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IFNγ INFLUENCES EPITHELIAL ANTI-VIRAL RESPONSES VIA HISTONE METHYLATION OF THE *RIG-I* PROMOTER

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Running Title

IFNγ priming mediates *RIG-I* epigenetic regulation

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ABSTRACT

The asthmatic lung is prone to respiratory viral infections that exacerbate the symptoms of the underlying disease. Recent work has suggested that a deficient Th1 response in early life may lead to these aberrant anti-viral responses. We investigated whether the inflammatory environment of the airway epithelium could modulate anti-viral gene expression via epigenetic mechanisms, in order to study the development of a long-term dysregulation of innate responses, which are a hallmark of asthma.

We primed AALEB, a human bronchial epithelial cell line, with IFNγ and IL13 and subsequently infected cells with Respiratory Syncytial Virus (RSV) and innate anti-viral genes expression and their epigenetic markers were analysed.

Priming epithelial cells with IFNγ reduced RSV viral load. Microarray analysis identified that IFNγ-priming enhanced *RIG-I* mRNA expression and this expression correlated with epigenetic changes at the *RIG-I* promoter that influenced its transcription. Using chromatin immunoprecipitation, we observed a reduction of trimethylated Histone3 Lysine9 (H3K9me3) at the *RIG-I* promoter. Addition of inhibitor BIX-01294 to this model indicated an involvement of lysine methyltransferase G9a in *RIG-I* epigenetic regulation.

These data suggest that prior exposure to IFNγ may leave an epigenetic mark upon the chromatin that enhances airway cells' ability to better resist infection possibly via epigenetic upregulation of *RIG-I*. These observations provide further evidence for a crucial role of IFNγ in the development of mature anti-viral responses within a model of respiratory infection. Further clinical validation is required to determine if this effect in early life leads to changes in anti-viral responses associated with asthma.

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Key Words: IFNγ, cytokine priming, *RIG-I*, epigenetic regulation of innate immune response, asthma.

Clinical Relevance

The airway inflammatory milieu at or before birth, may have important effects on the development of anti-viral innate immunity at the level of the epithelium. As rapid maturation of adaptive immunity from a tolerant (Th2) to an anti-infective (Th1) state occurs in the neonatal period, the presence of a Th1 milieu appears key to the development of effective anti-viral responses.

INTRODUCTION

The pulmonary innate immune system matures rapidly in early life (1), but the age of the first viral infection and hence the state of maturity of the immune system at the time are critical factors predisposing to both severe infection and to the risk of long term airways disease (2-4). Respiratory Syncytial Virus (RSV) is the most common cause of severe respiratory infection in new-born babies and each year leads to over 3 million hospitalizations and around 66,000 deaths worldwide each year (4). Some infants have an inherent predisposition to severe RSV infection and to a tendency to post-infectious wheezing which is associated with increased pro-inflammatory cytokine production (5). Previous studies have investigated the role of cytokines in the development of asthma early in life, particularly the Th2 cytokine, IL13. However, there are conflicting reports of the role of IL13 in asthma development. IL13 can drive normal human pediatric PBECs toward an asthmatic phenotype in vitro (6), Contrastingly, Williams et al. (7) have described deficient IL13 production in babies that later developed atopic disease. Furthermore, early life bacterial and viral infection increase IL13 responses (3). Gern et al. (8) examined peripheral

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blood responses to activation by phytohemagglutinin (PHA) in children and found that infants who wheezed with RSV infection had reduced PHA-induced IL13 at birth and babies with detectable PHA-induced IFNγ were less likely to wheeze in the first year.

Studies in murine models showed that production of IFNγ during RSV infection determines the outcome of subsequent RSV reinfections (9) and also mice infected for the first time as neonates have low IFNγ response compared to mice infected as adults. Upon reinfection IFNγ deficient mice developed enhanced lung response (2, 10).

Work from Copenhaver et al. (11) showed that cord blood IFNγ responses were inversely related to the frequency of subsequent viral respiratory infections. Juntti et al. (5) measured also cytokines responses to LPS stimulation in cord blood and found that IFNγ responses were lower among the children hospitalised for RSV infection. In their key message Juntti et al. states that infants with a severe RSV infection differ from healthy children in their innate immunity cytokine profile before the infection. Taken together these data suggest that early life lung exposure to interferons may influence antiviral immunity in the longer term more than exposure to IL13.

Recently the $COPSAC_{2000}$ study linked asthma development with the number of viral respiratory tract infections episodes in the first year of life, but not with a particular viral trigger (12). Airway epithelial cells are not only a physical barrier between the human body and the external environment (13), but also a key site in the innate immune response, being the first line of defence against lung pathogens. Activated epithelial cells are able to produce antiviral and pro-inflammatory cytokines that enhance the innate immune response and activate adaptive immunity, in the lung one key function of IFNγ is to upregulate antiviral gene transcription (14-16).

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Environmental exposures in early life are also crucial in influencing how immune responses are programmed pre- and postnatally (17). A study into the role of the environment in to the development of asthma and allergy was recently designed comparing Amish and Hutterite children. Amish community follow traditional farming practices whereas Hutterites live on large, highly industrialized, communal farms.

Taking advantage of their similar genetic ancestries and lifestyles the study showed that there was 4 and 6 times low incidence of asthma and allergic sensitization in Amish children and endotoxin levels in Amish house dust was 6.8 times as high suggesting one more that the Amish environment has a protective effect against asthma modulating innate immune response (18). von Mutius and Vercelli (19) describes this IFNγ centred model and cite “farm living” as an example of the environment characterised by intense microbial exposure, in which mothers live during pregnancy, that is protective against asthma and allergies for the children later in life. These combined exposures, which occur at a crucial time for programming immune responses, upregulate regulatory T (Treg) cell function and IFNγ production at birth, which in turn enhance innate immune responses (through increased expression of pattern-recognition receptors), and dampen Th2 cell dependent allergic inflammation in early childhood (19). The ability to produce high levels of IFNγ at birth may ensure effective responses to respiratory viral infections in early life, thereby counteracting the contribution of these infections to increased asthma susceptibility (19). The IFNγ priming in our cellular model mimic the increased IFNγ upregulation following microbial exposure and help us to identify the effects of IFNγ priming in the antiviral response early in life.

We investigated how IFNγ is able to modulate the innate immune response to RSV by the airway epithelium. We used an unbiased approach to identify modulation in gene

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transcription induced by IFNγ-primed epithelium and further investigated the mechanisms by which IFNγ-priming may modulate the epithelial response to viral infection. In addition to transcription factors, gene expression can also be modulated in the longer term via epigenetic mechanisms. Post-transcriptional modifications of nucleosomal histones in the chromatin, including methylation, leading to changes in gene transcription that affect the cellular phenotype and reflect the nature and timing of the environmental stimulus (20). We therefore investigated the potential epigenetic mechanisms employed by IFNγ to regulate anti-viral gene transcription.

Some of the results of these studies have been previously reported in the form of abstracts: (21, 22).

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METHODS

Cell culture and RSV infection

AALEB human bronchial epithelial cells (23) were cultured in BEGM and used at a passage number between 20 and 30 and plated in 24 well plates at approximately $2x10^4$cells per well. Primary Bronchial Epithelial cells (PBECs) isolated from bronchial brushes from healthy controls undergoing bronchoscopy. Subjects gave written informed consent and the study was approved by the South Central—Southampton B National Research Ethics Service (NRES) Committee (12/SC/0304). PBECs were kept in culture in flasks coated with collagen (PureCol, Nutacon, Netherlands) using Bronchial Epithelial Growth Medium (BEBM plus SingleQuots of Growth Supplements) (Lonza, Slough, UK). Cells at passage 2 or 3 were then plated in collagen coated 24 well plates at $1X10^4$ cells per well for subsequent experiments.

PBECs and AALEB cells prepared in 24 well plate and maintained for 48 h in starvation media (BEBM supplemented with ITS 1X (insulin, transferrin, selenium, Life Biotechnology, Thermo Fisher Scientific Waltham, MA USA) and 0.02% BSA (SIGMA Dorset, England). Where required, 24 h before viral infection, IL13 or IFNγ (both 10 ng/ml, R&D Systems, Abingdon, UK) were added to media.

Before RSV infection, cells were washed twice with DMEM (SIGMA, Dorset, England) supplemented with 2 mM Glutamine (SIGMA) before RSV subtype A Memphis 37 (Amsbio Abingdon, UK) (24) at MOI 1 was added to the wells required in a volume of 200 μl for 2 h at 37°C with gentle agitation. Then cells were washed twice with DMEM 2 mM Glutamine and cultured for 48 h in starvation media. Cells were harvested and stored in TriFast (peqGOLD PEQLAB, Southampton, UK) at -80 ^{o}C prior to RNA extraction.

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RNA extraction

RNA extraction was performed as per manufacturer's instructions (see online protocol).

Microarray analysis

Global gene expression was assessed using the Affymetrix GeneChip U133 Plus 2.0 (see online protocol).

GEO accession number: **GSE77154** (*reviewer access link:* *https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=utmbyqyqbnmrxix&acc=GSE77154*).

Epigenetic inhibitors

AALEB cells, were plated in 24 well plates at about $2x10^4$ cells per well can cultured for 48 h and then exposed to a range of epigenetic inhibitors for 6 hours in starvation media **(TABLE E1)**. IFNγ was then added at 10 ng/ml for 24 h and cells were collected in TriFast (peqGOLD PEQLAB, Southampton, UK) and RNA extraction was performed with standard protocol as stated earlier.

RT-qPCR

(see online protocol and **TABLE E2** for Taqman Gene expression assays)

Chromatin immunoprecipitation (ChIP)

ChIP was performed essentially as previously described (25) (see online protocol). Antibodies and PCR primers in **Tables E3** and **E4** respectively.

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Production of cell lysates and protein quantitation

For Western blot experiments see online protocol.

Statistical analysis

Data were analysed with software GraphPad Prism software version 6 using one-way ANOVA (Holm-Sidak's multiple comparisons test) for gene expression analysis and two-tailed Wilcoxon paired t-test for analysis of pull down isolated chromatin from ChIP experiments. Number of samples between 3 and 9 as indicated in the legend. Error bars represent Mean with SEM.

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RESULTS

Viral shedding is reduced in the presence of IFNγ.

In an attempt to investigate the effects of the local cytokine environment on the airway epithelium we first investigated the effects of Th1 (IFNγ) and Th2 (IL13) cytokines in modulating epithelial responses to viral infection. AALEB cells, a cell line derived from human airways epithelial cells, (23) previously used by Tesfaigzi's group (26) to study the link between p53 and IFNγ were used for these sets of experiments. AALEB cells, were pre-treated with IFNγ or IL13 (27), then cells were infected with RSV **(FIGURE 1A).** RSV efficiently replicated within the AALEB cells as measured by RT-qPCR **(FIGURE 1B).** IFNγ pre-treatment reduced RSV replication compared to un-primed cells but IL13 had no statistically significant effect on viral replication (p=0.23) (**FIGURE 1C).**

IFNγ-modulated genes are involved in the viral innate immune response.

Unbiased gene expression analysis using microarrays was then performed to identify innate immune genes that may be responsible for the priming effect of IFNγ on the respiratory epithelium. Gene expression changes were determined for the following conditions compared to the untreated control: IFNγ treated, RSV infected, IFNγ pre-treated and RSV infected. **(FIGURE 2A).**

As expected, several genes involved in inflammation, interferon response, and antigen presentation were upregulated following RSV infection in IFNγ-primed cells. As a consequence of IFNγ priming, amongst the top genes differentially expressed were the Interferon Stimulated Genes, ISGs: *MX1*, *IFIT1* and *IFIT3*, known to inhibit viral replication. We focused on the antiviral receptor *RIG-I* (also known as *DDX58*) because of its key role in

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the detection and eradication of replicating viral genomes (28). *RIG-I* is known to be upregulated in Hela cells stimulated by IFNγ (29) and similarly in intestinal cells (30). Furthermore, other groups have previously highlighted the robust link between *RIG-I* and viral load. Kato et al. demonstrated that *RIG-I*$^{-/-}$ mice are more susceptible to viral infections compared to control mice (31). In addition, siRNA-mediated knockout of *RIG-I* expression in human bronchial epithelial tissue showed reduced innate cytokine responses and rhinovirus replication (32). Similarly, Foronjy et al. stated that silencing *RIG-I* in A549 epithelium had a significant impact on RSV infection (33). Overall the work already published clearly shows an association between *RIG-I* expression and modulation in viral infection. Furthermore, genetic association studies have already linked polymorphisms in this gene to viral respiratory illness (34) and also to asthma exacerbations (35).

IFNγ upregulated the expression of *RIG-I* compared to both control cells and RSV infected cells **(FIGURE 2B)**. A further increase in *RIG-I* expression by IFNγ-primed RSV-infected cells, was also observed and confirmed by RT-qPCR **(FIGURE 3).** In addition to IFNγ effects on cells we investigated the effects of IL13 and no significant changes in *RIG-I* expression were seen when were primed with IL13 with or without exposure to RSV.

We further investigated the relevance of this observation of increased *RIG-I* expression in response to IFNγ in primary human bronchial epithelial cells (PBECS). Similarly to AALEB, IFNγ priming caused upregulation of *RIG-I* expression in PBECS 24 h after IFNγ treatment **(FIGURE 4A).** Intriguingly, in the PBECS this effect of IFNγ on *RIG-I* expression was still manifest up to 4 days after IFNγ was removed from the culture medium **(FIGURE 4B).** Furthermore IFNγ reduced viral load in IFNγ-primed RSV-infected PBECs compared to RSV control cells and IL13-primed RSV-infected PBECs **(FIGURE 4C)**. A further increase in *RIG-I* expression by IFNγ-primed RSV-infected PBECs, was also observed and confirmed by RT-

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qPCR **(FIGURE 4D).** We didn't reach significance in PBECs due to a small number of repeats (n=3) when we measured viral load and *RIG*-I following priming and RSV infection but these data indicated a long term effect of IFNγ priming in PBECS and suggest epigenetic regulation of *RIG-I* expression may play a role alongside any temporary transcriptional regulation. Due to their almost unlimited availability and similarity of *RIG-I* responses in PBECs following IFNγ priming, we opted to continue analysis of the epigenetic control of gene regulation in AALEB cells. Epigenetic analysis requires great amounts of chromatin and an immortalised cell line like AALEB would be an ideal model to use for this project.

Effects of IFNγ-priming on Epigenetic Regulation of *RIG-I* expression

We investigated if the increase of *RIG-I* transcription induced by IFNγ–priming correlated with epigenetic changes at the RIG-I promoter and hence focused on changes in the levels of histone methylation associated with increased *RIG-I* transcription. In association with an increase in *RIG-I* mRNA detected at 24 h following IFNγ priming, we saw a significant reduction of the methylation of the lysine 9 residue on histone 3 (H3K9me3) **(FIGURE 5)**, but not of lysine 4 (H3K4) or lysine 27 (H3K27) at the *RIG-I* promoter **(FIGURE 5B)**, in AALEB cells using Chromatin Immunoprecipitation (ChIP). Trimethylation of H3K9 is associated with transcriptional repression and hence its reduction following IFNγ priming is consistent with the observed increase in *RIG-I* expression. In addition we investigated histone acetylation of the lysine 9 residue (H3K9ac) as this also has a functional link to gene expression (36). In our cellular model H3K9ac was not altered by IFNγ, indicating that H3K9ac may not be involved in IFNγ-regulated *RIG-I* expression. In addition, RNApolII, an essential element in the transcription complex, recruited to the *RIG-I* promoter was detected at the promoter as expected but it did not change significantly with priming **(FIGURE 5C).**

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Functional effects of epigenetic markers on *RIG-I* expression

To further analyse the epigenetic factors influenced by IFNγ priming and to investigate their functional consequences we employed small molecule inhibitors of histone demethylases and methyltransferases **(TABLE E1)** to identify the role of histone methylation in *RIG-I* transcription **(FIGURE 6)**. First we employed the methyltransferase inhibitors WDR5-103 and DZNep. WDR5-103 inhibits methylation of lysine 4 on histone H3 (H3K4me3) (37), whilst DZNep inhibits trimethylation of lysine 27 on histone H3 (H3K27me3) (38). When cells where pre-treated either with WDR5-0103 or DZNep with or without IFNγ priming, there was no effect on *RIG-I* transcription compared to the untreated cells (data not shown) further confirming that H3K4me3 and H3K27me3 were not involved in *RIG-I* modulation by IFNγ.

Analysis of histone demethylase function on *RIG-I* epigenetic regulation

As H3K9 methylation appeared to be involved in these IFNγ-mediated epigenetic changes of the *RIG-I* promoter, we next explored if the histone-modifying enzymes involved in the regulation of H3K9 tri- and di-methylation were IFNγ-modulated. Unlike monomethylated H3K9 which promotes transcription, di- and tri-methylation of H3K9 are capable of preventing activation of gene expression (39). Several enzymatic complexes are required to modulate methylation that ultimately regulates transcription and ensures that epigenetic information is carried over through cellular replication (40). Based on our findings that IFNγ priming was associated with a reduction of tri-methylation of H3K9 at the *RIG-I* promoter we investigated two possible options: a) IFNγ removes methyl residues from H3K9me3 by recruiting demethylases, b) IFNγ inhibits the methyltransferases that add methyl groups to H3K9me1 and H3K9me2. To investigate which mechanism is used by IFNγ to modulate *RIG-I*

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transcription we used JIB-04 a pan inhibitor of the activity of the Jumonji family of histone demethylases (41) and BIX-01294 (diazepin-quinazolin-amine derivative) that inactivates the lysine methyltransferase G9a involved in H3K9me2 methylation (42). Additionally, for testing if Lysine-specific demethylase 1 (LSD1) was involved in mono- and di- demethylation of H3K9, we used Pargyline and Tranylcypromine as inhibitors (41, 43)

Pargyline and Tranylcypromine treatment did not cause any significant increase in *RIG-I* (data not shown) possibly indicating that lysine demethylation of mono and di methylation of H3K9 is not involved in IFNγ-mediated *RIG-I* regulation. Following treatment with 1 μM JIB-04, contrary to expectations, we observed a significant increase of *RIG-I* transcription in IFNγ-primed cells compared to cells treated with JIB-04 alone. The effects of this compound are dose dependent and the concentration we used (1 uM) was aimed at inhibiting JMJD2C (KDM4C) the enzyme responsible for demethylating H3K9me3 **(FIGURE 7A)**. We further analysed the effects of JIB-04 on the *RIG-I* promoter by ChIP. Intriguingly, no differences in H3K9me3 were detected at the *RIG-I* promoter in IFNγ primed cells following JIB-04 treatment (not shown). This result suggests that JIB-04 may be acting on *RIG-I* transcription through other regulatory mechanisms that do not involve H3K9me3. Similarly, no changes were observed for H3K4me3, H3K27me3, H3K9ac and RNApolII in the presence of JIB-04 (data not shown). The inhibitor JIB-04 without IFNγ priming, promotes an increase in transcription; this indicates that some of its effects are independent of IFNγ **(FIGURE 7A)**. Due to the purpose of this investigation, aimed a characterise regulation IFNγ-mediated, JIB-04 was not investigated any further.

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Involvement of lysine methyltransferase G9a in *RIG-I* epigenetic regulation

We next studied whether IFNγ modulates *RIG-I* via inhibition of methylation, possibly interfering with lysine methyltransferase G9a that uses mono-methylated H3K9 (H3K9me1) as substrate to add a second methyl group (H3K9me2). The activity of G9a leads to an increase of di-methylated H3K9 that is then the substrate for H3K9 trimethylation, a transcription repressive marker. Inhibiting G9a with BIX-01294 should therefore results in an increase of transcription. Treatment of AALEBs with BIX-01294 alone did not lead to any change in *RIG-I* expression at either the RNA or protein level. As expected, addition of BIX-01294 to IFNγ primed cells led to a significant increase in *RIG-I* expression at both the RNA and protein level compared to IFNγ alone **(FIGURE 7B and 7C)**. This suggests the involvement of lysine methyltransferase G9a in *RIG-I* epigenetic regulation by IFNγ. ChIP analysis revealed that the levels of H3K9me3, did not change after BIX-01294 treatment alone compared to untreated and with or without IFNγ pre-treatment **(FIGURE E1A).** We observed no correlation between *RIG-I* expression and the levels of H3K9me2 after BIX-01294 treatment **(FIGURE E1B).** Taken together these results suggest that these epigenetic inhibitors may stabilise the IFNγ-induced reduction in H3K9me3 leading to enhanced *RIG-I* transcription.

To demonstrate that the effects seen on *RIG-I* were specific, we measured the gene expression levels of a housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* and showed that its expression **(FIGURE E2A)** and levels of H3K9me3 or me2 **(FIGURE E2C and E2E)** were not affected by IFNγ priming or BIX-01294 treatment**.** The Toll Like Receptor 7 (*TLR7)* gene was expressed in AALEBs at barely detectable levels, but was also not affected by IFNγ priming or BIX-01294 treatment **(FIGURE E2B),** *TLR7* gene shows at the promoter high levels of repressive epigenetic marks H3K9me3 and me2 compared to

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GAPDH consistent with its transcription levels **(FIGURE E2D and E2F).** This indicates that the effects of the methyltransferase inhibitor BIX-01294 are gene specific and here we see an effect of this inhibitor on IFNγ regulated genes.

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DISCUSSION

We have demonstrated that IFNγ priming of epithelial cells reduces viral load and the enhanced *RIG-I* mRNA expression following IFNγ priming correlated with epigenetic changes at the *RIG-I* promoter linked with increased transcription. Global gene expression analysis using microarrays following the exposure of epithelial cells to IFNγ and RSV identified induction of many epithelial expressed anti-viral genes including the cytosolic viral sensor *RIG-I* known for its role in the detection and eradication of replicating viral genomes (28). *RIG-I* upregulation was also confirmed using RT-qPCR. IFNγ priming caused a reduction of transcriptionally repressive histone methylation, H3K9me3, at the *RIG-I* promoter. Furthermore, use of the inhibitors JIB-04 and BIX-01294 suggested that more than one mechanism may be involved in this IFNγ-mediated epigenetic regulation of *RIG-I*. The specific mechanism of IFNγ priming still needs to be elucidated but taken together these results suggest that exposure to IFNγ can prime the anti-viral response of the airway epithelium by epigenetic changes to key pathways.

Increasingly strong evidence suggests that the environment is capable of inducing innate immune memory and that epigenetic changes, including histone methylation, play an important role in the development and persistence of this memory (44). Ooi et al. (45) showed that mechanisms of epigenetic regulation involved in allergic airway inflammation were key in the development of a chronic asthmatic phenotype. Early life has already been shown to be a critical stage where allergens, poor air quality and repeated viral infections impacting on pre-existing genetic susceptibility can impact on the evolution of asthma (46, 47). In contrast, exposure to farming environments and endotoxins for instance can lead to higher levels of IFNγ pre- and post-natally and may be protective against an asthma

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phenotype (2, 17-19). Studies in mice have shown that IFNγ is required for viral clearance (2, 4) but there is also data showing that IFNγ can prevent replication (27). Our data do not provide an indication as to which of these mechanisms predominate in our model. IL13 has been linked to airway inflammation and remodelling but it seems to be a response to RSV rather than the cause of a severe response during viral infection (2, 4). Our cellular model data support these conclusions in that the addition of IL13 did not affect viral load or *RIG-I* expression, an RNA sensor known to have a pivotal role in the antiviral immune response (31). These data suggest that the “Th2 hypothesis”, an upregulated Th2 and a downregulated Th1 response drive the development of the disease (48), may be re-read as “a lack of a Th1 and not a Th2 predominant immune milieu per se that drives disease development”.

IFNγ regulates downstream genes through phosphorylation and activation of STAT1 homodimers which activate Gamma Activating Sequence (GAS) at the promoter of immune related genes and increases transcription by recruiting interferon regulatory factors (IRFs) (49). *RIG-I* gene is under the regulation of STAT1 (50) and in our model we show that the regulation of *RIG-I* by IFNγ is further influenced at the epigenetic level by a reduction of the repressive marker H3K9me3 leaving a signature on the histones. Interestingly, Robertson et al. 2008 (51) showed that 13% of STAT1 promoters do not contain H3K4me3 and similarly we did not find involvement of H3K4me3 and H3K27me3 in the regulation of *RIG-I* transcription suggesting that the Polycomb/Trithorax enzymatic complex (52) are not modulating *RIG-I* regulation by IFNγ.

This trimethylation of lysine 9 on histone 3 is associated with heterochromatin and inactive genes, although there are exceptions where methylation on lysine 9 contributes to the regulation of euchromatic genes (53-55). In our cellular model, where *RIG-I* is expressed

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even in unstimulated cells, the presence of H3K9me3 at low levels is permissive of transcription and its further reduction following IFNγ priming is linked with an increase of *RIG-I* gene expression (56). In an attempt to understand how IFNγ induces these epigenetic changes at the RIG-I promoter we used small molecule epigenetic inhibitors. The lysine methyltransferase inhibitor (BIX-01294) and the pan inhibitor of Jumonji histone demethylases (JIB-04) upregulated *RIG-I* expression in the presence of IFNγ, but neither of them indicate what the epigenetic changes caused by IFNγ priming are. JIB-04 induced a trend towards upregulation of *RIG-I* expression in AALEB cells in the absence of IFNγ. This observation may suggest a direct correlation between JMJD2C/KDM4C (the enzyme responsible for demethylating H3K9me3), which is inhibited by JIB-04, and *RIG-I* expression. Whilst in the presence of IFNγ, the effect of JIB-04 on *RIG-I* expression was additive, the primary aim of this investigation was to identify regulatory mechanisms that were mediated purely by IFNγ. We therefore concentrated on BIX-01294, which, in contrast to JIB-04, had significant effects on *RIG-I* expression only in IFNγ primed cells. BIX-01294 alone did not cause a reduction in the tri-methylation levels of H3K9 (H3K9me3) at the RIG-I promoter. Tri-methylation of H3K9 was reduced when cells were primed with IFNγ, but this was not further reduced in the presence of BIX-01294. This could indicate that these inhibitors may have other unidentified ways to potentiate the mechanisms used by IFNγ to regulate *RIG-I.*

Additionally we investigated whether IFNγ modulates *RIG-I* by interfering with lysine methyltransferase G9a (57) that uses mono-methylated H3K9 (H3K9me1) as substrate to add a second methyl group (H3K9me2). The activity of G9a physiologically leads to an increase of H3K9me2a substrate for H3K9me3 known to be a transcriptional repressor marker. When we consider that BIX-01294 inhibits G9a involved in H3K9me2 methylation, we would expect a reduction in the level of H3K9me2 in cells exposed to BIX-01294 although

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in our experiments this reduction did not reach significance. The removal of H3K9me2 did not increase *RIG-I* transcription on its own and the levels of H3K9me3 described earlier remained high, which may mask any effects produced by BIX-01294. Thus, even though we have inhibited demethylases and methyltransferases known to be associated with H3K9 methylation we saw no effect on baseline methylation. However, IFNγ reduced H3K9me3 of the *RIG-I* promoter and increased gene transcription in the presence of both inhibitors. Therefore, IFNγ may modulate H3K9 methylation and *RIG-I* transcription via a different mechanism and further work is required to elucidate this. It would also be informative to analyse other areas of the *RIG-I* promoter to have a clearer picture of the histone profile and possibly where the effects of the epigenetic inhibitors would be more evident. We may have not investigated the histone markers directly regulated by IFNγ and what we have shown here is a downstream effect and not the cause of change in transcription levels. These findings do not identify the mechanisms associated with IFNγ regulation, although the results strongly suggest that the cytokine environment may epigenetically modulate genes involved in the viral response and drive innate future responses.

There are limitations in this investigation that may have restrict our findings. The use of AALEB cells as an epithelial model instead of primary airways epithelial cells may have given us an incomplete or an imperfect picture of the epigenetic profile of *RIG-I* promoter. We also do not take into account the effects that other cytokines may have in conjunction with IFNγ on modulating gene expression. Additionally we analyse the effects of IFNγ priming after only after 24 hours and although it is enough to show an effect on *RIG-I* promoter, it is possible that longer time points may have been more informative.

The innate immune response and the Th1/ Th2 predisposition in the lung changes along the life course and these variations are evident in the interaction between host and

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environment and correlate to susceptibility to disease (18, 19). The inflammatory environment of naive epithelial cells may epigenetically modulate innate immune responses altering the levels of histone methylation and acetylation and hence potentially may lead to long term impacts on anti-viral immunity. The presence of a Th1 and Th2 milieu appear key to be involved in the development of innate immune responses. The effect of the cytokine milieu on the anti-viral responses rely on the environmental influences that modulate cytokine production, genetic background and on the timing of viral infections._ Further investigation using ChIP-seq analysis may identify other genes that are regulated in a similar fashion by cytokines and clarify mechanisms that once understood may provide a way to learn how to better modulate the innate system against viral infections and virus induced asthma.

We describe a potentially important regulatory mechanism that need to be explored further but may explain the ability of the airway immune environment to modulate gene transcription early in life to prime immune responses. The airway inflammatory milieu, driven by the maturing immune response to the environment, may have important effects on the development of epithelial anti-viral innate immunity and the consequent development of its long term dysregulation, a key feature of asthma.

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Figure Legends

FIGURE 1 Detection of RSV replication in AALEB cells. **(A)** Experimental design of IFNγ priming and viral infection on AALEB cells. AALEBs, after being plated, were primed with 10 ng/ml IFNγ (RSVIFNγ), 10 ng/ml IL13 (RSVIL13), or untreated (RSV) for 24 h before being exposed to RSV for 48 h. **(B)** Viral replication measured by RT-qPCR in AALEB cells exposed to RSV subtype A Memphis 37 at MOI 1 for 48h (RSV), with no virus quantified in UV–treated RSV cells (UV) and in the untreated cells control (UN). n=7, **(C)** IFNγ priming significantly reduced RSV replication. Virus replication was detected with Custom Taqman Assays (Life Technologies) specific for the N gene of the RSV genome. Values were normalized against *HPRT* and calculated using ΔCt method. n=7

FIGURE 2 (A) Heatmap depicting the log2 expression values for 100 genes with top peak fold changes in untreated AALEB cells (UNT), RSV infected (RSV), primed with IFNγ (IFNγ) and primed and infected (RSV+IFNγ). Hierarchical clustering was performed using Pearson correlation metric to calculate distances and clustered using complete linkage. The genes expression values were averaged and scaled across every row to indicate the number of standard deviations above (red) or below the mean (blue), denoted as the row Z-score. (**B)** Log2 expression values for *RIG-I* mRNA with top peak fold changes in untreated cells (UNT), RSV infected (RSV), primed with IFNγ (IFNγ) and primed and infected (RSVIFNγ) n=2.

FIGURE 3 Detection of *RIG-I* mRNA in AALEB cells measured by RT-qPCR after cytokine priming and RSV infection.
AALEB cells were untreated (UNT), primed with 10 ng/ml IL13 (IL13) or 10 ng/ml IFNγ (FNγ) for 24h. AALEB cells were untreated (RSV), primed with 10 ng/ml IL13 (RSVIL13) or 10 ng/ml IFNγ (RSVIFNγ) for 24 h before being exposed to RSV subtype A Memphis 37 at MOI 1 for 48 h. Values were normalized against *HPRT* and calculated using ΔCt method. n=7.

FIGURE 4 (A) Detection of *RIG-I* mRNA measured by RT-qPCR in PBECs exposed IFNγ priming (10 ng/ml) for 24 h, n=8. **(B)** Detection of *RIG-I* mRNA measured by RT-qPCR in PBECs being exposed to IFNγ (10 ng/ml) for 24 h and after being washed twice in PBS left in culture media for 4 additional days, n=6. IFNγ primed (IFNγ), or untreated (UNT). Values were normalized

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against HPRT gene and calculated using ΔCt method. **(C)** PBECs cells after being plated, were primed with 10 ng/ml IFNγ (RSVIFNγ), 10 ng/ml IL13 (RSVIL13), or untreated (RSV) for 24 h before being exposed to RSV subtype A Memphis 37 at MOI 1 for 48 h. Virus replication was detected with Custom Taqman Assays (Life Technologies) specific for the N gene of the RSV genome. n=3 **(D)** Detection of *RIG-I* mRNA in PBECs measured by RT-qPCR after cytokine priming and RSV infection. Values were normalized against HPRT and calculated using ΔCt method. n=3

FIGURE 5 Analysis of methylation and acetylation levels at lysines residues on histone 3 at the promoter of *RIG-I* gene on AALEB cells by Chromatin Immune Precipitation (ChIP). AALEBs primed with IFNγ (IFNγ) or left untreated (UNT). **(A)** Analysis of methylation levels at lysines 9 residue on histone 3 (H3K9me3). Antibodies against trimethylated Lysine 9 on histone (H3K9me3) were used, n=6. Other antibodies against the following modifications were used: **(B)** trimethylated Lysine 4 on histone 3 (H3K4me3), trimethylated Lysine 27 on histone 3 (H3K27me3) n=3, **(C)** acetylation on Lysine 9 on histone 3 (H3K9ac) and against RNA polymerase II (RNApolII), n=3. The pull down chromatin is amplified by qPCR with Custom Taqman Assays specific for *RIG-I* promoter sequence. Percentage of input is calculated as 100×2[Ct(input) – Ct(IP)], after adjusting the mean input Ct value for 1/20 starting material (fraction of input chromatin reserved). The levels of H3K27me3, and H3K4me3 do not change significantly. IFNγ causes a not significant reduction of H3K9ac and an increase of RNApolII.

FIGURE 6 Schematic representation of experimental conditions used to prime AALEB cells with IFNγ alongside the chemical compounds used to inhibit histone demethylases and methyltransferases.

FIGURE 7 Detection of *RIG-I* mRNA measured by RT-qPCR in AALEB cells exposed to epigenetic inhibitors. **(A)** AALEBs exposed to 1 μM JIB-04 for 6 h before IFNγ priming (10 ng/ml) for 24 h. JIB-04 exposure and IFNγ priming (JIB +IFNγ), IFNγ primed (IFNγ), exposed to JIB-04 only (JIB) or untreated (UNT), n=6. **(B)** AALEBs were exposed to 5 μM BIX-01294 for 6 h before IFNγ priming (10 ng/ml) for 24 h. BIX-01294 exposure and IFNγ priming (BIX +IFNγ),

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IFNγ primed (IFNγ), exposed to BIX-01294 only (BIX) or untreated (UNT). Both the inhibitors upregulate *RIG-I* expression when cells were also IFNγ primed. Values were normalized against *HPRT* and calculated using ΔCt method. n=7. **(C)** Detection of RIG-I protein levels measured by western blot following IFNγ priming and BIX-01294 treatment. Representative image of 3 independent experiments. Tubulin served as loading control, 40ug of lysate.

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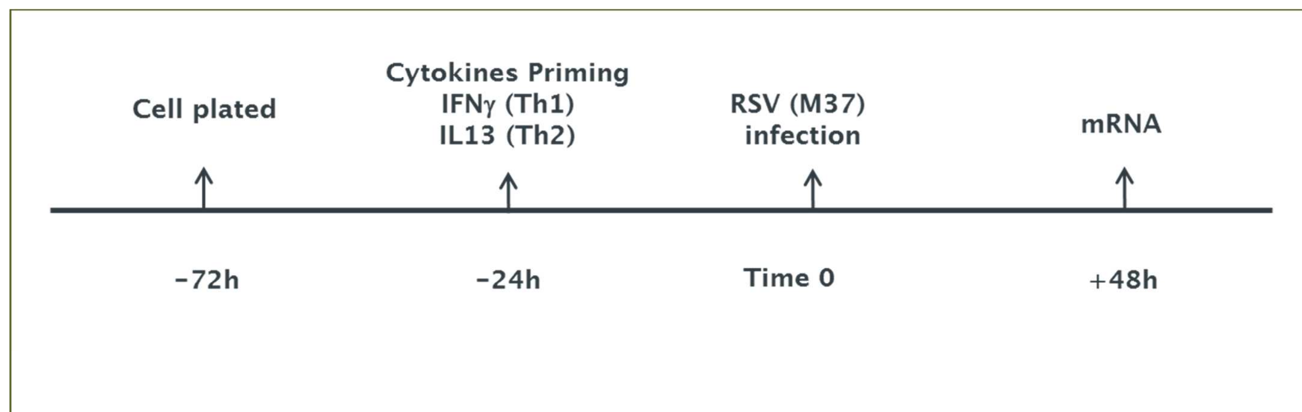
FIGURE 1

(A)



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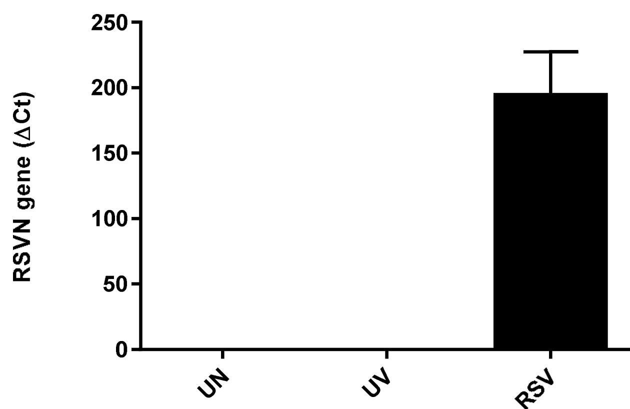

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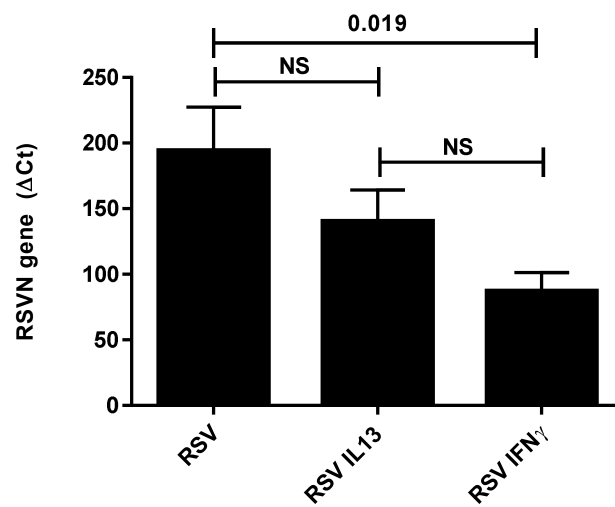

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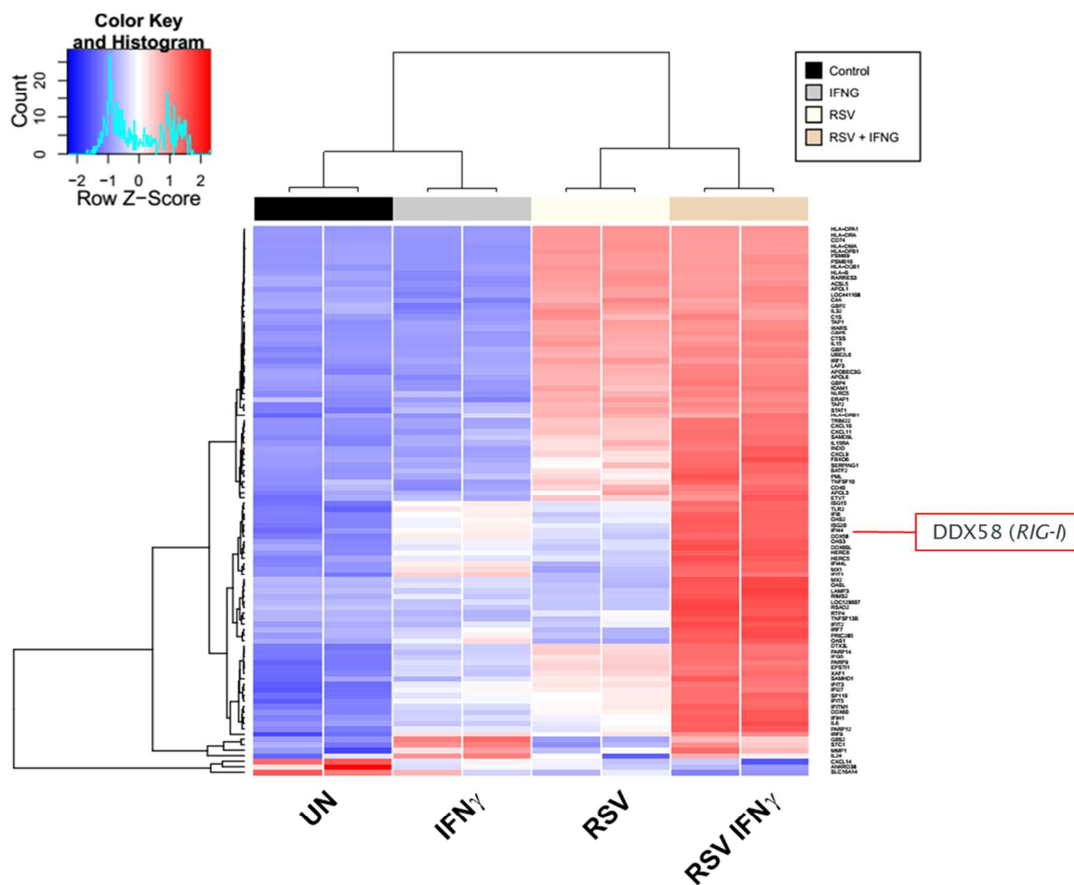
FIGURE 2

(A)



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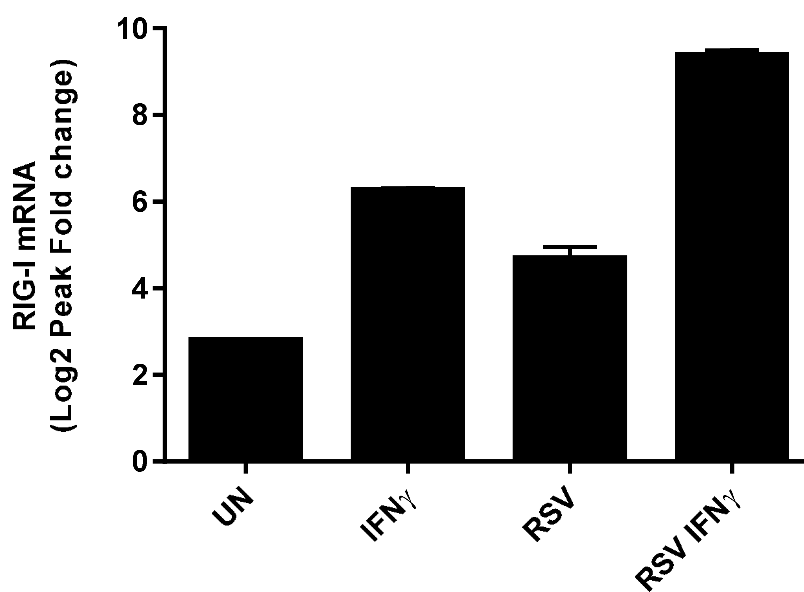

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FIGURE 3

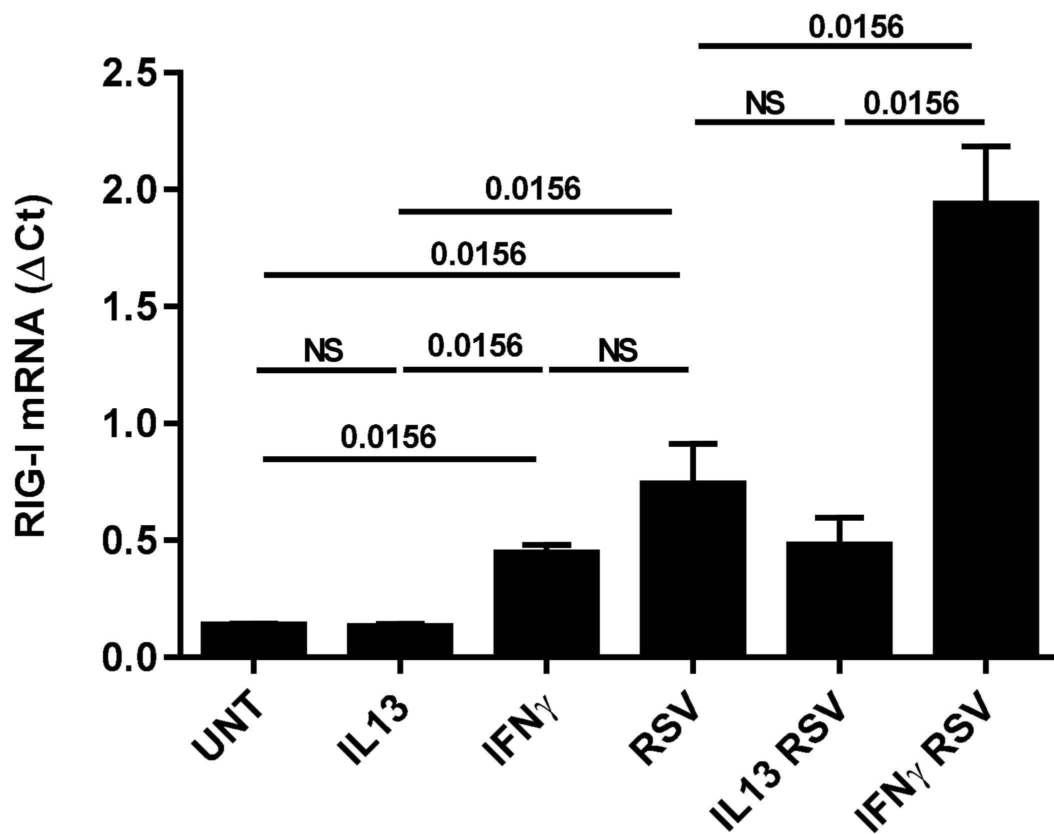

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FIGURE 4

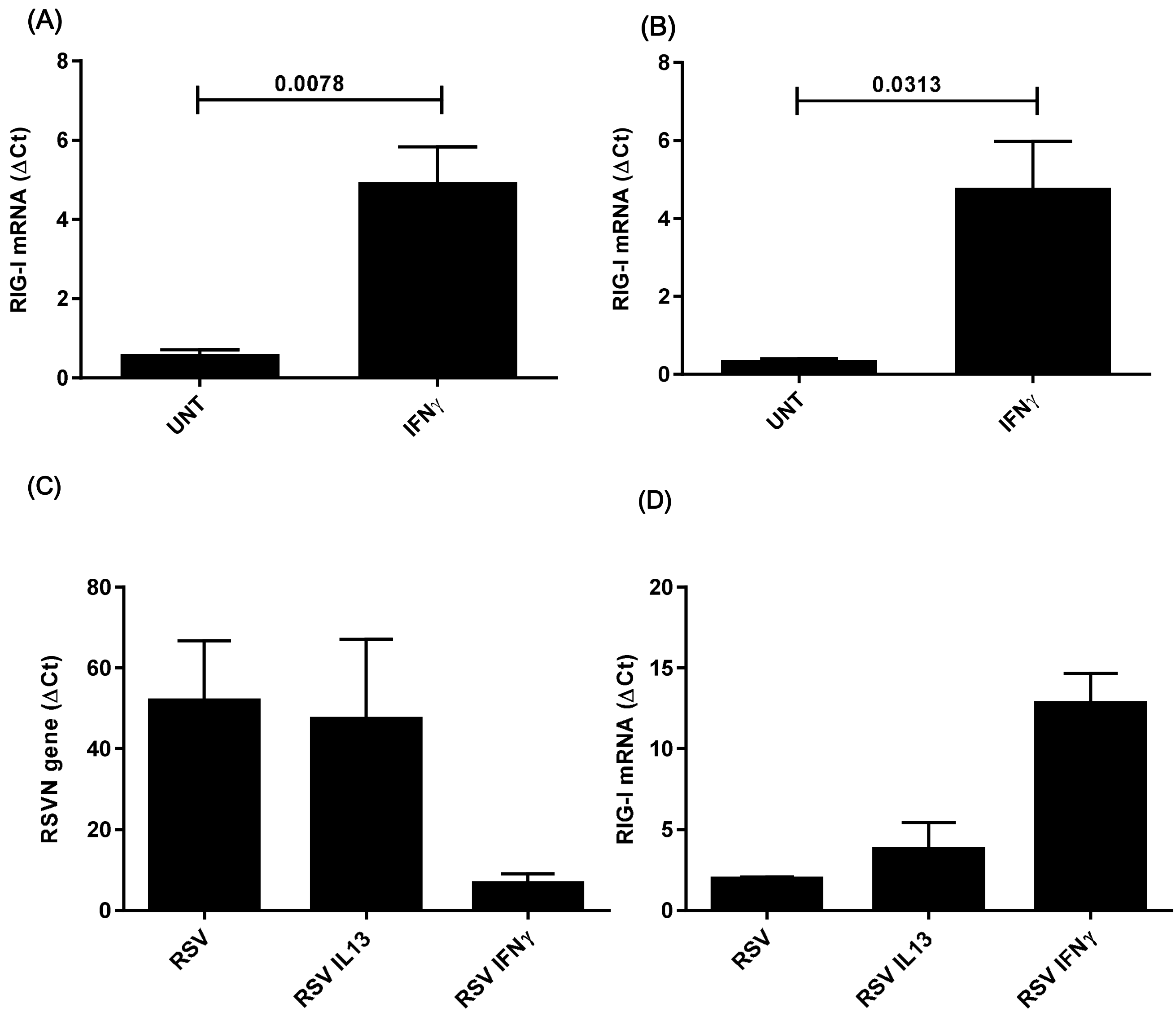

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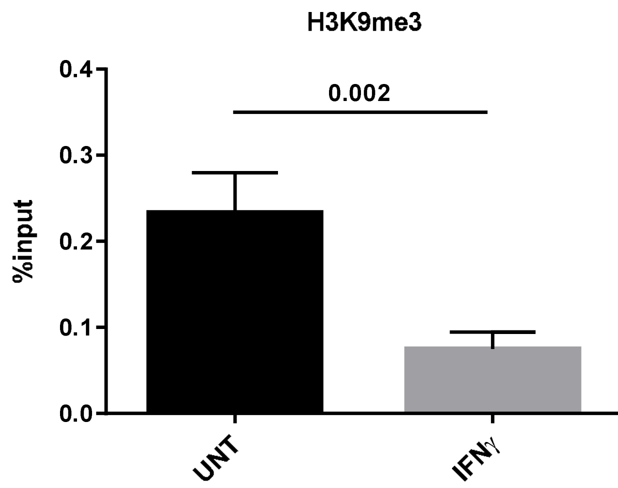
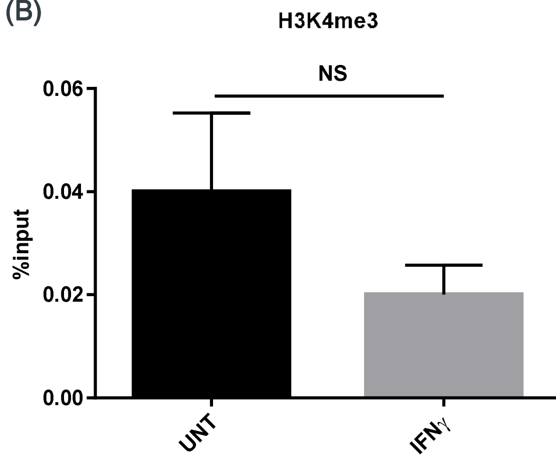
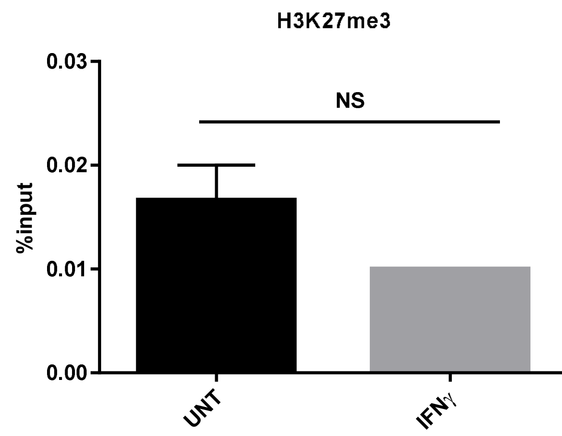
FIGURE 5

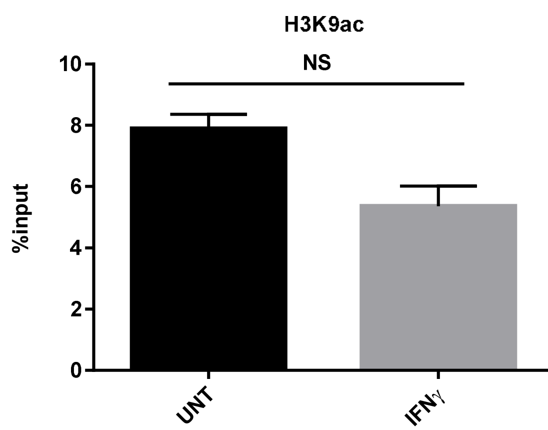
(A)

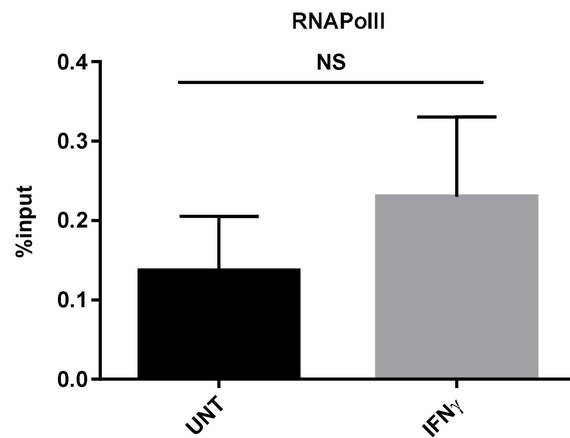


(B)









(C)

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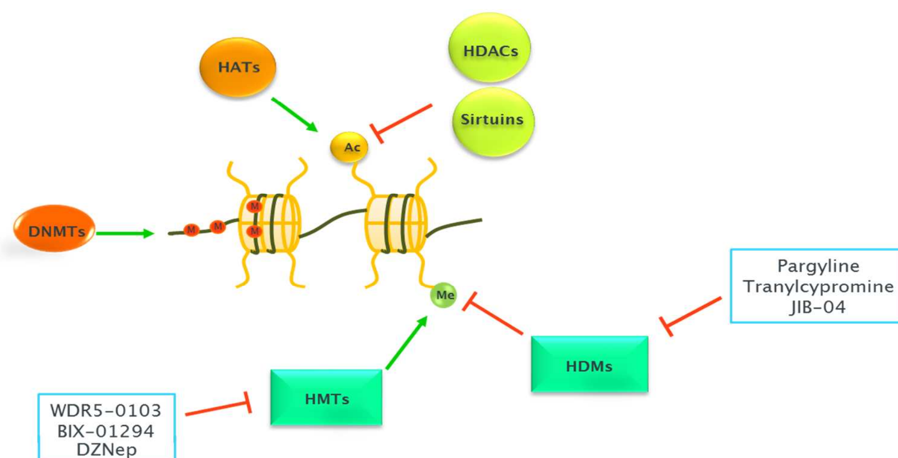
FIGURE 6



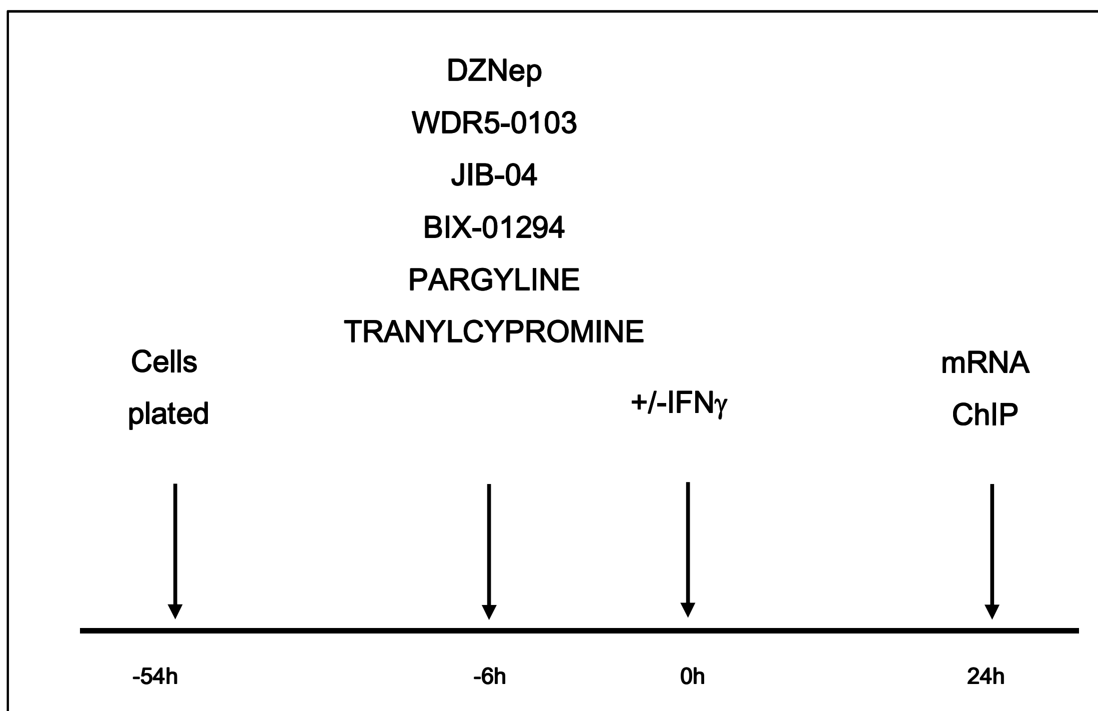


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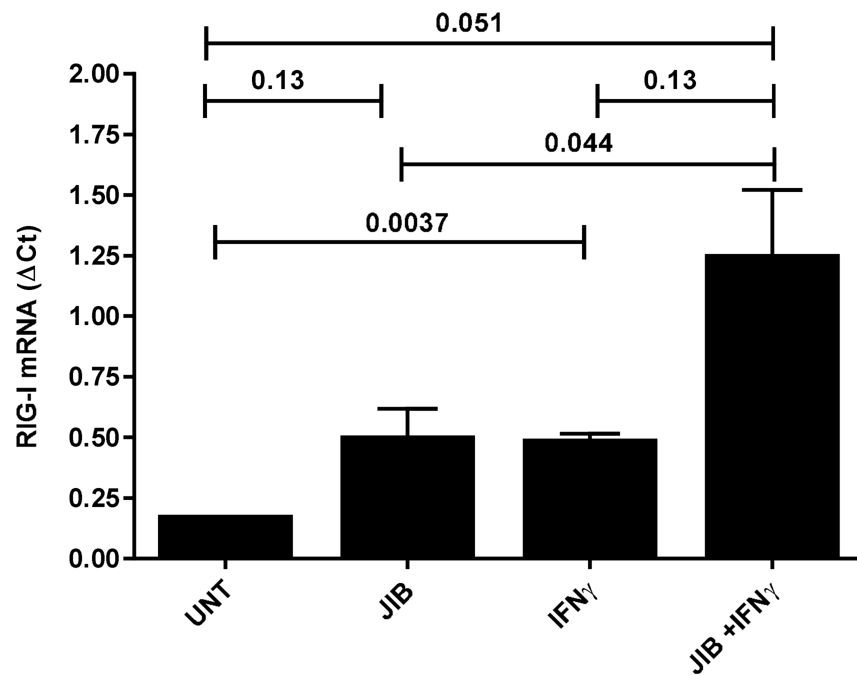
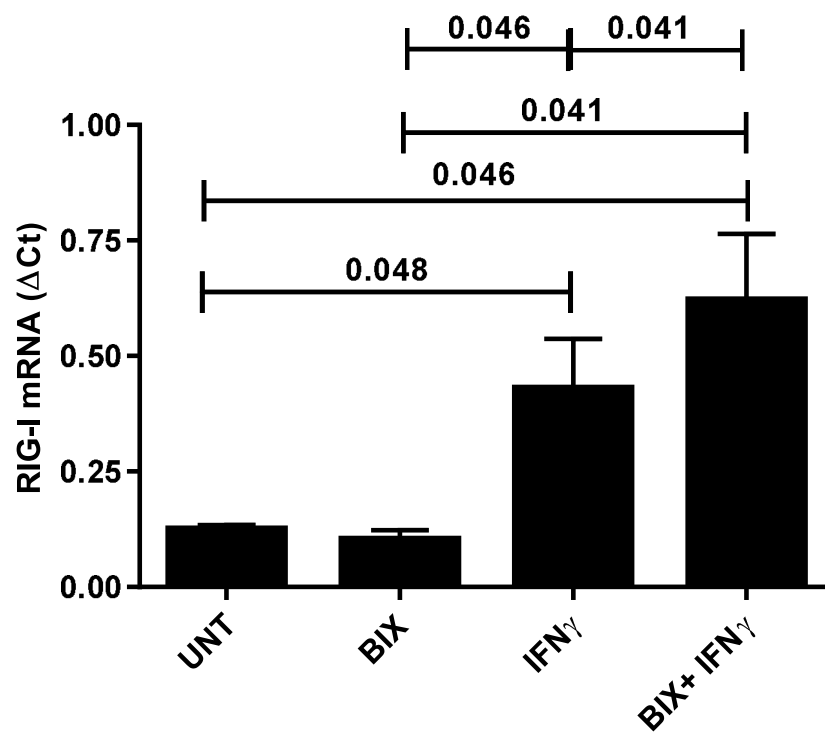
FIGURE 7

(A)



(B)



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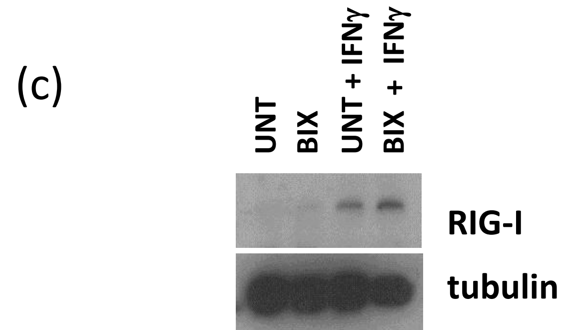

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IFNγ INFLUENCES EPITHELIAL ANTI-VIRAL RESPONSES VIA HISTONE METHYLATION OF THE *RIG-I* PROMOTER

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ONLINE SUPPLEMENT

EXTENDED MATERIAL AND METHODS

RNA extraction

Per 1 mL of TRIFast 0.2 mL of chloroform were added and the sample were centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase was precipitated at -20 °C for 30 minutes in the presence of 4 μg of RNase-free glycogen as a carrier with 0.5 mL of 100% isopropanol. The RNA was centrifuged at 12,000 × g for 20 minutes at 4°C and the pellet washed twice in cold 75% ethanol.

The RNA used for microarray gene expression analysis was further purified with RNeasy Mini Kit (Qiagen, Manchester, UK). As standard quality measurements RNA was analysed with Eukaryote Total RNA Nano Series II assay kit on 2100 Bioanalyzer (Agilent Technologies, Cheshire, UK). RIN values were above 9 for every sample.

Microarray analysis

Global gene expression was assessed using the Affymetrix GeneChip U133 Plus 2.0. Labeling and array hybridisations were performed according to standard protocols (Source BioScience,

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Berlin, Germany) in AALEB cells subjected to the following conditions: (1) untreated (2) IFNγ treated, (3) RSV infected and (4) IFNγ treated and RSV infected. Gene expression analysis was done in duplicate for every condition and RNA for each condition was the result of three independent experiments pooled together. Raw microarray gene expression data was normalized using Gene Chip Robust Multi-Array Analysis (GCRMA) (1) and subjected to quality control procedures. Fold changes were calculated by averaging 2 replicates and subtracting the log2 expression values from control. Only genes with actual fold changes greater than 2 or less than -2 were considered for functional enrichment analysis. Direct protein interaction networks (PINs) were generated using MetaCore™ (GeneGo, St. Joseph, MI, USA) and visualized in Cytoscape (version 3.2.1) (2) and pathways significantly over-represented for differentially expressed genes in a hypergeometic test (FDR-corrected p-value <0.05) were identified using ToppGene (3).

RT-qPCR

Total extracted RNA concentration was determined using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific Waltham, MA USA). 250ng of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) with random examers primers carried out accordingly to the manufacturer’s protocols. RNA expression was measured using TaqMan gene expression assays and RSV replication was detected with custom Taqman Assays as listed in **TABLE E2**.

For RT-qPCR, we employed TaqMan® universal PCR master mix, No AmpErase® UNG in a 7900HT fast real-time PCR system machine (both from Life Technologies, Thermo Fisher Scientific). The values were normalized against Hypoxanthine-guanine

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phosphoribosyltransferase *(HPRT1)* from the same manufacturer using comparative Ct method ($2^{-\text{Delta CT}}$).

Chromatin immunoprecipitation (ChIP)

Cells were plated in 10 cm petri dishes at concentration of $0.5X10^6$. 24-48 h later cells were treated for 6 h with the relevant inhibitor and then with IFNγ for further 24 h. Cells were crosslinked with 1 % final concentration of formaldehyde for 10 minutes. Crosslinking was stopped by the addition of glycine for 10 minutes to a final concentration of 125 mM. Cells washed three times with PBS, scraped and collected, and then resuspended in Lysis Buffer (25 mM Tris–HCl pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 % (v/v) Triton-X-100, 0.1 % (w/v) SDS) with proteases inhibitors (Roche, Basel, (1-4) Switzerland). The lysate was then sonicated with Soniprep 150 (MSE, London, UK) with 5 pulses of 30 seconds each. The sample was centrifugated and the supernatant resuspended in Lysis Buffer with proteinases inhibitors and aliquoted.

Every chromatin aliquot was immunoprecipitated with the chosen antibody (**TABLE E2**) and allowed to form complexes overnight at 4^oC with agitation. One chromatin aliquot was used as negative control to which no antibody was added and 50 μl of chromatin was also reserved as input. After the overnight antibody incubation, the immune complexes were incubated with Protein G-agarose beads (GE Healthcare) blocked with salmon sperm DNA and BSA (Life Technologies).

The Protein G agarose/antibody/protein/DNA complexes were washed three times with each of the following buffer: Lysis buffer; IP1 buffer (25 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 % (v/v) Triton-X-100, 0.1 % (w/v) SDS); IP2 buffer (10 mM Tris–HCl pH 8.0, 250

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mM LiCl, 0.5 % (v/v)NP-40, 0.5 % (w/v) sodium deoxycholate, 1 mM EDTA); and two washes with TE (10 mM Tris pH 8.0, 1 mM EDTA)

To purify precipitated DNA, complexes were incubated with DNase-free RNase (Sigma) for 30 min at 37°C, then 0.5 % SDS and Proteinase K (Sigma) were added to remove proteins and incubated 12 h at 37°C. After enzyme treatment, chromatin was incubated at 65°C for 6 h to reverse the cross-link. DNA was purified by phenol/chloroform extraction, precipitated with ethanol, and resuspended in TE.

Recovered DNA was amplified with Custom Taqman Assays (Life Technologies) specific for the promoter of the genes analysed. Primers and assay ID in **TABLE E4**:

Duplicates qPCRs were performed using an ABI 7900HT Fast Real-Time System (Life Technologies).

Production of cell lysates and protein quantitation

For Western blot experiments cells were lysed with 1% SDS + 1X protease inhibitor cocktail (Merck, Kenilworth, New Jersey, USA). 40ug of total protein lysate was loaded onto precast Polyacrylamide gel (Thermo Fisher Scientific) in reducing conditions and proteins transferred onto Hybond C Extra nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). RIG-I was detected with Anti-DDX58 (Ab140963 Abcam, Cambridge, UK) 1:500 and tubulin (sc-5286 Santa Cruz Biotechnology, Dallas, Texas) 1:1000. Detection was performed using horseradish peroxidase–conjugated secondary antibody goat anti-mouse IgG-HRP (sc-2005 Santa Cruz) 1:2000 in 5% non-fat milk/TBS-T and E enhanced chemiluminescence (ECL)-based system (Super Signal West Dura Kit, Thermo Fisher Scientific). Images were acquired using a Xograph X4 photographic film processor (Xograph Healthcare Ltd, Gloucestershire, UK).

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SUPPLEMENTAL TABLES AND FIGURES

TABLE E1 Epigenetic inhibitors

Inhibitor	Concentration used	Company
3-Deazaneplanocin A	0.5 μM	R&D Systems
JIB-04	1 μM	R&D Systems
Tranylcypromine	250 μM	Enzo Life Science
BIX01294	5 μM	Cambridge Bioscience
Pargyline	1.5 mM	Cambridge Bioscience
WDR5-0103	50 μM	Cambridge Bioscience

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TABLE E2 Taqman Gene expression assays (Applied Biosystems):

Gene name	Assay ID	Primers sequence (where available)
RSV (Perkins et al. 2005)	AIT95TB	Forward: CATCCAGCAAATACACCATCCA Reverse: TTCTGCACATCATAATTAGGAGTATCAA Probe: CGGAGCACAGGAGAT
RIG-I	Hs01061436_m1	
TLR7	Hs01933259_s1	
GAPDH	Hs02758991_g1	
HPRT	Hs02800695_m1	

TABLE E3 Antibodies (Abcam) used in ChIP experiments

Antibody	code
H3K4me3	ab1012
H3K9me3	ab8898
H3K9me2	mAbcam 1220
H3K27me3	ab6002
H3K9ac	ab10812
RNA polymerase II	ab5131

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TABLE E4 Taqman Gene assays for ChIP (Applied Biosystems):

Gene name	Assay ID	Primers sequence (where available)	
RIG-I	AJHSNWI		
TLR7		Forward Primer Reverse Primer Probe	CGACCTGATCTTTGTAGTTGGAA GGCAGGAAGTGTAGTCACAAAGAC TCCAGGGCTGGCTG
GAPDH		Forward Primer Reverse Primer Probe	CCTAATTATCAGGTCCAGGCTACAG CGGGAGGCGGCTTGA CTGCAGGACATCGTG

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Supplementary Figure Legends

FIGURE E1 Analysis of methylation levels at lysines residues on histone 3 at the promoter of *RIG-I* gene on AALEB cells by Chromatin Immune Precipitation (ChIP). Cells primed with IFNγ (IFNγ), untreated (UNT), exposed to BIX-01294 (BIX) and primed with IFNγ following exposure to BIX-01294 (BIX+IFNγ). **(A)** Antibodies against trimethylated Lysine 9 on histone (H3K9me3) n=3 and **(B)** dimethylated Lysine 9 on histone 3 (H3K9me2) n=3 were used. The pull down chromatin was amplified by qPCR with Custom Taqman Assays specific for RIG-I promoter sequence. Percentage of input was calculated as 100×2[Ct(input) – Ct(IP)], after adjusting the mean input Ct value for 1/20 starting material (fraction of input chromatin reserved).

FIGURE E2 Detection of *GAPDH* **(A)** and *TLR7* **(B)** mRNA measured by RT-qPCR in AALEB cells exposed to 5 μM BIX01294 for 6 h before IFNγ priming (10 ng/ml) for 24 h. Cells primed with IFNγ (IFNγ), untreated (UNT), exposed to BIX-01294 (BIX) and primed with IFNγ after exposure to BIX-01294 (BIX+IFNγ).
Analysis of methylation levels at lysines residues on histone 3 at the promoter of *GAPDH* and *TLR7* genes on AALEB cells by Chromatin Immune Precipitation (ChIP). Antibodies against trimethylated Lysine 9 on histone (H3K9me3) **(C and D)** and dimethylated Lysine 9 on histone 3 (H3K9me2) **(E and F)** were used. The pull down chromatin was amplified by qPCR with Custom Taqman Assays specific for *GAPDH* and *TLR7* promoter sequence. Percentage of input was calculated as 100×2[Ct(input) – Ct(IP)], after adjusting the mean input Ct value for 1/20 starting material (fraction of input chromatin reserved). n=3

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FIGURE E1

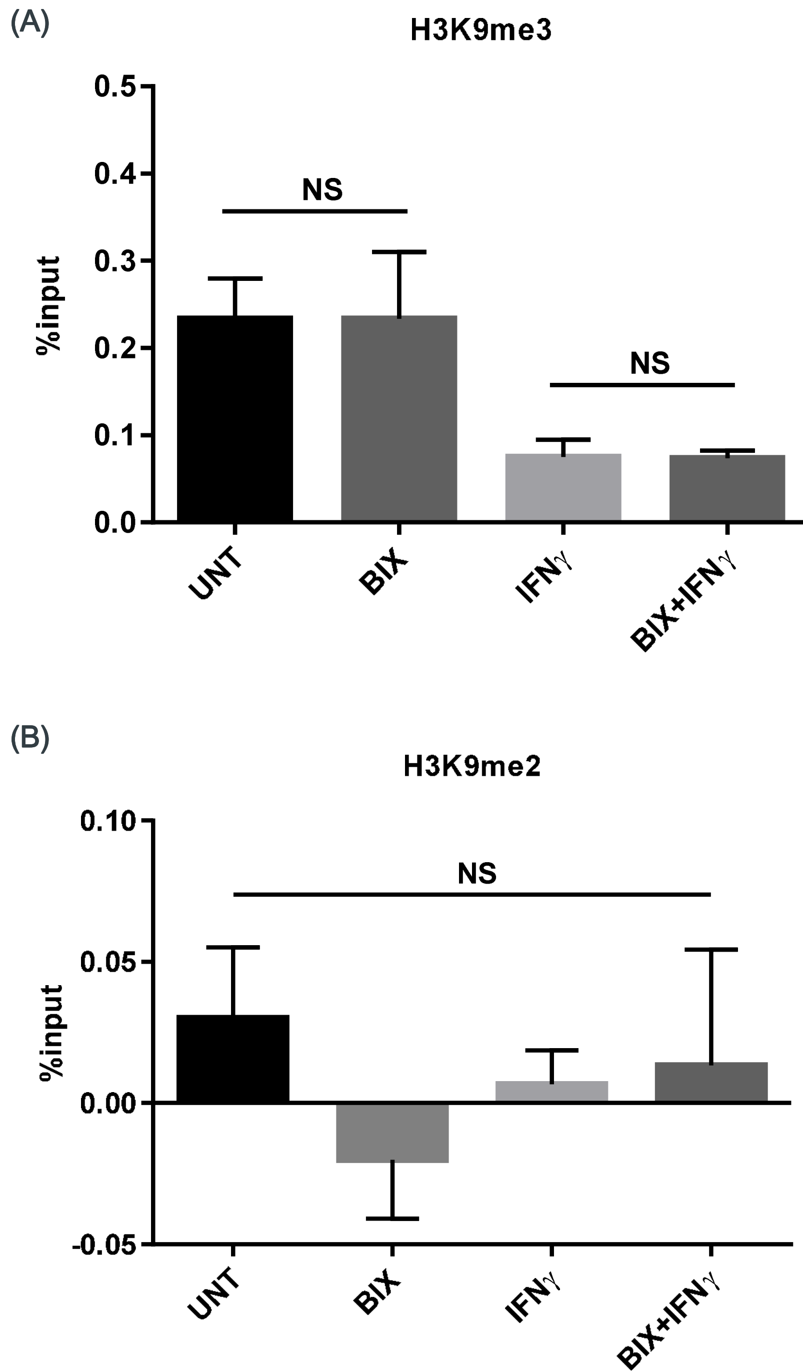

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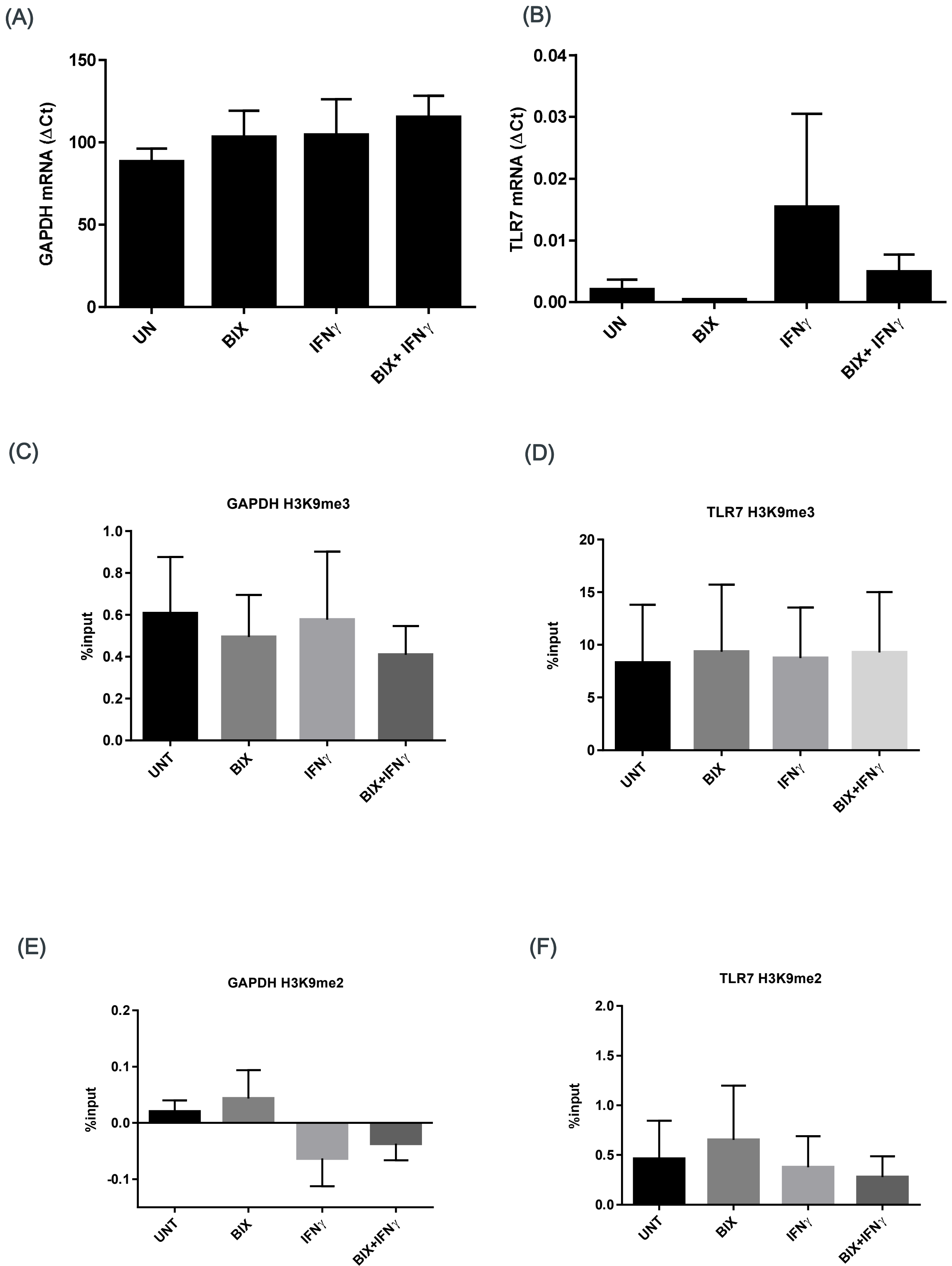
FIGURE E2



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