Zika virus (ZIKV) is an arthropod-borne virus, transmitted by Aedes mosquitoes, which belong to the Flaviviridae family and the Flavivirus genus. Recently, ZIKV has emerged as a public health threat because of its global transmission and link to severe congenital disorders, such as microcephaly, Guillain–Barré syndrome, and meningoencephalitis (1–3). ZIKV infection in placental macrophages has been reported to transmit the virus from mother to fetus (4). The 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 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, an enzyme that converts cholesterol to 25HC (5–7). Following stimulation of Toll-like receptors (TLRs) and 4, common sensors of viral and bacterial infection, CH25H is highly induced in mouse macrophages and dendritic cells. Moreover, its induction depends on interferon α receptor (IFNAR) interactions (7). Importantly, 25HC exerts antiviral activity against both enveloped and nonenveloped viruses in vitro (5, 6, 8–10), specifically by suppressing proteolytic activation of sterol regulatory element–binding proteins and acting as a ligand for liver X receptor (11–14). In these ways, 25HC promotes the induction of genes involved in cholesterol efflux, 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 both liver X receptors and sterol regulatory element–binding proteins (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). However, the details of this connection appear to differ in mouse and human cells. In murine models, type I IFN induces CH25H, linking these two biological
processes (7, 19, 20). On the other hand, in human hepatoma cell lines, CH25H does not appear to be a classic 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 induction of CH25H in humans are poorly understood. Achieving such an understanding is critical to design therapeutic agents to control pathogens.

In this study, using in vitro cell culture models of human 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, TNFα, and IL-6. Finally, we demonstrate a previously unknown role of pro-inflammatory cytokines in controlling lipid metabolism and further support 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 with ZIKV-infected THP-1 macrophages (Fig. 1C).

Given that ZIKV is known to trigger pattern recognition receptors, 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 using poly(I:C), lipopolysaccharide (LPS), and flagellin ligands, respectively (Fig. 1D), but only LPS robustly increased expression. In microglial cells, by contrast, only TLR3 ligand (poly(I:C)) significantly up-regulated CH25H (Fig. 1E).

Next we asked whether CH25H can be induced in mammalian cells other than macrophages. To assess this, we first mea-
sured CH25H transcript levels in human THP-1 monocytes upon inoculation with Zika virus. No significant change was observed (Fig. S2A). Because CH25H was 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 with mock-exposed monocytes (Fig. S2B).

We also measured CH25H expression in two human nonimmune cell lines: LX-2 hepatic stellate cells and Huh 7.5.1 hepatocytes (Fig. S2, C and D). 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 nonimmune cells but not in monocytes.

The endosomal compartment is involved in 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 with 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 with live virus in microglial cells (Fig. 2A, right panel). Because of this discrepancy in the dependence of CH25H gene expression on viral replication in the two tested cell lines, we examined whether the viral entry or sensing compartments are similar in both cell lines. To assess the necessity of the endosomal compartment for CH25H induction, we pretreated cells with a vacuolar type H+/H1001-ATPase inhibitor, bafilomycin, which prevents endosome acidification. The inhibitory effect of bafilomycin on ISG induction was first confirmed by qPCR (Fig. S3, A and B). We next added bafilomycin to ZIKV-infected THP-1 macrophages and microglial cells, resulting in blunted CH25H induction (Fig. 2B). Together, these results suggest that, although dependence on viral replication is different between THP-1 macrophages and microglial cells, the endosomal compartment is necessary for ZIKV-mediated CH25H expression in both cell lines.

CH25H expression occurs through the 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 β (TRIF) (24–27). To
Role of inflammatory cytokines in CH25H induction

To determine the role of MyD88 in ZIKV induction of CH25H, we utilized MyD88 KO THP-1 cells. 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 (Fig. S4A). 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 with WT controls (Fig. 3A). Likewise, ZIKV was unable to induce CH25H expression in MyD88-KO THP-1 cells compared with ZIKV-infected WT controls (Fig. 3B).

To determine the contribution of TBK1 activity, we treated THP-1 cells with the pharmacologic agent BX795, an inhibitor of TBK1 and other IκB kinase-related kinases, resulting in inhibition of IRF3 activation. First we examined the effect of BX795 on the phosphorylation of interferon regulatory transcription factor 3 (IRF3) by Western blotting. BX795-treated cells displayed substantially reduced levels of IRF3 phosphorylation in response to LPS stimulation (Fig. S4B). Then we assessed the expression of CH25H in BX795-treated cells following TLR stimulation or ZIKV infection. Treatment of BX795 completely abolished LPS- or ZIKV-induced CH25H compared with WT controls (Fig. 3A and B). However, there was no significant difference in MR766-mediated CH25H induction between WT and TRIF knockdown THP-1 cells generated by the CRISPR-Cas 9 system (data not shown).

We next investigated the role of TLR3 on Zika-mediated CH25H induction. After confirming the inhibitory effect of the TLR3 inhibitor on ISG induction (Fig. S4C), we infected macrophages with ZIKV in the presence of the TLR3 inhibitor. TLR3 inhibition partially reduced ZIKV-mediated CH25H (Fig. 3C), suggesting a cooperative role of other TLRs residing in the endosome. Altogether, these results suggest a role of MyD88 and TLR3 in ZIKV- and TLR-mediated CH25H induction in THP-1 macrophages.

ZIKV infection in human macrophages induces CH25H expression in a 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 IFNs with TLR stimulation and viral exposure. With TLR stimulation, transcript levels of type I (IFNα) and III (IFNλ) induction was negligible (S5, A–C). In addition, gene expression of pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) was also up-regulated with TLR3/4 agonists. Consistent with results from TLR agonist treatment, the inflammatory cytokines IL-1β, TNFα, and IL-6 were also up-regulated upon

Figure 3. CH25H expression occurs through TRIF and MyD88 signaling pathways. A and B, WT THP-1 macrophages were pretreated with either vehicle (Veh, DMSO) or BX795 (10 μM) for 1 h. In parallel, MyD88 KO THP-1 macrophages were also pretreated with DMSO for 1 h. Thereafter, both cell types were treated with LPS (A, 100 ng/ml) for 3 h or with MR766 (B, 1 m.o.i.) for 24 h. RNA was then isolated, and CH25H mRNA expression was measured by qPCR. C, THP-1 macrophages were pretreated with DMSO or TLR3 inhibitor (2.5 μM) for 1 h and then exposed to ZIKV (MR766, 1 m.o.i.) for 24 h. 24 h 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 ± S.E. from triplicate samples. **, p < 0.0021; ****, p < 0.0001 (two-tailed unpaired Student’s t test).
ZIKV infection in THP-1 macrophages without notable IFN gene induction (Fig. 4, D–F). However, transcription of both inflammatory cytokines and IFNs was induced by MR766 in microglial cells (Fig. 4, A–F). Secretion of these inflammatory cytokines from THP-1 macrophages (Fig. 4, G–I) and microglial cells (Fig. 4, J–L) was determined by ELISA.

To test the role of these cytokines in 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 (Fig. 5A). Notably, LPS-mediated IFNβ

**Figure 4.** Cytokine production with ZIKV exposure. A–F, THP-1 macrophages and microglial cells were inoculated with ZIKV (MR766, 1 m.o.i.) for 24 h. 24 h post-infection, cells were collected for selected pro-inflammatory cytokine and IFNs analysis by qPCR. HPRT or 2M was used as an endogenous control to normalize the target mRNA. The results are shown as means ± S.E. from data pooled from two independent experiments with triplicate experiments. *, p < 0.0332; **, p < 0.0021; ***, p < 0.0002; ****, p < 0.0001 (two-tailed unpaired Student’s t test). NS, not significant; Mφ, macrophages.

**Figure 5.** Type I IFNs are not required for TLR- and ZIKV-mediated CH25H induction. A and B, THP-1 macrophages were pretreated with vehicle (DMSO) or cycloheximide (100 μM) for 1 h and then treated with poly(I:C) (10 μM) or LPS (100 ng/ml) for 3 h. RNA was collected for CH25H expression for qPCR (A). LPS-treated cells were used for Western blot analysis of IFNβ protein with GAPDH as a loading control (B). MW, molecular weight. C–E, THP-1 macrophages were first pretreated with isotype antibody (IgG), IFN receptor subunit 2 (IFNAR2)–neutralizing antibody (C and D), or type I IFN–neutralizing antibody mixture (E) for 1 h, followed by treatment with poly (I:C) (C and D) or LPS for 3 h or inoculated with ZIKV (E, MR766, 1 m.o.i.) for 24 h. CH25H mRNA expression was measured by qPCR. HPRT or β, M was used as an endogenous control to normalize the target mRNA, and the results are shown as means ± S.E. from triplicate samples. *, p < 0.0332; **, p < 0.0021; ***, p < 0.0002 (two-tailed unpaired Student’s t test). NS, not significant; Mφ, macrophages.
production was abrogated in cycloheximide-treated cells (Fig. 5B), suggesting that IFNβ is potentially crucial for the induction of CH25H expression. To test this, we examined whether blockade of the IFN 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 classic ISG, with antibody treatment (Fig. 5D). In addition, we also examined the role of type I IFN on ZIKV-induced CH25H by pretreating macrophages with a type I IFN–neutralizing antibody mixture prior to ZIKV infection. The type I IFN–neutralizing antibody mixture had no effect on ZIKV-induced CH25H transcription (Fig. 5, E and F) but significantly reduced MX1 induction (Fig. S6A). To further confirm that ZIKV-induced CH25H is not dependent on IFN, we performed a supernatant transfer assay by addition of culture supernatant from ZIKV-infected macrophages to naïve macrophages. CH25H was only induced on macrophages upon ZIKV infection but not with addition of supernatant from infected macrophages (Fig. S6B), whereas MX1 was induced by both treatments (Fig. S6C). These results suggest that the induction of CH25H in human macrophages is not dependent on IFN and that CH25H may not be a classic ISG in human macrophages.

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

Because there is reduced gene expression of CH25H (Fig. 3A) and pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) (Fig. S4A) in MyD88 KO macrophages compared with the WT upon TLR stimulation, it is possible that pro-inflammatory cytokines may be responsible for CH25H induction. To test this, we examined the ability of supernatant from LPS-stimulated WT cells to increase CH25H induction in MyD88 KO cells. Notably, the level of CH25H mRNA was decreased in LPS-stimulated MyD88 KO THP-1 macrophages compared with the WT, and supernatant transfer from WT to MyD88 KO macrophages partially rescued CH25H expression (Fig. 6A). These results suggest that cytokines are partly necessary for induction of CH25H.

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

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

To understand the molecular basis of CH25H induction in human macrophages in response to ZIKV infection or TLR stimulation, we determined the transcription factor responsible for CH25H mRNA expression. Given a significant reduction in CH25H mRNA levels by treatment with a 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 the signaling cascade of CH25H gene transcription using BX795 treatment. We examined activation of mitogen-activated protein kinase (MAPK), NF-κB, ATF2/c-jun, ATF3, IRF3, and STAT because of their involvement in inflammatory signaling in response to pathogen-associated molecular patterns or cytokine stimulation (28–34).

Comparison of BX795-treated THP-1 macrophages with MyD88-KO and WT THP-1 macrophages showed that phosphorylation of p38 MAPK and NF-κB–p65 was not reduced in cells treated with BX795 following LPS stimulation but reduced in MyD88-KO macrophages. In contrast, there was a significant reduction in ATF3 and reduced phosphorylation of IRF3, SAPK/JNK, and STATs in BX795-treated cells compared with the WT control (Fig. S7). These results suggest that ATF3, IRF3, SAPK/JNK, and STAT1/2 may be involved in transcription of CH25H.

Additionally, ZIKV infection was sufficient to induce activation/phosphorylation of the STAT1, ATF3, and SAPK/JNK transcription factors but could not induce activation/phos-
Role of inflammatory cytokines in CH25H induction

Figure 7. Potential CH25H transcription factor(s) induced by viral infection and cytokine stimulation. A and B, THP-1 macrophages (A) or PBMC monocyte derived macrophages (B) were inoculated with ZIKV (MR766, 1 m.o.i.) for 24 h. MW, molecular weight. C, THP-1 macrophages were treated with pro-inflammatory cytokines for 3 h. Cell lysates were analyzed for potential transcription factor induction and GAPDH protein by Western blotting.

phorylation of IRF3 (Fig. 7A). Activation of STAT1 and ATF3 was also detectable in human primary monocyte-derived macrophages following MR766 infection (Fig. 7B). Similar results were obtained in cells stimulated with pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) (Fig. 7C). 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 toward boosting their anti-viral activity.

STAT1 plays a role as a key transcription factor for CH25H induction

Next we sought to define specific transcription factor(s) involved in CH25H induction. We first examined involvement of the SAPK/JNK pathway in CH25H induction by examining the effect of JNK inhibition on CH25H expression. Inhibition of SAPK/JNK activity by SP600125 was measured by assessing p-c-jun downstream of JNK along with a potential off-target effect on other transcription factors (IRF3, STAT, and Activating of transcription factors). SP600125 was highly specific in affecting the JNK pathway (Fig. S8A). JNK inhibition significantly decreased poly(I:C) and LPS-mediated induction of CH25H (Fig. S8B) but had no effect on cytokine-mediated CH25H induction (Fig. S8D). Surprisingly, the SAPK/JNK inhibitor increased MR766-mediated CH25H compared with vehicle treatment (Fig. S8C). Because the SAPK/JNK inhibitor only affected TLR-mediated CH25H induction, we asked whether this effect is due to the reduction of TLR-mediated CH25H partially relying on cytokines. To test this possibility, THP-1 macrophages were pretreated with vehicle (DMSO), the SAPK/JNK inhibitor (SP600125), or LPS. 3 h post-treatment, culture supernatants were transferred to DMSO- or SP600125-treated (pretreated for 1 h) THP-1 macrophages. As shown in Fig. S8E, this induced similar CH25H expression, demonstrating that SAPK/JNK inhibition reduces cytokines that 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, and 3 and Tyk2 (it arrests STAT phosphorylation), on induction of CH25H. The inhibitory effect (and its specificity in relation to potential transcription factors) of JAK inhibitor I was confirmed by Western blotting (Fig. S9A). As a positive control, JAK inhibitor I significantly decreased transcription of MX1 and MX2 (Fig. S9, B and C). Notably, this inhibitor significantly decreased the transcription levels of TLR, ZIKV infection, and cytokine-induced CH25H expression (Fig. 8, A–C). These results indicate that STAT proteins are necessary for 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 by STAT inhibitor experiments, we utilized a ChIP assay in macrophages treated with LPS to identify the binding of either ATF3 or STAT1 to the CH25H promoter. The ChIP–qPCR analysis of CH25H promoter sequences revealed LPS-mediated recruitment of Stat1 to the binding region of the CH25H locus (Fig. 8D). In addition, ChIP–qPCR analysis also revealed binding of ATF3 to the CH25H promoter sequence, but this binding was not statistically significant. Collectively, these experiments show that CH25H transcriptional activation is coupled to TLR activation through direct recruitment of STAT1 to the CH25H promoter, potentially supplemented by recruitment of ATF3.
Discussion

In this study, we took 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 identified an upstream sensing compartment (endosome) involved in ZIKV-induced CH25H expression. We described differences in the dependence of CH25H gene expression on viral replication in two tested cell lines. In microglial cells, intact ZIKV was required for CH25H induction; therefore, dsRNA formed during viral replication may be necessary to trigger CH25H expression. In THP-1 macrophages, heat-inactivated virus was able to induce CH25H. However, this induction was significantly lower than that caused by live virus, affirming the importance of intact viral particles. One potential explanation for differences in viral replication dependence 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; therefore, dsRNA formed during viral replication may be necessary to trigger CH25H expression. In THP-1 macrophages, heat-inactivated virus was able to induce CH25H. However, this induction was significantly lower than that caused by live virus, affirming the importance of intact viral particles. One potential explanation for differences in viral replication dependence 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; therefore, dsRNA formed during viral replication may be necessary to trigger CH25H expression. In contrast, for THP-1 macrophages, TLR3 activation can also induce CH25H expression, but its expression was robustly induced with TLR4, suggesting a role for pathogen-associated molecular patterns in induction of CH25H expression in this cell type. Furthermore, others have shown that Zika virus does not replicate in THP1 cells (46). In addition, inhibition of TLR3 signaling in THP1 cells decreased ZIKV-mediated induction of CH25H expression but did not completely abolish ZIKV-mediated CH25H induction. Taken together, our data from two different cell lines and the TLR3 inhibitor suggest signal sensing other than TLR3 may also be involved. Thus, further studies need to be done to identify other pattern recognition receptors involved in Zika-mediated CH25H induction.

Surprisingly, CH25H does not function as a classic ISG in human macrophages upon ZIKV infection; rather, inflammatory cytokines are involved in CH25H gene transcription. This is the first report demonstrating a role of pro-inflammatory cytokines in inducing CH25H gene expression during ZIKV infection. Our finding of 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: no IFNs were induced with ZIKV-infected THP-1 macrophages (Fig. 4, A–C); IFN and IFN receptor blockade were not sufficient to reduce TLR- and ZIKV-mediated CH25H (Fig. 5, C, E, and F); and culture supernatants from ZIKV-infected cells were sufficient for induction of MX1, a classic ISG, but failed to induce CH25H expression (Fig. S6). Recently, Zika virus NS5 protein has been reported to antagonize type I IFN production (45). In addition, pro-inflammatory cytokines such as IL-6, IL-1β, and TNFα induce expression of CH25H in THP-1 macrophages.

Antagonists for inhibiting inflammatory cytokines (IL-6, IL-1β, and TNFα) have been used in the treatment of inflammatory diseases, autoimmunity, and cancer (35). However, our findings regarding the role of these cytokines in antiviral immunity (CH25H induction) raise questions about how and when to block this cytokine to use these treatments to improve disease...
Role of inflammatory cytokines in CH25H induction

outcome and patient well-being because cytokine blockade will also render patients more susceptible to viral infection. Thus, our study suggests a need to revise the current approaches for treatment of inflammatory diseases, autoimmunity, and cancer with respect to viral infection. Wang et al. (31) reported that TNFα induced ISGs by directly transactivating the interferon-sensitive response element motif and NF-κB activation but is independent of the JAK–STAT pathway. In contrast to this report, our study indicates that IL-1β, TNFα, and IL-6 – induced CH25H depends on the JAK–STAT pathway. It remains unclear whether these cytokines are independent or work cooperatively with IFNs in induction of CH25H in human macrophages.

Previous studies have indicated that CH25H exerts 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 cytotoxic properties and induce apoptosis in various cell types (38–41). For example, 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 the HEK293 cell line induced 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 activates an integrated stress response pathway and induces cell death. Taken together, these findings highlight the importance of the 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 a mouse model, Park and Scott (7) reported previously that TLR-mediated CH25H expression is type I IFN- and STAT1-dependent. Moreover, Blanc (5) revealed the binding of STAT1 to CH25H promoter region directly using a ChIP assay. In this study, using human macrophages and a ChIP assay, we further confirmed that binding of STAT1 as well as weak binding of ATF3 to the CH25H promoter region drives its transcription. Although ATF3 binds weakly to the CH25H promoter, it is possible that it might play a role as a cotranscription factor. Interestingly, mouse studies have identified ATF3 as a negative regulator of the macrophage transcriptional response to inflammatory stimuli (28). In the absence of ATF3, the levels of CH25H and 25HC are increased (43). These differences raise further questions regarding how mouse models of disease affect therapeutic approaches for these diseases in humans. Recently, Sood et al. (44) identified ATF3 acting as a negative regulator of antiviral response in other mammalian cells. Nevertheless, this possible cell type dependence for the negative regulatory effect of ATF3 still needs to be investigated.

In conclusion, our results demonstrate, for the first time, that pro-inflammatory cytokines such as IL-1β, TNFα, and IL-6 induce CH25H in human macrophages. Intriguingly, this CH25H induction depends on STAT1 activation but is independent of IFN production. These findings will guide us to better understand how CH25H is regulated in human cells, providing further understanding of the connection between innate immunity and sterol metabolism and developing potential antiviral therapies based on the manipulation of inflammatory cytokines.

Experimental procedures

Ethics statement

Our study was performed in accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals. The procedures for Zika infection in human/mouse tissue cell lines and human primary monocytes/macrophages were approved by the institutional review board (IRB–HSR 16147, Flavivirus Immunopathogenesis).

Viruses, cells, and reagents

The 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 medium (Life Technologies) (37 °C, 5% CO₂) supplemented with 100 units/ml penicillin (Life Technologies), 100 ng/ml streptomycin (Life Technologies), 10% FBS (Atlas Biologicals), 2 mm l-glutamine (Life Technologies), 1× 2-mercaptoethanol (Life Technologies), and 1.25 g dextrose. Microglial cells (ATCC, CRL-3304) were cultured in medium (ATCC, 37 °C, 5% CO₂) and supplemented with penicillin (Life Technologies). Human recombinant IFNβ, TNFα, IL-1β, IFNα, and IL-6 were purchased from Peprotech. Phorbol 12-myristate 13-acetate (PMA) purchased from Invivogen, RNA Bee was purchased from Fisher Scientific, high-capacity RNA-to-DNA kit was purchased from Life Technologies, 2× SYBR Green Master Mix was purchased from Applied Biosystems. DMSO was purchased from Sigma.

Primary human cells

Human monocye-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 privacy protection by removing personal identifiable information from datasets. Monocytes were separated from PBMCs via plastic adherence. Monocytes were differentiated into macrophages with 10 ng/ml human macrophage colony stimulating factor (Biologic) in RPMI medium (Gibco) 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 medium before any treatment. RNA (using RNA Bee) or proteins were then collected for qPCR or Western blot analysis, respectively. THP-1 macrophages were treated with a panel of TLR agonists (Invivogen), including 1 μg/ml synthetic triacylated lipopeptide Pam3CysSerLys4.
TLR1/2 agonist, 1 × 108 mL heat-killed Listeria monocytogenes (a TLR2 agonist), 10 μg/mL poly(I:C) (a TLR3 agonist, high molecular weight), 1 μg/mL flagellin from Salmonella enterica serovar Typhimurium (a TLR5 agonist), 1 μg/mL FSL-1 (a TLR6/2 agonist), 1 μg/mL ssRNA40 (a TLR7 agonist), 2.5 μM CpG ODN1826 (a TLR9 agonist), and 100 ng/mL LPS (a TLR4 agonist) from Escherichia coli 0111:B4 (Sigma-Aldrich). The TLR3 inhibitor was obtained from Calbiochem (catalog no. 614310). After treatment, cells were washed twice with ice-cold Dulbecco’s PBS (Life Technologies). Cells were then lysed, and total RNA (using RNA Bee) was extracted and stored at −80 °C until use.

For RNA isolation, cDNA synthesis, and real-time quantitative PCR, macropheroes were washed twice with ice-cold Dulbecco’s PBS (Life Technologies). RNA was extracted using RNA Bee following the manufacturer’s instructions. 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 a high-capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR was performed on a StepOnePlus system (Applied Biosystems). Primers were used for target gene quantification using SYBR Green Master Mix (Applied Biosystems). Target gene expression was determined using the comparative cycle threshold (ΔΔCT) technique, and results were normalized to HPRT or β2M.

Western blots

Total cell lysates were harvested with radioimmuno precipitation assay buffer and 1× Halt protease inhibitor mixture (Thermo Scientific). Protein concentrations were determined using the Pierce BCA Protein Assay Kit. Cell lysates containing 50 μg of protein were reduced and denatured with sample buffer. Protein lysates were further denatured by boiling for 5 min, and run on 4%–15% SDS-PAGE for 1–2 h at 100 V. Protein was then transferred to a polyvinylidene difluoride membrane and blocked with 5% BSA in tris-buffered saline with tween buffer. The membrane was probed with specific antibodies. The membrane-bound antibodies were visualized with horseradish peroxidase–conjugated antibodies to rabbit IgG and developed with ECL (Santa Cruz Biotechnology). The membrane-bound antibodies were visualized with HRP-conjugated antibodies to rabbit IgG and developed with ECL (Santa Cruz Biotechnology). The membrane-bound antibodies were visualized with horseradish peroxidase–conjugated antibodies to rabbit IgG and developed with ECL (Santa Cruz Biotechnology). The membrane-bound antibodies were visualized with HRP-conjugated antibodies to rabbit IgG and developed with ECL (Santa Cruz Biotechnology). The membrane-bound antibodies were visualized with HRP-conjugated antibodies to rabbit IgG and developed with ECL (Santa Cruz Biotechnology).

Chromatin immunoprecipitation

The ChIP assay was performed using an EpiQuik ChIP kit according to the manufacturer’s instruction. Briefly, THP1 cells differentiated with PMA into macrophages were treated with LPS for the desired times. After treatment, cells were washed twice with PBS and 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

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

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