Metabolic regulators Nampt and Sirt6 serially participate in the macrophage interferon antiviral cascade

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Abstract
Molecular determinants underlying interferon (IFN)-macrophage biology can help delineate enzyme systems, pathways and mechanisms for enabling host-directed therapeutic approaches against infection. Notably, while the IFN antiviral response is known to be directly coupled to mevalonate-sterol biosynthesis pathway mechanistic insight for providing host pathway-therapeutic targets, remain incomplete. Here, we show that Nampt and Sirt6 are coordinately regulated upon immune activation of macrophages and contribute to the IFN-sterol antiviral response. In silico analysis of the Nampt and Sirt6 promoter regions identified multiple core immune gene-regulatory transcription factor sites, including Stat1, implicating a molecular link to IFN control. Experimentally, we show using a range of genetically IFN-defective macrophages that the expression of Nampt is stringently regulated by the Jak/Stat-pathway while Sirt6 activation is temporally displaced in a partial IFN-dependent manner. We further show that pharmacological inhibition of Nampt and small interfering RNA (siRNA)-mediated inhibition of Nampt and Sirt6 promotes viral growth of cytomegalovirus in both fibroblasts and macrophages. Our results support the notion of pharmacologically exploiting immune regulated enzyme systems of macrophages for use as an adjuvant-based therapy for augmenting host protective pathway responses to infection.
Introduction

Infection is a dynamically complex and multifaceted process requiring not only the avoidance of immune countermeasures but also the exploitation of host cellular networks and machinery by the pathogen. In many cases, parasitization by pathogens and especially by viruses requires remodeling of metabolic and energy resources for the successful production of progeny. Notably, the immune system has been found to cross regulate these resources and processes as an evolutionary selected countermeasure. For example, IFNγ induced consumption of tryptophan, by the Indoleamine 2,3 Dioxygenase (IDO) pathway, has been shown to inhibit replication of several intracellular organism including hCMV (1-5). More recently interferon regulation of the sterol biosynthesis pathway has been shown to be a central biosynthetic pathway targeted by the immune system for broad host-protection against infection.

In this scenario, Toll-like receptor activation of macrophages by pathogens leads to the production of type I interferons which coordinately regulate a marked and sustained reduction in the mevalonate-sterol biosynthetic pathway, and whereby a wide-spectrum of different human and animal viruses have been shown to be sensitive to suppression of the pathway (6-20). The currently known molecular pathways for down-regulating the sterol pathway involve the IFN induction of an hydroxylase enzyme (Ch25h) and its cognate regulatory metabolite, 25-hydroxycholesterol (25HC) that potently inhibits, at the protein level, the master transcription factor for sterol biosynthesis (SREBP2) (8, 21), and also the key regulated mevalonate reductase, HMGCR (22), and additionally IFN regulated microRNAs (miR342-5p), that coordinate changes in the enzymatic flux of the cholesterol pathway within the cell (6). However, there remains yet to be identified transcriptional or epigenetic mechanisms for suppression of SREBP2 and sterol biosynthesis.

More broadly, there is increasing evidence showing connections between immune signaling, such as interferon (IFN) signaling, and the regulation of sterol, sugar, and fatty acid metabolism (23-26). While the cell typically induces changes through rapid established routes such as the PI3K/AKT/mTOR signaling pathway, these changes are not sustained over a longer period time and do not support the increased needs for de novo lipogenesis. In the context of cellular stress and inflammation, Sirtuins (SIRTs) are known to play sustained roles in protecting against cellular stress through epigenetic control of metabolic pathways (27, 28). This includes the regulation of glycolytic and lipid metabolism by the nicotineamide adenine dinucleotide (NAD⁺)-dependent deacetylases SIRT1 and SIRT6 (29-32). Metabolic coupling is strictly dependent on NAD⁺ production through de novo biosynthesis from tryptophan or through the nicotineamide (NAM) salvage pathway, which is regulated by the rate-limiting enzyme nicotineamide phosphatidyltransferase (NAMPT). It is notable that NAD⁺-dependent activation of SIRT6 has been shown to repress the SREBF2 promoter (31), and thereby directly linking SIRT6 activity to sterol metabolism. However, whether NAMPT or SIRT6 are coordinately regulated by the IFN macrophage antiviral response is not known. Most notably this remains a central unanswered question to the notion of using macrophage interferon biology as a guiderail for identifying host-directed druggable targets as anti-infectives.

In the present report, we find that Nampt and Sirt6 are coordinately regulated upon immune activation of macrophages and contribute to the interferon antiviral response. The coupling to the IFN response is via direct transcriptional activation of NAMPT through the JAK/STAT signaling pathway. We show that pharmacological inhibition of Nampt and small interfering RNA (siRNA)-mediated inhibition of Nampt and Sirt6 enhances the viral growth of cytomegalovirus (mCMV) in both fibroblasts and macrophages. These findings support the proposition that immune regulated enzyme systems may be used as an adjuvant therapy for augmenting the host protective response to infection.
Results
Co-ordinate regulation of Nampt and Sirt6 are part of the interferon-metabolic antiviral response.

We first investigated whether murine Nampt and Sirt6 and human NAMPT and SIRT6 promoter regions contained any putative transcriptional binding sites (TFBS) for immune-regulatory transcription factors (Figure 1, Supplementary tables 1-4). By using the sequence analysis tool PROMO (33, 34), and manual procurement by comparing putative binding sites to published consensus binding sequences, an array of significant binding sites (restricted to 15% dissimilarity) for core immune-activated transcription factors, including AP-1, NFκB (defined here as DNA binding activity constituted either by p50 homodimer, a p50/p65 heterodimer, or a heterotetramer), RELA (p65 subunit of NFκB), GATA1 and GATA2, were identified within (-1kb upstream of) the murine Nampt and Sirt6 promoter regions (Figure 1). PROMO analysis of the human NAMPT and SIRT6 promoter regions identified similar binding sites, suggesting that the overall activation mode of these genes is conserved between humans and mice. Notably, several putative Signal Transducers and Activators of Transcription 1 (STAT1) sites were identified across the Nampt promoter region, suggesting Nampt expression might be driven directly by the activation of the JAK/STAT signaling pathway (Figure 1). A putative Oct cluster (OCT1/2/3/4) was also identified in the distal Nampt promoter region, in close proximity to putative NFκB, RELA, and STAT1 binding sites. While the promoter region of Sirt6 did not contain any putative STAT1 binding sites, it was dominated by putative binding sites for Activator protein 1 (AP-1), c-JUN, c-FOS, and NFκB. The AP-1 structure is a heterodimer composed of proteins belonging to the c-FOS, c-JUN, Activating transcription factor (ATF), and Jun dimerization protein (JDP) families (35, 36). Consistent with its reported activity, the putative AP-1 binding sites were found in close proximity to either c-FOS, c-JUN, or in areas containing cis-located c-FOS and c-JUN binding sites (Figure 1). AP-1, an early response transcription factor, has been reported to regulate gene expression in response to various stimuli, including cytokine stimulation and bacterial and viral infections (37).

The presence of several putative immune-gene regulatory transcription factor binding sites, prompted us to investigate whether Nampt and Sirt6 expression was induced by infection (Figure 2). The relative expression of Nampt and Sirt6 in mCMV-infected NIH-3T3 and p53 mouse embryonic fibroblasts (p53-MEFs) was measured using Quantitative reverse-transcriptase Polymerase Chain Reaction (qRT-PCR) (Figure 2A-B). mCMV infection of NIH-3T3 and p53-MEFs resulted in significantly higher levels of Nampt expression during the first 6 hours of infection (Figure 2A-B). While an early increased Sirt6 expression was not observed, a significantly higher expression was observed in NIH-3T3 after 10 hours of infection, indicating a delayed response. Similarly, the temporal expression profiles (over 24 hours) of Nampt and Sirt6 were investigated in mCMV infected bone marrow derived macrophages (BMDM). Following mCMV infection, cells were harvested every 2 hours until 10 hours post treatment (0 (0 hours after viral adsorption or poly(I:C) treatment), 2, 4, 6, 8, 10 hours) and at 24 hours, followed by transcriptomic profiling and modeling of their temporal expression. In these experiments, polynomial fitting of the smoothened data was used to determine whether the expression profiles of Nampt and Sirt6 changed significantly (where a R² > 0.9 indicated significant change) with time in infected BMDM. (Supplementary tables 5-6). In addition further statistical evaluation was performed by determining the p-value of the fitted model in relation to a horizontal flat line, where a significant p value predicted a temporal change and a non-significant p value is predicted of non fluctuation in expression. Consistent with the observations in NIH-3T3s and p53-MEFs, mCMV infection of BMDM resulted in a significant early and dynamic expression of Nampt (Figure 2C and Supplementary table 5), with a peak at
5 hours, followed by a steady decline. Moreover, similarly to the observations in NIH-3T3s and p53-MEFs, temporal expression analysis revealed that mCMV infected BMDM exhibited a delayed but continuous, albeit lower than Nampt, significant Sirt6 expression (Figure 2C and Supplementary table 6). Collectively, these results show that while Nampt and Sirt6 are both induced in response to mCMV infection, their response time differs from each other irrespective of cell type, indicative of potential differential transcriptional regulation.

**Nampt gene expression is activated by the Jak/Stat signaling pathway and induced by both type-I and type-II IFNs, while response of Sirt6 is indirect or restricted to type-I IFN response.**

The presence of the several putative STAT1 binding sites in the Nampt promoter region suggests that Nampt expression is induced in a JAK/STAT signaling pathway-dependent manner. To initially investigate this, the expression of Nampt mRNA was measured Tyk-2-deficient BMDM (Figure 3). Nampt mRNA synthesis was investigated in mCMV infected wild-type and Tyk2-deficient BMDM at 1-1.5 hours post infection and at 6-6.5 hours post infection (Figure 3A). The non-receptor tyrosine-protein kinase Tyk2 has been implicated in type-I IFN, IL-6, IL-10, and IL-12 signaling (38-42). Consistent with the identification of putative STAT1 binding sites in Nampt promoters, Nampt expression peaked downstream of Nampt activation of Tyk2-deficient (Tyk2−/−) BMDM resulting in a much-reduced Nampt synthesis, compared to infected wild-type cells, suggesting that Nampt is, at least partly, induced in a JAK/STAT pathway-dependent manner (Figure 3A).

The presence of putative STAT1 binding sites and the observed dependence of Nampt expression on TYK2 and on the JAK/STAT signaling pathway poses the question: **Whether the induced expression of Nampt and Sirt6 is dependent on type I IFN signaling?** To investigate this, the expression of Nampt and Sirt6 was assessed in polyinosinic-polycytidylic acid (poly(I:C)) treated Ifnb-deficient BMDM and compared to the response in mCMV infected Ifnb1-deficient (C57BL/6J Ifnb1−/−) BMDM (Figure 3B-C). Poly(I:C), a ligand of Toll like receptor 3 (TLR3), is structurally similar to double-stranded RNA and is, thus, used to simulate viral infections. Following mCMV infection or poly(I:C) treatment, cells were, as described above (Figure 2), harvested every 2 hours until 10 hours post treatment and at 24 hours, followed by transcriptomic profiling and modeling of their temporal expression. As in Figure 2C, mCMV infection of wild-type BMDM resulted in an early dynamic expression of Nampt. Similar to Nampt, mCMV infection of wild-type BMDM significantly induced, albeit at a lower level, the expression of Sirt6 expression peaking downstream of Nampt. Poly(I:C) treatment of wild-type BMDM, resulted in a significant temporal activation of both Nampt and Sirt6, indicating that the observed expression is a host-driven response to infection (Figure 3C, Supplementary tables 7-8). The level of Nampt activation following mCMV infection was significantly reduced in Ifnb1-deficient cells, suggesting that a robust Nampt expression response is IFNβ-dependent (Figure 3B). While the robustness in Nampt expression was lost, a small significant temporal change in the expression profile was observed, suggesting that Nampt expression is possibly governed by other factors or pathways including by other type I IFNs but that the magnitude of expression is strongly dependent on intact IFNβ-signaling. The early expression of Sirt6 was, however, not extensively altered in mCMV infected or poly(I:C) treated Ifnb-deficient cells. The expression was similar to wild-type cells up until 7 hours post infection and up until 5 hours post poly(I:C) treatment. This was followed by a reduction in expression, indicating that early but not late activation of Sirt6 expression following mCMV infection and poly(I:C) treatment is induced independently of IFNβ (Figure 3B-C and Supplementary tables 6 and 8). Together, these results indicate that Nampt and Sirt6 are coupled to the type I IFN response in macrophages.
Further in our BMDM, where stimulation with physiologically relevant concentration of IFNγ has been previously determined (47), IFNγ induced the expression of Nampt, peaking at 6 hours post treatment (Supplementary figure 1 and Supplementary table 9). Moreover, to further investigate the dependence of Nampt and Sirt6 expression on the type-II IFN response, the level of newly transcribed Nampt and Sirt6 mRNA was measured every 30 minutes, over a period of 8 hours, using reverse transcriptase-quantitative PCR (qRT-PCR) in BMDM stimulated with IFNγ (Figure 3D). Stimulation with IFNγ resulted, after 2 hours of infection, in an eight-times increase in de novo transcribed Nampt RNA levels, followed by a rapid drop in Nampt mRNA. Sirt6 mRNA levels were on the other hand not affected by IFNγ stimulation, further suggesting that it is not a type-II IFN stimulated gene. Notably, the increase in Nampt mRNA expression was followed by a drop in Srebf2 expression, consistent with previously published data from Blanc et al. (7).

The dependence on type-II IFN and JAK/STAT signaling was further investigated in wild-type and Stat1-deficient (Stat1−/−) p53-MEFs stimulated with IFNγ (Figure 3E-F). Steady state levels of Nampt mRNA was investigated at 1, 2, 4, 8, and 16 hours post treatment and compared to untreated (0h) cells (Figure 3E). IFNγ activated wild-type cells resulted, as early as 2 hours post treatment, in a significantly increased expression of Nampt compared to untreated cells (statistical significance depicted with #), suggesting that Nampt is a type-I IFN responsive gene. In the IFNγ activated Stat1-deficient (Stat1−/−) cells, a significantly increased Nampt expression, compared to the untreated Stat1-deficient control (not shown), was only observed at later time points (16 hours). This expression was significantly reduced at all time points compared to activated wild-type cells, indicating that Nampt is dependent on intact Stat1 signaling, consistent with the identification of putative STAT1 binding sites within the Nampt promoter region.

Moreover, loss of Stat1 resulted in a significantly reduced expression of Sirt6 compared to the wild-type p53-MEFs (Figure 3F). Notably, unlike Nampt, Sirt6 mRNA expression in wild-type cells did not increase statistically with time (compared to untreated control), suggesting that it is not a type-II IFN stimulated gene. Nor did the expression change significantly in Stat1-deficient cells to the respective untreated control. It is possible to speculate that Stat1 signaling is required for the basal, but not induced, Sirt6 expression. The absence of identified STAT1 binding sites in combination with the absence of gene induction with time further support the notion that this activation is indirect. Collectively, these results show that Nampt and Sirt6 are both induced in response to mCMV infection and suggest that Nampt is an interferon-stimulated gene (ISG), with Nampt expression being an immediate-response gene induced by type-I and type-II IFN in a JAK/STAT dependent manner.

**Intact type-I IFN signaling is required for strong infection-induced expression of upstream, but not downstream, TLR signaling pathway components.**

Infection with double-stranded DNA viruses, such as CMV, are known to trigger the common TLR signaling pathway that elicits the activation of NFκB and MAPK through the Myd88 adaptor (43), while other pathways, such as the IPS-1 and STING mediated pathways, induce type-I IFN synthesis (44-46) resulting in downstream target activation, e.g. Nampt and Sirt6. To explore the gene activation of factors belonging to these pathways, the temporal gene expression profiles of Myd88, p50 (Nfkb1), p65 (Rela), Trif (Ticam1), Rig-I (Ddx58), Mda5 (Ifih1), Ips-1 (Mavs), Sting (Tmem173), and cGas (Md21d1) were investigated in mCMV infected or poly(I:C) treated wild-type and Ifnb1-deficient BMDM (Figure 4 and Supplementary tables 10-27). Following mCMV infection of wild-type BMDM, a significant temporal activation of Myd88, p50, p65, Trif, Rig-I, Mda5, and Sting was observed (Figure 4A). The expression profile of Ips-1 was initially suppressed up until 5 hours post infection followed by an activation, while cGas exhibited an early activation between 1 and 3 hours post infection,
followed by a rapid drop in expression. Notably, the activation of Trif, which was absent until 3 hours post infection, was followed by a rapid increase in expression peaking at 7 hours. In Ifnβ1-deficient BMDM, mCMV infection resulted in a significant temporal change in expression of all genes but Trif. While the temporal expression of Myd88, Rig-I, Mda-5, and Sting was significantly changed over time, the level of expression was much reduced in these cells, suggesting that IFNβ-signaling is in part needed for the full induction of these genes. Notably, while the expression level of cGas was initially much higher in wild-type cells, the level expression after 5 hours dropped to similar levels as those observed in the Ifnβ1-deficient cells, suggesting that IFNβ is required for the early activation of this gene.

In poly(I:C) activated wild-type BMDM, significant temporal expression change was observed for Myd88, p50, Trif, Rig-I, Ips-1, and cGas, consistent with that observed in mCMV infected cells (Figure 4B), p50, Mda5, and Sting all exhibited an initial increase in expression between 1-3 hours post treatment, however, unlike Mda5 and Sting that did not significantly change, the level of p50 expression was reduced between 3-7 hours. The modeled temporal change in the p50 expression profile was, however, not significant. In Ifnβ1-deficient BMDM, a significant temporal change was observed for all genes with the exception of Trif. As in infected Ifnβ1-deficient BMDM, poly(I:C) treatment resulted in a reduced temporal expression for Myd88, Rig-I, Mda-5, and Sting. Moreover, Mda5 exhibited a repressed temporal profile, as compared to its expression in poly(I:C) treated wild-type cells. Notably, Ips-1 and cGas both exhibited an increased expression over time, with the expression of Ips-1 exceeding that observed in wild-type cells (Figure 4B).

Collectively, these results suggest intact IFNβ-signaling is not required for the expression of p50 and p65 following mCMV infection, but is required for the magnitude in expression of the upstream components (Myd88, Rig-I, Mda5, and Sting) of these pathways.

Inhibition of SIRT6 and NAMPT results in increased viral replication.

The observation that Nampt and Sirt6 were coordinately induced in macrophages by immune stimulation, either by infection or the ensuing interferon response, prompted us to test whether NAMPT and SIRT6 exhibit antiviral activity. To investigate whether Sirt6 and Nampt exhibit antiviral properties, mCMV replication was measured after siRNA mediated knockdown of Sirt6 and after pharmacologic inhibition or siRNA mediated knockdown of Nampt, with the highly specific non-competitive inhibitor FK866, respectively (48) (Figure 5, Supplementary figure 2). Consistent with the reported antiviral activity of human SIRT6 (49), mediated knockdown of murine Sirt6 resulted, in a siRNA concentration-dependent manner, in an increased viral replication (Figure 5A). Moreover, siRNA mediated knockdown and pharmacologic inhibition of murine Nampt also resulted in an increase in viral replication, respectively (Figure 5B-C). Together, these results indicate that Sirt6 and Nampt both display antiviral properties, providing druggable targets in bolstering interferon antiviral immunity linked to sterol metabolism.

Discussion

Here we demonstrate upon infection of macrophages the serial activation of Nampt and Sirt6. The observed rapid kinetics of Nampt induction shows a strict dependency on both type I and type II IFN signal activation of transcription and, thus, represents an immediate-early class of Interferon Simulated Genes (ISG). By contrast Sirt6 shows delayed induction kinetics and is only indirectly activated downstream of viral induced type I IFN signaling. In agreement, we find the Nampt promoter region contains multiple consensus Stat1 binding sites whereas these sites are absent in the Sirt6 promoter region. Notably, pharmacological inhibition of NAMPT enzymatic activity or knock-down of Nampt or Sirt6 result in increased viral replication
revealing anti-viral roles for these metabolic regulators in infection. Hence, in an apparent
orchestrated and coordinated manner Nampt enzymatically drives NAD⁺ production that is a
key rate-limiting co-factor for Sirt6 activation and thereby couples Sirt6 functions to the IFN
antiviral response (Figure 6).

We further find that temporal expression analysis of key pathway components of
the common TLR signaling pathway, which elicits the activation of NFκB and MAPK through
the Myd88 adaptor (43), and the IPS-1 and STING mediated pathways that induce type-I IFN
synthesis, revealed a part dependency on intact type I IFN signaling as loss of Ifnb1 resulted in
a reduced magnitude of expression (Myd88, Rig-1, Mda5, and Sting). The downstream signaling
components of these pathways, p50, p65, Trif, Ips-1, and cGas were on the other hand not
affected in the same way by the loss of Ifnb1. An activated expression profile was, however,
observed for Ips-1 and cGas, suggesting that intact type I IFN signaling might be required for
maintaining a regulated expression of these genes. As for p50 (NFκB), an initial increased
expression was observed followed by a gradual (mCMV infection) or rapid (poly(I:C)
treatment) declining expression. A similar expression profile was observed for p65 following
mCMV infection, but not poly(I:C) treatment. Notably, in recent years, SIRT6 has been shown
to inhibit NFκB expression (50-52) and NFκB target gene activation by interacting with p65
(Figure 6, Summary figure) (51, 53). It is possible that this mode of regulation is reflected in
the observed p50 and p65 expression profiles, nevertheless, further analysis would be required
to confirm this. Whether Sirt6 is regulated by NFκB in this system remains to be explored,
however, global profiling of p65 binding sites (by ChIP-seq) in TNFα-induced human
osteosarcoma U-2 OS cells (54) and TNFα-induced or poly(I:C) stimulated Detroit 562 cells
(55) did not identify SIRT6 as a NFκB/p65 target gene. Genome-wide profiling of p65-bound
sites after 3h and 6h of LPS treatment, have on the other hand identified NAMPT as a p65-
activated gene (56).

Together, these findings are consistent with observations, in other systems, that
are supportive of a potential antiviral role for Nampt and Sirt6 (49, 53, 57-60). In a systems-
level screen for ISGs with antiviral activity, human NAMPT was identified as one of several
type-I interferon stimulated genes that exhibited, in infected Huh-7 cells, antiviral activity
towards Venezuelan equine encephalitis virus (VEEV), a single-stranded RNA virus (57).
NAMPT has also been reported to exhibit anti-HIV-1 activity, interfering with both early events
of the life cycle (61) and Tat-induced HIV-1 long terminal repeat (LTR) transactivation (58,
59, 62). As for the role of SIRT6 in antiviral immunity this is less known. Koyuncu et al.
reported in a loss-of-function study, that loss of human Sirtuin activity, including SIRT6
activity, in infected fibroblast MRC5 cells resulted, by unknown mechanism, in significant
increases in viral titers (hCMV, HSV-1, Adenovirus, and Influenza A) (49). Moreover, a recent
report by Li et al. show that SIRT6 negatively regulates Dengue virus-induced inflammatory
responses by targeting the DNA binding domain of NFκB p65 (53). Notably, unlike our
observations reported here those reported by Koyuncu et al. (49), DENV replication was
reduced in HEK293T cells upon silencing of SIRT6 (53). It is possible that with the diverse
nature of SIRT6 the mechanisms by which it exerts its antiviral function differs depending on
cell type and viral strain, though the mechanism by which Nampt and Sirt6 exert antiviral
effects in these studies is not known. Nevertheless, together with our findings they support the
notion that NAMPT and SIRT6 constitute yet another way by which the macrophage can limit
productive viral infection.

A central mechanism of action worth noting is the reciprocal increase in de novo
Nampt mRNA expression, in IFNγ stimulated BMDM, is followed by a decrease in Srebf2
transcription. This is consistent with studies demonstrating IFN-antiviral suppression of
transcription of multiple members of the sterol biosynthesis pathway, in part mediated by a drop
in SREBP2 RNA transcription and protein levels (7). Mechanistic studies of IFN suppression
of macrophage sterol biosynthesis pathway have determined an approximately 40%
contribution by Ch25h and its cognate metabolite 25HC acting at the post-translational level,
and 40% by a post-transcriptional mechanism involving microRNA (miR342-5p). However,
these known mechanisms fail to account for the observed transcriptional effects on Srebf2
levels. In this regard, there is good evidence to show that Sirt6 binds to and regulates Srebf2
via transcription (6, 22). Figure 6 shows a schematic of a proposed mechanism for the anti-viral
activities of Nampt and Sirt6, mediated through epigenetic transcriptional suppression of Srebf2,
which encodes the master transcription factor sterol biosynthesis. Figure 6 also highlights the
other proposed molecular pathways for down-regulating the sterol pathway in macrophages
involving the generation of 25-hydroxycholesterol (25HC) and miR342-5p microRNA, both of
which contribute toward modulating the SREBP2 autoregulatory loop in response to interferon-
signaling (6, 19).

It is noteworthy that host-directed targeting of immune modulated cellular
pathways can be used as an innovative therapeutic intervention that also overcomes the antiviral
drug resistance (63). In this regard, we note that SIRT6 inhibitors are under development as
anti-cancer drugs (64-66). Some studies have investigated the efficiency of Ex527 (Selisistat),
a commercially available Sirtuin inhibitor (SigmaAldrich) (67, 68) and another proposed
approach in inhibiting SIRT6 activity is through administration of nicotinamide
(NAM/Vitamin B3), which in addition to being a NAD+ precursor, also acts as an endogenous,
non-competitive Sirtuin inhibitor (69). Thus, there is an opportunity for repurposing these
cancer drugs for potential antiviral therapy.

Author contributions
WD, KAR, and PG conceived and designed the experiments. WD and KAR performed the
experiments, and WD, KAR, and PG performed the data analysis. WJW performed the
statistical analysis of the time-course microarray analysis. BS contributed to the Tyk2-/-
experiments. WD and PG wrote the paper.

Conflict of interest
Authors declare no conflict of interest.

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Materials and Methods
Mice
C57BL/6 mice were housed in the specific pathogen-free animal facility at the University of
Edinburgh. Tyk2-/- mice were maintained under specific-pathogen-free conditions at the
Institute of Animal Breeding and Genetics, Department for Biomedical Sciences, University of
Veterinary Medicine Vienna, Vienna, Austria. The generation or source of knockout mouse
strains for Tyk2-/- has been described before (70). All procedures were carried out under project
and personal licenses approved by the Secretary of State for the Home Office, under the United
Kingdom’s 1986 Animals (Scientific Procedures) Act and the Local Ethical Review Committee at Edinburgh University.

Cell propagation and culture
NIH-3T3 (ATCC® CRL-1658™) immortalized cell line of embryonic mouse fibroblasts was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and grown in Dulbecco’s modified Eagle medium (DMEM) (Lonza, Vervier, Belgium), supplemented with 5% Calf Serum (CS) (Thermo Fisher Scientific, Waltham, MA, U.S.A.), 2mM glutamine (Lonza) and 50/μl of penicillin/streptomycin (Lonza). The p53-MEF immortalized cell lines, of p53−/− embryonic mouse fibroblasts (p53-MEFs , MB355(ATCC® CRT-2818™)) and Stat1−/− p53-MEFs, were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). p53-MEFs and Stat1−/− p53-MEFs were grown in DMEM (Lonza), supplemented with 5% fetal calf serum (FCS) (Thermo Fisher Scientific), 2mM glutamine (Lonza) and 100U/ml of penicillin/streptomycin (Lonza). BMDM were isolated and grown in DMEM/F-12 (Ham 1:1) and L-glutaMAX, supplemented with 10% Fetal Calf Serum (Lonza), 10% L929 and 100U/ml of penicillin/streptomycin. All cells were grown in accordance to standard procedures. BMDMs were differentiated with CSF-1 derived from L929 cells for 7 days prior to further treatment.

Viruses and Reporter Viruses
Wild-type murine cytomegalovirus (MCMV-C3X) has been previously described (71). The GFP-encoding MCMV (mCMV-GFP) has also been previously described (72). For RNA expression analysis, infection was done at a multiplicity of index (MOI) of 1 unless else specified.

RNAi and Assay for GFP-virus growth
siRNAs and “RISC-free” control siRNA were purchased from Dharmacon® RNAi Technologies (Thermo Fisher Scientific). miR-342-5p microRNA mimic were kindly gifted by Integrated DNA Technologies (WOS:000332467100005). The following siRNAs were used: “RISC-free” siRNA, SiGenome™ Control (Cat. No. D-001220-01-05); Mouse Sirt6 siRNA (deconvoluted), ON-TARGETplus siRNA Mouse Sirt6 (Cat. No. J-061392-09, J-061392-10, J-061392-11, J-061392-12); Mouse Nampt siRNA (deconvoluted), ON-TARGETplus siRNA Mouse Nampt (Cat. No. J-040272-09, J-040272-10, J-040272-11, J-040272-12); M54 siRNA, custom made order from Dharmacon® (5’-3’ sense strand sequence is AGAAGACGACTGAGCTA). Mimics and siRNA were transfected into cells (NIH-3T3), in a 96 well plate, using DharmaFECT1 (Thermo Fisher Scientific) using the reverse-transfection method and in accordance to the manufacturer’s recommendations. M54 siRNA and miR-342-5p microRNA mimic were transfected at a final concentration of 25nM and Sirt6 siRNA was transfected at a final concentration of 6.25, 12.5, and 25 nM/well. For the analysis of miR-342-5p inhibitor effects on virus replication, medium containing 3% delipidized serum (Bovine Serum, Lipid Depleted (Part number: S181L), VWR, UK) was used. After 48h, MCMV-GFP (MOI 0.025) was used for infection. The viral growth (fluorescence in each well) was measured using a POLARstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK) according to manufacturer’s recommendations. The RNAi and viral growth assay were set up as two independent experiments with 3 biological replicates per experiment (n=6). Virus replication slopes over the linear phase were calculated, from 68 hours to the end of the time course, and then normalized to control transfected wells. Statistical significance was determined using One-way ANOVA with a Dunnett’s multiple comparisons test. P-values of <0.05, <0.01 and <0.001 were considered significant.
**In-silico promoter analysis.**

In silico promoter analysis of the NAMPT and SIRT6 promoter regions was done using PROMO, a virtual laboratory for identification of putative transcriptional binding sites (33, 34). The promoter regions consisting of the 1kb upstream regions of murine and human NAMPT and SIRT6 were retrieved from the Mouse Genome Informatics (MGI) Web Site (73-75) and The National Center for Biotechnology Information (NCBI) (76) resource, and imported into the online PROMO analysis tool. Species specific (Mus musculus or Homo sapiens) transcription factors and transcription factor sites were chosen. For murine Nampt and Sirt6, 306 and 270 putative transcription factor binding sites within a dissimilarity margin less or equal than 15% were identified in the promoter regions, respectively. For human NAMPT and SIRT6, 444 and 436 putative transcription factor binding sites within a dissimilarity margin less or equal than 15% were identified in the promoter regions, respectively. All identified putative binding sites can be found in Supplementary Tables 1-4. From these, the most probably immune-regulatory and core transcription factor binding sites were identified via manual procurement by comparing putative binding site to publicly available/published consensus binding sequences for each transcription factor.

**IFN-γ treatment of p53-MEFs and isolation**

Wild-type and Stat1γ−/− p53-MEFs were plated, in a 24-well plate, at a cell density of 3x10⁵ cells/well and grown in DMEM (Lonza), supplemented with 5% fetal calf serum (FCS) (Thermo Fisher Scientific), 2mM glutamine (Lonza) and 100U/ml of penicillin/streptomycin (Lonza), for 24 hours prior to treatment with murine recombinant IFN gamma (IFNγ) (Perbio Science). The IFNγ was diluted in complete medium and added to cells at a final concentration of 10U/ml. Cells were harvested at 1, 2, 4, 8, and 16 hours post treatment for quantitative real-time PCR analysis using 350 µl Qiagen RTL Plus buffer (Qiagen RNeasy Plus kit) as per manufacturer’s recommendations.

**BMDM IFN-γ treatment, RNA labelling and isolation**

Incorporation of 4-thiouridine (Sigma-Aldrich, St. Louis, MI, U.S.A) into newly-transcribed RNA was undertaken as described by Dölken et al. (77) and Robertson et al. (6). In brief, at time zero medium was aspirated from all plates and 15ml of pre-warmed medium containing IFN-γ (final concentration of 10U/ml) or normal medium was added to the cultures. RNA labelling in BMDM during the first 30 minutes of the time course was undertaken by addition of 200µM 4-Thiouridine to the medium of appropriate plates at this time. After 30 minutes, to end the RNA labelling period, terminate transcription and lyse the cells, medium was aspirated from the labelled BMDM and replaced with 4mls of RLT lysis buffer (Qiagen, Hilden, DE). In parallel, 10ml of medium from the next BMDM cultures to be labelled was added to an appropriate volume of 4-thiouridine, mixed and immediately added back to the plate. BMDM cultures were then returned to the incubator. The above cycle of 4-thiouridine addition to BMDM cultures and transcriptional termination was repeated at 30-minute intervals until the end of the time course. Total RNA was isolated using RNeasy Midi kit (Qiagen) according to manufacturer’s instructions, quantitated using a Nanodrop (Thermo Scientific) and integrity was confirmed using an Agilent Bioanalyser (Agilent UK). Newly transcribed RNA (ntRNA) was then isolated as described in Dölken et al. (77) and Robertson et al. (6) and again quantitated using a Nanodrop, followed by qRT-PCR.

**Quantitative real-time PCR (qRT-PCR) Analyses of individual genes**

Cells were harvested in 350 µl Qiagen RTL Plus buffer (Qiagen RNeasy Plus kit) as per manufacturer’s recommendations. Total RNA was extracted from cells with RNeasy Plus kit (Qiagen) according to the manufacturer’s instructions and quantitated using a Nanodrop
(Thermo Scientific). All experiments were performed on three biological replicates/samples (n=3) and expression analysis were performed with three technical replicates/sample (n=3), unless else specified. Quantitative gene-expression analyses were performed using Taqman® Primer probe sets (Applied Biosystems, Warrington, UK). Mouse Assay ID: NAMPT: Mm00451938_m1, SIRT6: Mm01149042_m1, SREBF2: Hs01081784_m1, Actin Beta (ACTB). Temporal expression analysis (Affymetrix Mouse Gene 1.0ST microarray). Cells treated with IFN-β were harvested and frozen down at -80°C. The effect of FK866 treatment on viral growth was quantified by plaque assay on p53−/− MEF monolayers in 48-well plates using standard methodology. Statistical significance was determined using unpaired T test with Welch’s correction. P-values of <0.05, <0.01 and <0.001 were considered significant.

Pharmacological inhibition of NAMPT with FK866 and Viral Plaque Assay.

Pharmacological inhibition of NAMPT was done using FK866 (48) at a final concentration of 10μM. Briefly, NIH-3T3 cells were seeded in a 48 well plate at a cell density of 1x10^5 cells/well and infected with MCMV-GFP (MOI 0.1) the following day. After the adsorption period, the infection media was replaced with media with FK866 (10 nM) or without (control). Cells were assessed for viral growth (GFP signal) over a 4-day period at which point the cells were harvested and frozen down at -80°C. The effect of FK866 treatment on viral growth was quantified by plaque assay on p53−/− MEF monolayers in 48-well plates using standard methodology. Statistical significance was determined using unpaired T test with Welch’s correction. P-values of <0.05, <0.01 and <0.001 were considered significant.

Time-Course Microarray analysis

Temporal expression analysis for Nampt, Sirt6, and Stat1 in wild-type and IFNB−/− BMDM are based off previously generated microarray data published by Blanc et al. (21). Briefly, wild-type, and IFNB−/− BMDM were either infected with MCMV (MOI 1) or treated with or poly(I:C) (25μg/ml) or IFNγ (10U/ml). Cells infected with MCMV or treated with poly(I:C) were harvested at 0, 2, 4, 6, 8, 10, and 24 hours post-treatment for RNA isolation and microarray analysis (Affymetrix Mouse Gene 1.0ST microarray). Cells treated with IFNγ were harvested...
every 60 minutes for a total of 12 hours post-treatment for RNA isolation and microarray analysis (Affymetrix Mouse Gene 1.0ST microarray). In brief, the arrays were normalized using the gcRMA algorithm (80) and imported into Partek Genomics Suite (Partek, U.S.A) for downstream analysis (21). Temporal expression data for Nampt in MCMV-infected wild-type and Tyk2 

mice were cultivated for 7 days. Following 7 days, cells were infected with MCMV (MOI 1) and harvested 1-1.5 and 6-6.5 hours post infection followed by RNA isolation (RNeasy Midi kit, Qiagen) and microarray analysis (Affymetrix Mouse Gene 1.0ST microarray). Time course microarray analysis data are compliant with the National Centre for Biotechnology Information Gene Expression Omnibus (GEO) (81) under SuperSeries accession number GSE42505 (SubSeries numbers GSE42503, GSE42504) (GEO, http://www.ncbi.nlm.nih.gov/geo/). Macrophage microarray data will be deposited in Gene Expression Omnibus and will be accessible through GEO.

Statistical analysis of time-course microarray data

Prior to statistical analysis, gene expression data (0-24 hours post mCMV infection or poly(I:C) treatment) was smoothened by taking the mean of every consecutive pair of points, i.e. mean of score at 0 and 2 hours defined the score at 1 (hour) and mean of 2 and 4 hours defined the score at 3 (hours), 4 and 6 hours defined the score at 5 (hours), 6 and 8 hours defining the score at 7 (hours), and 8 and 10 hours defining the score at 9 (hours). Smoothing was not done beyond 10 hours as the gap to the next point at 24 hours was too large and, thus, excluded from analysis. This type of smoothing preserves the patterns in the data while removing some of the fine scale rapid changes. To investigate whether the gene expression changed over time, the mean smoothed data (or non-smoothened for the 12h-dataset) for each gene expression was compared to a straight horizontal line. This was achieved by fitting the data to an appropriate polynomial in time t, i.e. either a linear model \( y = \alpha + \beta t \), or a quadratic model \( y = \alpha + \beta t + \gamma t^2 \) or a cubic model \( y = \alpha + \beta t + \gamma t^2 + \delta t^3 \). If the coefficients other than the intercept (\( \alpha \)) were significant then the model and hence the gene expression, must vary with time. Which model was chosen depended on the model fit as given by the \( R^2 \) value – the higher the better. The great majority of the fits were very strong - \( R^2 > 0.9 \). To determine then if the model deviated significantly for the horizontal, the significance attached to each of the coefficients (\( \beta, \gamma, \delta \)) of the time variables was investigated. If anyone of these was significant at the p < 0.05 level or if any two were significant at the 0.05 < p < 0.1 level then we considered the model and the gene expression to vary significantly with time. Refer to Supplementary tables 5-29 for the results of the statistical analysis.

Figure legends

Figure 1. Putative transcription factor binding sites within the promoter regions of murine Nampt and Sirt6 and human NAMPT and SIRT6. In silico analysis using PROMO of the murine Nampt and Sirt6 and human NAMPT and SIRT6 promoter regions revealed putative binding sites for an array of different immune-regulatory transcription factors (TF) (Supplementary tables 1-4). The cut off for dissimilarity to the consensus TF binding-sequence was set to 15 % (* marks binding sites with low percentage, 0-4%, dissimilarity) and the identified string sequences were compared to the consensus binding site sequences for each transcription factor. Figure is not to scale.

Figure 2. Expression of Nampt and Sirt6 is upregulated by MCMV infection.
(A) Quantification of relative Nampt and Sirt6 mRNA expression in NIH-3T3 cells, at 6 and 10 hours, using RT-qPCR following mCMV infection with mock-infected cells serving as controls (n=3). (B) Quantification of relative Nampt and Sirt6 mRNA expression in p53-MEF cells, at 3 and 6 hours, using RT-qPCR following mCMV infection (n=3). ANOVA with Tukey post test was used to assess statistical significance. (C) Normalized temporal Nampt and Sirt6 expression (antilog) in mCMV infected bone marrow derived macrophages (BMDM). The expression was measured over the first 24 hours of infection using microarray and compared to timepoint 0. The expression levels between 0-10 hours were smoothened and fitted to a linear (+), quadratic (#), or cubic (*) polynomial on time and the statistical significance (p-values) was assessed. **+/##/*** p < 0.05, +/###/**** p < 0.01, and +++/#####/***** p < 0.001 were considered to be significant (ns, not significant). Bars represent standard error of the mean (SEM). * p < 0.05, ** p < 0.01, and *** p < 0.001 were considered to be significant (ns, not significant).

Figure 3. Expression of Nampt is induced by both type-I and type-II IFN, while response of Sirt6 is restricted to type-I IFN response. (A) Quantification of Nampt mRNA expression, using microarray, in wild-type (wt) and Tyk2−/− BMDM following mock- (control) and mCMV-infection. (B) Normalized temporal expression (antilog) of Nampt and Sirt6 in mCMV infected wild-type and Ifnb−/− BMDM. The expression was measured over the first 24 hours of infection using microarray and compared to timepoint 0. The expression levels between timepoints 0-10 hours post treatment were smoothened and fitted to a linear (+), quadratic (#), or cubic (*) polynomial on time to assess significance (p-values). **+/##/*** p < 0.05, +/###/**** p < 0.01, and +++/#####/***** p < 0.001 were considered to be significant (ns, not significant). (C) Normalized temporal expression (antilog) of Nampt and Sirt6 in poly(I:C) treated wild-type and Ifnb−/− BMDM. The expression was measured over the first 24 hours using microarray and compared to timepoint 0. The expression levels between timepoints 0-10 hours post treatment were smoothened and fitted to a linear (+), quadratic (#), or cubic (*) polynomial on time to assess significance (p-values). **+/##/*** p < 0.05, +/###/**** p < 0.01, and +++/#####/***** p < 0.001 were considered to be significant (ns, not significant). (D) Quantification of de novo synthesis of Nampt, Sirt6, and Srebf2 mRNA in IFNγ-stimulated BMDMs using qRT-PCR. Expression was measured every 30 min for a total of 8 hours. (E) Quantification of relative Nampt mRNA expression in wild-type and Stat1−/− p53-MEF cells, at 1, 2, 4, 8, and 16 hours, using RT-qPCR following IFNγ stimulation (n=3). Expression is relative to wild-type untreated (0h) cells, set as 1 (not shown). One-way ANOVA with a Tukey’s post test was used to assess statistical significance to untreated controls. One-way ANOVA with a Sidak’s multiple comparisons test was used to assess statistical significance between wild-type and Stat1−/− mutants. Bars represent standard error of the mean (SEM). Statistical significance between groups (wild-type and Stat1−/−) were depicted with *. Statistical significance in relation to untreated controls (wild-type and Stat1−/−, respectively) were depicted with #. * and # p < 0.05, ** and ## p < 0.01, *** and ### p < 0.001 were considered to be significant. (F) Quantification of relative Sirt6 mRNA expression in wild-type and Stat1−/− p53-MEF cells, at 1, 2, 4, 8, and 16 hours, using RT-qPCR following IFNγ stimulation (n=3). Expression is relative to wild-type untreated (0h) cells, set as 1 (not shown). One-way ANOVA with a Tukey’s post test was used to assess statistical significance to untreated controls. One-way ANOVA with a Sidak’s multiple comparisons test was used to assess statistical significance between wild-type and Stat1−/− mutants. Bars represent standard error of the mean (SEM). Statistical significance between groups (wild-type and Stat1−/−) were depicted with *. Statistical significance in relation to untreated controls (wild-type and Stat1−/−, respectively) were depicted with #. * and # p < 0.05, ** and ## p < 0.01, *** and ### p < 0.001 were considered to be significant.
Figure 4. Expression of upstream, but not downstream, TLR signaling pathway components is dependent on IFNβ/type-I IFN signaling.

(A) Normalized temporal expression (antilog) of Myd88, p50 (Nfkb1), p65 (Rela), Trif (Ticam1), Rig-I (Ddx58), Mda-5 (Ifih1), Ips-1 (Mavs), Sting (Tmem173), and cGas (Mb21d1) in mCMV infected wild-type and Ifnb−/− BMDM. The expression was measured over the first 24 hours of infection using microarray and compared to timepoint 0. The expression levels between timepoints 0-10 hours post treatment were smoothened and fitted to a linear (+), quadratic (#), or cubic (*) polynomial on time to assess significance (p-values). \*\*\*\* p < 0.01, and +++##/###/#### p < 0.001 were considered to be significant (ns, not significant).

(B) Normalized temporal expression (antilog) of Myd88, p50 (Nfkb1), p65 (Rela), Trif (Ticam1), Rig-I (Ddx58), Mda-5 (Ifih1), Ips-1 (Mavs), Sting (Tmem173), and cGas (Mb21d1) in poly(I:C) treated wild-type and Ifnb−/− BMDM. The expression was measured, as in A, over the first 24 hours using microarray and compared to timepoint 0. The expression levels between timepoints 0-10 hours post treatment were smoothened and fitted to a linear (+), quadratic (#), or cubic (*) polynomial on time to assess significance (p-values). \*\*\*\* p < 0.01, and +++##/###/#### p < 0.001 and +++##/###/#### p < 0.001 were considered to be significant (ns, not significant).

Figure 5. Inhibition of NAMPT and SIRT6 activity increases viral loads in MCMV infected cells. (A) Normalized replication slope of mCMV-GFP in Sirt6 siRNA-treated NIH-3T3. “RISC-free” siRNA served as negative control and miR342-5p siRNA served as positive control. (B) Normalized replication slope of mCMV-GFP in Nampt siRNA-treated NIH-3T3, with M54 serving as positive control. Bars represent standard error of the mean (SEM). Statistical significance was determined using One-way ANOVA with a Dunnett’s multiple comparisons test. \* p < 0.05, \*\* p < 0.01, and \*\*\*\* p < 0.001 were considered to be significant (ns, not significant). (C) mCMV-GFP replication (titer) in NIH-3T3 after pharmacological inhibition of NAMPT with FK866 (10nM final concentration). mCMV-GFP was propagated in NIH-3T3 fibroblasts and quantified by plaque assay on p53-MEF monolayers in 48-well plates. Statistical significance was determined using unpaired T test with Welch’s correction. \* p < 0.05 was considered to be significant.

Figure 6. Summary figure.

Supplementary figure legends:

Supplementary figure 1. Nampt expression in macrophages is Jak/Stat-dependent and induced by IFNγ stimulation.

Normalized temporal Nampt expression in IFNγ-treated BMDM. The expression was measured hourly over the first 12 hours of treatment using microarray, and the expression levels were fitted to a cubic polynomial on time and assessed for statistical significance (temporal change over 12 hours). \*\*\* p < 0.001 were considered to be significant.

Supplementary figure 2. Knock-down efficiency of Nampt and Sirt6 in NIH-3T3 cells.

(A) Quantification of relative Nampt mRNA expression in NIH-3T3 cells following using qRT-PCR following siRNA treatment (6.25nM, 12.5nM, and 25nM siRNA) (n=3). (B) Quantification of relative Sirt6 mRNA expression in NIH-3T3 cells following using qRT-PCR following siRNA treatment (6.25nM, 12.5nM, and 25nM siRNA) (n=3). One-way ANOVA with a Dunnett’s multiple comparisons test was used to determine statistical significance. Bars represent standard error of the mean (SEM). \* p < 0.05, \*\* p < 0.01, and \*\*\*\* p < 0.001 were considered to be significant (ns, not significant).
References


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