Title

MafF is an anti-viral host factor that suppresses transcription from Hepatitis B Virus and Epstein Barr Virus promoters

Running title

MafF restricts viral replication

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Abstract

Herein, we report that Maf bZIP transcription factor F (MafF) promotes host defense against infection with Hepatitis B virus (HBV). Suppression of MafF increased HBV pre-genomic RNA in HBV-infected primary hepatocytes. MafF inhibited the binding of the transcriptional activator, HNF-4α, at overlapping recognition sites in HBV core promoter. Mutations introduced at the MafF binding site abolished the physical interaction between MafF and the HBV promoter and counteracted MafF-mediated suppression of HBV replication. MafF expression was induced by IL-1β and TNF-α in an NF-κB-dependent manner. These findings are consistent with the identified induction of MafF expression in chronic HBV patients, notably during the immune clearance phase. Interestingly, MafF also suppressed expression of the trans-activator, BZLF1, that promotes lytic reactivation of Epstein Barr virus (EBV) infection. In conclusion, MafF is a novel anti-viral host factor which is inducible by inflammatory cytokines, and suppresses transcription from the promoters of susceptible DNA viruses.

Introduction

In the earliest stages of viral infection, the host initially detects and counteracts infection via induction of innate immune responses (1). Host restriction factors are essential components of the innate antiviral immune response; these factors serve critical roles in limiting virus replication before the adaptive immune response engages to promote virus clearance (2). These anti-viral restriction factors are typically induced by cytokines, including interferons (IFNs) (3), transforming growth factor-beta (TGF-β) (4), and interleukin-1-beta (IL-1β) (5). These restriction factors suppress viral replication by targeting the infection at various stages of the virus life cycle, including viral entry (6), transcription of the viral genome (7), viral RNA stability (8), translation of viral proteins (9), viral replication (10), and production of viral particles (11).

Approximately 250 million people worldwide are chronically infected with Hepatitis B virus (HBV). These patients are at high risk of developing life-threatening complications, including hepatic cirrhosis, hepatic failure, and hepatocellular carcinoma. Current treatments include nucleos(t)ide analogs that efficiently suppress HBV replication. However, an HBV replication intermediate, covalently closed circular DNA (cccDNA), persists in the nucleus. The cccDNA intermediate gives rise to progeny virus, and may lead to the development of drug-resistant mutants and/or relapsing HBV after drug withdrawal (12). As such, new strategies for HBV treatment are needed.

HBV has been identified in human remains from ~7000 years ago (13). This prolonged history and evolution has shaped HBV to be one of the most successful of the “stealth” viruses that can successfully establish infection while evading IFN induction (14). Although HBV can evade IFN induction, the majority of HBV-infected adults (90%) are ultimately able to clear the virus. This observation suggests that there are likely to be one or more IFN-independent host restriction factors that facilitate HBV clearance.

The small Maf proteins (sMafs) are a family of basic-region leucine zipper (bZIP)-type transcription factors. MafF, MafG and MafK are the three sMafs identified in vertebrate species (15). Homodimers of these factors act as transcriptional repressors, consistent with their lack of a transcriptional activation domain (16). Intriguingly, previous reports have documented induction of MafF in myometrial cells by inflammatory cytokines, including IL-1β and tumor necrosis factor alpha (TNF-α) (17). However, there have been no previous studies that have addressed a role for MafF in promoting an anti-viral innate immune response.

Epstein Bar Virus (EBV) is a human gamma herpesvirus that establishes primarily latent infections in B lymphocytes. Only a small percentage of infected cells switch from the latent stage into the lytic cycle and go on to produce progeny viruses (18). Although the mechanism of EBV reactivation *in vivo* is not fully understood, it is known to be elicited *in vitro* by treatment of latently infected B cells with chemical reagents, including 12-O-tetradecanoylphorbol-13-acetate (TPA), calcium ionophores, or sodium butyrate. Treatment with these reagents leads to the expression of two viral transcriptional regulatory genes, one encoding BZLF1 (also known as ZEBRA) and the other encoding BRLF1, required for the transition from the latent to the lytic productive stage of EBV [28].

Using an HBV reporter virus and an siRNA library, we performed functional siRNA screening to identify the host factors that influence the HBV life cycle. Based on the results of this screen, we identified MafF as a negative regulator of HBV infection. Further analysis revealed that MafF functions as a repressor of transcription at the HBV core promoter, thereby suppressing HBV replication. Interestingly, the antiviral effects of MafF also have an impact on the life-cycle of EBV, as MafF negatively regulated the transcription of BZLF1 viral gene required for the reactivation of EBV from dormant to lytic (productive) stage. This is the first study to report a role for MafF as an antiviral host factor that represses transcription from the promoters of susceptible viruses.

Results

1. MafF suppresses expression of the HBV/NanoLuc (NL) reporter virus

We screened for host factors that influence the HBV infection using the HBV/NL reporter virus (19) in combination with the siRNA Library. This approach facilitated testing of 2200 human genes for their influence on the HBV life cycle. The screen was performed in HepG2-C4 cells that express the HBV entry receptor, hNTCP (20). Non-targeting siRNAs, and siRNAs against h*NTCP*, were used as controls for each plate (Fig. 1A). Cellular viability was determined using the XTT assay; wells with ≥20% loss of cell viability were excluded from further evaluation. *MafF* was identified as an anti-HBV host factor based on independent trials and silencing with at least two different siRNA sequences, both of which provided greater than 2-fold induction of NanoLuc activity. Specifically, silencing of *MafF* expression with si-1 or si-3 resulted in 6-fold ($p<0.0001$) or 10-fold ($p<0.001$) increases in NanoLuc activity, respectively, compared to that observed in cells transfected with a control siRNA (Fig. 1B). The MafF-specific sequence, si-2, did not show a similar effect (Fig. 1B). This result was consistent with the fact that si-2 had a lower silencing efficiency with respect to MafF expression (Fig. 1C). Taken together, these findings suggest that MafF may suppress HBV infection.

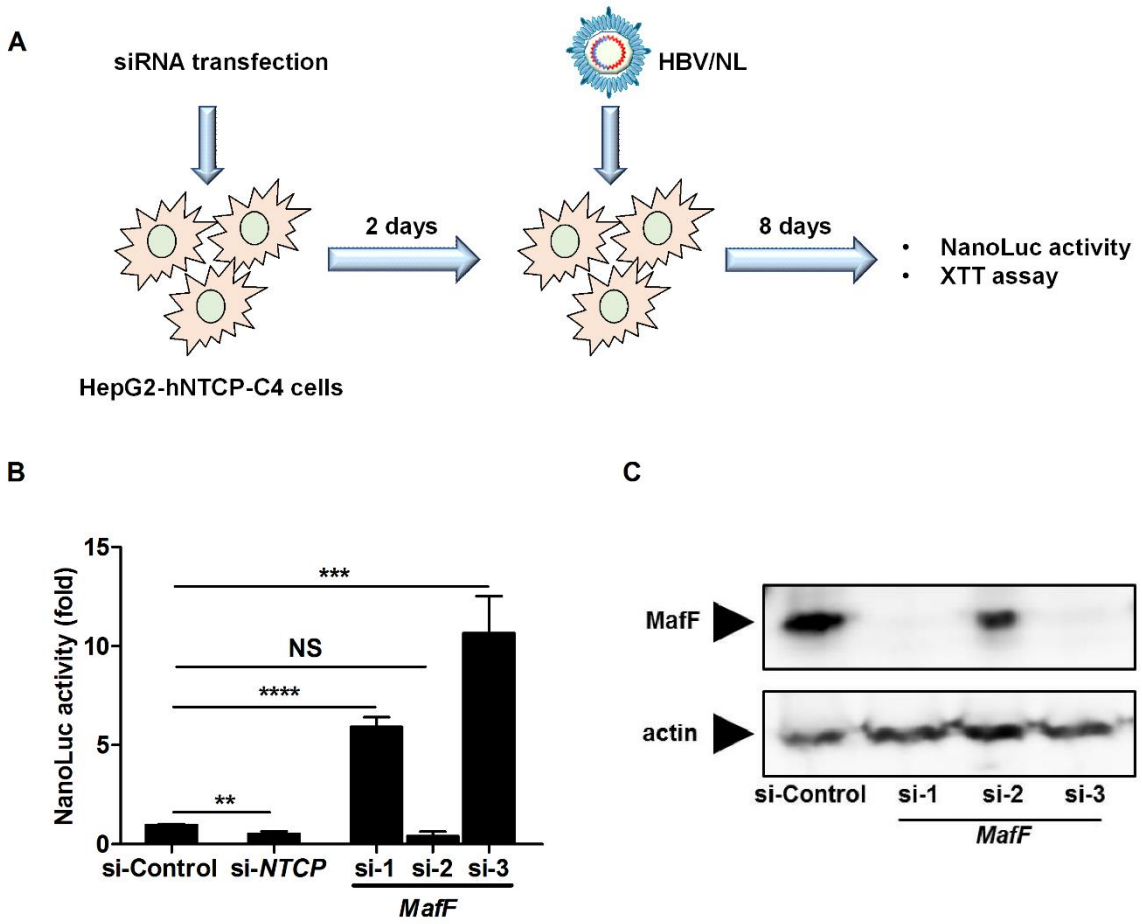

Figure 1. MafF suppresses HBV infection.

A. A schematic diagram showing the experimental approach used to screen the siRNA library. **B.** HepG2-hNTCP-C4 cells were transfected with control, *NTCP*, or *MafF*-targeting siRNAs (si-1, si-2, and si-3); two days later, transfected cells were infected with the HBV/NL reporter virus. At day 8 post-infection, luciferase assays were performed, and NanoLuc activity was measured and plotted as fold-difference relative to the mean luciferase levels in control siRNA-transfected cells. **C.** HepG2 cells were transfected with control or *MafF*-targeting siRNAs (si-1, si-2, and si-3); total protein was extracted two days later. Expression of MafF (upper panel) and actin (control; lower panel) was analyzed by immunoblotting with their respective antibodies. All assays were performed in triplicate and included three independent

experiments; data are presented as mean±standard deviation (SD); **p<0.01, ***p<0.001, ****p< 0.0001; NS, not significant.

2. MafF strongly suppresses HBV core promoter activity

The HBV/NL reporter system can be used to detect factors affecting the early steps of the HBV life cycle, from HBV entry through cccDNA formation, transcription and translation of HBV pgRNA (19). Silencing of *MafF* had no impact on cccDNA levels observed in cells infected with HBV (Fig. 2A); these results indicated that MafF suppressed the HBV life cycle at stage that was later than that of cccDNA formation. Given that MafF can induce transcriptional suppression (16), we analyzed the impact of MafF on various HBV promoters (core, X, preS1, and preS2) using the HBV/NL luciferase reporter system. We found that overexpression of MafF resulted in significant suppression of transcription from the HBV core promoter (approximately 8-fold; p<0.0001), and significant, albeit less of an impact on transcription from the HBV-X and preS1 promoters (both at approximately 2-fold, p<0.0001); overexpression of MafF had no significant impact on transcription from the preS2 promoter (Fig. 2B). Likewise, siRNA silencing of endogenous *MafF* enhanced HBV core promoter activity (Fig. 2C, p<0.0001). Since the NanoLuc gene in HBV/NL virus (Fig. 1B) is transcribed from an HBV core promoter (19), the findings presented in Fig. 1 and Fig. 2 collectively suggest that MafF-mediated suppression of HBV is mediated primarily by inhibition of transcription from the core promoter.

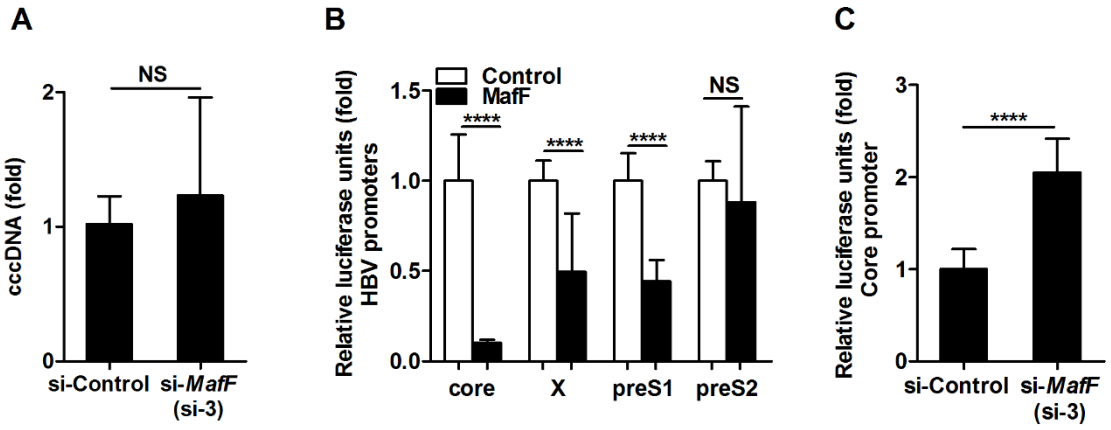

Figure 2. MafF suppresses the transcriptional activity of the HBV core promoter.

A. HepG2-hNTCP-C4 cells were transfected with control or *MafF*-targeting siRNA (si-3). Two days after transfection, the transfected cells were infected with HBV at 12,000 genomic equivalents (GEq) per cell. Eight days later, the cells were harvested, DNA was extracted, and cccDNA was quantified by real-time PCR. The data were normalized to expression of *GAPDH* and are presented as fold-change relative to control siRNA-transfected cells. **B.** HepG2 cells were co-transfected with a MafF expression vector or empty vector (control) together with firefly luciferase reporter plasmids with HBV promoters (core, X, S1, and S2) and the pRL-TK control plasmid encoding *Renilla* luciferase. Two days after transfection, the cells were harvested and evaluated by dual luciferase assay. **C.** HepG2 cells were transfected with control or *MafF*-targeting siRNA (si-3); 24 hours later, the cells were transfected with firefly luciferase reporter-HBV core promoter vector and the pRL-TK plasmid encoding *Renilla* luciferase. Two days later, the cells were lysed and evaluated by dual luciferase assay. For panels B and C, firefly luciferase data were normalized to *Renilla* luciferase levels; relative light units (RLUs) for firefly luciferase were plotted as fold differences relative to the levels detected in the control groups. All assays were performed in triplicate and included three independent experiments; data are presented as mean±SD. ****$p<0.0001$; NS, not significant.

3. <u>MafF suppresses HBV replication</u>

The HBV core promoter controls the transcription of the longest two HBV RNA transcripts, the precore and pgRNAs. HBeAg is translated from the HBV precore RNA, while translation of HBV pgRNA generates both the polymerase (Pol) and the capsid subunit; the pgRNA also serves as the template for HBV-DNA reverse transcription (21, 22). As such, we assumed that MafF served to inhibit HBV replication by controlling transcription of the HBV core promoter. In fact, overexpression of MafF resulted in significant suppression of the pgRNA titer of HBV genotypes A and D, as demonstrated by RT-qPCR (Fig. 3A, $p<0.0001$ for each genotype). Overexpression of MafF also suppressed the release of HBeAg as measured by ELISA (Fig. 3B, $p<0.0001$), as well as the intracellular accumulation of HBV core protein as detected by immunoblotting (Fig. 3C upper and lower panels; $p<0.05$ by densitometric analysis) and the level of HBV core-associated DNA as revealed by Southern blot (Fig. 3D).

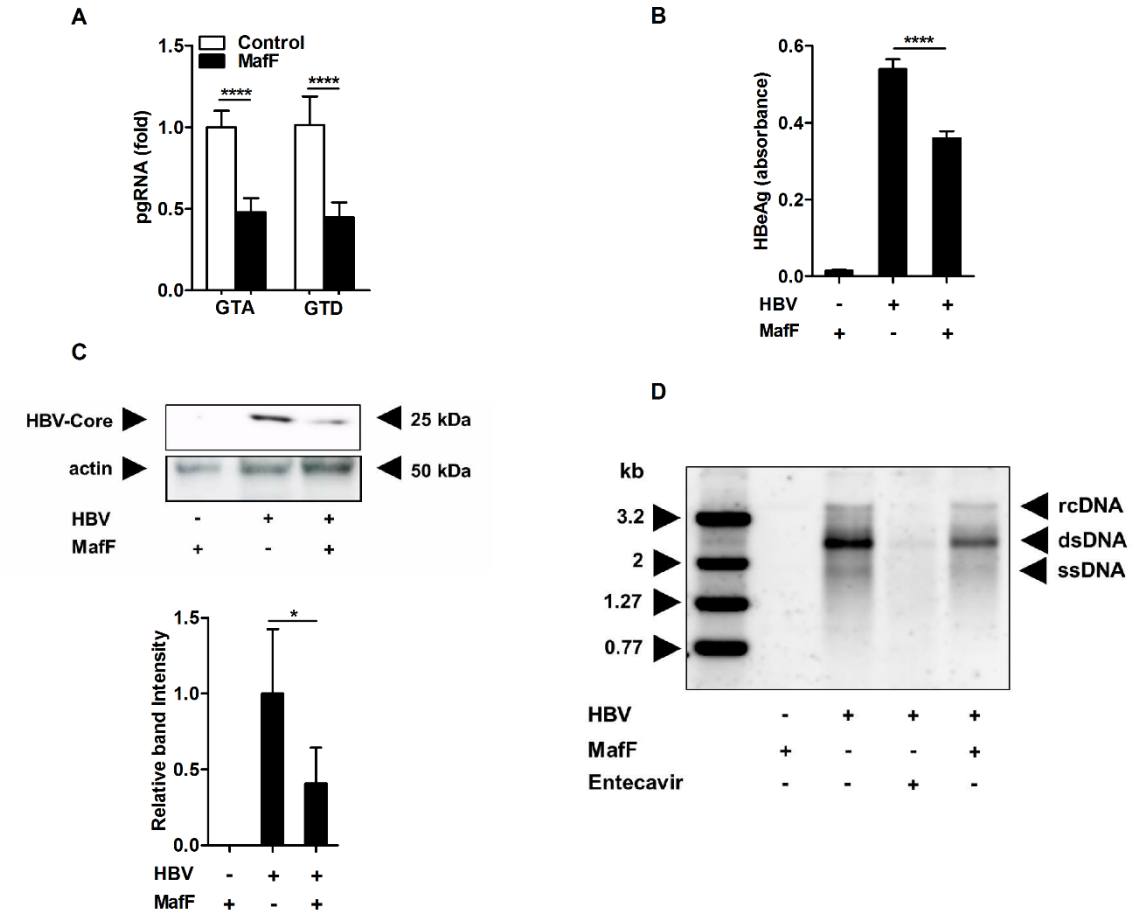

Figure 3. MafF suppresses HBV life cycle.

A. HepG2 cells were transfected with empty (control) or MafF expression vector together with expression vectors encoding HBV genotypes A and D. Two days later, the cells were harvested and the pgRNA expression was quantified by real-time RT-PCR. The data were normalized to the expression of *GAPDH* and are shown as the fold-change relative to control plasmid-transfected cells. **B–D:** HepG2 cells were transfected with empty (control) or MafF expression vectors together with an expression plasmid encoding HBV genotype D **(B)** At 2 days post-transfection, HBeAg in the cell culture supernatants were quantified by ELISA. **(C)** The intracellular levels of HBV core protein (upper left panel) and actin (loading control; lower left panel) were evaluated by immunoblotting; the intensities of the bands (right panel) were quantified by ImageJ software. **(D)** At 3 days post-transfection, the levels of

intracellular core-associated DNA were determined by Southern analysis; transfected cells treated with 10 µM entecavir were used as controls.

Data are presented as fold differences relative to the control plasmid-transfected cells. All assays were performed in triplicate and include results from three independent experiments; data are presented as mean±SD; *p<0.05, ****p<0.0001.

4. MafF binds to the HBV core promoter

We next analyzed the HBV core promoter for potential MafF binding sites using the JASPAR database of transcription factor binding sites (23). Toward this end, we identified the sequence 5'-TGGACTCTCAGCG-3' that corresponded to nucleotides (nts) 1667 to 1679 of the HBV-C_JPNAT genome (GenBank AB246345.1) in the enhancer 2 (EnhII) of the HBV core promoter. This motif was partially conserved among the various HBV genotypes (Fig. 4A) and was similar to a previously defined (16) Maf responsive element (MARE). The main difference between this predicted MafF binding site and that of the endogenous Maf recognition element (MARE; CTGA) was the sequence between the third and sixth nucleotides which was replaced by a complementary (GACT) sequence in the HBV promoter (Fig. 4A). As such, we evaluated the role of this predicted site with respect to HBV core promoter activity. By analyzing JASPAR matrix (23) profile MA0495.1, we found that the 10th and 12th nucleotides of the aforementioned predicted MafF binding region, which are A and C, respectively, are highly conserved residues. We disrupted the predicted HBV MARE sequence by introducing 2-point mutations (A1676C and C1678A) into the HBV core promoter. Interestingly, these mutations significantly counteracted the suppressive effect of MafF at the HBV core promoter (Fig. 4B, p<0.0001). Furthermore, ChIP analysis revealed that there was significantly less physical interaction between MafF and the HBV core promoter with the double mutation than was observed between MafF and the HBV wild type (WT) counterpart (Fig. 4C, p<0.05 for % of input and p<0.01 for fold enrichment). These results confirmed that MafF physically binds to the WT HBV core promoter at the predicted MafF binding site and thereby suppresses transcription. In its role as a transcriptional repressor, MafF is known to homodimerize or to heterodimerize with other small Mafs (MafG and MafK) that also lack transcriptional activation domains. We found that silencing

of *MafG* or *MafK* in the HepG2-C4 cells had no significant impact on HBV/NL infectivity (Supplementary Fig. 1A, B, and C). These data suggest that MafF most likely homodimerizes in order to suppress transcription from the HBV core promoter.

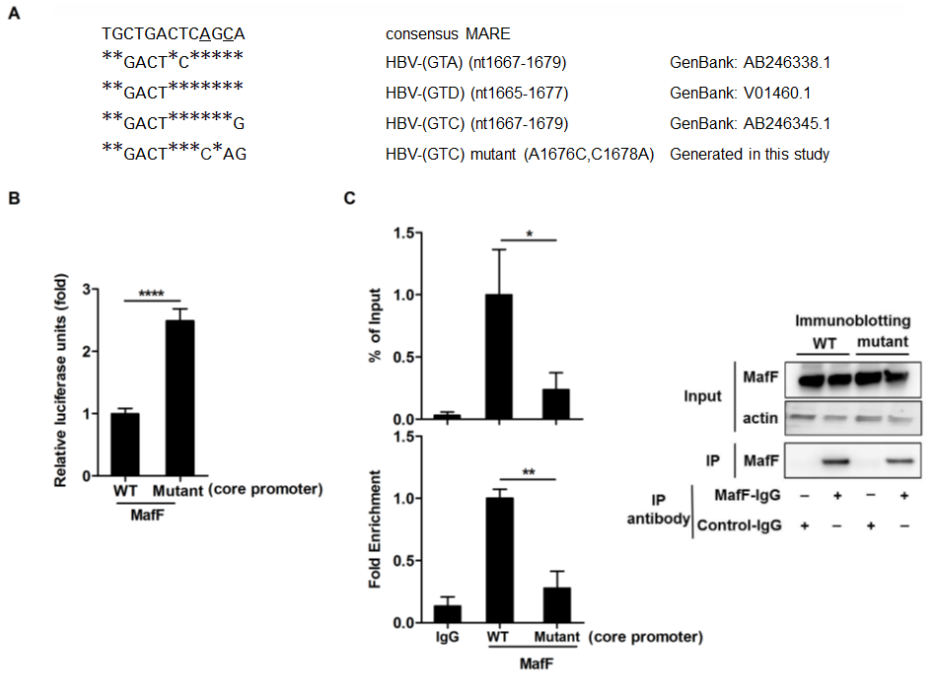

Figure 4. Physical interaction of MafF with HBV core promoter is required for transcriptional repression.

A. A schematic representation of the putative MafF binding site (presented as a consensus Maf recognition element [MARE] sequence) within enhancer 2 (EnhII) of the HBV core promoter from the four different HBV genotypes. A mutant construct was prepared by introducing two point mutations (A1676C and C1678A) into the MARE sequence identified in the wild-type (WT) core promoter. * means consensus sequence, underlined nucleotides are HBV nucleotides 1676A and 1678C mutated in this study. **B.** HepG2 cells were co-transfected with a MafF expression plasmid along with an HBV core promoter (WT or mutant)-reporter plasmid and pRL-TK encoding *Renilla* luciferase. At two days post-transfection, a dual luciferase assay was performed; firefly luciferase data were normalized relative to *Renilla* luciferase levels, and RLUs for firefly luciferase are plotted as fold differences relative to activity in the control group. **C.** 293FT cells were transfected with

either the WT or mutant HBV core promoter-luciferase reporter plasmid together with a MafF expression plasmid (at a ratio 1:4). At two days post-transfection, cell lysates were collected; two aliquots (1/10 volume each) were removed from each sample. One aliquot was used for the detection of MafF protein (Input) and actin (loading control) by immunoblotting (upper and middle right panels); the second aliquot was used for DNA extraction and detection of HBV core promoter (Input) by real-time PCR. The remaining cell lysates (each 8/10 of the original volume) were subjected to ChIP assay using either isotype control antibody (rabbit IgG) or rabbit anti-MafF IgG to detect MafF. Following immunoprecipitation (IP), 1/10 volume of each IP sample was analyzed by immunoblotting for MafF (lower right panel); each remaining IP sample was subjected to DNA extraction and real-time PCR assay in order to detect associated HBV core promoter DNA. The fraction of core promoter DNA immunoprecipitated compared to the input value was determined by real-time PCR and was expressed as percent of input (% of input) and as the fold enrichment over the fraction of *GAPDH* DNA immunoprecipitated. The assays of panel B and C were performed in triplicate and include data from three independent experiments. Data are presented as mean±SD; $*p<0.05$, $**p<0.01$, $****p<0.0001$.

5. MafF is a competitive inhibitor of hepatocyte nuclear factor (HNF)-4α binding to HBV EnhII

HNF-4α is a transcription factor that has been previously reported to bind HBV core promoter and to induce its transcriptional activity (24-26). We found that the predicted MafF binding site in the EnhII region overlaps with an HNF-4α binding site that is located between nucleotides 1662 to 1674 of the HBV C_JPNAT core promoter (27) (Fig. 5A). This finding suggests the possibility that MafF may compete with HNF-4α at these binding sites within the EnhII region. To examine this possibility, we constructed a deletion mutant of EnhII/Cp (EnhII/CpΔHNF-4α#2) that extends from nt 1591 to nt 1750; this construct includes the overlapping binding sites identified for MafF and HNF-4α (i.e., HNF-4α site #1 at nt 1662–1674) but lacks the second HNF-4α binding site (HNF-4α site #2 at nt 1757–1769) as shown in Fig. 5A. We performed a ChIP assay and found that the interaction between HNF-4α and

EnhII/CpΔHNF-4α #2 was significantly reduced in the presence of MafF (Fig. 5B, $p<0.01$ for % of input and $p<0.05$ for fold enrichment). Furthermore, MafF had no impact on the expression of HNF-4α (Fig. 5C). Together, these data indicated that MafF interacts directly with the HBV core promoter at the predicted binding site and suppresses the transcriptional activity of the HBV core promoter by competitive inhibition of HNF-4α binding at an overlapping site in the EnhII region.

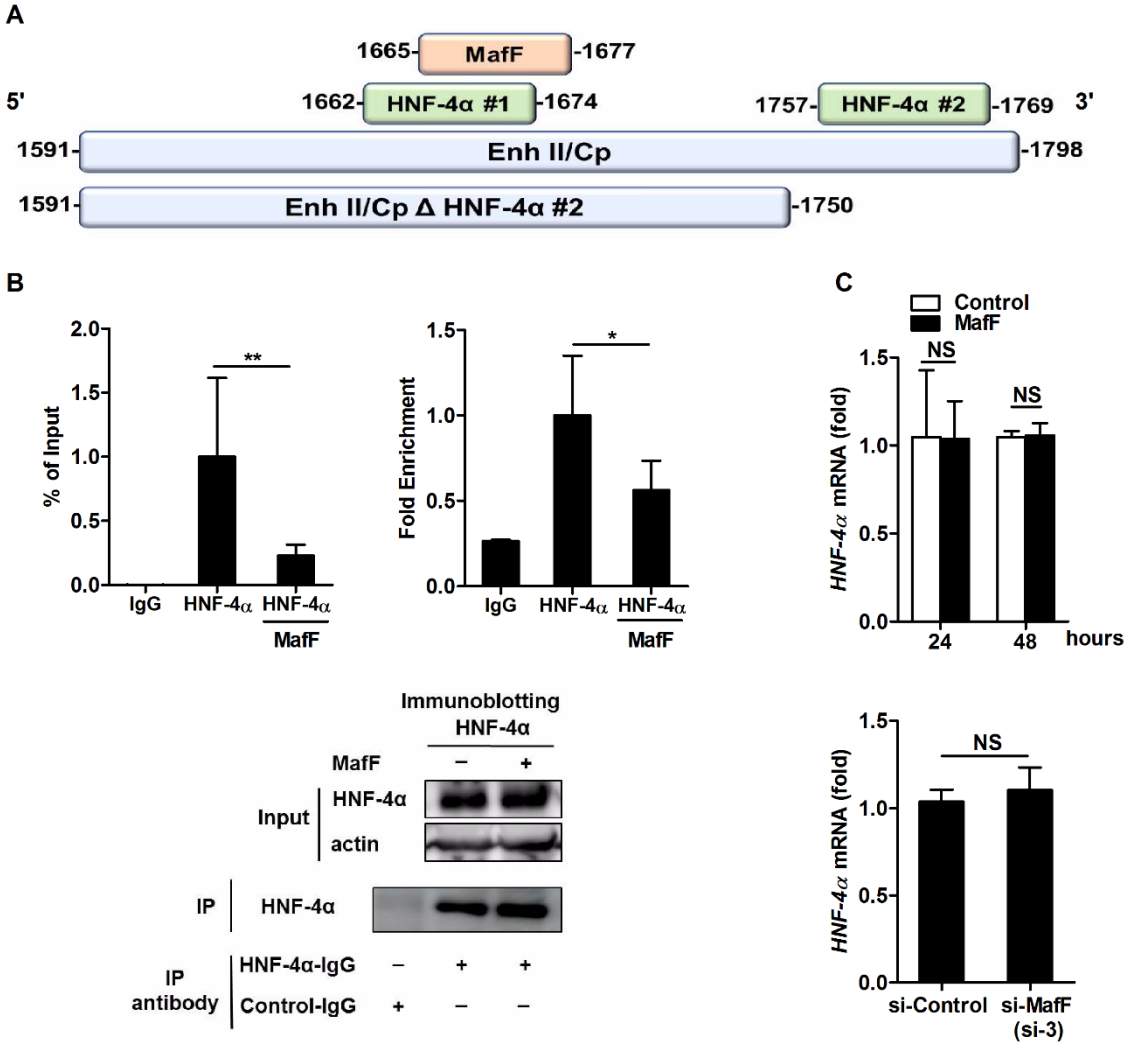

Figure 5. MafF competes with HNF-4α for binding to the HBV core promoter.

A. A schematic representation of the enhancer 2 (EnhII) and the basal HBV core promoter (Cp; nt 1591-1798) featuring the MafF binding site (nt 1667–1679) and the two HNF-4α

binding sites HNF-4α#1 (nt 1662–1674) and HNF-4α#2 (nt 1757–1769). A deletion mutant construct (EnhII/Cp Δ HNF-4α#2, nt 1591–1750) was prepared to eliminate HNF-4α#2. **B.** 293FT cells were co-transfected with the EnhII/Cp Δ HNF-4α#2-luciferase reporter plasmid, a FLAG-tagged HNF-4α expression plasmid, and a MafF (or control) expression plasmid at a ratio of 1:1:2. At two days post-transfection, cell lysates were collected and two aliquots (1/10 volume each) were removed from each sample. One aliquot was used for the detection of HNF-4α protein (Input) and actin (loading control) by immunoblotting (lower panel); the second aliquot was used for DNA extraction and detection of HBV core promoter (Input) by real-time PCR. The remaining cell lysates (each 4/10 of the original volume) were subjected to ChIP assay using isotype control antibody (rabbit IgG) or rabbit anti-HNF-4α IgG to precipitate FLAG-tagged HNF-4α. Following immunoprecipitation (IP), 1/5 volume of each IP sample was analyzed by immunoblotting to detect HNF-4α (lower panel) and each remaining IP sample was subjected to DNA extraction and real-time PCR assay for the detection of associated HBV core promoter DNA. The fraction of core promoter DNA immunoprecipitated compared to the input value was determined by real-time PCR and was expressed as percent of input (% of input) (upper left panel) and as the fold enrichment (upper right panel) over the fraction of *GAPDH* DNA immunoprecipitated. **C. Upper panel:** HepG2 cells were transfected with empty vector (control) or MafF expression vector. After 24 h or 48 h, total RNA was extracted and *HNF-4α* expression was quantified by real-time RT-PCR. The data were normalized to *GAPDH* expression and are presented as fold differences relative to the control cells. **Lower panel:** HepG2 cells were transfected with control or *MafF*-targeting siRNA (si-3) and *HNF-4α* expression was evaluated 48 h later as noted just above. All assays were performed in triplicate and data are presented from three independent experiments. Data are presented as mean±SD; *$p<0.05$, **$p<0.01$; NS, not significant.

6. <u>IL-1β and TNF-α-mediated induction of MafF expression *in vitro*</u>

Given these findings, we speculated that MafF expression might be induced in hepatocytes in response to HBV infection. Based on a previous report of the induction of MafF by both

IL-1β and TNF-α in myometrial cells (17), and the fact that both of these cytokines have been implicated in promoting host defense against HBV, we explored the possibility that MafF might be induced by one or more of these cytokines in our *in vitro* system. As shown in Fig. 6, addition of IL-1β or TNF-α resulted in significant induction of *MafF* mRNA expression in HepG2 cells (Fig. 6A, $p<0.0001$ for each cytokine); MafF protein was also detected at higher levels in HepG2 cells exposed to each of these cytokines (Fig. 6B). Furthermore, IL-1β suppressed HBV core promoter activity ($p<0.0001$); silencing of *MafF* partially counteracted IL-1β-mediated suppression ($p<0.001$; Fig. 6C). Taken together, these data indicate that MafF contributes at least in part to the suppressive effects of IL-1β on HBV infection via its capacity to suppress transcription from HBV core promoter.

NF-κB is a downstream regulatory factor that is shared by the IL-1β and TNF-α signaling pathways. We found that chemical inhibition of NF-κB activity with Bay11-7082 or BMS-3455415 suppressed the induction of *MafF* expression in response to IL-1β (Fig. 6D and E; $p<0.05$ for each of these inhibitors) and to TNF-α (Supplementary Fig. 2; $p<0.01$). These findings indicate that the IL-1β and TNF-α–mediated induction of *MafF* expression in hepatocytes is regulated by NF-κB signaling.

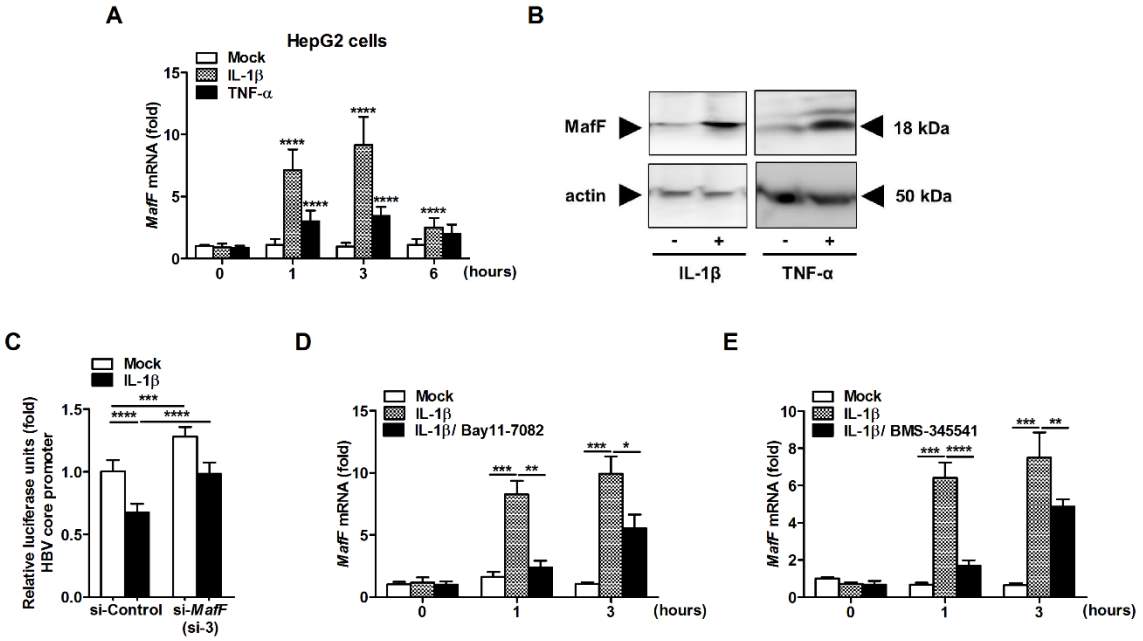

Figure 6. IL-1β and TNF-α induce MafF expression via NF-κB-mediated signaling. **A.** HepG2 cells were treated with IL-1β (1 ng/ml), TNF-α (10 ng/ml), or PBS (diluent control) for the times as indicated (hours). The cells then were lysed, total cellular RNA was extracted, and *MafF* mRNA was quantified by real-time RT-PCR. The data were normalized to the expression of *ACTB* and are shown as the fold-change relative to the mean of the control group. **B.** HepG2 cells were treated for 24 h with IL-1β, TNF-α, or PBS control as in A.; the cells then were harvested and total protein was extracted. Expression of MafF (upper panel) and actin (the loading control; lower panel) was analyzed by immunoblotting. **C.** HepG2 cells were co-transfected with an HBV core promoter-reporter plasmid and the pRL-TK plasmid (encoding *Renilla* luciferase) together with control or *MafF*-targeting siRNA (si-3). At 2 days post-transfection, the cells were treated with 1 ng/ml IL-1β or PBS (diluent control) for 3 h; a luciferase assay was then performed. Data were normalized to the *Renilla* luciferase activity, and the RLUs were plotted as fold differences relative to the mean of the luciferase activity of the PBS treated- control siRNA-transfected cells. **D–E.** HepG2 cells were pre-treated with NF-κB inhibitors Bay11-7082**,** BMS-345541, or DMSO (diluent control) for 1 h and then treated with 1 ng/ml IL-1β or PBS (control) for 1 and 3 h. Expression of MafF was quantified as described in A. All assays were performed in triplicate and including the results from three (panels A, B, and C) or two (E and F) independent experiments. Data are presented as mean±SD; *p<0.05, ***p*<0.01, ****p*<0.001, *****p*< 0.0001.

7. <u>MafF targets HBV infection in human primary hepatocytes</u>

To confirm the suppressive effects of MafF on HBV infection in a more physiological context, we silenced *MafF* expression in human primary hepatocytes (PXB cells) using two independent siRNAs, including si-3 which efficiently targets the *MafF* transcript, and si-2 which was associated with a negligible silencing efficiency (Fig. 1C and Fig. 7A, upper panels), followed by infection with HBV (genotype D). *MafF* silencing in response to si-3 resulted in significant induction of HBV-pgRNA (Fig. 7A, lower panels; $p<0.05$); administration of si-2 did not yield a similar effect. In all experiments, transcription of

pgRNA was inversely associated with expression of *MafF* (Fig. 7B, p=0.008); these findings confirmed the role of endogenous MafF with respect to the regulation of HBV-pgRNA transcription. To confirm our earlier findings documenting induction of *MafF* by IL-1β and TNF-α, we treated PXB cells with both cytokines and observed a significant increase in *MafF* mRNA (Fig. 7C, p<0.05 for each cytokine).

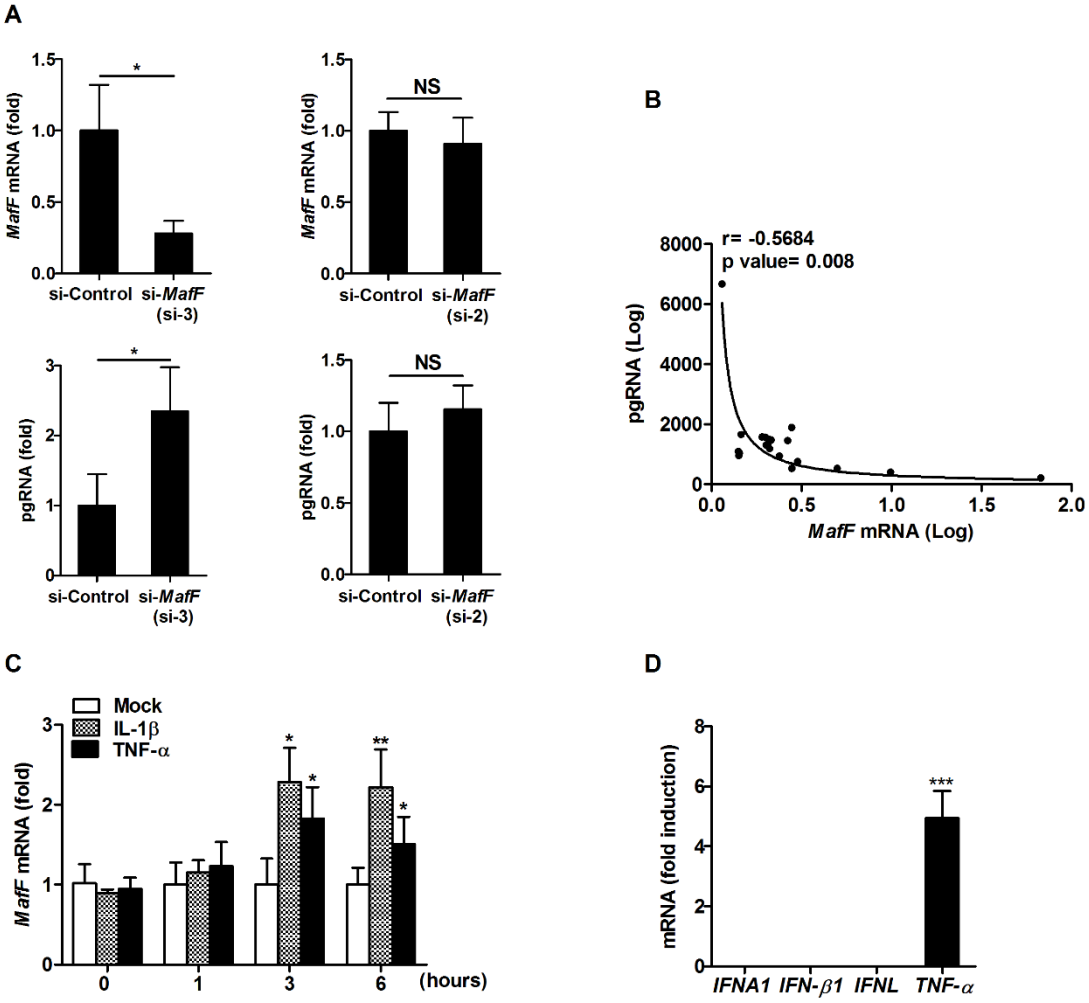

Figure 7. MafF suppresses HBV infection in primary human hepatocytes (PXB cells). **A.** Primary hepatocytes (PXB cells) were infected with HBV virions at 5,000 GEq per cell. After 3 days, the cells were transfected with control or *MafF*-targeting siRNAs (si-2 and si-3); at 4 days after transfection, total RNA was extracted. **Upper panel:** *MafF* expression level was quantified by real-time RT-PCR and normalized to the expression of *ACTB*. **Lower**

panel: Levels of pgRNA were quantified by real time RT-PCR using a standard curve quantification method. Data are presented as fold differences relative to the control siRNA-transfected cells. **B.** Correlation between expression of *MafF* mRNA and pgRNA in HBV-infected and siRNA transfected PXB cells as described in A. **C.** Primary hepatocytes (PXB) cells were treated with IL-1β (at 10 ng/ml), TNF-α (at 10 ng/ml), or PBS (diluent control) for the times indicated (hours). The cells then were lysed, total cellular RNA was extracted, and *MafF* mRNA was quantified by real-time RT-PCR. The data were normalized to the expression of *ACTB* and are shown as the fold change relative to the mean of the control group. All assays were performed in triplicate and include data from two independent experiments. Data are presented as mean±SD; *$p<0.05$, **$p<0.01$; NS, not significant.

8. ***MafF* expression is higher in HBV chronically infected patients with a positive correlation to *IL-1β* and *TNF-α* expression**

To explore a role for MafF in HBV infection in human subjects, we evaluated data from an open database (28), and found that *MafF* was expressed at significantly higher levels in patients with chronic HBV compared to healthy individuals (Fig. 8A, $p<0.0001$); this was notably the case in patients undergoing immune clearance HBV (Fig. 8B, $p<0.0001$). This result confirmed the induction of *MafF* expression during active inflammation associated with this infection. This observation was strengthened by the demonstration of positive correlations between the levels of *IL-1β* and *TNF-α* transcripts and those encoding *MafF* in the immune clearance patient subset (Fig. 8C, D). Interestingly, no correlations were observed between *MafF* expression and transcripts encoding IFNs (Fig. 8E, F, G, and H). These data suggest that MafF induction associated with chronic HBV disease was unrelated to induction of IFN signaling pathways.

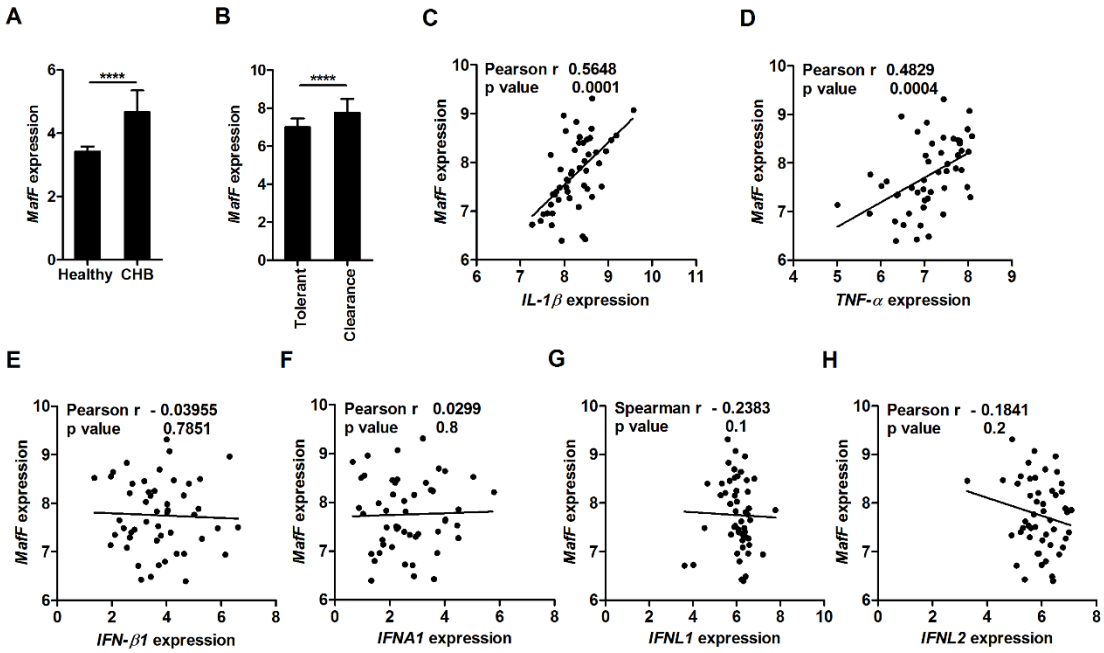

Figure 8. *MafF* expression is increased in patients with chronic HBV infections and is positively correlated to expression of *IL-1β* and *TNF-α* mRNAs

A. *MafF* mRNA levels in the liver tissue of patients with chronic hepatitis B infection (CHB; n=122) and healthy subjects (n=6, GSE83148). **B.** *MafF* mRNA levels in the liver tissue of immune-tolerant (n=22) and immune-clearance (n=50) HBV-infected patients (GSE65359). **C.-H.** Correlations between the expression of mRNAs encoding *MafF* and **C.** *IL-1β*, **D.** *TNF-α*, **E**. *IFN-β1*, **F.** *IFNA1*, **G**. *IFNL1*, and **H**. *IFNL2* in liver tissue of patients undergoing immune-clearance. In panels A and D, data are presented as the mean±SD; ****p<0.0001.

9. <u>MafF suppresses expression of BZLF1, a key player for switching from the latency to the lytic phase of EBV infection.</u>

To determine whether MafF-mediated restriction is limited to HBV or has broader antiviral effects, we explored the impact of MafF on another DNA virus, Epstein Barr virus (EBV). BZLF1 (ZEBRA) is a transcriptional activator with structural features of other members of the basic leucine zipper (b-Zip) family. BZLF1 expression triggered the reactivation of EBV from the latent to the lytic (productive) stage (29). In addition, it also activates transcription

from its own promoter (30). Previous reports indicated that ZII, positive regulatory element, in BZLF1 promoter is a binding site for AP1, CREB/ATF, and MafB b-Zip transcriptional activators (31). Since MARE is known to occur in close association with these transcription factors binding sites (32), we speculated that MafF may bind to and suppress transcription from BZLF1 promoter via ZII. A comparison between the MARE consensus sequence and ZII sequence is shown in Fig. 9A. In fact, we found that MafF suppressed transcription from the BZLF1 promoter; which was not observed when mutant BZLF1 promoter (mZII) in which b-Zip binding site is mutated, was used ((31); Fig. 9B, $p<0.05$). Similarly, stable silencing of MafF in Raji cells, an EBV-positive B cell line, using *MafF* shRNA (Fig. 9C) alone, without the use of chemical inducers such as 12-O-tetradecanoylphorbol-13-acetate (TPA), led to an increase in BZLF1 mRNA (Fig. 9D, $p<0.05$) and protein (ZEBRA protein, Fig. 9E). By contrast, MafF silencing had no impact on the latency stage gene, LMP1 (Fig. 9F). Overall, these data indicate that endogenous MafF may suppress reactivation of EBV lytic phase and the resulting EBV proliferation by the repression of BZLF1 transcription.

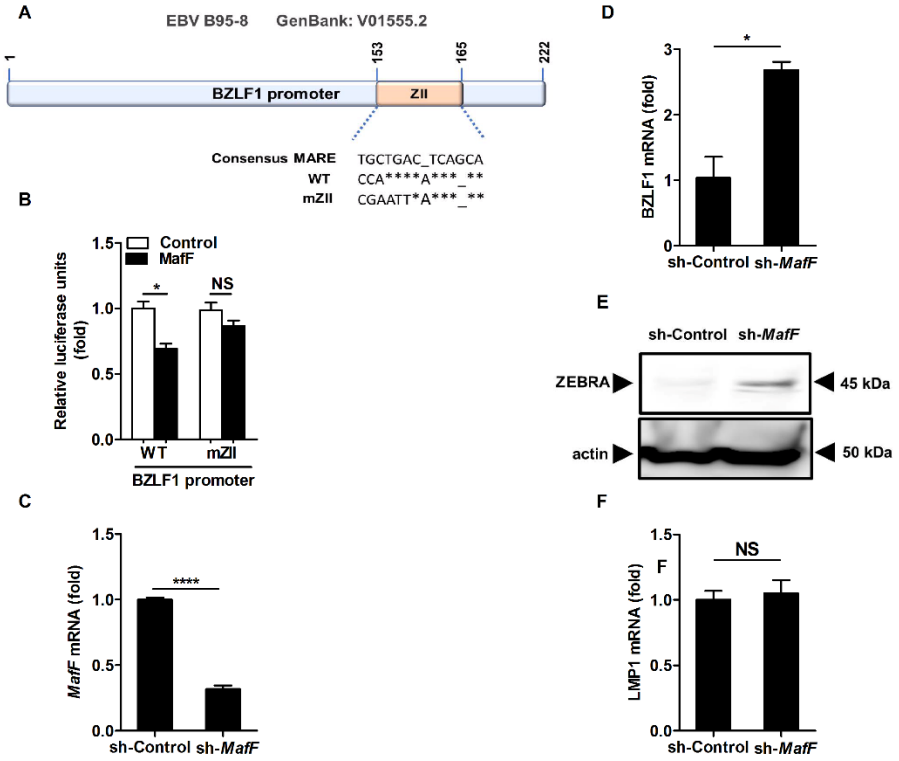

Figure 9. MafF suppresses expression of BZLF1, a key player of switch from latency to lytic phase

A. A schematic representation of the BZLF1 promoter of EBV B95-8 (nt 1-222), and a comparison between the MARE consensus sequence and b-Zip binding site (ZII) sequence of the wild type (WT) promoter and mutated (mZII) promoter. * and under bar (_) represent identical sequence and deletion, respectively. **B.** 293 FT cells were co-transfected with a MafF expression plasmid together with EBV BZLF1 promoter constructs, including WT (pzp-luc) and b-Zip binding site mutated (pzp-luc mZII))-reporter plasmids and pRL-TK which encodes *Renilla* luciferase. At two days post-transfection, a dual luciferase assay was performed; firefly luciferase data were normalized relative to *Renilla* luciferase levels; RLUs for firefly luciferase are plotted as fold differences relative to activity in the control group. **C-F.** Raji cells were stably transfected with control or *MafF*-targeting shRNA and selected with puromycin (2 µg/ml) for 10 days. **C, D, and F**. Total RNA was extracted and expression of *MafF,* BZLF1, and LMP1 was quantified by real-time RT-PCR. The data were normalized to the expression of the housekeeping gene *GAPDH*, and are presented as fold differences relative to the control cells. **E.** The expression of both ZEBRA (upper panel) and actin (the loading control; lower panel) were evaluated by immunoblotting. All assays were performed in triplicate and include data from three independent experiments. Data are presented as mean±SD; *p<0.05, ****p<0.0001; NS, not significant.

Discussion

The intrinsic, or innate immune response is mediated by cellular restriction factors. Many of these factors induced by cytokines (3-5) and serve to suppress different stages of the viral life cycle, from entry to virion release (2). Several host restriction factors can suppress transcription from DNA virus promoters (7, 33). In this work, we identified MafF as a new host restriction factor that can inhibit both HBV and EBV infections via transcriptional suppression at targeted viral promoters.

MafF is a member of the small Maf (sMaf) family of transcription factors, a group that includes MafG (34), MafK, and MafF (35). The sMafs are bZIP-type transcription

factors that bind to DNA at Maf recognition elements (MAREs). MAREs were initially defined as a 13-bp (TGCTGA(G/C)TCAGCA) or 14-bp (TGCTGA(GC/CG)TCAGCA) elements (36, 37). However, multiple studies (38-40) have presented findings suggesting heterogeneity within MARE sequences. Using the JASPAR database for transcription factor binding sites, we identified a sequence extending from nt 1667 to 1679 (TGGACTCTCAGCG) in the EnhII region of HBV as a potential MafF binding site. Both ChIP and functional analysis confirmed the importance of the interaction between MafF and this specific sequence in HBV core promoter; MafF binding at this site results in suppression of the transcriptional activity from HBV core promoter and inhibition of the HBV life cycle. Interestingly, the MafF binding site in the HBV core promoter was conserved among the multiple HBV genotypes; MafF-mediated suppression was also observed more broadly at core promoters from HBV genotypes A, C, and D.

The expression levels of sMafs serve as strong determinants of their overall function. An excess of sMafs may increase the level of homodimer formation and shift the balance toward transcriptional repression (41). Of note, sMafs can form homodimers or serve as partners for heterodimer formation with cap'n'collar (CNC) family proteins Nrf1, Nrf2, Nrf3, Bach1, and Bach2 (42). Furthermore, MARE consensus sites include an embedded canonical AP1 motif; as such, some Jun and Fos family factors can also heterodimerize with Maf/CNC proteins. Finally, large Maf proteins are also capable of binding at MARE elements (43, 44). Given the large number of possible homo- and heterodimeric combinations of proteins capable of binding to MAREs, transcriptional responses ranging from subtle to robust can be elicited at a single MARE site (44). The inflammatory response to virus infection facilitates the production of cytokine-inducible host factors that can repress transcription from factors binding at MAREs; these factors serve to suppress replication of DNA viruses that depend on the aforementioned network for the transcriptional activation at its promoters. Of note, our findings revealed that MafF expression is induced by IL-1β and TNF-α in primary hepatocytes (PXBs) and that this induction was mediated by NF-κB, an inducible transcription factor that is a central regulator of immune and inflammatory responses (45). Both IL-1β and TNF-α have been associated with protection against HBV. For example, a

polymorphism in the IL-1β–gene has been linked to disease progression in patients with HBV-related hepatitis (46), while TNF-α expression in hepatocytes induced by HBV (47) has been shown to decrease the extent of HBV persistence (48). We detected higher levels of *MafF* expression in patients with chronic HBV, especially among those in the immune clearance group, compared to healthy individuals. Moreover, we have also reported that silencing of *MafF* expression resulted in partial rescue of IL-1β-mediated suppression of transcription from the HBV core promoter. Taken together, these data suggest an important role for MafF with respect to the anti-HBV effects of these cytokines in HBV-infected patients.

Transcription driven from HBV core promoter is controlled by two enhancers, enhancer I (EnhI) and EnhII, the latter overlapping with the core promoter (EnhII/Cp); transcription is also modulated by a negative regulatory element (NRE) (49). Liver-enriched transcription factors, including C/EBPα, HNF-4α, HNF3, FTF/LRH-1, and HLF4, can interact with the EnhII/Cp region and thereby enhance the core promoter activity (50, 51). Negative regulation of HBV core promoter mainly takes place at the NRE, which is located immediately upstream of EnhII (52). Our analysis of the EnhII segment revealed an overlap between MafF and one of the HNF-4α binding sites located between nt 1662 to nt 1674. We identified MafF as a novel negative regulator of EnhII activity that acts via competitive inhibition of HNF-4α binding to the HBV core promoter at this site; we present this mechanism as a plausible explanation for MafF-mediated suppression of HBV infection.

MafF mediated significantly stronger suppression of transcription from HBV core promoter compared to its actions at HBV-X and PreS1; this is likely due to the aforementioned direct interaction between MafF and core promoter. MafF may not be involved in direct interactions with HBV-X and PreS1 promoters, but may play a role via interactions with other transcription factors that regulate transcription from these promoters. HBV core promoter regulates the expression of HBV-precore and pgRNA transcripts. The precore-RNA serves as the template for the translation of HBV-precore protein. HBV-pgRNA is translated into two proteins, HBc (the capsid-forming protein) and pol (polymerase); the HBV-pgRNA also serves as a template for HBV-DNA reverse

transcription and viral replication (53). MafF inhibits HBV replication via suppressing the production of HBV-pgRNA, thereby limiting the production of the corresponding replication-associated proteins (core and pol; Fig. 3). We showed here that HBV-pgRNA titers in HBV-infected primary hepatocytes (PXB) were higher in cells subjected to MafF silencing; levels of HBV-pgRNA were inversely correlated with MafF mRNA levels (Fig. 7A and B). These data confirmed the importance of endogenous MafF with respect to the regulation of HBV-pgRNA transcription and viral replication. The HBV precore protein is a well-known suppressor of the anti-HBV immune response (54-56). As such, suppression of HBV precore protein expression may promote a MafF-mediated recovery of the anti-HBV immune response and enhanced viral clearance.

We found here that silencing of MafF expression induced the transcription of BZLF1, resulting in increases in BZLF1 mRNA, and ZEBRA protein levels. These data suggest that MafF may have a strong role in preventing of EBV reactivation through suppression of BZLF1 expression. We described the induction of MafF expression by IL-1β and TNF-α inflammatory cytokines through NF-κB signaling in human hepatocytes. Interestingly, both TNF-α and NF-κb signaling were previously reported to promote EBV latency by negatively regulating BZLF1 transcriptional activity (57). Knowing that MafF expression was induced by the TNF-α in NF-κB dependent manner (Fig. 6), pauses MafF as a possible inducer for the suppression of BZLF1 transcriptional activity reported by this signaling pathway.

To summarize, the results of this work identified MafF as a novel anti-HBV and anti-EBV host factor. *MafF* expression was induced by both IL-1β and TNF-α in primary hepatocytes and also in patients with chronic HBV. Furthermore, MafF was shown to play an important role in the suppression of transcription from the HBV core promoter as well as the EBV BZLF1 promoter. Further analysis will be needed in order to determine whether the anti-viral function of MafF is effective against other DNA viruses as well as its impact on viral evasion mechanisms.

Materials and Methods

Cell culture

All the cells used in this study were maintained in culture at 37°C and 5% CO_2. HepG2, HepG2-hNTCP-C4, and HepAD38.7-Tet cell lines were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) GlutaMAX media (Gibco) as previously described (20). Primary human hepatocytes (Phoenixbio; PXB cells) were cultured as previously described (58). HEK 293FT cells were cultured in DMEM (Sigma), and Raji Burkitt's lymphoma cells were cultured in Roswell Park Memorial Institute media (Gibco) as previously described (8, 59).

siRNA library

A Silencer Select™ Human Druggable Genome siRNA Library V4 was used for screening of HepG2-hNTCP-C4 cells infected with the HBV/NL reporter virus. The siRNAs were arrayed in a 96-well format; siRNAs targeting the same genes with different target sequences were distributed across three plates (A, B, and C). The following plates from this siRNA library (2200 human genes) were screened: 1-1, 1-2, 1-3, 1-4, 2-1, 2-2, 2-3, 2-4, 3-1, 5-4, 6-2, 6-3, 9-2, 11-3, 11-4, 13-3, 15-1, 15-4, 19-1, 22-2, 25-3, 25-4, 26-1, 26-2, and 26-3. Cellular viability was determined using the XTT assay (Roche) according to the manufacturer's instructions. Wells with ≥ 20% loss of cell viability were excluded from further evaluation. Protocols for the preparation of HBV/NL and screening were as described previously (19).

Plasmid vectors

An HBV genotype D subtype ayw replicon (60) was obtained from Addgene. HBV Ae (genotype A),HBV D_IND60, and HBV C_JPNAT are 1.24 HBV replicons which were described previously (61). A MafF expression plasmid (pFN21AB8874) was purchased from Promega. To add a C-terminal HaloTag, the MafF-encoding sequence was subcloned into the PC14K HaloTag vector using the Carboxy Flexi system (Promega). Additional details regarding the construction of other plasmids are included in the Supplementary Materials.

DNA and RNA transfection

Plasmid DNA transfection was performed according to the manufacturer's guidelines, using Lipofectamine 3000 (Invitrogen) for HepG2 cells and Lipofectamine 2000 (Invitrogen) for HEK 293FT cells. Reverse siRNA transfection into HepG2-hNTCP-C4 was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific); forward siRNA transfection was performed in PXB cells only using Lipofectamine RNAiMAX or in HepG2 using Lipofectamine 2000 for siRNA/DNA co-transfection studies according to the respective manufacturer's guidelines. Silencer Select™ si-*MafF* (s-1, s24372; s-2, s24371; and s-3, s24370), si-*MafK* (s194858), si-*MafG* (s8419), and the negative control siRNA (#2) were purchased from Thermo Fisher Scientific.

Western blot analysis

Cells were lysed with PRO-PREP protein extraction solution (Intron Biotechnology). Protein samples were separated on a 12% gel via SDS-PAGE. Immunoblotting and protein detection were performed as previously reported (8). Primary antibodies included mouse monoclonal anti-HBc (provided by Dr. Akihide Ryo, Yokohama City University), anti-Halo-tag (Promega), anti-FLAG (M2, Sigma), Anti-EBV ZEBRA (BZ1 IgG, Santa Cruz), and anti-actin (Sigma), rabbit polyclonal anti-MafF (Protein Tech), and rabbit monoclonal anti-HNF-4α (Abcam). The band intensities were quantified by ImageJ software (NIH).

HBV preparation and infection

HBV and HBV/NL stocks used in this study were prepared as described previously (19, 20) For infection of HepG2-hNTCP-C4 cells, the cells first were reverse-transfected with *MafF* or negative control siRNAs two days prior to the HBV infection, and then infected 2 days later with inoculation of HBV or HBV/NL as described previously (19, 20); the experiment was terminated at 8 days post-infection. For PXB cells, the cells were first infected with HBV; at 3 days post-infection, the cells were transfected with the siRNAs, and the experiment was terminated at 7 days post-infection.

RNA extraction and quantitative real-time PCR

Isolation of total cellular RNA was performed with a RNeasy Mini kit (Qiagen) according to the manufacturer's guidelines and cDNA synthesis was performed using a Superscript VILO

cDNA Synthesis Kit (Thermo Fisher Scientific). Primer and probe sequences are included in the Supplementary materials.

DNA extraction and cccDNA quantification

For selective extraction of cccDNAs, HBV-infected HepG2-hNTCP-C4 cells were harvested and total DNA was extracted using a Qiagen DNA extraction kit according to the manufacturer's instructions but without the addition of Proteinase K as recommended by the concerted harmonization efforts for HBV cccDNA quantification reported in the 2019 International HBV meeting (62). Levels of cccDNA were measured by quantitative real-time PCR (qPCR) using the TaqMan Gene Expression Master Mix (Applied Biosystems), specific primers, and probe as described previously (63). Data were processed as $2^{(-\Delta\Delta Ct)}$ for quantification of cccDNA using *GAPDH* (via primer-probe set Hs04420697_g1; Applied Biosystems) as an internal normalization control.

Dual luciferase reporter assay

Firefly luciferase reporter plasmids carrying the entire HBV core promoter (nt 900 to 1817), the Enh1/X promoter (nt 950 to 1373), the preS1 promoter (nt 2707 to 2847), or the preS2/S promoter (nt 2937 to 3204) were constructed as previously reported (64). The WT (pzp-luc) and mutated BZLF1 (pzp-luc mZII) luciferase reporter plasmids were also as previously reported (65). HepG2 or 293FT cells were co-transfected with the firefly reporter vectors and the *Renilla* luciferase plasmid pRL-TK (Promega) as an internal control. At 48 h post-transfection, the cells were lysed and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). For experiments involving IL-1β, cells were treated for 3 h with IL-1β (1 ng/ml) at 48 h post-transfection followed by evaluation of dual luciferase activity.

Quantification of HBe antigen

Cell supernatants were harvested and an enzyme-linked immunosorbent assay (ELISA; Enzygnost HBe monoclonal, Siemens) was used to determine the levels of HBe antigen in the culture supertatants according to the manufacturer's instructions.

Southern blotting assay

HepG2 cells were co-transfected with MafF-encoding or control vectors together with the HBV ayw plasmid both with or without 5 μM entecavir (Sigma) as a control. At 3 days post-transfection, core-associated DNA was isolated from intracellular viral capsids as described previously (66). Southern blot analysis to detect HBV-DNAs was performed also as described previously (8)

Chromatin Immunoprecipitation (ChIP) assay

293FT cells were co-transfected with a MafF expression plasmid together a reporter plasmid harboring either the wild-type (WT) or mutated core promoter (substitution mutations in MARE) at a 4:1 ratio for assessment of the interactions between MafF and HBV core promoter. In other experiments, 293FT cells were co-transfected with plasmids encoding FLAG-tagged HNF-4α, EnhII/CPΔHNF-4α#2, and a MafF expression plasmid (or empty vector) at a 1:1:2 ratio for assessment of competitive binding of MafF and HNF-4α. At 48 h post-transfection, ChIP was carried out using a Magna ChIP G kit (Millipore) according to the manufacturer's instructions. Additional detailed information is included in the Supplementary materials.

Cytokine treatment and NF-κB inhibitors

Responses to IL-1β and TNF-α (R&D Systems) were evaluated in HepG2 cells (at 1 ng/ml and 10 ng/ml, respectively) and in PXB cells (both at 10 ng/ml) after 1, 3, and 6 h. For the experiments involving NF-κB inhibitors, Bay11-7082 and BMS-345541 (both from Tocris) were added to final concentrations of 10 μM and 5 μM, respectively. HepG2 cells were pre-treated for 1 h with each inhibitor followed by the addition of 1 ng/ml IL-1β (for 1 and 3 h) or 10 ng/ml TNF-α (for 1 h). All experiments included phosphate-buffered saline (PBS) as a diluent control for the cytokines and dimethyl sulfoxide (DMSO) as the diluent control for the NF-κB inhibitors.

Database

Transcriptional profiling of the patients with chronic HBV (CHB) (GSE83148), and of HBV patients with immune-tolerance and undergoing HBV clearance (GSE65359) were identified in the Gene Expression Omnibus public database. Expression data for *MafF* and for genes

encoding cytokines *IL-1β, TNF-α, IFNA1, IFN-β1, IFNL1, and IFNL2* were extracted by GEO2R.

Construction and stable transfection with shRNA.

The pLKO.1-puro lentivirus expression vector was digested with *AegI* and *EcoR*I and annealed with oligonucleotides including sh-MafF: 5'-aggacgaggtaccggtaGCCTTTTTAGATTGAGAGATTctcgagAATCTCTCAATCTAAAAA GGCtatttttgaattctagatcttga-3' or sh-control (67) that were ligated into the vector. For the production of lentivirus vectors, 293FT cells were transfected with expression plasmids encoding HIV-1 Gag-Pol, and VSV G, and shRNAs (sh-control, and sh-MAfF) using Lipofectamine 2000 (Invitrogen). Culture supernatants were collected at t = 60 hours. Raji cells were infected with the lentiviral constructs in the presence of 10 μg/mL Polybrene. Bulk selection of cells in with stable integration of the shRNA construct was performed by the addition of Puromycin (2μg/mL) to the culture supernatant.

Statistical analysis

The data were analyzed with algorithms included in Prism (v. 5.01; GraphPad Software, San Diego, CA). Tests for normal distribution of the data were performed. Two-tailed unpaired t tests, and Mann-Whitney U tests were used for statistical analysis of parametric and non-parametric data, respectively. The correlation coefficients were determined by Pearson or Spearman correlation analysis of parametric and non-parametric data, respectively. Values of $p \leq 0.05$ were considered statistically significant.

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Supplementary Materials

Plasmid Construction

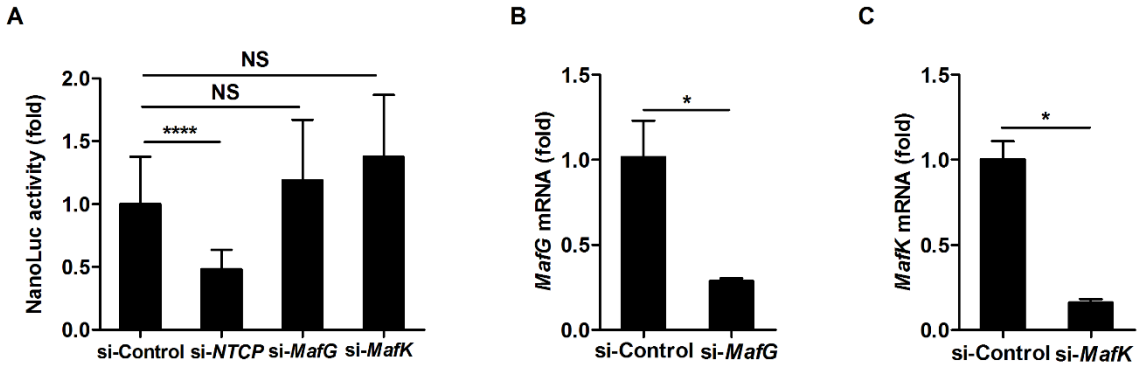
The reporter plasmid for the HBV core promoter mutant was generated by introducing two point mutations (A1676C and C1678A) at the MafF binding site (Fig. 4A). Briefly, several rounds of PCR amplification were performed using pGL4.10_Ce_xmut as the template; the resulting products were digested with *Hind*III and *EcoR*I and subcloned into restriction-digested pGL4.10 (Promega). The set of primers used in the construction of the mutated core promoter include forward primers 5'-TCGAGGAATTCGGGTACTTTACCACAGGAAC-3' and 5'-CTTGGACTCTCCGAAATGTCAACG-3' and reverse primers 5'-TTGCCAAGCTTGAACATGAGATGATTAGGC-3' and 5'-CGTTGACATTTCGGAGAGTCCAAG-3'. The sequence encoding HNF-4α was amplified from FR_HNF4A2 (Addgene; (68)) by PCR using primers including forward primer, 5'-AGCTAGGATCCACCATGCGACTCTCCAAAACC-3' and reverse primer 5'-GAGTCGAATTCTTACTTGTCGTCATCGTCTTTGTAGTCAGCAACTTGCCCAAAG CG-3'. The resulting amplification product was cloned into pCDNA3.1 (Invitrogen) to yield pcDNA3.1-HNF4A-FLAG. The reporter deletion mutant EnhII/CPΔHNF-4α #2 (Fig. 5A) was constructed using pGL4.10_Ce_xmut as the template and a primer set including forward primer 5'- TCGAGGGTACCGCCTGTAAATAGACCTATTG-3' and reverse primer 5'-CTAACAAGCTTTCCTCCCCCAACTCCTCCC-3'; the amplification product was subcloned into pGL4.10 using *Hind*III and *Kpn*I restriction enzymes. All constructs were validated by DNA sequencing. Plasmid DNAs used in transfection experiments were purified using the Purelink Plasmid Midi Kit (Invitrogen).

Real-time PCR primers

The relative levels of the *MafF*, *MafG*, and *MafK* mRNAs were determined using TaqMan Gene Expression Assay primer-probe sets (Applied Biosystems) Hs05026540_g1, Hs00536278_m1, and Hs00242747_m1, respectively; expression of *ACTB* (primer-probe set

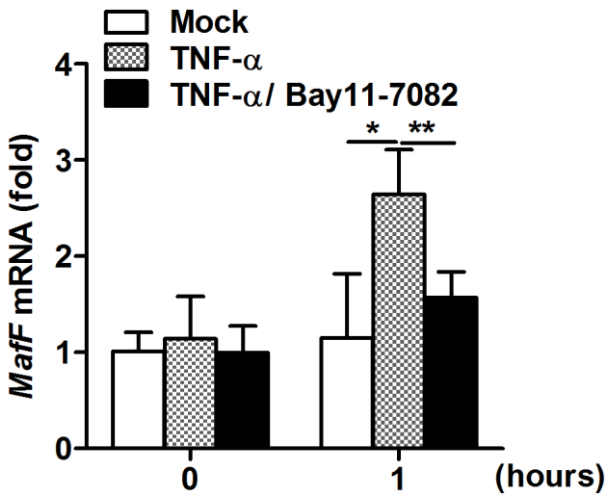
Hs99999903_m1) was used as an internal control for normalization. The quantification of pgRNA, *HNF-4α*, and mRNAs encoding EBV BZLF1 and LMP1 was performed using Power SYBR Green PCR Master Mix (Applied Biosystems); for these transcripts, expression of *GAPDH* was used as an internal control for normalization. Data were expressed as fold change relative to the mean of the control group. The set of primers used in these assays included the Precore forward primer 5'-ACTGTTCAAGCCTCCAAGCTGT-3' and reverse primer 5'-GAAGGCAAAAACGAGAGTAACTCCAC-3', *HNF-4α* (69) forward primer 5'-ACTACGGTGCCTCGAGCTGT-3' and reverse primer 5'-GGCACTGGTTCCTCTTGTCT-3'; *GAPDH* forward primer 5'-CTTTTGCGTCGCCAG-3' and reverse primer 5'-TTGATGGCAACAATATCCAC-3', EBV BZLF1 forward primer 5′-AAATTTAAGAGATCCTCGTGTAAAACATC-3′ and reverse primer 5′-CGCCTCCTGTTGAAGCAGAT-3′, EBV LMP1 forward primer 5′-CTGGTTCCGGTGGAGATGA-3′ and reverse primer 5′-CTGGTTCCGGTGGAGATGA-3′.

Supplementary Figure Legends



Supplementary Figure 1. Silencing of *MafG* and *MafK* has no impact on HBV/NL. A. HepG2-hNTCP-C4 cells were transfected with control, *NTCP*, *MafG*, or *MafK*-targeting siRNAs, followed two days later by infection with the HBV/NL reporter virus. At day 8 post-infection, the cells were evaluated by luciferase assay; NanoLuc activity was determined and plotted as fold differences relative to the mean luciferase activity of the control siRNA-

transfected cells. **B.–C.** HepG2 cells were transfected with control, *MafG*-targeting siRNA **(B)**, or *MafK*-targeting siRNA **(C)** for 2 days followed by the extraction of total RNA and the quantification of the corresponding mRNAs. The data were normalized to the expression of the *ACTB* and are presented as fold differences relative to the control siRNA-transfected cells. All assays were performed in triplicate and include data from three independent experiments. Data are presented as mean±SD; **p*<0.05, *****p*<0.0001; NS, not significant.



Supplementary Figure 2. Induction of *MafF* mRNA in response to TNF-α is mediated by NF-κB signaling. HepG2 cells were pretreated with Bay11-7082 or DMSO diluent control for 1 h and then treated with 10 ng/ml TNF-α or left untreated (mock) for 1 h. Cells were then lysed, total cellular RNA was extracted, and *MafF* mRNA was quantified by real-time RT-PCR. The data were normalized to the expression of the *ACTB* and shown as the fold change relative to the mean of the control group. All assays were performed in triplicate include data from three independent experiments. Data are presented as mean±SD; **p*<0.05, ***p*<0.01.

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