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SAMHD1 Deficient Human Monocytes Autonomously Trigger Type I Interferon

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Abstract

Germline mutations in the human *SAMHD1* gene cause the development of Aicardi-Goutières Syndrome (AGS), with a dominant feature being increased systemic type I interferon (IFN) production. Here we tested the state of type I IFN induction and response to, in *SAMHD1* knockout (KO) human monocytic cells. *SAMHD1* KO cells exhibited spontaneous transcription and translation of *IFN-β* and subsequent interferon-stimulated genes (ISGs) as compared to parental wild-type cells. This elevation of *IFN-β* and ISGs was abrogated via inhibition of the TBK1-IRF3 pathway in the *SAMHD1* KO cells. In agreement, we found that *SAMHD1* KO cells present high levels of phosphorylated TBK1 when compared to control cells. Moreover, addition of blocking antibody against type I IFN also reversed elevation of ISGs. These experiments suggested that *SAMHD1* KO cells are persistently auto-stimulating the TBK1-IRF3 pathway, leading to an enhanced production of type I IFN and subsequent self-induction of ISGs.

Keywords

SAMHD1; IFN; AGS; TBK1-IRF3; ISGs

1. Introduction

The sterile alpha motif HD domain 1 (*SAMHD1*) protein is best known as potently restricting HIV-1 infection of non-cycling cells such as macrophages, dendritic cells and resting CD4⁺ T cells (Arfi et al., 2008; Baldauf et al., 2012; Descours et al., 2012; Goujon et al., 2008; Goujon et al., 2003; Goujon et al., 2007; Hrecka et al., 2011; Laguetta et al., 2011; St Gelais et al., 2012). The ability of *SAMHD1* to block HIV-1 infection correlates with its

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dNTPase activity (Goldstone et al., 2011; Lahouassa et al., 2012; Powell et al., 2011), which is necessary to decrease levels of dNTPs in non-cycling cells. However, several known SAMHD1 mutations can decrease cellular levels of dNTPs without restricting HIV-1 infection, suggesting that the dNTPase activity of SAMHD1 is necessary but not sufficient to enable SAMHD1 to block HIV-1 infection (Cribier et al., 2013; Welbourn et al., 2013; Welbourn and Strebel, 2016; White et al., 2017; White et al., 2013b); rather the ability of SAMHD1 to block HIV-1 infection correlates with the phosphorylation state of T592 (Cribier et al., 2013; Welbourn et al., 2013; White et al., 2017).

SAMHD1 is comprised of a sterile alpha motif (SAM) and a histidine-aspartic acid (HD) domain. SAM domains are protein interaction modules that mediate contact with other SAM domains (Qiao and Bowie, 2005) or non-SAM domain-containing proteins (Qiao and Bowie, 2005), or that bind to a specific DNA sequences of DNA, acting as transcription activators or repressors (Qiao and Bowie, 2005). In contrast, the HD domain is a dGTP-regulated dNTPase that decreases cellular dNTP levels (Goldstone et al., 2011; Kim et al., 2012; Lahouassa et al., 2012; Powell et al., 2011), and also is necessary for SAMHD1 to oligomerize and bind to RNA (Bhattacharya et al., 2016; Brandariz-Nunez et al., 2013; Goncalves et al., 2012; Koharudin et al., 2014; Seamon et al., 2015; Tungler et al., 2013; White et al., 2013a; Yan et al., 2013). Although the HD domain has been reported to have nuclease activity, this remains incompletely understood (Antonucci et al., 2016; Beloglazova et al., 2013; Choi et al., 2015; Ryoo et al., 2014; Seamon et al., 2015).

Germline mutations in the human *SAMHD1* gene are responsible for the auto-inflammatory Aicardi-Goutieres syndrome (AGS), which mimics congenital infection and is manifested by increased production of type I interferon (IFN) (Crow et al., 2006a; Crow and Manel, 2015; Dale et al., 2010; du Moulin et al., 2011; Kretschmer and Lee-Kirsch, 2017; Leshinsky-Silver et al., 2011; Livingston and Crow, 2016; Ramantani et al., 2011; Rice et al., 2009; Thiele et al., 2010). If dysregulated IFN production is a dominant feature in the pathology, such a syndrome is now termed type I interferonopathy (Crow, 2014), where AGS is now classified, as are other AGS-like syndrome like ISG15 and USP18 deficiencies (Bogunovic et al., 2012; Hermann and Bogunovic, 2017; Meuwissen et al., 2016; Zhang et al., 2015). Besides SAMHD1, the AGS phenotype is associated with mutations in six additional genes, *TREX1* (AGS1)(Crow et al., 2006a), *RNASEH2A* (AGS2)(Crow et al., 2006b), *RNASEH2B* (AGS3)(Crow et al., 2006b), *RNASEH2C* (AGS4)(Crow et al., 2006b), *ADAR1* (AGS6)(Rice et al., 2012), and *IFIH1* (AGS7)(Rice et al., 2014). As of now, all proteins affected in the AGS syndrome are involved in nucleic acid metabolism, leading to the hypothesis that AGS is caused by inappropriate activation of type I IFN, following recognition of endogenous DNA and/or RNA species that can activate nucleic-acid-sensing pattern-recognition receptors (Roers et al., 2016).

Perhaps, the best-studied protein associated with AGS development is the 3' repair exonuclease 1 (TREX1) (Crow et al., 2006a; Hoss et al., 1999; Mazur and Perrino, 1999; Stetson et al., 2008). TREX1 is an essential negative regulator of the interferon-stimulatory DNA (ISD) response, an antiviral response triggered by innate sensing of intracellular DNA (Stetson et al., 2008). Loss-of-function mutations in *TREX1* are associated with the development of AGS (Crow et al., 2006a; Lehtinen et al., 2008; O'Driscoll, 2008). TREX1

metabolizes reverse-transcribed DNA from endogenous retroelements, preventing their accumulation. The accumulation of reverse-transcribed DNAs from endogenous retroelements triggers the type I interferon response (Stetson et al., 2008). Overall, these experiments suggested that AGS observed in individuals with *TREX1* mutations is triggered by the inability of TREX1 protein to degrade reverse-transcribed DNA from endogenous retroelements that are left in the cell, triggers the innate immune response. Moreover, it has recently been shown that SAMHD1 is involved in the restriction of endogenous retroelements in cycling cells and that this regulation is controlled by T592 phosphorylation, suggesting that the absence of SAMHD1 allows accumulation/replication of retroelements, which could trigger AGS or autoimmune diseases (Goodier, 2016; Herrmann et al., 2018; White et al., 2016; Zhao et al., 2013).

Inspired by the mechanism used by TREX1 to prevent type I IFN response and by the role of SAMHD1 in myeloid cells HIV biology in particular, we sought to test the hypothesis that absence of SAMHD1 in monocytes triggers spontaneous type I interferon response. In agreement with this hypothesis, here we demonstrate that stable KO of SAMHD1 expression indeed triggers the type I IFN response. Interestingly, SAMHD1 KO cells exhibited spontaneous transcription and translation of several interferon-stimulated genes (ISGs). By using an inhibitor of the catalytic activity of TBK1/IKK ϵ (BX795)(Clark et al., 2009), which blocks its phosphorylation, and hence IRF3 activation and IFN- β production, we were able to prevent spontaneous transcription and translation of ISGs in SAMHD1 KOs. In agreement, we found that SAMHD1 KO cells present high levels of phosphorylated TBK1 when compared to control cells. These experiments suggested that these cells persistently secrete type I IFN, causing autocrine stimulation. To test whether spontaneous transcription and translation of ISGs in SAMHD1 KOs is triggered by secreted type I IFN, we used an antibody against IFN α/β that blocks the ability of IFN α/β to stimulate the interferon receptor. Remarkably, the use of this antibody blocked the spontaneous transcription and translation of ISGs in the knockouts. Taken together, the results described below showed that SAMHD1 KO monocytes spontaneously trigger the type I IFN response. More specifically, the results suggest that SAMHD1 is shielding innate immune sensors from interaction with nucleic acids, thereby preventing the activation of the type I IFN response.

2. Materials and Methods

2.1 Cell lines

Human THP-1 cells (ATCC TIB-202) were grown in growth medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, 1 \times GlutaMAX (Gibco) and 1% penicillin-streptomycin (Gibco). All cells were cultured at 37 °C and 5% CO₂.

For the THP-1 SAMHD1 KO generation, two single guide RNAs (gRNAs) targeting unique sequences targeting exon 1 of the SAMHD1 gene were designed (gRNA1: ⁶⁶CTCAAACACCCCTTCCGCAG₈₆ and gRNA 2: ⁸⁸GGCAGACTGGTCCCCGGGCC₁₀₈) and cloned into the lentiCRISPR v1 plasmid, which contains a puromycin resistance cassette and Cas9 nuclease. Both gRNA constructs were functionally tested for their ability to mediate genomic insertion, deletion or inversions by isolating the genomic DNA from stably transduced and selected polyclonal population of THP-1 cells and performing a Surveyor

nuclease assay as per manufacturer's instructions. Puromycin resistant cells were selected from each gRNA vector transduction, and then sorted by fluorescence-activated cell sorting (FACS) into 96-well plates for clonal expansion. PCR of the SAMHD1 gene was used to analyze the sequences of the KO cell lines. Clone THP-1 SAMHD1 KO 1 has a deletion of 4 nucleotides in exon 1 which gives a SAMHD1 protein with a STOP codon at the beginning of the open reading frame [CCCCTTCCG- (82-87)-GGCAGACTGG], losing the first Met in the sequence. Clone SAMHD1 KO 1-3 has a 14 nucleotides deletion [AAACACCCCT-(78-92)-CTGGTCCCCG], also losing the first Met in the sequence.

2.2 Cell priming with IFN α and BX795 treatment

THP-1, THP-1 Contrail or THP-1 SAMHD1 KO cells were primed by incubation with 10, 100, 1000 or 5000 IFN Alpha A U/ml (Hu-IFN- α A; Hu-IFN- α 2a, Millipore) in normal RPMI 10% FCS medium for 30 minutes, 24 h or as indicated. After incubation, cells were collected for transcript (RNA) and protein expression of ISGs.

The TBK1/IKK ϵ catalytic inhibitor of phosphorylation BX795 (Invivogen), which prevents IRF3 activation and IFN- β production, was used at concentrations of 2 and 4 μ M in non-PMA treated cells for 48 h at 37 °C in RPMI culture medium containing 10% FCS. After incubation, cells were collected for protein expression analysis.

2.3 ISG Expression Analysis by qRT-PCR

RNA was extracted from THP-1 cells (Qiagen RNeasy) and reverse-transcribed (SuperScript III reverse transcriptase) according to the manufacturer's instructions. Levels of ISG expression (*IFN- β* , *IFIT1*, *MXA*), relative to the *ACTIN* RNA housekeeping gene, were analyzed by Eppendorf quantitative real-time PCR using SYBRTM Green PCR Master Mix (Applied Biosystems). The primer sequences used were as follows: *ACTIN* Forward 5' AACACCCAGCCATGTACGT'3 and Reverse 5' CGGTGAGGATCTTCATGAGGTAGT'3, *IFN- β* (NM002176.3) Forward 5' ACCTCCGAAACTGAAGATCTCCTA'3 and Reverse 5' TGCTGGTTGAAGAATGCTTGA'3, *IFIT1* (NM001548.4) Forward 5' TTGCCTGGATGTATTACCAC'3 and Reverse 5' GCTTCTTGCAAATGTTCTCC'3 and *MXA* (NM001144925.1) Forward 5' AGGACCATCGGAATCTTGAC'3 and Reverse 5' TCAGGTGGAACACGAGGTTC'3. Relative expression of the ISGs were calculated by the Ct method, with comparison with the mean value for the mock-treated THP-1 Control 1 cells.

2.4 ISG Protein Expression

One million THP-1 cells, undifferentiated or treated for 24 h with PMA (40ng/mL), were grown in six-well dishes, and lysed in 0.1 ml of whole-cell extract (WCE) buffer [50 mM Tris pH 8.0, 280 mM NaCl, 0.5% IGEPAL, 10% glycerol, 5 mM MgCl₂, 50 μ g/ml ethidium bromide, 50 U/ml benzonase (Roche)]. The extract was then incubated for 1 h at 4 °C, and the cell debris removed by centrifugation at 20,000 *g* for 1 h at 4 °C. The supernatant was then mixed with 5 \times protein-loading buffer and the samples were analyzed by SDS-PAGE and Western blotting with anti-ISG antibodies [anti-MXA (1:1000; Abeam), anti-MXB (1:1000; Novus Biologicals), anti-ISG15 (1:500; Santa Cruz Biotechnology), anti-SAMHD1

(1:1000; house made rabbit anti-SAMHD1) and anti-GAPDH (1:5,000; Ambion)] in a buffer containing 5% non-fat Milk in PBS-Tween 1%. Secondary antibodies against rabbit and mouse conjugated to IRDye 680LT or IRDye 800CW were obtained from Li-Cor (1:10,000 diluted). Protein concentrations were quantified by optical densitometry of the gel band, using the program Image J and normalizing to the GAPDH band intensity.

In the case of STAT1, STAT2, pSTAT1, pSTAT2, USP18 and pTBK1, cells were lysed RIPA buffer (Thermo Scientific), and protease/phosphatase inhibitor cocktail (Cell signaling technologies) and analyzed by western blot. The following antibodies were used: USP18 (Cell Signaling Technology D4E7, 1:1,000); Stat1 (Santa Cruz C-111, 1:1,000); Stat2 (Santa Cruz C-20, 1:1,000); phospho-Stat1 (Cell Signaling Technology 9171 L, 1:1,000); phospho-Stat2 (Millipore 07224, 1:1000); pTBK1 (Cell Signaling Technology 5483 1:1,000); GAPDH (Millipore MAB374, 1:20,000). Antibody binding was detected by enhanced chemiluminescence (Western Lightning, Perkin Elmer).

2.5 IFN bioassay

To examine the production of type I IFN the reporter cell line HEK293-ISRE-Luc was used (Saito et al., 2016). This reporter cell line carries IFN-stimulated response element within the promoter region driving the expression of luciferase. One day prior to use, HEK293-ISRE-Luc cells were plated on a 96-well flat bottom plate at 30,000 cells per well with 100 μ l of cell suspension. One day after, 100 μ l of culture supernatant from THP-1 cells was harvested and transferred into the 96-well flat bottom plate containing HEK293-ISRE-Luc cells for 24 h. In each experiment, the IFN standard curve was generated using a stock solution of recombinant interferon alpha A (Millipore). A working solution of IFN at 10,000 units per mL was used to generate 10-fold serial dilutions. These standard dilutions were added at 10 μ l per well in triplicates. The cells were placed in a tissue culture incubator after addition of supernatant samples and IFN standards. At the following day, the cells were lysed with 25 μ l of 1 \times buffer prepared from Luciferase Cell Culture Lysis 5 \times Reagent (Promega) for 20 minutes at -80°C . Twenty five μ l of cells lysates was transferred to an opaque 96-well plate and 100 μ l of the luciferase assay reagent (Promega) was added, followed by reading the relative luciferase units in a luminometer.

2.6 Statistical analysis

To compare the effects of each cell line in relation to its control, all data were analyzed using Student's t-test. Differences were considered statistically significant at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***), not significant (ns).

3. Results

3.1 THP-1 cells stably knocked out for SAMHD1 expression show increased basal transcription and translation of IFN-stimulated genes (ISGs).

Mutations in the human SAMHD1 gene trigger AGS, in an autosomal recessive fashion with the phenotype being characterized by cerebral vasculopathy and early stroke. Interestingly, this phenotype correlates with high levels of type I IFN and ISGs in the peripheral blood of these patients (Dale et al., 2010; Rice et al., 2009). In an attempt to model this human *in*

vivo phenotype in a traceable *in vitro* system, we stably knocked out SAMHD1 in a monocytic cell line.

We used human monocytic THP-1 cells and the CRISPR-Cas9 system to knockout *SAMHD1*. As shown in Figure 1A, three independent knockout clones were obtained (KO 1, KO 2, and KO 1-3). Expression of SAMHD1 was assayed by Western blotting using a specific antibody against SAMHD1. Two CRISPR control cell lines that retained normal SAMHD1 expression (THP-1 Control 1 and THP-1 Control 2) were also obtained (Fig. 1A). Next, we tested the basal transcription of different ISGs by qRT-PCR. For this purpose, we tested the basal transcription of *MXA* and *IFIT1* (Fig. 1B). Remarkably, basal transcription of *MXA* increased ~20-, ~7-, and ~40-fold for THP-1 SAMHD1 KO 1, THP-1 SAMHD1 KO 2, and THP-1 SAMHD1 KO 1-3 cells, respectively, when compared to THP-1 Control 1 cells (Fig. 1B). Similarly, *IFIT1* transcripts increased ~30-, ~6- and ~20-fold for THP-1 SAMHD1 KO 1, THP-1 SAMHD1 KO 2, and THP-1 SAMHD1 KO 1-3 cells, respectively (Fig. 1B). Expression of the different transcripts was normalized to actin (Fig. 1B). These experiments showed that stable SAMHD1 KO increased basal transcription of ISGs in THP-1 SAMHD1 KO clones.

Because the increased basal transcription of ISGs may be due to production of type I IFNs, we tested whether production of IFN- β is increased in SAMHD1 KO clones (Fig. 1C). Interestingly, the basal transcription levels of IFN- β increased 15-, 3-, and 25-fold in THP-1 SAMHD1 KO 1, THP-1 SAMHD1 KO 2 and THP-1 SAMHD1 KO 1-3 cells, respectively, when compared to THP-1 CRISPR control cells (THP-1 Control 1) (Fig. 1C). This result indicated that IFN- β is up-regulated in SAMHD1 KO clones.

To further determine whether the observed transcriptional activation translates into protein expression, we tested for *MXA*, *MXB*, and *ISG15* production in the three THP-1 SAMHD1 KO clones 1, 2, and 1-3 by Western blotting (Fig. 1D). As a control, and to ensure that the observed phenotype was not an artifact of the CRISPR/Cas9 procedure, we utilized two different THP-1 CRISPR control clones (THP-1 Control 1 and Control 2). As shown in Figure 1D, all tested THP-1 SAMHD1 KO clones expressed higher protein levels of *MXA*, *MXB* and *ISG15* when compared to THP-1 control clones or THP-1 wild-type (WT) cells. As a loading control, we measured expression of *GAPDH*. The results indicate that stable KO of SAMHD1 increases the basal levels of type I IFN, thereby increasing transcription and translation of ISGs. The bar graphs in Figure 1D show the fold-induction relative to THP-1 Control 1 cells normalized to *GAPDH*.

We next tested whether differentiation of THP-1 SAMHD1 KO cells to a non-cycling state with phorbol 12-myristate 13-acetate (PMA) changed the observed phenotype (Schwende et al., 1996). PMA-treated THP-1 SAMHD1 KO clones (Clones 1, 2, 1-3) still showed increased *MXA*, *MXB* and *ISG15* expression when compared to THP-1 CRISPR control clones (THP-1 Control 1 and Control 2) (Fig. 1E). These experiments suggested that absence of SAMHD1 increases basal transcription levels of ISGs in cycling and non-cycling cells.

Next we tested whether knocking out the expression of SAMHD1 in epithelial cells resulted in the increased expression of ISGs. For this purpose, using the CRISPR/Cas9 methodology, we prepared HT-1080 cells that are KO for the expression of SAMHD1 (Data not shown). Surprisingly, none of the HT-1080 SAMHD1 KO cells exhibited increased expression of the protein MxB. These results indicate that this phenotype may only occur in cells that are derived from the immune system lineages.

To assess whether the phenotype observed in THP-1 SAMHD1 KO cell lines changes in the presence of human serum, we measured expression of MxA (Fig. 1S). Interestingly, we observed a stronger phenotype in human serum when compared to fetal bovine serum (Fig. 1S). The use of human serum, which is a condition closer to in vivo, increases the strength of this phenotype suggesting that human serum must contain agents that accentuate this phenotype.

Altogether our results suggested that ISGs are upregulated in THP-1 SAMHD1 KO clones and this may be due to the constant production of type I IFNs by KO clones.

3.2 THP-1 cells stably knocked out for SAMHD1 show intact responsiveness to type I IFN.

The type I interferon response needs to be tightly regulated for preservation of immune homeostasis (Chen et al., 2017). Dysregulation of IFN responses are linked to immune and autoimmune disorders (Kretschmer and Lee-Kirsch, 2017). Because AGS is an immune disorder, we tested whether response to type I IFN is intact in SAMHD1 KO cells. Thus, we stimulated THP-1 SAMHD1 KO clones with 1000 U/ml of IFN α , and measured MXA and IFIT1 transcription 24 h later. As shown in Figure 2A, stimulation of THP-1 SAMHD1 KO clones 1, 2, and 1-3 with 1000 U/ml of IFN α triggered equally robust MXA transcription response when compared to THP-1 Control 1 cells. Similarly, induction of IFIT1 transcription was similar when compared to THP-1 Control 1 cells (Fig. 2A).

To test whether transcriptional activation in THP-1 SAMHD1 KO cells translates into increased protein expression, we performed the same experiment but measured protein expression by Western blotting. Thus, we stimulated THP-1 SAMHD1 KO cells with 1000 or 5000 U/ml of IFN α , and measured MXA, MxB and ISG15 protein expression 24 h later. As shown in Figure 2B, THP-1 SAMHD1 knockout cells (THP-1 SAMHD1 KO 1-3) showed similar levels of protein expression of MXA, MxB and ISG15 when compared to THP-1 Control 1 cells. In agreement with the transcription assay results, these experiments showed that basal translation of ISGs is likewise upregulated in THP-1 SAMHD1 KO cells. Similar results were obtained by using lower amounts of IFN α (Fig. 2C). Altogether these results indicated that SAMHD1 KO cells have normal responsiveness to type I IFN stimulation. This was important to document as type I IFN hypersensitivity was observed in patients with AGS-like disease, who lack USP18 or ISG15, a negative feedback regulators of type I IFN signaling (Meuwissen et al., 2016; Speer et al., 2016).

3.3 The small molecule BX795 inhibits spontaneous production of type I IFN in THP-1 SAMHD1 KO cells.

We sought to test whether spontaneous induction of ISGs in THP-1 SAMHD1 KO cells is due to persistent stimulation and production of type I IFN, which may auto-stimulate cells

and expression of ISGs. To this end, we used BX795, a small-molecule inhibitor of the catalytic activity of TBK1/IKK ϵ that blocks the latter's phosphorylation, preventing IRF3 activation and IFN- β production (Clark et al., 2009); and ultimately shuts down the TBK1-IRF3 pathway, which is used by several intracellular sensors to induce type I IFN response. As shown in Figure 3A, use of increasing BX795 concentrations attenuated spontaneous expression of ISGs (MXA, MXB and ISG15) in all the three THP-1 SAMHD1 KO clones (1, 2, 1-3) when compared to THP-1 Control 1 cells or WT THP-1 cells. Interestingly, treatment with 2 μ M BX795 for 48 h dramatically reduced expression of ISGs in THP-1 SAMHD1 KO clones. The bar graphs in Figure 3B represent fold-induction, normalized to GAPDH relative to the untreated KO clone (Mock). The results of these experiments suggested that spontaneous expression of ISGs in THP-1 SAMHD1 KO clones may be caused by persistent production of type I IFNs, which are responsible for the higher basal levels of ISGs in THP-1 SAMHD1 KO cells.

Because BX795 concentrations attenuated spontaneous expression of ISGs (MXA, MXB and ISG15) in THP-1 SAMHD1 KO clones, we tested the phosphorylation state of TBK1 in THP-1 SAMHD1 KO 1 cells. As shown in Figure 3C, the levels of phosphorylated TBK1 (pTBK1) are upregulated in the THP-1 SAMHD1 KO 1 cells when compared with the THP-1 control cells. This result suggests that the element or elements triggering the expression of ISGs in the THP-1 SAMHD1 KO cell lines are promoted by the phosphorylation of TBK1.

To examine the production of IFN by THP-1 SAMHD1 KO cell lines, we used the HEK293T-ISRE-Luc reporter cell line (Saito et al., 2016). The HEK293T-ISRE-Luc reporter cell line contains the interferon stimulated response element (ISRE) fuse to the luciferase gene. To validate the assay, an IFN α standard curve was generated (Fig. 3D). To measure the levels of IFN α in the THP-1 SAMHD1 KO cells, we incubated supernatants from THP-1 SAMHD1 KO cell, treated or not with 2 μ M of BX795, with HEK293T-ISRE-Luc for 24 h. As shown in Figure 3E, luciferase activity was higher for THP-1 SAMHD1 KO cells when compared to control cells and is reduced when treated with BX795.

3.4 Use of anti-IFN α / β prevents spontaneous expression of ISGs in THP-1 SAMHD1 KO cells.

To further characterize the spontaneous induction IFN- β and activation of the type I IFN pathway, we measured the expression of phosphorylated STAT1 and STAT2 (pSTAT1 and pSTAT2) in THP-1 SAMHD1 KO cells upon IFN α stimulation. For this purpose, we incubated THP-1 SAMHD1 KO 1 cells with increasing concentrations of IFN α for 30 min, and measured phosphorylated STAT1 and STAT2. As shown in Figure 4A and B, levels of STAT1 and STAT2 expression were higher in THP-1 SAMHD1 KO 1 cells than in THP-1 Control 1 cells. Similarly, pSTAT2 levels and pSTAT1 levels were higher in THP-1 SAMHD1 KO 1 cells than in THP-1 Control cells.

To test whether spontaneous transcription and translation of ISGs in SAMHD1 KO cells is triggered by secreted type I IFN, we used an antibody against IFN α / β that blocks the latter's ability to stimulate the interferon receptor. For this purpose, THP-1 SAMHD1 KO 1 and control cells were treated with 5 μ g/ml of anti-IFN α / β for 30 mins (Fig. 4B), and levels of

ISG15, UPS18, STAT1, STAT2, pSTAT1, and pSTAT2 were measured by Western blotting. As shown in Figure 4B, anti-IFN α/β antibodies significantly reduced expression of the interferon-stimulated genes ISG15 and USP18. Similarly, levels of phosphorylated STAT1 and STAT2 were also reduced to those in THP-1 Control 1 cells. These findings suggested that spontaneous expression of ISGs in THP-1 SAMHD1 KO clones is due to secreted type I IFN, which is persistently stimulating the type I IFN receptor.

4. Discussion

Compelling evidence in patients suggests that SAMHD1 is a negative regulator of the type I IFN response (Rice et al., 2009). AGS is a heritable disease linked to mutations of the SAMHD1 gene, causing afflicted individuals to exhibit increased production of type I IFN (Crow et al., 2015; Dale et al., 2010; du Moulin et al., 2011; Kretschmer and Lee-Kirsch, 2017; Leshinsky-Silver et al., 2011; Livingston and Crow, 2016; Rice et al., 2009; Thiele et al., 2010). However, the mechanism by which type I IFN is upregulated in patients with mutations or deletions of SAMHD1 is unknown. Consistently with these observations, SAMHD1 KO mice display increased levels of type I IFN (Maelfait et al., 2016a; Rehwinkel, 2014; Rehwinkel et al., 2013). In agreement with these observations, our experiments showed that stably knocking out SAMHD1 in THP-1 cells increased the basal levels of type I IFN, in a way replicating the phenotype seen in patients with AGS (Berger et al., 2011; Puigdomenech et al., 2013). The present work provided a model to study the spontaneous type I IFN stimulation that occurs in the absence of SAMHD1.

To demonstrate that THP-1 SAMHD1 KO cells spontaneously produce type I IFN, we measured transcript and protein levels of classic ISGs such as MXA and IFIT1. THP-1 SAMHD1 KO clones showed increased transcript and protein levels of the tested ISGs. This suggests that absence of SAMHD1 correlates with increased ISG transcription and translation. One possibility is that SAMHD1 degrades, or binds to, an as yet unidentified nucleic acid (RNA or DNA) with the ability to trigger innate immune sensors, e.g. RIG-I-like receptors (RLRs), cytosolic DNA sensors (CDSs) or the protein “stimulator of interferon” (STING) (Habjan and Pichlmair, 2015; Mankan et al., 2014; Wu and Chen, 2014). RLRs such as RIG-I and MDA5 are cytoplasmic RNA helicases that sense double-stranded RNA, leading to activation of type I IFN through the TBK1-IRF3 pathway (Fujita et al., 2007; Gleason et al., 2011; Hiscott, 2007; Tu et al., 2013; Yoneyama and Fujita, 2007). Similarly, CDSs sense damaged or pathogenic DNA, leading again to type I IFN production through the TBK1-IRF3 pathway (Ma et al., 2015a). STING protein on the other hand is activated by cyclic dinucleotides, leading also to a potent type I IFN response mediated by the TBK1-IRF3 pathway (Ma et al., 2015b; Tanaka and Chen, 2012). In this scenario, SAMHD1's role might be to degrade, or bind to, any of the aforementioned substrates (DNA and/or RNA), thereby preventing type I IFN activation. In the absence of SAMHD1, any or all of these intracellular substrates could conceivably activate type I IFN. (Maelfait et al., 2016b; Meuwissen et al., 2016).

AGS-like diseases are also developed by mutations that are involved in the type I IFN negative feedback regulation loop, as is the Ubiquitin-specific peptidase 18 (USP18) (Meuwissen et al., 2016; Taylor et al., 2018), which causes enhanced induction of ISGs after

stimulation with type I IFN, a phenotype known as hypersensitivity response to IFN. To understand whether the absence of SAMHD1 triggers type I IFN hypersensitivity, we stimulated the THP1-SAMHD1 KO cells with IFN α and measure the levels of different ISGs, observing that the cells lacking SAMHD1 behaved like THP1 control cells, suggesting that a negative feedback loop may not exist.

Since most of the known pathways sensing cytosolic RNA and DNA lead to type I IFN via the TBK1-IRF3 pathway, we decided to test whether inhibition of this pathway by the small-molecule inhibitor BX795 can abrogate the spontaneous type I IFN phenotype (Pokatayev et al., 2016). Interestingly, BX795 restored the native phenotype in THP-1 SAMHD1 KO cells. In agreement, we found that the levels of phosphorylated TBK1 in THP-1 SAMHD1 KO cells are upregulated when compared to control cells, suggesting that the TBK1-IRF3 pathway is activated in these cells. Overall, these results suggested that absence of SAMHD1 leads to activation of type I IFN by a signaling mechanism initiated by a cytosolic nucleic acid. This is consistent with growing evidence that all known defective genes associated with AGS are somehow involved in the metabolism of nucleic acids (Roers et al., 2016).

In agreement with the conclusion that nucleic acids are activating innate immune sensors in THP-1 SAMHD1 KO cells, it is well established that SAMHD1 binds to RNA and DNA (Goncalves et al., 2012; Tungler et al., 2013). Triggering of the type I IFN response in THP-1 SAMHD1 KO cells by RNA or DNA might be of self or non-self origin. A similar situation is observed in the case of the DNase protein TREX1 (O'Driscoll, 2008; Stetson et al., 2008), which binds to and subsequently degrades both self- and nonself- DNA species, thereby preventing activation of type I IFN response (Crow et al., 2015; Stetson et al., 2008; Yang et al., 2007). Future experiments will pursue the innate immune sensor that triggers the type I IFN response in THP-1 SAMHD1 KO cells.

Lastly this work explored whether the spontaneous induction of ISGs in THP-1 SAMHD1 KO cells is caused by IFN α/β autocrine stimulation. Interestingly, we observed that the use of anti-IFN α/β antibodies in the supernatant of THP-1 SAMHD1 KO cells prevents the induction of the type I IFN response. This result suggested that IFN α/β is produced and secreted by THP-1 SAMHD1 KO cells to subsequently stimulate the type I IFN receptor. Noteworthy was the fact that STAT1 and STAT2 levels are higher in THP-1 SAMHD1 KO cells than in control cells, also suggesting that the type I IFN pathway is active in THP-1 SAMHD1 KO cells.

The work reported here suggests that absence of SAMHD1 causes nucleic acids in the cytosol of THP-1 cells to trigger innate immune sensors that, in turn, use the TBK1-IRF3 pathway to activate the type I IFN response. These findings are a first step toward unraveling the complex molecular mechanism by which patients with SAMHD1 deficiencies exhibit high levels of type I IFN, and suggest the possibility of using the small-molecule inhibitor BX795 or an improved next-generation molecule to treat patients with early-stage AGS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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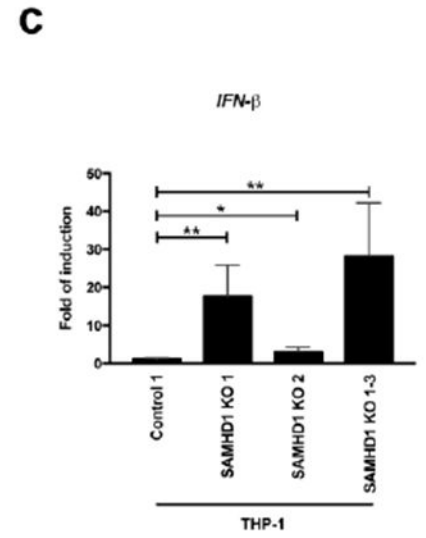
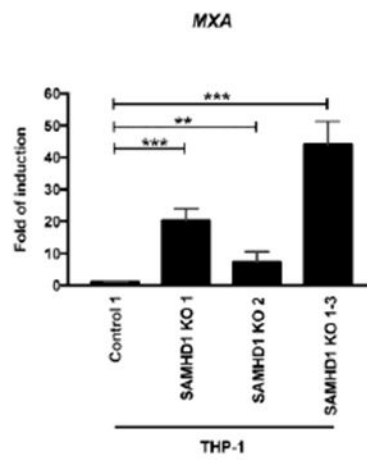
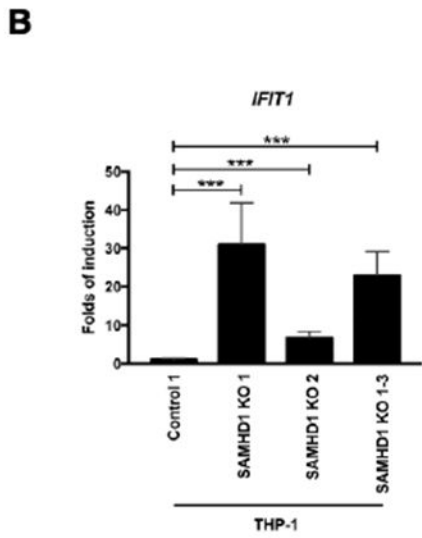
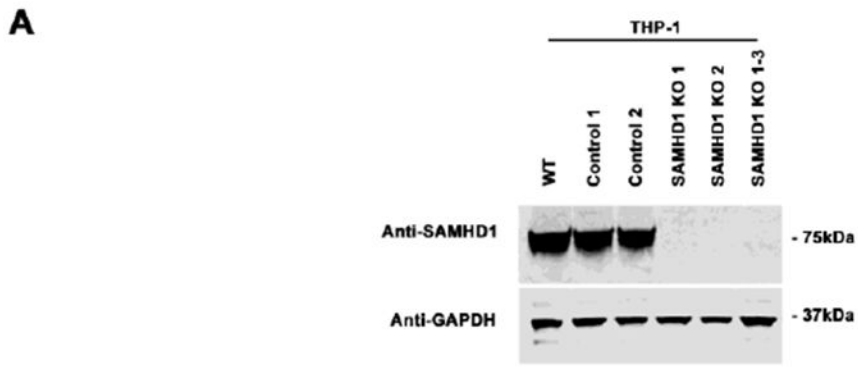
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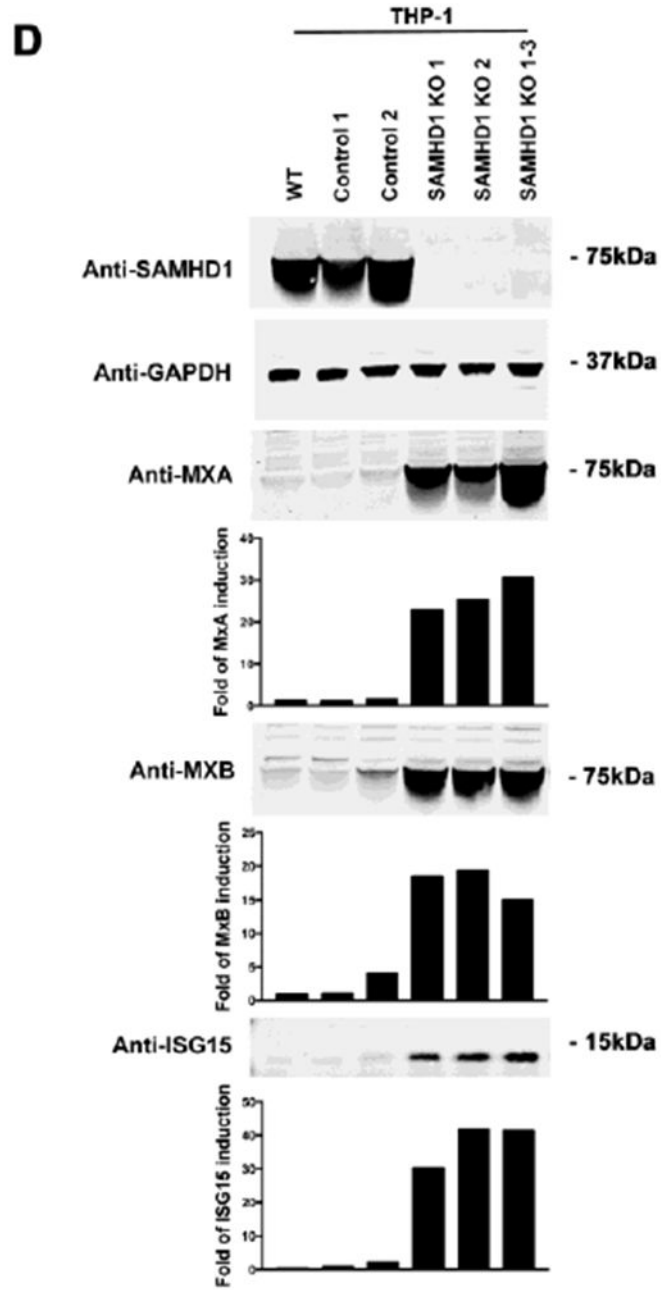
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Highlights

- SAMHD1 KO human cells replicate the phenotype observed in patients with Aicardi-Goutières Syndrome.
- SAMHD1 KO cells show spontaneous expression of type I IFNs and interferon-stimulated genes.
- Inhibition of the TBK1-IRF3 pathway abrogated the production of type I IFNs in SAMHD1 KO cells.
- Spontaneous expression of ISGs in SAMHD1 KO cells is due to secreted type I IFN.





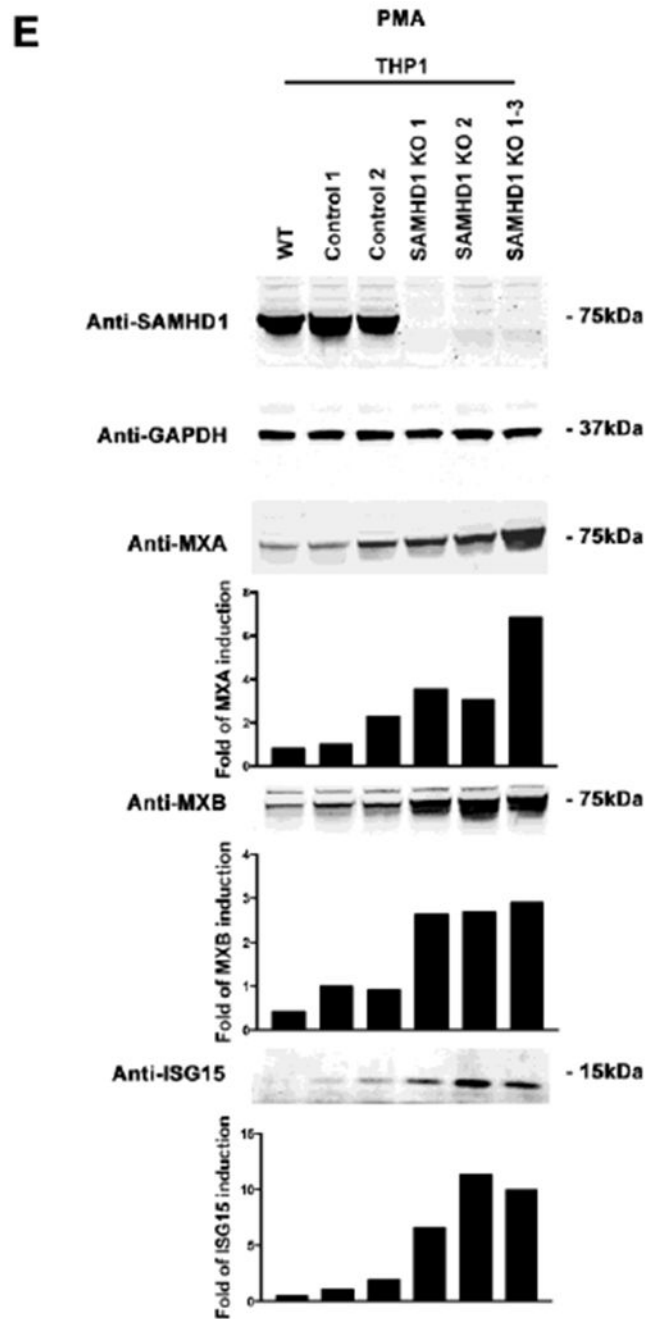
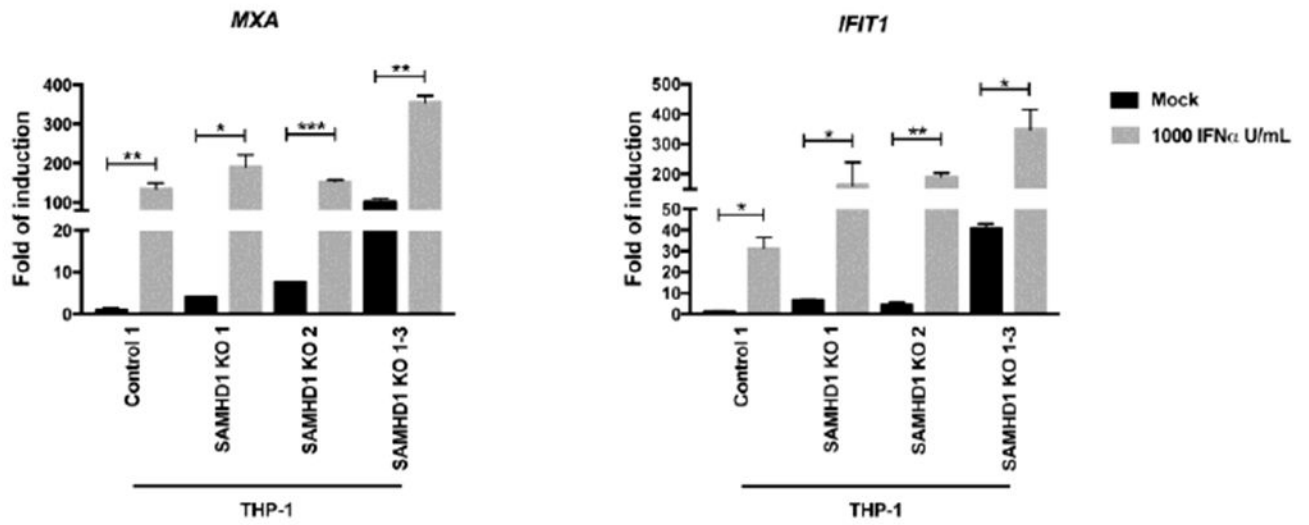


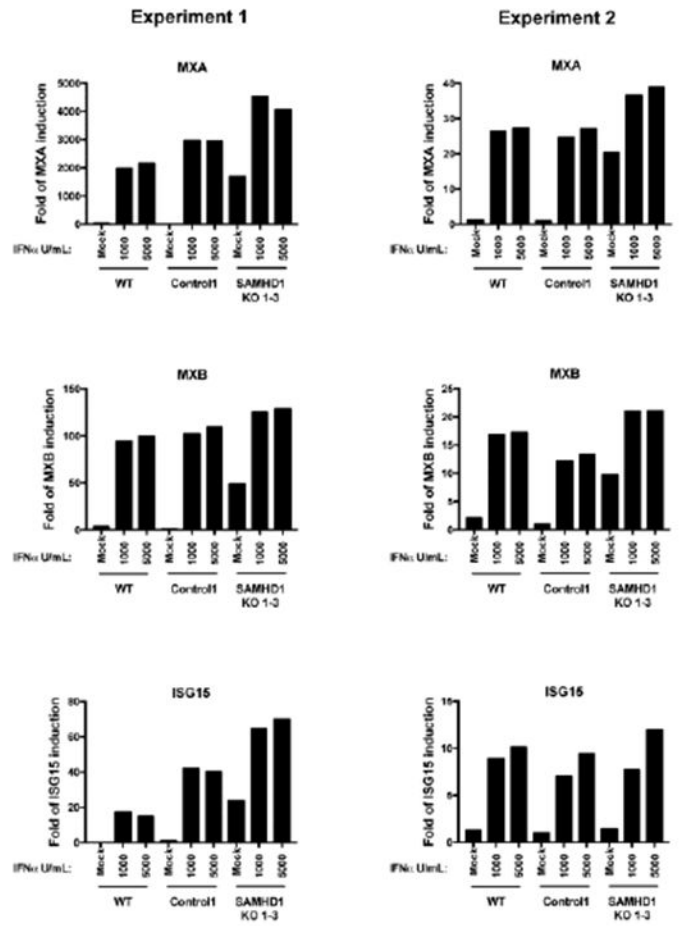
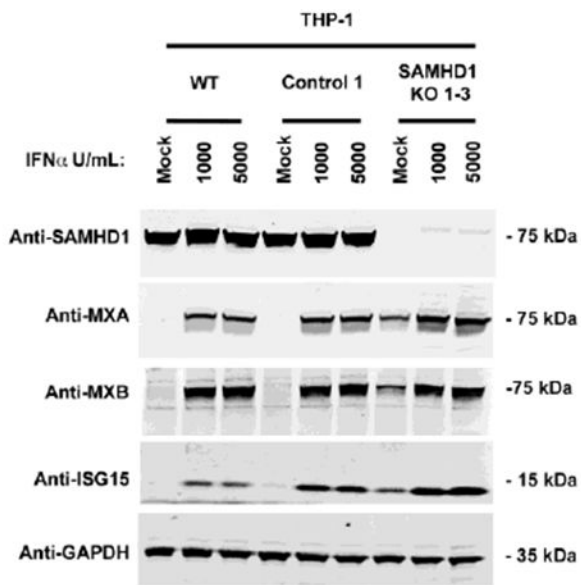
Figure 1. THP-1 cells stably knockout for SAMHD1 expression showed increased basal transcription and translation of interferon-stimulated genes (ISGs).

(A) SAMHD1 KO clones were prepared by using the CRISPR-Cas9 system. Three different THP-1 SAMHD1 KO clones (SAMHD1 KO 1, 2 and 1-3) were identified by Western blotting using antibodies against SAMHD1 (upper panel). THP-1 clones that underwent the CRISPR-Cas9 protocol but did not lose expression of SAMHD1 (Controls 1 and 2) were also identified (upper panel). In addition, a wild type THP-1 cell line (WT) was included. As a loading control, samples were also analyzed for GAPDH expression (upper panel). (B)

Transcript levels of MXA and IFIT1 were analyzed by qRT-PCR in the indicated THP-1 SAMHD1 KO clones, and were compared to THP-1 control cells using a specific set of primers for each indicated gene (lower panel). Transcript levels were normalized to actin, and fold-induction relative to the THP-1 Control 1 is shown. $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) using two tailed Student's t-test are shown. **(C)** Similarly the transcript levels of *IFN β* were measured. Transcript levels were normalized to actin, and fold-induction relative to the THP-1 Control 1 is shown. **(D)** MXA, MXB and ISG15 protein levels were analyzed by Western blotting in the indicated THP-1 SAMHD1 KO clones. Expression levels for THP-1 Control clones are also shown. As a loading control, samples were also analyzed for GAPDH. Quantification of band intensities normalized to GAPDH is shown. Experiments were repeated at least three times and a representative example is shown. **(E)** Protein levels of MXA, MXB and ISG15 were also analyzed in PMA-treated THP-1 SAMHD1 KO clones. Quantification of band intensities normalized to GAPDH is shown. Experiments were repeated at least three times and a representative example is shown.

A

B



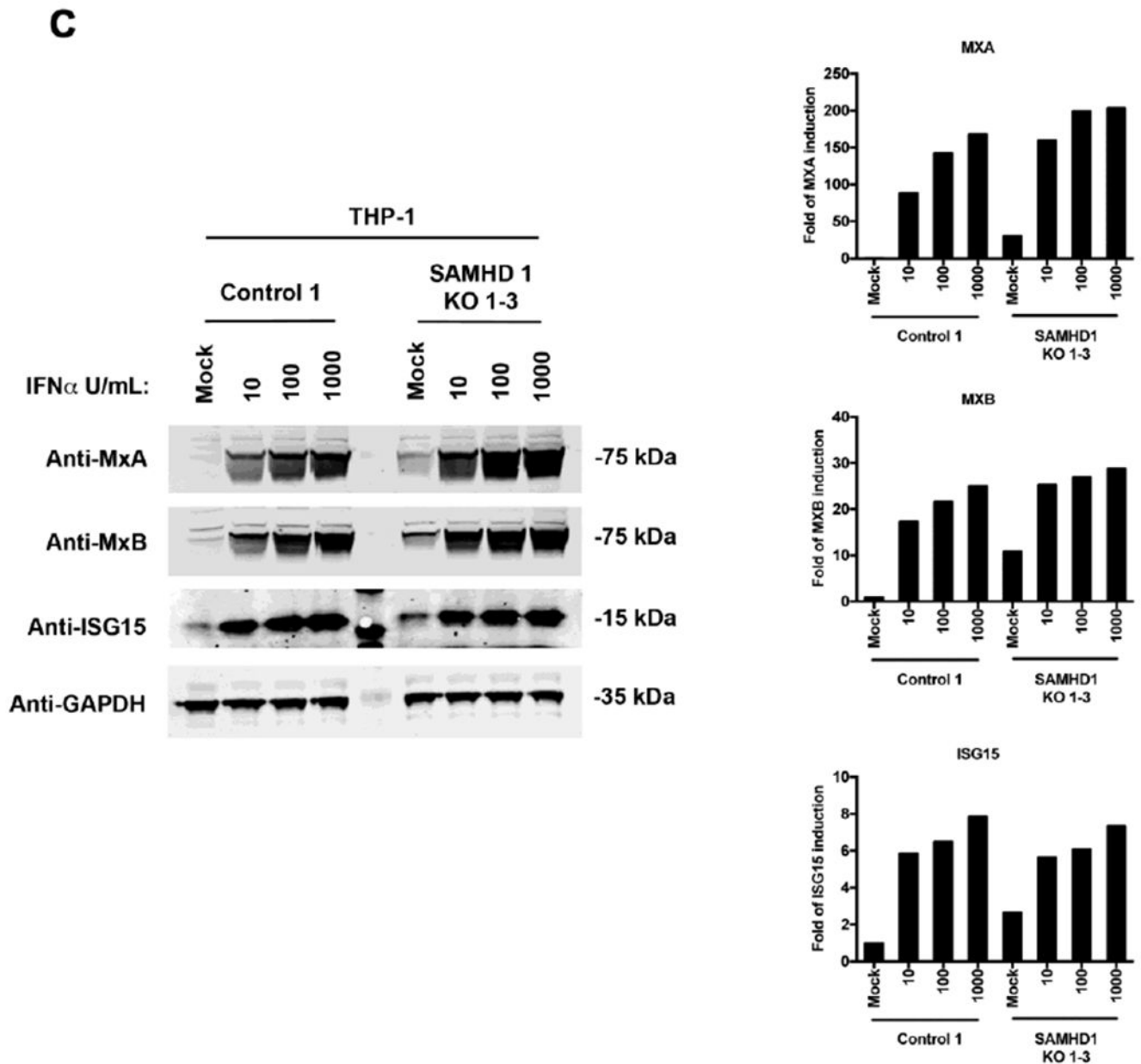


Figure 2. THP-1 cells stably knocked out for SAMHD1 show intact responsiveness to type I IFN. (A) Transcript levels for MXA and IFIT1 were determined in IFN α -treated THP-1 SAMHD1 KO clones. The indicated THP-1 SAMHD1 KO clones were treated with 1000 U/ml for 24 h, after which the cells were lysed and processed for RNA extraction. Transcription levels were determined by qRT-PCR using a set of specific primers for each indicated gene. Transcript levels were normalized to actin, and the fold-induction relative to THP-1 Control 1 is shown. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) or not significant (ns), using two-tailed Student's t-test are shown. (B) MxA, MxB and ISG15 levels were measured in THP-1 SAMHD1 KO 1-3 cells treated with the indicated concentrations of IFN α for 24 h (left panel). Protein levels were determined by Western blotting using the indicated antibodies (left panel). Expression of the different proteins was quantifying and

normalized to GAPDH. Experiments were repeated at least three times and two independent experiments are shown (right panel). (C) Similar experiments to measure the levels of MxA, MxB and ISG15 were performed using lower concentrations of IFN α (left panel). Expression of the different proteins was quantifying and normalized to GAPDH. Experiments were repeated at least three times and a representative experiment is shown (right panel).

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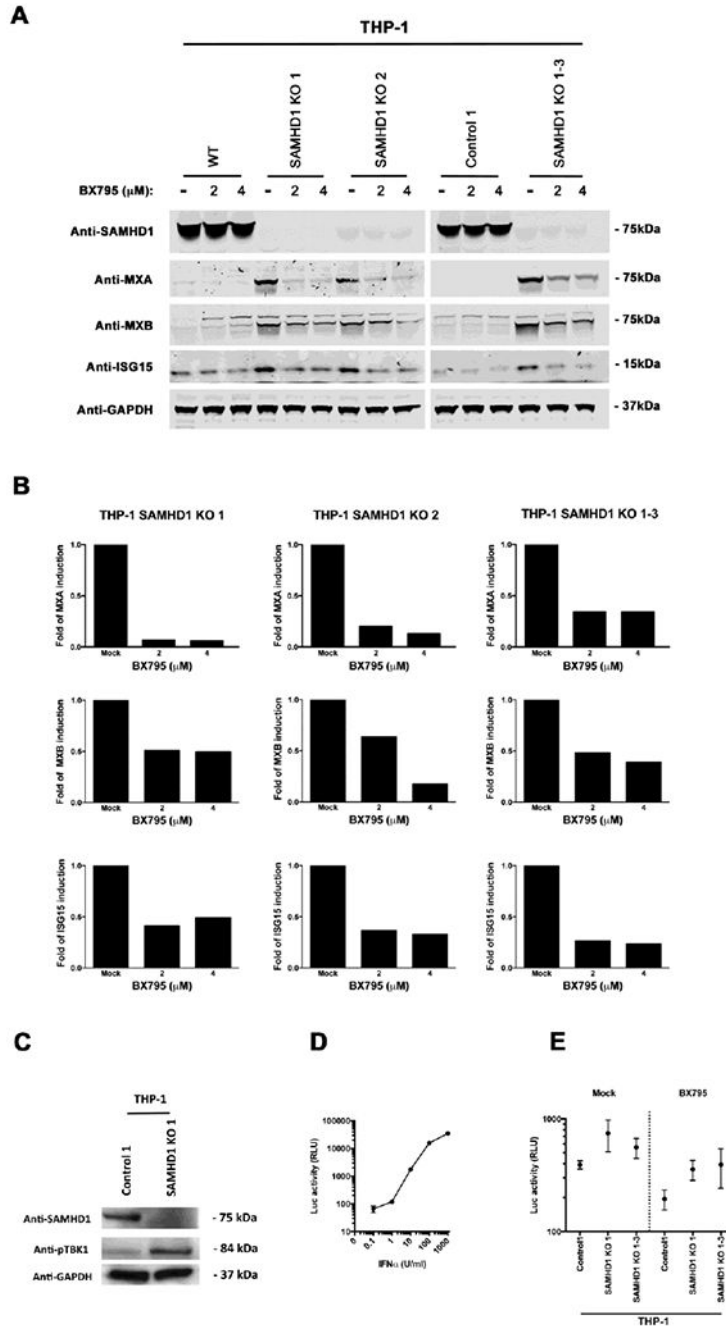


Figure 3. The TBK1 inhibitor BX795 prevents spontaneous expression of ISGs in THP-1 SAMHD1 KO cells.

(A) Expression of MxA, MxB and ISG15 in BX795-treated THP-1 SAMHD1 KO clones. THP-1 SAMHD1 KO clones were treated with the indicated concentrations of BX795 for 48 hours, after which the cells were lysed, and protein expression was measured by Western blotting using specific antibodies against MxA, MxB, and ISG15, respectively. As a loading control, samples were also analyzed for GAPDH. (B) Band quantification normalized to GAPDH is shown. Experiments were repeated three times for each KO cell line, and a

representative example is shown. **(C)** THP-1 SAMHD1 KO 1 cells were analyzed for the expression of phosphorylated TBK1 (pTBK1) by Western blotting. As a loading control, samples were also analyzed for GAPDH. **(D)** HEK293-ISRE-Luc cells were treated with the indicated concentrations of IFN α for 24 h. Subsequently, cells were lysed and analyzed for luciferase activity. **(E)** Supernatant harvested from THP-1 control, THP-1 SAMHD1 KO 1 and KO 1-3, treated or not with 2 μ M of BX975 for 48h, were used to treat HEK293-ISRE-Luc cells for 24 h. Subsequently, cells were lysed and analyzed for luciferase activity.

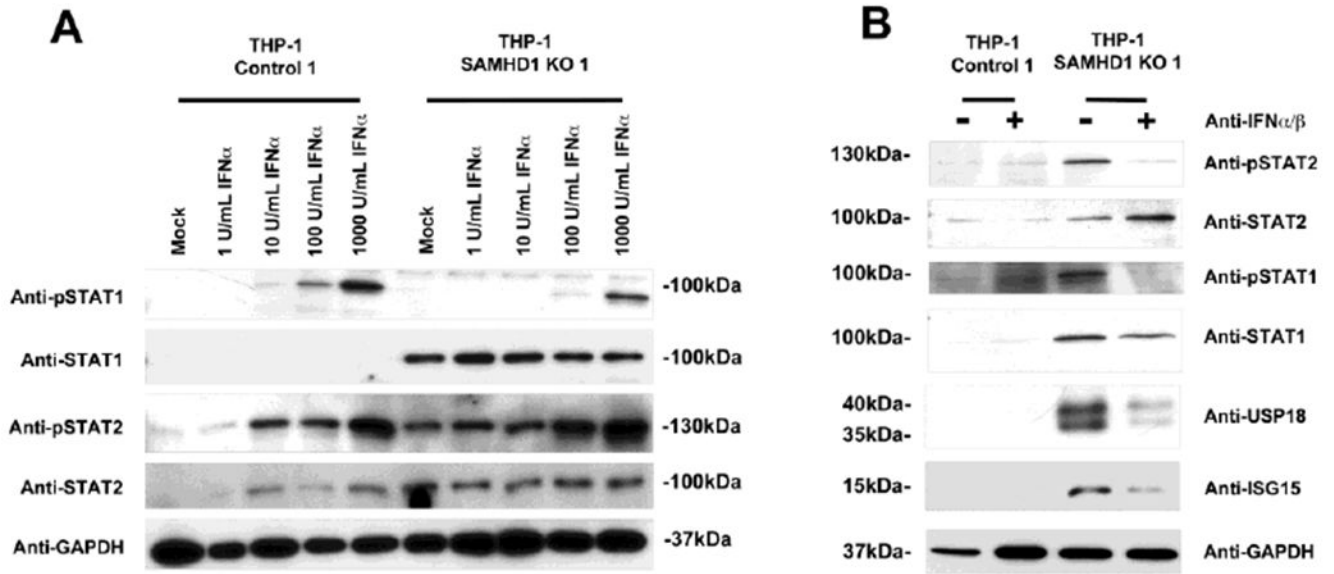


Figure 4. Anti-IFN α/β antibody prevents spontaneous production of ISGs in THP-1 SAMHD1 KO cells.

(A) THP-1 SAMHD1 KO 1 cells were treated with increasing amounts of IFN α . After 30 min, cells were lysed, and STAT1, STAT2, phosphorylated-STAT1 (pSTAT1), and phosphorylated-STAT2 (pSTAT2) expression was measured by Western blotting using specific antibodies. Similar analysis was performed in THP-1 Control 1 cells. As a loading control, samples were analyzed for GAPDH. (B) THP-1 SAMHD1 KO 1 cells were treated with IFN α for 30 min in the presence of anti-IFN α/β antibodies. The cells were then lysed and STAT1, STAT2, pSTAT1, pSTAT2, USP18 and ISG15 expression was measured by Western blotting using specific antibodies. As a loading control, samples were analyzed for expression of GAPDH. As a control, serum from sheep was used. Experiments were performed a least three times, and a representative is shown.