# Characterization of cholesterol 25-hydroxylase expression in human macrophages

By

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**Authentication**

I, Magoro Tshifhiwa, hereby declare that this thesis for a doctoral degree in Microbiology at the University of Venda, hereby submitted by me, has not been submitted previously for the degree at this or other university, that it is my own work in design and execution, and that all references contained therein have been duly acknowledged.

Signature:_____ Date_____

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## Dedication

This thesis is lovingly dedicated to my parents, Mrs Johanna and Joseph Magoro, with my gratitude for their support, and inspiration throughout my study.

## Abstract

**Background** Conversion of Cholesterol to 25-HydroxyCholesterol (25HC) by Cholesterol 25-hydroxylase (CH25H) has been shown to exert broad antiviral properties. Given its antiviral activities, CH25H is part of an increasingly appreciated connection between type I interferon (IFN-I) and lipid metabolism. Moreover, the details of this connection appear to differ in mouse and human cells. Nevertheless, the molecular basis for the induction of CH25H in humans is not known.

**Objective** Elucidation of signaling and transcriptional events for induction of CH25H expression is critical to design therapeutic antiviral agents.

**Materials and methods**: Wildtype THP-1 monocytic cell-line or THP-1 MyD88 Knockout cell-line were treated with PMA for 72 hours for differentiation into macrophages. Differentiated macrophages or Microglial cells were stimulated with either TLR-agonists, pro-inflammatory cytokine, or interferons, and CH25H mRNAs expression levels were measured by qPCR.

**Results** In this study, we show that CH25H is induced by Zika virus infection or TLR stimulation. Interestingly, CH25H is induced by pro-inflammatory cytokines including 1L-1$\beta$, TNF-$\alpha$, and IL-6, and this induction depends on STAT-1 transcription factor. Additionally, we have observed that ATF3 weakly binds to the CH25H promoter, suggesting co-operation with STAT-1. However, ZIKV induced CH25H was independent of type I interferon.

**Conclusion** This study has demonstrated for the first time that pro-inflammatory cytokines such as 1L-1$\beta$, TNF-$\alpha$, and IL-6 induce CH25H expression. Moreover, this provides further understanding to the connection between innate immunity and sterol metabolism and encourages the exploration of cytokines in antiviral immunity.

**Keywords**: Cholesterol 25-hydroxylase; 25-hydroxylase; pro-inflammatory cytokines; STAT-1; ATF3; TLR stimulation.

## Scientific communications related to this thesis

### Published articles

Lucas T. Jennelle, Aditya P. Dandekar, Tshifhiwa Magoro, Young S. Hahn (2017). Immunometabolic Signaling Pathways Contribute to Macrophage and Dendritic Cell Function. Critical Reviews™ in Immunology, 36(5): 379–394.

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### Conference presentations

Meeting/Conference Title: **Infectious diseases and biodefense research day**
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Place of meeting/Conference: **University of Virginia, USA**.
Title of presentation: **CH25H is not a classical interferon stimulated gene in human THP-1 macrophages**

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## Abbreviations

| | |
|---|---|
| 25HC | 25-hydroxycholesterol |
| AID | Activation-induced cytidine deaminase |
| ALRs | AIM2-like receptor |
| ASC | Apoptosis-associated speck-like protein containing a CARD |
| ATF | Activating transcription factor |
| ATP | Adenosine triphosphate |
| $B_2M$ | Beta-2-microglobulin |
| BCA | Bicinchoninic acid assay |
| BMDM | Bone marrow-derived macrophages |
| BSA | Bovine serum albumin |
| bZip | Basic-region leucine zipper |
| cAMP | Cyclic adenosine monophosphate |
| CARD | Caspase activation and recruitment domain |
| CCD | Coiled-coiled domain |
| CH25H | Cholesterol 25-hydroxylase |
| CLRs | C-type lectin receptor |
| CNS | central nervous system |
| CREB | cAMP response element-binding protein |
| DAMPs | Damage-associated molecular patterns |
| DBD | DNA binding domain |
| DD | Death domain |
| DENV | Dengue virus |
| DNA | Deoxyribonucleic acid |
| dsRNA | Double-stranded ribonucleic acid |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinases |
| FBS | Fetal Bovine Serum |
| GAF | IFN-γ activation factor |
| GAS | Gamma-activated sequence |

| | |
|---|---|
| GBS | Guillain-Barre syndrome |
| Gp130 | Glycoprotein 130 |
| HCV | Hepatitis C virus |
| HIV-1 | Human immunodeficiency virus-1 |
| HMG-CoA | 3-hydroxy-3-methylglutaryl coenzyme A |
| HPRT | Hypoxanthine phosphoribosyltransferase |
| HSV-1 | Herpes simplex virus-1 |
| IBD | Inflammatory bowel disease |
| IFNAR | Interferon alpha receptor |
| IFNGR | Interferon gamma receptor |
| IFN | Interferon |
| IFNα | Interferon alpha |
| IFNβ | Interferon beta |
| IFNγ | Interferon gamma |
| IFNλ | Interferon lammda |
| IL | Interleukin |
| IL-1β | Interleukin 1 beta |
| IL1RAcP | Interleukin 1 receptor accessory protein |
| IL-2 | Interleukin-2 |
| IL-6 | Interleukin 6 |
| IL-12 | Interleukin 12 |
| IL-6R | Interleukin 6 receptor |
| ISG | Interferon stimulated gene |
| ISGF3 | Interferon-stimulated gene factor 3 |
| ISREs | Interferon signaling response elements |
| JAK | Janus kinase |
| JNK | c-Jun amino (N)-terminal kinase |
| IRAK | Interleukin-1 receptor associated kinase |
| IRF | Interferon regulatory transcription factor |
| LBP | Lipopolysaccharide binding protein |
| LPS | Lipopolysaccharides |

| | |
|---|---|
| LXR | Liver X receptor |
| MAL | MyD88-adaptor-like |
| MAPK | Mitogen-activated protein kinase |
| M-CSF | Macrophage colony-stimulating factor |
| MDP | Muramyl dipeptide |
| memTNFα | transmembrane tumor necrosis factor alpha |
| MHV-68 | Murine gamma herpes virus 68 |
| MOI | Multiplicity of infection |
| mRNA | Messenger ribonucleic acid |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NFAT | Nuclear factor of activated T cells |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLK | Nemo-like kinase |
| NLRP | Nucleotide-binding oligomerization domain leucine rich repeat and pyrin |
| Nmi | N-Myc interacting protein |
| NLRs | NOD-like receptor |
| NS | Non-stimulated |
| PAMPs | Pathogen-associated molecular patterns |
| PBMCs | peripheral blood mononuclear cells |
| PI3K | phosphoinositide 3-kinase |
| PIP2 | phos-phatidylinositol-4,5-bisphosphate |
| PMA | Phorbol 12-myristate 13-acetate |
| POLY(I:C) | Polyinosinic:Polycytidylic acid |
| PRR | Pattern recognition receptor |
| qPCR | Quantity polymerase chain reaction |
| RIG-I | Retinoid acid-inducible gene-I |
| RIPA | Radioimmunoprecipitation assay |
| RLRs | Retinoid acid inducible gene (RIG)-I-like receptors |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |

| | |
|---|---|
| SAPK | Stress-activated protein kinases |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sIL-6R | Soluble interleukin 6 receptor |
| SODD | Silencer of death domain |
| SREBPs | Sterol regulatory element binding protein |
| ssRNA | single-stranded Ribonucleic acid |
| sTNFα | Soluble tumor necrosis factor alpha |
| STAT | Signal transducer and activator of transcription |
| TACE | TNFα-converting enzyme |
| TAD | Transcriptional activation domain |
| TBK1 | TANK-binding kinase |
| TIR | Toll/IL-1 receptor |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor alpha |
| TRAF3 | TNF receptor associated factor 3 |
| TRAF6 | TNF receptor associated factor 6 |
| TRADD | TNFR-associated death domain protein |
| TRIF | Toll/IL-1 receptor (TIR) domain-containing adapter-inducing interferon-β |
| TYK | Tyrosine kinase |
| UV | Ultraviolet |
| VZV | Varicellar zoster virus |
| WR | Working solution |
| ZIKV | Zika virus |

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# CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

## 1.1 INTRODUCTION

Viruses are obligate parasites that depend on the host cell to provide the energy and molecular precursors necessary for successful infection. Furthermore, they manipulate lipid metabolism through synthesis of cholesterol to favor their own replication (Heaton and Randall, 2011; Goodwin et al., 2015). Cholesterol and components of the cholesterol biosynthetic pathway have fundamental roles in all mammalian cells viability and proliferation. Cholesterol is one of the most abundant lipids in mammalian cells, constituting up to 25% of the plasma membrane lipid and maintains both membrane fluidity and rigidity (Cyster et al., 2014). Moreover, Cholesterol-rich micro-domains (rafts) in the plasma membrane mediates cell signaling, and cholesterol distribution in membranes affects viral entry, replication, and budding (Simons and Gerl, 2010; Heaton and Randall, 2011; Chukkapalli et al., 2012). It also functions as a precursor for steroid hormones and bile acids. Dysregulation of cholesterol homeostasis is associated with a number of human disease states, including atherosclerosis, which is associated with high cholesterol levels (Lewington et al., 2007; Howe et al., 2016)

Mammalian hosts have developed a metabolic countermeasure, through the upregulation of cholesterol 25-hydroxylase (CH25H), which arrests cholesterol biosynthesis. Murine cholesterol-25-hydroxylase (ch25h) is an interferon stimulated gene that encodes an enzyme that converts cholesterol to 25-hydroxycholesterol (25HC). CH25H and its metabolite, 25HC, have been shown to exert broad antiviral activity against enveloped and non-enveloped viruses in tissue culture. CH25H is highly induced in mouse macrophages and dendritic cells following stimulation with TLR3/4 agonist, and its induction is dependent on interferon alpha receptor (IFNAR) interactions (Park and Scott, 2010). 25HC regulates cholesterol biosynthesis by suppressing sterol regulatory element

binding proteins (SREBPs) proteolytic activation and acting as a ligand for liver X receptor (LXR) activation, promoting induction of genes which are involved in cholesterol efflux.

## 1.2 LITERATURE REVIEW

### 1.2.1 Cholesterol homeostasis regulation by LXR and SREBP

In mammals, lipid biosynthesis is controlled by a family of transcription factors called sterol regulatory element binding proteins (SREBPs). As shown in **figure 1.1**, there are three isoforms of SREBPs in mammalian cells namely SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are produced from a single gene (SREBF1), whereas SREBP-2 is encoded by a separate gene (SREBF2) (Walker and Näär, 2012). SREBP-1 is responsible for transcription of genes involved in fatty acid synthesis, while SREBP-2 mediates transcription of genes involved in cholesterol biosynthesis and uptake (Schroepfer, 2000; Ye and DeBose-Boyd, 2011). SREBPs are found in inactive states in the endoplasmic reticulum (ER) and must be escorted by SREBP cleavage-activating protein (SCAP) to the Golgi for proteolytic activation. Activated SREBPs will then translocate to the nucleus to induce the transcription of target genes (**Figure 1.2**). This process is regulated by cholesterol and oxysterols through retention of SREBPs in the ER (Ye and DeBose-Boyd, 2011; Cyster et al., 2014). In addition, liver X receptors (LXR) $\alpha$ and $\beta$ are also transcription factors that regulate cholesterol absorption, transport and elimination. Oxysterols are also physiologic ligands for LXRs. In macrophages, activation of LXR will induce the expression of genes that are involved in cholesterol efflux, ABCA1 and ABCG1, and genes that inhibit cholesterol loading (IDOL) and synthesis (Shibata and Glass, 2010). LXR activation has also been attributed to produce anti-inflammatory mediators by inhibiting the expression of NF-kB dependent inflammatory gene transcription. In addition to their roles in metabolic pathways, inappropriate regulation of these transcription factors has been linked with disorders related to metabolism

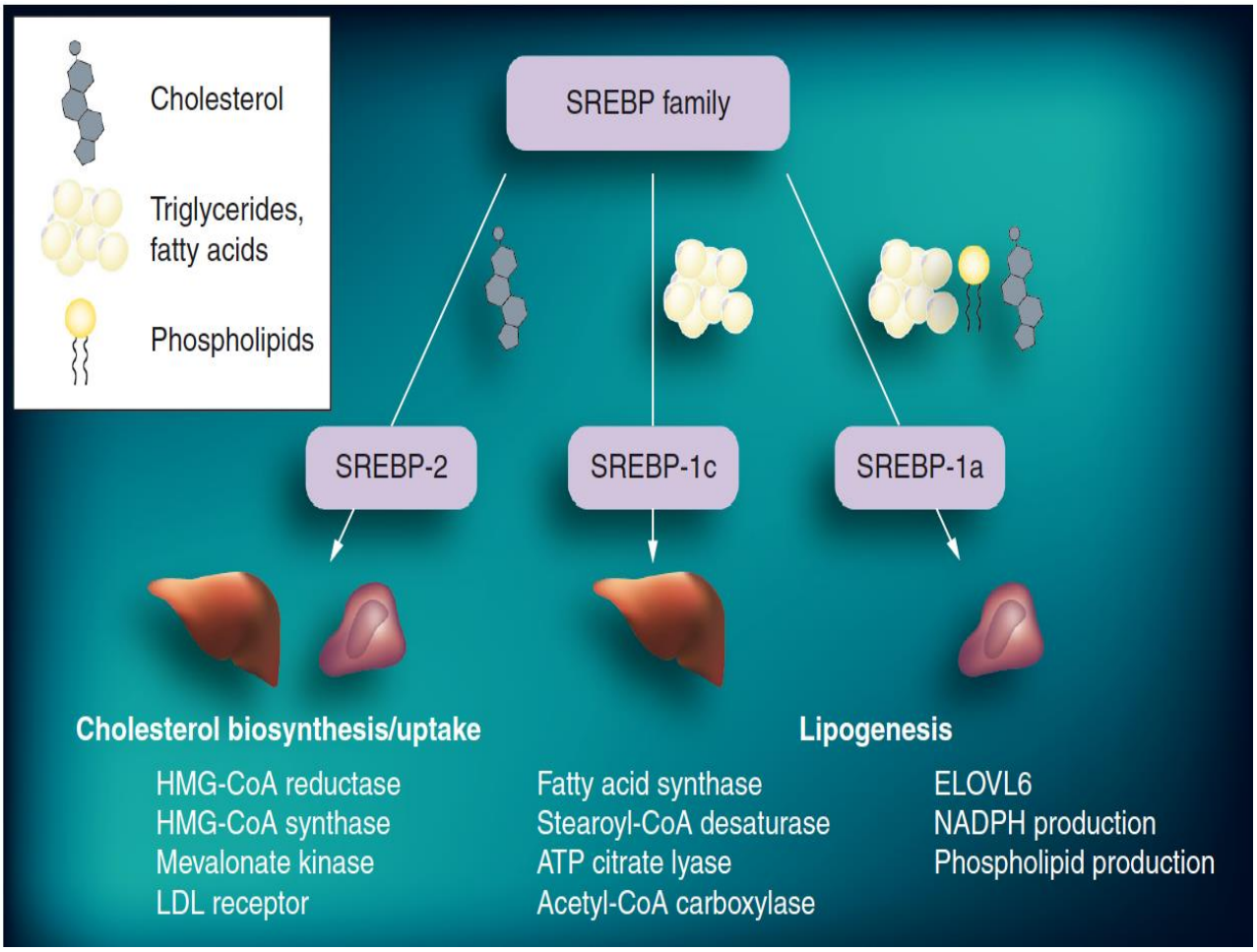


**Figure 1.1**. Members of the SREBP family of transcription factors, their preferred classes of target genes and predominant expression patterns (Walker and Näär, 2012).

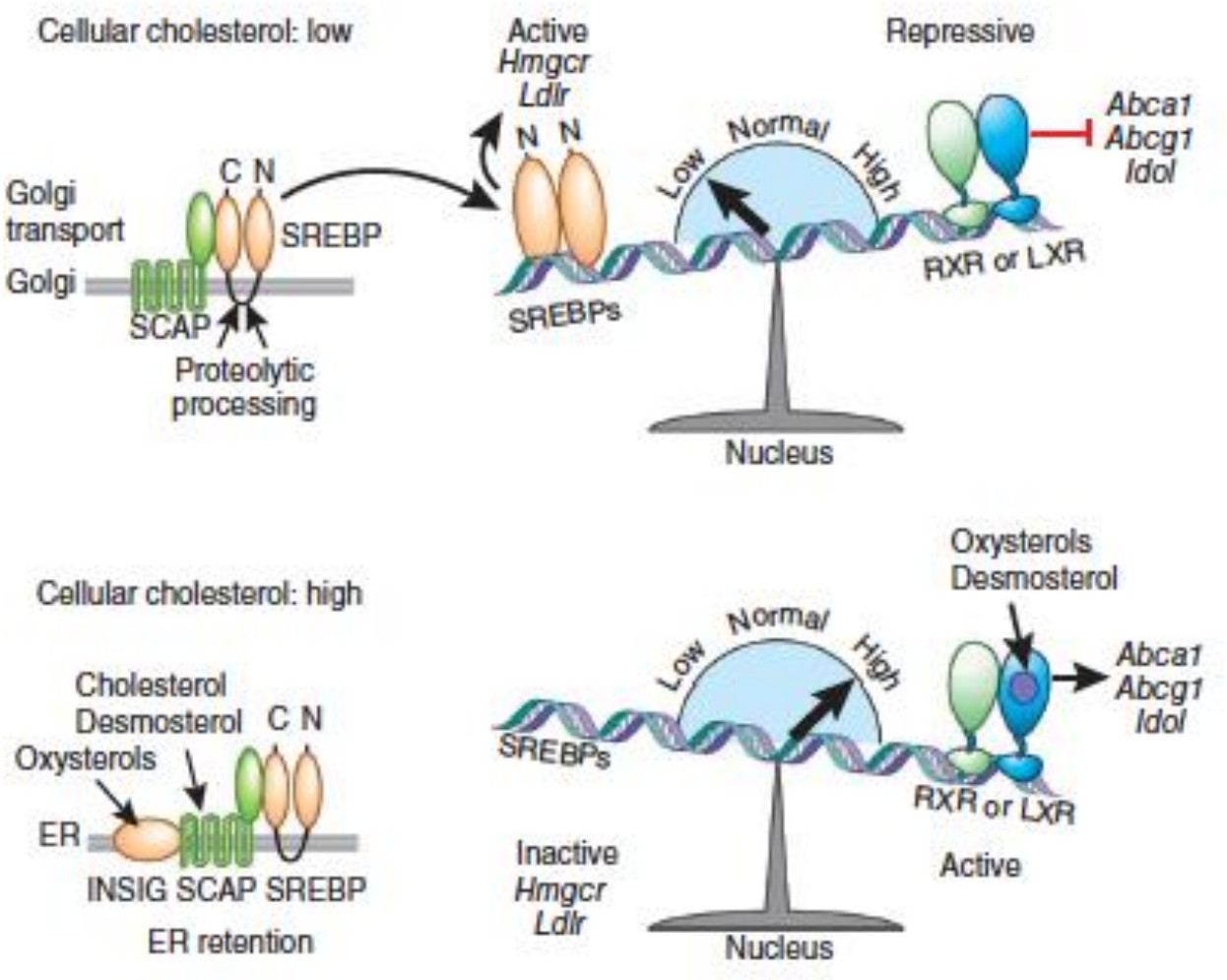


**Figure 1.2. Cholesterol homeostasis regulation by LXR and SREBP**. When cellular cholesterol is low, SREBP in ER is transported by SCAP protein to the Golgi for proteolytic activation. The amino terminal domain of SREBP will then translocate to the nucleus to active genes responsible for cholesterol biosynthesis (HMG CoA reductase) and cholesterol uptake (LDL receptor). Under the same condition, LXR will recruit a corepressor to inhibit the expression of genes responsible for cholesterol efflux (ABC1, and ABCG1), and genes that inhibit cholesterol uptake (IDOL, responsible for LDL receptor degradation). Under conditions where cellular cholesterol is high, cholesterol and some oxysterols inhibit SREBP migration to the Golgi for activation, while oxysterols binding to LXRs to induce the transcription of target genes, ABCA1, ABCG1 and IDOL (Spann and Glass, 2013).

### 1.2.2 Production of 25-hydroxycholesterol

Bile acid synthesis can occur through two pathways, namely, the classic (neutral) or the alternative (acidic) pathway. The classic pathway is the major pathway that occurs in the liver and accounts for approximately 90% of bile acid synthesis. The alternative pathway produces the remaining 10% of bile acids and is primarily extrahepatic (Ferdinandusse and Houten, 2006). About 17 individual enzymes are required for a full synthesis of bile acids and these enzymes occur in multiple intracellular compartments that include the cytosol, endoplasmic reticulum (ER), mitochondria, and peroxisome (Russell, 2003). Cholesterol has four rings and an eight-carbon side chain, and it carries a 3β-hydroxyl group. Similar to a parent molecule, cholesterol, all bile acids have a 3-hydroxyl group (**Figure 1.3**). The initial step in the classical pathway is initiated by the enzymatic addition of a 7α-hydroxyl group by cholesterol 7α-hydroxylase (CYP7A1) an ER localized enzyme, forming 7α-hydroxycholesterol. An alternative pathway is initiated by hydroxylation of cholesterol at the 27 position by mitochondrial sterol 27-hydroxylase (CYP27A1) (Chiang, 2009).

Hydroxylation of cholesterol at 25 position on the side chain produces 25-hydroxycholesterol. To be converted into bile acids, 25-hydroxycholesterol must undergo 7α-hydroxylation by enzymatic action of 7α- hydroxylase (CYP7B1) (Figure 3). The 25-hydroxylation reaction is catalyzed by the cholesterol 25-hydroxylase (CH25H), a multi-transmembrane endoplasmic reticulum (ER) protein (Cyster et al., 2014). 27-hydroxylase (CYP27A1) can also hydroxylate cholesterol at carbon 25 to generate 25-hydroxycholesterol (Diczfalusy, 2013). Unlike 25-hydroxylase enzymes, which contribute only modestly to bile acid synthesis in the mouse, about 25% of the bile acid pool originates from oxysterols produced by sterol 27-hydroxylase (Ferdinandusse and Houten, 2006). 25HC has several roles in innate immunity, and these roles are briefly described below.

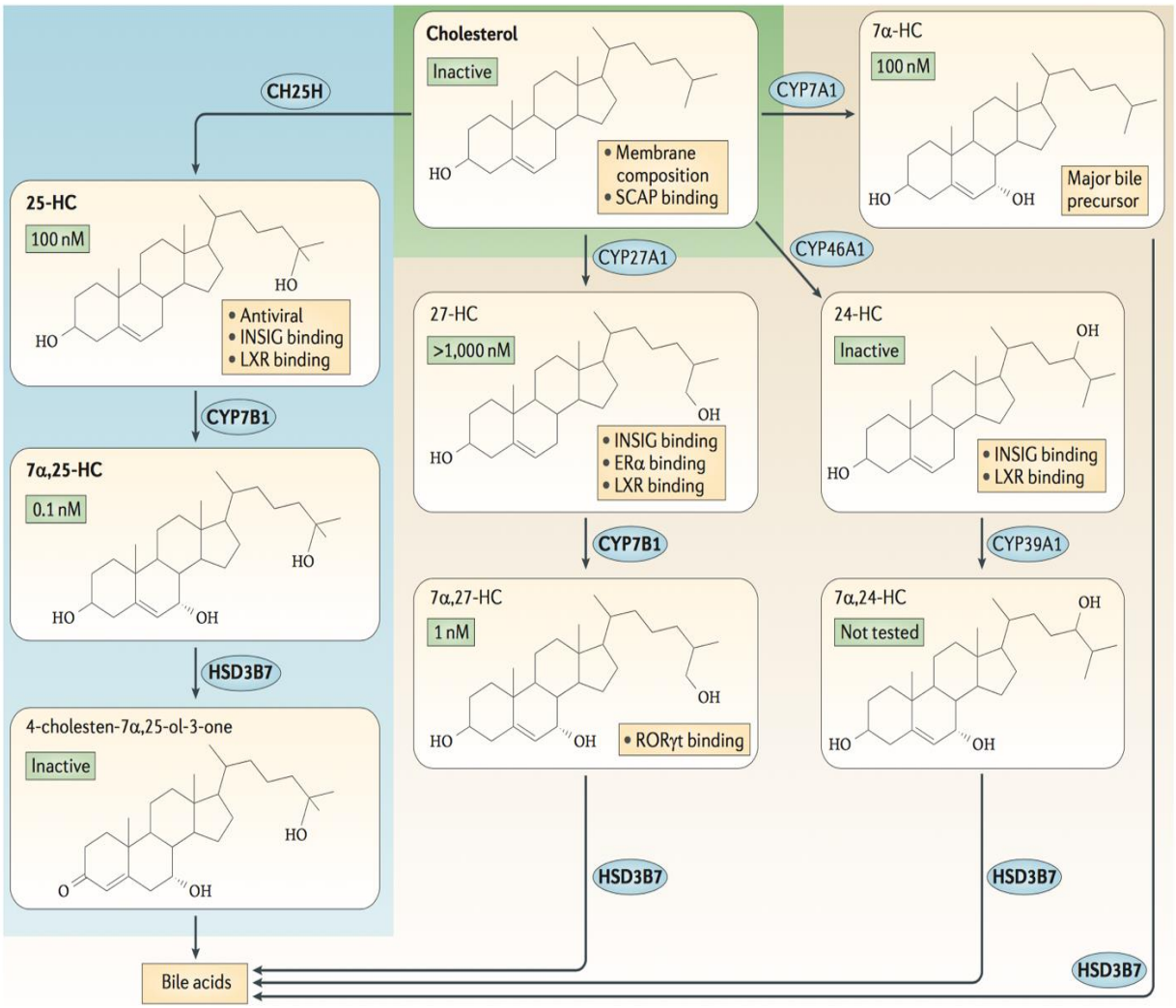


**Figure 1.3. Enzymatic steps involved in the generation of oxysterols from cholesterol** (Cyster et al., 2014). Cholesterol is converted to bile acids. Key regulated enzymes, CYP7A1, CYP27A1, CYP7B1, and HSD3B7 in the pathways are indicated. CYP7A1 initiates the classic bile acid biosynthetic pathway. CYP27A1 initiates the alternative pathway.

### 1.2.3 Cholesterol 25-hydroxylase (CH25H) and 25-hydroxycholesterol functional activity

The CH25H gene encodes an ER-associated enzyme that convert cholesterol to 25HC. The soluble factor 25-HC is an important regulatory molecule of the cholesterol biosynthesis pathway and it is also a substrate in bile synthesis (Lund et al., 1998). 25-HC suppress proteolytic activation of sterol regulatory element binding proteins (SREBPs)

which controls levels of key regulatory enzymes in cholesterol synthesis, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and HMG-CoA synthase. As a result, cholesterol biosynthesis is halted. Under the same condition, 25HC activates LXRs, and this activation leads to the upregulation of several metabolic genes, such as the ABCA1, ABCG1 and IDOL, which is capable of removing and inhibiting cholesterols loading (Adams et al., 2004; Radhakrishnan et al., 2007; Cyster et al., 2014; Reboldi et al., 2014).

Several studies using mouse and human cells showed that CH25H/25HC had broad antiviral activity against wide range of pathogenic viruses, such as Zika virus, Ebola virus, Rift Valley fever virus, Nipah virus, and Russian spring-summer encephalitis virus, human immunodeficiency virus (HIV)-1, herpes simplex virus, vesicular stomatitis virus, Influenza A (H1N1), herpes simplex virus-1 (HSV-1), varicellar zoster virus (VZV) murine gamma herpes virus 68 (MHV-68), and hepatitis C virus (HCV) (Blanc et al., 2013; Xiang et al., 2015; Li et al., 2017). As shown in **figure 1.4**, Although 25HC had broad antiviral activity against a wide range of virus, its mode of action seems to differ depending on virus-host context.

25-HC has also been shown to play role in adaptive immunity. Blanc et al. (2013) showed that exposure of naïve B cells with 25-hydroxycholesterol suppressed IL-2-mediated stimulation of B cell proliferation, repressed activation-induced cytidine deaminase (AID) expression, and blocked class switch recombination, leading to markedly decreased IgA production. 25-HC has also been found to promote macrophage foam cell formation (Gold et al., 2012) , and to elevate the production of some inflammatory cytokines (Koarai et al., 2012; Gold et al., 2014). In addition, it has also been found to control the differentiation of monocytes into macrophages, and to antagonize sterol response element-binding protein (SREBP) processing to reduce IL-1β transcription and to broadly repress IL1-activating inflammasomes (Reboldi et al., 2014).

Blanc et al. (2013) identified 25HC as the only macrophage-synthesized and -secreted oxysterol in mouse macrophages by utilizing quantitative metabolome profiling of all

naturally occurring oxysterols upon infection or IFN-stimulation. Using transcriptional regulatory-network analyses, genetic interventions and chromatin immunoprecipitation experiments they demonstrated that Stat1 directly coupled Ch25h regulation to IFNβ in mouse macrophages. Although previous studies, have shown antiviral properties of 25HC. Harmful effects of 25HC may still exist. Moreover, (Zou et al., 2011) showed that increased CH25H expression promotes survival of L. monocytogenes-infected cells and increases sensitivity of the host to infection.

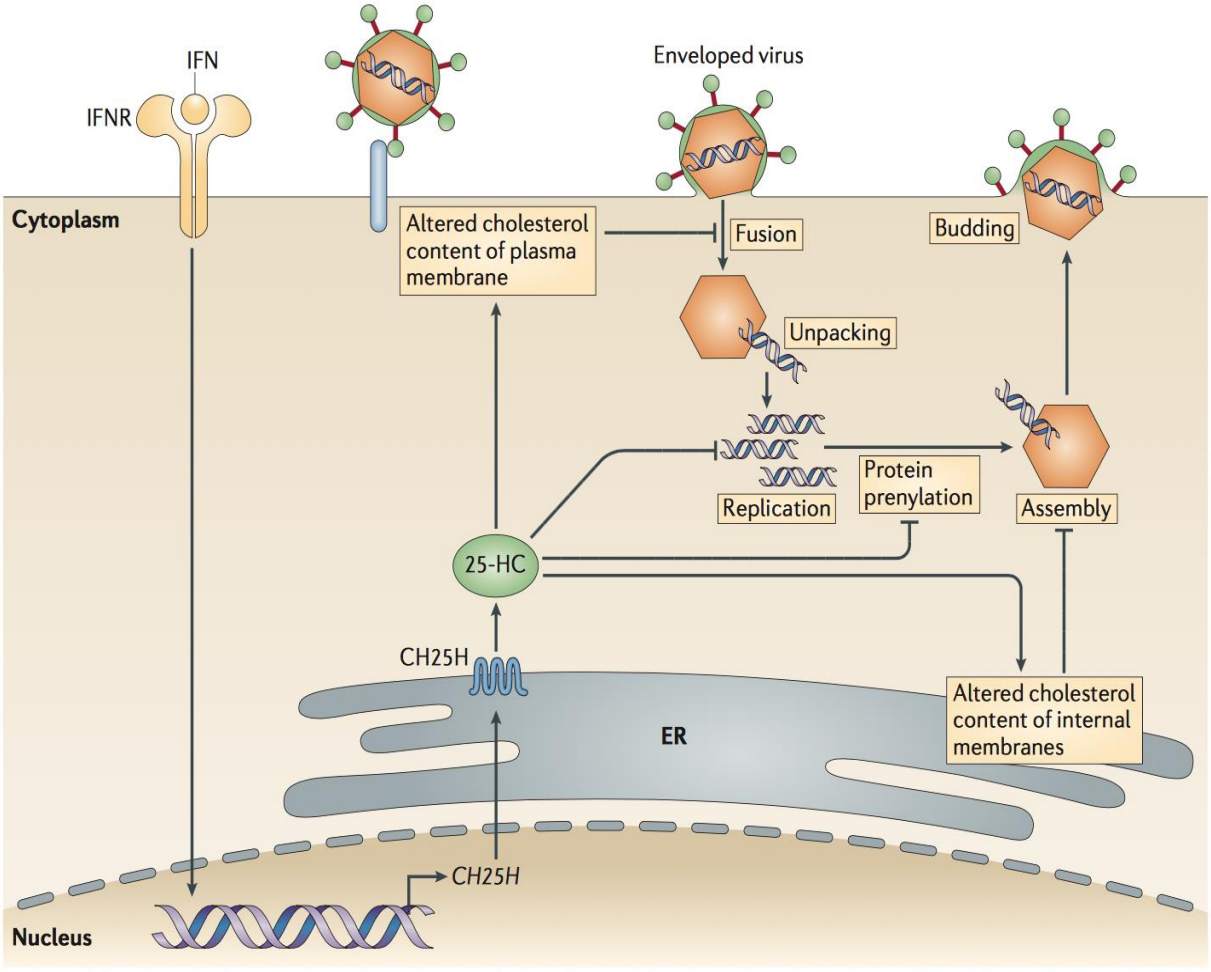


**Figure 1.4**. **Induction of CH25H/25HC and its mode of action**. interferon binds to their cognitive Interferon receptor (IFNR) and activate the induction of CH25H, which encodes cholesterol 25-hydroxylase and promotes the production of 25-HC from cholesterol. studies on 25HC differed in their conclusions about the mechanism of action, and have suggested that 25-HC can have the following antiviral effects: alter plasma membrane lipids (particularly cholesterol content) to inhibit

viral fusion and entry; inhibit viral replication; inhibit viral and endogenous protein prenylation which is important for viral replication and assembly; and alter the cholesterol distribution in internal membranes to inhibit the formation of membrane-associated 'factories' that are involved in viral assembly and packaging (Cyster et al., 2014)

### 1.2.4 Overview of innate immunity

The innate immune system detects conserved structures among pathogens, which are called pathogen-associated molecular patterns (PAMPs), and initiates mechanisms to eliminate potentially infectious threats. PAMPs are recognized by pattern recognition receptors (PRR) expressed by immune cells and nonprofessional immune cells that survey both the extracellular and intracellular space for conserved microbial determinants (Takeuchi and Akira, 2010; Kawai and Akira, 2011). PRRs are also responsible for recognizing endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). As illustrated in **table 1.1**, there are five classes of pattern recognition receptors, which includes the transmembrane proteins such as toll-like receptor (TLR) and C-type lectin receptors (CLRs), together with cytoplasmic proteins retinoid acid inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain NOD-like receptor (NLRs) and the AIM2-like receptors (ALRs) (Takeuchi and Akira, 2010; Moresco et al., 2011; Brubaker et al., 2015). TLR receptors survey for the presence of pathogen-associated molecular patterns in the extracellular space and within endosomes. The NLRs, RLRs, and ALRs are located in the cytoplasm, where they survey for the presence of intracellular pathogens (Brubaker et al., 2015). Herein, we will discuss TLRs in depth only.

**Table 1.1. Pattern recognition receptors and their specific ligands** (Takeuchi and Akira, 2010; Brubaker et al., 2015)

| Pattern recognition receptors | Localization | Ligand |
|---|---|---|
| **Toll-like receptors** | | |
| TLR1 | Plasma membranes | Triacyl lipoprotein |
| TLR2 | Plasma membranes | Lipoprotein |
| TLR3 | Endolysosome | dsRNA |
| TLR4 | Plasma membranes | Lipopolysacharide |
| TLR5 | Plasma membranes | Flagellin |
| TLR6 | Plasma membranes | Diacyl lipoprotein |
| TLR7 (human TLR8) | Endolysosome | SsRNA |
| TLR9 | Endolysosome | CpG-DNA |
| TLR10 | Endolysosome | Unknown |
| TLR11 | Plasma membranes | Profilin-like molecule |
| | | |
| **RIG-I-like receptors** | | |
| RIG-I | Cytoplasm | Short dsRNA, 5'triphosphate dsRNA |
| MDA5 | Cytoplasm | Long dsRNA |
| LGP2 | Cytoplasm | Unknown |
| | | |
| **NOD-like receptors** | | |
| NOD1 | Cytoplasm | iE-DAP |
| NOD2 | Cytoplasm | Muramyl dipeptide (MDP) |
| | | |
| **C-type lectin receptors** | | |
| Dectin-1 | Plasma membranes | β-Glucan |
| Dectin-2 | Plasma membranes | β-Glucan |
| MINCLE | Plasma membranes | SAP130 |
| | | |
| **ALR** | | |
| AIM2 | | |
| IFI16 | | |

### 1.2.5 Toll-like receptors

All ten Toll-like receptors discovered in humans are composed of the N-terminal domain and the cytoplasmic domain. The cytoplasmic domains of toll-like receptors are homologous to interleukin-1 receptor and are termed Toll/IL-1 receptor (TIR) domain. The TIR domain is conserved in all TLRs, but the extracellular domain is unique to the individual TLR as it confers specificity for ligand recognition (Szabo et al., 2006; Brubaker et al., 2015). These receptors are expressed by immune and non-immune cells and are

responsible for the recognition of different PAMPs expressed by pathogen. Ligand binding to TLRs leads to the synthesis and secretion type 1 interferons and inflammatory cytokines such as TNFα, IL-6 and IL-1β (**Figure 1.5**). As shown in **table 1.1**, recognition of PAMPs by TLRs occurs in various cellular compartment, such as the plasma membrane, endosomes, lysosomes and endo-lysosomes. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR 10 are expressed on the cellular membrane, while TLRs 3,7, 8 and 9 are found in the endosomal compartment allowing site-specific recognition of pathogens. Intracellular TLRs, including TLR3, TLR7, TLR8, and TLR9 located in the endosomal compartment, and are capable of detecting foreign nucleic acids. These receptors are able to distinguish between host and foreign nucleic acids, because host nucleic acid is usually absent from endosomal compartment (Blasius and Beutler, 2010; Chow et al., 2015). Furthermore, TLRs have different adaptors proteins located at the surface or in the internal cell compartments to respond to activation and recruit downstream signaling components, such adaptors include myeloid differentiation primary response gene 88 (MyD88), Toll/IL-1 receptor (TIR) domain-containing adapter-inducing interferon-β (TRIF, also known as TICAM1), MyD88-adaptor-like (MAL) (also known as TIRAP), and TRIF-related adaptor molecule (TRAM) (**Figure 1.5**) (Kawai and Akira, 2006; Kawai and Akira, 2010). The engagement of TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR11 with their respective ligands recruits MyD88 adaptor molecule. As shown in **figure 1.5**, all TLRs utilizes the myD88 dependent pathway with the exception of TLR3 which uses myD88 independent pathway/TRIF dependent pathway. Furthermore, Only TLR4 have the capability to recruits both MyD88 and TRIF. Mal and Tram adaptors functions as a sorting adaptor that bridges MyD88 and TRIF to the TIR domain to facilitate downstream signaling (Mcgettrick and Neill, 2004; Takeda and Akira, 2005; Kawai and Akira, 2006; Kumar et al., 2009).

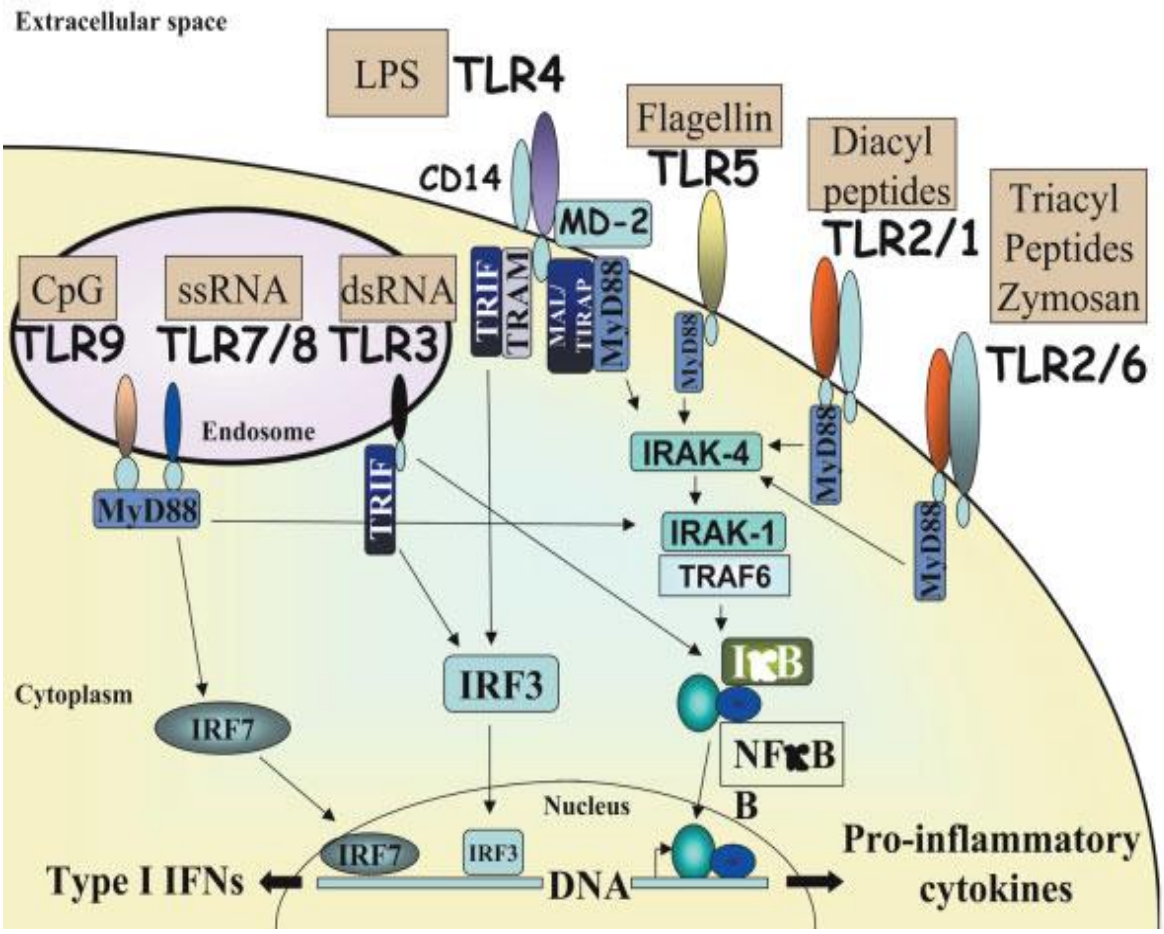


**Figure 1.5. The relationship between Toll-like receptors (TLRs), their ligands, co-receptors, adaptors and intracellular signaling pathways** (Szabo et al., 2006). PAMP recognition by cell surface TLRs. The TLR4 response to LPS requires a multireceptor complex consisting of LPS-binding protein (LBP), CD14, and MD-2. The detection of LPS is first achieved through the actions of LPS-binding protein (LBP), which is present in the extracellular fluids, not shown on the figure. Then, monomer of LPS will bind to CD14 which exists as a glycosylphosphatidylinositol-anchored protein at the plasma membrane of many mammalian cell. the LPS present in complex with CD14 can be transferred to MD-2 and then associates with TLR4. LPS complexes follow two different sets of pathways. The LPS complex initially transmits signals for the early-phase activation of NF-κB by recruiting the TIR domain– containing adaptors TIRAP (Mal) and MyD88 (MyD88-dependent pathway). The second activity of LPS-complex is to promote its internalization into the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 and late-phase NF-κB for the induction of type I interferon (TRIF-dependent pathway). Both early- and late-phase activation of NF-κB is required for the induction of inflammatory cytokines. As shown in the Figure, TLR2 is in association with TLR1 or TLR6. TLR2-

TLR1 and TLR2-TLR6 heterodimers recognize triacylated and diacylated lipopeptide, respectively. TLR5 recognizes flagellin, and two of the three lipid chains of the triacylated lipopeptide interact with TLR2, and the third chain binds the hydrophobic channel of TLR1 (absent from TLR6). Similar to TLR4, TLR5, TLR2-TLR1 and TLR2-TLR6 induce NF-κB activation through recruitment of TIRAP and MyD88. PAMP recognition by intracellular TLRs: TLR3, TLR7, and TLR9 are localized in the endosome and detect viral nucleic acids. TLR7/8 binds ssRNA, and TLR9 binding to its ligand CpG oligode- oxynucleotide (CpG-A) induces IFN by activating the transcription factor IRF-7 via the adaptor protein MyD88. TLR3 binds dsRNA, and though TRIF induces IFN by activating transcription factor IRF-3.

### 1.2.6 TRIF-Dependent pathway

As already stated above, TLR3 utilizes the TRIF dependent pathway compared to other TLRs that uses myD88 signaling pathway, with the exception of TLR4 which uses both adaptor proteins. Firstly, we will discuss theTLR3 signaling through TRIF pathway: TLR3-mediated signaling is triggered by double-stranded RNA (dsRNA) and in response to this stimulation it will recruit an adaptor protein called TRIF (Okahira et al., 2005; Szabo, Chang et al., 2007; Blasius and Beutler, 2010; Takeuchi and Akira, 2010). Although both TLR3 and TLR4 are able to recruit TRIF, the mechanism is different. TLR3 directly interacts with TRIF, whereas TLR4 selectively recruit TRAM to link TRIF to TLR4 (Kawasaki and Kawai, 2014).

TRIF is in association with TNF receptor-associated factor 6 (TRAF6) and RIP-1, and upon TLR3 activation this complex will associate with TRAF3 to activate two IKK-related kinases, TANK-binding kinase (TBK1) and IKKε (also known as IKK-i) (**figure 1.6**) (Kumar et al., 2009). TRAF3 links TLR adaptors and downstream important kinases for activation of interferon regulatory factor (IRF) to induce interferon production (Kawai and Akira, 2006; Kawai and Akira, 2010). Activated TBKI and IKKε will then phosphorylate interferon regulatory factor 3 (IRF-3). Phosphorylated IRF-3 will translocate to the nucleus and coordinate the expression of genes responsible for type 1 interferon (Kumar et al., 2009). In addition, TRAF6 and RIP-1 can also active nuclear factor kB (NF-kB) and MAPKs to induce pro-inflammatory cytokines production such as tumor necrosis factor-a TNFα, IL-

12, IL-1, IL-6, and chemokines (Szabo, Mandrekar et al., 2007; Bowie and Unterholzner, 2008; Blasius and Beutler, 2010; Kawai and Akira, 2011).

Secondly, we will elaborate the TLR4 signaling through TRIF pathway: As stated above, TLR4 is unique in that it utilizes both MyD88 and TRIF adaptor protein in a sequential order. Immediately after receptor ligation, the receptor engages TIRAP/MyD88 to initiate signaling from the cell surface and then undergoes endocytosis and engages TRAM/TRIF-dependent signaling from endosomes (**Figure 1.7**) (Newton and Dixit, 2012; Brubaker et al., 2015). The Toll- interleukin 1 receptor domain-containing adaptor protein (TIRAP)-MyD88 pathway induces cytokines, and the TRAM/TRIF-dependent pathway induces IFN. Inhibiting TLR4 endocytosis, through vacuolar type H+-ATPase inhibitor (which prevents endosome acidification), disrupts the TRAM–TRIF pathway. Thus, delivery of TLR4 and TRAM to endosomes are necessary for the activation of the IRF3 signaling pathway (Kagan et al., 2008). The plasma membrane-localized lipid phos-phatidylinositol-4,5-bisphosphate (PIP2) is commonly implicated in the regulation of LPS responses. PIP2 regulates, the localization of the sorting adaptor TIRAP, its ability to induce myddosome formation, and the endocytosis of CD14 and TLR4 (Kagan and Medzhitov, 2006; Kagan, 2017). As such, the switch between the two TLR4 mediated pathways may be caused by a drop of phos-phatidylinositol-4,5-bisphosphate (PIP2) concentrations from the membrane during endocytosis (Botelho et al., 2000), which releases the TIRAP-MyD88 complex from TLR4, thereby enabling TLR4 to interact with TRAM–TRIF in endosomes (Murphy et al., 2009).

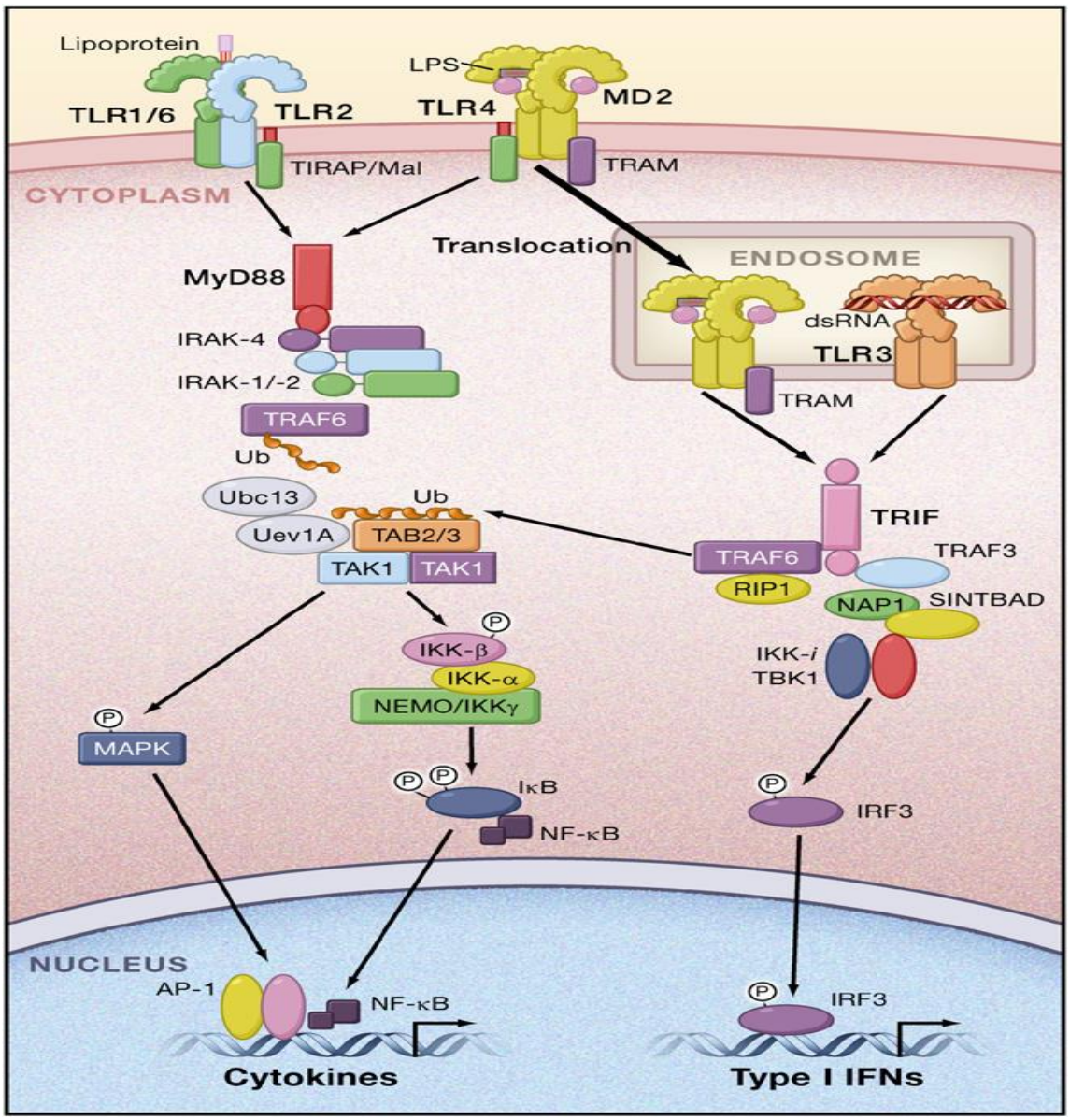


**Figure 1.6. TLR2, TLR3, and TLR4 Signaling Pathways**. TLR4, TLR2 and TLR1-TLR6 localize to the cell surface, and TLR3 localize in the endosome compartment. TIRAP conducts the signal from TLR4 to MyD88, and TRAM mediates the signal from TLR4 to TRIF. TLR engagement, except for TLR3, induces formation of the Myddosome, which contains MyD88, IRAK1 and IRAK4. Ligand binding to TLR3 will lead to the activation of TRIF-dependent pathway. TRAF6 and RIP1 activate NF-kB, whereas TRAF3 is responsible for phosphorylation of IRF3 by TBK1/IKK-i (Takeuchi and Akira, 2010).

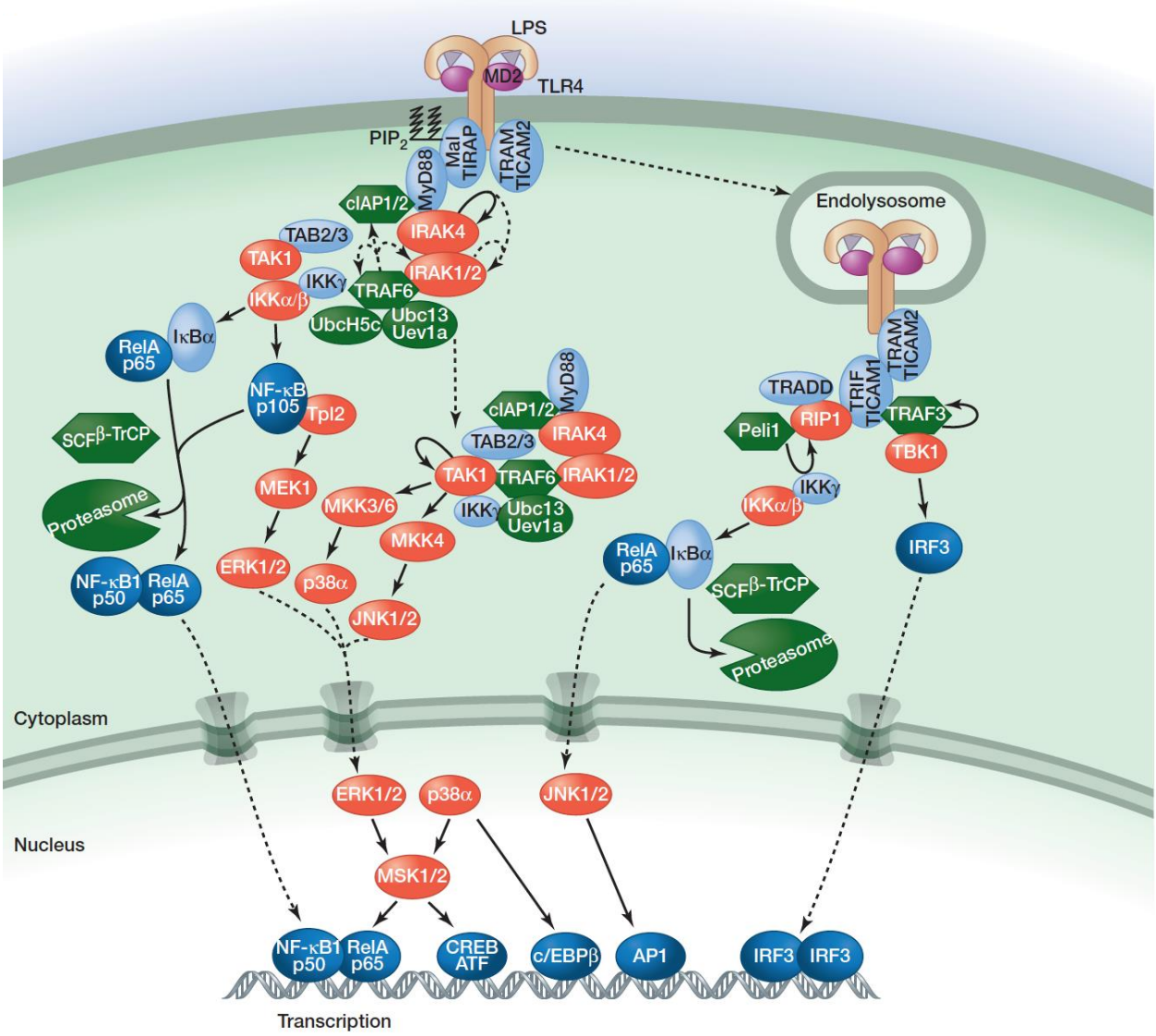


**Figure 1.7. Signaling by LPS, a TLR4 ligand.** TLR4 activates two pathways: The TIRAP/MyD88 pathway, which initiate signaling from the cell surface and the TRAM/TRIF-dependent pathway which signal from the endosomes. The figure depicts the requirement of TLR4 internalization to endosomes for the exchange of the TIRAP- MyD88 signaling complex with the TRAM–TRIF signaling complex (Newton and Dixit, 2012).

### 1.2.7 MyD88 dependent pathway

MyD88 adaptor is composed of Toll/IL-1 receptor (TIR) domain in the C-terminal portion and a death domain (DD) in the N-terminal portion (Akira and Takeda, 2004). This adaptor connects with the cytoplasmic portion of TLR through their C-terminal portion (TIR-TIR interaction) and upon stimulation they also interact with interleukin-1 receptor associated

kinase (IRAK)-4 through their death domain to form complexes called myddosome. Activated IRAK-4 will then recruit other two IRAK family members, namely IRAK-1 and IRAK-2, leading to their activation and phosphorylation. IRAKs then dissociate from the complex and associate with TRAF6 (**Figure 1.6**) (Akira and Takeda, 2004; Mcgettrick and Neill, 2004; Takeuchi and Akira, 2010).

TRAF6 is activated via the process called ubiquitination. These process targets proteins for degradation by proteasomes, by attaching ubiquitin to a target protein through the K48 or K63 lysine residues on ubiquitin. However, covalent attachment to the target protein via K63 lysine on ubiquitin does not result in the proteosomal degradation (Lowe et al., 2006). TRAF6 is an E3 ubiquitin ligase conjugating K63-linked polyubituitin chain on TRAF6 itself, and then act directly on E2 complex called Ubc13 and Uev1A (Lu et al., 2008; Takeuchi and Akira, 2010). As shown in **Figure 1.6 & 1.8**, Ubiquitinated TRAF6 recruits and bind TAB-1 (transforming growth factor-β-activated kinase-1 (TAK-1)-binding protein-1) and TAB-2. This complex then phosphorylates and activate TAK-1, which will then activate kinases upstream of p38 and JNK and the inhibitory kB (IkB) kinase (IKK) complex leading to NF-kB activation. Activation of NF-kB leads to the production of cytokines (Mcgettrick and Neill, 2004). A brief description of transcription factors, kinases, and pro-inflammatory cytokines which have been tested for the regulation of CH25H in this study are detailed in the next pages.

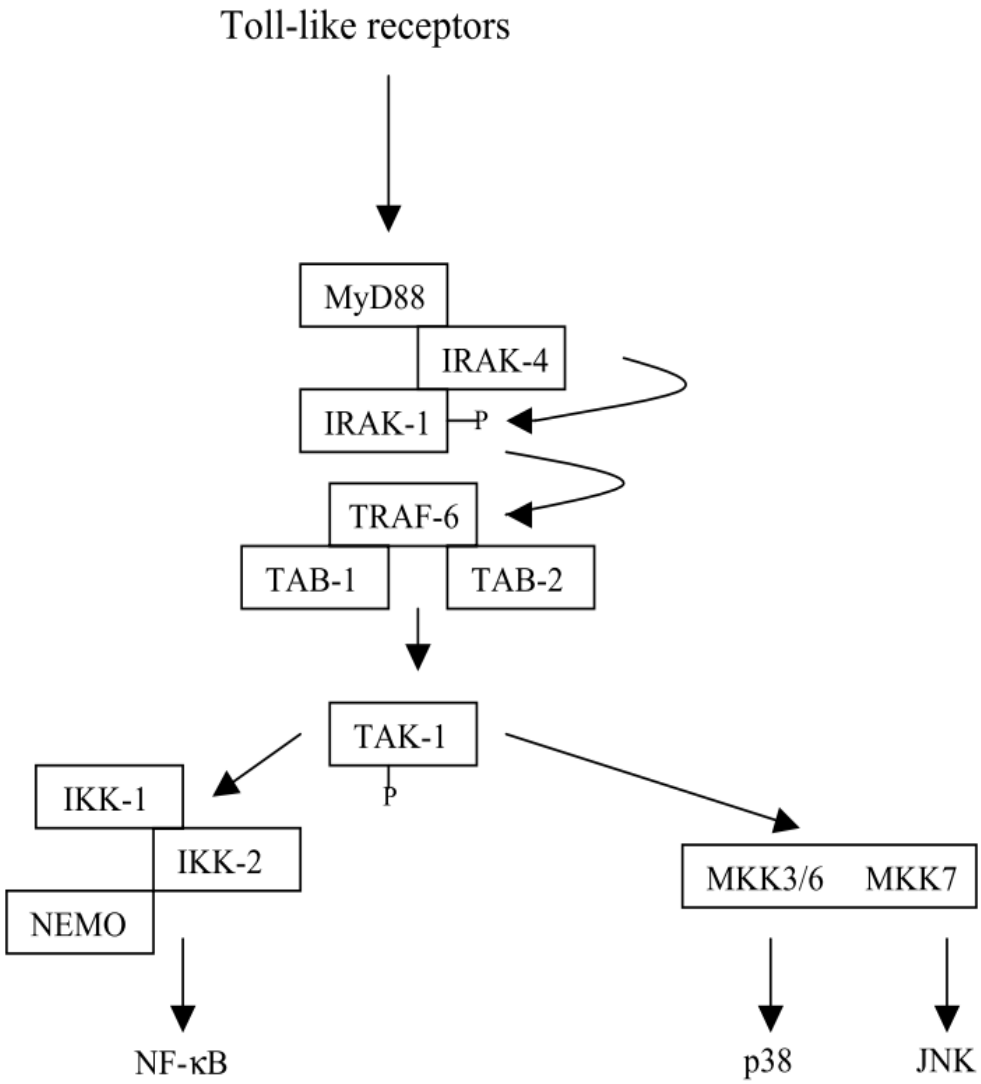


**Figure 1.8. MyD88 dependent pathway**. After ligand binding to receptor that relies on MyD88 adaptor, this adaptor (MyD88) will interacts with IL-1 receptor-associated kinase-4 (IRAK-4), which then recruits and phosphorylates IRAK-1. Activated IRAK-1 then interacts with TRAF-6, which in turn recruits and bindsTAB-1and TAB-2. This complex leads to the phosphorylation and activation of the kinase, TAK-1, which activates kinases that activates p38, JNK, and NF-kB (Mcgettrick and Neill, 2004).

### 1.2.8 Mitogen activated protein kinase pathway

Mitogen-activated protein kinase (MAPK) are evolutionarily conserved family of serine/threonine (Ser/Thr) kinases that transduce signals from the cell-surface membranes to the nucleus in response to a wide range of stimuli such as stress, mitogens, hormones, or neurotransmitters to receptor tyrosinekinases, growth factors, cytokines, thereby controlling fundamental cellular processes such as growth, proliferation, differentiation, migration, metabolism rate, survival and apoptosis (Wada and Penninger, 2004; Plotnikov et al., 2011). This transduction of extracellular signals is achieved through sequential phosphorylation of the three core kinases; MAPK, a MAPK activator (MAPK kinase, MKK or MEK)), and a MAPKK activator (MAPKK kinase, MEKK), and often also additional upstream (MAP4K) and downstream (MAPKAPK) components (Figure 9) (Dhillon et al., 2007; Roskoski Jr, 2012). As shown in **Figure 1.9**, there are two classes of MAPKs in mammals; Conventional MAPKs, which comprises the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38 isoforms ($\alpha$, $\beta$, $\gamma$, and $\delta$), ERK5 and atypical MAPKs, which comprises ERK3/4, ERK7, and Nemo-like kinase (NLK) (Cargnello and Roux, 2011). ERK1/2, JNKs, and p38 isoforms are the most extensively studied groups of mammalian MAPKs. ERK1/2 are activated in response to growth stimuli, while both JNKs and p38-MAPK are simultaneously activated in response to a variety of cellular and environmental stresses are called stress kinases, (Wada and Penninger, 2004). Stress kinases, JNKs, and p38 isoforms will only be reviewed herein.

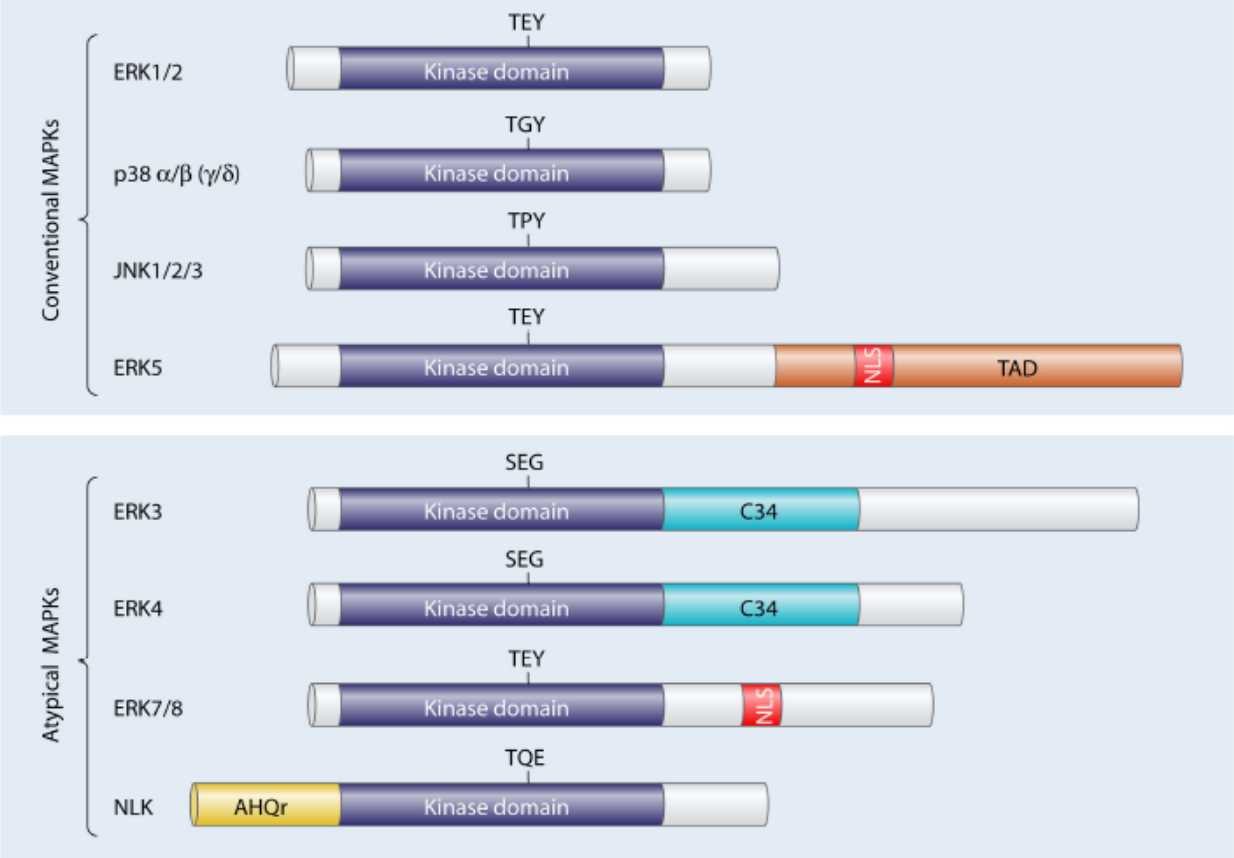


**Figure 1.9. Schematic representation of the overall structures of conventional and atypical MAPKs**. All MAPKs contain a Ser/Thr kinase domain flanked by N- and C-terminal regions of different lengths. Different additional domains are also present in some MAPKs, including a transactivation domain (TAD), a nuclear localization sequence (NLS), a region conserved in ERK3 and ERK4 (C34) (Cargnello and Roux, 2011)

### *1.2.8.1 JNK pathway*

JNKs (also known as stress-activated protein kinases; SAPKs) are ubiquitously expressed, and the JNK stress pathways is predominately activated by cytokines, UV radiation, growth factor deprivation, DNA-damaging agents, certain G-protein coupled receptors and serum (Chang and Karin, 2001). Three distinct genes encode JNK/SAPKs: JNK1/SAPKα, JNK2/SAPKβ and JNK3/SAPKγ. JNK1 and 2 are ubiquitously expressed, whereas JNK3 expression is restricted to the brain, heart and testis (Dong et al., 2002;

Dhillon et al., 2007; Bogoyevitch et al., 2010). JNK is an important mediator of apoptosis and cell proliferation. As shown in **figure 1.10**, JNKs are normally activated by MKK7 and MKK4 (which are both present in the cytoplasm and in the nucleus), which in turn phosphorylate transcription factors such as c-Jun, ATF-2, p53, Elk-1/3, and nuclear factor of activated T cells (NFAT). These phosphorylated transcription factors will then regulate the expression of specific sets of genes to mediate cell proliferation, differentiation or apoptosis. JNK proteins are also involved in cytokine production, and inflammatory response (Raman et al., 2007; Plotnikov et al., 2011; Lei et al., 2014). MEK1 and MEK2 are structurally related to MKK4 and 7, but MEKs do not activate JNKs and, conversely, MKK4 and 7 do not activate ERKs (Wada and Penninger, 2004). In certain settings MKK7 and MKK4 may also activate the p38 Pathway, although JNK/SAPKs are the preferred substrates (Pearson et al., 2001). To activate the JNK pathway, the MKK4 and MKK7 need to be activated by upstream activators such as ASK1, HPK1, MLK-3, or Ste11/Byr 2-homologues MKKK1-4, as well as proteins such as TAK-1 and TPL-2 (Wada and Penninger, 2004; Cargnello and Roux, 2011)

### *1.2.8.2 The P38 MAPK pathway*

The p38 MAPK itself is a 38kD polypeptide that exists in four isoforms (α, β, γ, and δ) (**Figure 1.10**). Of the four isoforms, p38α is the best characterized and is expressed in most cell types. The p38 MAPK and JNK pathways can cross talk at several levels. In mammalian cells, the p38 isoforms are strongly activated by environmental stresses and inflammatory cytokines but not appreciably by mitogenic stimuli. MKKs such as MKK3 and MKK6 activate P38 (Kuida and Boucher, 2004; Cuenda and Rousseau, 2007; Munoz and Ammit, 2010). These MKKs are specific as they do not activate ERK1/2 or JNK. However, MKK3 is somewhat selective, as it preferentially phosphorylates the p38α and p38β isoforms, while MKK6 activates all p38 isoforms (Roux and Blenis, 2004). The MAPK kinase kinase (MKKK) that activates MKK6 and MKK3 appears to be cell type and stimulus specific. However, Several MKKKs such as MLKs, ASK1, TAK-1 and some members of the MEKK family have been implicated in the regulation of p38MAPK signaling (Cuenda and Rousseau, 2007).

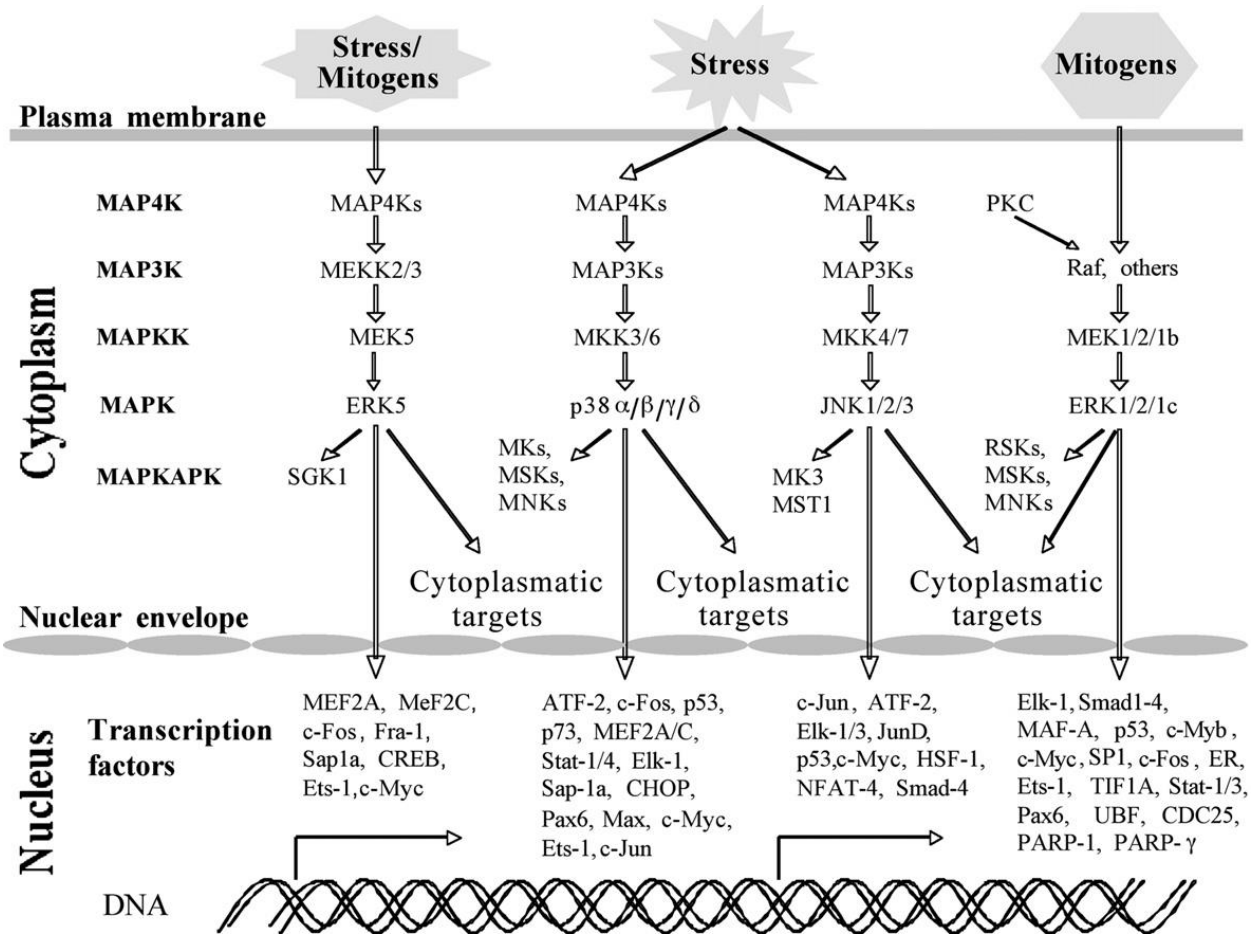


**Figure 1.10. Schematic representation of the MAPK cascades and their nuclear targets**. The MAPK pathway is organized in 3-tiered architecture: MAPKKKs, MAPKK, and MAPK. 4 main subgroups compose the MAPK family, that is, ERK (ERK1/ ERK2), c-Jun (JNK/SAPK), p38 MAPK (p38α, p38β, p38γ and p38δ), and ERK5. Stimulants such as mitogens and stress of the cascades are mentioned, although each cascade can be activated by additional stimulations. Stimulants promote the activation of different MAPK pathways, which in turn phosphorylate and activate the five subgroups of MAPKAPKs, including RSK, MSK, MNK, MK2/3, and MK5 (Plotnikov et al., 2011).

### 1.2.9 Activating transcription factor 3 (ATF3)

Activating transcription factor (ATF) 3 belongs to the activating transcription factor/ cyclic adenosine monophosphate (cAMP) responsive element-binding protein (ATF/CREB) family of transcription factors, which share a basic-region leucine zipper (bZip) element, bind to specific DNA via the basic region in this domain and form homodimers or heterodimers with other bZIP containing proteins via the leucine zipper region (Thompson et al., 2009). This protein family has at least ten members which includes ATF1, CREB, CREM, ATF2, ATF3, ATF4, ATF5, ATF6, ATF7, and B-ATF (Jadhav and Zhang, 2017). ATF3 is a stress-inducible gene, its basal expression is low in most cell types, but rapidly and transiently elevated in response to many different stimuli such as hypoxia, proinflammatory cytokines, nitric oxide, high concentrations of glucose, palmitate, and ER stress (Jang et al., 2013; Lee et al., 2013; Boespflug et al., 2014; Kim et al., 2017). Moreover, ATF3 has been shown to homodimerize or heterodimerize with other ATF/CREB proteins, including ATF2, c-Jun, Jun B, and Jun D and function as either a transcriptional activator or repressor. In bone marrow-derived macrophages (BMDM), activation of various TLRs increases ATF3 protein expression, suggesting its role in TLR signaling. Recently, ATF3 has been shown to be counter-regulatory immune transcription factor, which is induced by Toll-like receptor (TLR) signaling and, in turn, limits the inflammatory response by controlling the expression of a number of cytokines and chemokines (**Figure 1.11**). In addition, ATF3 expression in macrophages is necessary for controlling basal IFN-β expression, as well as the magnitude of IFN-β production following TLR activation (Gilchrist et al., 2008; Thompson et al., 2009; Boespflug et al., 2014; Labzin et al., 2015).

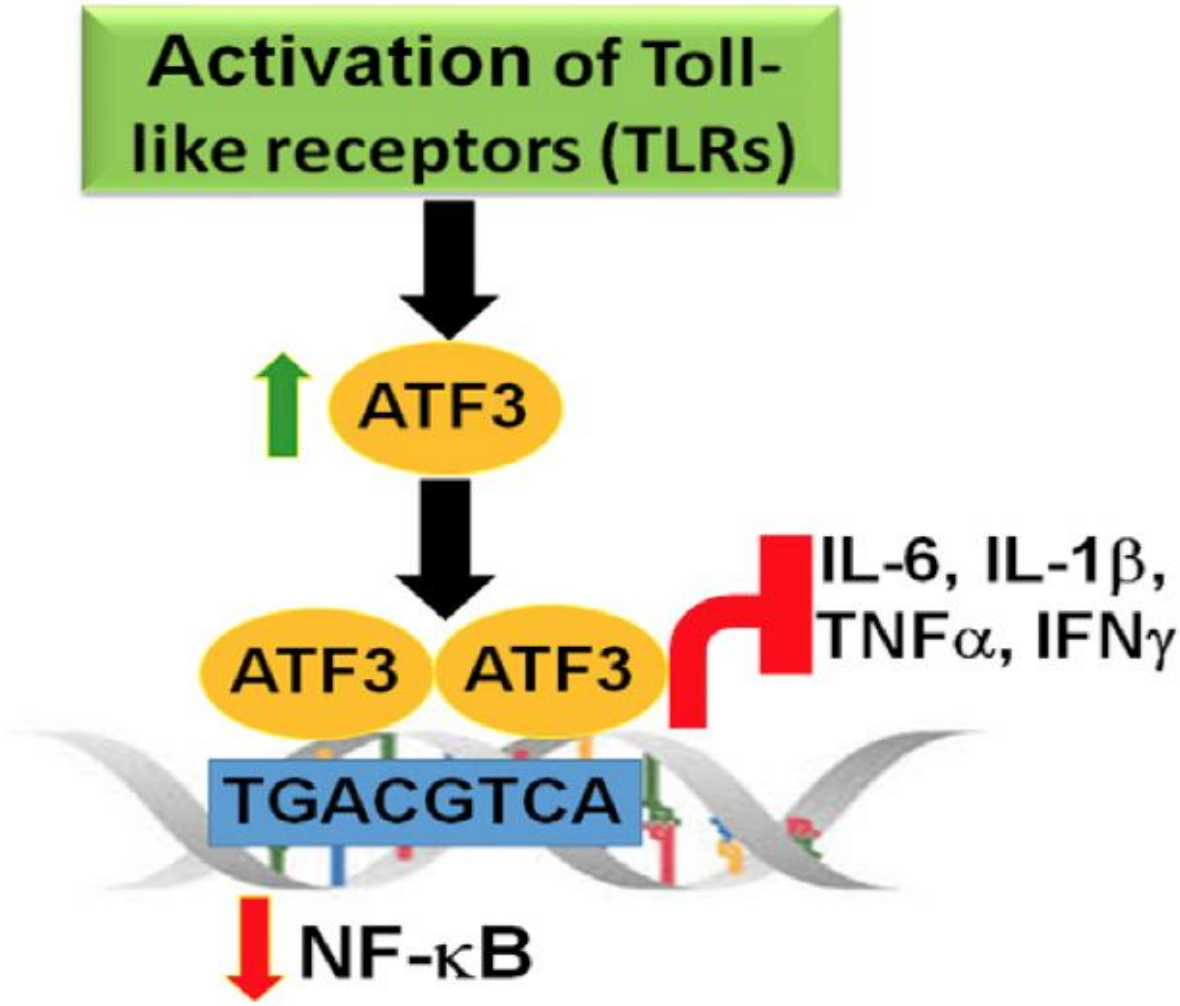


**Figure 1.11. ATF3 as negative regulator of the inflammatory response**. ATF3 basal expression is low in non-stimulated immune cells but rapidly elevated in response Toll-like receptors (TLRs) activation. Thereafter, ATF3 forms dimers and binds to its consensus binding sequence, TGACGTCA, on the promoter of nuclear factor-kB (NF-kB), which result in repression of its expression, as well as downstream targets such as IL-6, IL-β, TNFα, and IFNγ. Adapted from (Jadhav and Zhang, 2017).

### 1.2.10 Signal Transducer and Activator of Transcription (STAT) protein

Signal Transducer and Activator of Transcription (STAT) factors are a group of cytoplasmic transcription factors that possess the ability to transduce signals from the cell membrane to the nucleus to activate gene transcription. They respond to extracellular cytokine and growth factor signals in a wide range of cell types and tissues. STAT1 and STAT2 were the first STAT genes to be identified in the interferon (IFN) signal transduction pathways in mammalian cells. To date, there are seven different members of the STAT family, namely, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 that are encoded by distinct genes (Horvath, 2000; Ihle, 2001; Lim and Cao, 2006).

All STAT proteins in mammals range in size from 750 and 850 amino acids, and despite functional differences in individual STAT proteins, they possess several conserved domains of STAT proteins, which have an imperative function for STAT activation. As shown in **figure 1.12**, these conserved domains include the amino-terminal domain (NH2), the coiled-coiled domain (CCD), the DNA binding domain (DBD), the linker domain and the SH2/tyrosine activation domain. In contrast to the conserved domains, the carboxy-terminal transcriptional activation domain (TAD) is quite divergent and contributes to STAT specificity (Kisseleva et al., 2002). This domain is involved in the communication with other transcription factors and co-activators facilitating transcriptional activation. The amino (N)-terminal domain plays a role in stabilizing Stat dimer-dimer interactions required to facilitate the formation of STAT tetramer or oligomer (Brierley and Fish, 2005; Kim and Lee, 2007), whereas the coiled-coil domain mediates interactions with proteins, including IFN regulatory factor-9 (IRF-9), N-Myc interacting protein (Nmi), and the transcription factor c-Jun. The DNA-binding domain is structurally homologous to the immunoglobin-like DNA binding domain, and binds to DNA as a dimer. This domain also contains several β-sheets that are folded similarly to those found in the DNA-binding domains of the transcription factors, NF-κB or p53. The linker domain connects the DNA-binding domain with the SH2 domain. SH2 domain is the most highly conserved domain among STATs, and is critical for receptor binding and Stat dimerization (Horvath, 2000; Brierley and Fish, 2005; Lim and Cao, 2006; Kim and Lee, 2007)

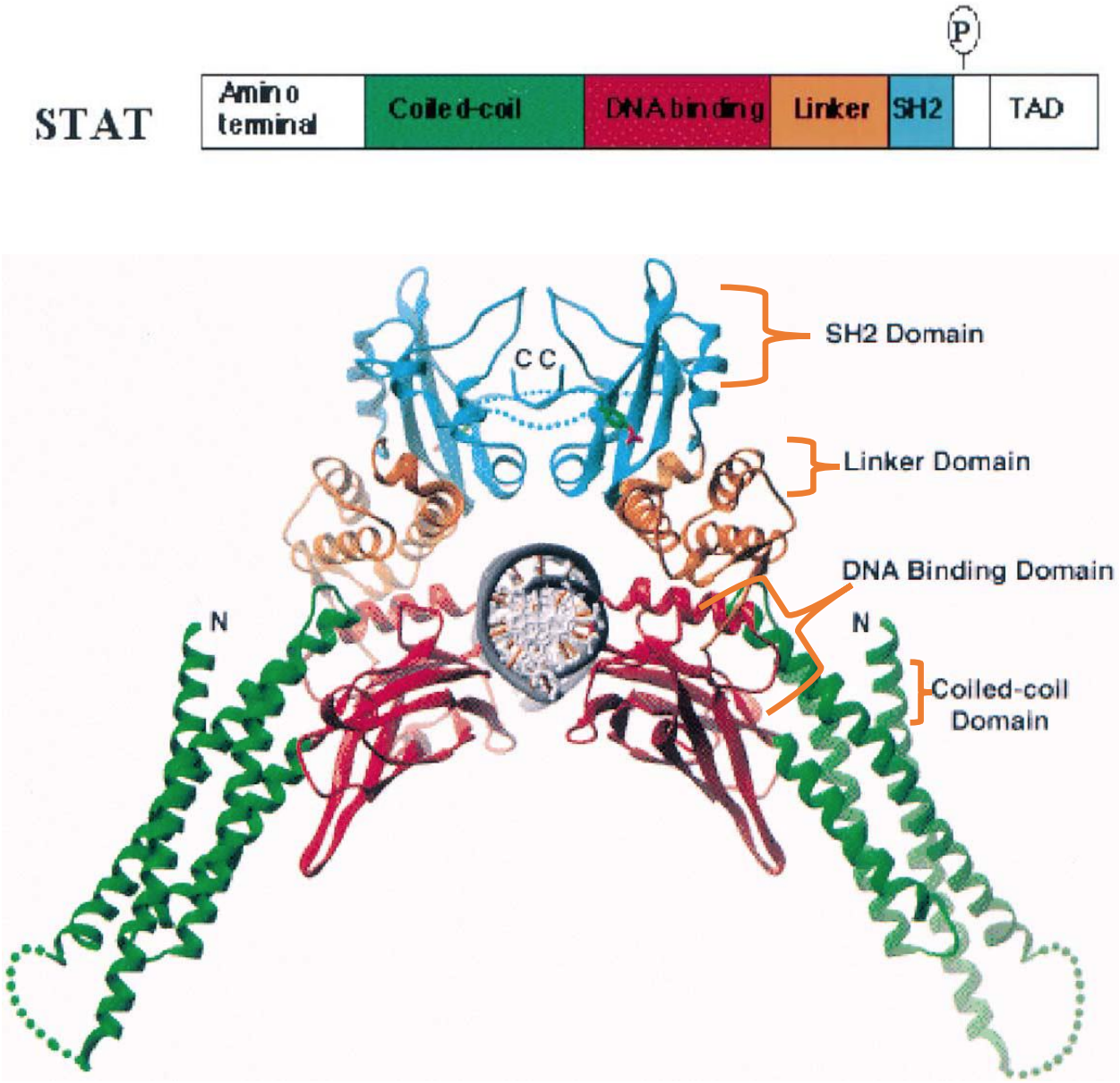


**Figure 1.12. STAT1 structure**. The coiled-coil domain is shown in green, the DNA-binding domain is shown in red, the linker domain in orange and the SH2 domain and the tyrosine-phosphorylated tail segments are shown in cyan. Two disordered loops in the coiled-coil domain and one connecting the SH2 domain are represented as dotted lines. The DNA backbone is colored in gray. 'N' and 'C' indicate the N- and C-termini of the Stat1 core dimer. The carboxy-terminal transcriptional activation domain (TAD) is conserved in function but not in sequence (Kisseleva et al., 2002)

### 1.2.11 Pro-inflammatory cytokine and interferons

#### *1.2.11.1 Interferon signaling: The JAK-STAT signaling pathway*

Interferons (IFNs) were discovered in 1957 by Isaacs and Lindenmann as an agent that inhibited the replication of influenza virus (Isaacs and Lindenmann, 1957). Interferons are proteins produced and secreted by cells following cellular detection of PAMPs by pattern-recognition receptors, which then help regulate the activity of the immune system. Following pathogen detection and interferon production and secretion, interferons can induce cell-intrinsic antimicrobial state in infected (autocrine) and neighbouring (paracrine) cells to attenuate the spread of an infectious pathogen (Ivashkiv and Donlin, 2014). IFNs engage signaling cascade through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway, which lead to the transcriptional regulation of hundreds of IFN-stimulated genes (Schneider et al., 2014).

IFNs are classified into three distinct major categories, type I (α, β,ω, ε, κ, ν, τ); type II (γ), and type III IFNs [λ1, λ2, λ3, which are also known as interleukin-29 (IL-29), IL-28A, and IL-28B, respectively] (Platanias, 2005; Fish and Platanias, 2014). Recently, IFNλ4 has been added to the type III interferon family (Chow and Gale, 2015). As shown in **Figure 1.13**, IFNs are classified according to the receptor complex they signal through. In humans and mice, the IFN-a genes are composed of more than 13 subfamily genes (13 in humans and 14 in mice), whereas only a single IFN-β gene is found. IFNα and β are by far the most studied type I IFNs. Type I IFNs constitute the largest IFN class and engages the ubiquitously expressed IFNAR (IFNα receptor) complex that is composed of IFNAR1 and IFNAR2 (Sadler and Williams, 2008; Schneider et al., 2014; Paludan, 2016). There is sufficient evidence that shows that although all type I interferons bind to the same receptor, and appear to activate a common set of signaling elements, they are differences between IFN-β and other type I interferons in their biological outcomes (Kaur and Platanias, 2013). The type II class of IFNs binds the IFNγ receptor (IFNGR) complex, which is composed of IFNGR1 and IFNGR2, and mediates broad immune responses to pathogens other than viruses. Type III IFNs signal through receptors containing IFNLR1

(IFNλ receptor 1; also known as IL-28Ra) and IL-10R2 (also known as IL-10Rβ) (Platanias, 2005).

All interferons upon IFN binding to their cognitive cell surface receptors, they induce a signaling transduction cascade through the JAK/STAT pathway. As shown in **Figure 1.13**, binding of type I and III interferon to their receptor will induces the activation of Janus family (JAK1, Janus kinase 1 and TYK2, tyrosine kinase 2) kinases, which in turn phosphorylate tyrosine residues in the intracellular tail of the interferon receptors (Sadler and Williams, 2008; Schneider et al., 2014). In the absence of a stimulus, the cytoplasmic domain of the IFN receptor is bound by an inactive JAK kinases. The phosphorylated tyrosine residues of the interferon receptor will then serve as docking sites for recruitment and phosphorylation of the signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2). Activated STAT1 and STAT2 will then undergo heterodimerization. The STAT1-STAT2 heterodimer complex will then recruit and associate with IFN-regulatory factor 9 (IRF9) to form the active transcriptional complex ISGF3 (van Boxel-Dezaire et al., 2006; McNab et al., 2015). Binding of type II IFN to the IFNGR1/2 complex leads to the phosphorylation and activation JAK1 and JAK2 to induce STAT1 homodimerization. STAT1 homodimers form the IFN-γ activation factor (GAF). Both ISGF3 and GAF complexes translocate into the nucleus, and binds to specific promotor elements denoted as interferon signaling response elements (ISREs) or gamma-activated sequence (GAS) to induce IFN-stimulated stimulated genes (ISGs). Beyond the canonical pathways, type I IFNs are reported to activate all seven STAT family members in different cell types, leading to the formation of many hetero- and homodimer pairs (van Boxel-Dezaire et al., 2006; Chow and Gale, 2015). As shown in **Figure 1.14**, although the JAK-STAT pathway is the most studied pathway, this signaling pathway alone is not sufficient to explain all the biological effects of type I IFNs. The phosphoinositide 3-kinase PI3K and mitogen-activated protein kinase (MAPK) pathways can also be activated downstream of IFNAR and have emerged as critical additional components of IFN-induced signal transduction (Katsoulidis et al., 2005; Kaur et al., 2005; McNab et al., 2015).

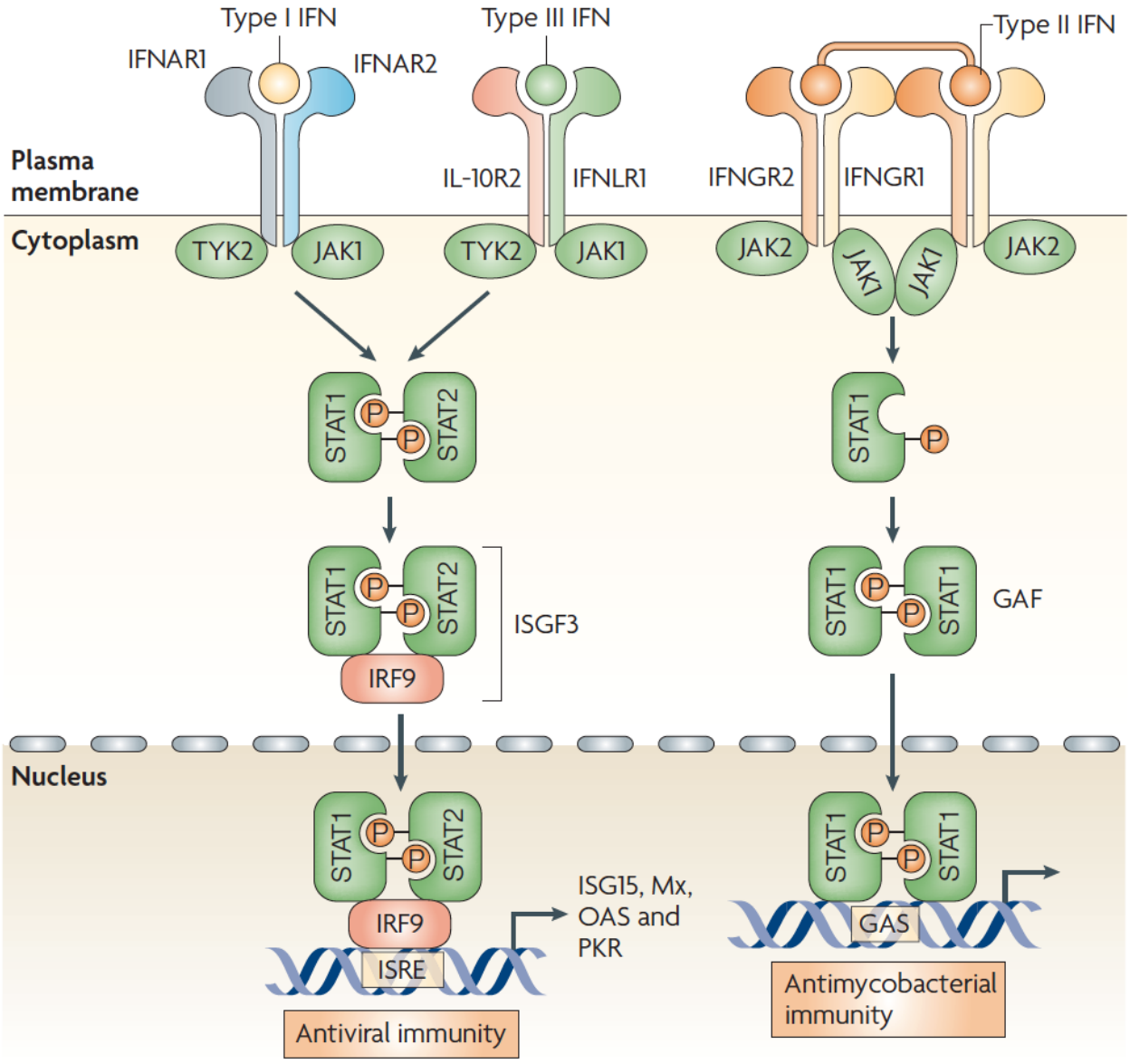


**Figure 1.13. Interferon receptor signaling**. The three different classes of IFNs signal through distinct receptor complexes on the cell surface: type I IFNs initiate signaling through IFNAR1 and IFNAR2 heterodimers; type III IFN through IL-10R2 and IFNLR heterodimers; and type II IFN through IFNGR1 and IFNGR2 heterodimers. Binding of both type I and type III IFNs to their cognitive receptors, triggers phosphorylation of JAK1 and TYK2, which in turn phosphorylate tyrosine residues in the intracellular tail of the interferon receptors. This phosphorylation leads to the recruitment and phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2

associate to form a heterodimer. The STAT1-STAT2 complex recruits associate with IFN-regulatory factor 9 (IRF9) to form IFN-stimulated gene factor 3 (ISGF3) complex. Binding of type II IFN to their cognitive receptors leads to phosphorylation of JAK1 and JAK2 tyrosine kinases, and transphosphorylation of the receptor chains leads to recruitment and phosphorylation of STAT1, which then binds to another STAT1, forming a complex called IFN-γ activation factor (GAF). Both ISGF3 and GAF complexes translocate into the nucleus, and binds to specific promotor elements denoted as interferon signaling response elements (ISREs) or gamma-activated sequence (GAS) to induce IFN-stimulated stimulated genes (ISGs). ISGs are the ultimate antiviral effectors of the interferon signaling. (Sadler and Williams, 2008).

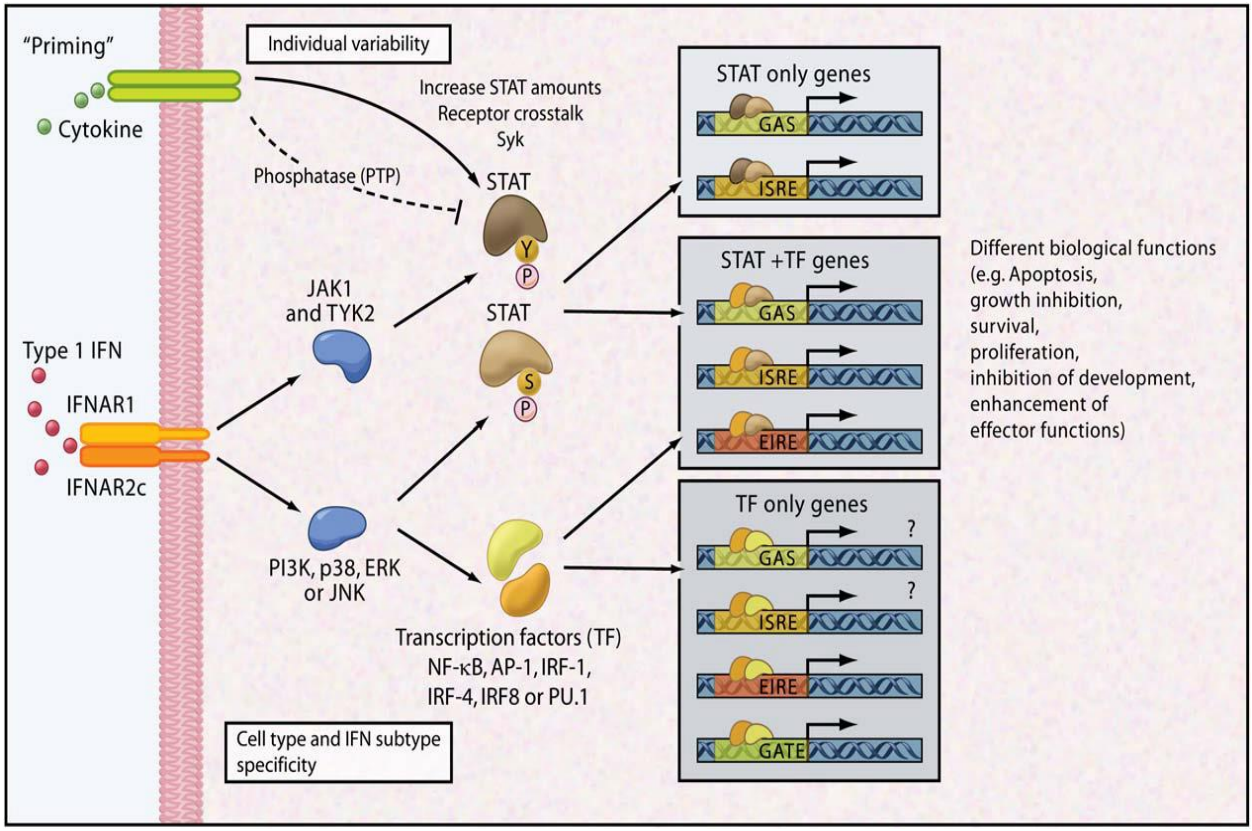


**Figure 1.14. The Complexity of Type I IFN-Dependent Signaling**. Upon binding of type I IFNs to their cognitive receptor (IFNAR), divergence from the canonical pathway, multiple downstream signaling pathways can be induced, leading to a diverse range of biological effects (van Boxel-Dezaire et al., 2006).

### *1.2.11.2 Interleukin-6 (IL-6) signaling*

Interleukin-6 (IL-6) is a pleiotropic cytokine that not only have biological activities in immune regulation and inflammatory response (pro- as well as anti- inflammatory properties) but also affects hematopoiesis, metabolism, and organ development (HEINRICH et al., 2003; Kishimoto, 2010; Su et al., 2017). IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-alpha and IL-1, and activation of IL-1ra and IL-10.

As stated above, IL-6 can be secreted by several cells in response to PAMPs upon detection by pattern recognition receptors, such as Toll-like receptors. However, other cytokines, such as IL-1β and tumor necrosis factor have the ability to induce IL-6 expression (Hunter and Jones, 2015).

IL-6 utilizes two mechanisms to mediate its biological effects, namely Classical IL-6 receptor signaling and IL-6 trans-signaling. Classical IL-6 receptor signaling occurs in cells that express IL-6 receptor (IL-6R, CD126) and gp130 (CD130), while IL-6 trans-signaling can act on cells that lack IL-6R (Wolf et al., 2014; Hunter and Jones, 2015). It is noteworthy that although gp130 is ubiquitously expressed, only a few cell types, such as neutrophils, T-cells, monocytes/macrophages, podocytes, hepatocytes and glomerular epithelial cells, express IL-6R on the cell surface, and therefore can directly respond to IL-6 (Scheller and Ohnesorge, 2006; Su et al., 2017).

Classical IL-6 receptor signaling: On target cells, IL-6 first binds to IL-6R on the cell surface, and then associates with two molecules of signal-transducing membrane protein gp130 and initiate signal transduction (**Figure 1.15**) (Murakami et al., 1993; Schaper and Rose-John, 2015). The first event in the signal transduction is the activation of JAK tyrosine kinase family members, which then lead to the recruitment and activation of STATs, which induce activation of various genes (Taga and Kishimoto, 1997; Scheller et al., 2011). As shown in **figure 1.16**, pathways that regulates cell proliferation and differentiation, such as GTPase Ras, and its effector Raf, and Mitogen-activated protein

kinase cascade are also induced by IL-6, but the JAK-STAT signaling pathway is best understood (Taga and Kishimoto, 1997). However, the crosstalk between these signaling pathways still needs to be elucidated.

IL-6 trans-signaling: In addition to the membrane-bound receptor, a soluble form of the IL-6R (sIL-6R) has been detected in human body fluids such as serum and urine (Novick et al., 1989; Su et al., 2017). As shown in **figure 1.17**, IL-6R soluble form is released from the cell surface by proteolysis and splicing of IL6R mRNA, and can bind IL-6 to form an agonistic complex (Chalaris et al., 2011). IL-6/sIL-6R complex, similar to the classical IL-6 receptor signaling associate with gp130 and initiate signal transduction (Scheller and Ohnesorge, 2006). Interestingly, cells which only express gp130 can respond to IL-6 bound to the sIL-6R. The functional activity of soluble IL-6R as an agonist under physiological conditions is still not clear, since relatively high concentrations of gp130 has been found in human blood. This soluble gp130 can act as an antagonist by neutralizing the IL-6/sIL-6R complexes (**Figure 1.17**) (Jostock et al., 2001; Scheller and Ohnesorge, 2006; Schaper and Rose-John, 2015).

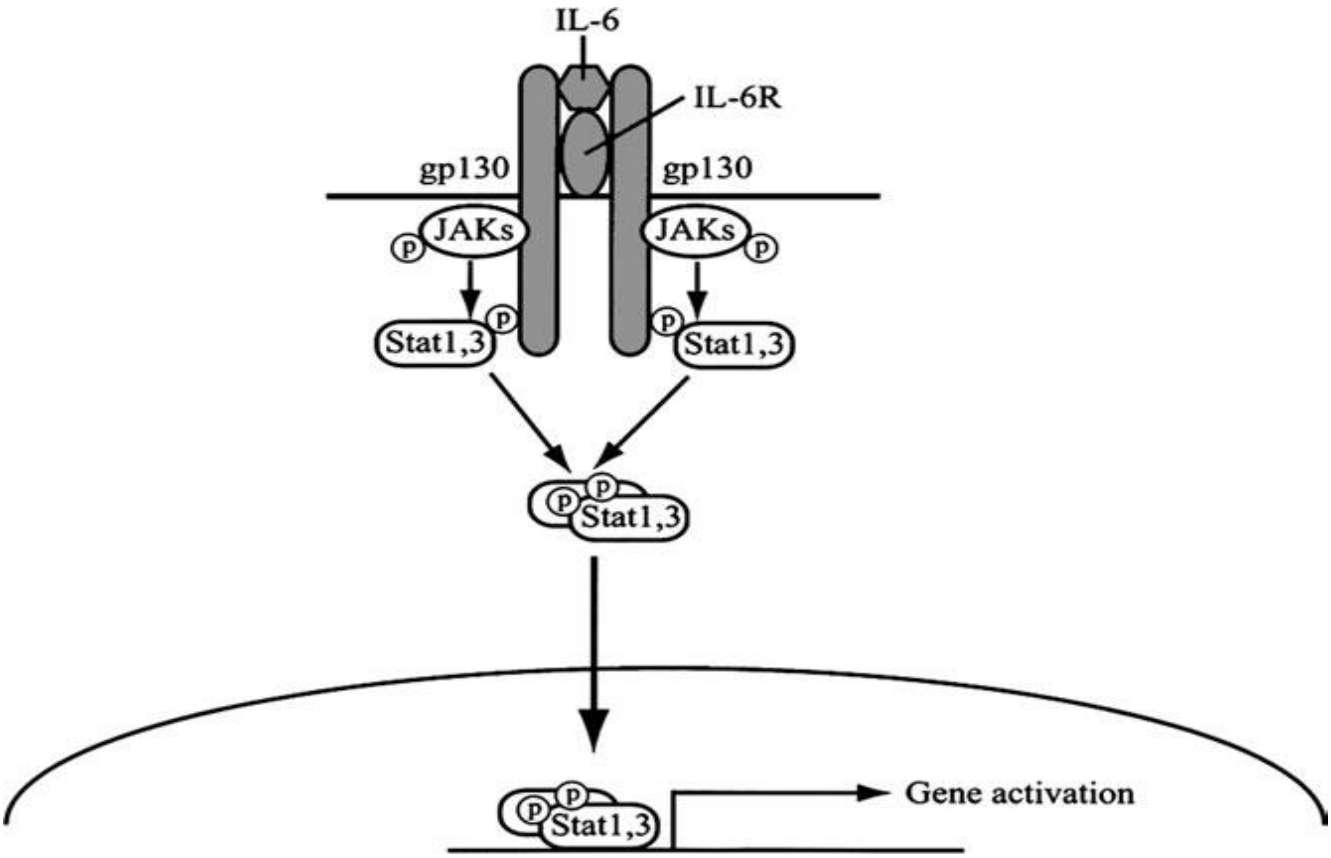


**Figure 1.15. IL-6 signal transduction**. Upon IL-6-bindind to its receptor IL-6R and homodimers of gp130, it leads to the activating (phosphorylating) JAKs, and Stat1 and Stat3. Thereafter, activated Stat1 and Stat3 form homodimers or heterodimers, which induce activation of various genes (Kishimoto, 2010)

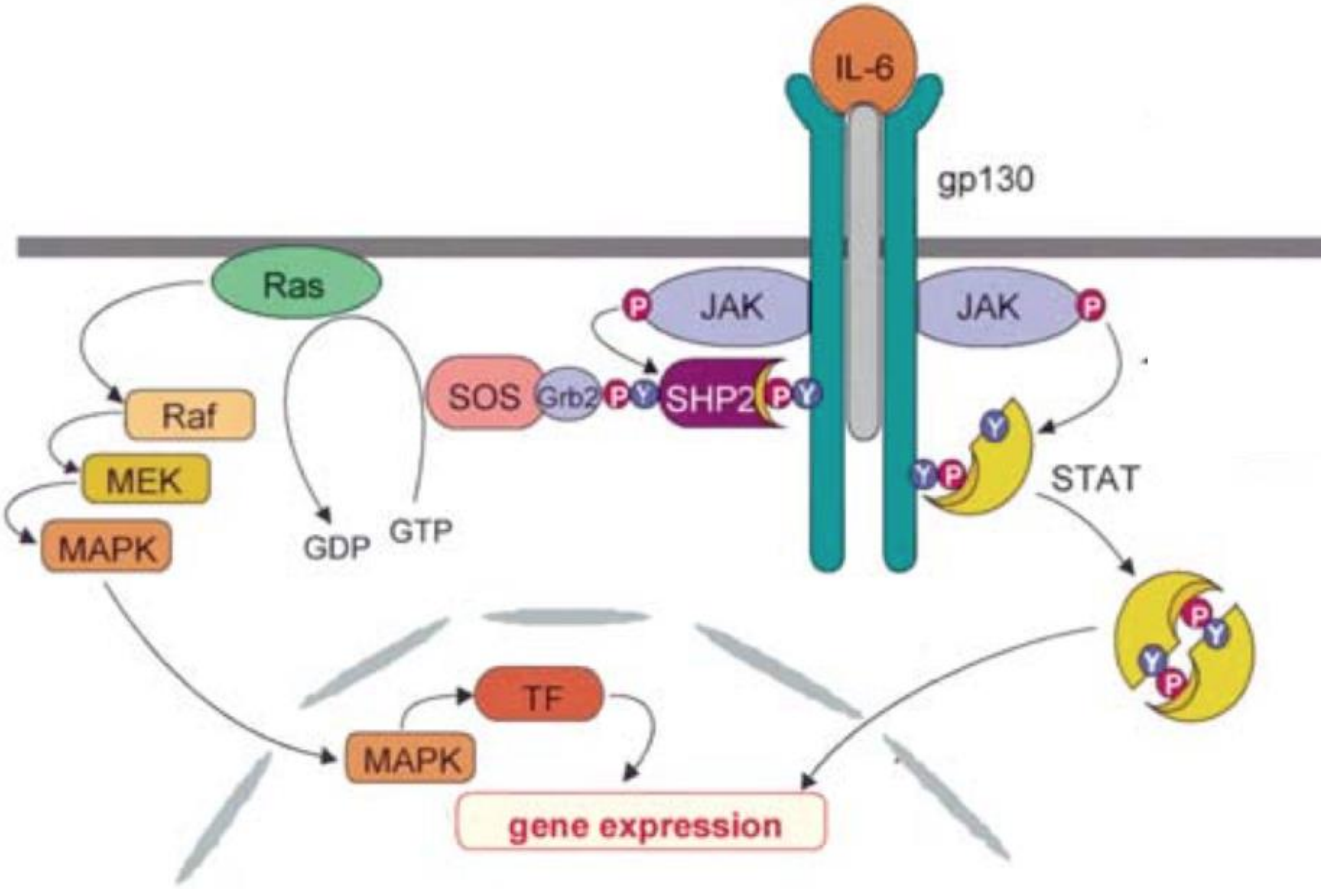


**Figure 1.16**. IL-6 activates the JAK/STAT pathway and the MAPK cascade. Adapted and modified from (HEINRICH et al., 2003).

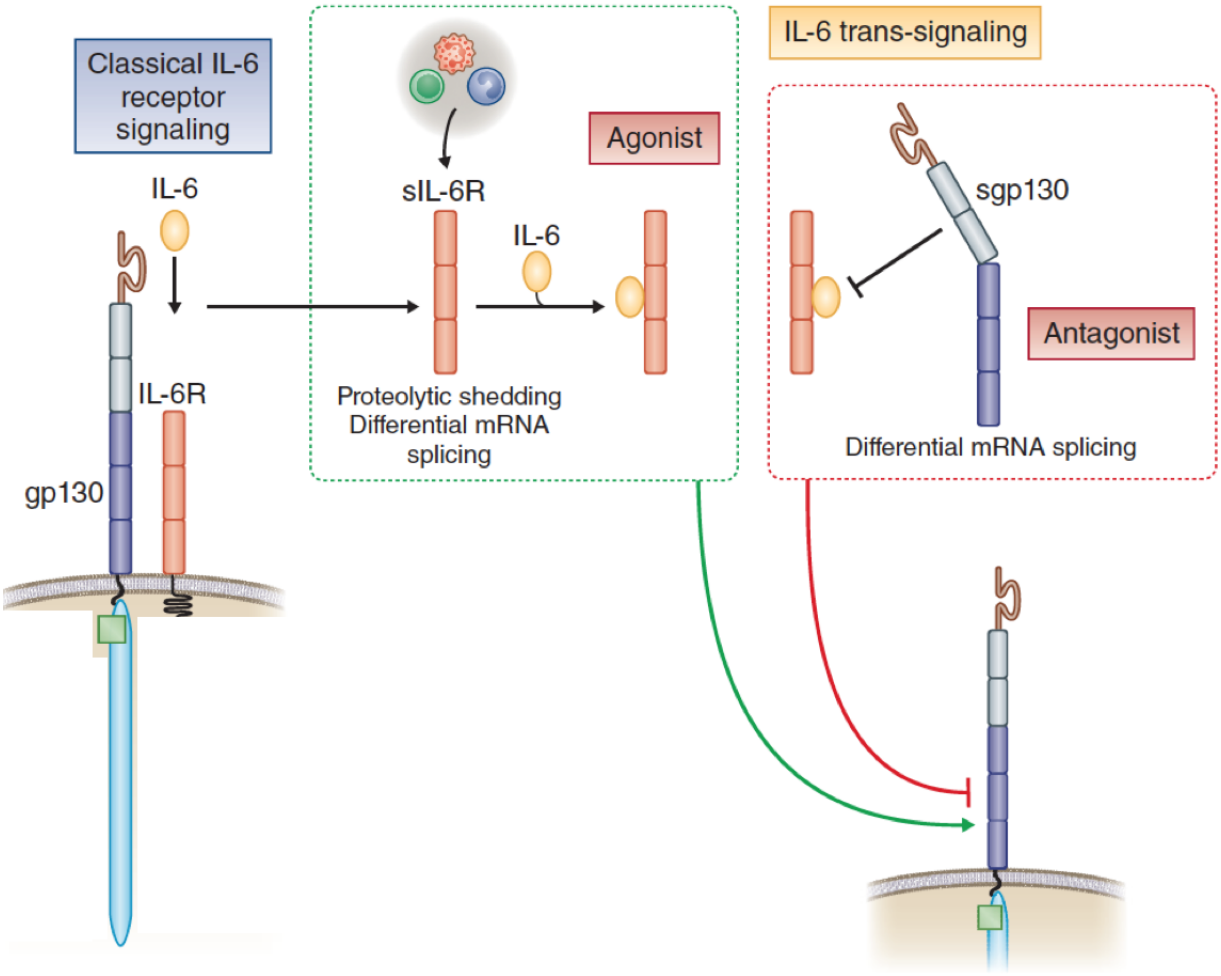


**Figure 1.17. Two IL-6 modes of action and the functional activity of the soluble gp130 (sgp130).** The two modes of IL-6 activation are presented as classical IL-6 receptor signaling, which is activation via the membrane-bound IL-6R, and the L-6 trans-signaling, which is sIL-6R-mediated. Sgp130 antagonizes IL-6/sIL-6R complexes. Adapted and modified from (Hunter and Jones, 2015).

### *1.2.11.3 Tumor necrosis factor alpha (TNF$\alpha$) signaling*

Tumour necrosis factor alpha (TNF$\alpha$ ) was originally described as a circulating factor that can cause necrosis of tumours but has since been identified as a potent cytokine that mediates inflammatory responses and innate immunity on various cell types. Moreover, Dysregulation of TNF production has been implicated in the pathogenesis of several

diseases, including rheumatoid arthritis, alzheimer's disease, cancer, diabetes, psoriasis, sespsis and inflammatory bowel disease (IBD) (Bradley, 2008; Wong et al., 2008; Horiuchi et al., 2010). Although TNFα can be produced by other cell types, it is produced chiefly by activated macrophages and T lymphocytes as a 26 kDa trans-membrane protein, pro-TNF, which requires proteolytic cleavage by TNFα-converting enzyme (TACE, also named ADAM17) to release a soluble 17 kDA TNFα (Black et al., 1997; Bradley, 2008; Horiuchi et al., 2010; Kalliolias and Ivashkiv, 2016). This soluble TNFα (sTNFα) can acts in a paracrine manner, distinct from the transmembrane TNFα which exerts its biological function in a cell-to-cell contact fashion (Perez et al., 1990). The soluble TNF-α mediates its biological activities by triggering Type 1 and 2 TNF receptors (TNF-R1 and TNF-R2, respectively). Moreover, transmembrane TNF-α (memTNF) also binds to TNF-R1 and TNF -R2, but its biological activities are supposed to be mediated mainly through TNF-R2 (Grell et al., 1995; Wajant et al., 2003). TNF-R1 is constitutively expressed in most tissues, whereas expression of TNF-R2 is highly regulated and is typically found in cells of the immune system. Moreover TNFR-1 bears conserved cytoplasmic death-domain motifs, while TNFR2 lacks a death domain and thus is unable to induce programmed cell death directly.

As shown in **figure 1.18a**, homotrimers of TNF bind to homotrimeric TNFRs to induce signalling. In unstimulated TNFR1, the cytoplasmic domain is pre-associated with a cytoplasmic protein designated silencer of death domain (SODD), and the binding of TNF to its receptor causes a conformational change in the receptor, leading to the dissociation of the inhibitory protein SODD from the intracellular death domain (Jiang et al., 1999). Following the dissociation of SODD, TNFR1 through its death domain recruits the adaptor protein TNFR-associated death domain protein (TRADD) and the assembly of distinct signalling complexes, termed complexes I, IIa, IIb and IIc, which lead to distinct functional outcomes (Bradley, 2008; Kalliolias and Ivashkiv, 2016). As shown in **Figure 1.18b**, in contrast to TNFR1 that recruit the TRADD, TNFR2 recruits TNFR-associated factor 2 (TRAF2), triggering the formation of complex I. Similar to TNFR1 complex I, TNFR2 complex 1 formation leads to downstream activation of NFκB and MAPKs (Sabio and Davis, 2014).

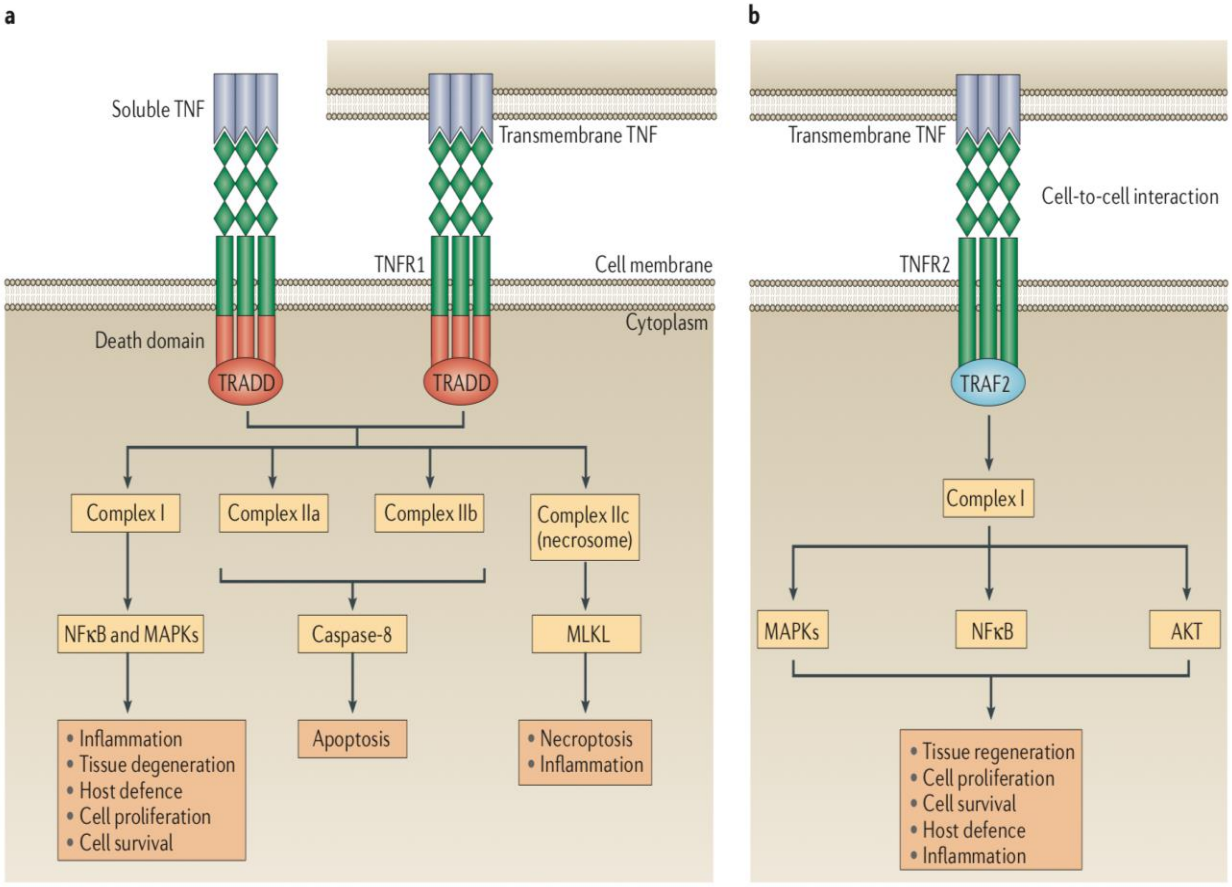


**Figure 1.18. Membrane-bound TNF and soluble TNF signaling pathways**. Membrane-bound TNF and soluble TNF (sTNF) both bind to TNFR1 and TNFR2. However, sTNF predominantly stimulates TNF-R1 and has limited signaling capacities on TNFR2. (Kalliolias and Ivashkiv, 2016).

### *1.2.11.4 Interleukin-1 beta (IL-1β) production and signaling*

IL-1 is the term for a family of polypeptide mediators that now includes 11 members: IL-1α, IL-1β, the IL-1 receptor antagonist (IL-1Ra), IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL-1Hy2 (Turner et al., 2014), but only IL-1β will be described in details here. Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine that is crucial for host-defense responses to infection and injury (Lopez-castejon and Brough, 2011). However, IL-1β is not just a player of the innate immune response, but involved in the development, proliferation and differentiation of T helper cells and B cells. Thus, they play a huge role at the interface of innate and adaptive immune responses (Martin and Wesche, 2002). Of

the 11 IL-1 family members, IL-1β It is the best characterized and the most studied. IL-1 receptor and the human TLRs shares an intracellular TIR (Toll IL-1 receptor) signaling-domain, which is required for initiating intracellular signaling. Thus, enabling TLR-like transcriptional responses in cells not expressing TLRs (Dinarello, 2013; Gaidt and Hornung, 2017). In response to TLR, activated complement components, other cytokines (such as TNF-α), and IL-1 itself, IL-1β is produced as an inactive 31 kDa precursor, termed pro-IL-1b, within the cytosol, where they await proteolytic cleavage to gain bioactivity, and released into the extracellular space. This maturation step is most commonly executed by the cysteine protease Caspase-1, whose activity is controlled by a cytosolic activation platform called the inflammasome. Four main key components of the macromolecular complex (inflammasomes) have been characterized: NLRP1 (NALP1); NLRP3 (NALP3, cryporin); NLRC4 (IPAF) and AIM2 (Lopez-castejon and Brough, 2011; Szabo and Csak, 2012; Garlanda et al., 2013; Piccioli and Rubartelli, 2013; Anders, 2016; Gaidt and Hornung, 2017). They have different ligand recognition sites and utilization of adapter molecules but all results in the activation of caspase-1. Since NLRP3 is the most fully characterized member of the inflammasome family, we will only review pathways that lead to the action of NLRP3 and the release of IL-1β. The inflammasome complex is formed with or without the contribution of an adapter molecule, such as the apoptosis-associated speck like CARD domain containing protein (ASC) (Szabo and Csak, 2012).

As shown in **figure 1.19**, in addition to the well know pathway that pro-IL-1β is expressed by inflammatory cells after activation of Toll-like receptors (TLR), by pathogen products or factors released by damaged tissues (DAMPs) such as uric acid crystals, other signals exist, including exogenous ATP, which trigger the assembly of the inflammasome leading to activation of caspase-1 that in turn processes pro-IL-1β to its mature form (Gram et al., 2012; Piccioli and Rubartelli, 2013). This assembly of the inflammasome occurs following a drop in the intracellular potassium which is triggered by the binding of ATP to purinergic receptor P2X7. The binding of ATP to this receptor opens the potassium channel which results in potassium efflux and pannexin recruitment to induce caspase-1 activation by the inflammasome (Joosten et al., 2013; Ranson et al., 2017). Pannexin is a membrane

pore that allows the delivery of extracellular PAMPs and DAMPs into the cytosol. Since the presence of ATP is crucial, in differentiated monocyte-derived macrophages, there is little or no processing of the IL-1β precursor without this exogenous ATP. In addition to extracellular ATP activating inflammasome, some studies suggest that phagosomal materials (crystals, crystalline material, particles, protein aggregates), and reactive oxygen species (ROS) contribute to inflammasome activation (O'neill, 2008; Bauernfeind et al., 2011; Szabo and Csak, 2012; Gaidt and Hornung, 2017).

Similar to its family member IL-1α, mature IL-1β signals through its receptor IL-R which is composed of two subunits, Interleukin 1 receptor type I (IL-1RI) and Interleukin 1 receptor accessory protein (IL1RAcP) (**Figure 1.20**) (Rosenzweig et al., 2014). IL-1R is constitutively expressed on many cell types. Activated IL-1R through its cytosolic Toll/interleukin-1 receptor (TIR) domain recruit the adaptor protein Myeloid differentiation primary response gene 88 (MyD88) which in turn activates Interleukin-1 receptor-associated kinase 4 (IRAK4) and Interleukin-1 receptor-associated kinase 1 (IRAK1). IRAK1, in turn recruits and activate TRAF6 which results in the activation of Nuclear factor kappa-B (NF-kB) and the mitogen-activated protein kinases (MAPKs) (Symons et al., 2006; Acuner Ozbabacan et al., 2014). These transcription factors induce expression of multiple genes, including pro-inflammatory cytokines, which contribute to an immune response towards various pathogens (Rosenzweig et al., 2014)

In addition to IL-1RI, there is a decoy receptor made up of IL-1RII and IL-1RAcP which also binds IL-1β but this receptor (IL-1RII) lacks an intracellular activation domain (Mantovani et al., 2001). An absent of this intracellular activation domain renders this receptor inactive, and thus signaling is not induced following ligand binding. Moreover, IL-1RII acts as a decoy receptor, sequestering IL-1, inhibiting its activity by preventing IL-1 from binding to the signaling IL-RI (**Figure 1.21**) (Orlando et al., 2000; Turner et al., 2014). Another member of the IL-1 family members that have an inhibitory role, is an IL-1R antagonist (IL-1Ra), which is synthesized and released in response to the same stimuli that lead to IL-1 production. Moreover, it also binds to the signaling receptor with similar affinity to the IL-1β but prevents the recruitment of IL-1RAcP, thereby inhibiting

signal transduction (Mantovani et al., 2001; Akdis et al., 2011; Rosenzweig et al., 2014; Turner et al., 2014).

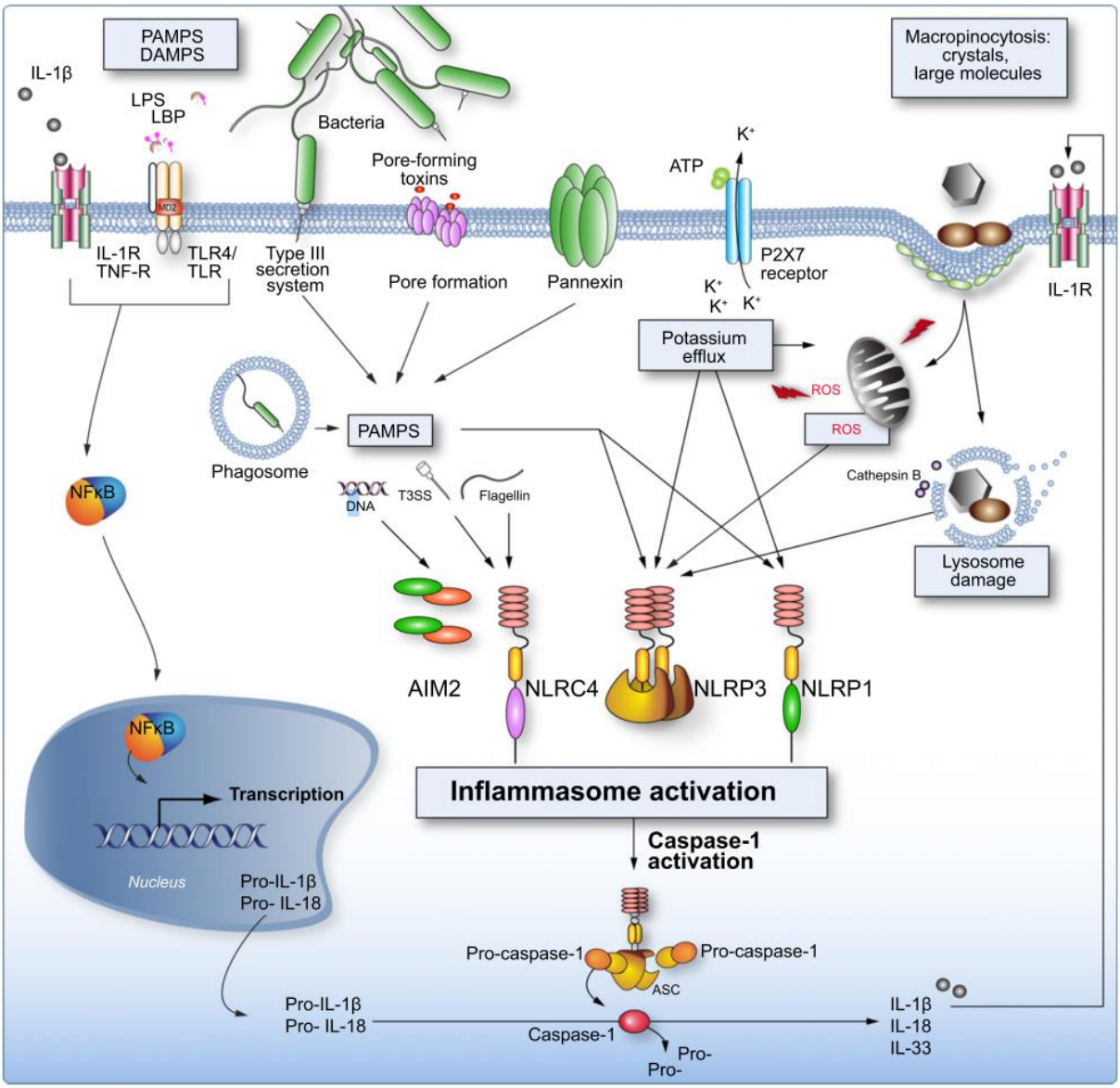


**Figure 1.19. Synthesis and the release of IL-1β.** The first in IL-1β production is the synthesis of pro-IL-1β after activation of Toll-like receptors, by pathogen products or factors released by damaged tissues (DAMPs), or by other cytokines such as TNF-α or IL-1β it-self. The second signal is the activation of inflammasome which results in the activation of caspase-1. Caspase-1 is activation by the inflammasome will induce the activation of pro-IL-1β into its mature form IL-1β (Szabo and Csak, 2012).

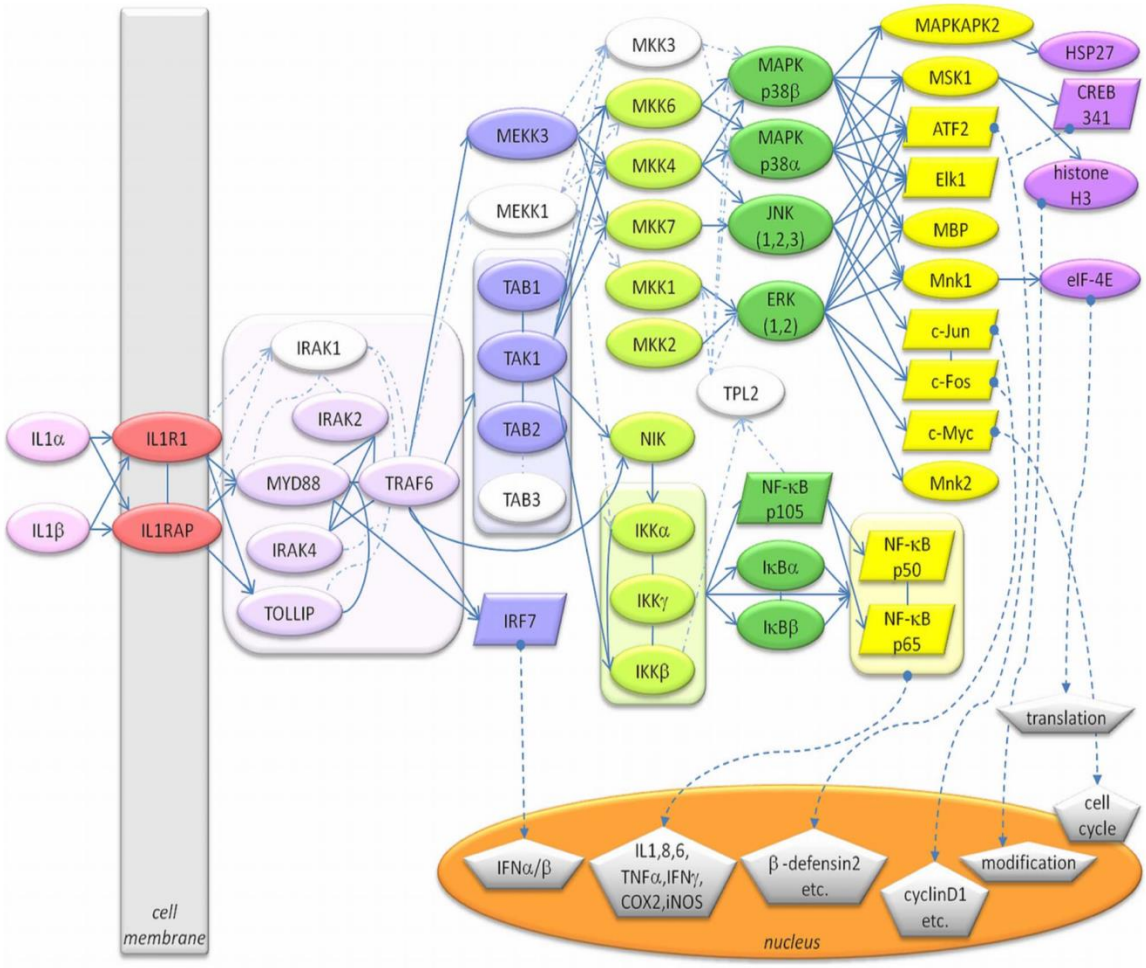


**Figure 1.20. Mechanism of interleukin-1 signaling**. Binding of IL-1α or IL-1β to its receptor IL-1R, which associates with the IL-1RAcP at the cell surface, and signals via the TIR adaptor and recruit MyD88 which initiate a signal transduction cascade, which results in the activation of transcription factors, Nuclear factor kappa-B (NF-kB) and the mitogen-activated protein kinases (MAPKs) (Acuner Ozbabacan et al., 2014).

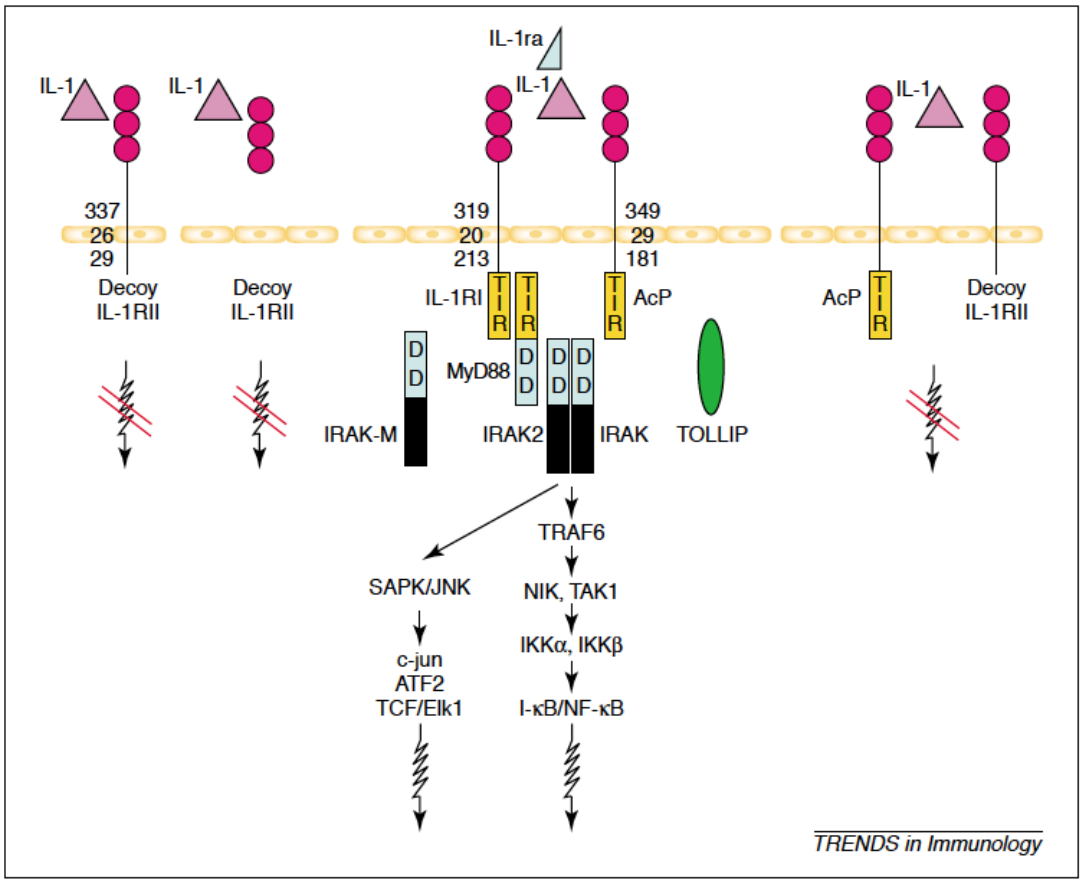


**Figure 1.21. The type II interleukin-1 receptor (IL-1RII) as a decoy**. IL-1 bind to its receptor IL-1RI, which associates with the IL-1RAcP at the cell surface, and signals via the TIR adaptor and activate MyD88 cascade which results in the activation of transcription factors. In contrast to the IL-RI, in membrane-bound or released form, IL-1RII captures IL-1 and prevents it from forming a signaling receptor complex (Mantovani et al., 2001).

### 1.3 Study rationale, Aims, and Objectives

One molecular mediator of innate antiviral immunity that has attracted much attention recently is Cholesterol-25-hydroxylase (CH25H). CH25H is an enzyme that converts cholesterol to 25-hydroxycholesterol (25HC) (Park and Scott, 2010; Blanc et al., 2013; Anggakusuma et al., 2015). Following stimulation of toll-like receptors (TLRs) 3 and 4, common sensors of viral and bacterial infection, CH25H is highly induced in mouse macrophages and dendritic cells. Moreover, its induction is dependent on interferon alpha receptor (IFNAR) interactions (Park and Scott, 2010). Importantly, 25HC exerts antiviral activity against both enveloped and non-enveloped viruses in *in vitro* (Blanc et al., 2013; Anggakusuma et al., 2015; Xiang et al., 2015; Shrivastava-ranjan et al., 2016; Li et al., 2017), specifically by suppressing proteolytic activation of sterol regulatory element binding proteins (SREBPs) and acting as a ligand for liver X receptor (LXR) (Adams et al., 2004; Radhakrishnan et al., 2007; Cyster et al., 2014; Reboldi et al., 2014). In these ways, 25HC promotes induction of genes involved in cholesterol efflux, thereby limiting cellular cholesterol content available for viral replication. Moreover, 25HC also exerts its antiviral activity by activating the integrated stress response, which is independent of the LXRs and SREBPs (Shibata et al., 2013). The details of these pathways can be found on our review article, tittled ‘’ Immunometabolic Signaling Pathways Contribute to Macrophage and Dendritic Cell Function’’ (attached in appendix).

Given its antiviral activities, CH25H is part of an increasingly appreciated connection between type I interferon (IFN-I) and lipid metabolism (Blanc et al., 2011; Keyel et al., 2012; Xiang et al., 2015; York et al., 2015). Importantly, however, the details of this connection appear to differ in mouse and human cells. In murine models, IFN-I induces CH25H, thereby linking these two biological processes (Bauman et al., 2009; Diczfalusy et al., 2009; Park and Scott, 2010). On the other hand, in human hepatoma cell lines CH25H does not appear to be a classical interferon stimulated gene (ISG); rather, it is induced in response to TLR3/4 agonists to restrict viral replication (Xiang et al., 2015). Thus, the precise transcriptional regulatory events for the induction of CH25H in humans are poorly understood. Achieving such understanding is critical to further design therapeutic agents to control pathogens.

Zika virus (ZIKV) is an arthropod-borne virus, transmitted by Aedes mosquitoes, which belongs to the *Flaviviridae* family and the *Flavivirus* genus. Recently, ZIKV has emerged as a public health threat due to its global transmission and link to severe congenital disorders, such as microcephaly, Guillain-Barre syndrome (GBS), and meningoencephalitis (Carteaux et al., 2016; Mlakar et al., 2016; Oliveira Melo et al., 2016). ZIKV infection in placental macrophages has been reported to transmit the virus from the mother to fetus (Quicke et al., 2016). Host immune responses to ZIKV infection have not been fully elucidated. At present, there is no vaccine or antiviral drug to combat ZIKV. With these concerns we thought to use ZIKV as avirus model system for the study of CH25H regulation in human macrophages. With this concerns in mind, we AIM:

To investigate the precise molecular mechanism by which ZIKV or TLR activation induce the expression of CH25H in human macrophages. To scrutinize this aim we proposed two specific objectives:

1. To determine whether a pathogen (ZIKV), TLR-stimulation, or secretory molecules (cytokines) induce the expression of CH25H mRNA.
2. To determine whether the NF-kB, MAPK, ATF3, IRFs, or the JAK-STAT signaling transduction pathways are involved in the regulation of CH25H expression.

To achieve these goals, we used microglial cells (human central nervous system-resident macrophage cell-line), and THP-1 macrophages (human monocytes-derived macrophage cell-line). These cell-lines are easily grown, maintained, and frequently used to model macrophages since primary macrophages cannot be readily expanded ex-vivo. Isolation of primary macrophages requires blood donation and limited cell numbers represent a barrier to the use of primary cells. The human macrophage model was ideal for the study of CH25H regulation, since in mouse models, CH25H was mostly expressed in tissue with resident macrophages.

# CHAPTER TWO: MATERIALS AND METHODS

This chapter explains in details all the materials and methods used in this thesis:

## 2.1 Virus, Cells, and Reagents

Zika Virus strain (MR766) was a gift from by Dr. Michael Gale.The JFH-1 strain of HCV was kindly provided by Takaji Wakita. Dengue Virus serotype 2 was provided by Dr. Daniel Engel (University of Virginia, Charlottesville, USA). Human recombinant IFNβ, TNFα, IL-1β, IFNλ, and IL-6 were purchased from Peprotech, Phorbol 12-myristate 13-acetate (PMA) (Invivognen), RNA Bee (Fisher Scientific), High capacity RNA-to-DNA kit (Life technologies), 2X SYBR green master mix (Applied Biosystems).

## 2.2 SDS-PAGE buffer preparation

### 2.2.1 10X Tris/ Glycine/ SDS buffer (BIO-RAD)

1x working solution buffer was prepared by adding about 100ml of (10x) Tris buffer concentrate to a 900 ml nanopure water. This mixture was then Mixed thoroughly, and a final concentration of a 1x solution is 25mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3. This buffer was then stored at 4°c until use.

### 2.2.2 10X Tris/ Glycine buffer, Transfer buffer (BIO-RAD)

1x working solution buffer was prepared by an addition of about 100 ml buffer concentrate (10x) to 200ml methanol and 700 ml nanopure water. This Mixture was then mixed thoroughly, and a final concentration of a 1x solution is 25 mM Tris, 192 Glycine and 20% methanol, pH 8,3. This buffer was then stored at 4°c until use.

## 2.3 1x TBST solution

1X TBST buffer was prepared by dissolving 6.05g Tris and 8.76g Nacl in 800ml of nanopure water. Thereafter, the pH was adjusted to 7.6 with 1M HCl. Following pH adjustment to 7.6, additional nanopore water was added to make volume up to 1L. Then,

100ul of Tween was added. The final concentration of 1X TBST is 50 mM Tris-Cl, pH 7.6; 150 mM NaCl, 0.1% Tween. This buffer was then stored at 4°c until use.

## 2.4 THP-1 cells and Microglial cells background

### *2.4.1 THP-1 cells*

THP-1 is a human monocytic cell line derived from the peripheral blood of a one-year old human male with acute monocytic leukemia, a disease that leads to the unimpeded growth of monocytes. Upon differentiation with phorbol esters this cell-line demostrate the properties of the primary macrophages

### *2.4.2 Microglial cells*

Microglia are a type of neuroglia (glial cell) located throughout the brain and spinal cord. Microglia account for 10–15% of all cells found within the brain. As the microglia suggests, these cells are small—the smallest of all the neuroglia. As the resident macrophage cells, they act as the first and main form of active immune defense in the central nervous system (CNS)

HMC3 (ATCC® CRL-3304™) cell-line used in this study retain the properties of primary microglial cells. The HMC3 cell line was established through SV40-dependent immortalization of a human fetal brain-derived primary microglia culture.

## 2.5 Maintenance of cell culture

### 2.5.1 THP-1 macrophages

THP-1 cells (ATCC) (**Figure 2.1**) and THP-1 MyD88 KO cells (Invivogen) were cultured in RPMI media (Life technologies) (37°c, 5% CO2), supplemented with 100units/ml penicillin (Life technologies), 100ng/ml streptomycin (Life technologies), 10% Fetal Bovine Serum (FBS) (Atlas biologicals), 2mM L-glutamine (Life technologies), 1x 2-mercaptoethanol (Life technologies), and 1.25g dextrose. The cells were split after two or three days in order to keep the cells within optimal density (not exceeding 1,0 x $10^6$ cells/ml) as they can undergo certain morphological changes if excessively stressed.

Differentiated wild-type THP-1 macrophages and THP-1 MyD88 KO macrophages: 1,0 x $10^6$ THP-1 monocytes were plated in 12 well-plate in RPMI media (supplemented as above) with 100nm PMA for 3 days at 37°c, 5% CO2, to become fully differentiated macrophages (**Figure 2.2**) before use in experiments. After 3 days with PMA, THP-1 macrophages were rested for 24hrs in PMA free RPMI media and then used for experiments.

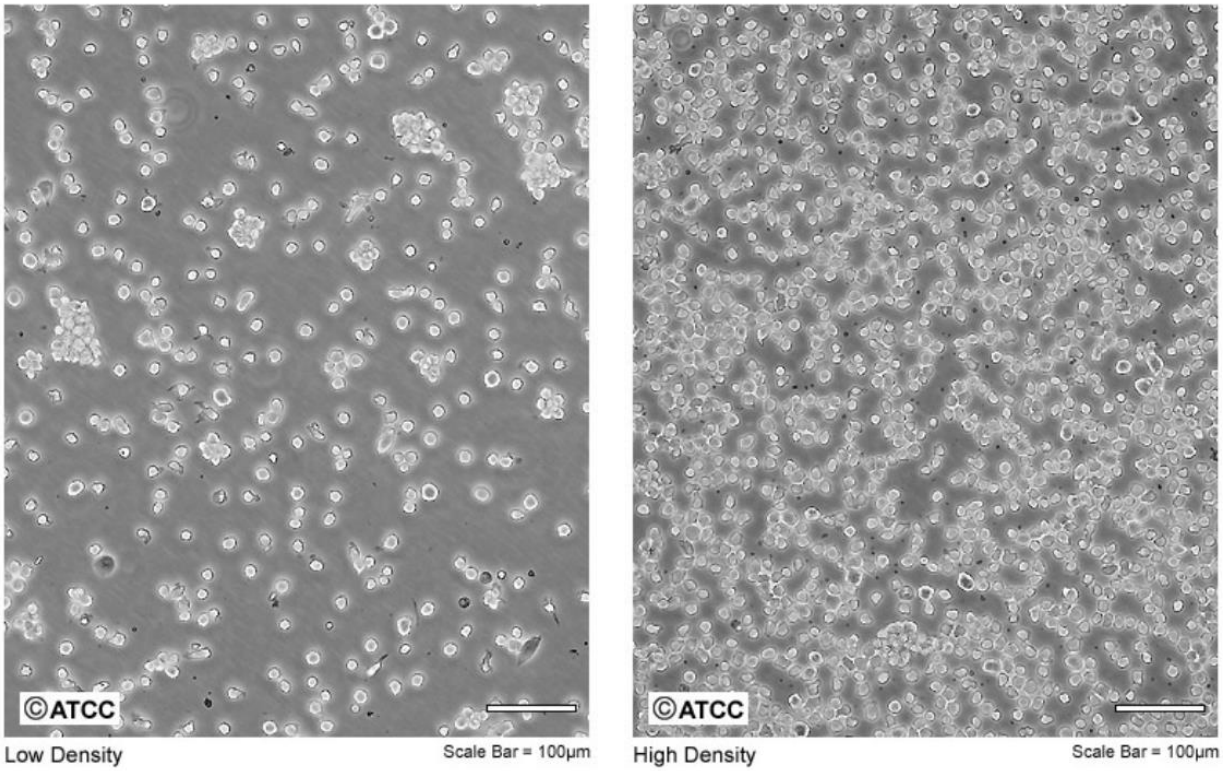


**Figure 2.1. Low and high density Undifferentiated THP-1 monocytes**. Adapted from ATCC.

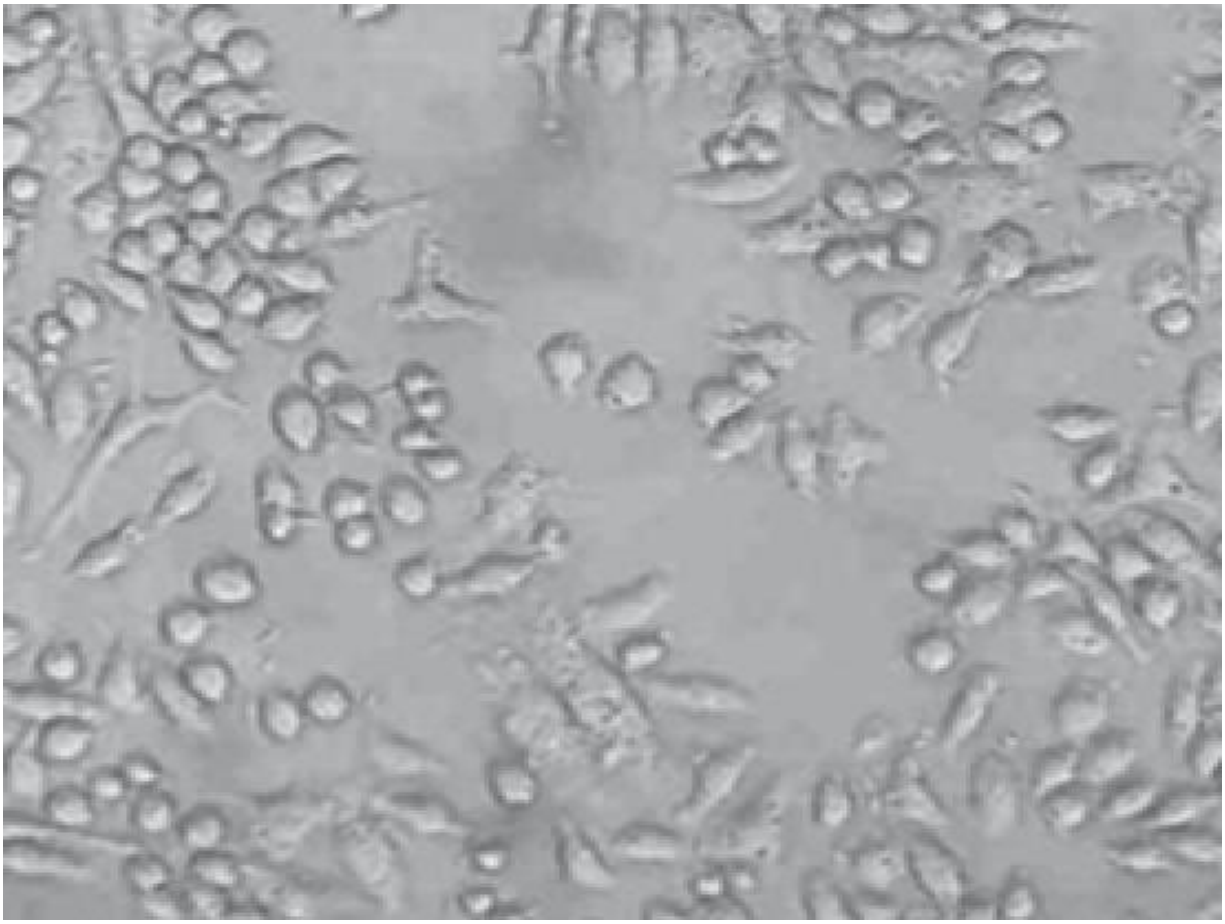

**Figure 2.2.THP-1 macrophages**. THP-1 monocytes were treated with phorbol 12-myristate 13-acetate (PMA) for differentiation into macrophages. Adapted from ATCC.

### 2.5.2 Microglial Cells

Microglial Cells (ATCC, HMC3, CRL-3304) were cultured in Eagle's Minimum Essential medium (EMEM) (ATCC 30-2003) (37°c, 5% CO2), and supplemented with penicillin (Life technologies) (**Figure 2.3**). About 300 000 Microglial Cells were plated in a 6 well-plate overnight before any experiments at 37°c, 5% CO2.

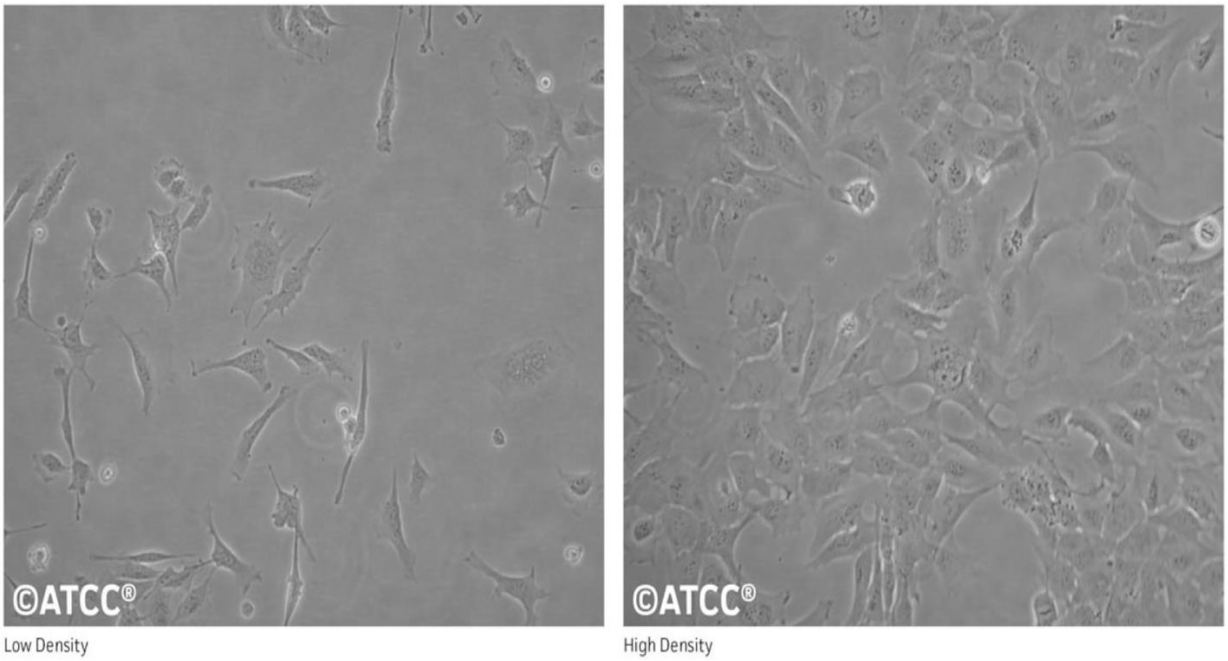


**Figure 2.3. Low and high-density microglial cells**. Adapted from ATCC.

### 2.5.3 Primary Human Cells

Human monocyte-derived macrophage: peripheral blood mononuclear cells (PBMCs) were isolated from leukopacks using standard ficoll procedures. Monocytes were separated from PBMCs via plastic adherence. Monocytes were differentiated into macrophage with 10 ng/ml human M-CSF (Biolegend) in RPMI (GIBCO) media with 10% FBS for 7 days prior to experimental use.

## 2.6 Stimulation with TLR ligands

THP-1 cells were stimulated for 3 days with 100 ng/ml PMA for differentiation into THP-1 macrophages, and rested for a day with PMA negative media before any treatment. RNA (using RNA bee) or Proteins were then collected for qPCR or Western analysis, respectively. THP-1 macrophages were treated with a panel of TLR agonists (Invivogen), including 1 µg/ml Pam3CSK4 (TLR1/2 agonist), 1 × 108/ml HKLM (TLR2 agonist), 10 µg/ml poly I:C (TLR3 agonist) High molecular weight, 1 µg/ml flagellin from Salmonella typhimurium(TLR5 agonist), 1 µg/ml FSL-1 (TLR6/2 agonist), 1 µg/ml ssRNA40 (TLR7 agonist), and 2.5 µM CpG ODN1826 (TLR9 agonist). 100 ng/ml of LPS (TLR4 agonist)

from Escherichia coli 0111:B4 (Sigma-Aldrich). After treatment cells were washed twice with ice cold dulbecco's phosphate buffered saline DPBS (Life technologies). Cells were then lysed and total RNA (using RNA Bee) were extracted and then stored at -80°c until use.

RNA Isolation, cDNA synthesis and Real-time Quantitative PCR: Macrophages were washed twice with ice cold dulbecco's phosphate buffered saline DPBS (Life technologies). RNA was extracted using RNA Bee following the manufacture's instruction. RNA concentrations were measured by a nano-drop 2000 spectrophotometer (Thermo Scientific). 1 μg/ml of the isolated total RNA was used as a template for cDNA synthesis using High capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR was performed on a StepOnePlus system (Applied Biosystems). Primers in table 2 were used for target gene quantification using SYBR green master mix (Applied Biosystems). Target genes expression were determined using comparative cycle threshold (ΔΔCT) technique and results normalized to HPRT or B2M.

**Table 2.1. Primer sequences for target genes.**

| | **Forward (5'-3')** | **Reverse (3'-5')** |
|---|---|---|
| **ISGs** | | |
| MX1 | GGTGGTCCCCAGTAATGTGG | CGTCAAGATTCCGATGGTCCT |
| MX2 | CAGAGGCAGCGGAATCGTAA | TGAAGCTCTAGCTCGGTGTTC |
| IFITM1 | CAACACCCTCTTCTTGAACTGG | GCCGAATACCAGTAACAGGATG |
| **IFNs** | | |
| IFNα | TGATGAATGCGGACTCCATCT | GACAACCTCCCAGGCACAAG |
| IFNβ | GAGCTACAACTTGCTTGGATTCC | CAAGCCTCCCATTCAATTGC |
| IFNγ | TCGGTAACTGACTTGAATGTCCA | TCGCTTCCCTGTTTTAGCTGC |
| IFNλ1 | CTGCCACATTGGCAGGTTCA | AGACAGGAGAGCTGCAACTC |
| | | |
| CH25H | TACAACTTC CCT TGG TCC ACT | TGTCCCAGT GTG TAA AGT ACG G |
| IL-1β | GCCAATCTTCATTGCTCAAGTGT | AAGTCATCCTCATTGCCACTGT |
| IL-6 | TCAATATTAGAGTCTCAACCCCCA | GAAGGCGCTTGTGGAGAAGG |
| TNF-α | TGAAAGCATGATCCGGGACG | TGGGGAACTCTTCCCTCTGG |

**Table 2.2. CH25H ChIP assay Primer sequences**

| | Forward (5'-3') | Reverse (3'-5') |
|---|---|---|
| **CH25H** | | |
| Pair 1 | CATACGTGGGCTTTTGCCTG | GAGGACGAGTTCTGGTGGTG |
| Pair 2 | GATCCACCCTGACTTCTCGC | GAGTGGTCCTCCACGGAAAG |
| Pair 3 | AACTGCAACTTCGCTCCGTA | CAGCAGTCCCGAGTCTTAGG |
| Pair 4 | TTCCCGCCCCAAATTGAGAT | GGTCTGTAGCTCGGGTGTTT |
| Pair 5 | TAACTGCAACTTCGCTCCGT | AGCAGTCCCGAGTCTTAGGG |
| Pair 6 | TGCCGGGTTGCTAGCTTATT | GGAAGAAGGGCGACTGTAGG |

### 2.7 Chromatin Immuno-precipitation assay

The ChIP assay was performed using an EpiQuik chromatinimmunoprecipitation kit according to the manufacturer's instruction. Briefly, THP1 cells differentiated with PMA into macrophages were treated with LPS for desired time point. After treatment cells were washed twice with PBS and were fixed with 1% formaldehyde. Protein-DNA complexes were immunoprecipitated with STAT-1 or ATF3 antibody (cell signaling technology). A negative control antibody was used with normal mouse IgG or HA tagged. DNA from these samples was subjected to PCR analyses with CH25H promoter-specific primers. An input control was used with amplification of soluble chromatin prior to immunoprecipitation.

### 2.8 Pierce BCA protein assay

Total cell lysates were harvested with RIPA buffer and, 1x Halt protease inhibitor cocktail (Thermo Scientific). The concentration of the total proteins extract was determined using the Pierce BCA protein assay reagent kit. Manufacturer's instructions were strictly followed. A standard curve was prepared for each assay using suitable serial dilutions of

a 2mg/ml bovine serum albumin (BSA) solution to give concentrations of 1500ug/ml, 1000ug/ml, 750ug/ml, 500ug/ml, 250ug/ml, 1250ug/ml, and 25ug/ml. The standard and test samples were diluted in extraction buffer (RIPPA buffer). To dilute test samples, about 5ul of the test sample was added to 25ul of RIPPA buffer. Then, about 25ul of each sample and standards were placed in a 96 well plate. Thereafter, about 200ul of working reagent (WR), provided with the kit, was added to each well with thorough mixing of the well by pipetting up and down. Thereafter, the plate was covered and incubated at 37°C for 30 minutes. Following incubation at 37°C, the absorbance was read at 562nm using a ----microplate reader. The protein concentration of the experimental samples was calculated from the standard curve.

### 2.9 Western blots

Total cell lysates were harvested with RIPA buffer and, 1x Halt protease inhibitor cocktail (Thermo Scientific). Protein concentrations were determined using Pierce BCA protein assay kit. Cells lysates containing 50ug of protein were reduced and denatured with sample buffer (Laemmli sample buffer with β-ME). Protein lysates were further denatured by boiling for 5 min, and ran on 4-15 % SDS-PAGE for 1-2 hours at 100v. Protein were them transferred to polyvinylidene difluoride membrane and blocked with 5% BSA in TBST buffer. The membrane was Probed with specific antibodies. The membrane-bound Abs were visualized with horseradish peroxidase–conjugated Ab to rabbit IgG or mouse IgG, and developed with ECL (Santa Cruz Biotechnology). SAPK/JNK (#9252), p38 MAPK (#8690), p44/42 MAPK (Erk1/2) (#4695), Phospho-SAPK/JNK (#4668), Phospho-p38 MAPK (#4511), Phospho-c-Jun (#3270), c-Jun (#9165), IRF-3 (#11904), Phospho-IRF-3 (#29047), Stat1 (#9172), Phospho-Stat1 (#9167), NF-κB p65 (#8242), Phospho-NF-κB p65 (#3033) were purchased from Cell Signaling.

### 2.10 Virus experiments

macrophages were infected with either ZIKV or HCV or Dengue at a multiplicities (MOI) of infection 1 pfu/cell. RNA analysis and global protein translation analysis were done as described in section **2.6** and **2.9**.

### 2.11 Statistical analysis

Statistical significance was determined using two-tailed Student's t test. Analysis was performed using GraphPad Prism version 7.00 (GraphPad Software Inc., La Jolla, CA, www.graphpad.com). Data was presented as mean ± SEM and compared using t-tests and values of $p < 0.05$ were regarded as statistically significant. Asterisks denotes $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$.

# CHAPTER THREE: THE ROLE OF ZIKV INFECTION OR TLR STIMULATION AND THEIR SECRETORY MOLECULES ON CH25H EXPRESSION (Objective 1)

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The following chapter is included in part in "Tshifhiwa Magoro, Aditya P. Dandekar, Lucas T. Jennelle, Rohan Bajaj, Gabriel Lipkowitz, Angelina R. Angelucci, Pascal O. Bessong, Young S. Hahn. IL-1β/TNF-α/IL-6 inflammatory cytokines promote STAT1-dependent induction of CH25H in zika virus-infected human macrophages. Journal of Biological Chemistry." (manuscript submitted, attached in appendix)

## 3.1 Introduction

The main aim of the work presented in this chapter was to investigate the precise mechanism by which ZIKV or TLR-stimulation induce CH25H in human macrophages. Although ZIKV infection has been reported to infect placental macrophages and transmit the virus from the mother to fetus (Quicke et al., 2016), however, whether ZIKV infection of THP-1 macrophages and microglial cells will induce CH25H remain elusive. In addition, TLR stimulation in mammalian cells has been show to induce CH25H, contrary to mouse cells, this expression was independent of interferons and IFNAR interactions. Thus, we thought to determine whether a pathogen (ZIKV) or TLR-stimulation or their secretory molecules (cytokines) induce the expression of CH25H mRNA in human macrophages. Materials and methods used in this chapter are detailed in chapter two.

## 3.2 Results

### 3.2.1 ZIKV infection in macrophages induces the expression of CH25H.

ZIKV is known to robustly infect macrophages, among other cells. To begin our investigation, we first examined the ability of ZIKV to induce CH25H expression following

infection of primary human macrophages. Indeed, CH25H mRNA levels were elevated in human primary macrophages following ZIKV infection (**Figure 3.1A**). Next, we extended our findings to THP-1 macrophages (a cell culture model of human monocyte-derived macrophages) and microglial cells (a cell culture model of human central nervous system-resident macrophages). ZIKV infection of these cell-lines, like primary macrophages, significantly induce CH25H expression (**Figure 3.1B, C**). Having established that MR766 ZIKV strain (Uganda strain) induces CH25H in macrophages, we expanded our findings to THP-1 macrophages using the current circulating ZIKV strain that has caused an epidemic in Brazil (Fortaleza strain, Brazil). Indeed, like the Uganda strain, Fortaleza significantly induced CH25H expression in THP-1 macrophages, as compared to mock (**Figure 3.2**). Having established that ZIKV can induce CH25H, we sought to find out if other viruses of the *flaviviridae* family could also induce CH25H expression. To test this, we used Hepatitis C virus (HCV) and Dengue virus (DENV). As shown in **figure 3.3&3.4** both HCV and Dengue virus significantly induced CH25H expression. However, the kinetics between HCV and Dengue virus differs. With Dengue virus CH25H was induced earlier as in 12 hours, but at 24 hours it goes down below the baseline levels.

Given that CH25H converts cholesterol to 25HC, we also investigated the role of 25HC in anti ZIKV immunity. To assess this, we investigated at which step of ZIKV life cycle was inhibited by 25HC. First, we examined the effect of 25HC on viral entry. Microglial cells were pretreated with various concentrations of 25HC for 8 hr and then infected with ZIKV for 48 hr. At 48 hr post-infection, the intracellular ZIKV RNA was measured by qPCR, and as shown in (**Figure 3.5**), ZIKV-infection was sensitive to 25HC treatment. Secondly, we also examined the effect of 25HC on post viral entry. To test this, 25HC was added to THP-1 microglial cells at 3 hr post ZIKV-infection. At 48 hr post-infection, the intracellular ZIKV RNA was also measured by qPCR. As shown in (**Figure 3.6**), zika infection was reduced, but this reduction was not statistically significant. All these together suggests that 25HC effectively blocks viral entry.

Having established that CH25H can be induced by ZIKV-infection and its product 25HC plays a role in anti ZIKV immunity. We sought to determine a signaling transduction

pathway which is involved in CH25H induction. Given that ZIKV is known to trigger pattern recognition receptors (PRRs), in particular TLRs (Hamel et al., 2015), which in turn cause downstream gene induction, we next examined the role of specific toll-like receptors in inducing CH25H expression. To this end, we treated macrophages with a panel of nine TLR ligands. In THP-1 macrophages, CH25H gene expression was induced upon stimulation with TLR3 (Poly I:C), TLR4 (LPS), and TLR5 (Flagellin) ligands (**Figure 3.1D**), though only Poly (I:C) and LPS robustly increased expression. In microglial cells, by contrast, only TLR3 ligand (Poly I:C) significantly upregulated CH25H (**Figure 3.1E**).

Next, we then asked whether CH25H can be induced in mammalian cells other than macrophages. To assess this, transcript levels of CH25H were firstly measured in human THP-1 monocytes upon inoculation with Zika virus, and no significant change was observed (**Figure 3.7A, B**). Since CH25H was robustly induced by TLR3/4 ligands in macrophages, we used TLR3 and 4 ligands to further investigate the mechanism in THP-1 monocytes. With TLR stimulation, no significant changes were observed on CH25H transcription levels compare to mock (**Figure 3.7C**). Secondly, expression of CH25H was also assessed in two human non-immune cell lines: LX-2 hepatic stellate cells and Huh 7.5.1 hepatocytes (**Figure 3.7D, 3E**). In contrast to monocytes, treatment of these hepatic cell lines with either Poly (I:C) or LPS significantly induced CH25H mRNA. These results suggest that while ZIKV infection and TLR3/4 (3 hr exposure) stimulation can induce CH25H expression in macrophages and human non-immune cells, these stimuli cannot in monocytes.

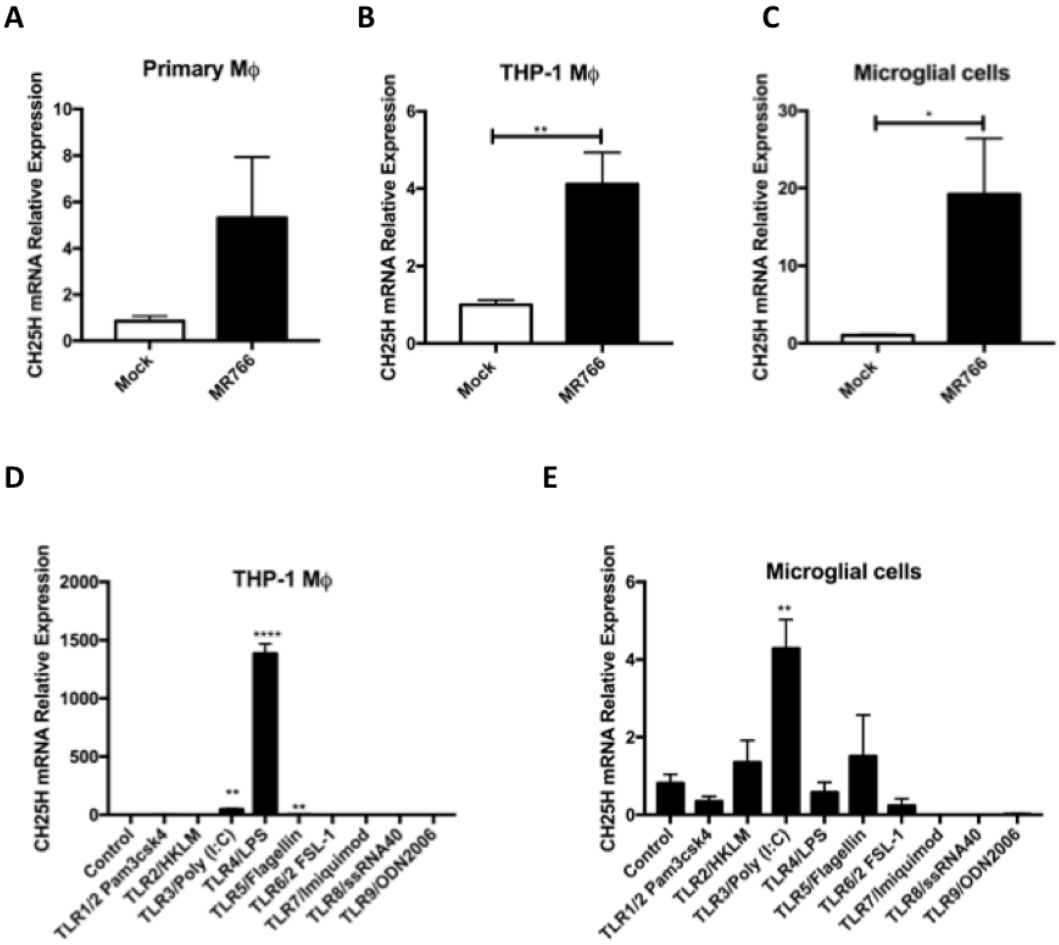


**Figure 3.1. Expression of CH25H with ZIKV exposure and TLR stimulation in human primary, monocytes-derived, and resident macrophages**. (**A-C**) Primary, THP-1 macrophages, and microglial cells were inoculated with ZIKV (MR766, MOI 1) for 24 hr, CH25H mRNA expression was measured by qPCR. (**D and E**) THP-1 macrophages and microglial cells were stimulated with a control (media only) and a panel of TLR agonist (TLR1-9) for 3 hr, CH25H mRNA expression was measured by qPCR. HPRT was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

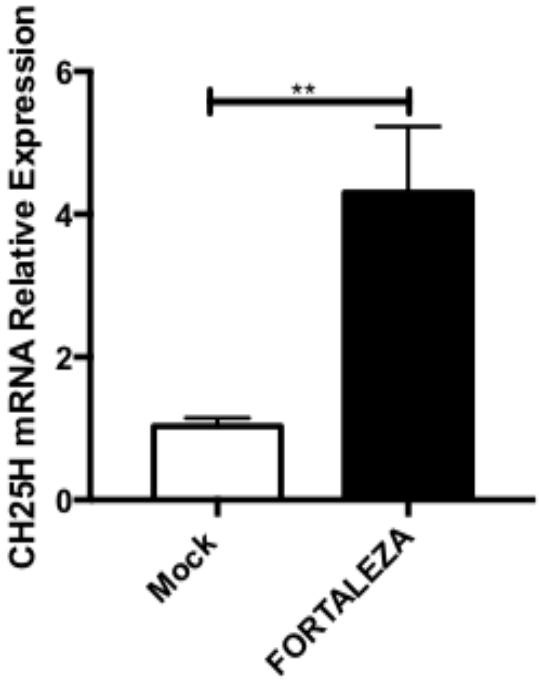


**Figure 3.2. Expression of CH25H with Fortaleza ZIKV exposure.** THP-1 macrophages were inoculated with ZIKV (MR766, MOI 1) for 24 hr, and CH25H mRNA expression was measured by qPCR. HPRT was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

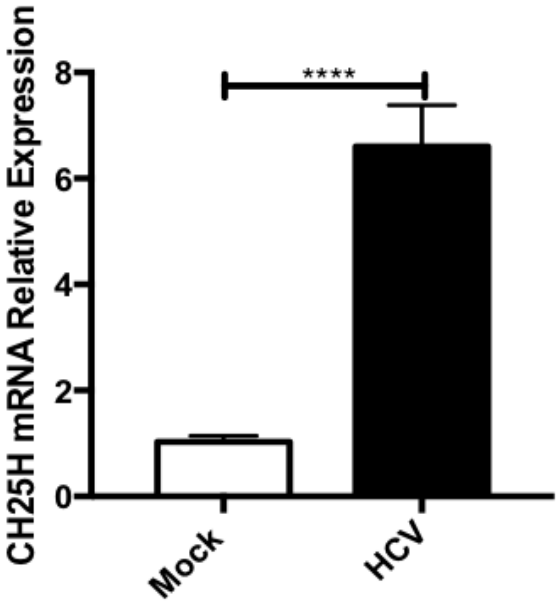


**Figure 3.3. Expression of CH25H with HCV exposure.** THP-1 macrophages were inoculated with HCV (JFH1, MOI 1) for 24 hr, and CH25H mRNA expression was measured by qPCR. HPRT was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

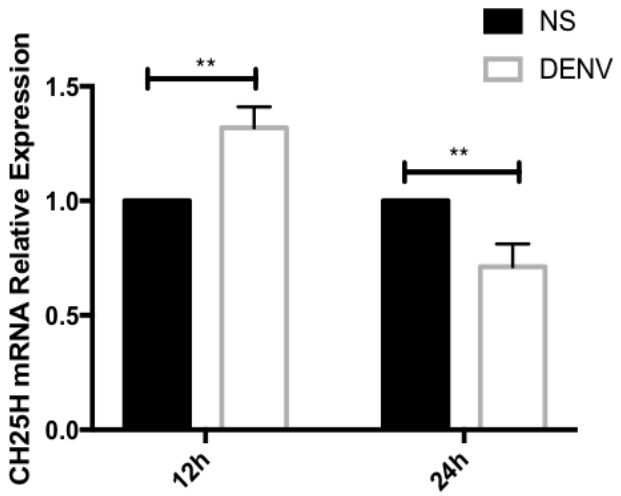


**Figure 3.4. Expression of CH25H with Dengue Virus exposure.** THP-1 macrophages were inoculated with Dengue virus (MOI 1) for 12 and 24 hr, and CH25H mRNA expression was measured by qPCR. HPRT was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test). Note: NS, Non-stimulated (mock); DENV, Dengue Virus.

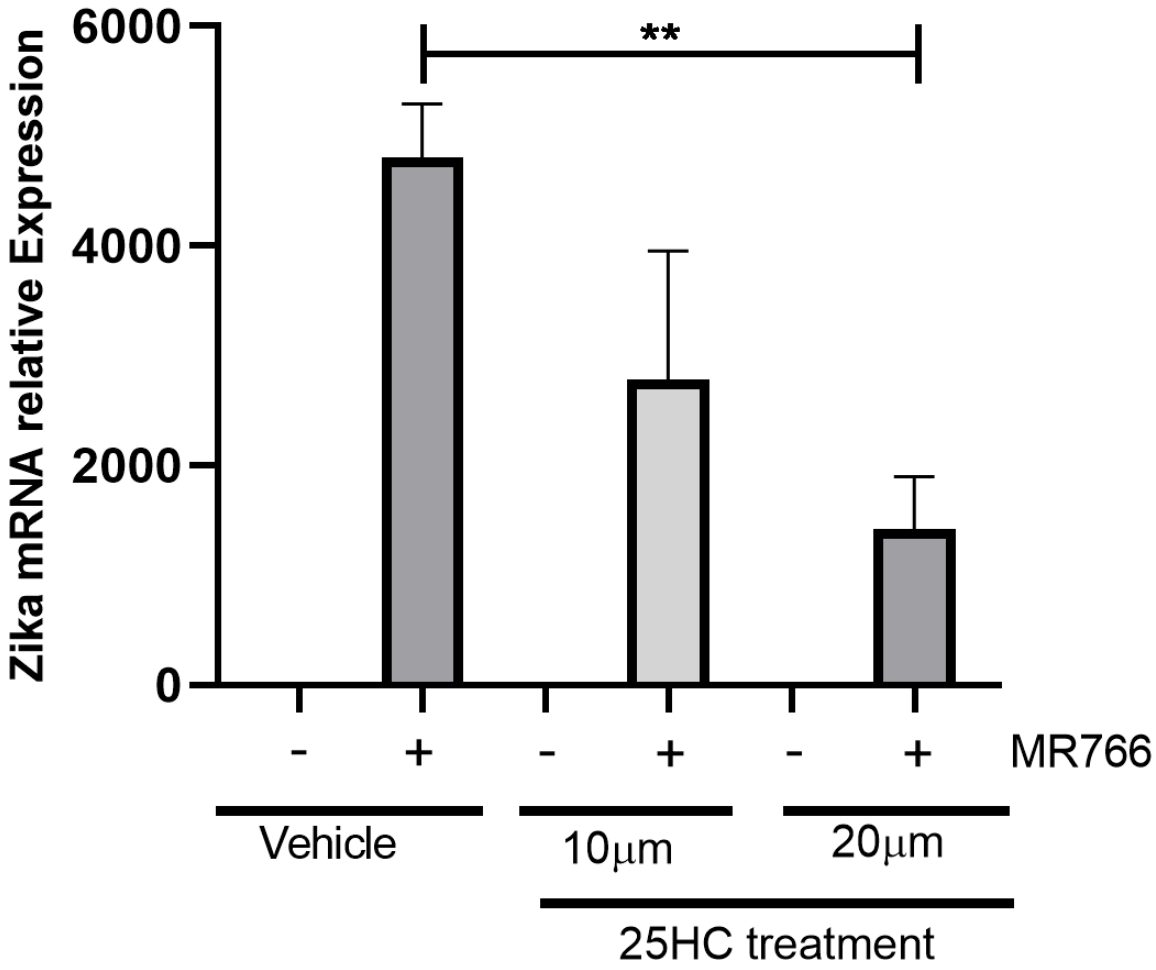


**Figure 3.5. Zika infection with 25HC pre-treatment.** Microglial cells were either exposed to vehicle (ethanol) or 25HC for 8 hr. Thereafter, cells were inoculated with ZIKV (MR766, MOI 1) for 48 hr. At 48 hr post-infection, the intracellular ZIKV RNA was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

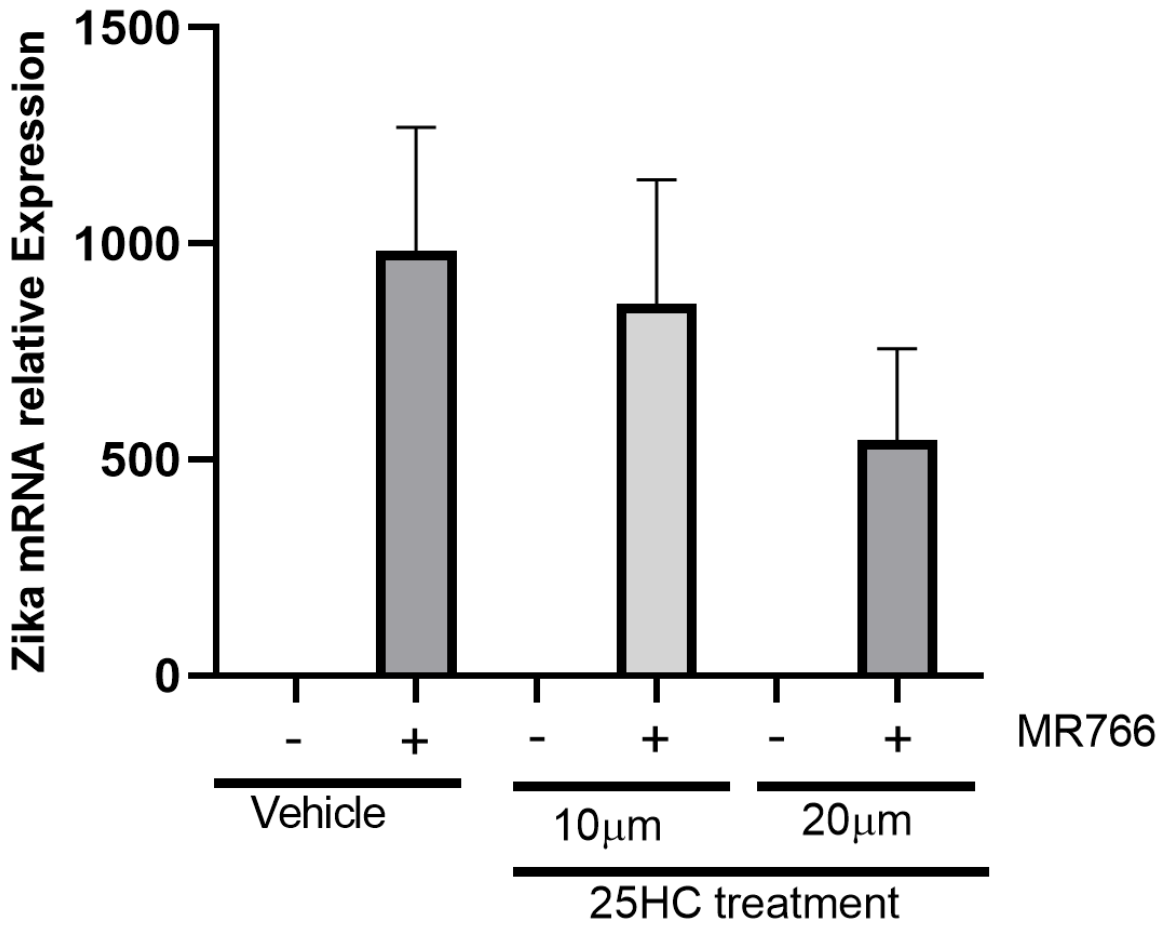


**Figure 3.6. Zika infection with 25HC post treatment.** Microglial cells were either exposed to vehicle (ethanol) or inoculated with ZIKV (MR766, MOI 1) for 3 hr. Thereafter, cells were treated with 25HC. At 48 hr post-infection, the intracellular ZIKV RNA was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

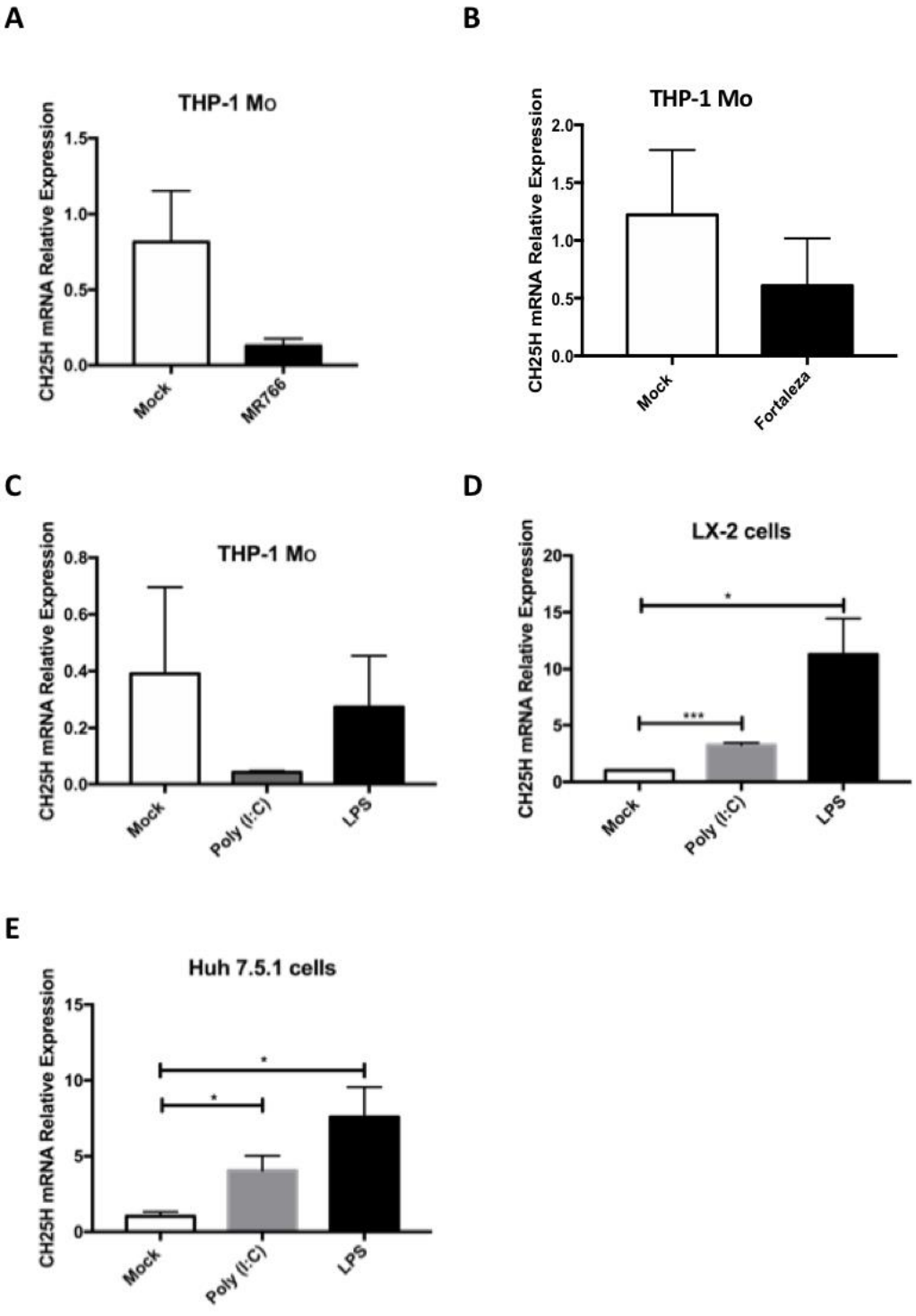

**Figure 3.7. TLR-stimulation induce CH25H expression in non-immune cells, but not in monocytes.** (**A-B**) THP-1 monocytes were inoculated with ZIKV (MR766, & Fortaleza, MOI 1) for 24 hr, CH25H mRNA expression was measured by qPCR. (**C-E**) THP-1 monocytes, LX-2 cells and Huh 7.5.1 cells were treated with poly (I:C) (10 μm) or LPS (100ng/ml) for 3 hr, CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

### 3.2.2 ZIKV replication and endosomal compartment are involved on the induction of CH25H transcript.

Having established that ZIKV infection can induce CH25H in THP-1 macrophages and microglial cells, we next sought to gain more insights into this induction. After all, it is not clear whether ZIKV can productively infect THP-1 macrophages and microglial cells, and whether this upregulation of CH25H is due to rapid viral replication. To address whether viral replication is required for Zika virus mediated CH25H gene expression, we used heat-inactivation, a well-characterized method of virus inactivation (Pfaender et al., 2015). Compared to live virus, Heat-inactivated virus robustly induced CH25H in THP-1 macrophages at significantly lower levels (**Figure 3.8A**, left panel). In parallel, heated-inactivated virus was not able to induce CH25H compare to live virus in microglial cells (**Figure 3.8A**, right panel). Due to this observed discrepancy in dependence of CH25H gene expression on viral replication in the two tested cell-lines. we examined if the viral entry or sensing compartment are similar in both cell-lines. To assess the necessity of the endosomal compartment on CH25H induction, we pre-treated cells with a vacuolar type H+-ATPase inhibitor bafilomycin, which prevents endosome acidification. The inhibitory effect of bafilomycin was confirmed by qPCR (**Figure 3.9A, B**), through measuring two classical ISGs, IFITM1 and MX1. Exposure of THP-1 macrophages and microglial cells with ZIKV in the presence of bafilomycin blunted CH25H induction (**Figure 3.8B**, left or right panel). All these together suggests that although there are differences regarding dependence on viral replication for induction CH25H gene expression in both cell-lines,

the endosomal compartment is necessary for ZIKV- mediated CH25H expression in both cell-lines.

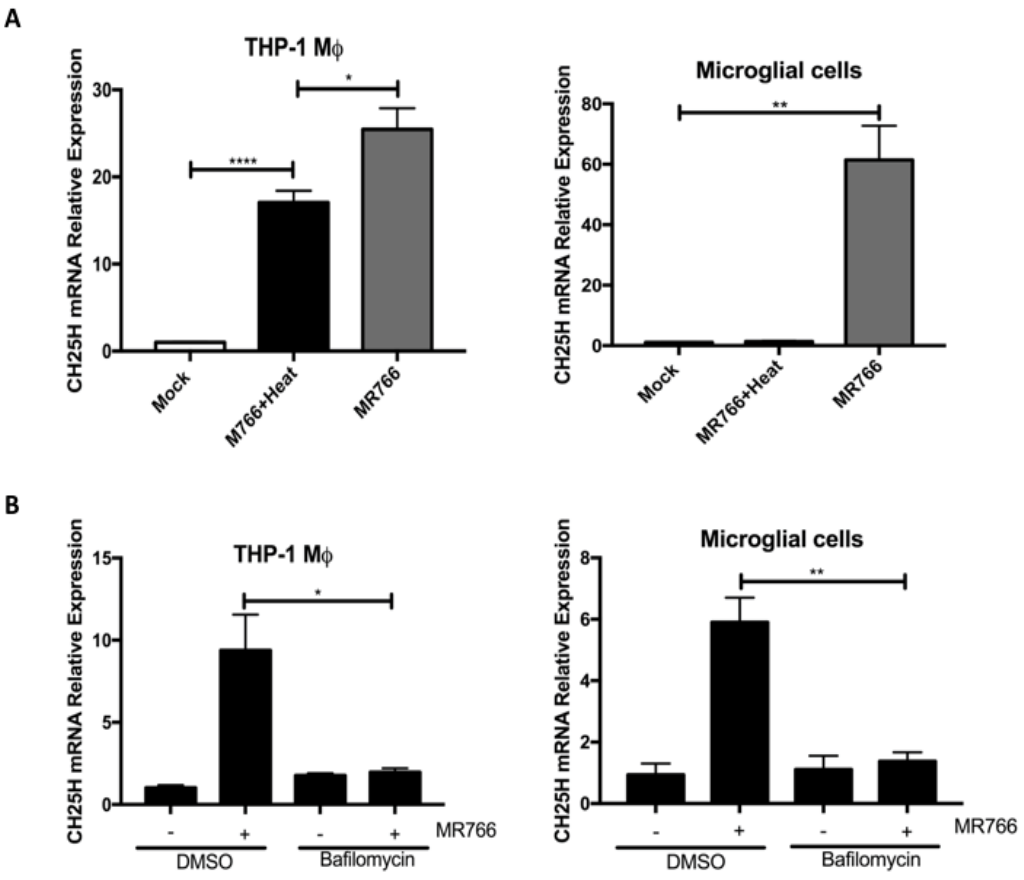


**Figure 3.8. Viral replication and the endosomal compartment are important on ZIKV mediated CH25H**. (**A**) THP-1 macrophages and microglial cells were exposed to either mock (non-virus media, or heat-inactivated, or live ZIKV (MR766, MOI 1) for 24 hr. Cells we then lysed, harvested, RNA isolated and subjected to qPCR to determine CH25H expression. (**B**) THP-1 macrophages and microglial cells were pretreated with DMSO or bafilomycin $A_1$ (5μm) for 1 hr, and then exposed to ZIKV (MR766, MOI 1) for 24 hr, in the presence of continued drug treatment. RNA was then isolated and CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as

means ± SEM from triplicate samples (three independent experiments). *p < 0.0332, **p < 0.0021, and ***p < 0.0002, ****p < 0.0001 (two-tailed unpaired Student's t test).

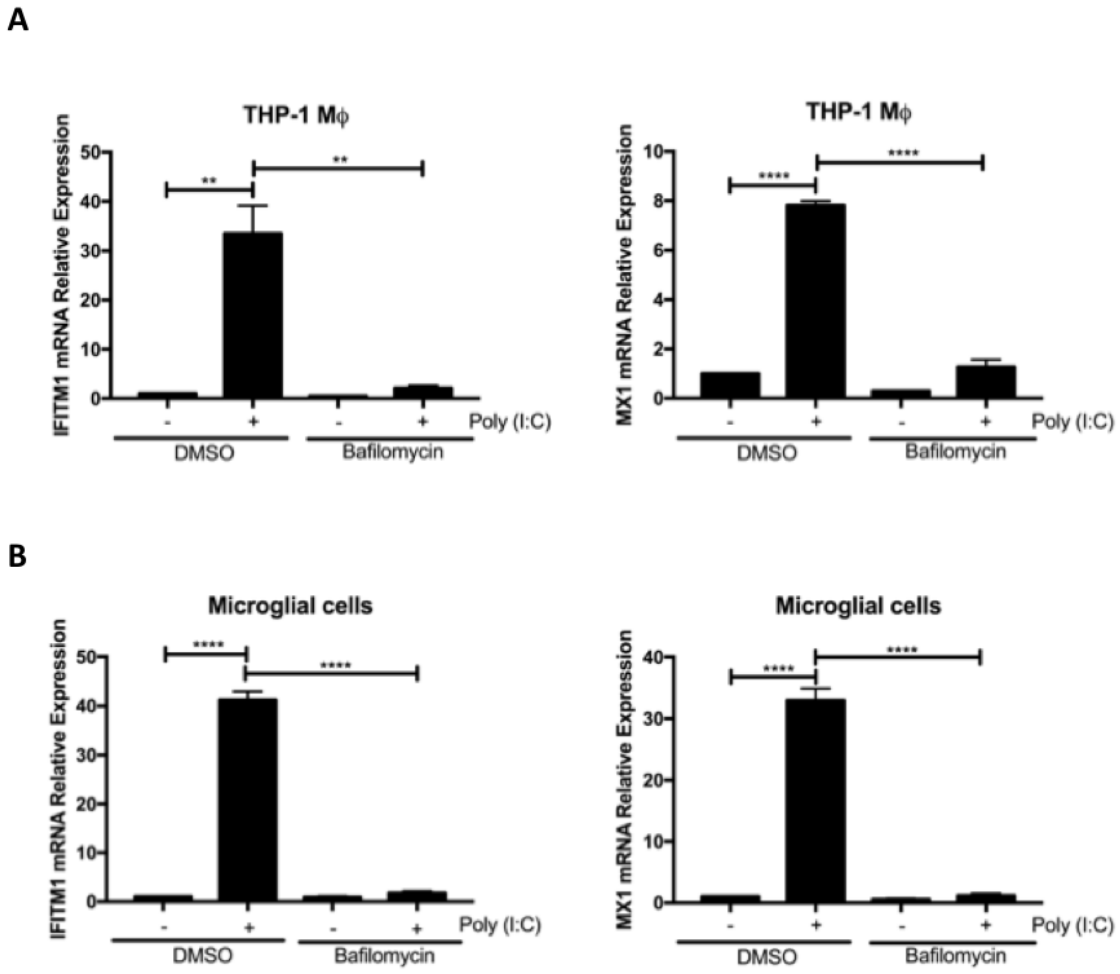


**Figure 3.9. The functional activity of the endosomal compartment is required for Poly (I:C) mediated induction of ISGs.** (**A and B**) THP-1 macrophages and human microglial cells were pre-treated with DMSO or bafilomycin $A_1$ (5μm) for 1 hr, and then treated with poly (I:C) (10 μm) for 24 hr, IFITM1 and MX1 mRNA expression were measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). *p < 0.0332, **p < 0.0021, and ***p < 0.0002, ****p < 0.0001 (two-tailed unpaired Student's t test).

### 3.2.3 CH25H expression occurs through TRIF/MyD88-dependent signaling pathway.

Having established that ZIKV mediated CH25H in THP-1 macrophages and microglial cells relies on the endosomal compartment, we sought to investigate a potential role of TLR signaling responsible for CH25H induction. In general, Signal transduction events initiated by TLRs rely mainly on two adaptor molecules, namely, MyD88 and TRIF (Kawai and Akira, 2010; Takeuchi and Akira, 2010; Kawasaki and Kawai, 2014; Brubaker et al., 2015)**.** To determine the role of MyD88 in ZIKV or TLR3/4-mediated CH25H induction in macrophages, we utilized MyD88-knock out (KO) THP-1 cells from INVIVOGEN. First, we confirmed efficient reduction in MyD88-dependent signaling by measuring gene expression of classical inflammatory cytokines. As expected, LPS-induced TNF$\alpha$, IL-1$\beta$, and IL-6 were significantly reduced in MyD88-KO THP-1 cells (**Figure 3.10A**). Then, we assessed whether this affected cells' ability to express CH25H upon TLR or ZIKV stimulation. Indeed, LPS-induced CH25H expression was dramatically reduced in MyD88-KO THP-1 cells compared to wild type controls (**Figure 3.11A**). Likewise, ZIKV was unable to induce CH25H expression in MyD88-KO THP-1 cells, unlike in wildtype controls (**Figure 3.11B**).

To similarly probe the contribution of the TRIF pathway, we treated THP-1 cells with the pharmacologic agent BX795, which attenuates the TRIF pathway by inhibiting TBK1. First, we indirectly confirmed the abrogation of TRIF pathway by measuring the degree of IRF3 phosphorylation with western blotting; as expected, BX795-treated cells displayed substantially reduced levels of IRF3 phosphorylation (**Figure 3.10B**). Then, we assessed whether TRIF signaling impairment reduced the cells capacity to express CH25H upon TLR stimulation or ZIKV infection. Indeed, the inhibition of TRIF signaling pathway completely abolished LPS or ZIKV-induced CH25H, compared to wild-type controls (**Figure 3.11A, B**). Given TLR3 relies only on the TRIF pathway, we next investigated the role of TLR3 on Zika mediated CH25H. To do so, we treated cells with TLR3 inhibitor, confirming its inhibitory effect by qPCR (**Figure 3.10C**). Then, we infected macrophages with ZIKV in the presence of TLR3 inhibitor. Indeed, as with general TRIF pathway inhibition, TLR3 inhibition significantly, though not completely, reduced ZIKV-mediated

CH25H (**Figure 3.10C**). Altogether, these results suggest an important role for TRIF, MyD88 and TLR3 in ZIKV and TLR-mediated CH25H induction in THP-1 macrophages.

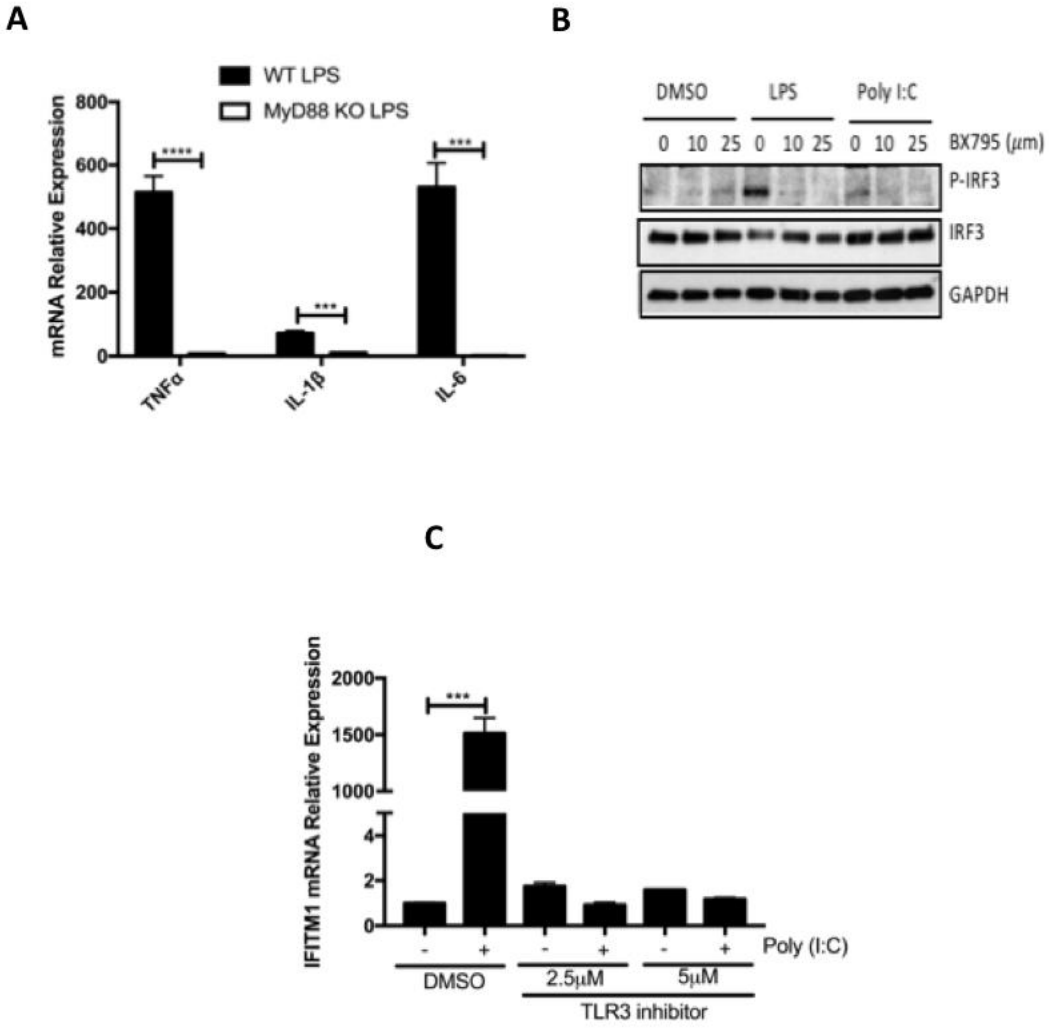


**Figure 3.10. Knock-down or Knock-out efficiency of TRIF and MyD88 signaling pathways.** (**A**) Wild-type and MyD88 KO THP-1 macrophages were treated with LPS (100ng/ml) for 3 hr. At 3 hr post-treatment TNFα, IL-1β and IL-6 mRNA expression was measured by qPCR. (**B**) THP-1 macrophages pretreated with DMSO or various concentrations of TBK1 inhibitor (BX795) for 1 hr were analyzed for phospho-IRF3, IRF3 total, and GAPDH by Western blotting. (**C**) THP-1 macrophages were pre-treated with DMSO or various concentrations of TLR3 inhibitor for 1 hr. Thereafter, cells were treated with poly (I:C) (10 μm) for 24 hr. IFITM1 mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent

experiments). *p < 0.0332, **p < 0.0021, and ***p < 0.0002, ****p < 0.0001 (two-tailed unpaired Student's t test).

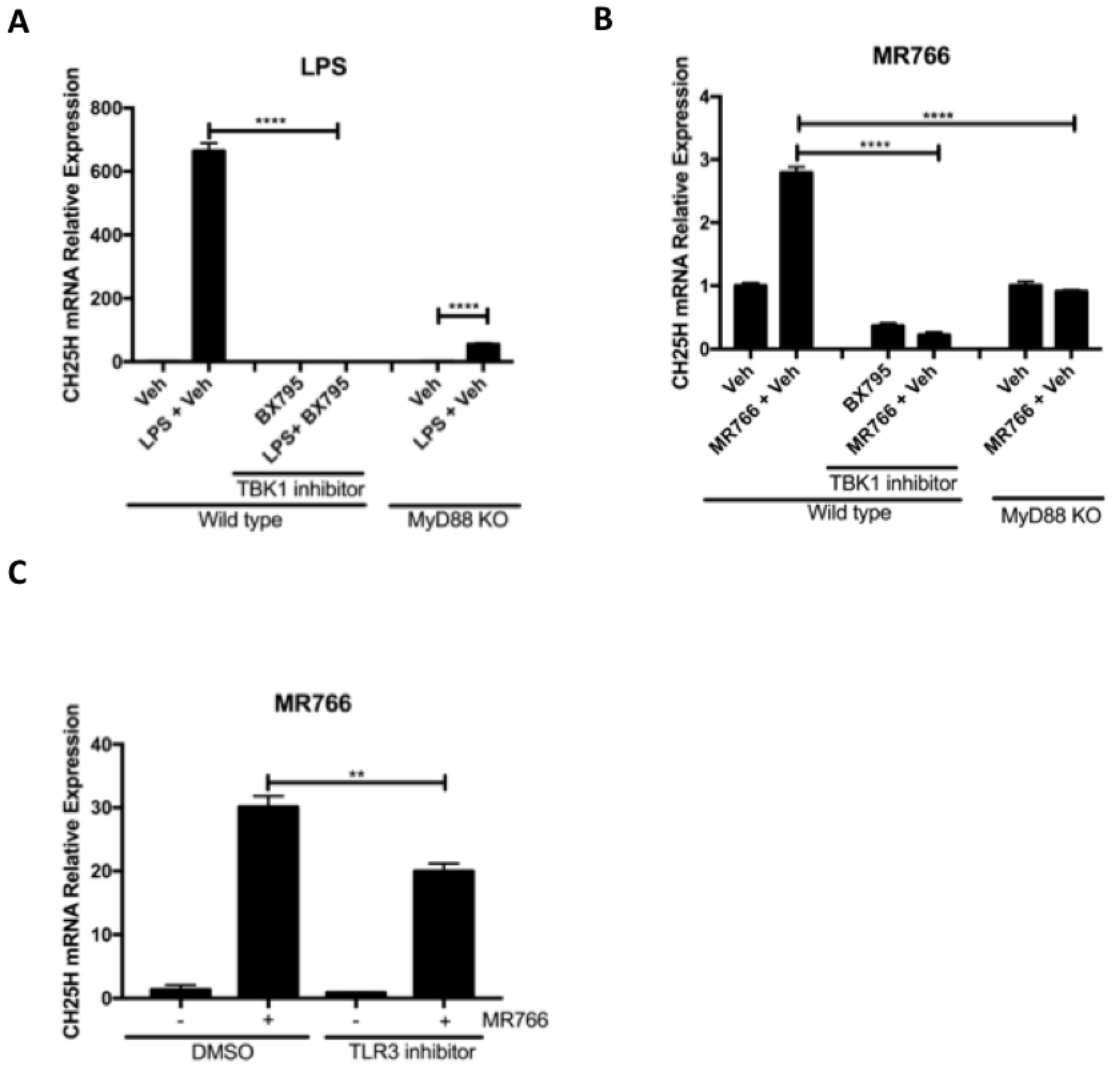


**Figure 3.11. CH25H expression occurs through TLR3, TRIF and MyD88 signaling pathways**. (**A**) Wild-type THP-1 macrophages were pre-treated with either Veh (DMSO) or BX795 (10μm) for 1 hr. in parallel, MyD88 KO THP-1 macrophages were also pre-treated with DMSO for 1 hr. Thereafter, both cell types were treated with LPS (100ng/ml) for 3 hr, CH25H mRNA expression was measured by qPCR. (**B**) Wild-type THP-1 macrophages were pre-treated with either Veh (DMSO) or BX795 (10μm) for 1 hr. in parallel, MyD88 KO THP-1 macrophages were also pre-

treated with DMSO for 1 hr. Thereafter, both cell types were exposed to ZIKV (MR766, MOI 1) for 24 hr, CH25H mRNA expression was measured by qPCR. (**C**) THP-1 macrophages were with pretreated with DMSO or TLR3 inhibitor (2.5μm) for 1 hr, and then exposed to ZIKV (MR766, MOI 1) for 24 hr. At 24 hr post-infection cells were collected for CH25H analysis by qPCR. HPRT was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples. $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

### 3.2.4 ZIKV induces CH25H expression in Type I IFN-independent manner.

We next examined the role of type I IFN or other inflammatory cytokines in inducing CH25H expression. To this end, we first assessed the production of a subset of pro-inflammatory cytokines and interferons with TLR stimulation and viral exposure. With TLR stimulation, transcript levels of type I (IFNβ) and III (IFNL1) interferons were upregulated, whereas induction of type II (IFNγ) interferons was negligible (**Figure 3.12A-C**). IFNα was not significantly increased with either TLR3/4 agonist. Classical pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) were also upregulated with TLR3/4 agonists. Consistent with synthetic TLR ligands, inflammatory cytokines IL-1β, TNFα, and IL-6 were also upregulated upon ZIKV infection in THP-1 macrophages (**Figure 3.13A-F**), though interferon induction was notably absent. Both inflammatory cytokines and interferons were induced by MR766 in microglial cells.

To test the role of these cytokines on CH25H induction in macrophages, we initially limited cytokine levels by inhibiting translation using cycloheximide. Cycloheximide treatment significantly inhibited the induction of CH25H in poly (I:C)-stimulated macrophages with less effect on CH25H induction in LPS-stimulated cells (**Figure 3.14A**). Notably, LPS-mediated IFNβ production was abrogated in CHX treated cells (**Figure 3.14B**), suggesting that IFNβ is potentially crucial for the induction of CH25H expression. Next, we examined the role of recombinant interferon beta in CH25H expression in macrophages. As shown in **Figure 3.15**, IFNβ significantly induced CH25H transcript. We then expanded our findings to two human non-immune cell lines: LX-2 hepatic stellate cells and Huh 7.5.1 hepatocytes. As shown in **Figure 3.16A,** in hepatocytes, IFNβ had

no effect on CH25H transcript, but MX1 and ISG15 **(Figure 3.16C**), two classical ISGs were significantly upregulated in this cell-line. In LX-2 hepatic stellate cells, by contrast, IFNβ had the ability to induce CH25H, MX1 and ISG15 mRNA levels (**Figure 316B, D**)

Next, we examined if the blockade of interferon receptor was enough to abrogate CH25H induction in macrophages. IFNAR2 neutralization had no significant effect on poly (I:C) or LPS induced CH25H (**Figure 3.14C**). The inhibitory effect of the neutralizing antibody for IFNAR2 was confirmed by reduction of MX1, a classical interferon stimulated gene with antibody treatment (**Figure 3.14D**). In addition, we also examined the role of type I interferons themselves on ZIKV-induced CH25H. Macrophages were pre-treated with type I interferon neutralizing antibody cocktail before being inoculated with ZIKV. As shown in **Figure 3.14E, F**, **and 3.17A** type I interferon neutralizing antibody cocktail had no effect on ZIKV-induced CH25H transcript but significantly reduced classical ISG, MX1, induction. To further confirm that ZIKV-induced CH25H is not dependent on interferons we performed a supernatant transfer assay. To perform this, culture supernatant from macrophages inoculated with ZIKV were transferred to naïve macrophages. As shown in **Figure 3.17B**, CH25H was only induced upon inoculation with ZIKV but not with supernatant from treated macrophages, whereas MX1 was induced by both treatments (**Figure 3.17C**). These results suggest that the induction of CH25H in human macrophages is not dependent on interferons, thus, CH25H is not a classical ISG.

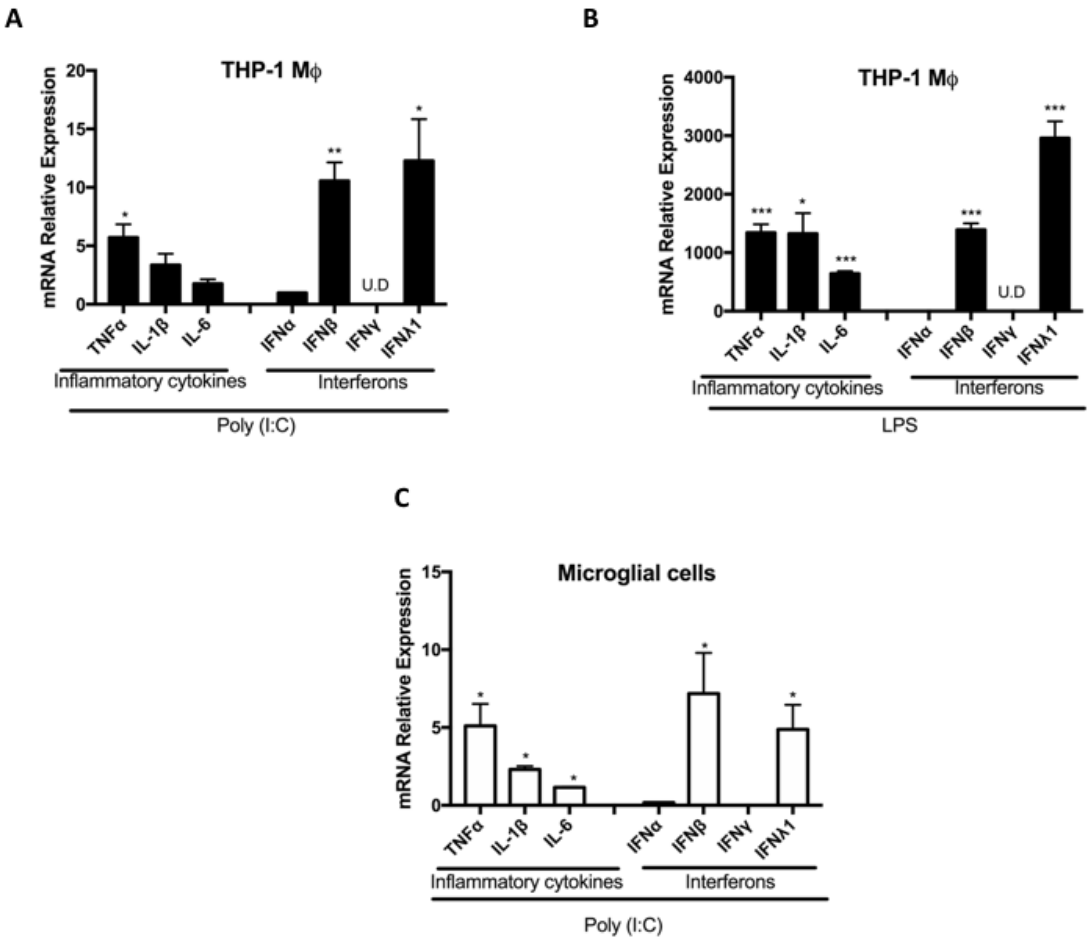


**Figure 3.12. Cytokine production with TLR-stimulation.** (**A-C**) THP-1 macrophages or microglial cells were treated with poly (I:C) (10 μm) or LPS (100ng/ml) for 3 hr. At 3 hr post-treatment cells were collected for selected pro-inflammatory cytokines and interferons analysis by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

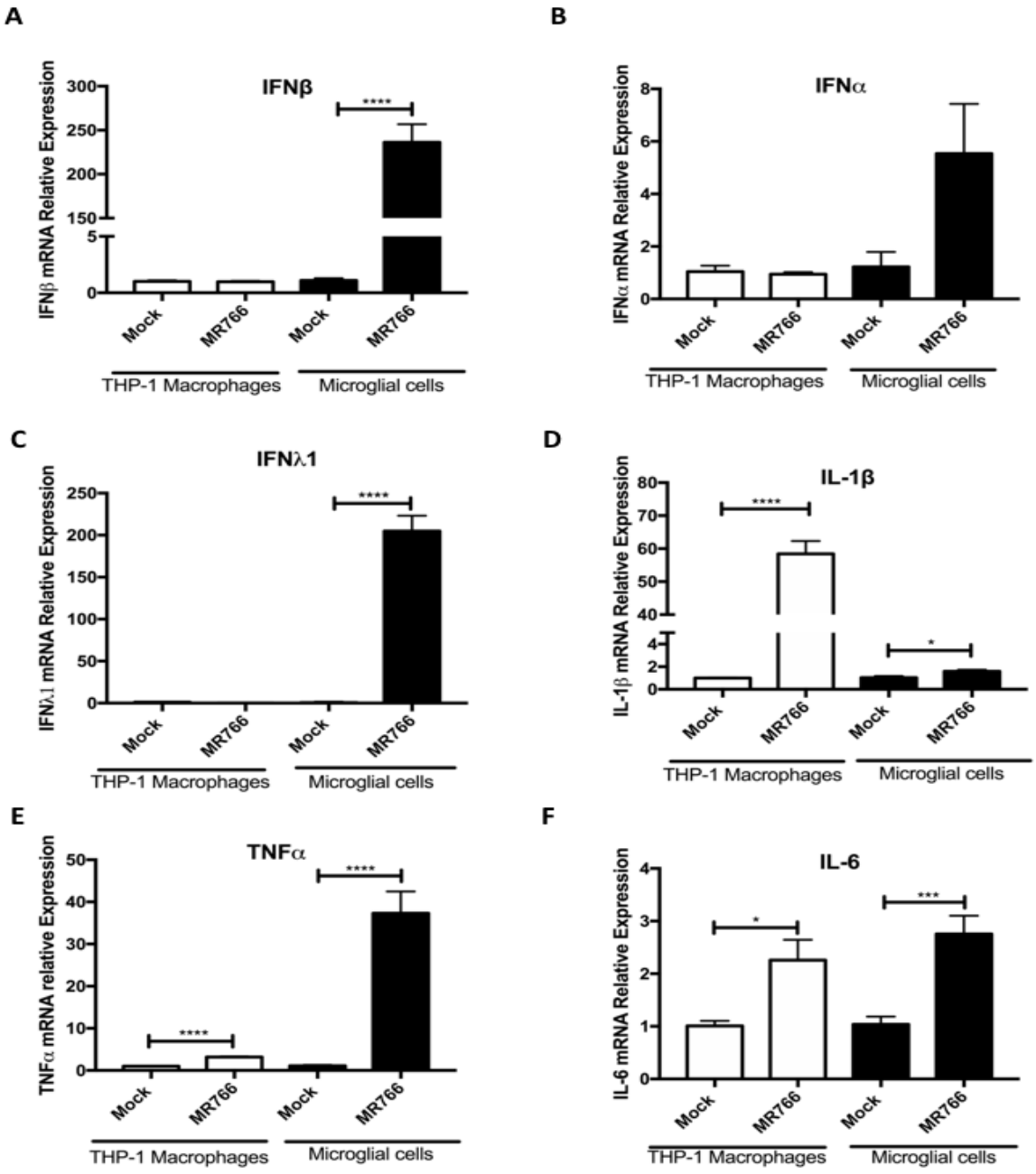


**Figure 3.13. Cytokine production with ZIKV exposure.** (**A-F**) THP-1 macrophages and microglial cells were inoculated with ZIKV (MR766, MOI 1) for 24 hr. At 24 hr post-treatment cells were collected for selected pro-inflammatory cytokines and interferons analysis by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

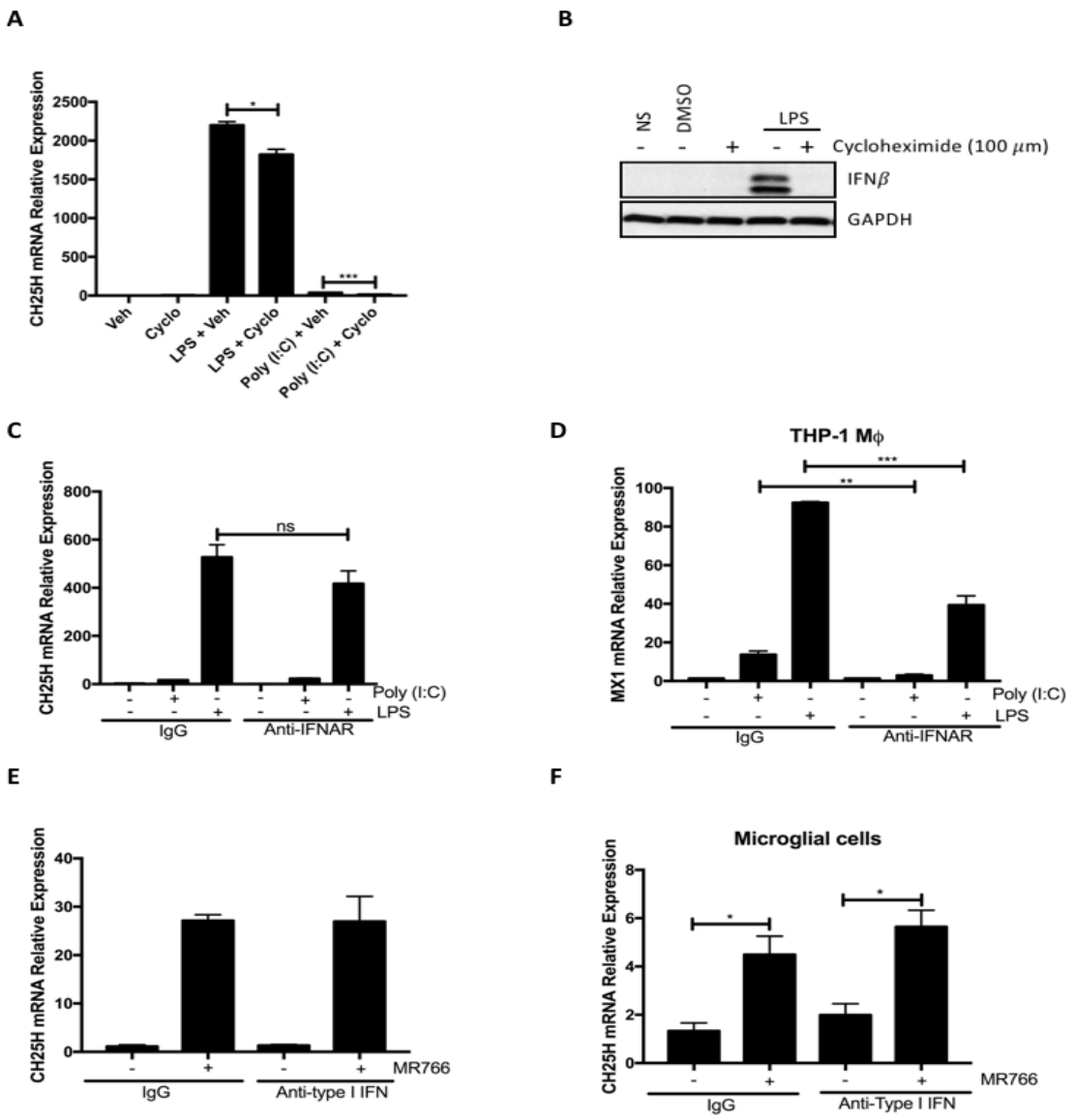


**Figure 3.14. Type I interferons are not required for TLR and ZIKV-mediated CH25H**. (**A**) THP-1 macrophages were pre-treated with veh (DMSO) or cycloheximide (100 μm) for 1 hr, and then treated with poly (I:C) (10 μm) or LPS (100ng/ml) for 3 hr, in the presence of continued drug treatment. Cells were collected for CH25H qPCR analyses at 3 hr post-treated. (**B**) THP-1 macrophages were pre-treated with media, or DMSO or cycloheximide (100 μm) for 1 hr, and then treated with LPS for 3 hr, in the presence of continued drug treatment. Expression of IFNβ was examined by western blot, GAPDH was used as a loading control. (**C**-**E**) THP-1 macrophages were first pre-treated with isotype antibody (IgG) or (**C and D**) Interferon receptor subunit 2 (IFNAR2) neutralizing antibody or (**E**) type I interferons neutralizing antibody cocktail for 1 hr. Thereafter, cells were treated with (**C and D**) poly (I:C) or LPS for 3 hr or (**E**) inoculated with ZIKV

(MR766, MOI 1) for 24 hr, in the presence of continued antibody treatment. CH25H or MX1 mRNA expression was measured by qPCR. (**F**) Microglial cells were first pre-treated with isotype antibody (IgG) or type I interferons neutralizing antibody cocktail for 1 hr. Thereafter, cells were inoculated with ZIKV (MR766, MOI 1) for 24 hr, in the presence of continued antibody treatment. CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

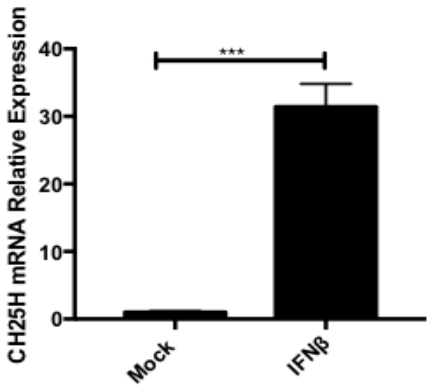


**Figure 3.15. CH25H mRNA expression with type I interferons in THP-1 macrophages.** THP-1 macrophages were treated with media (mock) or IFNβ for 3 hr, CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

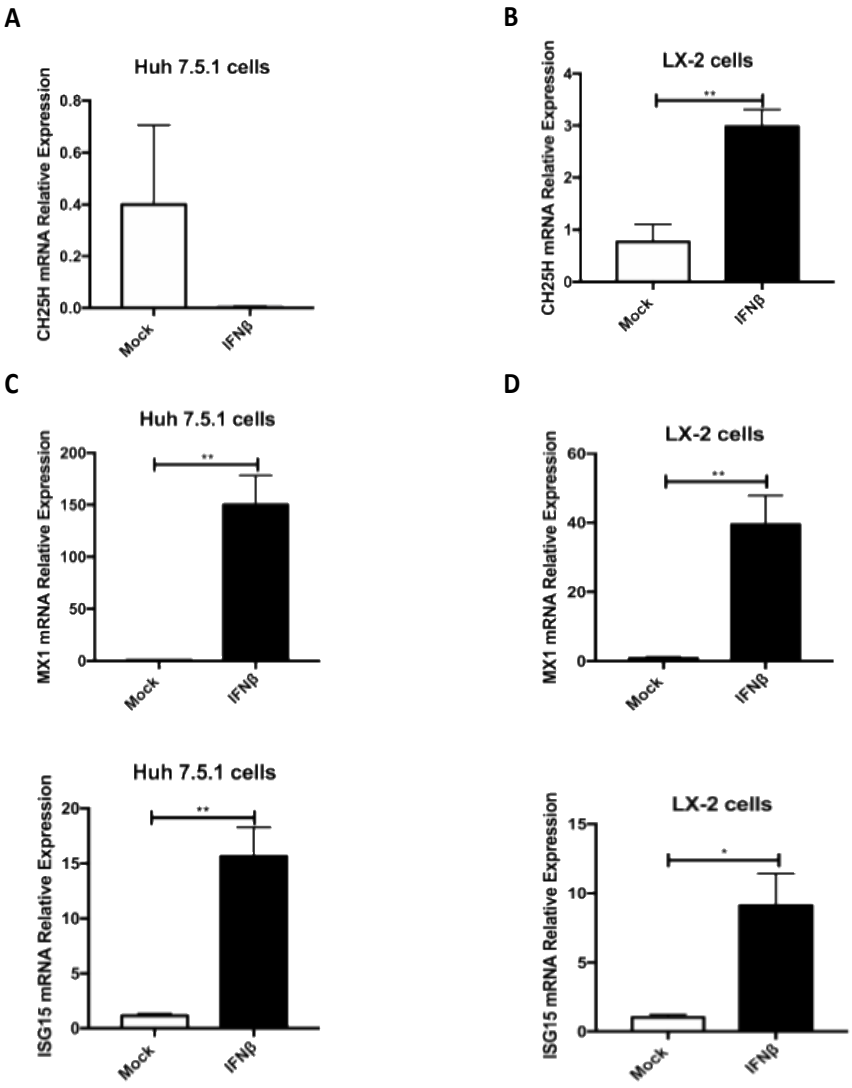


**Figure 3.16. induction of CH25H and classical ISGs in mammalian non-immune cells.** LX-2 hepatic stellate cells or Huh 7.5.1 hepatocytes were treated with media (mock) or IFNβ for 3 hr, CH25H, MX1, ISG15 mRNA expression were measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

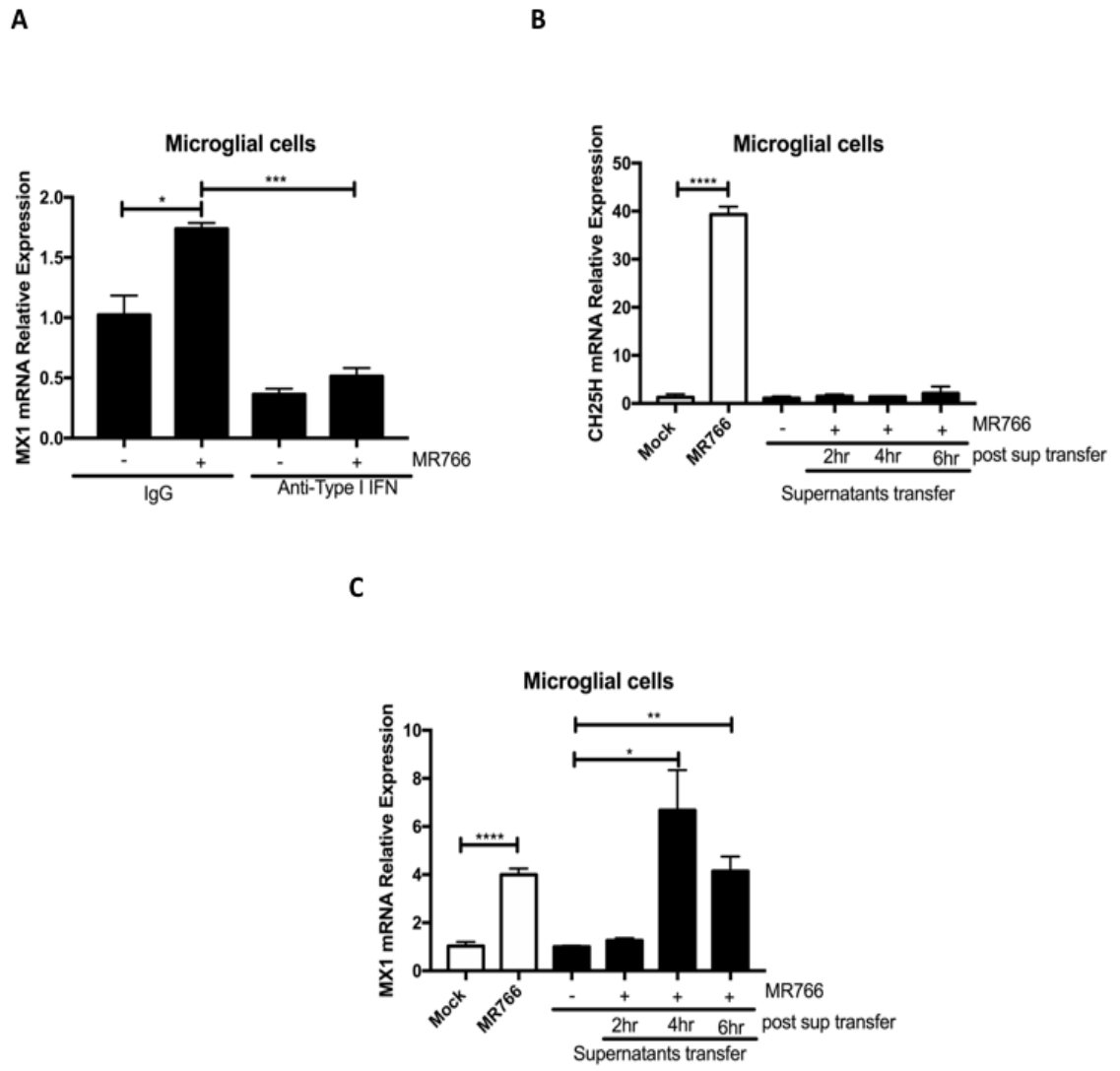


**Figure 3.17. Expression of MX1 with an actual treatment and a Supernatants transfer treatment.** (**A**) Microglial cells were pre-treated with isotype antibody (IgG) or type I interferons neutralizing antibody cocktail for 1 hr, and then treated with ZIKV (MR766, MOI 1) 24 hr. MX1 mRNA expression was measured by qPCR. (**B and C**) Microglial cells were treated with media (mock) or ZIKV (MR766, MOI 1) for 2 hr. At 2 hr post-infection unbound virus was washed twice with PBS and cells were supplemented with new media until 24 hr. At 24 hr post-treatment, culture supernatants from both treatments were transferred to naive THP-1 macrophages for 2, 4 and 6 hr. Both the actual treated and supernatant treated cells were analyzed for (**B**) CH25H or (**C**) MX1 mRNA expression by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three

independent experiments). *p < 0.0332, **p < 0.0021, and ***p < 0.0002, ****p < 0.0001 (two-tailed unpaired Student's t test).

### 3.2.5 Induction of CH25H in human macrophages with pro-inflammatory cytokine stimulation

Given cytokine gene expression was reduced in the absence of MyD88 adaptor, we next hypothesized that if CH25H expression is dependent on pro-inflammatory cytokines its expression should be reduced when treated with depleted supernatant from Myd88 KO macrophages. To test this, we treated either wild-type or MyD88 THP-1 macrophages with either PBS or LPS for 3 hr. At 3 hr post-treatment, we transferred culture supernatants from wild-type THP-1 macrophages to fresh MyD88 KO THP-1 macrophages for 3 hr, with the notion that if cytokines are sufficient this treatment should rescue CH25H to the level of wild-type. LPS-induced CH25H in MyD88 KO THP-1 macrophages was significantly lower than in wild-type, and supernatant transfer from wild-type to MyD88 KO macrophages partially rescued CH25H compared to wild-type (**Figure 3.18A**), demonstrating that cytokines are necessary for the induction of CH25H, though not sufficient for complete rescue. Indeed, knockout of MyD88 adaptor led to a reduction in pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) compare to wild-type (**Figure 3.10A**)

We further examined the effect of these inflammatory cytokines on inducing CH25H gene expression in human macrophages by measuring CH25H mRNA level in human macrophages in response to stimulation with human recombinant pro-inflammatory cytokines (IL-1β, TNFα, and IL-6). As shown in **Figure 3.18B**, CH25H levels in THP-1 macrophages were elevated by recombinant pro-inflammatory cytokine treatment. Next, we sought to further assess their role in TLR-mediated CH25H. As shown in **Figure 3.19A-C**, individual blockade of these cytokines had no effect on poly (I:C) and LPS mediated CH25H. Taken together these results suggest that inflammatory cytokines such as IL-1β, TNF-α, and IL-6 can induce CH25H expression in human macrophages, but their individual blockade is not enough to abrogate LPS and Poly (I:C) induced CH25H expression.

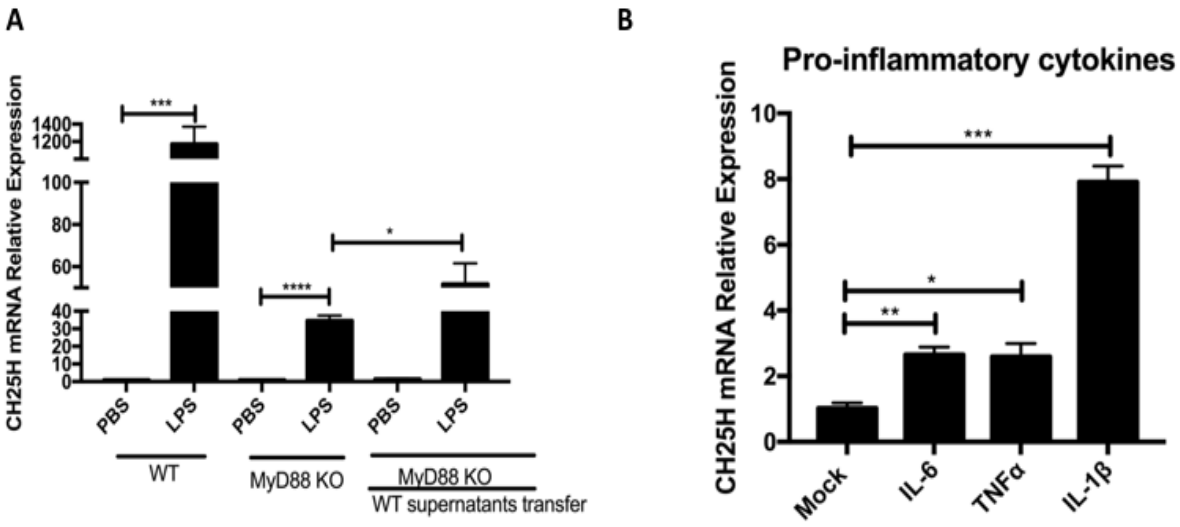


**Figure 3.18. Induction of CH25H in human macrophages with pro-inflammatory cytokine stimulation.** (**A**) Wild-type and MyD88 THP-1 macrophages were treated with either PBS or LPS for 3 hr. At 3 hr post-treatment, culture supernatants from wild-type THP-1 macrophages were transferred to fresh MyD88 KO THP-1 macrophages for 3 hr. Both the actual treatment and supernatant treatment were analyzed for CH25H mRNA expression by qPCR. (**B**) THP-1 macrophages were treated with media (mock) or selected pro-inflammatory cytokines for 3 hr, CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

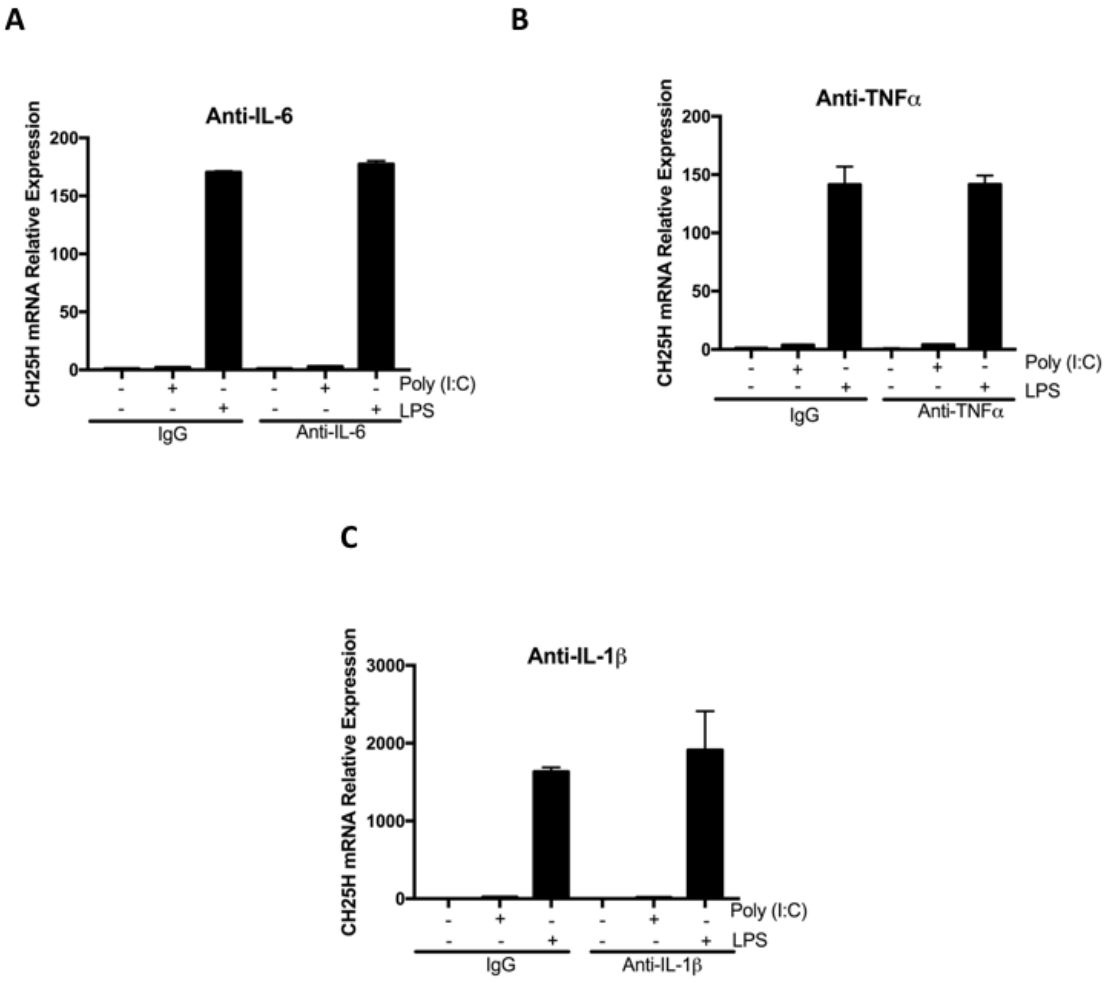


**Figure 3.19. The redundancy of individual cytokine on TLR-mediated CH25H expression**. (**A-C**) THP-1 macrophages were pre-treated with isotype antibody (IgG) or (**A**) anti-IL-6 or (**B**) anti-TNFα or (**C**) anti-IL-1β neutralizing antibody for 1 hr, and then treated with poly (I:C) (10 μm) or LPS (100ng/ml) for 3 hr, in the presence of continued antibody treatment. At 3 hr post-treatment cells were collected for CH25H analysis by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

### 3.3 Discussion and conclusion

Data on the regulation of cholesterol 25-hydroxylase in human macrophages are incosistent. In this study, we have taken a comprehensive approach to characterize the regulation of CH25H in human macrophages. Consistent with findings in murine models, our findings reveal that CH25H is robustly induced by TLR3/4 and ZIKV exposure in human immune and non-immune cells. We have identified an upstream sensing compartment (endosome) and sensing molecule (TLR-3 receptor) for ZIKV induced CH25H. Interestingly, we noticed discrepancy in dependence of CH25H gene expression on viral replication in two tested cell-lines. In microglial cells, ZIKV replication was required for CH25H induction, which suggests the necessity of dsRNA, which is formed during viral replication, for viral sensing. Similarly, in THP-1 macrophages, although heat-inactivated virus was able to induce CH25H, it was significantly lower than the live virus which also suggests the importance of viral replication. One potential explanation for differences on viral replication dependency between the two tested cell-lines could be based on the TLR signaling cascade. In microglial cells, only TLR3 was able to induce CH25H expression, suggesting the need for dsRNA formation to induce CH25H expression, whereas in THP-1 macrophages, although TLR3 activation can also induce CH25H expression, its expression was robustly induced with TLR4, suggesting PAMPs in the induction of CH25H expression in this cell type. In addition, inhibition of TLR3 signaling significantly decreased ZIKV-mediated induction of CH25H expression. However, since TLR-3 receptor blockade does not completely abolish ZIKV-mediated CH25H induction, it will be intriguing to investigate the effects of other TLRs which reside in the endosomal compartment. Moreover, similar to TRIF, MyD88 adaptor molecule plays a role in CH25H induction and the endosomal TLRs that rely on this adaptor molecule should be further investigated for a role in CH25H induction.

Corroborating Xiang observations (Xiang et al., 2015), we have found that CH25H is not a classical ISG in human cells, but rather inflammatory cytokines are involved in CH25H gene transcription. This conclusion is supported by the following reasons: no interferons were induced with ZIKV exposure in THP-1 macrophages (**Figure 3.13**); interferons and interferon receptor blockade was not enough to reduce TLR and ZIKV mediated CH25H;

culture supernatants from TLR and ZIKV treated cells was enough to induce MX1, a classical ISG, but fail to induce CH25H expression. Interestingly, in contrast to Xiang 's findings in mammalian cells, we found that recombinant type I interferon induced CH25H in THP-1 macrophages, microglial cells, LX-2 cell but not in Huh7.5.1 cell-line (**Figure 3.16**). In addition, pro-inflammatory cytokines such as IL-6, 1L-1β, and TNF-α also shows the ability to induce CH25H in THP-1 macrophages. This is the first report demonstrating the role of pro-inflammatory cytokines in inducing CH25H gene expression. However, the redundancy of all these cytokines on TLR mediated CH25H shows that the regulation of CH25H in human cell is complex. However, individual blockade of these cytokines may not be enough since other cytokines can also compensate for this loss. This conclusion is supported by the following observation: 1) complete blockade of cytokines with cycloheximide reduced LPS and Poly (I:C) mediated CH25H (**Figure 3.14A)**; 2) supernatant transfer from wild-type to MyD88 KO macrophages partially rescued CH25H compared to wild-type (**Figure 3.18A**), suggesting the role of cytokines. Nevertheless, TNF-alpha have been linked with the production of other antiviral genes (ISGs), However, this is the first report demonstrating the role of pro-inflammatory cytokines in inducing CH25H.

Antagonist that inhibit inflammatory cytokines (IL-6, 1L-1β, and TNF-α) are used in the treatment of inflammatory diseases, autoimmunity and cancer (Hunter and Jones, 2015). However, our findings on the role of these cytokines in antiviral immunity (CH25H induction), has raised questions about how and when to block this cytokine to improve disease outcome and patient wellbeing, since their blockade will also render patients more susceptible to viral infection. Thus, our study suggests a need to revise the current approaches for the treatment of inflammatory diseases, autoimmunity and cancer with respect to viral infection.

Previous studies have indicated that CH25H possess its antiviral properties through production of an antiviral molecule, 25HC (Diczfalusy, 2013; Liu et al., 2013; Li et al., 2017). Our study shows that CH25H is induced by mammalian cells, suggesting production of 25HC. Using 25HC, we have shown that 25HC inhibit ZIKV viral entry

(**Figure 3.5**). Our findings contribute to a rapidly growing body of evidence about the recently proposed mechanism of action of 25HC on inhibition of viral entry (Liu et al., 2013; Tani et al., 2016; Li et al., 2017) and post-entry immune processes (Blanc et al., 2013; Anggakusuma et al., 2015; Xiang et al., 2015; Shrivastava-ranjan et al., 2016). Although previous studies, including ours, have shown antiviral properties of 25HC. This mediator has been shown to possess detrimental or cytotoxic properties and induce apoptosis in various cell types (Ares et al., 1997; Ayala-Torres et al., 1997; Choi et al., 2008; Trousson et al., 2009). Moreover, Zou and colleagues showed that infection by the bacterium Listeria monocytogenes upregulated CH25H in an interferon-β dependent manner in mouse macrophages and this increase promotes survival of L. monocytogenes-infected cells and increases sensitivity of the host to infection (Zou et al., 2011).

Recently, 25HC has been shown to activate an integrated stress response (Shibata et al., 2013). In our study, an ectopic expression of CH25H in hepatic cell and HEK293 cell-line induce cell death. Although we did not extensively investigate these apparent findings, to reason about the possible pathway that might induce cell death, it is tempting to speculate that during viral infection, 25HC might be produced to contain viral infection, but if overwhelm by viral infection, it will activate an integrated stress response pathway and induce cell death. All these together underscore the importance of understanding the CH25H induction pathway, providing immunotherapeutic strategies to develop anti-viral agents. However, this assumption should be interpreted more carefully and warrants future studies to confirm these findings.

In conclusion, our results have demonstrated for the first time that pro-inflammatory cytokines such as 1L-1β, TNF-α, and IL-6 induce CH25H expression. This study provides further understanding to the connection between innate immunity and sterol metabolism and encourages the exploration of cytokines in antiviral immunity.

# CHAPTER FOUR: THE ROLE OF ATF3 AND JAK-STAT SIGNALING TRANSDUCTION PATHWAY ON CH25H EXPRESSION (Objective 2)

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The following chapter is in ncluded in part in “Tshifhiwa Magoro, Aditya P. Dandekar, Lucas T. Jennelle, Rohan Bajaj, Gabriel Lipkowitz, Angelina R. Angelucci, Pascal O. Bessong, Young S. Hahn. IL-1β/TNF-α/IL-6 inflammatory cytokines promote STAT1-dependent induction of CH25H in zika virus-infected human macrophages. Journal of Biological Chemistry.” (manuscript submitted, attached in appendix)

## 4.1 Introduction

Having established that TLR, ZIKV and cytokines can induce CH25H expression, the main aim presented in this chapter was to identify a transcription adaptor molecule that regulates this expression in human macrophages. Considering their roles in innate immunity, transcription factors such as MAPK, NF-kB, ATF2/c-jun, ATF3, IRF3, and STAT, can be induced following TLR stimulation, ZIKV infection and cytokine stimulation. Nevertheless, one or more of these transcription factors are likely to control the regulation of CH25H expression. Materials and methods used in this chapter are detailed in chapter two.

## 4.2 Results

### 4.2.1 Activation of p-STAT1, p-JNK, ATF3 by macrophages following ZIKV infection.

To understand a molecular basis of CH25H induction in human macrophages in response to ZIKV infection or TLR stimulation, we sought to determine a transcription factor responsible for CH25H mRNA expression. Although Involvement of STAT1 transcription factor has been demonstrated in the induction of mouse CH25H expression (Blanc et al., 2013), its role in human macrophages is yet to be understood. Given a significant

reduction of CH25H mRNA level by treatment with TBK1 inhibitor (BX795), it is likely that transcription factors affected by BX795 treatment could play a role in inducing CH25H expression. To this end, we attempted to define signaling cascade of CH25H gene transcription using BX795 treatment. We examined the activation of MAPK, NF-kB, ATF2/c-jun, ATF3, IRF3, and STAT considering their involvement in the inflammatory signaling in response to pathogen associated molecular patterns (PAMP) or cytokine stimulation (Katsoulidis et al., 2005; Symons et al., 2006; Thompson et al., 2009; Newton and Dixit, 2012; Turner et al., 2014; Labzin et al., 2015; Wang et al., 2016)

Comparison of BX795 treated THP-1 macrophages with THP-1 MyD88 knockout and wild type THP-1 macrophages showed that phosphorylation of p38 MAPK and NF-KB-p65, were not reduced in cells treated with BX795 following LPS stimulation but were reduced in MyD88 knockout macrophages. In contrast, there was a significant reduction of ATF3, and reduced phosphorylation of IRF3, SAPK/JNK, and STATs in BX795 treated cells compared to wild type control (**Figure 4.1**). These results suggest, ATF3, IRF3, SAPK/JNK, and STATs may be involved in transcription of CH25H.

Additionally, ZIKV infection was sufficient to induce activation/phosphorylation of ATF3, SAPK/JNK, and STATs transcription factors, but could not induce activation/phosphorylation of IRF3 (**Figure 4.2A**). Similar results are obtained in cells stimulated with pro-inflammatory cytokines (IL-1$\beta$, TNF-$\alpha$, IL-6) (**Figure 4.2B**). Taken together, cytokines and ZIKV-mediated CH25H induction likely occurs through one of these transcription factors and/or synergistic effect of several of these transcription factors combined. Thus, it is critical to identify a specific transcription factor involved in the expression of CH25H to target a specific pathway to reprogram macrophages towards boosting their anti-viral activity.

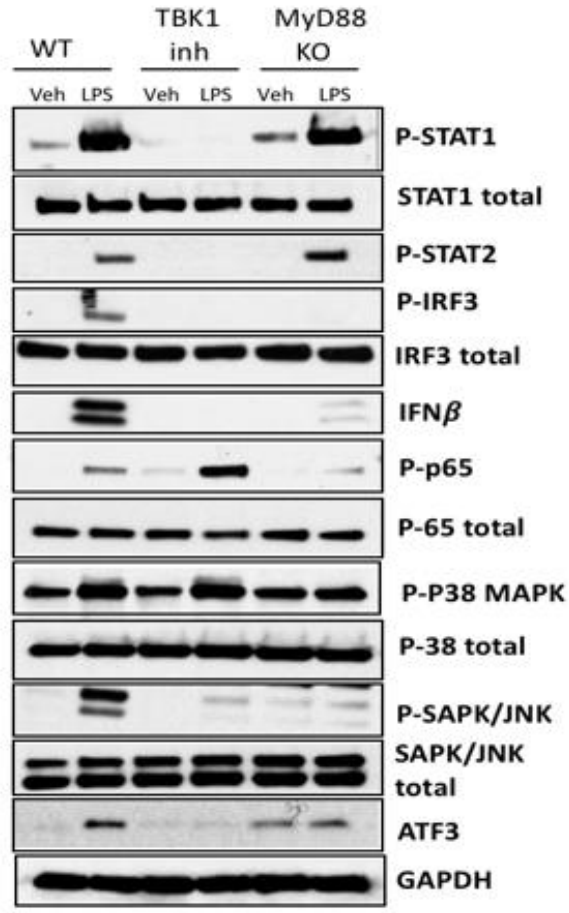


**Figure 4.1. Potential CH25H transcription factors in mammalian cells with TBK1 inhibitor.** Wild-type and MyD88 THP-1 macrophages were treated with a vehicle for 1 hr. in parallel, wild-type thp-1 macrophages were pretreated with TBK1 inhibitor (BX795, 10μm) for 1 hr, and then treated with LPS for 3 hr, in the presence of continued drug treatment. Wild-type and MyD88 THP-1 macrophages were also treated with LPS for 3 hr. Expression of potential transcription factors was examined by western blot, GAPDH was used as a loading control.

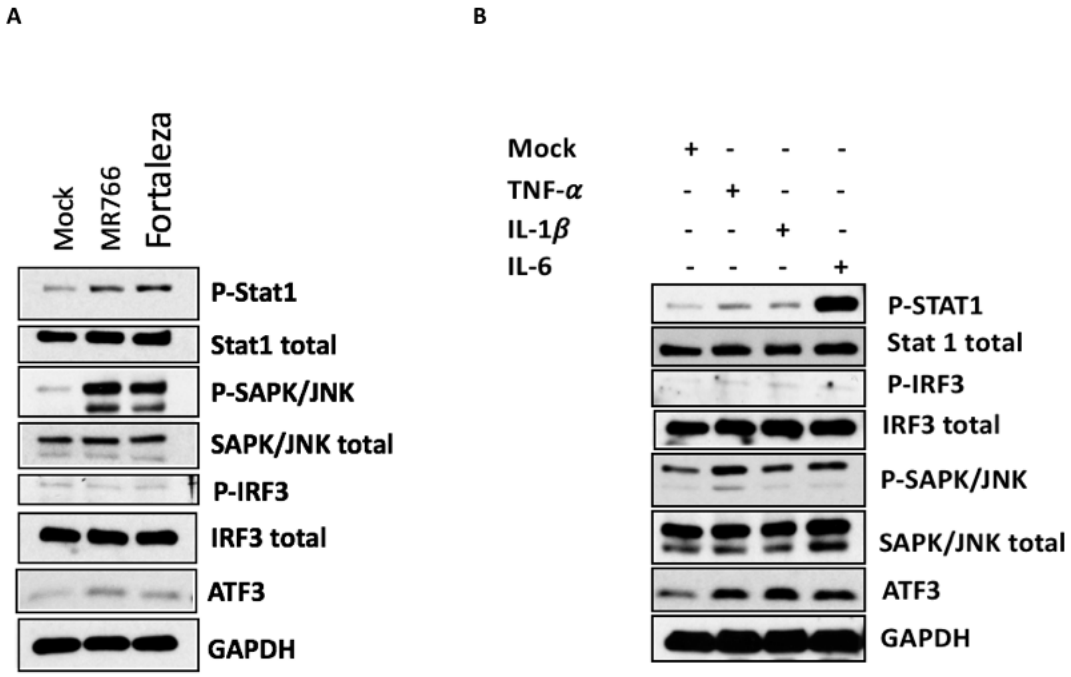


**Figure 4.2. Potential CH25H transcription factor/s are also induced by viral infection and cytokine stimulation**. (A and B) THP-1 macrophages inoculated with (A) ZIKV (MR766, or Fortaleza, MOI 1) for 24 hr, or (B) treated with pro-inflammatory cytokines for 3 hr were analyzed for potential transcription factors induction, and GAPDH expression by Western blotting.

### 4.2.2 The SAPK/JNK pathway is not necessary for the induction of CH25H

Next, we sought to pinpoint a transcriptional pathway involved in induction and expression of CH25H. First, we addressed whether the SAPK/JNK pathway is required for the expression of CH25H by TLR stimulation, cytokine stimulation, or viral infection. To do this, we examined the effect of JNK inhibition on CH25H expression. The reduction of SAPK/JNK (with SP600125) was indirectly assessed by assessing p-c-jun, downstream of JNK (**Figure 4.3A**). The specificity of SP600125 was also assessed in relation to other potential transcription pathway targets, indeed, SP600125 was highly specific for the JNK pathway (**Figure 4.3A**). JNK inhibition significantly decreased the poly (I:C) and LPS-mediated induction of CH25H but had no significant effect on cytokine-mediated CH25H induction (**Figure 4.3B&D**). Surprisingly, SAPK/JNK inhibitor increased MR766-mediated CH25H compared to vehicle treatment (**Figure 4.3C**). Since SAPK/JNK inhibitor only

effected TLR-mediated CH25H induction, we asked if this effect is due to the reduction of cytokines, as shown in **Figure 3.12A and 3.16A**, TLR-mediated CH25H partially relies on cytokines. To test this possibility, THP-1 macrophages were pre-treated with vehicle (DMSO) or SAPK/JNK inhibitor (SP600125) (10 μm) or LPS for 3 hr. At 3 hr post-treatment, culture supernatants were transferred to DMSO or SP600125 (pre-treaded for 1 hr) treated THP-1 macrophages for 3 hr. As shown in **Figure 4.3E**, LPS supernatants transferred to vehicle or sp600125 pre-treated macrophages induced similar CH25H expression (rescuing deficient CH25H induction in SP600125-treated macrophages), demonstrating that SAPK/JNK inhibition reduces cytokines which contribute to CH25H induction. Altogether this suggests that SAPK/JNK does not play a direct role as a transcription factor in CH25H induction but rather that SAPK/JNK participates in the production of cytokines that indirectly mediate CH25H induction.

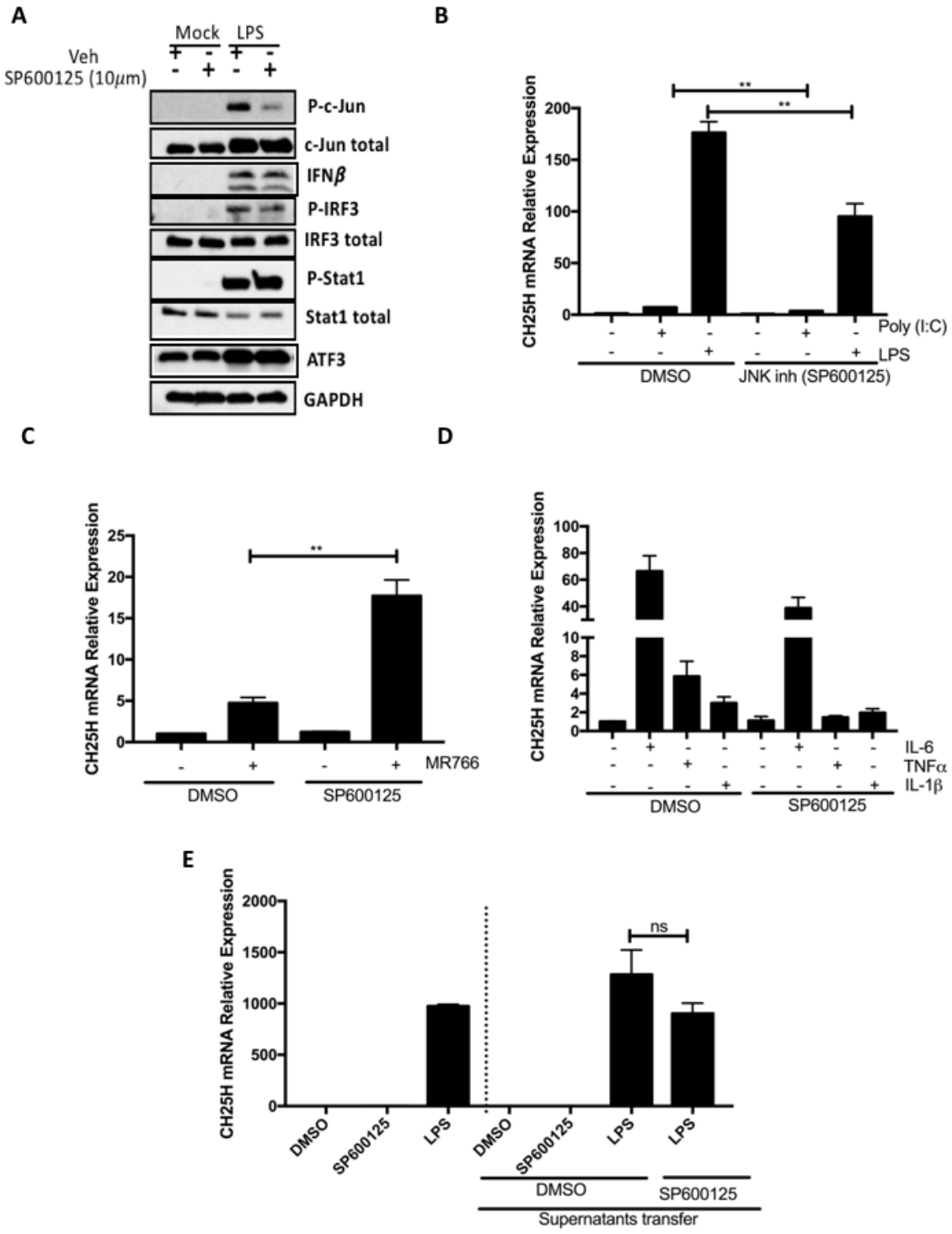


**Figure 4.3. SAPK/JNK pathway is necessary for only TLR-mediated CH25H expression in human macrophages.** (**A**) THP-1 macrophages were pre-treated with vehicle (DMSO) or SAPK/JNK inhibitor (SP600125) (10 μm) for 1 hr, and then treated with LPS for 3 hr. At 3 hr post-

treatment, cells were lysed and analyzed for phospho-c-Jun, c-Jun total, IFNβ, Phospho-IRF3, IRF3 total, Phospho-STAT1, STAT1 total, ATF3, and GAPDH expression by Western blotting. (**B-D**) THP-1 macrophages were pre-treated with vehicle (DMSO) or SAPK/JNK inhibitor (SP600125) (10 μm) for 1 hr, and then treated with (**B**) poly (I:C) (10 μm) or LPS (100ng/ml) for 3 hr or with (**C**) ZIKV (MR766, MOI 1) for 24 hr, or (**D**) with pro-inflammatory cytokines for 3hr, in the presence of continued drug treatment. CH25H mRNA expression was measured by qPCR. (**E**) THP-1 macrophages were pre-treated with vehicle (DMSO) or SAPK/JNK inhibitor (SP600125) (10 μm) or LPS for 3 hr. At 3 hr post-treatment, culture supernatants were transferred to DMSO or SP600125 (pre-treaded for 1 hr) treated THP-1 macrophages for 3 hr. CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples. $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

### 4.2.3 The JAK/STAT and ATF3 pathways are necessary for the induction of CH25H expression

To address whether the JAK/STAT pathway is required for the expression of CH25H in response to TLR or cytokine stimulation, or viral infection, we examined the effect of JAK inhibitor I (a highly potent, ATP-competitive inhibitor of JAK1, 2, 3 and Tyk2 (arrest STATs phosphorylation), on the induction of CH25H. The inhibitory effect (and its specificity in relation to potential transcription factors) of JAK inhibitor I was confirmed by western blot (**Figure 4.4A**). As a positive control, JAK inhibitor 1 significantly decreased transcription of MX1 and MX2 (**Figure 4.4B and C**). Notably, this inhibitor significantly decreased the transcription level of TLR, MR766 and cytokine-induced CH25H expression (**Figure 4.5A-C**). These results indicate that STAT proteins are necessary for the maximal induction of CH25H.

Next, we screened ATF3 for a potential role in CH25H regulation by using several commercially available ATF3 inhibitory drugs. However, pharmacological inhibitor experiments proved insufficient to define the potential role of ATF3 in CH25H transcriptional regulation in THP-1 macrophages. Nevertheless, to scrutinize ATF3 as a potential CH25H transcription factor, in cooperation with STAT as suggested through STAT inhibitor experiments, we utilized a chromatin immunoprecipitation (ChIP) assay in

macrophages treated with LPS to identify the binding of either ATF3 or STAT1 to CH25H promoter. The ChIP-qPCR analysis of CH25H promoter sequences revealed an LPS-mediated recruitment of Stat1 to the binding region of the Ch25H locus (**Figure 4.5D**). In addition, ChIP-qPCR analysis also revealed the binding of ATF3 to CH25H promoter sequence, but this binding was not statistically significant. Collectively, these experiments show that CH25H transcriptional activation is coupled to the TLR-activation through direct recruitment of STAT1 to the CH25H promoter, potentially supplemented by recruitment of ATF3.

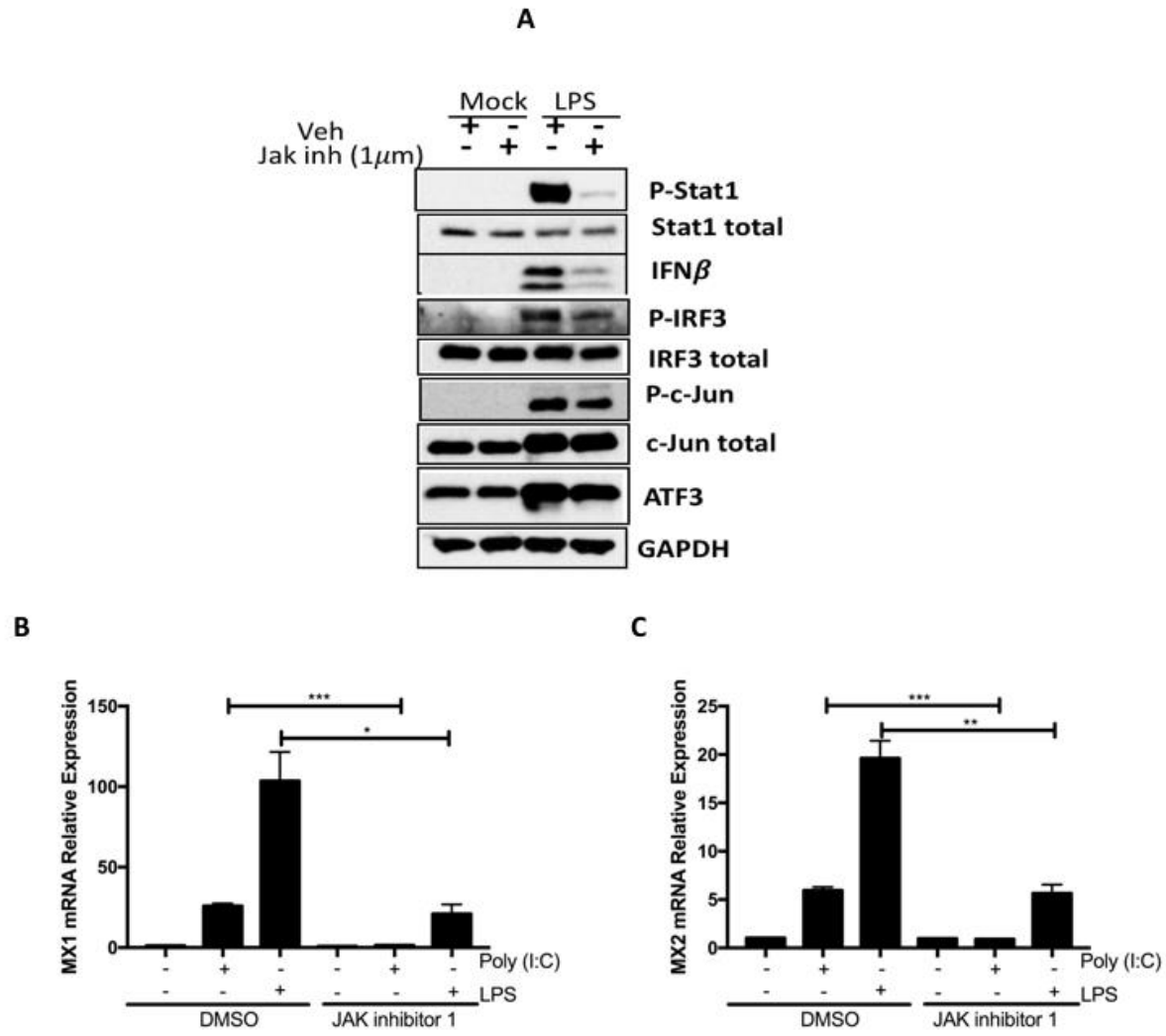


**Figure 4.4. JAK/STAT signaling pathway regulates the transcription of classical interferon stimulated genes**. (**A-C**) THP-1 macrophages were pre-treated with vehicle (DMSO) or JAK

inhibitor 1 (1 μm) for 1 hr, and then treated with LPS (100ng/ml) or poly (I:C) (10 μm) for 3 hr, in the presence of continued drug treatment. (**A**) At 3 hr post-treatment, cells were lysed and analyzed for phospho-STAT1, STAT1 total, IFNβ, phospho-c-Jun, c-Jun total, Phospho-IRF3, IRF3 total, ATF3, and GAPDH expression by Western blotting. (**B** and **C**) cells were collected and analyzed for MX1 or MX2 mRNA expression by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (three independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

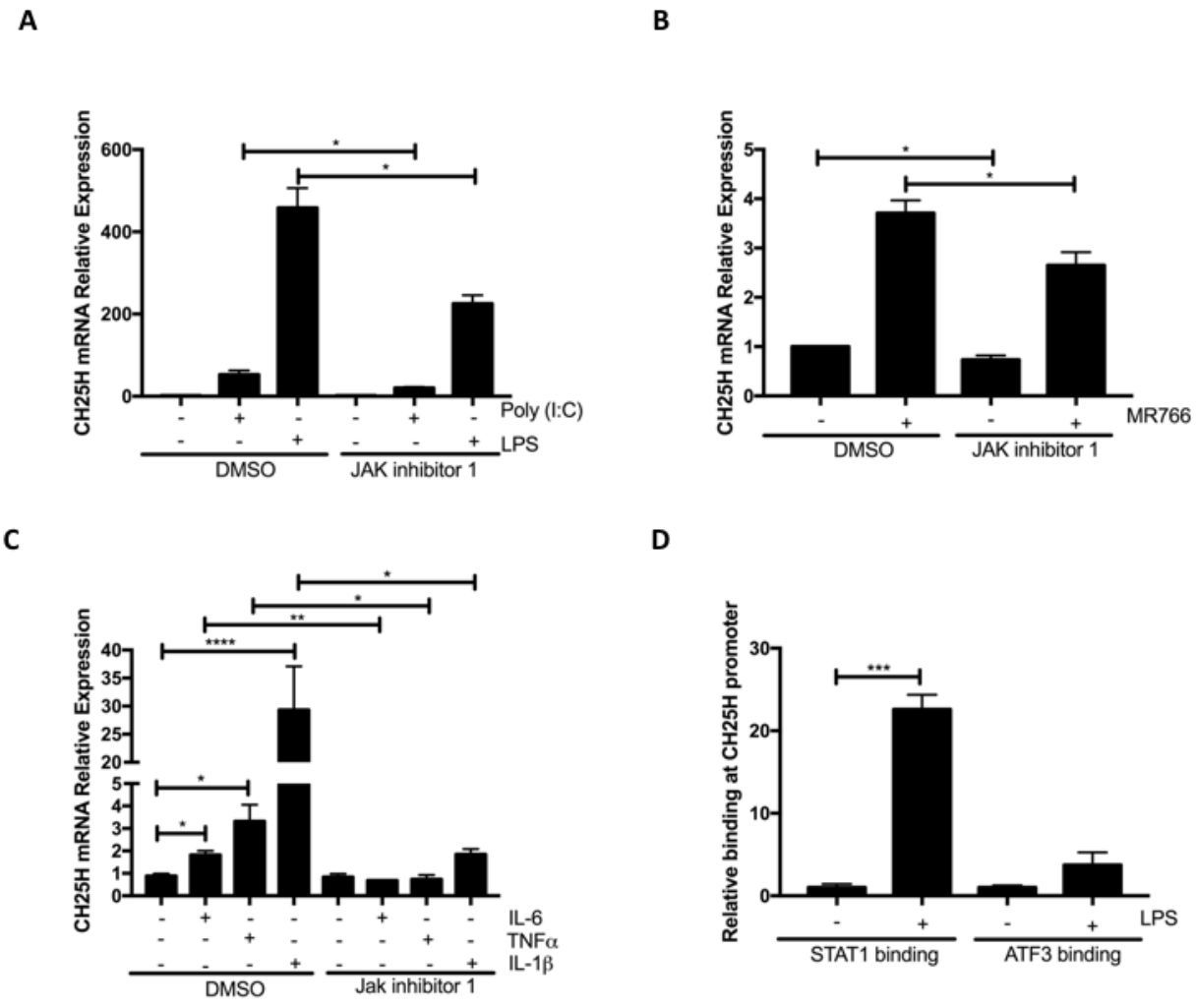

**Figure 4.5. STAT1 regulates CH25H expression in human macrophages.** (**A-C**) THP-1 macrophages were pre-treated with vehicle (DMSO) or JAK inhibitor 1 (1 μm) for 1 hr, and then treated with (**A**) poly (I:C) (10 μm) or LPS (100ng/ml) for 3 hr or with (**B**) ZIKV (MR766, MOI 1) for 24 hr, or (**C**) with pro-inflammatory cytokines for 3hr, in the presence of continued drug treatment. CH25H mRNA expression was measured by qPCR. (**D**) THP-1 macrophages were treated with media or LPS for 3 hr, and CHIP analysis for STAT1 and ATF3 binding was performed at CH25H promoter.

### 4.3 Discussion and conclusions

In this chapter we took a comprehensive approach to further deduce a signaling transduction cascade in the induction of CH25H by determining potential transcription factor/s. Previously, in mouse models, IFNAR-JAK-STAT signaling has been shown to be the cardinal pathway through which ISGs are induced, in particular, CH25H (Park and Scott, 2010; Blanc et al., 2013). Moreover, Park and Scott previously reported that TLR-mediated CH25H expression is type I IFN and STAT1-dependent (Park and Scott, 2010). In addition, Blanc revealed the binding of STAT1 to CH25H promoter region directly, using a ChIP assay (Blanc et al., 2013). Consistent with findings in murine models, In the present study, in human macrophages, using ChIP assay we further confirmed the binding of STAT1 as well as the weak binding of ATF3 to CH25H promoter region. Although ATF3 weakly binds to CH25H promoter, it is possible that it might be playing a potential role as a transcription co-factor. Interestingly, mouse studies have identified ATF3 as a negative regulator of the macrophage transcriptional response to inflammatory stimuli (Labzin et al., 2015). In addition, in the absence of ATF3, the levels of CH25H and 25HC are increased (Gold et al., 2012). These differences further raise questions of how best mouse models of disease inform studies of human diseases and therapeutic approaches. Recently, Sood and colleagues have also identified ATF3 acting as a negative regulator of antiviral response in other mammalian cells (Sood et al., 2017).Nevertheless, whether there is specific cell type-dependency for the negative regulatory effect of ATF3 still needs to be investigated.

To further show the role of these transcription factors in CH25H induction, ZIKV infection and cytokine stimulation was sufficient to induce activation/phosphorylation of ATF3 and

STATs transcription factors. However, this induction was reduced with the neutralization of STATs protein supporting a role in CH25H induction.

The identification or further confirmation of these potential transcription factor lessen the burden imposed by detrimental or cytopathic properties of CH25H/25HC suggested by others (Ares et al., 1997; Ayala-Torres et al., 1997; Choi et al., 2008; Trousson et al., 2009). The knockout or knock down of these transcription factors can reverse the effect of these molecules. However, since 25HC has also been shown to possess antiviral properties, activation or phosphorylation which can lead to the induction of CH25H/25HC which has obvious potential to affect viral replication.

In conclusion, we further confirm the role of STAT-1 transcription factor in the induction of CH25H in human macrophages. In addition, our findings suggest that an additional transcription factor, ATF3, may be involved, along with STAT-1, in induction of CH25H. These findings will guide us to better understand the regulation of CH25H in human cells. This study provides further understanding to the connection between innate immunity and sterol metabolism.

# CHAPTER FIVE: FINAL REMARKS AND FUTURE AIMS

## 5.1 Final remarks

While it has been shown that CH25H/25HC inhibit viral replication, it has been of great importance to study the CH25H/25HC induction pathway due to its detrimental or cytotoxic properties and the induction of apoptosis that has been shown in various cell types. In this study we have added new players into the CH25H signaling transduction cascade. While regulation of CH25H has been shown murine models, it has not been extensively studied in human models. In human cells, our findings introduce pro-inflammatory cytokines such as 1L-1β, TNF-α, and IL-6 as inducers of CH25H expression. These findings further strengthen the connection between innate immunity and cholesterol metabolism. Due to this study, now we know that inhibiting these inflammatory cytokines to treat inflammatory disease has a negative impact on a patient wellbeing with respect to viral infection. In this study we have interrogated the transcription factors involved in the induction of CH25H, and now we can demonstrate that STAT1 and ATF3 seems to be a player in this pathway. Now we can suggest that using STAT1 and ATF3 antagonists or knocking them out we might arrest the detrimental effect of CH25H. Nevertheless, stimulating CH25H effectors such as 1L-1β, TNF-α, and IL-6 might arrest viral replication.

In this study we further confirmed that 25HC reduced viral entry. Although we didn't interrogate further, it has been suggested that 25HC is likely to interfere with cholesterol biosynthesis, thus inhibit viral entry or viral replication. With its role in the manipulation of cholesterol content, 25HC cannot be used soley as antiviral agent it can also be used to treat cardiovascular diseases. Having shown that 25HC can arrest viral replication we further propose that 25HC could potentially be used as an antiviral therapeutic as it has in vitro activity against a number of viruses.

Although differences on the fold change which are seen between experiments are a major concern. Differences on the cells passage number might have a role on the differences seen with some of the experiments with same treatment.

### 5.2 Future work

From the work presented in this thesis, we have gained significant new knowledge about CH25H regulation in human macrophages. Prior to these studies, we had few investigations of CH25H regulation in mammalian cells. In addition, we also present for the first time the role of pro-inflammatory cytokines on CH25H regulation. Nevertheless, result presented herein, has opened several avenues for further investigations. For instance, the widespread role of pro-inflammatory cytokines in CH25H regulation during inflammation. Although TLR mediated CH25H induction seems to partially rely on cytokines, the redundancy of interferons and classical pro-inflammatory cytokines warrant further investigations. It would be of interest to further analyze the role of these mediators on the regulation of CH25H protein.

Although the potential involvement of the NF-kB, and other members of the MAPK pathway, in particular P38, have been ruled out on the regulation of CH25H the involvement of JNK/SAPK pathways on CH25H expression warrant reviews of this pathways as well. Our thought is based on the fact that JNK/SAPK seems to work on the cytokine branch, since its blockade only reduced TLR mediated not ZIKV mediated CH25H which does not rely on cytokines. In addition, LPS supernatants were transferred in the presence of the JNK/SAPK inhibitor and CH25H induction was not blunted, ruling out JNK/SAPK as a significant transcription factor in this context. NF-kB and P38 regulate cytokine production, and are likely to play a role on cytokine branch of CH25H regulation.

Although we have LPS STAT1 ChIP data it is of paramount importance to have ChIP data with ZIKV, since this will affirm the role of STAT1 on CH25H regulation. In addition, it would be of great interest to also perform these experiments in STAT1 knock-out cell line to add more insights into the regulation of CH25H. Moreover, the use of ATF3 ChIP assay

suggests its role in CH25H induction, however, definitive conclusions cannot be made since ATF3 knock out or knock down assays were not conducted.

Gold's group, in mouse cells, showed the production of 25-hydroxycholesterol (25HC) by immune cells in response to infection. In addition, they further demonstrated that 25HC amplifies the production of immune mediators, in addition to interfering with viral entry and replication. Furthermore, presence of 25HC was harmful in the setting of infection with influenza because the production of these immune mediators leads to damage to the host (Gold et al., 2014). In this study we have observed a significant reduction of viral entry with 25HC treatment, and it will be of great importance to interrogate 25HC treatment impact on the level of pro-inflammatory cytokines (feed-forward loop) and determine the detrimental or beneficial impact of this treatment in our settings.

Although we have focused on MAPK, NF-kB, ATF2/c-jun, ATF3, IRF3, and STATs as potential transcription factors, other transcription factors that can be induced by innate immunity are likely to be contributors to CH25H induction. Although our study focused heavily on inhibitors and agonists, it would be of importance to validate these findings genetically using knockout cell lines. We attempted to address this to the degree possible through limited studies with a MyD88 knock out cell line.

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# Appendix

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# Immunometabolic Signaling Pathways Contribute to Macrophage and Dendritic Cell Function

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**ABSTRACT:** Understanding of antigen-presenting cell (APC) participation in tissue inflammation and metabolism has advanced through numerous studies using systems biology approaches. Previously unrecognized connections between these research areas have been elucidated in the context of inflammatory disease involving innate and adaptive immune responses. A new conceptual framework bridges APC biology, metabolism, and cytokines in the generation of effective T-cell responses. Exploring these connections is paramount to addressing the rising tide of multi-organ system diseases, particularly chronic diseases associated with metabolic syndrome, infection, and cancer. Focused research in these areas will aid the development of strategies to harness and manipulate innate immunology to improve vaccine development, anti-viral, anti-inflammatory, and anti-tumor therapies. This review highlights recent advances in APC "immunometabolism" specifically related to chronic viral and metabolic disease in humans. The goal of this review is to develop an abridged and consolidated outlook on recent thematic updates to APC immunometabolism in the areas of regulation and crosstalk between metabolic and inflammatory signaling and the integrated stress response and how these signals dictate APC function in providing T-cell activation Signal 3.

**KEY WORDS:** macrophage, metabolism, ER stress, interferon, cholesterol

**ABBREVIATIONS: 25-HC**, 25-hydroxycholesterol; **ABC**, ATP-binding cassette; **APC**, antigen-presenting cell; **CD**, cluster of differentiation; **CH25H**, cholesterol 25-hydroxylase; **DC**, dendritic cell; **IFN**, interferon; **IL**, interleukin; **IFNAR**, type I IFN receptor; **IRE1α**, inositol-requiring enzyme 1α; **IRF**, interferon regulatory factor; **LXR**, liver X receptor; **MHC**, major histocompatability complex; **miR**, microRNA; **NF-κB**, nuclear factor κB; **NLRP,** NOD-, LRR-, and pyrin-domain-containing protein; **SREBP**, sterol regulatory element-binding protein; **TLR**, Toll-like receptor; **TNF**, tumor necrosis factor; **UPR**, unfolded protein response; **XBP1**, X-box-binding protein 1

## I. INTRODUCTION

T-cell activation by pathogens proceeds in an ordered sequence; recognition of antigen through the T-cell receptor (TCR) in the context of "self" major histocompatability complex (MHC) (Signal 1); co-stimulation through TCR accessory molecules CD28, CD80/CD86, or intercellular adhesion molecule 1 (ICAM-1) (Signal 2); and a more enigmatic Signal 3 involving inflammatory cytokines provided by antigen-presenting cells (APCs). The requirement for Signal 3 in $CD8^+$ T cells for acquisition of cytotoxic function has been well established such that interleukin-12 (IL-12) can provide Signal 3 and endow $CD8^+$ cells with cytotoxic ability, opposing the development of tolerance that occurs in the absence of this cytokine. Subsequently, IL-1β or type I interferons (IFNs) have been shown to act as Signal 3 for $CD4^+$ T cells. The involvement of IL-12, IL-1β, and IFN α/β strongly implicates upstream Toll-like receptor (TLR)-driven signaling in APCs as a critical component providing Signal 3 to T cells (Fig. 1A).

A critical component of APC activation involves signaling mediated by recognition of pathogen-associated molecular patterns (PAMPs) by cell-surface and endosomal pattern-recognition receptors (PRRs). Among the most well-characterized PRRs are the members of the TLR family. Ultimately, many inflammatory stimuli activate inflammasomes and type I IFN downstream of TLR signaling (Fig. 1B), Inflammation is only one function of APCs;

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many APCs, particularly those of myeloid origin, are highly metabolic. Increased metabolic activity in APCs occurs due to their role in phagocytosing and degrading dead and dying cells. Through their functions in the removal of apoptotic and necrotic cells, APCs encounter and metabolize bolus deliveries of lipids and cholesterol. APCs also produce and secrete a large amount of cytokines, which can induce endoplasmic reticulum (ER) stress. How APCs integrate this complex milieu of activating signals from multiple systems is not well understood.

Understanding of APC immune activation and metabolism have advanced independently, but major contemporary breakthroughs have demonstrated that these functions are related and are often co-regulated. Manipulation of either function to achieve reciprocal effects in the other pathway is a mechanism used by APCs and pathogens alike to maintain homeostasis or facilitate pathogen replication. The goal of this review is to begin to assimilate these conceptual advances into a systems-level understanding of APC activation, metabolism, and function and to incorporate insights provided through application of advanced molecular methods that have emerged recently among these disciplines into a unified concept of APC "immunometabolism." Section II of this review examines the transcriptional control of crosstalk between macrophage metabolism and inflammation. Section III provides an overview of ER stress responses in APC function. Section IV highlights recent developments in the regulation of inflammation by specific bioactive metabolites and microRNAs (miRs). Section V briefly introduces several specific studies describing integration of metabolism, inflammation, and ER stress.

## II. LIPID-ACTIVATED TRANSCRIPTION FACTORS AND MACROPHAGE PHEONTYPE

### A. Transcriptional Control of Macrophage Metabolism and Inflammatory Responses

The nuclear receptors liver X receptors (LXRs) are ligand-activated transcription factors, the most well-described function of which is activation of metabolic gene expression in response to cholesterol metabolites.[1] A role has emerged for TLRs and LXRs in a complex reciprocal crosstalk between the immune and metabolic systems at the level of APCs, particularly macrophages.

#### *1. LXRs and Macrophage Metabolism*

The two receptors of the LXR family, α and β, are similar in sequence (77% amino acid similarity in DNA- and ligand-binding domains), but different in tissue distribution, with LXRβ expressed ubiquitously and LXRα being restricted to highly metabolically active sites including macrophages and the liver.[1,2] The principle role of LXRs is to remove excess cholesterol at the cellular and organism levels through the process of "reverse cholesterol transport" involving trafficking peripheral cholesterol to the liver through high-density lipoproteins for excretion in the bile and feces.[2] This is mediated through up-regulation of LXR target genes involved in cholesterol efflux (ATP-binding cassette transporters including ABCA1) and apolipoprotein cholesterol acceptors. In addition to cholesterol efflux, LXRs regulate fatty acid synthesis through up-regulation of sterol regulatory element-binding protein (SREBP)-1c[3,4] and fatty acid synthase (FAS).[5] Carbohydrate metabolism is regulated by LXRs through suppression of hepatic gluconeogenesis and induction of tissue uptake of glucose.[2,6] LXRs also act as glucose sensors regulating signaling in response to glucose through binding glucose directly and induction of carbohydrate response element binding protein.[7,8]

#### *2. LXRs and Macrophage Inflammatory Responses*

##### *a. LXR Crosstalk with TLRs*

Numerous studies have demonstrated crosstalk between the LXRs and inflammatory signaling through TLRs.[2] Under inflammatory conditions, TLR3 or TLR4 negatively affect cholesterol efflux through IFN regulatory factor 3 (IRF3)-mediated suppression of LXR-induced expression of cholesterol transporters.[9] In this way, inflammation

contributes directly to foam cell development and atherosclerosis. Intriguingly, this relationship appears to be, at least partially, bidirectional because activation of LXRs reduces inflammatory gene expression induced by TLR4, IL-1β, or tumor necrosis factor α (TNF-α) signaling.[10,11] An interesting interpretation of these results has been presented recently suggesting that TLR activation decreases cholesterol efflux in a feedforward mechanism to potentiate further amplification of TLR signaling and the inflammatory response itself as an integrated response to pathogens.[12] Ultimately, upon resolution of infection and diminution of TLR agonists, established cholesterol accumulation induces LXR activation as a natural "brake" to restore the system to homeostasis (Fig. 1C). A constant low-level barrage of TLR agonists or inflammasome triggers (theoretically provided by cholesterol crystal deposition or inflammatory modified lipoproteins) then could circumvent this brake, establishing chronic inflammatory diseases.[12] To fully understand the impact of transcriptional crosstalk between inflammation and metabolism, it is necessary to examine proposed mechanisms of this crosstalk.

### b. *Mechanisms of Immune Modulation by LXRs: Direct and Indirect Effects*

Mechanisms of transcriptional activation of LXRs are relatively well described. LXRs belong to the class of nuclear receptors that bind the promoters

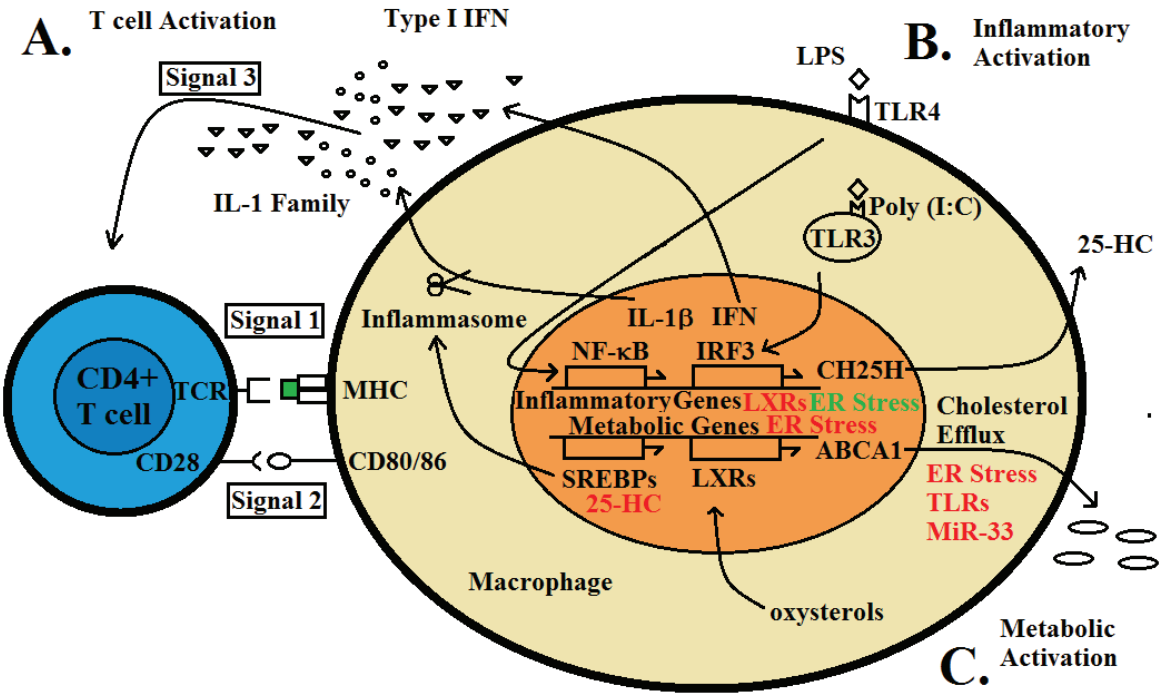


**FIG. 1:** Immunometabolic signaling pathways contribute to regulation of T-cell responses by APCs. (A) Activation signaling for CD4+ T cells; Signal 1 TCR engagement by the MHC:peptide complex; Signal 2 co-stimulatory activation through CD28; and Signal 3-activating cytokine signal provided by IL-1 family members or type I IFN. (B) Inflammatory activation by PAMPS through TLR3 or TLR4; signaling through NF-κB, or IRF3; and induction of inflammatory gene expression. (C) Metabolic activation of transcription factors through oxysterols produced upon cholesterol accumulation (LXR) and induction of metabolic gene expression. Inhibitory interactions are presented in red and are described in the text. Activating interactions are presented in green and are also described in the text.

of target genes containing LXR response elements (LXREs) in heterodimeric association with retinoid X receptors.[1,2] Nuclear receptors basally recruit co-repressors to these promoters, inhibiting gene expression, which is further reinforced by histone deacetylases (HDACs) and chromatin-modifying factors.[13,14] Particular corepressors on inflammatory genes include silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor.[13,15–20] Upon ligand binding, co-repressors are released and exchanged for co-activators to activate transcription of target genes.[14,21–24]

Despite being the focus of intense research, the mechanism of inhibition of TLR-signaling induced gene expression (of genes lacking LXREs and thus without direct binding by LXRs) by these receptors has remained somewhat nebulous. An elaborate mechanism known as "trans-repression" has been described for LXR control of inflammatory gene expression involving SUMOylation by SUMO2 or SUMO3 and involving E3 ligase HDAC4.[11,19,25–27] In this model, under normal conditions, the promoters of inflammatory genes (including inducible nitric oxide synthase 2 [iNOS or NOS2]) are bound by repressors including HDACs, NCOR, or SMRT, preventing attachment of co-activators. Upon inflammatory activation of TLR4, a signaling cascade results in ubiquitination and degradation of the corepressor complex by the 19S proteasome. Concomitant activation of nuclear factor κB (NF-κB) binding to sequence-specific elements in inflammatory gene promoters and recruitment of co-activators induces target gene expression.[1,2] Ligand-induced SUMOylation of LXR by SUMO2 or SUMO3 is proposed to allow LXR binding to the corepressor complex, preventing its degradation by the proteasome and thus maintaining repression of gene expression in the presence of inflammatory stimuli.[1,2,18]

A very recent study[28] has presented a competing theory explaining the anti-inflammatory effects of LXR activation on TLR signaling. In this model, transcriptional activation of LXRs is linked directly to both cholesterol homeostasis and inflammatory repression through direct transcriptional activation of the LXR target cholesterol transporter ABCA1. This work showed that ABCA1 affects inflammatory repression by reduction of lipid raft cholesterol content reducing recruitment of myeloid differentiation primary response gene 88 (MYD88) and TNF receptor associated factor 6 (TRAF6), therefore inhibiting TLR activation-induced phosphorylation of mitogen-activated protein kinases and activation of NF-κB and ultimately leading to reduced expression of inflammatory target genes.[28] The relevance of this model was demonstrated for activation of TLR2, TLR4, and TLR9, but not TLR3. Support for this model of LXR-mediated inflammatory repression has been provided by earlier studies demonstrating that ABCA1 and ABCG1 can negatively regulate TLR signaling by depleting lipid rafts where TLR signaling complexes are assembled.[29–31] Several studies have been published recently identifying non-coding regulatory miRs (miR-33a and miR-33b) produced from parent genes of the SREBP family.[31–34] The SREBPs regulate fatty acid (SREBP-1c) and cholesterol (SREBP-2) biogenesis and miR-33 appears to reinforce these effects to elevate cellular cholesterol by targeting and reducing ABCA1 expression.[32–34] Further contemporary support for the model has been presented in a recent study reporting that miR-33 can enhance inflammatory gene expression by modulating lipid rafts and NF-κB activation induced by TLR4 agonists in a mechanism involving ABCA1 and ABCG1.[31]

Using technical advances in promoter-enrichment-quantitative mass spectrometry, studies have recently begun to identify LXR corepressors that are recruited specifically during the contraction phase of transcriptional activation induced by LXR agonists (8-16Hrs).[14] This work demonstrated that nuclear receptor coactivator 5 (NCOA5), a corepressor that interacts with LXR, is only recruited to target gene (ABCA1) promoters upon LXR ligand stimulation during this contraction phase. Intriguingly, TLR3 (but not TLR4) agonists could enhance recruitment of NCOA5 and repression of gene expression at early time points only in the presence of LXR ligand, providing an additional layer of complexity to LXR/TLR crosstalk.

According to the corepressor–coactivator exchange and trans-repression mechanisms outlined above, LXRs can affect gene expression in either

direct or indirect fashion. It is therefore not surprising that putative LXREs have been identified in a limited number of inflammatory genes themselves, potentially allowing for more direct control of inflammation by LXRs. Specifically, TNF-α and IFN-γ genes have been reported to contain LXREs and to respond to ligand stimulation.[35,36] Although these reports are isolated, it will be interesting to see how direct regulation of inflammatory genes by LXRs is integrated contextually into the complex crosstalk that has been demonstrated to exist between LXRs and innate immunity. To establish thematic understanding of APC effects on T-cell activation and Signal 3, it is pertinent to emphasize direct effects of metabolic regulators on Signal 3 itself.

### *c. LXRs and Signal 3*

The mechanisms and effects described thus far demonstrate how LXRs can affect inflammatory signaling. In the context of metabolic effects on APC function, specifically T-cell Signal 3 provided by inflammasomes and type I IFNs, it is noteworthy that several direct effects of LXRs have been described. Inflammasome activation proceeds in a two-signal cascade. An initial priming stimulus through TLR/MYD88 activates NF-κB as a first signaling cascade. NF-κB activation induces expression of IL-1β, IL-18, and NOD-, LRR-, and pyrin domain-containing 3 (NLRP3). A multi-protein complex termed the inflammasome is assembled from NLRP3 and apoptosis-associated speck-like protein (ASC). A second signaling cascade activated via a variety of damage-associated molecular patterns (i.e., adenosine triphosphate, reactive oxygen species [ROS] or PAMPs, and involving cell surface receptors such as pannexins and potassium channels) activates NLRP3 and the cleavage function of the inflammasome. Once activated, the inflammasome cleaves Pro-caspase 1 to the mature Caspase-1, which subsequently cleaves pro-proteins of IL-1β and IL-18 to the mature forms that can be secreted and function in inflammation and T-cell activation. In an early study on the anti-inflammatory effects of LXRs, LXR activation was shown to reduce both lipopolysaccharide (LPS)-induced expression of IL-1β and IL-1β-induced expression of IL-6 and iNOS.[10] A recent report indicates that LXRs can similarly regulate IL-18. In that study, LXR activation was shown to inhibit both LPS-induced gene and protein expression of IL-18 and also processing of Pro-IL-18 by regulating Pro-caspase-1 expression and activation. LXR ligand activation also induced inhibitory IL-18 binding protein (IL-18BP).[37] Having reviewed transcriptional control of macrophage metabolism and inflammatory responses by LXRs, the next section highlights a specific example of feedback regulation by a bioactive cholesterol metabolite acting on another major metabolic transcription factor, SREBP-1.

## B. Regulation of Macrophage Inflammatory Pathways by Bioactive Lipid Metabolites

Through interaction between TLR signaling and transcription factors such as LXRs, inflammatory signaling in macrophages can affect macrophage metabolism. As described above, metabolic signaling can also affect reciprocal changes in inflammation. Although the LXRs couple cholesterol metabolism to the inflammatory response, another recently described mechanism couples lipogenesis to inflammation and, importantly, directly affects components of Signal 3 through the NLRP inflammasome and involves a signaling bioactive lipid mediator. A recent report demonstrated that SREBP-1a protected mice from lethal challenge with LPS and lethal bacterial-induced sepsis.[38] The mechanism was shown to involve a direct interaction between SREBP-1a and inflammasome component NLRP-1a (Fig. 1C). SREBP-1a induction of NLRP-1a was shown to be important for Caspase-1 activation and secretion of IL-1β and IL-12. Surprisingly, SREBP-1a was shown to be a direct target of NF-κB, allowing LPS-induced inflammation to activate lipogenesis. A subsequent study identified an unexpected activity of an IFN-induced metabolic enzyme, cholesterol 25-hydroxylase (CH25H), producing a bioactive signaling lipid mediator, 25-hydroxycholesterol (25-HC), and suppressing IL-1β-mediated inflammation downstream of type I IFN. A series of studies had previously demonstrated that 25-HC inhibits maturation, translocation, and resultant activation of SREBPs,

critical control nodes of cholesterol and fatty acid biosynthesis, through proteasomal degradation mediated by insulin-induced gene 1 (INSIG1).[39-43] In this study, the absence of CH25H and resultant elimination of 25-HC was shown to allow exaggerated inflammasome activity with overproduction of IL-1 family members due to loss of regulation by SREBP.[44] These studies identified 25-HC as a critical component of IFN-induced inhibition of inflammasome signaling with a dual role in regulation of lipogenesis through SREBPs.

## III. ROLE OF STRESS RESPONSES IN APC FUNCTION OF MACROPHAGES AND DENDRITIC CELLS

### A. Connection among Metabolic, ER, and Oxidative Stress in APC Functions

As discussed in the previous section, control of lipid metabolism is crucial for proper functioning of immune cells. The transcription factors SREBP1 and LXR are involved in macrophage and T-cell function and crosstalk through effects on metabolism and TLR and inflammasome signaling.[45,46] Metabolic disturbance accompanied by immune changes can also exert a significant effect on cellular stress responses. There are a number of metabolic byproducts and lipids, which can trigger activation of the ER stress response. As with TLR signaling, there exists a bidirectional relationship between ER stress responses and lipid metabolism. Conversely, ER stress can trigger activation of different factors and pathways involved in lipid metabolism. Links between X-box-binding protein 1 (XBP1), a downstream target of the unfolded protein response (UPR) sensor inositol-requiring enzyme 1α (IRE1α), and metabolism have been studied extensively in the context of liver function. XBP1 regulates transcription of many genes involved in fatty acid synthesis, including SCD-1 (stearoyl-CoA desaturase-1), ACC2 (acetyl-CoA carboxylases 2), and DGAT2 (diacyl glycerol acyl transferase). Upon exposure to tunicamycin (which disrupts glycosylation of newly synthesized proteins, resulting in ER stress), liver tissue exhibited down-regulation of lipid metabolic pathways of many genes such as FAS, SREBP1, PGC-1α (peroxisome proliferator-activated receptor coactivator), CEBPα (CCAAT-enhancer-binding protein α), and PPARα (peroxisome-proliferator-activated receptor α).[47] Acute ER stress can also modulate cholesterol metabolism in human hepatoma cells by causing ABCA1 redistribution to tubular perinuclear compartments.[48] Considering these strong connections between metabolic signaling and ER stress, it is not surprising that ER stress signaling affects the antigen-presenting functions of macrophages and dendritic cells (DCs).

### B. Understanding ER Stress Responses

The molecular signals released during infection or disease states are capable of eliciting stress responses within immune cells. Many studies have described different roles of specific stresses in aiding immune responses generated through APCs. The following section reviews the contribution and manipulation of oxidative stress and ER stress toward optimal or defective functioning of macrophages and DCs.

The ER is a large vesicular compartment that is actively involved in protein folding and protein trafficking within cells. It is also critical to the proper function of other organelles and multiple signaling cascades. An increase in secreted and membrane-embedded protein translation or a decrease in protein-folding capacity can result in a buildup of unfolded or misfolded proteins in the ER lumen, a condition known as ER stress. UPR is an adaptive intracellular signaling pathway that responds to ER stress by attenuating global protein translation and degrading unfolded proteins. Canonical UPR signaling is initiated by activation of three ER membrane-bound transducers: IRE1α, Activating Transcription Factor 6 (ATF6), and Double-Stranded RNA-Activated protein kinase-like ER kinase (PERK). Through transcriptional and translational reprogramming, the UPR is a cellular mechanism for stressed cells to adapt to and survive ER stress conditions. APCs, by virtue of their secretory demand, rely heavily on ER functioning and subsequent UPR signaling. Their reliance on ER makes them susceptible and sensitive to ER imbalance that compromises ER function. Understanding how the

UPR affects specific functions at the cellular level and host-related factors that affect this are of great importance and this is discussed below.

## C. ER Stress in APCs

### 1. ER Stress in Regulation of APC Maturation and Differentiation

High-mobility group box-1 (HMGB1) is a late inflammatory cytokine secreted by myeloid cells as well as NK cells. Studies have demonstrated the role of HMGB1 as an immunoregulatory molecule involved in DC maturation and differentiation. Recently, a study revealed that silencing of XBP1 in HMGB1-treated DCs decreased the expression of MHCII, CD80, and CD86 and resulted in a decrease in TNF-α production.[49] Silencing of XBP1 also abrogated the APC function of DCs, leading to reduced levels of IFN-γ in T cells. Another target for ER stress is protein tyrosine phosphatase 1B (PTP1B); a tyrosine phosphatase involved in STAT3 dephosphorylation. Whereas ER stress activates PTP1B in skeletal muscle,[50] loss of PTP1B causes a reduction in the DC maturation markers MHCII, CD80, and CD86 and leads to defective podosome formation in DC upon LPS stimulation.[51] Differentiation of monocytes into macrophages is an important event in the initiation of immune responses. Induction of ER stress in monocytes leads to attenuation of macrophage differentiation capacity. THP1, a human monocytic cell line pretreated with an ER stress inducer, displayed no alteration of forward and side scatter and no increase of CD11b and CD68 expression level.[52] Upon TLR signaling and immune activation, suppression of the C/EBP homologous protein pathway of UPR signaling is important for macrophage survival during the immune response.[53]

### 2. ER Stress in APC Antigen-Presenting Functions (Signal 1)

Potential effects of ER stress on antigen presentation are suggested from the observation that peptides derived from intracellular or extracellular pathogens are transported to the ER for association with MHC molecules. The translocation pathway of MHC peptide toward the cell surface is also initiated at the ER vesicular interface. Recently, a study described the role of HMG-CoA reductase degradation protein 1 (Hrd1), an ER-resident E3 ubiquitin ligase, in MHC-II expression on DCs,[54] possibly by degrading transcription factor B lymphocyte-induced maturation protein 1 (BLIMP1). Although this study ruled out the potential role of ER stress in Hrd1-mediated MHC-II expression in DC, there might be a link between the ER stress pathway IRE1α-XBP1 and degradation of BLIMP1. Regarding MHC-I expression, ER-associated degradation (ERAD) is used by DCs to generate peptides for cross-presentation. Moreover, the IRE1α-XBP1 branch of the ER stress response has been implicated in cross presentation. In $CD8a^+$ DCs, deletion of XBP1 leads to excessive endonuclease activity of IRE1α.[55] Subsequently, activated IRE1α degrades mRNAs such as lysosomal-associated membrane protein 1 (LAMP1), TAP-binding protein (TAPBP), and ER-Golgi intermediate compartment protein 3 (ERGIC3), which are involved in the cross-presentation of antigen. Autophagy-mediated antigen processing is another important arm of MHC-I cross-presentation.[56] Microtubule-associated protein 1A/1B-light chain 3 (LC3), a key component of the autophagy response, is activated upon ER stress. Phosphorylation of PERK induces an elevation in LC3 processing, thereby contributing to initiation of autophagy.[57]

### 3. ER Stress in Cytokine Production by APCs (Signal 3)

The role of ER stress responses in cytokine production has been widely studied in macrophages. ER stress has been shown to amplify cytokine production upon stimulation with TLR ligands. TLR2 and TLR4 ligands induce IRE1α activation in mouse J774 macrophages.[58] Furthermore, activation of IRE1α in macrophages inhibited XBP1 splicing. IL-1β transcription was shown to be induced by IRE1α in a glycogen synthase kinase 3β (GSK3β)-dependent manner. GSK3, in turn, inhibits XBP1 splicing and thereby the transcriptional activity of XBP1s at inflammatory target genes, including TNF-α.[59] Upon stimulation, IRE1α signaling is activated in a

ubiquitination-dependent manner by TRAF6. This prevents the dephosphorylation by protein phosphatase 2A (PP2A) and subsequent inactivation.[60] ATF4, one of the downstream targets of the PERK pathway of UPR signaling, binds directly to the IL-6 promoter.[61] The ER stress response is also involved in the production of IFNs and IFN-stimulated genes (ISGs) through phosphorylation and nuclear translocation of IRF3 (Fig. 1B). Treatment with the ER stress inducer thapsigargin induces activation of stimulator of IFN genes (STING), an ER-resident protein. STING-induced IRF3 phosphorylation mediated by TANK-binding kinase 1 (TBK1) in turn induces transcription of ISGs. Retinoic acid-inducible gene I (RIG-I)-like receptor signaling also modulates IFN-β levels through a mechanism involving ER stress.[62]

In DCs, XBP1 can act as a double-edged sword. Under normal conditions, XBP1 enhances lipid metabolism in an ER-stress-dependent manner in response to inflammatory stimuli.[63] This activity is necessary for optimum cytokine production by DCs by expanding the ER and Golgi compartment. Conversely, during tumor progression, DCs exposed to ROS reprogram XBP1 activity, thereby impairing lipid metabolism resulting in enhanced acquisition of immunosuppressive phenotype in DCs.[63] Thymic stromal lymphopoietin (TSLP) produced by epithelial cells, as well as DCs themselves, acts on DCs to drive differentiation of T-Helper 2 (TH2) cells.[64] Chemical induction of ER stress using tunicamycin (TM) or thapsigargin in conjunction with dectin-1 increased TSLP secretion from mDCs. This secretion of TSLP in mDCs is dependent on the IRE1α and PERK pathway of ER stress response because siRNA against these targets abrogated the production of TSLP by mDCs in a mechanism dependent on IL-1β production by the IRE1α branch of ER stress pathway.[65] This demonstrates that specific control of different arms of the UPR is crucial for optimal functioning of antigen presentation in macrophages and DCs. Given transcriptional crosstalk and the role of ER stress in macrophage metabolism and inflammatory responses, studies elucidating a connection between bioactive lipids, miRs, and the IFN response would be crucial for the development of therapeutic agents.

## IV. IFNS AND STEROL METABOLISM

In addition to transcriptional regulation of inflammation and metabolism by LXRs and the ER stress response, a contribution of bioactive metabolites to these processes has been described. Numerous microbial pathogens target host cell lipid metabolism to attain essential structural components required for replication. Cholesterol in particular, but also fatty acids and other metabolites, have been shown to be critical for replication of a number of human pathogens, viral, bacterial, and parasitic. It is attractive to speculate that host innate immune responses may have co-evolved to re-direct or override pathogen co-opted metabolic pathways to aide pathogen elimination. Compelling contemporary evidence for the existence of such pathways of specifically anti-viral immunity has been presented recently, elaborating a link between type I IFNs and cholesterol metabolism[66] and independently for the putative IFN-stimulated cholesterol metabolism gene product CH25H and its enzymatic product 25-HC in the context of viral infection. New discoveries and the adaptation of new technologies in pursuit of understanding the intricate regulation of these pathways has prompted re-evaluation of this simplistic view of the host response as fundamentally reactionary and instead a concept emerges of an evolutionary "arms race" of actions and reactions by both host and pathogen in an ancient, yet undoubtedly still evolving, fight for survival.[67]

### A. Lipid Signaling in Inflammatory Processes: Sterol Metabolism and Type I IFN

While evidence was accumulating steadily from multiple studies implicating TLRs and inflammatory signaling in transcription factor regulation of host metabolic pathways in APCs, a landmark study demonstrating this effect in anti-viral immunity was published in the journal *PLOS Biology* in 2011.[66] Using a time-series analysis of microarray data in murine bone-marrow derived macrophages (BMDMs) either infected with virus or treated with IFN-γ, the investigators demonstrated selective, coordinated negative regulation of the complete sterol

pathway.[66] Gene and protein expression of all major sterol pathway nodes were reduced by viral infection. IFNs were shown to be sufficient for down-regulation of the sterol pathway, whereas other inflammatory cytokines including IL-1β, TNFα, and IL-6 could not mediate this effect. IFN-induced down-regulation of the sterol pathway resulted in reduced steady-state concentrations of free cholesterol and other major sterols in virus-infected or IFN-treated cells. Experiments confirmed that biochemically simulating the cellular IFN response to virus by inhibition of the sterol pathway is anti-viral both *in vitro* and *in vivo* and additional experiments implicated the proximal isoprenoid branch of sterol biosynthesis, particularly geranylgeranyl transferase type II, as the specific target of the cellular response against virus. Indeed, geranylgeraniol itself alleviated the anti-viral activity of IFN-β. The investigators demonstrated down-regulation of sterol biosynthesis for diverse enveloped and non-enveloped DNA and RNA viruses. Molecular mechanistic experiments demonstrated that type I IFN, type I IFN receptor (IFNAR), and tyrosine kinase 2 (Tyk2) receptor signaling are required for the reduction of sterol biosynthesis in a mechanism that involves transcriptional and translational down-regulation of sterol response element binding protein 2 (SREBP2). Inhibition of SREBP2 is proposed to be the terminal effector mechanism of the IFN response as a master switch allowing compounding of small negative changes in multiple intermediates to affect pathway inhibition of sterol synthesis. The investigators described a two-step model for anti-pathogenic inhibition of sterol biosynthesis in which a first signal is provided by pathogen activation of PRRs (including TLRs) inducing a type I IFN response. The second signal is then provided by type I IFN signaling through IFNAR impinging upon SREBP2 activation. Presciently at the time, the investigators noted that their notion of anti-viral activity of inhibiting SREBP2 implicated "negative feedback on SREBP-2 via oxysterol metabolites." In short order, just such an interaction was indeed described in the context of multiple viruses for the oxysterol 25-HC.

A fascinating update to this general theory has been elaborated recently in a study demonstrating that virus infection or type I IFN treatment of macrophages altered the balance of lipid metabolism to reduce *de novo* synthesis specifically and increase import of cholesterol and fatty acids in an IFNAR-dependent manner.[68] Experimentally engineering an altered balance in the "set point" of lipid metabolism in mice by targeting SREBP cleavage-activating protein (SCAP) and thereby reducing lipid biogenesis relative to import phenocopied virus infection or type I IFN effects in reducing lipid synthesis and provided protection against viral infection. Remarkably, anti-viral activity was shown to be mediated by spontaneous and specific induction of a type I IFN response which was IFNAR-dependent in the absence of SCAP and ultimately discovered to be mediated by effects on SREBP2. In human and mouse studies, knockdown of SREBP2 spontaneously induced a type I IFN response that protected against multiple viruses in vitro. Finally, the mechanism of spontaneous induction of type I IFN was shown to involve cyclic GMP-AMP (cGAMP) synthase (cGAS), STING, TBK1, and IRF3.[68] Importantly, in cells that were infected or exposed to IFN, compensatory increases in lipid import opposing decreased synthesis essentially maintained total lipid levels in cells. This prompted the investigators to propose a new theory that "acutely decreasing synthesized cholesterol appears to provide a novel "danger" signal that activates a type I IFN-mediated anti-viral response" and "reprogramming of lipid metabolism is to alter the balance between lipid synthesis and scavenging, rather than to decrease lipid pool sizes." This suggests that lipids delivered to cells by exogenous uptake are less favorable to invading pathogens than *de novo* synthesized lipids, a provocative conclusion that suggests that there are still many unanswered questions in the interplay between host metabolism and anti-pathogen inflammatory responses. The unique interaction between sterols and IFN signaling suggested control by bioactive lipid mediators. The next section highlights a newly described example of metabolic regulation by the aforementioned 25-HC.

## B. Regulation of Macrophage Anti-Viral Activity by Bioactive Lipid Metabolites

CH25H and its enzymatic product, 25-HC, comprise a unique example of a metabolic circuit with

an emerging and seemingly independent role in immunity. The most historically well-defined role of 25-HC is inhibition of cholesterol biogenesis through inhibitory effects on HMG-CoA reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway.[42,43,69] 25-HC also regulates SREBPs through interactions involving SCAP and INSIG1.[39–43]

Unexpectedly, an exciting new role for CH25H and 25-HC in innate immunity has been described recently. In a series of studies, a molecular pathway was delineated in which murine macrophages significantly up-regulated CH25H in response to TLR stimulation, leading to production and secretion of 25-HC.[70,71] Humans voluntarily injected with a TLR4 agonist also demonstrated increased serum 25-HC.[70] Notably, studies in mice demonstrated the strongest TLR activation-induced effect on CH25H in tissues with substantial resident macrophage populations.[71,72] This pathway was subsequently confirmed in DCs and shown to involve TLR3/4, TIR-domain-containing adapter-inducing IFN-β (TRIF), IRF3/NF-κB, IFN-β, IFNAR, and Janus kinase and signal transducer and activator of transcription 1 (JAK/STAT1), ultimately inducing CH25H responsible for converting cholesterol to 25-HC, which is then secreted[73,74] (Fig. 1B). Another study has raised the possibility that IRF1 may also cooperate with STAT1 in the induction of CH25H.[75]

Before elaboration of the CH25H/25-HC molecular circuit, pioneering studies demonstrated anti-viral activity of this oxysterol against multiple viruses.[76–80] After the description of this circuit, in rapid succession, evidence accumulated demonstrating anti-viral activity of 25-HC generalizable to a wide array of essentially unrelated viruses that may also be relevant to other side-chain-substituted derivatives of cholesterol.[42,75,81–84] Most of these effects involve SREBP modulation by 25-HC, but an additional activity against oxysterol-binding proteins (OSBPs) and OSBP-related proteins (ORPs) involving phosphatidylinositol 4-kinase (PI4K) has also been described.[83,84] An anti-viral effect of 27-hydroxycholesterol (27-HC) and 24-hydroxycholesterol (24-HC) has also been demonstrated.[42,81] Notably, a number of studies have identified anti-viral activity of CH25H/25-HC against HCV potentially involving multiple mechanisms.[43,69,78,79,85–87] Finally, an exciting study has been published recently examining the non-coding transcriptome in HCV-infected cells in the presence and absence of 25-HC treatment, demonstrating that 25-HC alters the miR environment of these cells. miR-185 and miR-130b were shown to be induced by 25-HC treatment in infected cells and to inhibit HCV through a mechanism involving regulation of hepatic lipid metabolism and virus-induced lipid microenvironments (miR-185 and miR-130b) and an effect on IFNs (miR-130b).[88] Having reviewed the crosstalk among IFN signaling, cholesterol biosynthesis, and cholesterol metabolites in anti-viral immunity, the next section highlights an exciting new development in small, non-coding RNA regulation of these processes.

## C. Prospective: Regulating the Regulators–Sterol Biogenesis and miRs

Concurrently with more thorough description of the interaction between host metabolism and immunity, the discovery of anti-viral pathways in invertebrates involving small RNA-mediated gene silencing and their correlates among the non-coding portion of the human genome, regulatory small RNAs, including miRs, have led to a paradigm shift in our understanding of the regulation of diverse cellular processes, including immune responses. This unique confluence of small RNAs, biochemical pathway regulation, and immune responses prompted studies investigating whether miRs might function to reinforce the IFN-mediated down-regulation of sterol biosynthesis elucidated in previous studies.[66] Using systemic global analysis of RNA turnover via 4-thiouridine labeling, a unique regulatory mechanism involving cellular miR control of metabolic pathways that function in anti-viral immunity was uncovered.[89] Upon IFN treatment of murine BMDMs, the investigators confirmed the previously described reduction in synthesis and abundance of sterol pathway transcripts including a major effect on *SREBF2* (the gene encoding SREBP2). miR analysis identified increased synthesis and abundance of miR-155 and miR-342 in IFN-treated and mCMV-infected cells that was sensitive to type I

IFN and IFNAR. miR-342 induction by IFN was shown to be regulated coordinately with the transcript from which the miR is derived, namely Ena-vasodilator stimulated phosphoprotein gene (EVL). miR-342 was shown to reduce the abundance of major transcripts of the sterol biosynthesis pathway through a regulatory interaction involving direct binding to the *SREBF2* promoter with downstream reduction in immature and mature SREBP2 protein. miR-342 was also shown to reduce repressive function of the *SREBF2*-derived miR (miR-33), which was previously shown to target ABCA1 and ABCG1. Expectedly, miR-342 reduced levels of cholesterol metabolites in cells upon overexpression. Similar to the previous study, the miR-342-induced down-regulation of sterol metabolism resulted in broad-spectrum anti-viral activity.[76,77] These studies from multiple groups[42,68,89] have effectively combined insights from the integrated understanding of macrophage inflammatory pathways and metabolic signaling to make remarkable progress in advancing conceptual understanding of immunometabolism and present a compelling argument for describing innate immunity and metabolism as an integrated system.

## V. SYSTEMS-LEVEL INTEGRATION: LIPID METABOLISM AND ER STRESS

We have examined metabolism and ER stress and described how both biological systems impinge upon inflammation and immunity. However, metabolism, ER stress, and inflammation can also affect one another. Indeed, examples of integrative crosstalk have begun to emerge recently. The LXR target gene lysophosphatidylcholine acyltransferase 3 (Lpcat3) has been shown to regulate lipid-induced ER stress and inflammatory activation of proto-oncogene tyrosine-protein kinase Src (c-Src) through control of membrane composition and saturation.[90] Macrophage fatty acid binding protein-4 (aP2) has been shown to act as a major regulator mitigating lipid-induced ER stress involving PERK and XBP-1 through actions on the LXR pathway and LXR targets including SCD-1 and FAS.[91] In turn, ER stress has been shown to be a major contributor to hepatic steatosis through activation of SREBPs[92] and ER stress can also inhibit cholesterol efflux and synthesis through LXR-independent effects on ABCA1 and effects on HMGCR.[93] Therefore, it is appropriate and essential to view macrophages as major integrators of independent and dependent signals in the immune, metabolic, and ER stress responses.

## VI. CONCLUDING REMARKS: OUTLOOK

According to the model developed by Tall and Yvan-Charvet,[12] TLR- and IFN-mediated inflammation is initially a protective response to pathogens and feedforward inhibition of cholesterol efflux (Fig. 1B) and biogenesis and promotion of cholesterol uptake prolong and strengthen the TLR signal. Eventually, accumulation of cholesterol overrides the TLR effect and induces homeostatic activation of LXRs to remove cholesterol and restore balance in the system (Fig. 1C). Crosstalk between the systems is likely an evolutionary check on a futile cycle of simultaneous lipid biogenesis and catabolism, operating in response to conflicting signals in opposing pathways. According to this interpretation, prolonged low-level stimulation of TLRs (perhaps by cholesterol crystals, as suggested in Tall and Yvan-Charvet[12]) and possibly accentuated by ER stress (Fig. 1B) could produce a dangerous pathogenic cycle as has been described in atherosclerosis. Here, it has been observed that different areas of immunological foci, specifically atherosclerotic plaques, have distinctly different macrophage profiles with more inflammatory cells toward the interior of plaques yet, paradoxically, abundant anti-inflammatory macrophages toward the plaque periphery.[94] This could be explained by temporal effects because the macrophages toward the plaque periphery could be those that have activated restorative metabolic activities such as LXRs, whereas those in the plaque core are subject to the inhibition of LXR induced by TLRs. The ultimate result of this crosstalk may prove pathogenic when the inflammatory activities in core macrophages are incapable of eliminating the offending stimulus and the anti-inflammatory macrophages (which have enhanced tissue-building and matrix-depositing capability) at the plaque periphery actively promote pathogen invasion or inflammatory activation and sustenance of the inflammatory TLR signal in the plaque core.

The emerging understanding of reciprocal interactions between inflammation and metabolism at the level of transcriptional control, ER stress, bioactive lipid mediators, and miRs are theoretically complex and involve an extensive network of checks and balances (Fig. 1). The multi-dimensional nature of this integrated-systems-level inter-relatedness has intriguing implications for the exquisite and intricate control of these pathways in homeostasis and underscores the involvement of major regulatory nodes such as LXRs, SREBPs, and IRE1α. Just as importantly, understanding how disturbances in the system are exploited acutely during pathogen infection can lend important insights into systems-level disturbance engendered during chronic inflammatory diseases. Indeed, many of the major breakthroughs discussed in this review involve astute targeted perturbations in the inflammatory network producing unexpected but highly informative corrections achieved through metabolic reprogramming. Although these discoveries advance our understanding of the system itself and its regulation, they have dubious implications against the backdrop of the steady march of chronic diseases. The outlook presented in Tall and Yvan-Charvet,[12] specifically regarding atherosclerosis but likely applicable to a number of chronic diseases with an inflammatory component, is particularly inspiring. A new outlook of the systems-level integration of inflammatory and metabolic inputs, informed by the breakthroughs highlighted in this review, suggests a reason for optimism. Namely, increased understanding of these connections can empower researchers and physicians to make targeted interventions in these diseases that may achieve signal amplification and graded improvements in both measures, inflammation and metabolism, to ultimately improve health-related outcomes.

Empowering research to move forward by producing a consolidated theoretical model is important to encourage development of the theory, but must also acknowledge deficiency in current understanding. Although there is a reason for optimism, the outlook must be presented with caution. Many of the studies in this review focus on disease models and systemic perturbations in mouse systems. There are reported differences in regulation and expression of miR-33 in mouse and human.[31] Similarly, regulation of inflammasome components by SREBPs has been suggested to be an artefact of back-crossing deficits in in-bred mouse strains.[95] At least one report has challenged IFN sensitivity of CH25H in humans[87] and, in particular, many aspects of CH25H and 25-HC expression and regulation are not understood in humans, where protein expression of CH25H is reported to be low.[85] With these concerns in mind, acknowledgment must be made of studies that include a focused effort to realize applicability of findings across experimental models and consider translational impact. The excellent study by the Bensinger group achieves these goals laudably, melding studies in knock-out mice with *in vitro* experiments in primary human cells, including cells from patients with relevant human in-born errors of metabolism.[68] It is the goal of the authors that careful application of the theoretical model developed in this review in studies designed from the outset with an understanding of application and translation to human disease may allow rapid advancement of these concepts to the clinical setting and allow physicians and researchers to continually improve and update our understanding of immunometabolism.

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# Zika virus requires STAT1 for interferon-independent induction of cholesterol 25-hydroxylase

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Running title: *Role of inflammatory cytokines in CH25H induction*

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**ABSTRACT**

**Zika virus (ZIKV) is an enveloped, single-stranded positive-sense RNA virus of the Flaviviridae family that has emerged as a public health threat due to its global transmission and link to microcephaly. Currently, there is no vaccine for this virus. Conversion of Cholesterol to 25-Hydroxycholesterol (25HC) by Cholesterol 25-Hydroxylase (CH25H) has been shown to exert broad antiviral properties. However, the molecular basis for the induction of CH25H in humans is not known. Elucidation of signaling and transcriptional events for induction of CH25H expression is critical to design therapeutic antiviral agents. In this study, we show that CH25H is induced by ZIKV infection or TLR stimulation. Interestingly, CH25H is induced by pro-inflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), and this induction depends on signal transducer and activator of transcription 1 (STAT1) transcription factor. Additionally, we have observed that cyclic AMP-dependent transcription factor (ATF3) weakly binds to the CH25H promoter, suggesting co-operation with STAT1. However, ZIKV induced CH25H was independent of type I interferon. These findings provide an important information for understanding how ZIKA induces innate inflammatory responses and promotes the expression of anti-viral CH25H protein.**

Zika virus (ZIKV) is an arthropod-borne virus, transmitted by Aedes mosquitoes, which belongs to the *Flaviviridae* family and the *Flavivirus* genus. Recently, ZIKV has emerged as a public health threat due to its global transmission and link to severe congenital disorders, such as microcephaly, Guillain-Barre syndrome (GBS), and meningoencephalitis (1–3). ZIKV infection in placental macrophages has been reported to transmit the virus from mother to fetus (4). Host immune responses to ZIKV infection have not been fully elucidated. At present, there is no vaccine or antiviral drug to combat ZIKV. Thus, it is critically important to study the regulation of inflammatory responses to the virus in human macrophages.

One molecular mediator of innate antiviral immunity that has attracted much attention recently is CH25H, which is an enzyme that converts cholesterol to 25HC (5–7). Following stimulation of toll-like receptors (TLRs) 3 and 4, common sensors of viral and bacterial infection, CH25H is highly induced in mouse macrophages and dendritic cells. Moreover, its induction is dependent on interferon alpha receptor (IFNAR) interactions (7). Importantly, 25HC exerts antiviral activity against both enveloped and non-enveloped viruses in *in vitro* (5,6,8–10), specifically by suppressing proteolytic activation of sterol regulatory element binding proteins (SREBPs) and acting as a ligand for liver X receptor (LXR) (11–14). In these ways,

25HC promotes induction of genes involved in cholesterol efflux, thereby limiting cellular cholesterol content available for viral replication. Moreover, 25HC also exerts its antiviral activity by activating the integrated stress response, which is independent of the LXRs and SREBPs (15).

Given its antiviral activities, CH25H is part of an increasingly appreciated connection between type I interferon (IFN-I) and lipid metabolism (8,16–18). Importantly, however, the details of this connection appear to differ in mouse and human cells. In murine models, type I IFN induces CH25H thereby linking these two biological processes (7,19,20). On the other hand, in human hepatoma cell lines, CH25H does not appear to be a classical interferon stimulated gene (ISG); rather, it is induced in response to TLR3/4 agonists to restrict viral replication (8). Thus, the precise transcriptional regulatory events for the induction of CH25H in humans are poorly understood. Achieving such an understanding is critical to further design therapeutic agents to control pathogens.

In the present study, using *in vitro* cell culture models of macrophages, we provide insight into the upstream sensing mechanisms that trigger CH25H induction upon ZIKV infection. Furthermore, we identify STAT1 as a transcription factor that regulates CH25H gene transcription. Additionally, we demonstrate that induction of CH25H is not specific to type I interferons, but can also be mediated by pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6. Finally, we demonstrate a previously unknown role for pro-inflammatory cytokines in controlling lipid metabolism and further support a substantial cross-talk between innate immune signaling and lipid metabolic pathways in macrophages.

## Results

### *ZIKV infection in macrophages induces expression of CH25H*

ZIKV robustly infects macrophages, among other cells (4,21). To begin our investigation, we first examined the ability of ZIKV to induce CH25H expression following infection of primary human macrophages. Indeed, CH25H mRNA levels were elevated in human primary macrophages following ZIKV infection (**Fig 1A**). Next, we extended our findings to THP-1 macrophages (a cell culture model of human monocyte-derived macrophages) and microglial cells (a cell culture model of human central nervous system-resident macrophages). ZIKV infection of these cell-lines, like primary macrophages, significantly induced CH25H expression (**Fig 1B and C**). Notably, a higher level of CH25H induction was detectable in microglial cells following ZIKV infection compared to ZIKV-infected THP-1 macrophages (**Fig 1C**). Having established that MR766 ZIKV strain (Uganda strain) induces CH25H in macrophages, we expanded our findings to THP-1 macrophages using the current circulating ZIKV strain that has caused an epidemic in Brazil (Fortaleza strain, Brazil). Indeed, like the Uganda strain, Fortaleza significantly induced CH25H expression in THP-1 macrophages, as compared to mock (**S1 Fig**). Given that ZIKV is known to trigger pattern recognition receptors (PRRs), in particular TLRs (22), which in turn cause downstream gene induction, we next examined the role of specific toll-like receptors in inducing CH25H expression. To this end, we treated macrophages with a panel of nine TLR ligands. In THP-1 macrophages, CH25H gene expression was induced upon stimulation of TLR3, TLR4, and TLR5 with Polyinosinic-polycytidylic acid (Poly (I:C)), lipopolysaccharide (LPS), and Flagellin ligands respectively (**Fig 1D**), though only Poly (I:C) and LPS robustly increased expression. In microglial cells, by contrast, only TLR3 ligand (Poly I:C) significantly upregulated CH25H (**Fig 1E**).

Next, we asked whether CH25H can be induced in mammalian cells other than macrophages. To assess this, we first measured CH25H transcript levels in human THP-1 monocytes upon inoculation with Zika virus. No significant change was observed (**S2A Fig**). Since CH25H was robustly induced by TLR3/4 ligands in macrophages, we used these ligands to investigate CH25H regulation in THP-1 monocytes. Upon TLR stimulation, no significant changes in CH25H transcription levels were observed compared to mock exposed monocytes (**S2B Fig**). We also measured CH25H expression in two human non-immune cell lines: LX-2 hepatic stellate cells and Huh 7.5.1 hepatocytes (**S2C and S2D Fig**). In contrast to monocytes, treatment of these hepatic cell lines with either Poly (I:C) or LPS significantly induced CH25H mRNA. These results suggest that ZIKV infection or TLR3/4 stimulation can induce

CH25H expression in macrophages and human non-immune cells but not in monocytes.

***ZIKV replication and endosomal compartment are involved on the induction of CH25H transcript***

Having established that ZIKV infection can induce CH25H in THP-1 macrophages and microglial cells, we next sought to gain more insights into this induction. To determine the requirement for viral replication in Zika virus-induced CH25H gene expression, we used heat-inactivation, a well-characterized method of virus neutralization (23). Compared to live virus, heat-inactivated virus robustly induced CH25H in THP-1 macrophages, albeit at lower levels (**Fig 2A, left panel**). By contrast, heated-inactivated virus was not able to induce CH25H compared to live virus in microglial cells (**Fig 2A, right panel**). Due to this observed discrepancy in the dependence of CH25H gene expression on viral replication in the two tested cell-lines, we examined if the viral entry or sensing compartments are similar in both cell-lines. To assess necessity of the endosomal compartment for CH25H induction, we pre-treated cells with a vacuolar type H+-ATPase inhibitor bafilomycin, which prevents endosome acidification. The inhibitory effect of bafilomycin was confirmed by qPCR (**S3A and S3B Fig**). Exposure of THP-1 macrophages and microglial cells to ZIKV in the presence of bafilomycin blunted CH25H induction (**Fig 2B, left and right panel**). Together, these results suggest that though dependence on viral replication is different, the endosomal compartment is necessary for ZIKV- mediated CH25H expression in both cell-lines.

***CH25H expression occurs through TRIF/MyD88-dependent signaling pathway***

Having established that ZIKV-mediated CH25H induction in THP-1 macrophages and microglial cells relies on the endosomal compartment, we investigated a potential role of TLR signaling in CH25H induction. In general, signal transduction events initiated by TLRs occur through either of two adaptor molecules: myeloid differentiation primary response 88 (MyD88) or Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon beta (TRIF) (24–27)**.** To determine the role of MyD88 in ZIKV induction of CH25H, we utilized MyD88-knock out (KO) THP-1 cells from INVIVOGEN. First, we confirmed efficient reduction in MyD88-dependent signaling by measuring gene expression of classical inflammatory cytokines. As expected, LPS-induced TNFα, IL-1β, and IL-6 were significantly reduced in MyD88-KO THP-1 cells (**S4A Fig**). Then, we assessed the expression of CH25H upon TLR stimulation or ZIKV infection. Indeed, LPS-induced CH25H expression was dramatically reduced in MyD88-KO THP-1 cells compared to wild type controls (**Fig 3A**). Likewise, ZIKV was unable to induce CH25H expression in MyD88-KO THP-1 cells compared to that in ZIKV-infected wild-type controls (**Fig 3B**).

To probe the contribution of the TRIF pathway, we treated THP-1 cells with the pharmacologic agent BX795, which attenuates the TRIF pathway by inhibiting TBK1. First, we indirectly confirmed the abrogation of TRIF pathway by measuring the degree of interferon regulatory transcription factor 3 (IRF3) phosphorylation by western blotting; as expected, BX795-treated cells displayed substantially reduced levels of IRF3 phosphorylation in response to LPS stimulation (**S4B Fig**). Then, we assessed whether TRIF signaling impairment reduced cells capacity to express CH25H upon TLR stimulation or ZIKV infection. Indeed, the inhibition of TRIF signaling pathway completely abolished LPS or ZIKV-induced CH25H, compared to wild-type controls (**Fig 3A and B**). Given TLR3 signals solely through the TRIF pathway, we next investigated the role of TLR3 on Zika-mediated CH25H induction. To do so, we treated cells with TLR3 inhibitor, confirming the inhibitory effect by qPCR (**S4C Fig**). Then, we infected macrophages with ZIKV in the presence of TLR3 inhibitor. Indeed, as with general TRIF pathway inhibition, TLR3 inhibition significantly, though not completely, reduced ZIKV-mediated CH25H (**Fig 3C**), suggesting co-operative role of other TLRs residing in the endosome. Although this suggestion is in contrast with **figure 1D&E** which suggests no role for other TLRs inhibiting the endosome, differences on macrophage exposure duration may play a significant role in the differences observed between ZIKV and TLR-agonist exposed macrophages. Altogether, these results suggest a role for TRIF, MyD88 and TLR3 in ZIKV and TLR-mediated CH25H induction in THP-1 macrophages.

***ZIKV induces CH25H expression in Type I IFN-independent manner***

We next examined the role of type I IFN or other inflammatory cytokines in inducing CH25H expression. To this end, we first assessed the production of a subset of pro-inflammatory cytokines and interferons with TLR stimulation and viral exposure. With TLR stimulation, transcript levels of type I (IFNβ) and III (IFNL1) interferons were upregulated, whereas induction of type II (IFNγ) interferons was negligible (**S5A-C Fig**). Interferon lambda 2 and 3 were also not increased with TLR3/4 stimulation (data not shown). IFNα was not significantly increased with either TLR3/4 agonist. Classical pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) were also upregulated with TLR3/4 agonists. Consistent with synthetic TLR ligands, inflammatory cytokines IL-1β, TNFα, and IL-6 were also upregulated upon ZIKV infection in THP-1 macrophages (**Fig 4A-F**), though interferon induction was notably absent. Both inflammatory cytokines and interferons were induced by MR766 in microglial cells.

To test the role of these cytokines on CH25H induction in macrophages, we initially limited cytokine levels by inhibiting translation using cycloheximide (CHX). Cycloheximide treatment significantly inhibited the induction of CH25H in poly (I:C)-stimulated macrophages with less effect on CH25H induction in LPS-stimulated cells (**Fig 5A**). Notably, LPS-mediated IFNβ production was abrogated in CHX treated cells (**Fig 5B**), suggesting that IFNβ is potentially crucial for the induction of CH25H expression. To test this, we examined if the blockade of interferon receptor was enough to abrogate CH25H induction. IFNAR2 neutralization had no significant effect on poly (I:C) or LPS induced CH25H (**Fig 5C**). The inhibitory effect of the neutralizing antibody for IFNAR2 was confirmed by reduction of MX1, a classical interferon stimulated gene with antibody treatment (**Fig 5D**). In addition, we also examined the role of type I interferons themselves on ZIKV-induced CH25H. Macrophages were pre-treated with type I interferon neutralizing antibody cocktail before being inoculated with ZIKV. As shown in **Fig 5E and F**, type I interferon neutralizing antibody cocktail had no effect on ZIKV-induced CH25H transcript but significantly reduced classical ISG, MX1, induction (**S6A Fig**). To further confirm that ZIKV-induced CH25H is not dependent on interferons we performed a supernatant transfer assay. To perform this, culture supernatant from macrophages inoculated with ZIKV were transferred to naïve macrophages. As shown in **S6B Fig**, CH25H was only induced upon inoculation with ZIKV but not with supernatant from treated macrophages, whereas MX1 was induced by both treatments (**S6C Fig**). These results suggest that the induction of CH25H in human macrophages is not dependent on interferons, thus, CH25H is not a classical ISG.

***Induction of CH25H in human macrophages with pro-inflammatory cytokine stimulation***

Given cytokine gene expression was reduced in the absence of MyD88 adaptor, we next hypothesized that if CH25H expression is dependent on pro-inflammatory cytokines its expression should be reduced when treated with depleted supernatant from Myd88 KO macrophages. To test this, we treated either wild-type or MyD88 THP-1 macrophages with either PBS or LPS for 3 hr. At 3 hr post-treatment, we transferred culture supernatants from wild-type THP-1 macrophages to fresh MyD88 KO THP-1 macrophages for 3 hr, with the notion that if cytokines are sufficient this treatment should rescue CH25H to the level of wild-type. LPS-induced CH25H in MyD88 KO THP-1 macrophages was significantly lower than in wild-type, and supernatant transfer from wild-type to MyD88 KO macrophages partially rescued CH25H compared to wild-type (**Fig 6A**), demonstrating that cytokines are necessary for the induction of CH25H, though not sufficient for complete rescue. Indeed, knockout of MyD88 adaptor led to a reduction in pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) compare to wild-type (**S4A Fig**)

We further examined the effect of these inflammatory cytokines on inducing CH25H gene expression in human macrophages by measuring CH25H mRNA level in human macrophages in response to stimulation with human recombinant pro-inflammatory cytokines (IL-1β, TNFα, and IL-6). As shown in **Fig 6B**, CH25H levels in THP-1 macrophages were elevated by recombinant pro-inflammatory cytokine treatment. Taken together these results suggest that inflammatory cytokines such as IL-1β, TNF-α, and IL-6 are involved on CH25H expression in human macrophages.

***Activation of p-STAT1, p-JNK, ATF3 by macrophages following ZIKV infection***

To understand a molecular basis of CH25H induction in human macrophages in response to ZIKV infection or TLR stimulation, we determined a transcription factor responsible for CH25H mRNA expression. Given a significant reduction of CH25H mRNA level by treatment with TBK1 inhibitor (BX795), it is likely that transcription factors affected by BX795 treatment could play a role in inducing CH25H expression. To this end, we attempted to define signaling cascade of CH25H gene transcription using BX795 treatment. We examined the activation of mitogen-activated protein kinase (MAPK), NF-kB, ATF2/c-jun, ATF3, IRF3, and STAT because of their involvement in the inflammatory signaling in response to pathogen associated molecular patterns (PAMP) or cytokine stimulation (28–34).

Comparison of BX795 treated THP-1 macrophages with THP-1 MyD88 knockout and wild type THP-1 macrophages showed that phosphorylation of p38 MAPK, NF-KB-p65, were not reduced in cells treated with BX795 following LPS stimulation but were reduced in MyD88 knockout macrophages. In contrast, there was a significant reduction of ATF3, and reduced phosphorylation of IRF3, SAPK/JNK, and STATs in BX795 treated cells compared to wild type control (**S7 Fig**). These results suggest, ATF3, IRF3, SAPK/JNK, and STAT 1/2 may be involved in transcription of CH25H.

Additionally, ZIKV infection was sufficient to induce activation/phosphorylation of STAT1, ATF3, and SAPK/JNK transcription factors, but could not induce activation/phosphorylation of IRF3 (**Fig 7A**). Similar results are obtained in cells stimulated with pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) (**Fig 7B**). Taken together, ZIKV-mediated CH25H induction likely occurs through one of these transcription factors and/or synergistic effect of several of these transcription factors combined. Thus, it is critical to identify a specific transcription factor involved in the expression of CH25H to target a specific pathway to reprogram macrophages towards boosting their anti-viral activity.

***The JAK/STAT and ATF3 pathways are necessary for the induction of CH25H expression***

Next, we sought to pinpoint a transcriptional pathway involved in induction and expression of CH25H. To address whether the SAPK/JNK pathway is required for the expression of CH25H by TLR stimulation, cytokine stimulation, or viral infection, we examined the effect of JNK inhibition on CH25H expression. The reduction of SAPK/JNK (with SP600125) was indirectly assessed by assessing p-c-jun, downstream of JNK (**S8A Fig**). The specificity of SP600125 was also assessed in relation to other potential transcription pathway targets, indeed, SP600125 was highly specific for the JNK pathway (**S8A Fig**). JNK inhibition significantly decreased the poly (I:C) and LPS-mediated induction of CH25H but had no effect on cytokine-mediated CH25H induction (**S8B and S8D Fig**). Surprisingly, SAPK/JNK inhibitor increased MR766-mediated CH25H compared to vehicle treatment (**S8C Fig**). Since SAPK/JNK inhibitor only effected TLR-mediated CH25H induction, we asked if this effect is due to the reduction of cytokines, as shown in **Fig 5A and 6A**, TLR-mediated CH25H partially relies on cytokines. To test this possibility, THP-1 macrophages were pre-treated with vehicle (DMSO) or SAPK/JNK inhibitor (SP600125) (10 μm) or LPS for 3 hr. At 3 hr post-treatment, culture supernatants were transferred to DMSO or SP600125 (pre-treaded for 1 hr) treated THP-1 macrophages for 3 hr. As shown in **S8E Fig**, LPS supernatants transferred to vehicle or SP600125 pre-treated macrophages induced similar CH25H expression (rescuing deficient CH25H induction in SP600125-treated macrophages), demonstrating that SAPK/JNK inhibition reduces cytokines which contribute to CH25H induction. Altogether this suggests that SAPK/JNK does not play a direct role as a transcription factor in CH25H induction but rather that SAPK/JNK participates in the production of cytokines that indirectly mediate CH25H induction.

To address whether the JAK/STAT pathway is required for the expression of CH25H in response to TLR or cytokine stimulation, or viral infection, we examined the effect of JAK inhibitor I, a highly potent ATP-competitive inhibitor of JAK1, 2, 3 and Tyk2 (arrest STATs phosphorylation), on the induction of CH25H. The inhibitory effect (and its specificity in relation to

potential transcription factors) of JAK inhibitor I was confirmed by western blot (**S9A Fig**). As a positive control, JAK inhibitor 1 significantly decreased transcription of MX1 and MX2 (**S9B and S9C Fig**). Notably, this inhibitor significantly decreased the transcription level of TLR, ZIKV infection and cytokine-induced CH25H expression (**Fig. 8A-C**). These results indicate that STAT proteins are necessary for the expression of CH25H.

Next, we screened ATF3 for a potential role in CH25H regulation by using several commercially available ATF3 inhibitory drugs (data not shown). However, pharmacological inhibitor experiments proved insufficient to define the potential role of ATF3 in CH25H transcriptional regulation in THP-1 macrophages. Nevertheless, to scrutinize ATF3 as a potential CH25H transcription factor, in cooperation with STAT as suggested through STAT inhibitor experiments, we utilized a chromatin immunoprecipitation (ChIP) assay in macrophages treated with LPS to identify the binding of either ATF3 or STAT1 to CH25H promoter. The ChIP-qPCR analysis of CH25H promoter sequences revealed an LPS-mediated recruitment of Stat1 to the binding region of the Ch25H locus (**Fig 8D**). In addition, ChIP-qPCR analysis also revealed the binding of ATF3 to CH25H promoter sequence, but this binding was not statistically significant. Collectively, these experiments show that CH25H transcriptional activation is coupled to the TLR-activation through direct recruitment of STAT1 to the CH25H promoter, potentially supplemented by recruitment of ATF3.

**Discussion**

In this study, we have taken a comprehensive approach to characterize the regulation of CH25H gene expression in human macrophages following ZIKV infection. Consistent with findings in murine models, CH25H is robustly induced by ZIKV infection as well as TLR3/4 stimulation in human macrophages. We have identified an upstream sensing compartment (endosome) and sensing molecule (TLR-3 receptor) involved in ZIKV-induced CH25H expression. Interestingly, we identified differences in dependence of CH25H gene expression on viral replication in two tested cell-lines. In microglial cells, ZIKV replication was required for CH25H induction and thereby dsRNA, formed during viral replication may be necessary for viral sensing to trigger CH25H expression. Similarly, in THP-1 macrophages, although heat-inactivated virus was able to induce CH25H, this induction was significantly lower than that with the live virus which also suggests the importance of viral replication. One potential explanation for differences on viral replication dependency between the two tested cell-lines could be based on the TLR signaling cascade. In microglial cells, only TLR3 was able to induce CH25H expression, suggesting the need for dsRNA formation to induce CH25H expression, whereas in THP-1 macrophages, although TLR3 activation can also induce CH25H expression, its expression was robustly induced with TLR4, suggesting PAMPs in the induction of CH25H expression in this cell type. In addition, inhibition of TLR3 signaling significantly decreased ZIKV-mediated induction of CH25H expression. However, since TLR-3 receptor blockade does not completely abolish ZIKV-mediated CH25H induction, it will be intriguing to investigate the effects of other TLRs which reside in the endosomal compartment. Moreover, similar to TRIF, MyD88 adaptor molecule plays a role in CH25H induction and the endosomal TLRs that rely on this adaptor molecule should be further investigated for a role in CH25H induction.

Surprisingly, CH25H is not a classical ISG in human blood-derived macrophages upon ZIKV infection but rather inflammatory cytokines are involved in CH25H gene transcription. This is the first report demonstrating the role of pro-inflammatory cytokines in inducing CH25H gene expression during ZIKV infection. Our finding on IFN-independent CH25H expression is supported by a previous report of IFN-independent expression of CH25H in human hepatocytes (8). This conclusion is based on the following justifications: 1) no IFNs were induced with ZIKV exposure in THP-1 macrophages (**Fig 4A-C**); 2) IFN and IFN receptor blockade were not sufficient to reduce TLR and ZIKV mediated CH25H; 3) culture supernatants from ZIKV-infected cells were sufficient for the induction of MX1, a classical ISG, but failed to induce CH25H expression. In addition, pro-inflammatory cytokines such as IL-6, 1L-1β, and TNF-α induce the expression of CH25H in THP-1 macrophages.

Antagonists for inhibiting inflammatory cytokines (IL-6, 1L-1β, and TNF-α) have been used in the treatment of inflammatory diseases, autoimmunity and cancer (35). However, our findings on the role of these cytokines in antiviral immunity (CH25H induction) raise questions about how and when to block this cytokine to improve disease outcome and patient well-being, since their blockade will also render patients more susceptible to viral infection. Thus, our study suggests a need to revise the current approaches for the treatment of inflammatory diseases, autoimmunity and cancer with respect to viral infection. Wang and colleagues report that TNF-α induced ISGs through directly transactivating the ISRE motif and NF-κB activation but are independent of the JAK-STAT pathway (31). In contrast to this report, our studies indicate that 1L-1β, TNF-α, and IL-6, induced CH25H was dependent on the JAK-STAT pathway. It remains unclear whether these cytokines are independent or cooperatively work with IFNs in the induction of CH25H in human macrophages.

Previous studies have indicated that CH25H possesses its antiviral properties through production of an antiviral sterol, 25HC (9,36,37). Our study shows that CH25H is induced by mammalian cells, suggesting production of 25HC. Although previous studies have shown antiviral properties of 25HC, it has also been shown to possess detrimental or cytotoxic properties and induce apoptosis in various cell types (38–41). The increased expression of CH25H in mouse macrophages after infection with Listeria monocytogenes promotes survival of infected cells, resulting in enhanced susceptibility of the host to infection (42). Recently, 25HC has been shown to activate an integrated stress response (15). In our study, ectopic expression of CH25H in hepatic cells and HEK293 cell-line induce cell death (data not shown). Given these findings, it is tempting to speculate that during viral infection, 25HC production may reflect a response to contain viral infection, but in case of overwhelming viral infection, it will activate an integrated stress response pathway and induce cell death. All these together underscore the importance of CH25H induction pathway, providing immunotherapeutic strategies to develop anti-viral agents.

IFNAR-JAK-STAT signaling has been shown to be the cardinal pathway through which ISGs are induced, in particular, CH25H (5,7). In mouse model, Park and Scott previously reported that TLR-mediated CH25H expression is type I IFN and STAT1-dependent (7). Moreover, Blanc revealed the binding of STAT1 to CH25H promoter region directly, using a ChIP assay (5) In the present study, in human macrophages, using ChIP assay we further confirmed the binding of STAT1 as well as the weak binding of ATF3 to CH25H promoter region, thereby drives its transcription. Although ATF3 weakly binds to CH25H promoter, it is possible that it might be playing a potential role as a co-transcription factor. Interestingly, mouse studies have identified ATF3 as a negative regulator of the macrophage transcriptional response to inflammatory stimuli (28). In addition, in the absence of ATF3, the levels of CH25H and 25HC are increased (43). These differences further raise questions of how best mouse models of disease has an impact on human diseases and therapeutic approaches. Recently, Sood and colleagues have also identified ATF3 acting as a negative regulator of antiviral response in other mammalian cells (44).Nevertheless, whether there is specific cell type-dependency for the negative regulatory effect of ATF3 still needs to be investigated.

In conclusion, our results have demonstrated for the first time that pro-inflammatory cytokines such as 1L-1β, TNF-α, and IL-6 induce CH25H. Intriguingly, this CH25H induction depends on STAT1 activation but is independent of IFN production. These findings will guide us to better understand the regulation of CH25H in human cells. This study provides further understanding to the connection between innate immunity and sterol metabolism and encourages the exploration of cytokines in antiviral immunity.

## Materials and Methods

### Ethics statement

Our study was performed in accordance with the National Institute of Health guideline for the Care and Use of Laboratory Animals. Procedures for zika infection in human/mouse tissue cell lines and human primary monocyte/macrophages were approved by the Institutional Review Board (IRB-HSR #16147; Flavivirus Immunopathogenesis).

**Viruses, Cells, and Reagents**

Uganda isolate (strain MR766) and Brazil isolate (strain Fortaleza) were obtained from Dr. Michael Gale. THP-1 cells (ATCC) and THP-1 MyD88 KO cells (Invivogen) were cultured in RPMI media (Life technologies) (37°c, 5% CO2), supplemented with 100units/ml penicillin (Life technologies), 100ng/ml streptomycin (Life technologies), 10% Fetal Bovine Serum (FBS) (Atlas biologicals), 2mM L-glutamine (Life technologies), 1x 2-mercaptoethanol (Life technologies), and 1.25g dextrose. Microglial Cells (ATCC) were cultured in median (ATCC) (37°c, 5% CO2), and supplemented with penicillin (Life technologies). Human recombinant IFNβ, TNFα, IL-1β, IFNλ, and IL-6 were purchased from Peprotech, Phorbol 12-myristate 13-acetate (PMA) (Invivognen), RNA Bee (Fisher Scientific), High capacity RNA-to-DNA kit (Life technologies), 2X SYBR green master mix (Applied Biosystems). DMSO.

**Primary Human Cells**

Human monocyte-derived macrophage: peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors (Virginia Blood Services, Richmond, VA) by lymphocyte gradient centrifugation (Cedarlane Laboratories, Burlington, NC). All samples were anonymized for a privacy protection by removing personally identifiable information from data sets. Monocytes were separated from PBMCs via plastic adherence. Monocytes were differentiated into macrophage with 10 ng/ml human M-CSF (Biolegend) in RPMI (GIBCO) media with 10% FBS for 7 days prior to experimental use.

**Stimulation with TLR ligands**

THP-1 cells were stimulated for 3 days with 100 ng/ml PMA for differentiation into THP-1 macrophages, and rested for a day with PMA negative media before any treatment. RNA (using RNA bee) or Proteins were then collected for qPCR or Western analysis, respectively. THP-1 macrophages were treated with a panel of TLR agonists (Invivogen), including 1 μg/ml Pam3CSK4 (TLR1/2 agonist), 1 × 108/ml HKLM (TLR2 agonist), 10 μg/ml poly I:C (TLR3 agonist) High molecular weight, 1 μg/ml flagellin from Salmonella typhimurium (TLR5 agonist), 1 μg/ml FSL-1 (TLR6/2 agonist), 1 μg/ml ssRNA40 (TLR7 agonist), and 2.5 μM CpG ODN1826 (TLR9 agonist). 100 ng/ml of LPS (TLR4 agonist) from Escherichia coli 0111:B4 (Sigma-Aldrich). After treatment cells were washed twice with ice cold dulbecco's phosphate buffered saline DPBS (Life technologies). Cells were then lysed and total RNA (using RNA Bee) were extracted and then stored at -80$^{o}$c until use.

RNA Isolation, cDNA synthesis and Real-time Quantitative PCR: Macrophages were washed twice with ice cold dulbecco's phosphate buffered saline DPBS (Life technologies). RNA was extracted using RNA Bee following the manufacture's instruction. RNA concentrations were measured by a nano-drop 2000 spectrophotometer (Thermo Scientific). 1 μg/ml of the isolated total RNA was used as a template for cDNA synthesis using High capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR was performed on a StepOnePlus system (Applied Biosystems). Primers in table 1 were used for target gene quantification using SYBR green master mix (Applied Biosystems). Target genes expression were determined using comparative cycle threshold (ΔΔCT) technique and results normalized to HPRT or B2M.

**Western blots**

Total cell lysates were harvested with RIPA buffer and, 1x Halt protease inhibitor cocktail (Thermo Scientific). Protein concentrations were determined using Pierce BCA protein assay kit. Cells lysates containing 50ug of protein were reduced and denatured with sample buffer. Protein lysates were further denatured by boiling for 5 min, and ran on 4-15 % SDS-PAGE for 1-2 hours at 100v. Protein were them transferred to polyvinylidene difluoride membrane and blocked with 5% BSA in TBST buffer. The membrane was Probed with specific antibodies. The membrane-bound Abs were visualized with horseradish peroxidase–conjugated Ab to rabbit IgG or mouse IgG, and developed with ECL (Santa Cruz Biotechnology). SAPK/JNK (#9252), p38 MAPK (#8690), p44/42 MAPK (Erk1/2) (#4695), Phospho-SAPK/JNK (#4668), Phospho-p38 MAPK (#4511), Phospho-c-Jun (#3270), c-Jun (#9165), IRF-3 (#11904), Phospho-IRF-3 (#29047), Stat1 (#9172), Phospho-Stat1 (#9167),

NF-κB p65 (#8242), Phospho-NF-κB p65 (#3033) were purchased from cell signaling.

**Chromatin Immuno-precipitation**

The ChIP assay was performed using an EpiQuik chromatinimmunoprecipitation kit according to the manufacturer's instruction. Briefly, THP1 cells differentiated with PMA into macrophages were treated with LPS for desired time point. After treatment cells were washed twice with PBS and were fixed with 1% formaldehyde. Protein-DNA complexes were immunoprecipitated with STAT1 or ATF3 antibody (cell signaling technology). A negative control antibody was used with normal mouse IgG or HA tagged. DNA from these samples was subjected to PCR analyses with CH25H promoter-specific primers. An input control was used with amplification of soluble chromatin prior to immunoprecipitation.

**Virus Experiments**

Macrophages were infected with ZIKV a multiplicity of infection of 1 pfu/cell. RNA analysis and protein translation analysis were done as described above.

**Acknowledgement**

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**Competing interests**

The authors declare that they have no competing interests

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**Figures**

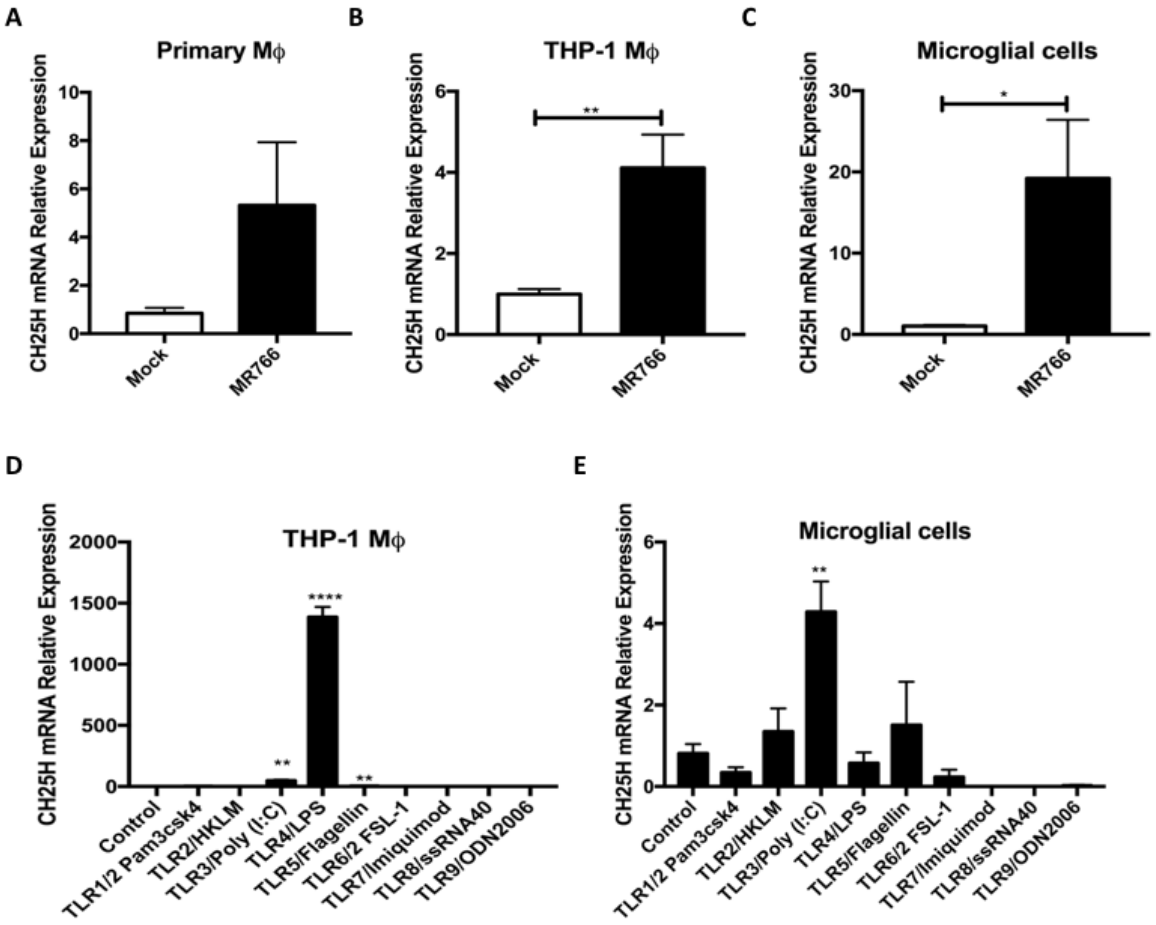


**Fig 1. Expression of CH25H with ZIKV exposure and TLR stimulation in human primary, monocytes-derived, and resident macrophages**. (**A-C**) Primary, THP-1 macrophages, and microglial cells were inoculated with ZIKV (MR766, 1 MOI) for 24 hr, CH25H mRNA expression was measured by qPCR. (**D and E**) THP-1 macrophages and microglial cells were stimulated with a control (media only) and a panel of TLR agonist (TLR1-9) for 3 hr, CH25H mRNA expression was measured by qPCR. HPRT was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples. $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

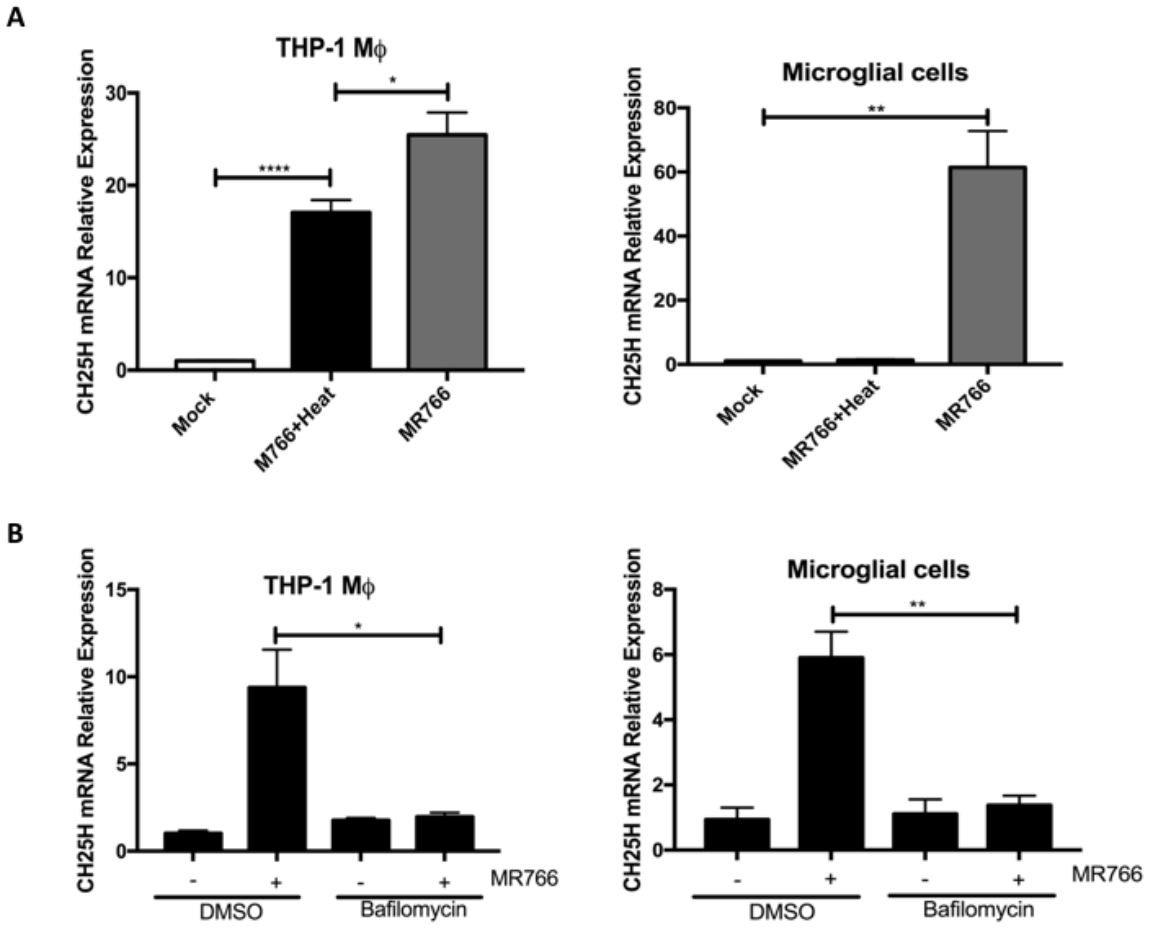


**Fig 2. Viral replication and the endosomal compartment are important for ZIKV-mediated CH25H**. (**A**) THP-1 macrophages and microglial cells were exposed to either mock (uninfected media), or heat-inactivated, or live ZIKV (MR766, 1 MOI) for 24 hr. Cells we then lysed, harvested, RNA isolated and subjected to qPCR to determine CH25H expression. (**B**) THP-1 macrophages and microglial cells were pretreated with DMSO or bafilomycin $A_1$ (5μM) for 1 hr, and then exposed to ZIKV (MR766, 1 MOI) for 24 hr, in the presence of continued drug treatment. RNA was then isolated and CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples. $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

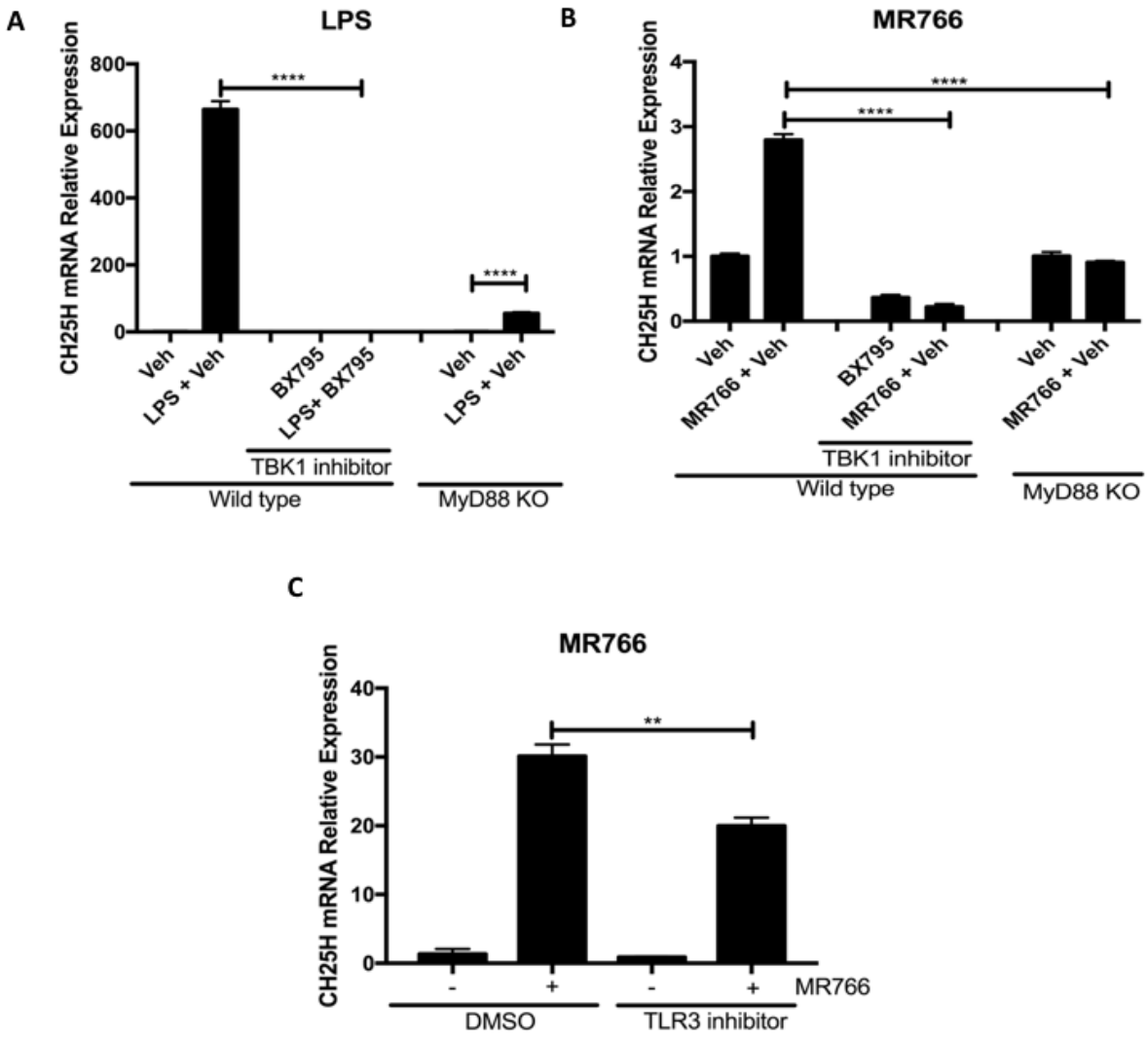


**Fig 3. CH25H expression occurs through TLR3, TRIF and MyD88 signaling pathways**. (**A** and **B**) Wild-type THP-1 macrophages were pre-treated with either Veh (DMSO) or BX795 (10 μM) for 1 hr. in parallel, MyD88 KO THP-1 macrophages were also pre-treated with DMSO for 1 hr. Thereafter, both cell types were treated with (**A**) LPS (100 ng/ml) for 3 hr, (**B**) or with MR766 (1 MOI) for 24 hr, in the presence of continued drug treatment. RNA was then isolated CH25H mRNA expression was measured by qPCR. (**C**) THP-1 macrophages were pretreated with DMSO or TLR3 inhibitor (2.5 μM) for 1 hr, and then exposed to ZIKV (MR766, 1 MOI) for 24 hr. At 24 hr post-infection cells were collected for CH25H analysis by qPCR. HPRT was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples. $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

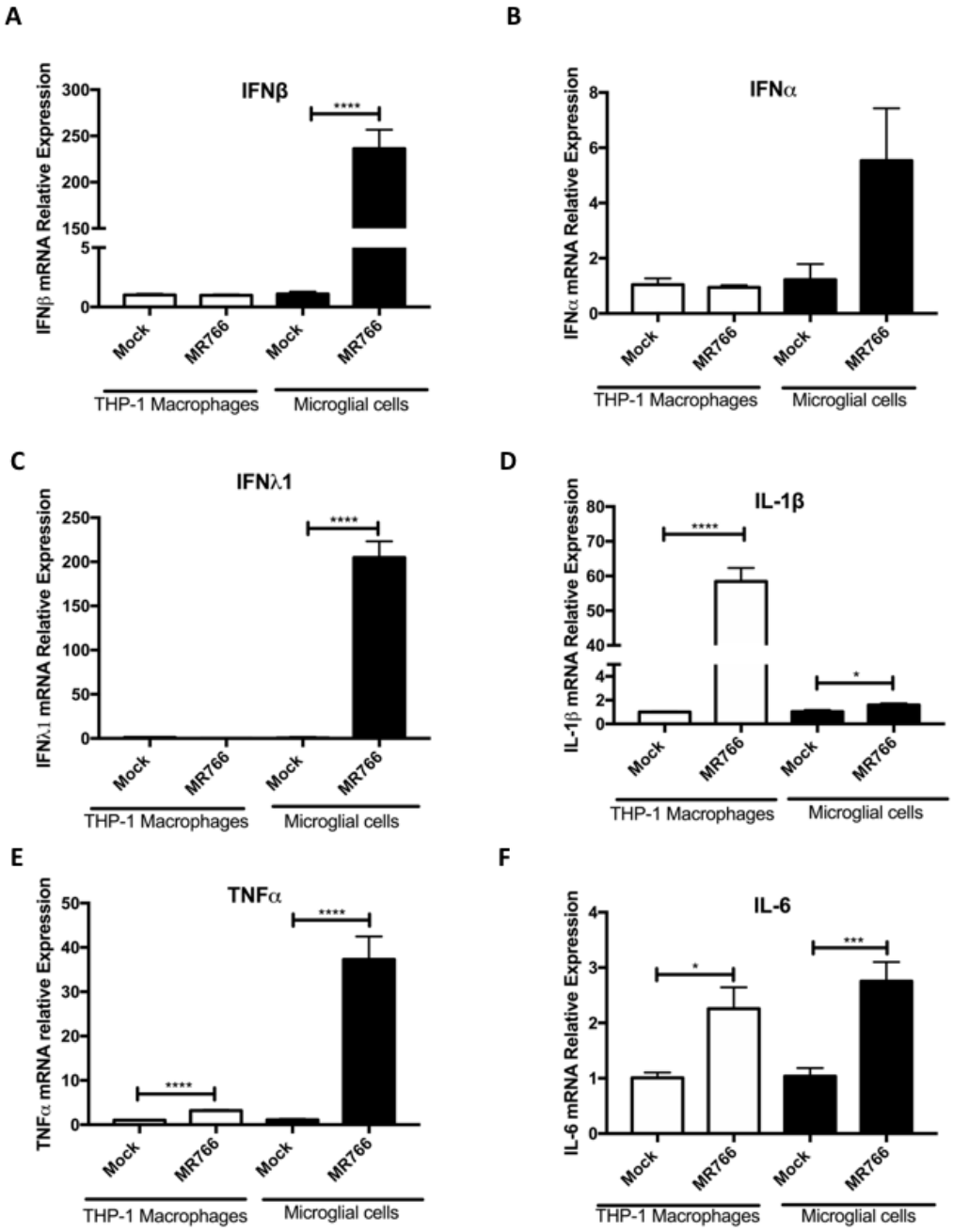


**Fig 4. Cytokine production with ZIKV exposure.** (**A-F**) THP-1 macrophages and microglial cells were inoculated with ZIKV (MR766, 1 MOI) for 24 hr. At 24 hr post-infected cells were collected for selected pro-inflammatory cytokines and interferons analysis by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples (two independent experiments). $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

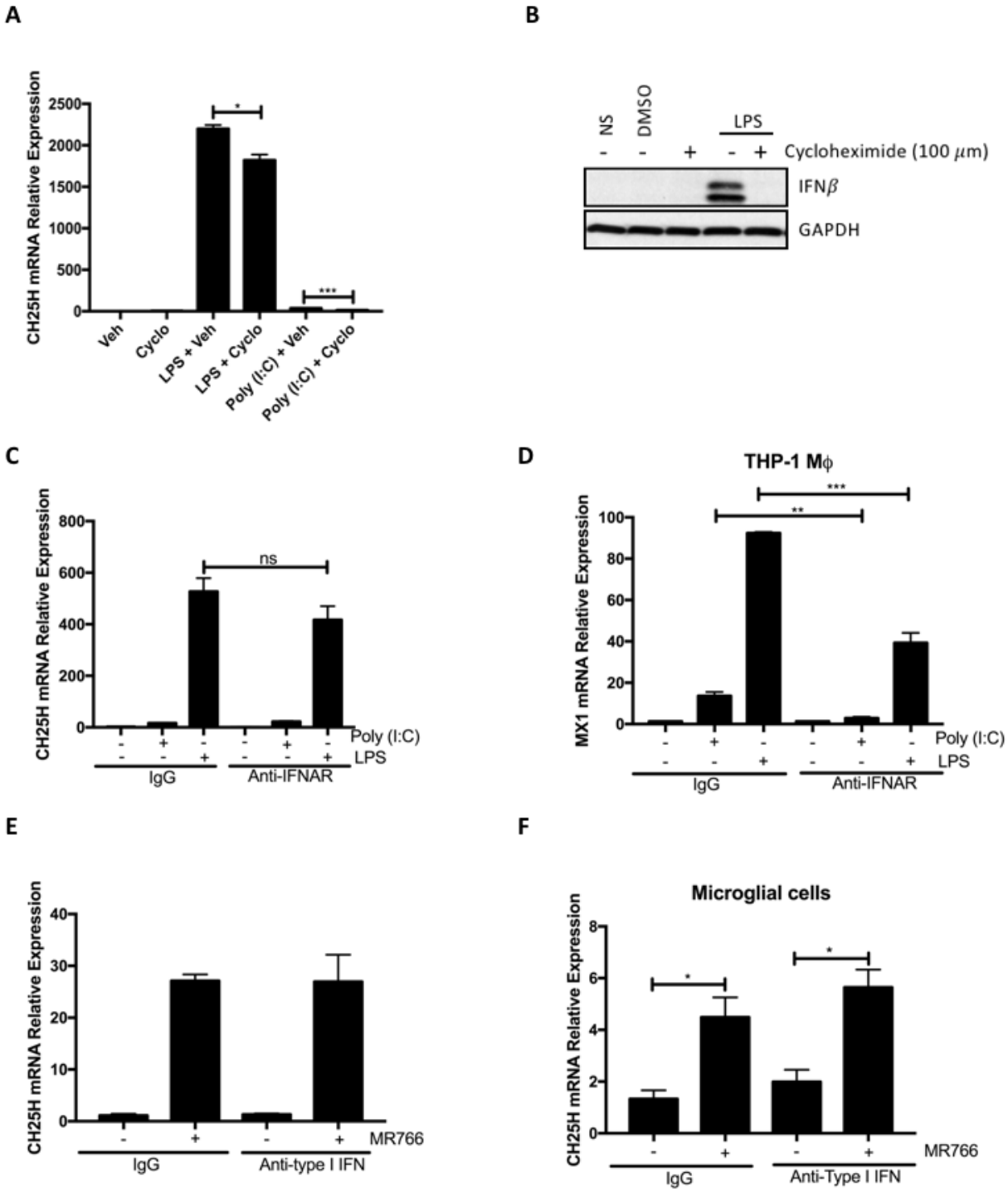


**Fig 5. Type I interferons are not required for TLR and ZIKV-mediated CH25H induction**. (**A**) THP-1 macrophages were pre-treated with veh (DMSO) or cycloheximide (100 μM) for 1 hr, and then treated with poly (I:C) (10 μM) or LPS (100 ng/ml) for 3 hr, in the presence of continued drug treatment. Cells were collected for CH25H qPCR analyses at 3 hr post-treated. (**B**) THP-1 macrophages were pre-treated with media, or DMSO or cycloheximide (100 μM) for 1 hr, and then treated with LPS for 3 hr, in the presence of continued drug treatment. Expression of IFNβ was examined by western blot, GAPDH was used as a loading control. (**C-E**) THP-1 macrophages were first pre-treated with isotype antibody (IgG) or

(**C and D**) Interferon receptor subunit 2 (IFNAR2) neutralizing antibody or (**E**) type I interferons neutralizing antibody cocktail for 1 hr. Thereafter, cells were treated with (**C and D**) poly (I:C) or LPS for 3 hr or (**E**) inoculated with ZIKV (MR766, 1 MOI) for 24 hr, in the presence of continued antibody treatment. CH25H or MX1 mRNA expression was measured by qPCR. (**F**) Microglial cells were first pre-treated with isotype antibody (IgG) or type I interferons neutralizing antibody cocktail for 1 hr. Thereafter, cells were inoculated with ZIKV (MR766, 1 MOI) for 24 hr, in the presence of continued antibody treatment. CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples. $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

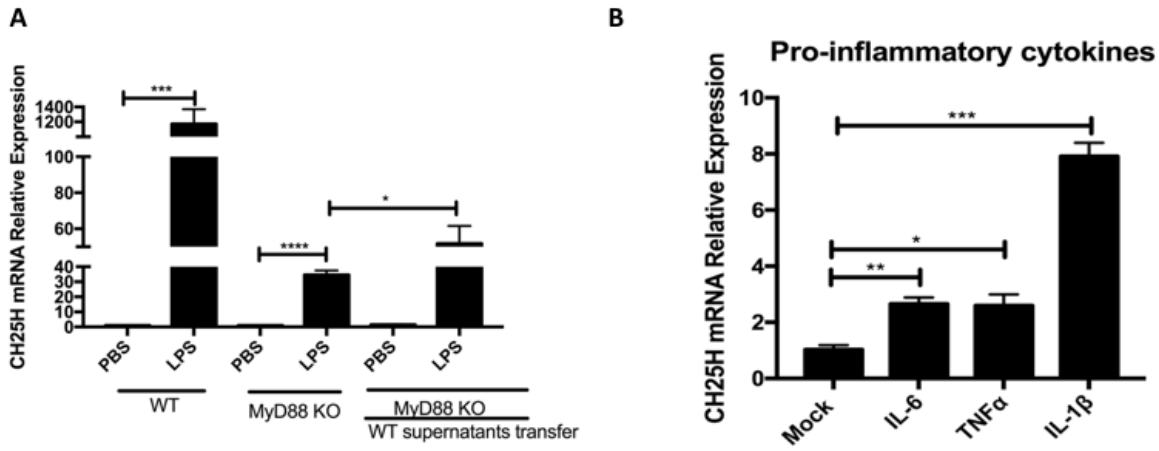


**Fig 6. Induction of CH25H in human macrophages with pro-inflammatory cytokine stimulation.** (**A**) Wild-type and MyD88 KO THP-1 macrophages were treated with either PBS or LPS for 3 hr. At 3 hr post-treatment, culture supernatants from wild-type THP-1 macrophages were transferred to fresh MyD88 KO THP-1 macrophages for 3 hr. Both the actual treatment and supernatant treatment were analyzed for CH25H mRNA expression by qPCR. (**B**) THP-1 macrophages were treated with media (mock) or selected pro-inflammatory cytokines for 3 hr, CH25H mRNA expression was measured by qPCR. HPRT or $\beta_2$M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± SEM from triplicate samples. $^{*}p < 0.0332$, $^{**}p < 0.0021$, and $^{***}p < 0.0002$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test).

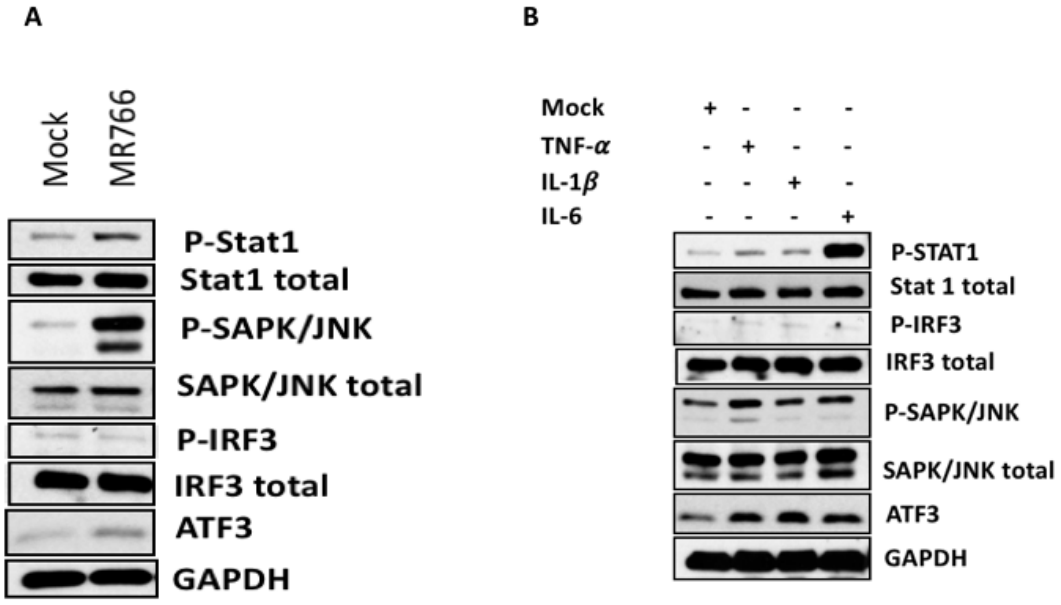


**Fig 7. Potential CH25H transcription factor(s) induced by viral infection and cytokine stimulation.** (**A and B**) THP-1 macrophages inoculated with (A) ZIKV (MR766, 1 MOI) for 24 hr, or (**B**) treated with pro-inflammatory cytokines for 3 hr were analyzed for potential transcription factors induction, and GAPDH protein by Western blotting.

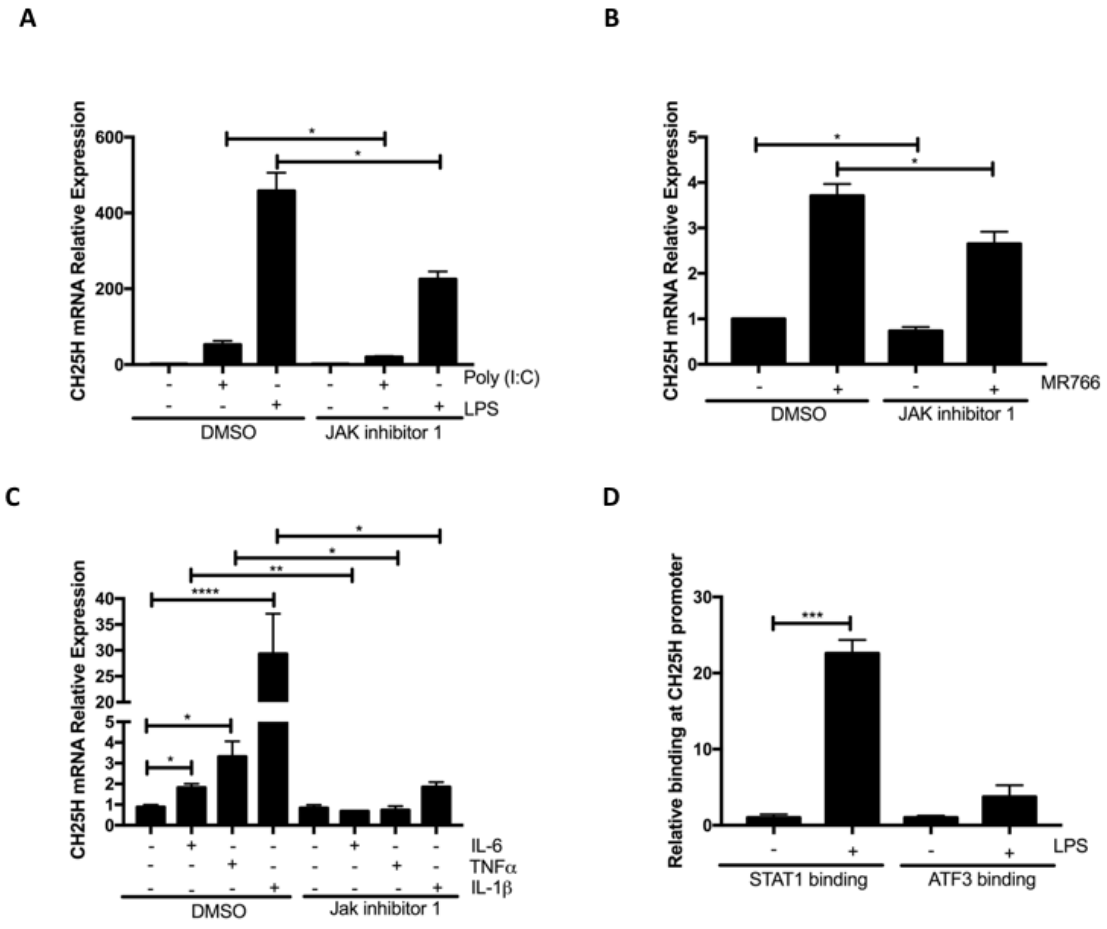


**Fig 8. STAT1 regulates CH25H expression in human macrophages.** (**A-C**) THP-1 macrophages were pre-treated with vehicle (DMSO) or JAK inhibitor 1 (1 μM) for 1 hr, and then treated with (**A**) poly (I:C) (10 μM) or LPS (100 ng/ml) for 3 hr or with (**B**) ZIKV (MR766, 1 MOI) for 24 hr, or (**C**) with pro-inflammatory cytokines for 3hr, in the presence of continued drug treatment. CH25H mRNA expression was measured by qPCR. (**D**) THP-1 macrophages were treated with media or LPS for 3 hr, and CHIP analysis for STAT1 and ATF3 binding was performed at CH25H promoter.