

LETTER TO THE EDITOR

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# Differential regulation of IFN $\alpha$ , IFN $\beta$ and IFN $\epsilon$ gene expression in human cervical epithelial cells

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## Abstract

Interferon $\epsilon$  (IFN $\epsilon$ ) is a unique type I IFN that has distinct functions from IFN $\alpha/\beta$ . IFN $\epsilon$  is constitutively expressed at mucosal tissues, including the female genital mucosa, and is reported to be modulated by estrogen and seminal plasma. However, its regulation by cytokines, including TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-17, IL-22 and IFN $\alpha$ , which are commonly present in the female genital mucosa, is not well documented in freshly isolated primary cervical cells from tissues. We determined the effect of these cytokines on gene expression of type I IFNs in an immortalized endocervical epithelial cell line (A2EN) and in primary cervical epithelial cells. Several pro-inflammatory cytokines were found to induce IFN $\epsilon$ , and TNF $\alpha$  induced the strongest response in both cell types. Pretreatment of cells with the I $\kappa$ B inhibitor, which blocks the NF- $\kappa$ B pathway, suppressed TNF $\alpha$ -mediated IFN $\epsilon$  gene induction and promoter activation. Expression of IFN $\alpha$ , IFN $\beta$ , and IFN $\epsilon$  was differentially regulated in response to various cytokines. Taken together, our results show that regulation of these IFNs depends on cell type, cytokine concentration, and incubation time, highlighting the complexity of the cytokine network in the cervical epithelium.

Dear Editor,

Interferon $\epsilon$  (IFN $\epsilon$ ) is a unique, functionally distinct type I IFN [1, 2]. Unlike IFN $\alpha/\beta$ , whose expression is nearly undetectable at baseline, IFN $\epsilon$  is expressed at basal constitutive levels in mucosal tissues, including the female genital mucosa. IFN $\epsilon$  gene expression is not induced in response to activation of several Toll-like receptors, nor is it activated by IRF3, IRF5 or IRF7 [3]. While the *in vitro* anti-viral activity of IFN $\epsilon$  is weaker than that of IFN $\alpha$  [4–6], IFN $\epsilon$  efficiently clears viral infections *in vivo* by recruiting lymphocytes and by increasing the numbers and functions of cytotoxic T cell subsets in mucosal tissues [1]. In IFN $\epsilon$ –/– mice, reduced NK cell numbers in the female genital tissues are associated with increased susceptibility to HSV2 and chlamydial infection [3]. Of considerable relevance, HIV-negative

sex workers with frequent exposure to semen have an increased level of IFN $\epsilon$  gene expression in the cervical epithelium and increased numbers of immune effectors cells in their cervical tissues [7], suggesting that IFN $\epsilon$  has a protective role in reducing HIV susceptibility by modulating the mucosal immune response.

Expression of IFN $\epsilon$  is reported to be regulated by estrogen and seminal plasma [3, 7, 8]; however, the effect of individual cytokines on IFN $\epsilon$  expression is less well documented. Matsumiya and colleagues demonstrated that TNF $\alpha$  at a concentration of 10 ng/ml or higher induces IFN $\epsilon$  mRNA expression by near twofold in HeLa cells, a cervical cancer cell line, although TNF $\alpha$  did not induce the IFN $\epsilon$  promoter activity in this study [9]. Interestingly, the expression of other type I IFNs including IFN $\alpha$ , IFN $\beta$ , IFN $\kappa$  and IFN $\omega$ , is not induced by TNF $\alpha$  in HeLa cells [9]. In this study, we examined the effect of cytokines commonly found in the mucosa during either a healthy or an inflammatory state, on IFN $\epsilon$  expression in A2EN cells, an immortalized endocervical cell line with limited passages [10], and in primary cervical epithelial cells (CECs)

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derived from various donors. Note that the protein levels were not assessed in this study because there is no reliable ELISA kit for IFN $\epsilon$ . Finally, we also determined the involvement of the NF- $\kappa$ B pathway in TNF $\alpha$ -mediated IFN $\epsilon$  gene induction and promoter activation.

## Results and discussion

A2EN cells or CECs were treated with stated cytokines at 1 or 10 ng/ml for 4 or 24 h before RNA extraction. Gene expression of IFN $\alpha$ , IFN $\beta$ , or IFN $\epsilon$  was determined by RT-PCR analysis. As expected, the baseline of IFN $\alpha$  or IFN $\beta$  was low in A2EN and CECs. The IFN $\epsilon$  mRNA level was 315- and 23-fold more abundant than IFN $\alpha$  and IFN $\beta$  mRNAs in A2EN cells, respectively, and 97-fold and 26-fold more abundant than IFN $\alpha$  and IFN $\beta$  mRNA levels in CECs, respectively (data not shown). Pro-inflammatory cytokines such as TNF $\alpha$  (1 ng/ml) and IL-1 $\beta$  at 1 and 10 ng/ml significantly induced IFN $\epsilon$  by 7.7 and 4.6-fold, respectively. IL-1 $\beta$  had a moderate effect on IFN $\alpha$  expression (twofold induction) in A2EN cells (Fig. 1a). Gene induction of IFN $\alpha$  and IFN $\epsilon$  by TNF $\alpha$  was also found in CECs (Fig. 1b). Interestingly, TNF $\alpha$  suppressed IFN $\beta$  expression by 40–50% in A2EN cells. IFN $\beta$  suppression by TNF $\alpha$  at 1 ng/ml for 4 h incubation in CECs was observed; however, treatment of TNF $\alpha$  for 24 h induced IFN $\beta$  in CECs.

IL-6 or IL-8, which are often elevated during inflammation at the mucosa, was noted to induce IFN $\epsilon$  in A2EN cells after 24 h incubation, but the induction was not significant when various experiments were compared (Fig. 1c). The effects of IL-6 and IL-8 on IFN $\epsilon$  induction were not prominent in CECs. Interestingly, IL-8 significantly suppressed IFN $\beta$  in A2EN cells (25–77% suppression) and CECs (61% suppression). IL-8 treatment for 4 h also suppressed IFN $\alpha$  in A2EN cells (53–71% suppression) but the significant suppression of IFN $\alpha$  was observed in CECs after the 1 ng/ml for 24 h incubation (64% suppression).

IL-17 and IL-22 play a crucial role in mucosal immune homeostasis [11]. In A2EN cells, IL-17 slightly elevated the IFN $\alpha$  mRNA level by 1.8-fold but had a trend to suppress expression of IFN $\beta$  ( $p=0.057$ ) (Fig. 1c). In CECs, IL-17 significantly suppressed IFN $\alpha$  and  $\beta$  after

4 h incubation. The suppression of IFN $\epsilon$  by IL-17 was observed but not significant ( $p=0.07$ ). IL-22 has similar but distinct functions compared to IL-17 [11]. In A2EN cells, IL-22 significantly suppressed IFN $\alpha$  and  $\beta$  at both concentrations after the 4 h incubation. IL-22 continued to suppress IFN $\beta$  after incubation for 24 h. The suppressive effect of IL-22 on IFN $\epsilon$  was limited at 10 ng/ml for 4 h incubation. IL-22 did not have a significant impact on IFN $\alpha$  expression but slightly induced IFN $\beta$  or IFN $\epsilon$  (1 ng/ml for 24 h) in CECs. IL-22 at 10 ng/ml significantly suppressed IFN $\beta$  after 4 h incubation, indicating that IL-22-mediated IFN $\beta$  gene regulation depended on the cytokine concentration and the incubation time.

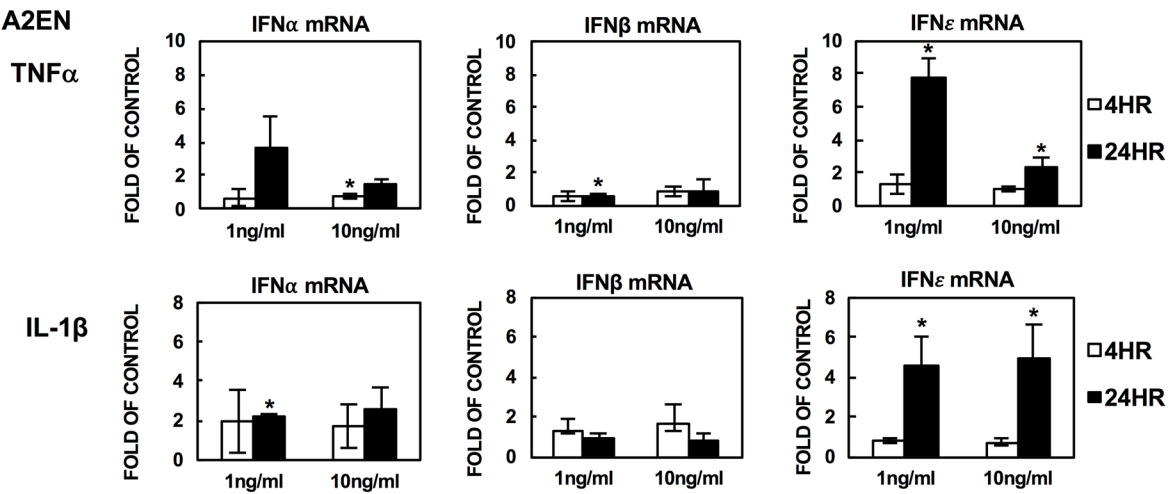
IFN $\alpha$ , induced in response to infection or Toll-like receptor activation, is known to induce type I IFNs [12]. Because type I IFN signaling can be cell-type specific [13], we determined the effect of IFN $\alpha$  on expression of IFNs in A2EN cells and CECs. IFN $\alpha$  moderately induced all three IFNs by 2–3 fold in A2EN cells after 24 h treatment although only induction of IFN $\alpha$  was statistically significant (Fig. 1c). IFN $\alpha$  slightly induced IFN $\alpha$  but had no effect on IFN $\epsilon$  in CECs. Regulation of IFN $\beta$  by IFN $\alpha$  appeared to be time and concentration dependent. IFN $\alpha$  at 10 ng/ml suppressed IFN $\beta$  expression by 60% after 4 h incubation, but there was a trend of IFN $\beta$  induction after 24 h incubation.

TNF $\alpha$  is known to activate the transcription factor NF $\kappa$ B [14] although the previous report showed that it did not induce the IFN $\epsilon$  promoter activity in HeLa cells [9]. To determine whether the NF- $\kappa$ B pathway was involved in TNF $\alpha$ -mediated IFN $\epsilon$  gene expression, primary cervical cells were treated with the I $\kappa$ B kinase (IKK) inhibitor, BAY 11-0782, to block NF- $\kappa$ B activation. BAY 11-0782 blocked TNF $\alpha$ -mediated IFN $\epsilon$  induction, suggesting the role for the NF $\kappa$ B pathway in IFN $\epsilon$  gene regulation (Fig. 2a). Note that the suppression of IFN $\epsilon$  induction by BAY 11-0782 was not due to cytotoxicity as determined by using the CytoTox-Glo Cytotoxicity assay (data not shown) (Promega). We confirmed that the IKK inhibitor blocked NF $\kappa$ B p65 serine-536 phosphorylation in response to TNF $\alpha$  stimulation (Fig. 2b). We then generated a DNA construct with a luciferase reporter gene under the control of IFN $\epsilon$  promoter (nt – 1000 to

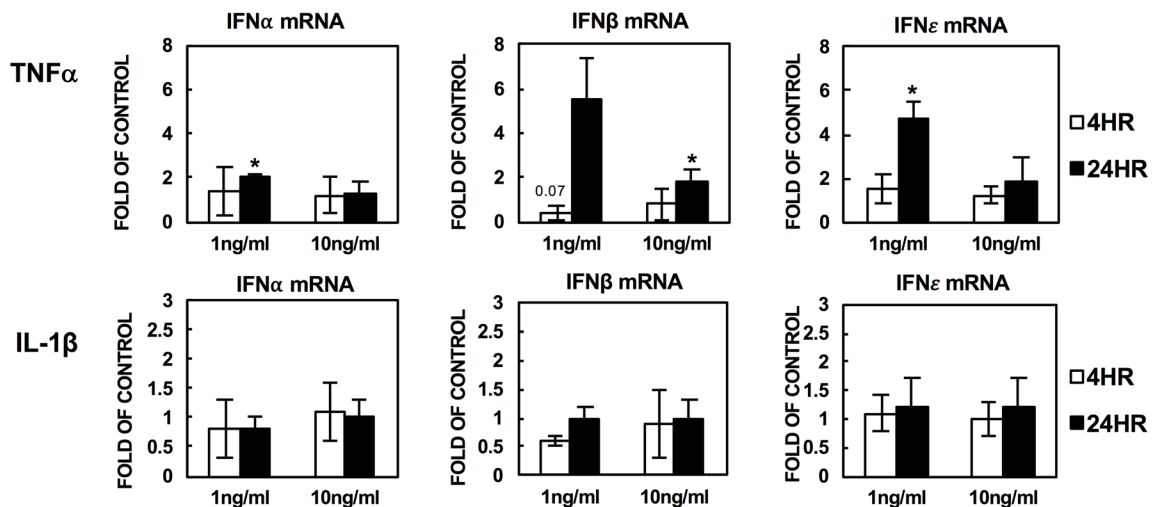
(See figure on next page.)

**Fig. 1** Regulation of IFN $\alpha$ , IFN $\beta$  and IFN $\epsilon$  by various cytokines in A2EN cells and primary cervical cells. A2EN or primary cervical epithelial cells were treated with stated cytokines at 1 or 10 ng/ml for 4 or 24 h. Total RNAs were prepared and gene expression of IFN $\alpha$ , IFN $\beta$  and IFN $\epsilon$  was measured by real-time RT-PCR. Induction of each gene (as fold-change relative to untreated control) was calculated using the  $\Delta\Delta$ CT method as described in "Materials and methods". The value of 1 indicates the level of gene expression of untreated control cells. Data for TNF $\alpha$  and IL-1 $\beta$  are mean  $\pm$  SD of four independent experiments in A2EN cells (**a**) and of experiments from primary cervical cells from different donors (**b**). Average of fold-changes of other cytokines-treated samples relative to untreated samples from four different experiments were summarized in **c**. Numbers in bold represents significant changes (\* $p < 0.05$ ). † $p=0.057-0.07$

**a A2EN**



**b CEC**

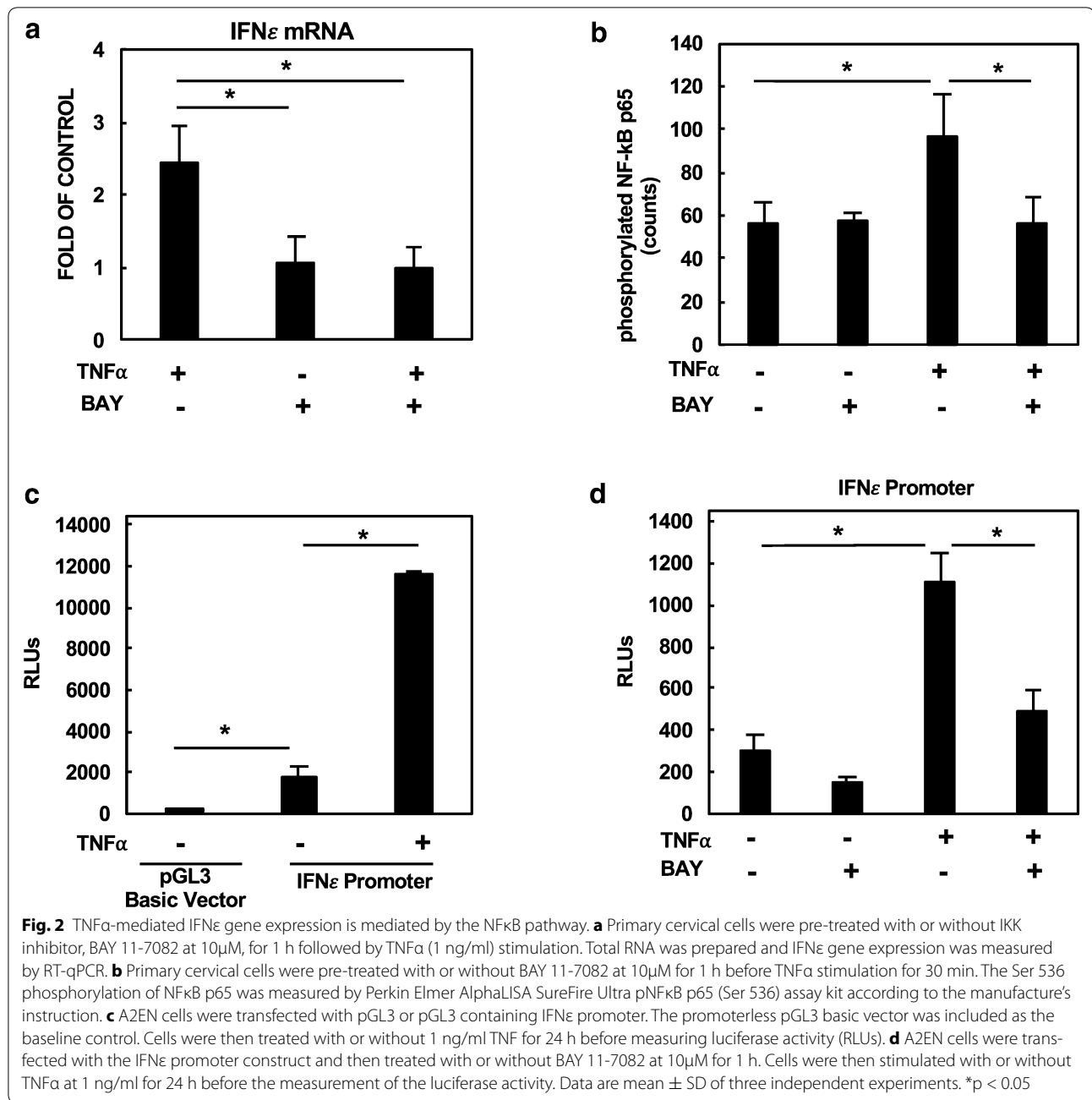


**c**

A2EN	IFNα				IFNβ				IFNε				Fold Change
	1ng/ml		10ng/ml		1ng/ml		10ng/ml		1ng/ml		10ng/ml		
	4HR	24HR	4HR	24HR	4HR	24HR	4HR	24HR	4HR	24HR	4HR	24HR	
TNFα	0.7	3.7	0.8*	1.5	0.6	0.6*	0.8	0.9	1.3	7.7*	1	2.4*	
IL-1β	1.9	2.2*	1.7	2.6	1.3	0.9	1.6	0.8	0.8	4.6*	0.7	5*	
IL-6	1.9	1.7	2.1	1.2	1.2	0.7	0.8	1	0.8	4.3	1.2	4	
IL-8	0.5*	1	0.3*	0.8	0.4*	0.8*	0.2*	1.1	0.9	2.4	0.8	2.9	
IL-17	1.9	1.5*	1.5	1.6	1	1.1	0.5†	1.5	1	1.8	0.8	1.3	
IL-22	0.6*	0.7	0.3*	1.2	0.6*	0.6*	0.3*	0.8*	1.3	0.7	0.4*	1.7	
IFNα	0.8	2.4	1.9	2.8*	1.1	2.4	1	2.2	1.2	3.4	0.9	1.8	

CEC	IFNα				IFNβ				IFNε				Fold Change
	1ng/ml		10ng/ml		1ng/ml		10ng/ml		1ng/ml		10ng/ml		
	4HR	24HR	4HR	24HR	4HR	24HR	4HR	24HR	4HR	24HR	4HR	24HR	
TNFα	1.4	2*	1.2	1.3	0.4†	5.5	0.8	1.8*	1.5	4.7*	1.2	1.9	
IL-1β	0.8	0.8	1.1	1	0.6	1	0.9	1	1.1	1.2	1	1.2	
IL-6	3	1.4	2.1	2.9	2.2	1.1	1.6	1.9	2.1	1.5	1.7	1.6	
IL-8	6	0.4*	5.3	1.3	1.9	0.4*	2.6	0.9	1.3	1	1.8	1	
IL-17	0.7	1	0.4*	0.6	0.5*	0.7	0.3*	1	1	1.1	0.5†	1.3	
IL-22	1	1.4	0.5	1.4	1	1.8*	0.4*	0.5	1.1	2.9†	1	0.7	
IFNα	1.3	1.3	0.7	1.3*	0.8	2.6	0.4*	3	0.9	1.4	0.9	0.9	



+ 20). The pGL3 with IFN $\epsilon$  promoter construct had a low basal luciferase activity compared to the promoterless pGL3 basic vector, and TNF $\alpha$  significantly induced the IFN $\epsilon$  promoter activity (Fig. 2c). Importantly, BAY 11-0782 blocked TNF $\alpha$ -mediated activation of the IFN $\epsilon$  promoter, suggesting the involvement of the NF $\kappa$ B pathway in IFN $\epsilon$  gene regulation (Fig. 2d).

Our results showed that TNF $\alpha$  induced IFN $\epsilon$  gene expression in both A2EN and CECs through NF- $\kappa$ B signaling. The degree of induction in these cells was more pronounced than that previously reported in HeLa cells [9].

Additionally, we observed TNF $\alpha$ -mediated IFN $\alpha$  induction, which is absent in HeLa cells [9]. The discrepancy may be due to the limited passage of A2EN and CECs, which could preserve a more primary cell-like immune response. Activation of the IFN $\epsilon$  promoter by TNF $\alpha$  was not observed in the study by Matsumiya et al. [9]. The discrepancy may be due to the different promoter region (Matsumiya, nt - 497 to + 296 vs nt - 1000 to + 20 in this study) or different cell types. NF- $\kappa$ B binding sites have been identified at the - 53 and - 854 nt position in the proximal promoter of IFN $\epsilon$  [2], which were included

in our promoter construct. Our study suggests the role for NF $\kappa$ B in IFN $\epsilon$  gene regulation. Because both TNF $\alpha$  and IL-1 $\beta$  activate NF- $\kappa$ B, p38 MAPK, and JNK [14, 15], further analysis of the IFN $\epsilon$  promoter function and of the role of transcriptional factors and kinases involved in IFN $\epsilon$  gene expression will produce a better understanding of IFN $\epsilon$  regulation by cytokines at mucosa.

Although TNF $\alpha$ -mediated induction of IFN $\alpha$  and IFN $\epsilon$  was consistent between A2EN and CECs, induction of IFN $\epsilon$  in response to various cytokines was noted to be stronger in A2EN cells than CECs. Similarly, suppression of IFNs by IL-22 was apparent in A2EN cells. Differential profiles of IFN regulation between A2EN and CECs may be due to the tissue origin (e.g. ectocervix or endocervix), the presence of HPV E6/E7 in A2EN cells, or cells at different differentiation states. A2EN is an endocervical cell line, representing a uniform cell population, whereas CECs were composed of heterogeneous epithelial cells from cervical tissues, mostly ectocervix. It is possible that some cell populations in CECs may not be responsive to stimulation.

In summary, our study demonstrated that TNF $\alpha$  and IL-1 $\beta$  were potent inducers of IFN $\epsilon$  and that the NF- $\kappa$ B pathway was involved in TNF $\alpha$ -mediated IFN $\epsilon$  gene regulation. Expression of IFN $\alpha$ , IFN $\beta$  and IFN $\epsilon$  was regulated differentially in response to specific cytokines, and gene regulation of IFNs by specific cytokines can be concentration-, time-, and cell type-dependent.

## Materials and methods

### Reagents

Keratinocyte Serum Free Media (1 $\times$ ) (KSFM) and all reagents for transfection and reverse transcription were purchased from Thermo Fisher Scientific (Waltham, MA). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IL-17A, IL-22, IL-1 $\beta$  and IL-8 were purchased from Shenandoah Biotechnology (Warwick, PA). Human IL-6 and TNF $\alpha$  were purchased from R&D Systems (Minneapolis, MN). IFN $\alpha$ 2a was purchased from PBL assay science (Piscataway, NJ).

### Cell culture

The immortalized epithelial cell line A2EN cells were generated from endocervical tissues of healthy donors as described previously [16]. A2EN cells (passage 19) were maintained in KSFM supplemented with 5 ng/ml epidermal growth factor, 50  $\mu$ g/ml bovine pituitary extract and 0.2 mM CaCl<sub>2</sub> at 37 °C in a 5% CO<sub>2</sub> humidified incubator. Cells were limited to 5–6 passages. Primary cervical cells were prepared based on published protocols with modification [16]. Briefly, cervical tissues without gross pathology were obtained from women undergoing therapeutic hysterectomy. Human subject protection received IRB

approval from Rutgers-New Jersey Medical School. All donors were HIV negative. Cervical tissues were cut into 2 mm<sup>3</sup> pieces and digested with collagenase IV at 37 °C for 90 min. After passing through a 70  $\mu$ m cell strainer, the cells were collected by centrifugation and washed with PBS. Isolated cervical cells were cultured overnight at 37 °C in a CO<sub>2</sub> incubator to allow epithelial cells to attach. Non-attached cells were removed by washing off non-adherent cells extensively. Adherent epithelial cells were cultured until cells reached their confluency. Primary human cervical epithelial cells were maintained in RPMI in the presence of 10% FBS.

Cervical cells were stained with the cervical epithelial marker CK-19 and analyzed by FACS to confirm their purity. Cells less than five passages were used in the experiments.

### Real-time PCR

Total RNA was isolated from cells using TRIzol<sup>®</sup> (Life Technologies, CA). To synthesize first-strand cDNA, 1000 ng of total RNA, oligo d(T)<sub>16</sub> (25  $\mu$ g/ml) and dNTP (0.5 mM) were incubated at 65 °C for 5 min and quick-chilled on ice. RT was performed at 42 °C for 50 min and 70 °C for 15 min using SuperScript III Reverse Transcriptase. The PCR reaction contained cDNA equivalent to 30 ng of RNA input, 200 nM primer sets, and SYBR Green Master Mix (QIAGEN, Valencia, CA), and was run in a StepOnePlus real-time PCR system (Life Technologies, Carlsbad, CA). PCR conditions included 95 °C denaturation for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s. PCR products were quantified and normalized relative to the amount of GAPDH cDNA products as described by Schmittgen et al. [17]. Relative quantification of gene expression was calculated using the  $\Delta\Delta$ Ct (Ct, threshold cycle of real-time PCR) method according to the formula:  $\Delta$ Ct = Ct<sub>GAPDH</sub> - Ct<sub>target</sub>,  $\Delta\Delta$ Ct =  $\Delta$ Ct<sub>control</sub> -  $\Delta$ Ct<sub>IFN</sub>, Ratio = 2<sup>- $\Delta\Delta$ Ct</sup>. Primer sequences were: IFN $\epsilon$  forward (5'-AGC ACT CAT GGG ACT GGA ACT GGA AG-3'), reverse (5'-CAG GTG CTG TAG TCC TGG TT-3'); IFN $\alpha$  forward (5'-CAC ACA GGC TTC CAG GCA TTC-3'), reverse (5'-TCT TCA GCA CAA AGG ACT CAT CTG-3'), IFN $\beta$  forward (5'-GAG CTA CAA CTT GCT TGG ATT CC-3') reverse (5'-CAA GCC TCC CAT TCA ATT CC-3'); GAPDH forward (5'-GCA CCA CCA ACT GCT TAG CAC-3'), reverse (5'-TCT TCT GGG TGG CAG TGA TG-3').

### Promoter construct and transfection

The IFN $\epsilon$  promoter DNA that comprised the 5'-flanking region nt - 1000 to + 20 of the IFN $\epsilon$  gene was synthesized and cloned in the Promega pGL3 luciferase reporter basic vector (no promoter) by Genescript. The

DNA sequence of the construct was confirmed. To assess the IFN $\epsilon$  promoter activity, the pGL3 or pGL3 with IFN $\epsilon$  promoter region was transfected into A2EN (0.125 $\mu$ g plasmid DNA) by Lipofectamine 2000 and incubated for 24 h. Cells were pre-treated with or without the IKK inhibitor BAY-11-7082 for 1 h before stimulation with TNF $\alpha$  at 1 ng/ml. Cells were cultured for 24 h before lysis in passive lysis buffer (Promega, Madison WI). Luciferase activity [in relative light units (RLUs)] was measured on a 2300 EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA).

### Statistical analysis

Statistical comparisons were performed by using two-tailed paired-Samples t test (GraphPad Prism 5.01, LaJolla, CA). A *p* value of < 0.05 was considered significant.

### Abbreviations

IFN: interferon; CECs: cervical epithelial cells; ELISA: enzyme-linked immunosorbent assay; h: hour; TNF- $\alpha$ : tumor necrosis factor; IL-1 $\beta$ : interleukin-1 $\beta$ ; NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK: mitogen-activated protein kinase; JNK: c-Jun-N-terminal kinase.

### Authors' contributions

JC designed the study, collected tissues, prepared primary cervical cells, conducted the experiments and prepared the manuscript. CT assisted in experiments using primary cervical cells. JK, and TS performed RT-PCR in A2EN cells in some experiments. SF assisted in data analysis. AJQ participated in experimental design and data discussion. PL and DH helped tissue collection and data discussion. TLC oversaw the project, designed experiments and prepared the manuscript. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interests.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Consent for publication

Not applicable.

### Ethics approval and consent to participate

Human subject protection received the IRB approval from Rutgers-New Jersey Medical School. No patient identifiers were associated with tissue; thus, the study was not considered human subjects research.

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