of TLR9.

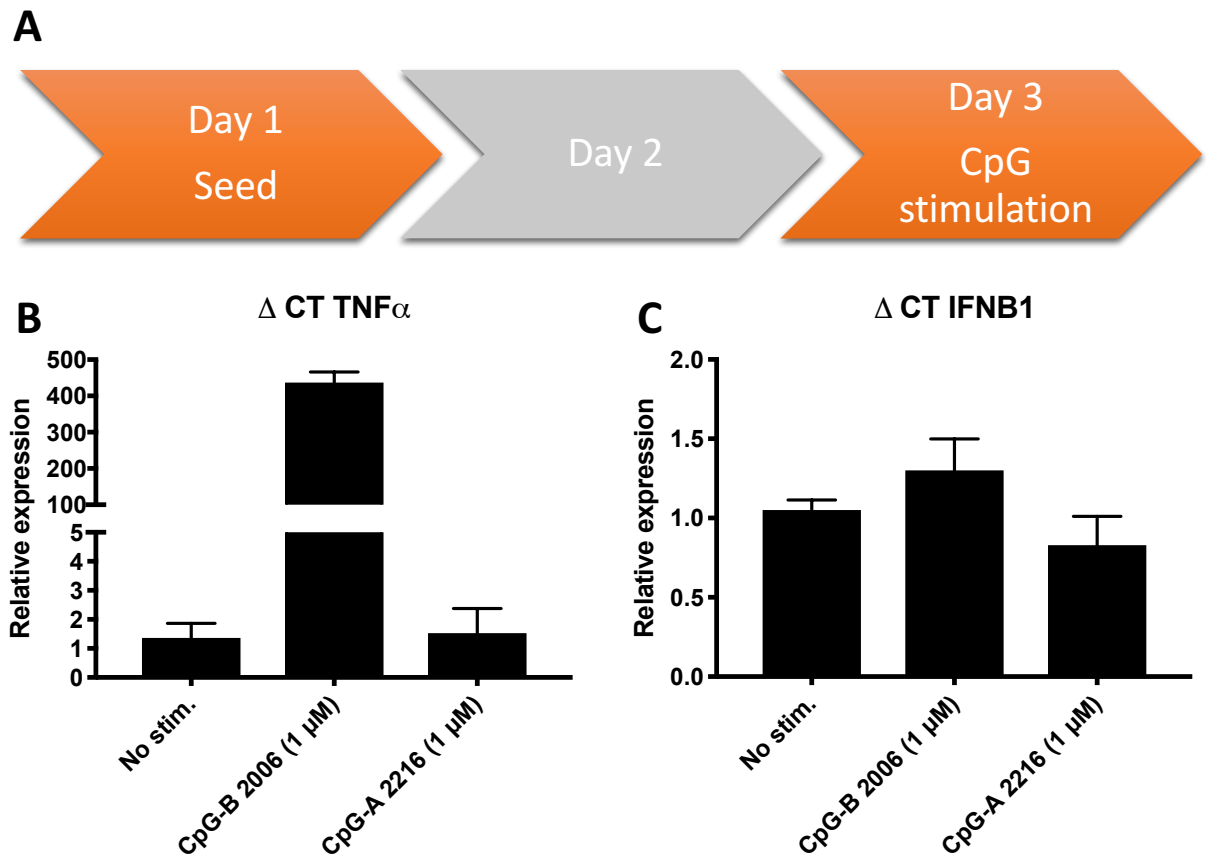


Figure 4.3 Testing CpG ODNs in HEK293XL/TLR9 cells. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα and C) IFNB1 mRNA induction in HEK293XL cells overexpressing TLR9 left untreated (No stim.) or stimulated with Lipofectamine LTX-complexed CpG-B 2006 (1 µM) or CpG-A 2216 (1 µM) for 2h. CpG was complexed with transfection reagent Lipofectamine LTX to deliver CpG intracellularly. Fold induction of TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells and normalized to the housekeeping gene TATA-binding protein (TBP). Results are presented as the mean with SD of two biological replicates.

4.1.4 PMA-differentiated THP-1 TLR9 mCherry cells induced marked IFNB1 expression in response to TLR4 ligand LPS

Since CpG failed to induce IFNB1 in THP-1 TLR9 mCherry cells it was suspected that this could be due to a defect in their ability to induce this cytokine. Wild type THP-1 cells do not express endogenous TLR9, but TLR4[91]. These cells were therefore included as a control cell line to investigate whether THP-1 TLR9 mCherry cells could induce IFNB1 in a similar way as wild type THP-1 cells in response to TLR4 ligand LPS. To determine this, THP-1 TLR9 mCherry cells and wild type THP-1 cells were differentiated with PMA (40 ng/ml) for 3 days before cells were left untreated or treated with Doxycycline (1 µg/ml) for 48 hours to induce expression of TLR9. The cells were then stimulated with either Lipofectamine LTX-complexed

CpG-B 2006 (1 μM) or LPS (100 ng/ml) for 2 hours before they were assayed for TNFα and IFNB1 mRNA induction by qRT-PCR. Figure 4.4 A illustrates the treatment schedule of the cells. Wild type THP-1 cells did not induce any of the cytokines tested in response to CpG-B 2006 (Figure 4.4 B and C). In line with previous results, THP-1 TLR9 mCherry cells induced TNFα, but not IFNB1, mRNA after treatment with Doxycycline and CpG-B 2006 (Figure 4.4 B and C). Both cell lines induced a potent expression of TNFα and IFNB1 in response to LPS, demonstrating that THP-1 TLR9 mCherry cells are fully capable of inducing an IFNB1 response. THP-1 TLR9 mCherry cells gave a bigger IFNB1 mRNA induction compared to wild type THP-1 cells, while it's opposite for TNFα mRNA induction (Figure 4.4 D and E). Combined, these results demonstrate that THP-1 TLR9 mCherry cells mount a TNFα response to CpG-B 2006 indicating that they express functional TLR9. They fail to induce IFNB1 in response to CpG-B 2006, although they are fully capable of inducing IFNB1 to other stimuli like LPS. This indicates that these cells have fully functional signaling pathways leading to IFNB1-induction.

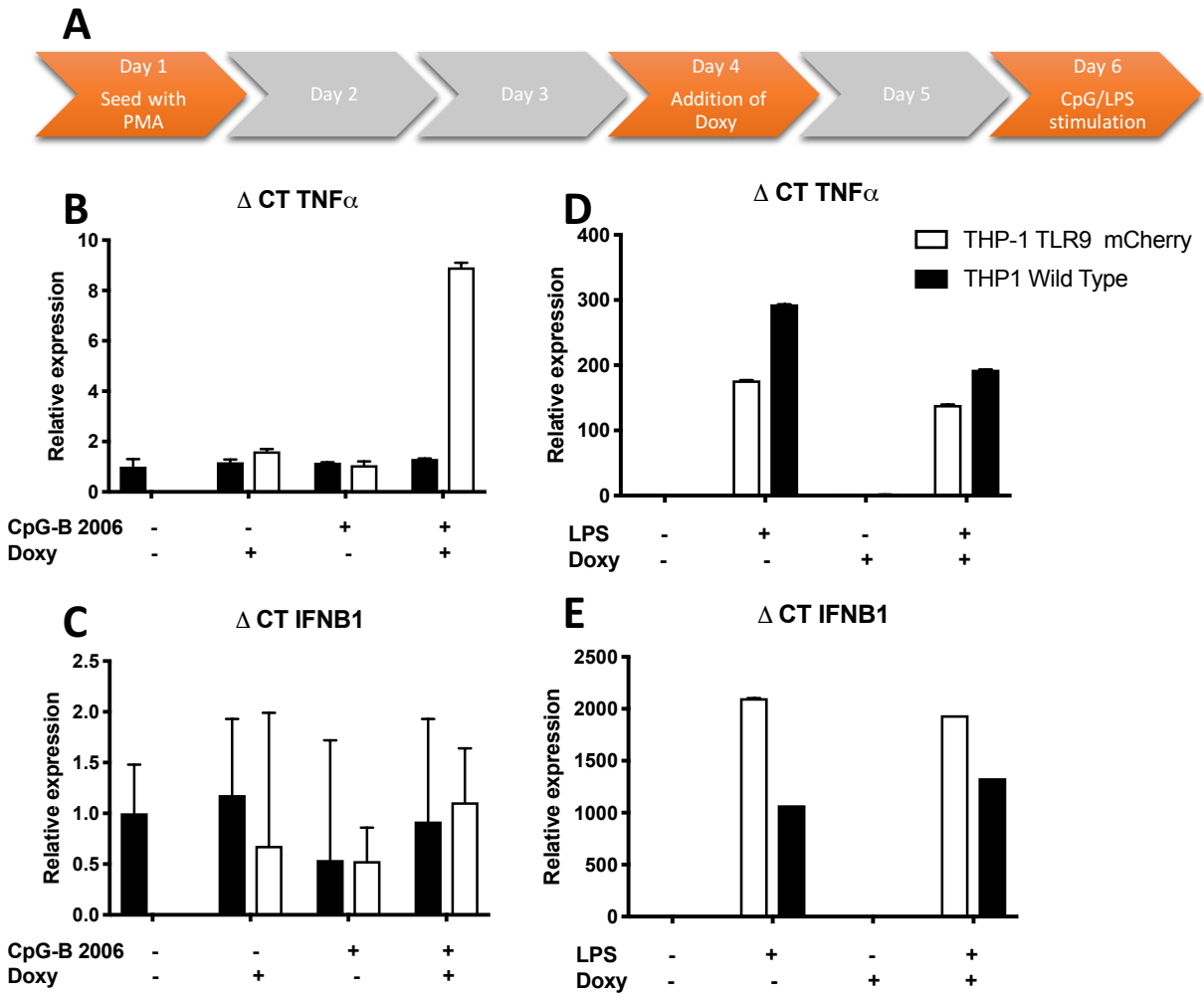


Figure 4.4 Stimulation of PMA-differentiated THP-1 TLR9 mCherry cells and wild type (WT) THP-1 cells with Lipofectamine LTX-complexed CpG-B 2006 or LPS A) Timeline illustrating the workflow and procedure from day to day. B, C, D, E) THP-1 TLR9 mCherry cells and WT THP-1 cells were assessed for their ability to induce TNFα and IFNB1 mRNA in response to B, C) stimulation with CpG-B 2006 (1 μM) for 2h and D, E) stimulation with TLR4 ligand LPS (from E.coli, 100 ng/ml) for 2h. THP-1 TLR9 mCherry cells and WT THP-1 cells were differentiated with PMA (40 ng/ml) for 3 days before respective samples were treated with Doxycycline (1 μg/ml) for 48h to induce expression of TLR9 prior to CpG and LPS stimulation. CpG was complexed with transfection reagent Lipofectamine LTX to deliver CpG intracellularly. Fold induction of TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

4.1.5 PMA-differentiated THP-1 TLR9 mCherry cells induce TNFα and IL-12B, but not IFNB1, in response to higher concentrations of CpG-B 2006

Since there had not been detected any increase in IFNB1 mRNA level in response to CpG, it was investigated if this cytokine could be induced using higher CpG-concentrations than 1 μM. It was also interesting to compare the cytokine induction in response to CpG alone and CpG complexed with Lipofectamine LTX in order to investigate if LTX really was needed to deliver CpG intracellularly. THP-1 TLR9 mCherry cells were differentiated with PMA (40 ng/ml) for 3 days before Doxycycline (1 μg/ml) was added for 48 hours to induce expression of TLR9. Cells were stimulated with CpG-B 2006 (1 μM, 5 μM, 10 μM) or CpG-A 2216 (10 μM) for 2

hours both with and without LTX. Further, the cells were assayed for TNFα, IL-12B, and IFNB1 mRNA induction by qRT-PCR. Figure 4.5 A illustrates the treatment schedule of the cells. IL-12B was included to determine if other proinflammatory cytokines than TNFα also were induced in response to CpG.

The results revealed that inflammatory cytokines such as TNFα and IL-12B were induced in response to increasing concentrations of CpG-B 2006. Potent responses were observed in response to CpG-B 2006 when the concentration was increased to 10 μM. Interestingly, this increase seemed to be independent of LTX, implying that LTX is not necessary to deliver CpG-B 2006 to endosomal TLR9 at these concentrations (Figure 4.5 B and C). A higher concentration of CpG-A 2216 did not induce any marked increase in TNFα or IL-12B (Figure 4.5 B and C). Higher concentrations of CpG, both 2006 and 2216, did not induce noticeable changes in IFNB1 mRNA levels (Figure 4.5 D). These results show that THP-1 TLR9 mCherry cells respond well to 10 μM CpG-B 2006 independently of Lipofectamine LTX, but fail to induce an IFNB1 response even to high concentrations of this ligand. Lipofectamine LTX was excluded from the following experiments.

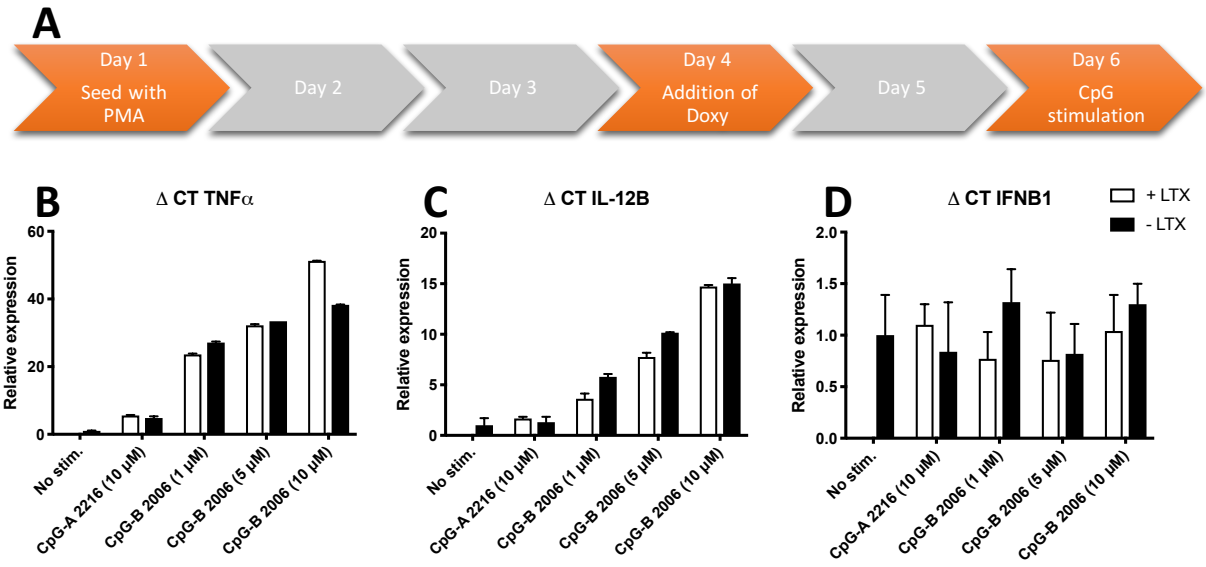


Figure 4.5 CpG stimulation of PMA-differentiated THP-1 TLR9 mCherry cells with and without Lipofectamine LTX. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα, C) IL-12B and D) IFNB1 mRNA induction in PMA-differentiated THP-1 TLR9 mCherry cells in response to stimulation with CpG-A 2216 (10 μM) or increasing concentrations of CpG-B 2006 (1 μM, 5 μM, 10 μM) with and without Lipofectamine LTX for 2h. THP-1 TLR9 mCherry cells were differentiated with PMA (40 ng/ml) for 3 days before Doxycycline (1 μg/ml) was added for 48h to induce expression of TLR9 prior to CpG stimulation. Fold induction of TNFα, IL-12B, and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

4.1.6 Induction of TNFα and IL-12B, but not IFNB1, peaks after 3 hours incubation time with CpG-B 2006 in PMA-differentiated THP-1 TLR9 mCherry cells

Both TNFα and IFNB1 are known to be induced rapidly in response to LPS in macrophages[92]. 2 hours is, however, a short stimulation time, and longer periods of CpG stimulation of THP-1 TLR9 mCherry cells were therefore tested to determine if IFNB1 may be induced at later time-points. THP-1 TLR9 mCherry cells were therefore differentiated with PMA (40 ng/ml) for 3 days before Doxycycline (1 µg/ml) was added for 48 hours to induce expression of TLR9. The cells were then incubated with CpG-A 2216 (10 µM) for 1, 2, 3, 4 and 5 hours and with CpG-B 2006 (10 µM) for 1, 2, 3 and 4 hours before they were assayed for TNFα, IL-12B and IFNB1 mRNA induction by qRT-PCR. Figure 4.6 A illustrates the treatment schedule of the cells.

These results revealed a potent induction of TNFα and IL-12B mRNA after 3 hours of incubation with CpG-B 2006. Interestingly, both TNFα and IL-12B were observed to be induced in this experiment in response to CpG-A 2216. The responses were, however, weaker than the TNFα and IL-12B expression observed in response to CpG-B 2006. TNFα and IL-12B induction in response to CpG-A 2216 was also observed at later time-points, peaking at 4 hours after stimulation. (Figure 4.6 B and C). IFNB1 mRNA was not triggered by either CpG-B 2006 nor CpG-A 2216 at any of the time points tested in this experiment (Figure 4.6 D) in line with previous experiments.

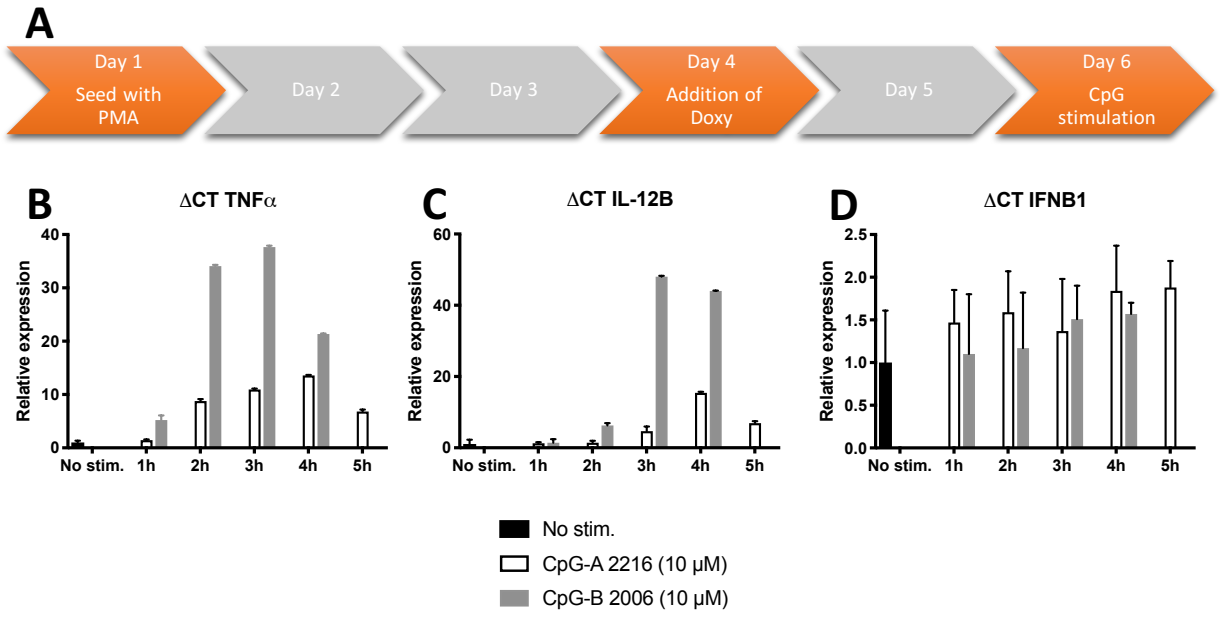


Figure 4.6 PMA-differentiated THP-1 TLR9 mCherry cells incubated with CpG at different time-points. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα, C) IL-12B and D) IFNB1 mRNA induction in PMA-differentiated THP-1 TLR9 mCherry cells in response to stimulation with CpG-A 2216 (10 µM) for 1, 2, 3, 4 and 5h or stimulation with CpG-B 2006 (10 µM) for 1, 2, 3 and 4h. THP-1 TLR9 mCherry cells

were differentiated with PMA (40 ng/ml) for 3 days before Doxycycline (1 μg/ml) was added for 48h to induce expression of TLR9 prior to CpG stimulation. Fold induction of TNFα, IL-12B, and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

4.1.7 Signaling from TLR9 is impaired in PMA-differentiated THP-1 TLR9 mCherry cells pretreated with the endocytosis inhibitor Dynasore

Cells take up CpG via clathrin-dependent endocytosis and TLR9 encounter CpG in endosomes[48]. Dynamin is a protein responsible for fission of clathrin-coated vesicles formed during endocytosis[50]. By using an inhibitor of dynamin, Dynasore, it was possible to investigate whether signaling from TLR9 was dependent on dynamin or not. This was done in PMA-differentiated THP-1 TLR9 mCherry cells that were treated with Doxycycline (1 μg/ml) for 48 hours to induce expression of TLR9. Cells were then treated with Dynasore (100 μM) for 30 minutes to inhibit dynamin before stimulation with CpG-B 2006 (10 μM) for 3 hours. Figure 4.7 A illustrates the treatment schedule of the cells. The cells were subsequently assessed for TNFα, IL-12B, and IFNB1 mRNA induction by qRT-PCR. The results showed that CpG-induced TNFα and IL-12B mRNA levels were significantly reduced in samples pretreated with Dynasore compared to mock-treated samples (Figure 4.7 B, C). Expression of IFNB1 mRNA remained unchanged after dynamin inhibition and CpG stimulation (Figure 4.7 D) which is in line with previous experiments. These results suggest an endosomal localization of TLR9 since CpG-induced responses are impaired when dynamin is inhibited.

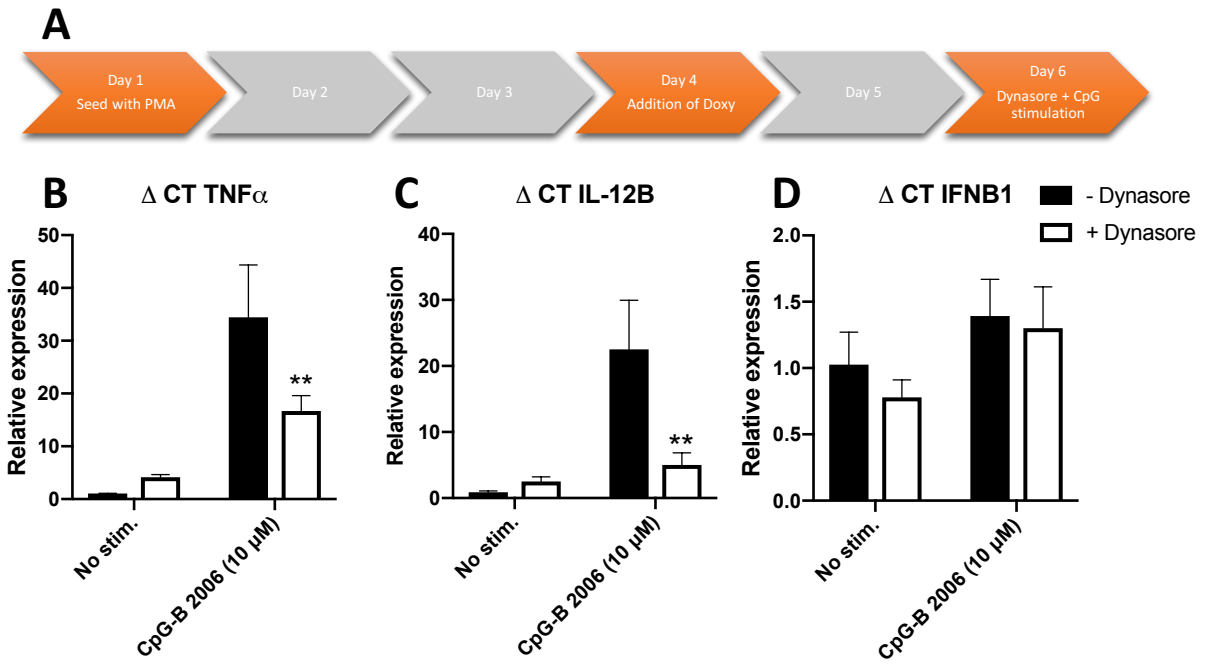


Figure 4.7 Inhibition of dynamin in PMA-differentiated THP-1 TLR9 mCherry cells. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα, C) IL-12B and D) IFNB1 mRNA induction in PMA-differentiated THP-1 TLR9 mCherry cells after treatment with the dynamin inhibitor, Dynasore (100 µM), for 3,5h together with CpG-B 2006 (10 µM) stimulation the last 3h. THP-1 TLR9 mCherry cells were differentiated with PMA (40 ng/ml) for 3 days before Doxycycline (1 µg/ml) was added for 48h to induce expression of TLR9 prior to inhibition of dynamin and CpG stimulation. Fold induction of TNFα, IL-12B, and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). Results are presented as the mean with SD of three individual experiments. Statistical analysis includes Two-way ANOVA with Sidak's multiple comparisons test. ** P<0,006 (TNFα); ** P<0,001 (IL-12B) vs. uninhibited samples.

4.2 Characterization of TLR9 responses in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells

Differentiation of THP-1 TLR9 mCherry cells using PMA gave a potent induction of TNFα and IL-12B in response to CpG, but no IFNB1 induction. This raised the question if another differentiation protocol could provide a better IFNB1 induction in response to CpG. Hence, differentiation using GM-CSF and IL-4 was performed. GM-CSF and IL-4 make THP-1 cells adopt an immature dendritic cell (DC)-like morphology[84]. When doing experiments with GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells, it was observed that they did not become adherent, and remained in suspension. These cells also continued to proliferate after differentiation, which is in line with the literature[84].

4.2.1 GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells induce TNFα and IFNB1 in response to CpG-B 2006

To investigate whether differentiation of THP-1 TLR9 mCherry cells using GM-CSF and IL-4 gave a better starting point for studying signaling from TLR9, the cells were differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for 5 days in total. Fresh cytokines were replenished on day 3 as well as addition of Doxycycline (1 μg/ml) to induce expression of TLR9. The cells were stimulated with increasing concentrations of CpG-B 2006 (0,5 μM, 1 μM, 5 μM, 10 μM) for 3 hours. Cells were also stimulated with CpG-B 2006 (10 μM) for 1, 2, 3, 4 and 5 hours before they were assayed for TNFα and IFNB1 mRNA induction by qRT-PCR. Figure 4.8 A illustrates the treatment schedule of the cells. The results showed that increasing concentrations of CpG-B 2006 gave increasing mRNA induction of both IFNB1 and TNFα when THP-1 TLR9 mCherry cells were differentiated with GM-CSF and IL-4 (Figure 4.8 B and C). Potent responses were observed in response to CpG-B 2006 at a concentration of 10 μM. CpG-induced responses peaked after 3 hours for both cytokines (Figure 4.8 D and E). These results show that THP-1 TLR9 mCherry cells differentiated with GM-CSF and IL-4 induce marked levels of both TNFα and IFNB1 in response to CpG-B 2006 after 3 hours.

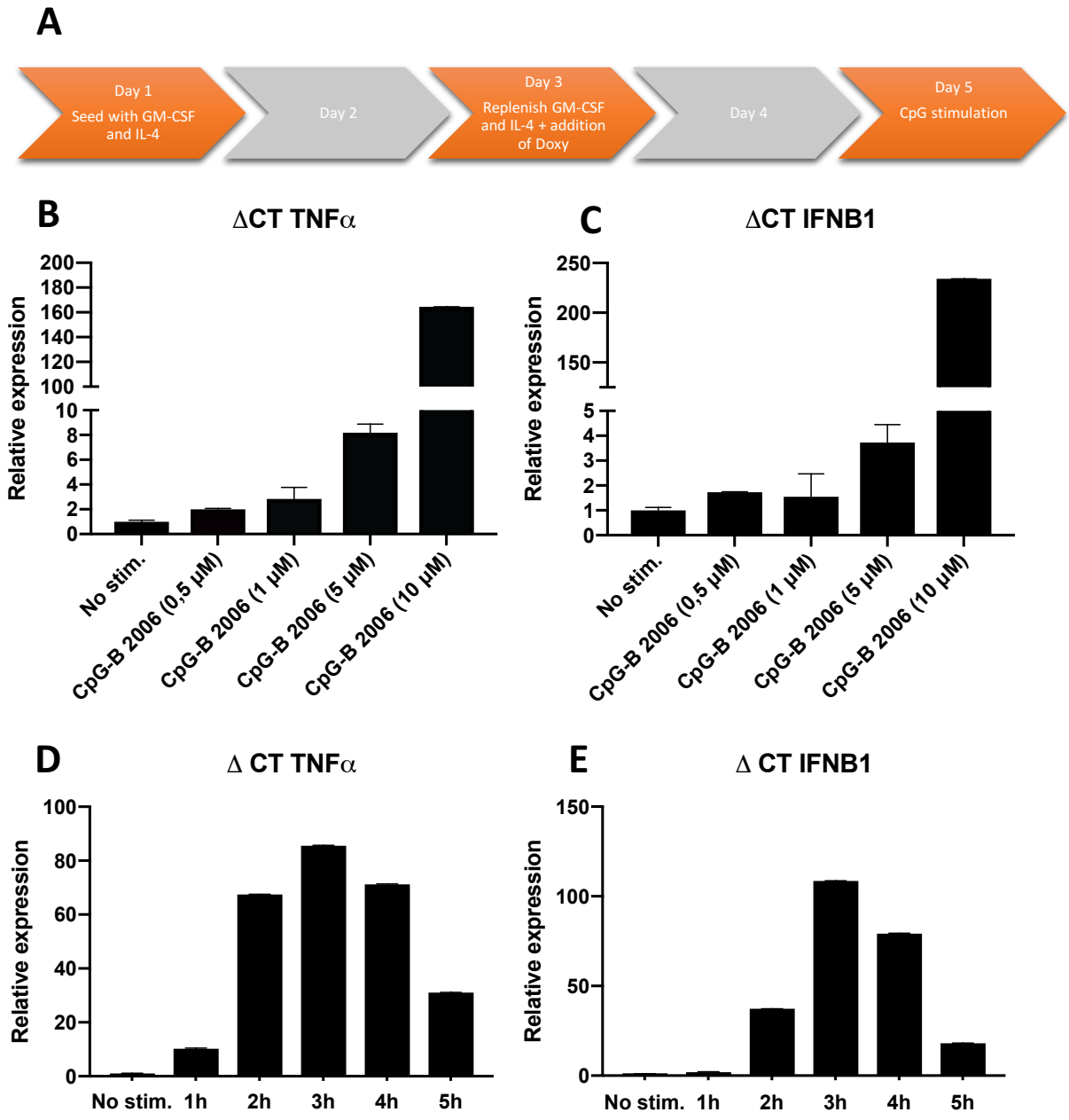


Figure 4.8 GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells stimulated with CpG at different concentrations and time-points. A) Timeline illustrating the workflow and procedure from day to day. B, C, D, E) TNFα and IFNB1 mRNA induction in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells in response to B, C) stimulation with increasing concentrations of CpG-B 2006 (0,5 µM, 1 µM, 5 µM, 10 µM) for 3h and D, E) stimulation with CpG-B 2006 (10 µM) for 1, 2, 3, 4 and 5h. The cells were differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for 5 days with addition of Doxycycline (1 µg/ml) the last 48h to induce the expression of TLR9 prior to CpG stimulation. Fold induction of TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

4.2.2 GM-CSF and IL-4-differentiation of THP-1 TLR9 mCherry cells in combination with PMA

Suspension cells can be more difficult to transfect with siRNA molecules than adherent cells[93]. Since the goal for this thesis was to characterize a cell line optimized for studying trafficking and signaling from TLR9, it was important that the model cell line was possible to transfect with siRNA molecules. THP-1 TLR9 mCherry cells differentiated with GM-CSF and IL-4 are suspension cells, thus it was of great interest to try to make these cells adherent but at the same time keep the immature DC-like morphology. To try this, PMA was added to the system to determine if GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells could adopt adherent properties.

THP-1 TLR9 mCherry cells were differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for 5 days in total with freshly added cytokines and Doxycycline (1 μg/ml) on day 3. PMA (60 ng/ml) was added 20 hours prior to stimulation to make the cells adherent before they were stimulated with CpG-B 2006 (1 μM) for 3 hours. Figure 4.9 A illustrates the treatment schedule of the cells. Cells were then assayed for TNFα and IFNB1 mRNA induction by qRT-PCR. The results revealed that CpG-induction of TNFα and IFNB1 were higher in samples with PMA compared to samples without PMA (Figure 4.9 B and C). However, both cytokines exhibit some basal secretion in unstimulated samples, indicating that PMA in itself may trigger low levels of cytokine production in these cells. Additionally, when looking at the cells in the microscope after PMA treatment, less than half of the cells were attached, suggesting that the cells were a heterogeneous mix.

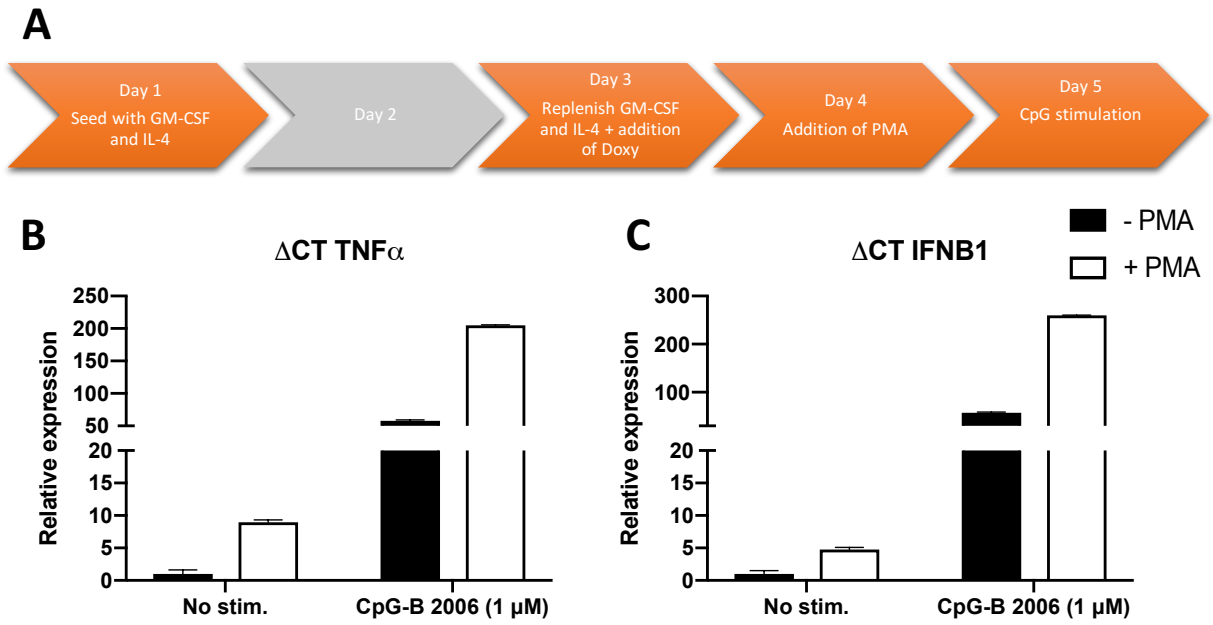


Figure 4.9 CpG stimulation in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells with PMA treatment 20h prior to stimulation. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα and C) IFNB1 mRNA induction in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with CpG-B 2006 (1 µM) for 3h. The cells were differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for 5 days with addition of Doxycycline (1 µg/ml) the last 48h to induce the expression of TLR9. PMA (60 ng/ml) was added to respective samples 20h prior to CpG stimulation. Fold induction of TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

Another attempt in generating attached cells that still signaled sufficiently included trying PMA-treatment before GM-CSF and IL-4-differentiation. The purpose of trying this was to obtain attached cells that could potentially be transfected with siRNA before they were differentiated into immature DC-like cells with GM-CSF and IL-4. IFNB1 was the only cytokine investigated in this experiment since earlier experiments with PMA had given a poor IFNB1 mRNA induction. THP-1 TLR9 mCherry cells were seeded with different concentrations of PMA (20, 40, 60 ng/ml) for 2 days with AllStar negative control siRNA transfection for 24 hours on day 2. AllStar was complexed with Lipofectamine RNAiMAX to deliver siRNA to the cells. After 48 hours with PMA and 24 hours with siRNA treatment, the cells were exposed to GM-CSF (100 ng/ml), IL-4 (200 ng/ml) and Doxycycline (1 µg/ml) for an additional 2 days. Cells were stimulation with CpG-B 2006 (10 µM) for 3 hours before they were assayed for IFNB1 mRNA induction by qRT-PCR. Figure 4.10 A illustrates the treatment schedule of the cells. The results revealed poor induction of IFNB1 in response to CpG. Even though there is a small increase after treatment with 20 ng/ml PMA (Figure 4.10 B), this experimental setup is not adequate to use as a protocol for knock-down experiments. The cells were attached at all PMA concentrations tested, however, they looked macrophage-like and not

immature DC-like. Combined, these results demonstrate that PMA-involvement in the GM-CSF and IL-4 differentiation protocol, only makes the protocol more complicated. PMA does not provide any beneficial aspects in terms of generating attached cells which also induce potent cytokine responses. Hence, PMA was excluded from future experiments.

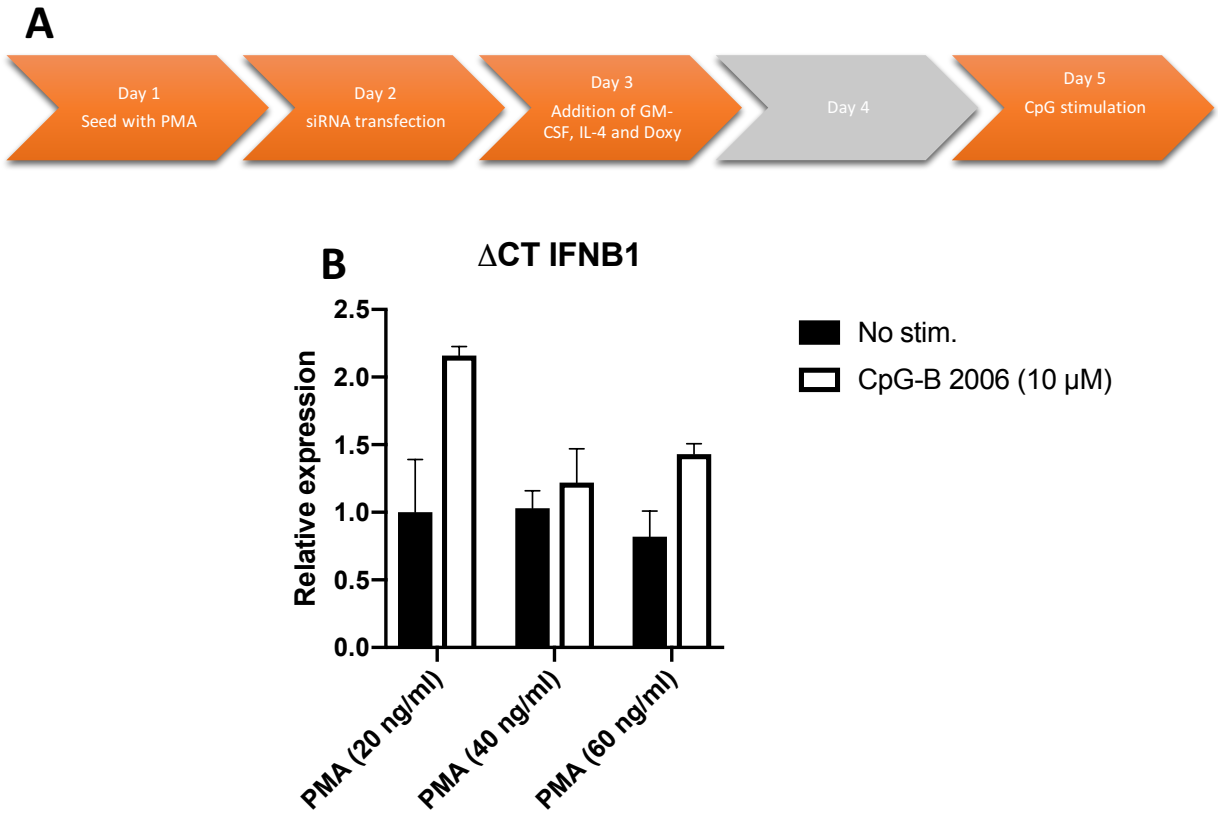


Figure 4.10 CpG stimulation in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells pretreated with PMA. A) Timeline illustrating the workflow and procedure from day to day. B) IFNB1 mRNA induction in THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with CpG-B 2006 (10 µM) for 3h. THP-1 TLR9 mCherry cells were treated with PMA (20 ng/ml, 40 ng/ml, 60 ng/ml) for 48h, with AllStar negative control siRNA transfection (16nM) on day 2 for 24h. siRNA was complexed with Lipofectamine RNAiMAX to deliver siRNA to the cells. The cells were then differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for another 48h. Doxycycline (1 µg/ml) was added together with GM-CSF and IL-4 to induce the expression of TLR9 prior to CpG stimulation. Fold induction of IFNB1 mRNA-levels was assessed by qRT-PCR relative to PMA 20 ng/ml untreated cells (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

4.2.3 Knock-down of Rab39a in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells

So far, differentiation of THP-1 TLR9 mCherry cells using GM-CSF and IL-4 had provided a potent induction of TNFα and IFNB1 in response to CpG. However, these cells were non-adherent which could potentially complicate siRNA transfection. Nevertheless, it was interesting to see if these cells actually could work as a model system to knock-down specific mRNA-molecules. Rab39a is a small GTPase suspected to be involved in the regulation of

TLR9 trafficking and signaling (Grøvdal, Unpublished data). Hence, silencing of this protein could potentially reveal if Rab39a is involved in this mechanism or not.

THP-1 TLR9 mCherry cells were therefore differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for 5 days with replenish of cytokines on day 3. The cells were treated with siRNA oligos targeting Rab39a (#5, #6, #7) (16 nM), AllStar negative control siRNA (16 nM) or exposed to only transfection reagent RNAiMAX for 24 hours. siRNA was complexed with Lipofectamine RNAiMAX to deliver siRNA to the cells using the forward transfection technique. Doxycycline (1 μg/ml) was added 48 hours prior to CpG stimulation to induce the expression of TLR9. The cells were then stimulated with CpG-B 2006 (5 μM) for 3 hours before they were assayed for Rab39a, TNFα and IFNB1 mRNA induction by qRT-PCR. Figure 4.11 A illustrates the treatment schedule of the cells. A potent knock-down was considered to be around 50% compared to AllStar.

The results showed that IFNB1 and TNFα mRNA levels were slightly decreased for oligo #5 and #7 after stimulation with CpG-B 2006 compared to AllStar (Figure 4.11 C, D). However, the poor knock-down of Rab39a in this experiment was considered as insufficient to conclude if Rab39a affects signaling from TLR9 (Figure 4.11 B). Ideally, AllStar and RNAiMAX should give the same mRNA induction of cytokines and the fact that these two are not equal generates some uncertainty regarding the results. Oligo #6 has an off-target; thus, it was decided to disregard this oligo. Together these results indicate that this experimental setup was not ideal to perform siRNA transfection and examine the role of Rab39a regarding TLR9 signaling.

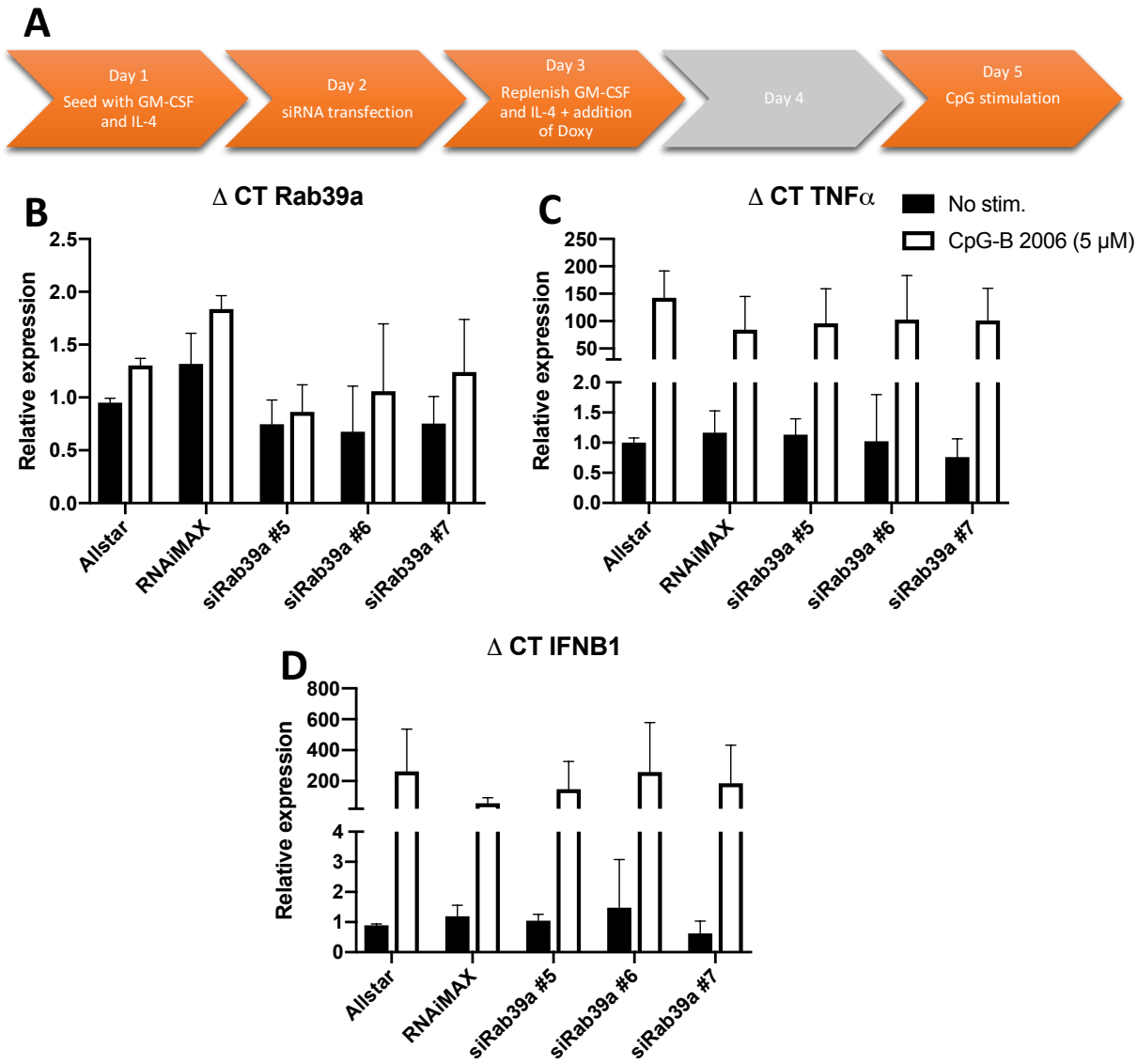


Figure 4.11 Knock-down of Rab39a in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells prior to CpG stimulation. A) Timeline illustrating the workflow and procedure from day to day. B) Rab39a, C) TNFα and D) IFNB1 mRNA induction in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with CpG-B 2006 (5 µM) for 3h. THP-1 TLR9 mCherry cells were differentiated with GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) for 5 days with siRNA transfection on day 2 for 24h. The cells were treated with only RNAiMAX or transfected with AllStar negative control siRNA (16 nM) or siRNA oligos targeting Rab39a (#5, #6, #7) (16 nM). siRNA was complexed with Lipofectamine RNAiMAX to deliver siRNA to the cells using forward transfection technique. Doxycycline (1 µg/ml) was added the last 48h to induce the expression of TLR9 prior to CpG stimulation. Fold induction of Rab39a, TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to AllStar (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). The results are presented as the mean with SD of three individual experiments. Statistical analysis includes Two-way ANOVA with Sidak's multiple comparisons test.

4.3 Characterization of TLR9 responses in undifferentiated THP-1 TLR9 mCherry cells

Due to problems with getting a proper Rab39a knock-down in THP-1 TLR9 mCherry cells differentiated with IL-4 and GM-CSF, we had to look for other setups as potential model systems for studying trafficking and signaling from TLR9. Earlier in this project, it was

observed that undifferentiated THP-1 TLR9 mCherry cells gave some induction of TNFα in response to low concentrations of CpG (1 μM) (Figure 4.2 B). Additionally, in an experiment testing differentiation with IL-3, undifferentiated THP-1 TLR9 mCherry cells were included, and it was observed that undifferentiated cells provided a potent IFNB1 and TNFα induction in response to higher concentrations of CpG (10 μM) (Appendix II). These results enabled the idea that undifferentiated cells might be an option as a model system to study knock-down of Rab39a. This would provide a much shorter and simpler protocol with fewer steps and treatments. Undifferentiated THP-1 TLR9 mCherry cells carry the same monocytic morphology as wild type THP-1 cells and they are suspension cells[77].

4.3.1 CpG-B 2006 induces TNFα and IFNB1 expression after 24 and 48 hours with Doxycycline treatment in undifferentiated THP-1 TLR9 mCherry cells

Until now, the cells had been exposed to Doxycycline for 48 hours prior to CpG stimulation. Results from our group showed that TLR9 was expressed in undifferentiated THP-1 TLR9 mCherry cells after 24 hours incubation time with Doxycycline (Cemalovic, Grøvdal, Unpublished data). Therefore, it was interesting to investigate if TLR9 also signaled at this time point to optimize the protocol even more. Undifferentiated THP-1 TLR9 mCherry cells were seeded with Doxycycline (1 μg/ml) for 24 hours or 48 hours prior to stimulation. The cells were stimulated with CpG-B 2006 (10 μM) for 3 hours before they were assayed for TNFα and IFNB1 mRNA induction by qRT-PCR. Figure 4.12 A illustrates the treatment schedule of the cells. The results showed that 48 hours incubation time with Doxycycline gave a stronger induction of TNFα and IFNB1 compared to 24 hours incubation time in response to CpG. However, 24 hours incubation time gave a marked induction of the two cytokines (Figure 4.12 B and C). The results indicate that Doxycycline treatment for 24 hours before CpG stimulation should be adequate to use in knock-down experiments to evaluate the effect of Rab39a silencing regarding TLR9 signaling. This was also beneficial because it shortened the protocol even more and reduced the number of days from siRNA treatment to CpG stimulation.

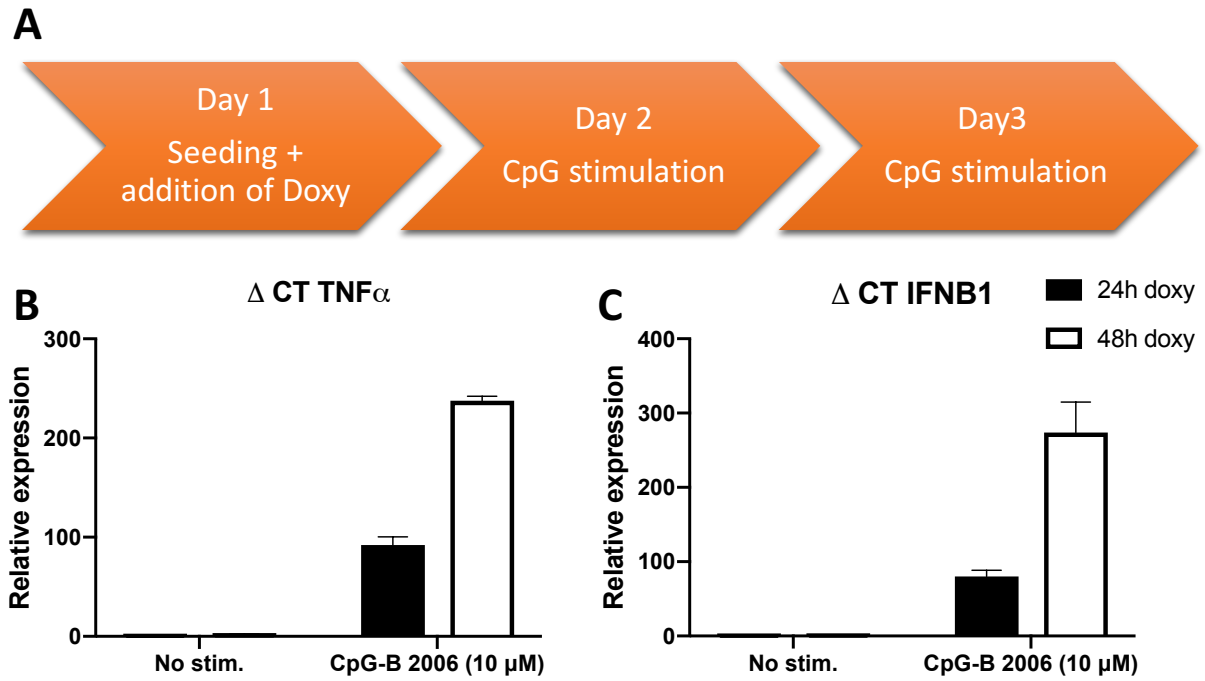


Figure 4.12 CpG stimulation in undifferentiated THP-1 TLR9 mCherry cells after TLR9 induction for 24h and 48h. A) Timeline illustrating the workflow and procedure from day to day. B) TNFα and C) IFNB1 mRNA induction in undifferentiated THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with CpG-B 2006 (10 µM) for 3h. Doxycycline (1 µg/ml) was added for 24h or 48h to induce the expression of TLR9 prior to CpG stimulation. Fold induction of TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to untreated cells and normalized to the housekeeping gene TATA-binding protein (TBP). The results are presented as the mean with SD of three biological replicates.

4.3.2 Knock-down of Rab39a in undifferentiated THP-1 TLR9 mCherry cells

With a new experimental setup, it was time to test if undifferentiated THP-1 TLR9 mCherry cells could work as a model system to study trafficking and signaling from TLR9. Due to their non-adherent properties, the transfection reagent Lipofectamine 3000 (LF3000) was used to deliver siRNA to the cells. LF3000 is considered more effective in transfecting suspension cells compared to RNAiMAX.

At this time, siRNAs targeting Rab39a was not available in the lab. Hence, the experimental setup was tested out with siRNA targeting Rab11a. The results of this experiment revealed a potent knock-down of Rab11a (Appendix III). Based on these promising results, it was interesting to investigate if it was possible to obtain a potent knock-down of Rab39a in these cells. Undifferentiated THP-1 TLR9 mCherry cells were plated and transfected with siRNA oligos targeting Rab39a (#5, #7) (32 nM), AllStar negative control siRNA (32 nM) or exposed to only LF3000 for 48 hours. Forward transfection technique was performed. Doxycycline (1 µg/ml) was added 24 hours prior to lysis before the cells were assayed for Rab39a mRNA induction by qRT-PCR. Figure 4.13 A illustrates the treatment schedule of the cells. The results

revealed some, but not a potent knock-down of Rab39a. Oligo #7 gave better silencing of Rab39a than oligo #5 compared to AllStar (Figure 4.13 B). These results indicate that this experimental setup was not optimal to generate a proper knock-down of Rab39a.

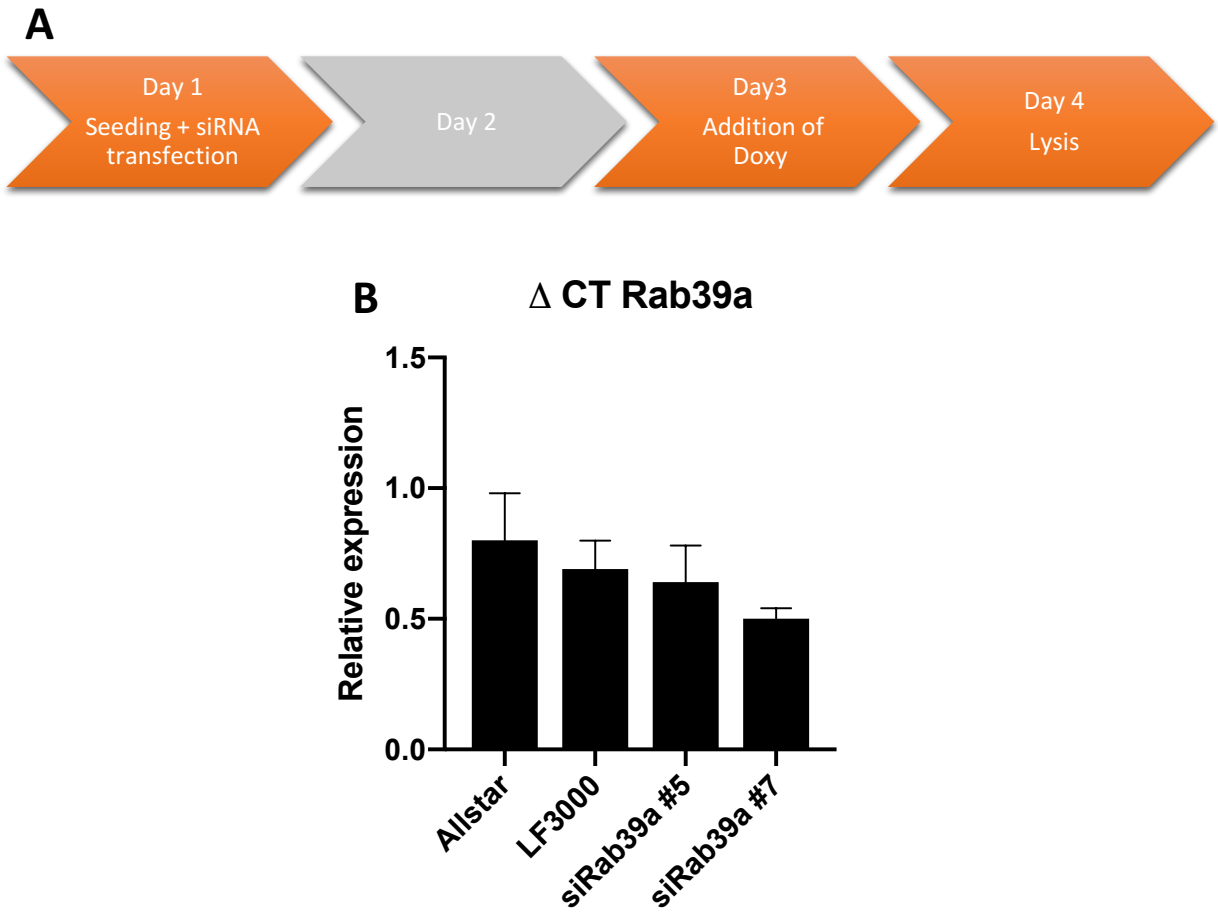


Figure 4.13 Knock-down of Rab39a in undifferentiated THP-1 TLR9 mCherry cells with forward transfection technique. A) Timeline illustrating the workflow and procedure from day to day. B) Rab39a mRNA induction in undifferentiated THP-1 TLR9 mCherry cells treated with only LF3000, AllStar negative control siRNA (32 nM) or siRNA oligos targeting Rab39a (#5, #7) (32nM). SiRNA was complexed with Lipofectamine 3000 to deliver siRNA to the cells using forward transfection technique. The cells were exposed to siRNA for 48h before Doxycycline (1 μg/ml) was added for 24h. Fold induction of Rab39a mRNA-levels was assessed by qRT-PCR relative to AllStar and normalized to the housekeeping gene TATA-binding protein (TBP). The results are presented as the mean with SD of three biological replicates.

In order to obtain a better knock-down of Rab39a, undifferentiated THP-1 TLR9 mCherry cells were tried transfected using the LF3000 manufacturer's protocol together with reverse transfection technique. Undifferentiated THP-1 TLR9 mCherry cells were transfected with siRNA oligos targeting Rab39a (#5, #7) (36 nM), AllStar negative control siRNA (36nM) or exposed to only LF3000 for 48 hours. Doxycycline (1 μg/ml) was added 24 hours prior to lysis before the cells were assayed for Rab39a mRNA induction by qRT-PCR. Figure 4.14 A illustrates the treatment schedule of the cells. The results from this experiment showed a potent knock-down of Rab39a, where oligo #7 provided a slightly better knock-down than oligo #5

compared to AllStar (Figure 4.14 B). This result suggests that Rab39a can be silenced in undifferentiated THP-1 TLR9 mCherry cells using reverse transfection technique in combination with the LF3000 manufacturer's protocol. Hence, undifferentiated THP-1 TLR9 mCherry cells could work as a model system to examine the role of Rab39a concerning TLR9 signaling.

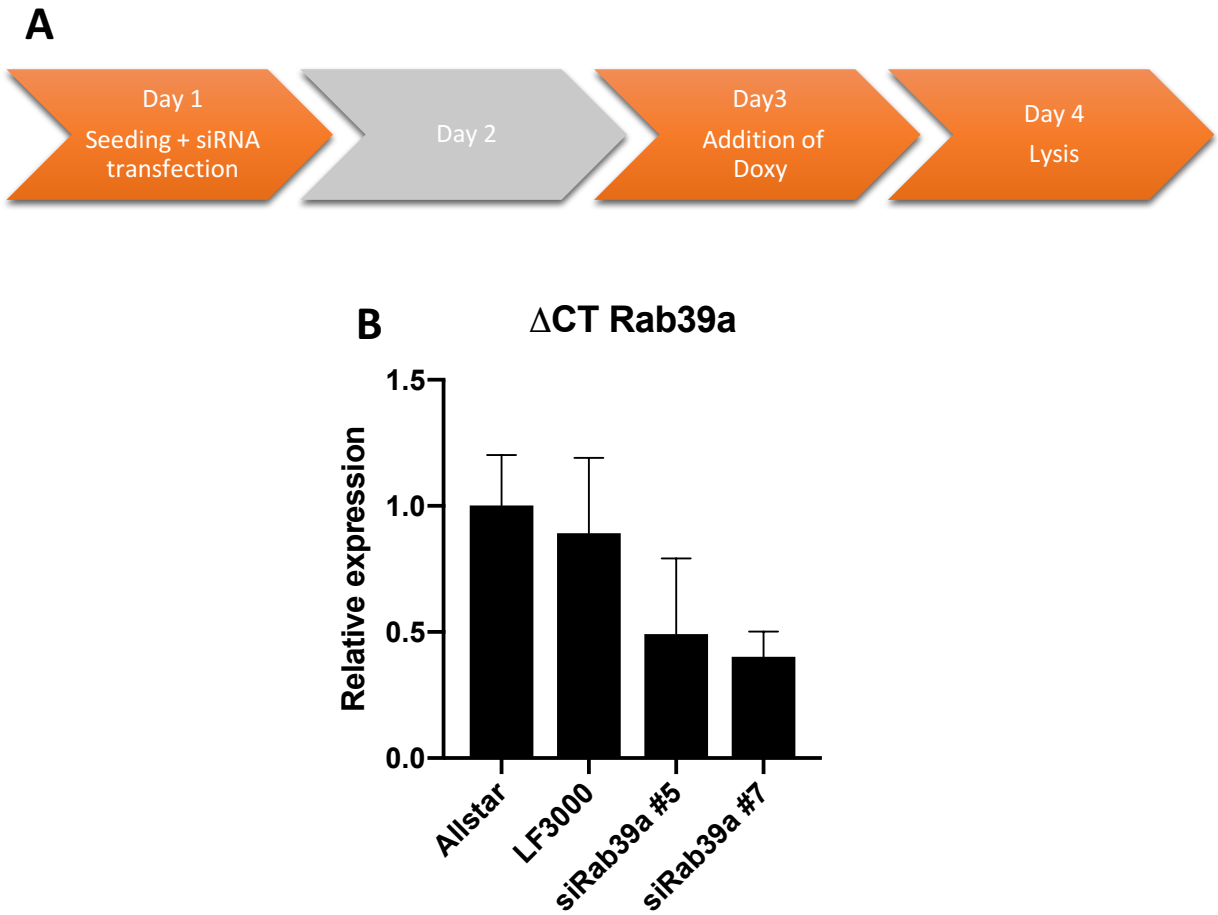


Figure 4.14 Knock-down of Rab39a in undifferentiated THP-1 TLR9 mCherry cells with reverse transfection technique. A) Timeline illustrating the workflow and procedure from day to day. B) Rab39a mRNA induction in undifferentiated THP-1 TLR9 mCherry cells treated with only LF3000, AllStar negative control siRNA (36 nM) or siRNA oligos targeting Rab39a (#5, #7) (36 nM). SiRNA was complexed with Lipofectamine 3000 to deliver siRNA to the cells using reverse transfection technique. The cells were exposed to siRNA for 48h before Doxycycline (1 µg/ml) was added for 24h. Fold induction of Rab39a mRNA-levels was assessed by qRT-PCR relative to AllStar and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

The LF3000 manufacturer's protocol together with the reverse transfection technique had provided the best knock-down of Rab39a in undifferentiated THP-1 TLR9 mCherry cells. Therefore, this experimental setup was used to determine if Rab39a was involved in the regulation of TLR9 signaling. Undifferentiated THP-1 TLR9 mCherry cells were plated and transfected with siRNA oligos targeting Rab39a (#5, #7) (36 nM), AllStar negative control siRNA (36nM) or exposed to only LF3000 for 48 hours. Doxycycline (1 µg/ml) was added 24

hours prior to stimulation with CpG-B 2006 (10 μM) for 3 hours. Figure 4.15 A illustrates the treatment schedule of the cells. Further, cells were assayed for Rab39a, TNFα and IFNB1 mRNA induction by qRT-PCR. The results showed a potent knock-down of Rab39a with oligo #7 (Figure 4.15 B) which reflects the knock-down obtained in the previous experiment (Figure 4.14). Surprisingly, the results revealed an increasing tendency of CpG-induced TNFα and IFNB1 mRNA after Rab39a knock-down compared to AllStar (Figure 4.15 C and D). However, the fact that AllStar and LF3000 are not equal for TNFα and IFNB1 generates some uncertainty regarding the results. Oligo #5 shows some basal level of IFNB1 and TNFα, indicating that the increase observed for these cytokines when stimulated with CpG is not fully trustworthy. These results suggest that undifferentiated THP-1 TLR9 mCherry cells have potential as a model cell line to study intracellular signaling from TLR9. Interestingly, knock-down of Rab39a seemed to increase mRNA levels of TNFα and IFNB1 in response to CpG. Further investigations is, however, needed to verify these findings.

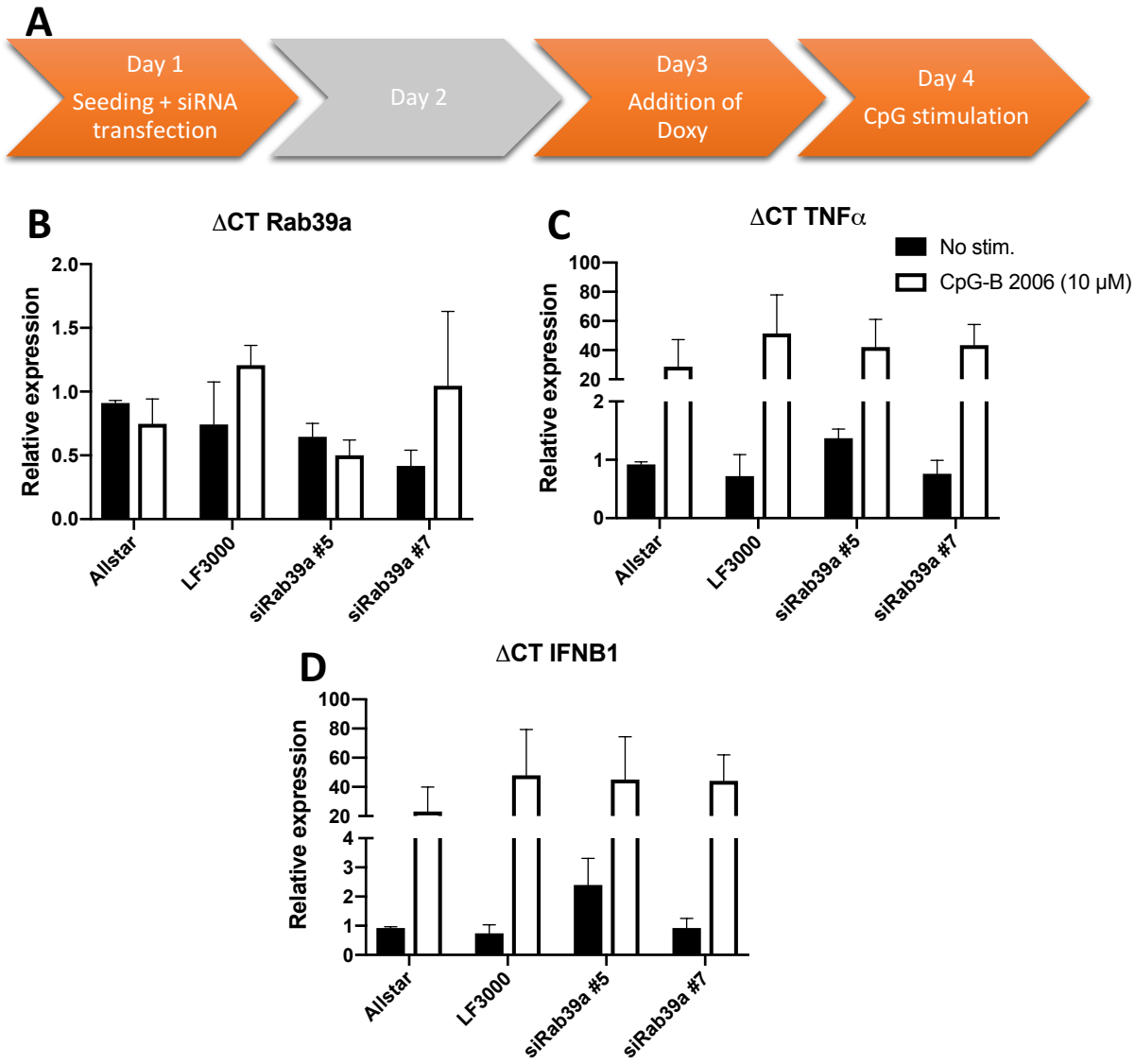


Figure 4.15 Knock-down of Rab39a in undifferentiated THP-1 TLR9 mCherry cells prior to CpG stimulation. A) Timeline illustrating the workflow and procedure from day to day. B) Rab39a, C) TNFα and D) IFNB1 mRNA induction in undifferentiated THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with CpG-B 2006 (10 μM) for 3h. Undifferentiated THP-1 TLR9 mCherry cells were treated with only LF3000, AllStar negative control siRNA (36 nM) or siRNA oligos targeting Rab39a (#5, #7) (36 nM). SiRNA was complexed with Lipofectamine 3000 to deliver siRNA to the cells using reverse transfection technique. The cells were exposed to siRNA for 48h before Doxycycline (1 μg/ml) was added for 24h prior to CpG stimulation. Fold induction of Rab39a, TNFα and IFNB1 mRNA-levels were assessed by qRT-PCR relative to AllStar (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). The results are presented as the mean with SD of three individual experiments. Statistical analysis includes Two-way ANOVA with Sidak's multiple comparisons test.

The results obtained during the work with this project regarding different differentiation protocols and cytokine responses from TLR9 are summarized in table 4.1, while results regarding siRNA transfection of THP-1 TLR9 mCherry cells and knock-down of Rab39a are summarized in table 4.2. The results showed that PMA-differentiated THP-1 TLR9 mCherry cells induced TNFα, but not IFNB1, in response to CpG-B 2006. Both GM-CSF and IL-4-

differentiated and undifferentiated THP-1 TLR9 mCherry cells induced TNFα and IFNB1 in response to CpG-B 2006. However, THP-1 TLR9 mCherry cells left undifferentiated was observed to provide the best starting point for siRNA transfection experiments. The LF3000 manufacturer's protocol in combination with the reverse transfection technique generated a potent knock-down of Rab39a. This experimental setup revealed that CpG-induced TNFα and IFNB1 showed an increasing tendency after knock-down of Rab39a.

Table 4.1 Summarizing table of differentiation protocols, CpG stimulation and subsequent cytokine responses observed in this project. For cytokine responses, (+) indicates a marked response, (-) indicates no response and (+/-) indicates some, but not a strong response.

Differentiation protocol	Resulting cell type	Adherence properties	Doxycycline induces TLR9 expression	CpG stimulation		Cytokine responses	
				CpG-B 2006	CpG-A 2216	TNFα	IFNB1
PMA	Macrophage-like	Adherent	-	-	-	-	-
			-	+	-	-	-
			-	-	+	-	-
			+	-	-	-	-
			+	+	-	+	-
			+	-	+	+/-	-
GM-CSF and IL-4	Immature DC-like	Non-adherent	-	-	-	-	-
			+	-	-	-	-
			+	+	-	+	+
Undifferentiated	Monocytes	Non-adherent	-	-	-	-	-
			-	+	-	-	-
			-	-	+	-	-
			+	-	-	-	-
			+	+	-	+	+
			+	-	+	-	-

Table 4.2 Summarizing table of siRNA transfections performed in this project. The table lists different differentiation protocols, transfection reagents, transfection techniques and siRNA concentrations used and % knock-down of Rab39a obtained in the experiments performed during the work with this project.

Differentiation protocol	Resulting cell type	Adherence properties	Transfection reagent	Transfection technique	siRNA concentration	Rab39a Knock down	
						siRab39a #5	siRab39a #7
GM-CSF + IL-4	Immature DC-like	Non-adherent	Lipofectamine RNAiMAX	Forward	16 nM	24%	18%
Undifferentiated	Monocytes	Non-adherent	Lipofectamine 3000	Forward	32 nM	20%	37,5%
				Reverse	36 nM	51%	60%

5 Discussion

5.1 Distinct differentiation protocols provide varying cytokine induction in response to CpG

In order to characterize and establish a potent model system to study intracellular trafficking and signaling from TLR9, it was important to investigate if the receptor was able to signal in THP-1 cells with inducible expression of TLR9. This project started with differentiating the cells with PMA, providing a macrophage-like morphology. After TLR9 induction using Doxycycline and stimulation with CpG-B 2006 it was observed a strong TNFα response, but no IFNB1 response. Why IFNB1 is not detected after CpG stimulation is difficult to say, but PMA-differentiated THP-1 cells might not express transcription factor IRF7 which promotes the production of type I IFNs downstream of TLR9. PMA-differentiated THP-1 cells are frequently used when studying TLR4. TLR4 uses different adaptor proteins and downstream signal transducers to promote the production of type I IFNs compared to TLR9. PMA-differentiated THP-1 TLR9 mCherry cells induced marked levels of IFNB1 in response to LPS, indicating that these cells are fully capable of inducing IFNB1 in response to other TLR ligands like LPS. This might indicate that proteins downstream of TLR9 are responsible for the absent IFNB1 response. TLR9 uses the same adaptor protein, MyD88, to induce both proinflammatory cytokines and type I IFNs. Since it was observed a TNFα response, it is more likely that there is something downstream of MyD88 which is responsible for the poor IFNB1 response. However, both undifferentiated and GM-CSF and IL-4-differentiated cells provided a strong induction of IFNB1 in response to CpG, indicating that PMA does something to the cells that makes them unable to produce IFNB1 in response to CpG.

In contrast to PMA differentiation, GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells induced marked levels of both IFNB1 and TNFα in response to CpG. This differentiation protocol provided an immature DC-like morphology[84]. GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells remained as suspension cells which are considered more difficult to transfect[93]. Since the goal for this thesis was to characterize a model cell line that could be transfected with siRNA, it was hypothesized that the non-adherent properties of these cells could complicate siRNA transfection. Therefore, it was attempted to generate adherent cells which at the same time signaled stably through TLR9. PMA was added to the system to try to make the cells attached after they were differentiated into immature DC-like cells. A 20 hours

incubation time with PMA generated high levels of both IFNB1 and TNFα in response to CpG compared to samples without PMA. However, less than half of the cells were attached. The reason why it was detected elevated cytokine levels in samples with PMA compared to samples without PMA might be that PMA in itself can induce cytokines, like TNFα[94]. This can mask any induction by subsequent stimuli. A higher basal level of both IFNB1 and TNFα was also observed in unstimulated samples treated with PMA. This indicates that the increased levels of cytokines observed in PMA samples after CpG- stimulation are not completely reliable. This underlines the importance of rest time in PMA-free medium before CpG stimulation[82].

Undifferentiated cells were also observed to provide a potent IFNB1 and TNFα mRNA induction in response to CpG after 24 and 48 hours incubation time with Doxycycline. In the very beginning of this project, undifferentiated THP-1 TLR9 mCherry cells were assessed for their ability to induce IFNB1 and TNFα in response to CpG. In this experiment, it was only observed a small increase in TNFα and no increase in IFNB1. However, at a later stage in the process, it was detected a strong induction of both cytokines in response to CpG in undifferentiated cells. These opposing results are most likely due to the fact that in the first experiment, the cells were stimulated with a CpG concentration of 1 uM while in the later experiment a concentration of 10 uM was used. In any case, these promising results appointed undifferentiated cells as a potential system for studying TLR9 trafficking and signaling.

5.2 CpG-induced responses in THP-1 TLR9 mCherry cells are dynamin-dependent

CpG is said to be taken up by the cells in a clathrin-dependent manner where dynamin is responsible for vesicle fission from the plasma membrane[48,50]. These vesicles with CpG will fuse with endosomes and lysosomes where TLR9 is found expressed and activated by CpG. During the work with this project, it was investigated whether signaling from TLR9 was dependent on dynamin by using the dynamin-inhibitor Dynasore. After pre-incubation with Dynasore and CpG stimulation, it was observed significantly decreased mRNA levels of proinflammatory cytokines represented by TNFα and IL-12B. This indicates that signaling from TLR9 is dependent on dynamin, at least in the case of proinflammatory cytokines. This also suggests an endosomal localization of TLR9, which is in line with the literature[48]. Regarding mRNA levels of IFNB1, it can't be concluded if dynamin is important or not for this signaling pathway since this experiment was performed in PMA-differentiated THP-1 TLR9 mCherry cells. The poor results for IFNB1 are most likely due to PMA and not necessarily that

induction of IFNB1 is not dependent on dynamin. In order to investigate how Dynasore treatment affects the induction of IFNB1, this experiment should have been carried out in undifferentiated or immature DC-like cells. Even though this experiment showed a significant reduction in mRNA levels of proinflammatory cytokines after Dynasore treatment, this inhibitor can display cytotoxic features[95]. An assay such as the lactate dehydrogenase (LDH) assay should have been conducted to examine the level of potential dead cells after treatment with Dynasore.

5.3 Differentiation protocols might complicate siRNA transfection in THP-1 TLR9 mCherry cells

Preliminary experiments from the group have shown that Rab39a might be involved in regulating trafficking of TLR9 to different endosomal compartments. The attempt in making adherent immature DC-like cells which still signaled sufficiently was not successful. Knock-down of Rab39a was therefore tried out in GM-CSF and IL-4-differentiated THP-1 TLR9 mCherry cells despite their properties as suspension cells. This experimental setup turned out to provide a poor knock-down of Rab39a. Hence it is difficult to determine how silencing of Rab39a might affect signaling from TLR9 based on this experiment. siRNA treatment was done on day 2 after seeding with GM-CSF and IL-4. The cells were exposed to siRNA for 24 hours before the medium was changed and fresh GM-CSF and IL-4 were added for an additional 2 days prior to CpG stimulation. GM-CSF and IL-4-differentiated THP-1 cells show slightly decreased proliferative activity, but not fully arrest of cell proliferation[84]. Hence, when the cells were given a new dose of cytokines on day 3 after siRNA treatment this could contribute to cell proliferation and give rise to a new pool of cells which have not been treated with siRNA. Thus, some cells would still express functional Rab39a and this might explain the insufficient knock-down results in this experiment. It could have been an idea to perform siRNA treatment after the cells were fully differentiated with GM-CSF and IL-4, making sure that all cells were treated with siRNA. The poor knock-down might also be caused by a bad transfection rate because these cells are suspension cells. Therefore, another transfection reagent like Lipofectamine 3000 could have been tested out which is supposed to be more effective in transfecting suspension cells.

Knock-down of Rab39a was also performed in undifferentiated THP-1 TLR9 mCherry cells. Using undifferentiated cells shortened the protocol a lot and previous issues with differentiation and siRNA treatment would not be a problem. Both forward and reverse transfection techniques

were tested out, with reverse transfection in combination with the LF3000 manufacturer's protocol generating the best knock-down of Rab39a. Surprisingly, knock-down of Rab39a in undifferentiated cells seemed to increase levels of IFNB1 and TNFα mRNA compared to AllStar after CpG stimulation. However, it was observed some discrepancy between AllStar and LF3000. In general, AllStar and only transfection reagent sample should give the same induction of cytokines since none of these treatments should activate the system. Any distinct difference between these two samples generates some uncertainty regarding the results. In the knock-down experiment done in undifferentiated cells, AllStar is lower than LF3000 for TNFα and IFNB1 after CpG stimulation. This might indicate that the increased levels of IFNB1 and TNFα observed with Rab39a oligos compared to AllStar might be due to a combined effect of siRNA and CpG. Oligo #5 also exhibit some basal level of both cytokines, particularly IFNB1, which might indicate that the increase in stimulated samples is unspecific.

Due to the poor knock-down of Rab39a in GM-CSF and IL-4-differentiated cells, it can't be concluded if Rab39a affects trafficking and signaling from TLR9 based on this experiment. Knock-down of Rab39a in undifferentiated cells with reverse transfection technique combined with the LF3000 manufacturer's protocol showed an increasing tendency of TNFα and IFNB1 mRNA-levels compared to AllStar. However, the results are somewhat uncertain due to differences between AllStar and LF3000. But, if the observed tendency is genuine, knock-down of Rab39a does not seem to reduce the induction of IFNB1 and TNFα as expected, but rather increase the induction of these cytokines. This is a surprising and interesting finding. One can speculate that when Rab39a is silenced, CpG will accumulate in a signaling endosome providing increased mRNA levels of IFNB1 and TNFα. However, these findings should be verified by additional methods and more research.

5.4 Undifferentiated THP-1 TLR9 mCherry cells as a model system for studying trafficking and signaling from TLR9

Characterizing and establishing a stable and robust model system to study TLR9 signaling and trafficking in THP-1 TLR9 mCherry cells had its challenges along the way. Since TLR9 is predominantly expressed in pDCs, but this cell line is rare in the human blood, it was of great interest to try to differentiate the THP-1 TLR9 mCherry cells into a cell line more similar to pDCs. Nevertheless, based on the result in this project it was found that undifferentiated cells gave the simplest starting point for studying this. These cells were characterized as potent inducers of both IFNB1 and TNFα in response to CpG. Using undifferentiated cells eliminated

the need for any differentiation step which shortened and simplified the protocol. Also, using undifferentiated cells reduced the exposure to external reagents which may have unwanted effects. In this way, the system became less artificial. Even though undifferentiated THP-1 TLR9 mCherry cells are not pDC-like, they are still human immune cells that are closer related to pDCs than for instance HEK cells which have been widely used in several previous studies on TLR9. However, their property as suspension cells can contribute to some difficulties when trying to transfect these cells. This can be considered as a weakness of this cell line since transient transfection of siRNAs is a quite useful technique to study how proteins are involved in a cellular mechanism.

Based on the results obtained in this project, undifferentiated THP-1 TLR9 mCherry cells were determined to provide beneficial aspects in terms of shortening and simplifying the protocol. This could aid in its potential to serve as a model cell line to study intracellular trafficking and signaling from TLR9. However, with additional experiments testing different transfection reagents and time points for siRNA transfection, GM-CSF and IL-4-differentiated cells might also have the potential to serve as a model cell line.

In this study, cytokine responses from TLR9 have only been examined by measuring RNA. Future experiments could include verifying these findings at the protein level, for instance by ELISA and Western blot. Here, the THP-1 TLR9 mCherry cell line has mainly been used to study signaling from TLR9. These cells can also be used in experiments investigating the trafficking of TLR9. The mCherry tag is particularly convenient for fluorescent microscopy where one could study TLR9 localization before and after CpG stimulation. Additionally, one could investigate by microscopy how knock-down of Rab39a affects trafficking and localization of TLR9 intracellularly. In this project, THP-1 TLR9 mCherry cells have been characterized regarding TLR9 signaling. By additional optimization and experiments, this cell line has the potential to serve as a model system to study intracellular signaling and trafficking of TLR9. This can again contribute to valuable knowledge in understanding and elucidating how TLR9 is involved in human disease.

6 Conclusion

In this study, PMA-differentiated THP-1 TLR9 mCherry cells induced TNFα, but not IFNB1 mRNA in response to CpG. However, CpG was found to induce potent mRNA levels of IFNB1 and TNFα in THP-1 TLR9 mCherry cells differentiated with GM-CSF and IL-4 or left undifferentiated. siRNA experiments revealed that undifferentiated cells provided the best knock-down of Rab39a when the cells were transfected with oligos using reverse transfection technique and the Lipofectamine 3000 manufacturer's protocol. This experimental setup was therefore used as a model system to examine the role of Rab39a concerning TLR9 signaling. Surprisingly, knock-down of Rab39a gave an increasing tendency of IFNB1 and TNFα in comparison to AllStar negative control siRNA. However, these results need to be confirmed and findings should therefore be investigated in more detail and examined by additional methods.

7 References

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RNeasy® Mini Kit, Part 1

The RNeasy Mini Kit (cat. nos. 74104 and 74106) can be stored at room temperature (15–25°C) for at least 9 months.

For more information, additional and more detailed protocols, and safety information, please refer to the *RNeasy Mini Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- If purifying RNA from cell lines rich in RNases, or tissue, add either 10 µl β-mercaptoethanol (β-ME), or 20 µl 2 M dithiothreitol (DTT)*, to 1 ml Buffer RLT. Buffer RLT with β-ME or DTT can be stored at room temperature for up to 1 month.
- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.
- Remove RNA*later*®-stabilized tissue from the reagent using forceps.
- For RNeasy Protect Mini Kit (cat. nos. 74124 and 74126), please start with the *Quick-StartProtocol RNAlater RNA Stabilization Reagent, RNAlater TissueProtect Tubes, and RNeasy Protect Kits*.

* This option not included for cells in handbook; handbook to be updated.

1. **Cells**: Harvest a maximum of 1 x 10^7 cells, as a cell pellet or by direct lysis in the vessel. Add the appropriate volume of Buffer RLT (see Table 1).
   Tissues: Do not use more than 30 mg tissue. Disrupt the tissue and homogenize the lysate in the appropriate volume of Buffer RLT (see Table 1). Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting, and use it in step 2.
2. Add 1 volume of 70% ethanol to the lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer up to 700 µl of the sample, including any precipitate, to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.

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Optional: For DNase digestion, follow steps 1–4 of "On-column DNase digestion" in *Quick-StartProtocol RNeasy Mini Kit, Part 2*.

4. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
5. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
6. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*.

   Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 1 min to dry the membrane.
7. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x *g* to elute the RNA.
8. If the expected RNA yield is >30 µg, repeat step 7 using another 30–50 µl of RNase-free water, or using the eluate from step 7 (if high RNA concentration is required). Reuse the collection tube from step 7.

Table 1. Volumes of Buffer RLT for sample disruption and homogenization

Sample	Amount	Dish	Buffer RLT	Disruption and homogenization
Animal cells	<5 x 10^6	<6 cm	350 µl	Add Buffer RLT, vortex (≤1 x 10^5 cells); or use QIAshredder, TissueRuptor®, or needle and syringe
	≤1 x 10^7	6–10 cm	600 µl	
Animal tissues	<20 mg	–	350 µl*	TissueLyser LT; TissueLyser II; TissueRuptor, or mortar and pestle followed by QIAshredder or needle and syringe
	≤30 mg	–	600 µl	

* Use 600 µl Buffer RLT for tissues stabilized in RNA*later*, or for difficult-to-lyse tissues.

RNeasy® Mini Kit, Part 2

The RNeasy Mini Kit (cat. nos. 74104 and 74106) can be stored at room temperature (15–25°C) for at least 9 months.

For more information, additional and more detailed protocols, and safety information, please refer to the *RNeasy Mini Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

On-column DNase digestion

Notes before starting

- If using the RNase-Free DNase Set for the first time, prepare DNase I stock solution by injecting 550 µl RNase-free water into the DNase I vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.
- For long-term storage of DNase I stock solution, divide it into single-use aliquots and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze aliquots after thawing.

1. Add 350 µl Buffer RW1 to RNeasy column, close lid, centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard flow-through.
2. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube. Centrifuge briefly.
3. Add DNase I incubation mix (80 µl) directly to RNeasy column membrane, and place on benchtop (20–30°C) for 15 min.
4. Add 350 µl Buffer RW1 to RNeasy column, close lid, centrifuge for 15 s at ≥8000 x *g*. Discard flow-through. Continue with step 5 of "RNA purification from cells/tissue samples" in *Quick-StartProtocol RNeasy Mini Kit, Part 1*, or step 4 of "RNA cleanup" (below).

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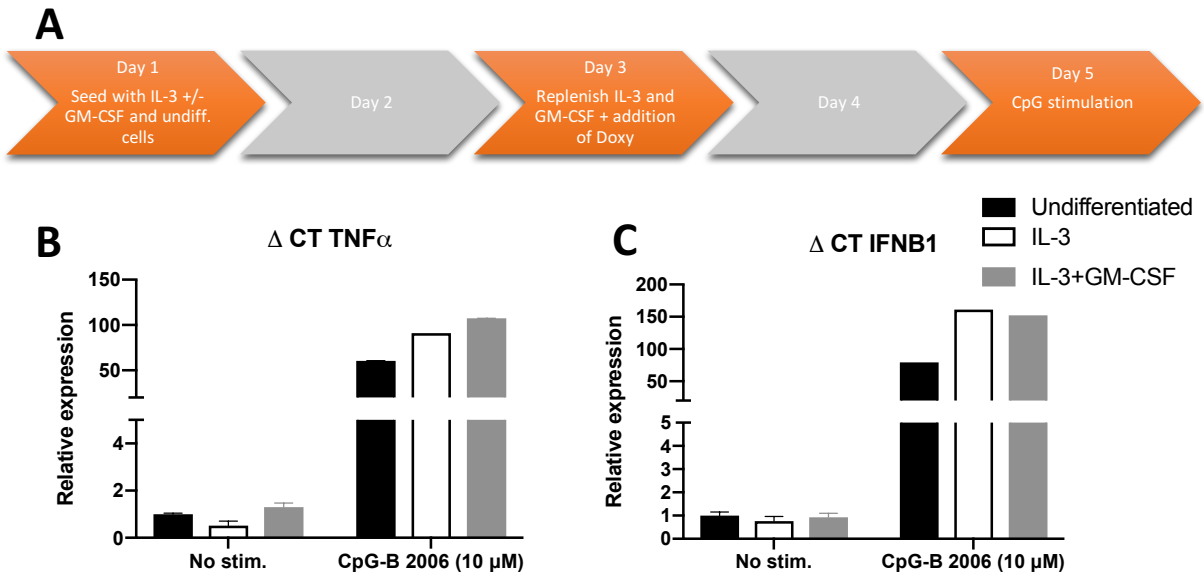
Appendix II

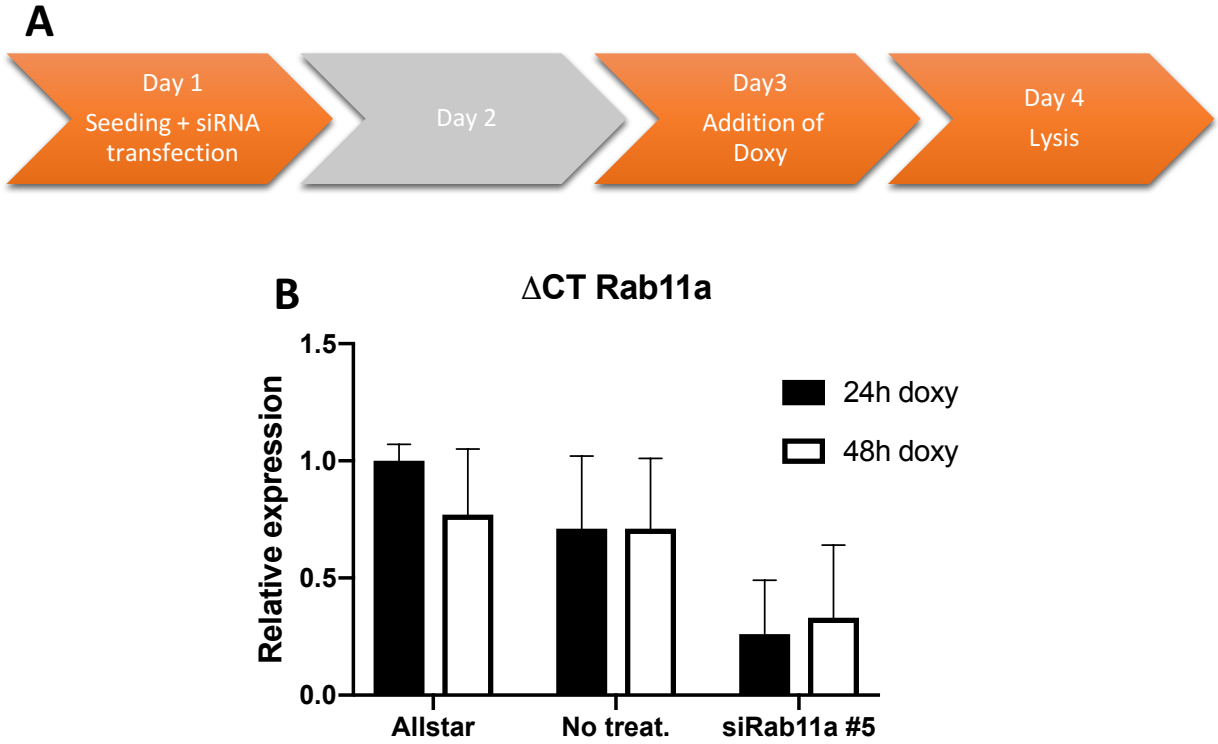
Experiment showing that undifferentiated THP-1 TLR9 mCherry cells induce marked IFNB1 and TNFα in response to CpG-B 2006.



A) Timeline illustrating the workflow and procedure from day to day. B) TNFα and C) IFNB1 mRNA induction in THP-1 TLR9 mCherry cells left untreated (No stim.) or stimulated with CpG-B 2006 (10 µM) for 3h. The cells were differentiated with IL-3 (200 ng/ml) alone or in combination with GM-CSF (100 ng/ml) for 5 days, or left undifferentiated. Doxycycline (1 µg/ml) was added the last 48h to induce the expression of TLR9 prior to CpG stimulation. Fold induction of IFNB1 and TNFα mRNA-levels were assessed by qRT-PCR relative to untreated cells (No stim.) and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

Appendix III

Experiment showing knock-down of Rab11a with TLR9 induction for 24 and 48 hours.



A) Timeline illustrating the workflow and procedure from day to day. B) Rab11a mRNA induction in undifferentiated THP-1 TLR9 mCherry cells treated with only LF3000 or siRNA; AllStar negative control (32 nM) or siRab11a #5 (32 nM). SiRNA was complexed with Lipofectamine 3000 to deliver siRNA to the cells using forward transfection technique. The cells were exposed to siRNA for 48h before Doxycycline (1 µg/ml) was added for 24h or 48h. Fold induction of Rab11a mRNA-levels was assessed by qRT-PCR relative to AllStar and normalized to the housekeeping gene TATA-binding protein (TBP). Error bars represent the SD of two technical replicates.

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