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Respiratory syncytial virus infection induces a subset of types I and III interferons in human dendritic cells

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Abstract

Whether respiratory syncytial virus (RSV) induces severe infantile pulmonary disease may depend on viral strain and expression of types I and III interferons (IFNs). These IFNs impact disease severity by inducing expression of many anti-viral IFN-stimulated genes (ISGs). To investigate the impact of RSV strain on IFN and ISG expression, we stimulated human monocyte-derived DCs (MDDCs) with either RSV A2 or Line 19 and measured expression of types I and III IFNs and ISGs. At 24h, A2 elicited higher ISG expression than Line 19. Both strains induced MDDCs to express genes for IFN- β , IFN- α 1, IFN- α 8, and IFN- λ 1–3, but only A2 induced IFN- α 2, - α 14 and - α 21. We then show that IFN- α 8 and IFN- α 14 most potently induced MDDCs and bronchial epithelial cells (BECs) to express ISGs. Our findings demonstrate that RSV strain may impact patterns of types I and III IFN expression and the magnitude of the ISG response by DCs and BECs.

Keywords

respiratory syncytial virus; interferon subtype; interferon; interferon stimulated gene; dendritic cell; bronchial epithelial cell; innate immunity

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Introduction

Respiratory Syncytial Virus (RSV) is the most frequent cause of viral bronchiolitis and pneumonia in infants. In addition to the immediate morbidity and mortality associated with severe RSV lower respiratory tract infection, it may also predispose infants to childhood asthma, and even mild RSV infection exacerbates existing asthma (Lotz et al., 2013). RSV infection occurs via inhalation of virus-containing droplets, which infect bronchial epithelial cells in small foci (Johnson et al., 2007). In the upper airway, dendritic cells reside beneath the epithelium and extend their dendrites towards the surface to sample inhaled antigens. Once DCs detect viral infection, they serve two functions: migration to local lymph nodes to initiate adaptive immunity, and expression of inflammatory mediators, including types I and III interferons (IFNs).

Types I and III IFNs are key inducers of an anti-viral state in response to viral infection. Human type I IFNs include IFN- β and functional genes for 12 subtypes of IFN- α that share 70–80% amino acid identity (Genin et al., 2009). All type I IFNs signal through the IFNAR1/2 receptor complex, which is expressed ubiquitously. Type III IFNs include IFN- λ 1, - λ 2, - λ 3, and the recently described IFN- λ 4 (Lazear et al., 2015; O'Brien et al., 2014). IFN- λ 2 and IFN- λ 3 share 96% amino acid identity, but are divergent from IFN- λ 1, and IFN- λ 4 shares only approximately 29% identity with other IFN- λ s (Lazear et al., 2015; O'Brien et al., 2014). Type III IFNs signal through a receptor dimer comprised of the IFN- λ R1 and IL-10R2 chains; expression of *IFNLR1* is largely limited to cells of epithelial lineage (Sommereyans et al., 2008). Although they signal through distinct receptors, types I and III interferons share a common signaling pathway that induces autocrine and paracrine expression of more than 600 genes, collectively referred to as interferon-stimulated genes (ISG) (Schoggins et al., 2011). Many ISGs code for proteins that directly inhibit viral replication and protect against spread of viral infection to neighboring cells. The importance of types I and III IFNs during RSV infection is emphasized by the strong inhibition of IFN induction and signaling mediated by the two earliest transcribed of the eleven RSV gene products, NS1 and NS2 (Barik, 2013; Spann et al., 2004).

Human monocyte-derived DCs (MDDCs) are often used as a model for myeloid DCs (mDCs). Similar to mDCs, MDDCs can be infected with RSV *in vitro* and produce IFNs and pro-inflammatory cytokines (Chi et al., 2006; Guerrero-Plata et al., 2006). Previously we demonstrated that the A2 strain of RSV induces human MDDCs to express types I and III IFNs (Chi et al., 2006), and that these together may affect the adaptive response to RSV. In two studies in mice, immune responses to two commonly used RSV strains were compared (Lukacs et al., 2006; Moore et al., 2009). The authors showed that strains A2 and Line 19 induced high and low expression of IFN- α respectively (Moore et al., 2009). Here, using the same two RSV strains, we asked which types I and III IFNs, ISGs and pro-inflammatory cytokines were induced in human MDDCs in response to RSV and whether the specific types I and III IFNs produced by MDDCs affect the anti-viral ISGs expressed by these cells as well as airway epithelial cells.

Materials and Methods

Viruses

RSV strains A2 (Lewis et al., 1961) and Line 19 (Herlocher et al., 1999) were grown in Vero cells for 6–8 days at an input multiplicity of infection (MOI) of 0.01 PFU/cell using OptiPro serum free medium (Life Technologies, Grand Island, NY) supplemented with 4 mM L-glutamine (Life Technologies). Procedures for isolating RSV from the cells were as described in Le Nouën *et al.* (Le Nouen et al., 2009) unless otherwise stated. Cells were harvested by scraping, vortexed to liberate surface-associated virus and clarified by centrifugation. The virus was purified by centrifugation through discontinuous sucrose gradients in a Beckman SW32Ti rotor at 121000 x g for 1.5 hours to remove cytokines and other cell derived macromolecules. Virus was harvested from the interface between the sucrose layers, diluted in RPMI 1640 (Life Technologies) supplemented with 2 mM L-glutamine and pelleted at 8,000 x g for 2 hours in a Sorvall SS-34 rotor to remove the sucrose. Pellets were snap frozen in RPMI 1640 and stored at –80°C until use. Viral titer was determined by plaque assay on Vero cells. RSV was UV-inactivated using a dose of 2400 mJ/cm² with a UVC 500 crosslinker (Hoefer Instruments, Holliston, MA).

Cells

Elutriated monocytes from healthy adult donors were obtained from the NIH Clinical Center Blood Bank and purified using CD14+ MACS beads (Miltenyi Biotech, Auburn, CA). CD14+ cells were differentiated into MDDCs by culturing them at 3x10⁵ cells/ml with IL-4 (1000 U/ml, Peprotech, Rocky Hill, NJ) and GM-CSF (800 U/ml, Immunex, Seattle, WA) for seven days in medium consisting of RPMI 1640 (Life Technologies) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 20 µg/ml gentamicin (Life Technologies). After seven days, MDDCs were stained with monoclonal antibodies against HLA-DR Alexa 680 (clone IV.3, conjugated in house), CD11c PE (clone B-ly6), and CD209 PerCP-Cy 5.5 (clone DCN43, both BD Biosciences, San Jose, CA) to check for purity. Data were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed with Flowjo 9.0 (Treestar Software, Ashland, OR). After seven day of differentiation, cells were >95% CD11c⁺HLA-DR⁺.

MDDCs were harvested after seven days, washed three times in medium to remove cytokines and re-cultured at 1x10⁶ cells/ml alone or with live RSV A2 or Line 19 at an MOI of 3, an equal volume of UV-inactivated virus or the indicated concentrations of type I (PBL, Piscataway, NJ) or type III IFNs (R&D Systems, Minneapolis, MN). In some experiments recombinant B18R (the vaccinia virus soluble type I IFN receptor, eBioscience, San Diego, CA) was added at 0.1 µg/ml to block type I IFNs from binding to their receptor. MDDCs were incubated at 37°C for the time points specified in the text and figure legends and then harvested and stained for flow cytometry or lysed using RLT buffer for RNA isolation (Qiagen). Cell culture supernatants were also harvested and stored at –80°C until use. This study was approved by the Institutional Review Boards of the NIH and the U.S. Food and Drug Administration.

The BEAS-2B bronchial epithelial cell line (CRL 9609, ATCC, Manassas, VA) was grown using the BEGM Bullet Kit (Lonza, Walkersville, MD) according to the manufacturer's instructions and maintained without antibiotics. Cells were seeded in 12-well plates with 20 µg/ml gentamicin and grown until 90% confluence. They were incubated at 37°C with type I or type III IFNs at the concentrations indicated for 24 hours and harvested as described for MDDCs.

Quantitative Real-time PCR

RNA was isolated using RNeasy spin columns including on column DNase digestion (Qiagen, Germantown, MD). RNA was either reverse transcribed using the RT² first strand kit (SA Biosciences, Frederick, MD) and gene expression detected using toll like receptor (TLR) signaling pathway RT² profiler plates with SYBR green master mix (SA Biosciences), or using Superscript III Supermix (Life Technologies) followed by gene expression assays (Life Technologies). All qRT-PCR was performed using the 7900HT or Viia 7 system (both Applied Biosystems) and fold change of gene expression was calculated using the Cq method. Gene expression of the twelve functional type I IFN subtypes and the three type III IFNs was measured individually using the novel qRT-PCR assay developed in the lab, as previously described (Hillyer et al., 2012). Succinate dehydrogenase complex, subunit A (*SDHA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were the two most consistent housekeeping genes (HKG) in MDDCs, data not shown and (Hillyer et al., 2012; Mane et al., 2008) *GAPDH* and ubiquitin C (*UBC*) were the most consistent in epithelial cells. *GAPDH* was used to determine comparative levels of expression for the RT² profiler studies in MDDCs (*SDHA* was not available in the arrays) and for all PCR experiments on epithelial cells, while *SDHA* was used for gene expression and IFN assays in MDDCs.

RSV RNA synthesis in MDDCs was measured using a qRT-PCR assay specific for conserved sequences within the M genes of both strains (forward primer: 5'-GCAAATATGGAAACATACGTGAACAA-3', nt 3,255-80; reverse primer: 5'-GGCACCCATATTGTAAGTGATGCA-3', nt 3,370-47; probe: 5'-CTTCACGAAGGCTCCACATATAMRA-3', nt 3,282-301; nucleotide numbering refers to the RSV A2 sequence (Genbank accession number M74568). This method measures both genomic and antigenomic RNA, as well as mRNA expression of the RSV M gene.

Measurement of cytokine proteins

Cytokines were measured using a Milliplex Map human cytokine/chemokine kit (Millipore, Billerica, MA). Data were acquired using Bioplex (Biorad, Hercules, CA) and concentrations determined from a standard curve (Prism 5.0, Graphpad, La Jolla, CA). IFN-α (all subtypes except IFN-α21), IFN-β, and IFN-λ (all three types) were measured by ELISA (PBL Interferon Source, Piscataway, NJ). In all assays, the lower limit of detection was calculated as: blank+ (2 x SD of blank).

Statistics

Differences in gene expression of each IFN type/subtype at single time points (i.e., at 4 hours, 8 hours, and 24 hours) were evaluated using a mixed effects model with the donor as

a random effect and the virus (control, live or UV-killed RSV strains A2 or Line 19) as the fixed effect. Pairwise comparisons were made among seven pairs of interest: between control and live A2, control and Line 19, control and UV A2, control and UV Line 19, UV A2 and live A2, UV Line 19 and Line 19, and live A2 and Line 19. No comparisons to the control were performed for RSV-M gene since these cells were uninfected and the gene was unquantifiable. The false discovery rate was controlled at 0.05 to adjust for multiplicity at each time point using the method of (Benjamini and Hochberg, 1995).

Statistical differences in cytokine, chemokine and interferon secretion were determined with Prism 6.0 (Graphpad, La Jolla, CA) using the Friedman test with Dunn's correction for multiple comparisons.

Results

The A2 RSV strain induces higher expression of innate immune response genes in primary human MDDCs than the Line 19 RSV strain

To first determine MDDC expression of innate immune genes in response to RSV infection, we stimulated MDDCs from three donors with live or UV-killed RSV strains A2 or Line 19, or medium (mock) for 24 hours. We then measured expression of a panel of 84 innate immune response genes with a qRT-PCR based array. *SDHA* was the most consistent housekeeping gene (HKG) in MDDCs (data not shown and (Hillyer et al., 2012; Mane et al., 2008)). However, *SDHA* was not available in the array kit used for Figure 1, so *GAPDH*; the next most consistent HKG was used for normalization in this one experiment, *SDHA* is used for MDDCs in all other figures. Figure 1A shows that live virus of each strain induced expression of a similar set of genes that included DC activation markers, TLR pathway signaling intermediaries, inflammatory mediators, and chemokines. UV killed virus of either strain induced little to no gene expression by MDDCs (Figure 1B). Among genes expressed 2.5-fold above mock in response to RSV, *IRF1*, *IL6*, and *CXCL10* were consistently expressed at higher levels in response to A2 than Line 19 (Figure 1B) suggesting that A2 induced a greater IFN and pro-inflammatory response in MDDCs than Line 19.

To further investigate the responses to the two RSV strains, we performed a more detailed analysis of MDDCs from 18 donors, including the original three. We previously showed that only a small proportion of MDDCs (2 – 15%) are robustly infected with RSV (Chi et al., 2006; Le Nouen et al., 2009) and most of the RSV particles in MDDC cultures are cell-associated rather than in the supernatant (Chi et al., 2006). In addition, RSV binding to the cell surface and/or internalization is required to stimulate pattern recognition receptors on MDDCs. For these reasons, rather than comparing the kinetics of A2 and Line 19 virus particle production, we compared the levels of the virus gene expression by qRT-PCR of the two strains using the RSV matrix (M) gene as a target.

As shown in Figure 2A, at 24 hpi RSV A2-infected MDDCs expressed similar levels of RSV-M to those infected with Line 19. However, despite considerable donor variability, A2 induced statistically higher expression of a set of ISGs (*DDX58*, *MDA-5*, *IRF1* and *IRF7*) and *NFKB1* (presented in Figure 2A, all on a log₁₀ scale). Analysis of the MDDC

supernatants revealed that while CXCL10 and CCL2 protein expression increased significantly above mock in response to RSV A2 or Line 19, expression was generally higher in each donor in response to A2, and that CCL5, IL-6 and TNF- α proteins were significantly induced only in response to infection by the A2 strain (Figure 2B). Although CXCL8 expression was slightly increased in several donors in response to A2, this was not statistically significant. Additionally, RSV A2 induced higher production of CXCL10, CCL2 and IL-6 than did UV-killed A2 virus, indicating that live virus is necessary to induce expression of these pro-inflammatory mediators. Neither of the RSV strains induced expression of IL-10, IFN- γ , or IL-12 p70 (data not shown) or enhanced surface expression of CD80, CD83, or HLA-DR (data not shown).

We then measured expression of types I and III IFN mRNA transcripts in all 18 donors and found that both RSV strains induced levels of expression of *IFNA1*, *IFNA8*, *IFNB1* and *IFNL1-3* significantly above the mock-infected controls (Figure 3A). While these IFN genes were more highly expressed in response to A2 than Line 19 in many individual donors, only *IFNB1*, *IFNL1*, *IFNL2* and *IFNL3* were statistically different between the strains (Figure 3A). Only A2 significantly induced expression of genes for three IFN- α subtypes: *IFNA2*, *IFNA14* and *IFNA21* (Figure 3A). Line 19 induced expression of these three subtypes above mock levels in some but not all donors, this reflects greater donor to donor variability in the IFN- α response to Line 19. The seven remaining IFN- α subtypes were not induced above mock by either strain of RSV (Figure 3A). We therefore conclude that the IFN- α gene expression patterns we observe may be induced by both RSV strains with greater variability than IFN- β and - λ s. We detected IFN- α , IFN- β and IFN- λ protein in MDDC supernatants only in response to A2 (Figure 3B), again with considerable donor variability.

The kinetics of ISG expression differ according to RSV strain

Since the innate response is initiated earlier than 24 hpi, we explored the timing of ISG and IFN induction in response to live A2 or live Line 19. In a subset of nine of the 18 donors used for Figures 2 and 3, we measured responses to RSV at 4 and 8 hpi. Figure 4A and B show that while there were no differences in RSV-M gene expression between the two RSV strains, Line 19 induced earlier expression of a subset of ISGs than A2, and earlier expression of *IFNB1* and *IFNL1* as well (Figure 4C). Taken together with the 24 hpi data shown in Figures 2 and 3 these analyses show earlier induction of a subset of ISGs and IFNs in response to Line 19, with a later but stronger induction in response to A2.

IFN- α subtypes differentially induce ISG expression

RSV induced expression of both types I and III IFNs, but functional IFN λ R is generally restricted to epithelial cell rich tissues (Sommerreyns et al., 2008; Witte et al., 2009). We first confirmed the primary importance of type I IFNs by measuring ISG expression at 24 hpi in MDDCs cultured with A2 or Line 19 (MOI = 3) in the presence or absence of recombinant B18R, a soluble type I IFN receptor which is encoded by vaccinia virus. In each of the four donors, B18R suppressed ISG expression (Figure 5A), however this suppression was not complete (Figure 5B) and was generally greater for A2 than Line 19 (e.g. *CXCL10*, A2 median suppression 81%, range 66.9 – 86.1%, Line 19 median suppression 60.1%, range 35.1 – 66.9%). *MX1*, *DDX58* and *IRF7* also had similarly non-overlapping ranges of

suppression. The lesser and more variable suppression observed in response to Line 19 was likely due to overall lower expression of ISGs compared to A2 (Figure 5A and B). Similar results were shown for CXCL10 protein expression (Figure 5C) in response to A2 (median suppression 81.8%, range 61.8 – 87.2%) or Line 19 (median suppression 71.0%, range 42.1 – 98.5%). In the four donors tested, B18R did not affect levels of A2 *RSV-M* (median 104.9%, range 82.8–117.9%), but enhanced levels of Line 19 *RSV-M* (median 119.5%, range 107.8–142.8%).

We then asked whether the IFNs induced by RSV (namely IFN- α 1, IFN- α 2, IFN- α 8, IFN- α 14, and IFN- β) differentially induce expression of ISGs in MDDCs by stimulating them for 24 hours with increasing doses of each of these rhIFNs, or IFN- α 4 for comparison to a non-RSV responsive IFN. Repetition of these experiments showed that IFN- α 2, - α 8 and - α 14 were consistently the most potent IFNs tested (Figure 5D). Surprisingly, IFN- β , which has the highest affinity for IFNAR1 and IFNAR2 (Schreiber and Piehler, 2015), was generally less potent than these IFNs at low doses. Consistent with the low expression of functional IFN- λ R on immune cells (Witte et al., 2009), the type III IFNs were less potent inducers of ISG expression than the type I IFNs (Figure 5D).

We then investigated whether the hierarchy of IFN potency was applicable to bronchial epithelial cells, the primary target for RSV infection. We stimulated BEAS-2B bronchial epithelial cells with a panel of rhIFNs that included IFN α 10 and IFN α 21 as high and low affinity comparators, respectively. Figure 5E shows that the overall hierarchy of expression of ISGs by BEAS-2B bronchial epithelial cells among the IFN- α subtypes was similar to that in MDDCs, but that comparative potency of IFN- β was higher and more consistent with its known receptor affinity. Consistent with the high expression of membrane bound IFN- λ R on lung epithelial cells (Mordstein et al., 2010; Sommereyns et al., 2008), IFN λ s, at 10-fold higher concentrations, stimulated the BEAS-2B cells to express most of the ISGs at similar levels as the less potent type I IFNs (Figure 5E).

In summary, using human MDDCs, we demonstrated that 1) RSV induces expression of a subset of IFN subtypes, 2) The type I IFNs expressed in response to RSV are potent inducers of ISGs in both DCs and epithelial cells, 3) ISG expression was higher in response to A2 than line 19 at 24 hpi, 4) Type I and III IFN and ISG expression levels and kinetics differed dependent on donor and RSV strain, and 5) Overall a greater diversity of IFNs was induced in response to RSV A2 compared to Line 19 (Figures 3 and 5).

Discussion

We explored early innate immune responses of human MDDCs to RSV and characterized expression of types I and III IFNs, ISGs and pro-inflammatory cytokines in response to two commonly used RSV strains. We used RSV strains A2 and Line 19 to explore the recent observation that among these two strains, Line 19 induced severe disease in inbred mice which was characterized by lower expression of IFN- α , and higher expression of mucin and IL-13 dependent airway hyperreactivity than the RSV A2-infected mice (Lukacs et al., 2006; Moore et al., 2009). The RSV strains, A2 and Line 19, were isolated from infected infants over 50 years ago. RSV A2 was isolated in 1961 in Australia (Lewis et al., 1961), is well

characterized, and has been used extensively *in vitro* and in animal models. RSV Line 19 was first described in 1999 (Herlocher et al., 1999) as a several decades old clinical isolate at the University of Michigan. However, due to its sequence similarity, Line 19 may have been originally derived from the Long strain, (Melero and Moore, 2013) a 1957 isolate from Baltimore (Chanock et al., 1957). While these two laboratory strains have been serially passaged over many years, phenotypic characterization of recent clinical isolates in mice confirm that RSV A2 is representative of clinical isolates 3–12 and 3–2, which induced little mucin and did not induce IL-13 and that Line 19 is representative of clinical isolates 2–20, 12–35 and 3–4, which induce IL-13, high levels of mucin expression and airway hyper-reactivity in mice (Stokes et al., 2011). This study demonstrates that A2 and Line 19 are at opposite ends of a wide spectrum of host responses to different RSV strains.

Line 19 may elicit early expression of IFN and ISG genes because it is detected by PRRs better or more quickly than A2, infects cells more quickly, replicates more quickly, is less efficient at suppressing IFNs or a combination of these. Moore and colleagues showed that mice infected with a strain A2 virus chimera that expresses the Line 19 F (fusion) protein had higher viral load at day 4 and lower expression of IFN- α at 24 hpi than those infected with A2 with A2F (Moore et al., 2009). This suggests that the Line 19 F protein permits more rapid infection and viral replication, perhaps associated with inefficient activation or better suppression of IFN- α , which may at later time points lead to a disconnect between viral load and the type I IFN response. The effects of this disconnect appear to be sustained, since despite equal viral titers by Day 6, the A2-Line 19F chimera-infected mice expressed higher levels of IL-13, and had more mucus secretion and airway hyperreactivity than those with A2 F protein. In a second study, these authors showed that higher fusion activity of RSV A2-Line19 mutants correlated with increased early viral load. In addition, two amino acid mutations unique to Line 19F were strongly associated with higher fusion activity (Hotard et al., 2015).

The inverse relationship between IFN- α and IL-13 observed in these reports is consistent with evidence of mutual antagonism between IFN- α and the type 2 response (Essayan et al., 1999; Huber et al., 2010; Schandene et al., 1996). For example, asthmatic children with fewer exacerbations of wheezing expressed the highest levels of IFN- α in response to rhinovirus *ex vivo* (Teach et al., 2015). In addition, STAT1 deficient mice, which cannot respond to type I, II or III IFNs, have augmented Th2 responses after RSV infection (Durbin et al., 2002). Mechanisms by which IFN- α suppress type 2 responses include suppressed expression of GATA3 (Huber et al., 2010) and the high-affinity IgE receptor (Gill et al., 2010), and suppression of STAT6 signaling by SOCS1, a classical ISG (Fukuyama et al., 2009) (Dickensheets et al., 1999). Conversely, IL-4 or IL-13 may induce expression of SOCS1, which suppresses signaling of STAT1 and STAT2 (Hebenstreit et al., 2005; Hebenstreit et al., 2003). Once T cells are polarized, however, type I IFNs enhance expression of IL-4 (Hillyer et al., 2013), and may therefore adversely amplify type 2 inflammation associated with a viral respiratory infection.

We and others have previously observed that RSV infects MDDCs and myeloid DCs (mDCs) *in vitro* (Bartz et al., 2003; Chi et al., 2006; de Graaff et al., 2005; Le Nouen et al., 2009). Similar to epithelial cells, only live virus stimulates maturation (Bartz et al., 2003; de

Graaff et al., 2005; Le Nouen et al., 2009), expression of inflammatory cytokines and chemokines (Figure 2 and (Chi et al., 2006; Guerrero-Plata et al., 2006; Munir et al., 2008), and types I and III IFNs (Figure 3 and (Bartz et al., 2003; Chi et al., 2006; Munir et al., 2008). Here we show that RSV induces *IFNB1*, *IFNL1-3*, and a select subset of IFN- α subtypes that is similar to those expressed by the human U937 cell line in response to Sendai virus (Zaritsky et al., 2015).

In vivo, mDCs reside adjacent to the respiratory epithelium (Holt, 2005) where they respond to infection by expressing pro-inflammatory cytokines and types I and III IFNs before migrating to local lymph nodes to initiate an adaptive response. Types I and III IFNs canonically signal through STAT1/STAT2/IRF9 and induce transcription of a similar pattern of ISGs (Bolen et al., 2014; Jilg et al., 2014). Recent evidence, however, supports non-redundant functional roles for each, particularly at sub-saturating levels, which are likely more relevant to initial exposure of the respiratory mucosa to small doses of a viral pathogen contained in micro-droplets. For example, sub-saturating combinations of IFN- β and IFN- λ 1 synergize towards suppression of vesicular stomatitis virus *in vitro* (Voigt and Yin, 2015), and additively enhance and sustain expression of many ISGs (Novatt et al., 2016). In RSV infection of MDDCs, using B18R to block type I IFNs (Figure 5), we have shown that the majority of ISG induction relies on the presence of type I IFNs, however this does not exclude the possibility that optimal ISG expression requires - α , - β and - λ IFNs in combination. In nasopharyngeal wash samples from infants hospitalized for RSV associated bronchiolitis, IFN- λ expression correlates positively both with anti-viral ISG expression and with the clinical score index of disease severity, but not with viral load. It should be noted however, that type I IFNs were not measured in this study, so it is unclear whether IFN- λ or another IFN was responsible for ISG expression (Selvaggi et al., 2014).

One impediment towards dissecting individual roles for IFN- α subtypes is that they are often expressed in groups rather than individually. Recently, Zaritsky and colleagues reported that after infection with Sendai virus, the U937 human monocytic cell line expresses multiple subsets of the 12 IFN- α , depending on the infectious dose used and autocrine/paracrine IFN stimulation through IFNAR1/2 (Zaritsky et al., 2015). One subset was IFNAR-independent and was comprised of IFN- α 1, -2, and - α 8 (three of the five IFN- α subtypes we observed), and another subset was IFNAR-dependent and included IFN- α 14 and - α 21 (the fourth and fifth IFN- α subtypes we observed). In this context, our comparison of RSV A2 and Line 19 infection of MDDCs suggests that at biologically relevant infectious doses of virus, the later waves of IFN- α subtypes may be a response to the early IFN- β autocrine/paracrine stimulation and ongoing viral replication.

Finally, we observed that expression of a set of anti-viral ISGs was most potently induced by IFN- α 2, - α 8, and - α 14 in MDDCs, and by IFN- α 8, - α 14 and IFN- β in respiratory epithelial cells. As reported by Lavoie et al, this hierarchy of potency is not necessarily consistent with the known affinities of the IFN- α subtypes for IFNAR1 or IFNAR2 (Lavoie et al., 2011). Instead, relative anti-viral potency varies according to cell type and pathogen. In addition, since respiratory epithelial cells do not express IFN- α in response to RSV (Okabayashi et al., 2011; Spann et al., 2004), it is likely that their expression by mDC enhance the local anti-viral state. As modeled in Figure 6, the greater consistency by which these IFN- α

subtypes are induced in response to A2 strain may explain why similar clinical strains are associated with a milder disease phenotype than those represented by Line 19.

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Highlights

- RSV induces expression of a subset of IFN subtypes in human DCs
- The type I IFNs expressed are potent inducers of ISGs in DCs and BEAS-2B cells
- ISG and IFN expression levels and kinetics differed dependent on RSV strain

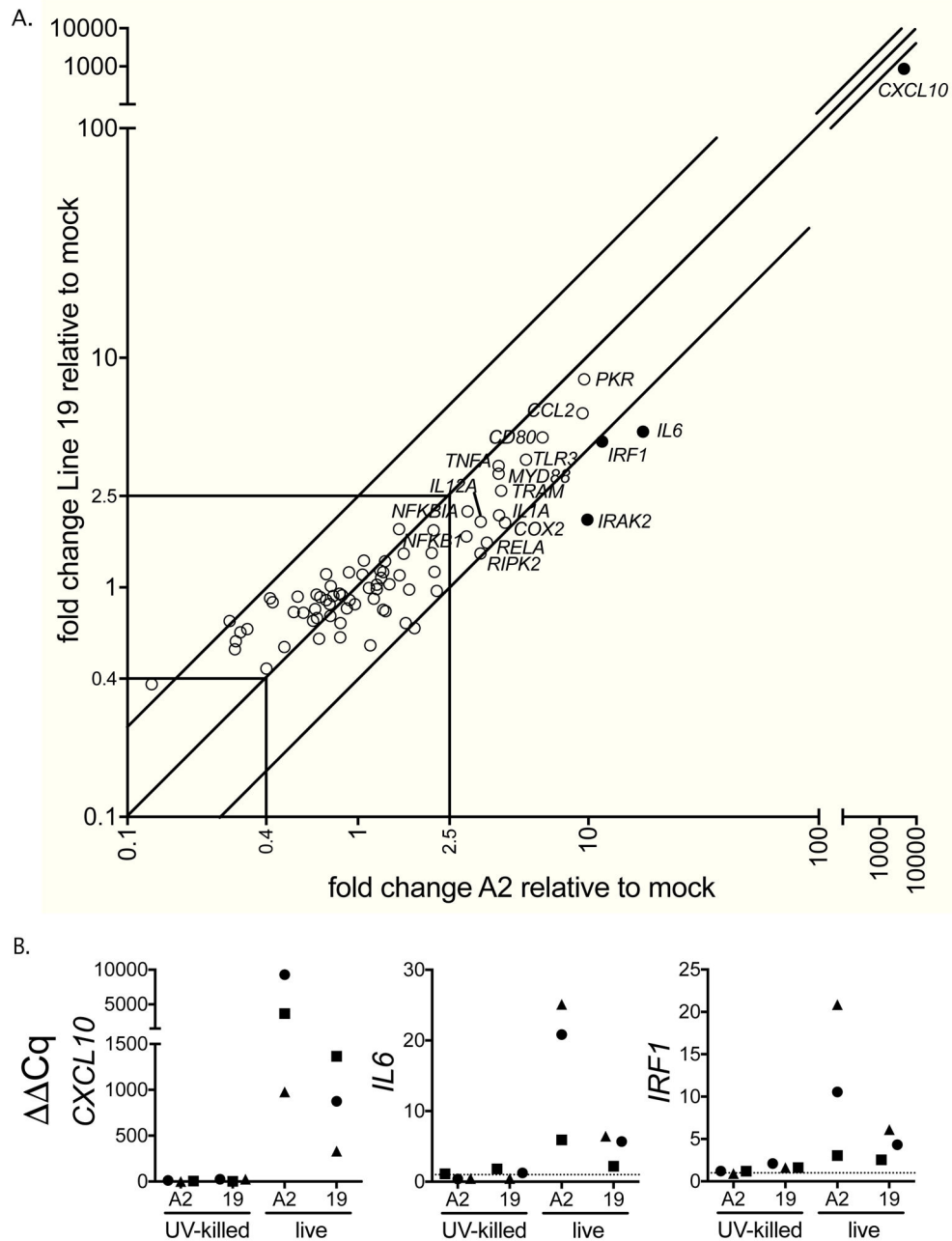


Figure 1. At 24 hours, innate immune gene expression is higher in response to A2 than Line 19 MDDCs from three donors were infected with either live or UV-inactivated RSV strain A2 or Line 19 (MOI = 3) for 24h. Gene expression was measured by qRT-PCR using a TLR pathway gene array. Data are expressed as mean fold change from mock-infected cells normalized to *GAPDH*. A. Live A2 and live Line 19 induce expression of similar genes, but live A2 induces higher expression of some TLR pathway genes in MDDCs compared to live Line 19. Each point represents one gene of 84 examined, and shows the mean of the data for the three donors. Genes upregulated > 2.5 fold by RSV infection (either strain) are labeled, diagonal lines represent 2.5 fold change in gene expression between the two strains of RSV

examined, specific genes expressed > 2.5 fold higher in response to A2 compared to Line 19 are indicated as filled circles. B. Live virus is required to induce gene expression. Data shown are from the same three donors in response to live A2 or Line 19, or UV-inactivated A2 or Line 19. Each individual donor is represented by a different symbol. The dotted line represents gene expression in mock-infected cells.

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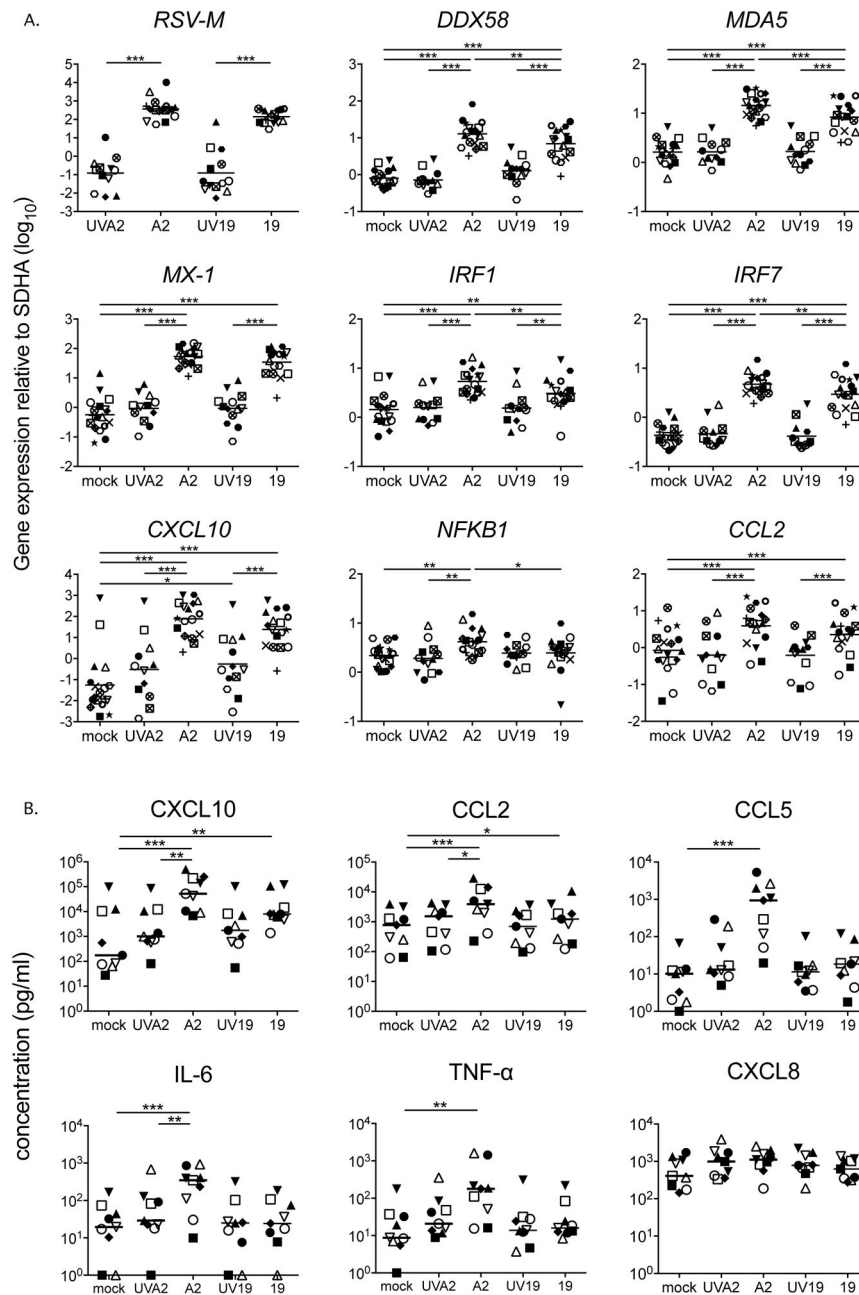


Figure 2. At 24 hours, expression of ISGs and inflammatory cytokines are higher in A2 compared to Line 19 infected MDDCs
 MDDCs from 18 donors were infected with either live or UV-inactivated RSV A2 or Line 19 (MOI = 3) for 24h. A. Gene expression by MDDCs from all 18 donors (UV-A2 12 donors, UV-Line 19 13 donors) was quantified by real time PCR. Gene expression was calculated relative to the housekeeping gene *SDHA* and is plotted on a \log_{10} scale. Each shape represents one donor. All p values were calculated using a mixed effects model (see methods for further details). Adjusted p values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B. Cytokines and chemokines were quantified by multiplex bead assay in 9 of 18 donors. Each shape represents one donor. Horizontal line indicates the median. Statistical differences were

determined by Friedman test with Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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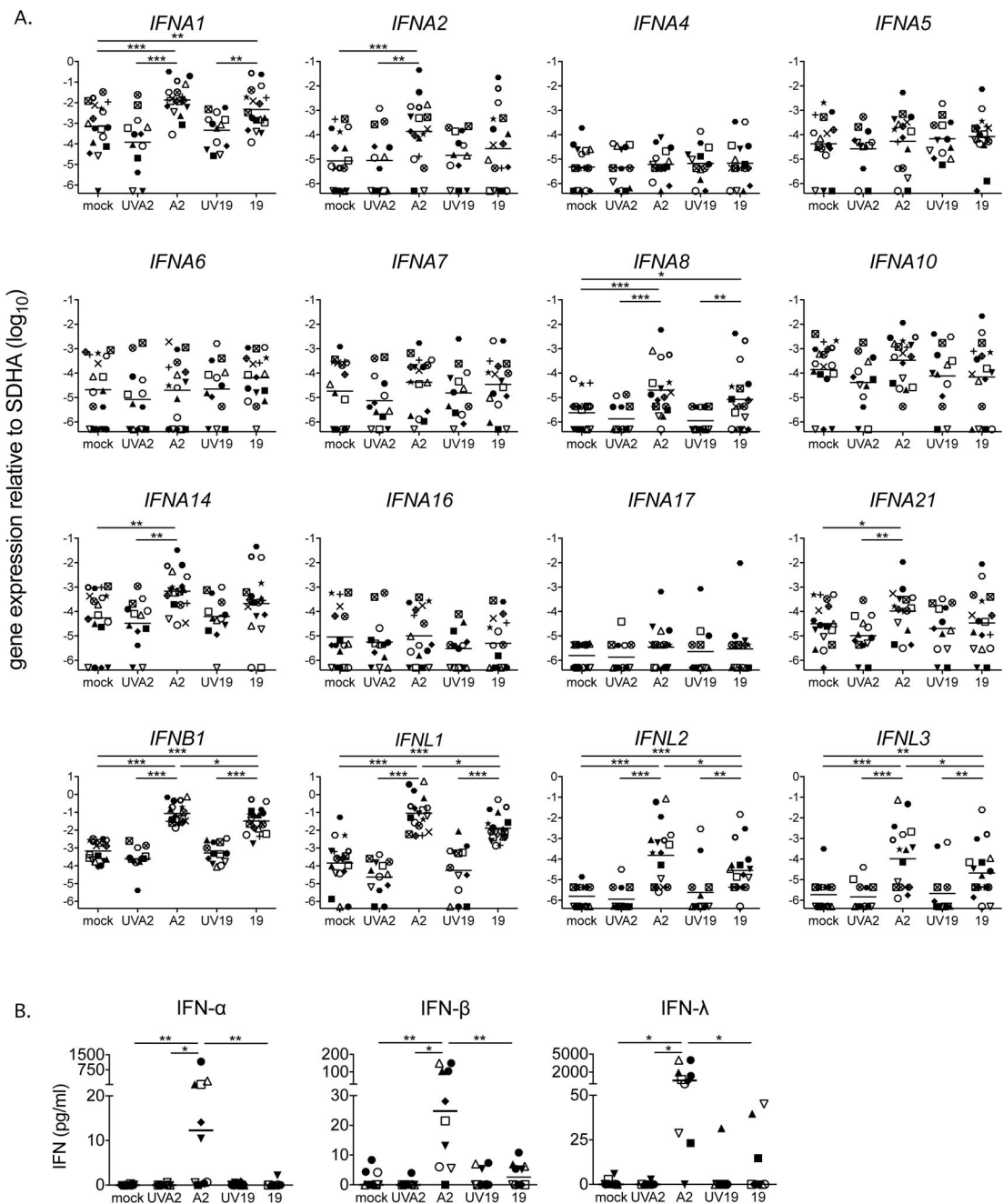


Figure 3. At 24 hours, RSV induces expression of a subset of type I and III IFNs

A. IFN subtype expression by MDDCs from 18 donors after exposure to A2 or Line 19 was quantified using a qRT-PCR assay that discriminates among each of the twelve unique subtypes of IFN- α and detects IFN- β and the three IFN- λ subtype genes. Expression of *IFNA1*, *IFNA8*, *IFNB1*, *IFNL1*, *IFNL2* and *IFNL3* was increased in response to both RSV strains. *IFNA2*, *IFNA14* and *IFNA21* were significantly increased in response to A2, and in many donors, in response to Line 19. Gene expression was calculated relative to *SDHA* and is shown on a \log_{10} scale. Each shape represents one donor. Mock, A2, line 19, n=18, UVA2 and UV19 n=13. Statistical differences were calculated using a mixed effects model (see

methods for further details). Adjusted p values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B. The levels of type I and III IFNs were measured by ELISA. RSV strain A2, but not Line 19 induced IFN- α , - β and - λ expression. Each shape represents one donor. Horizontal line indicates the median. All statistical differences were determined by Friedman test with Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$.

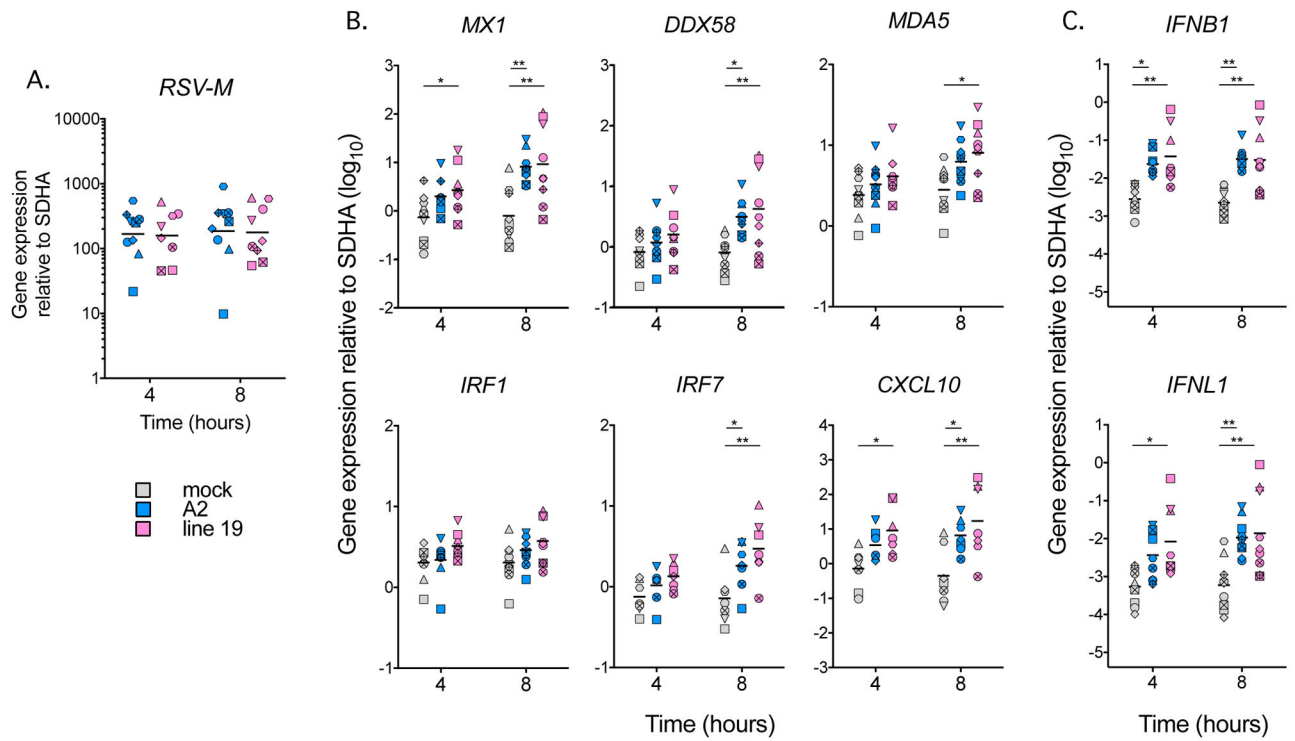


Figure 4. The kinetics of RSV-M, ISG and IFN expression differ dependent on RSV strain MDDCs from nine donors (eight donors at 4 hpi and 24 hpi Line 19) were exposed to live virus of each strain (MOI = 3) for 4, 8 or 24 hours and the RNA harvested. qRT-PCR was used to measure gene expression over time relative to *SDHA*. Each shape represents a different donor. Data are shown on a log₁₀ scale. A. *RSV-M*, B. ISG, C. *IFNB1* and *IFNL1* expression. Statistical differences were calculated using a mixed effects model at 4h and 8h as shown * p < 0.05, ** p < 0.01, *** p < 0.001 (see methods for further details).

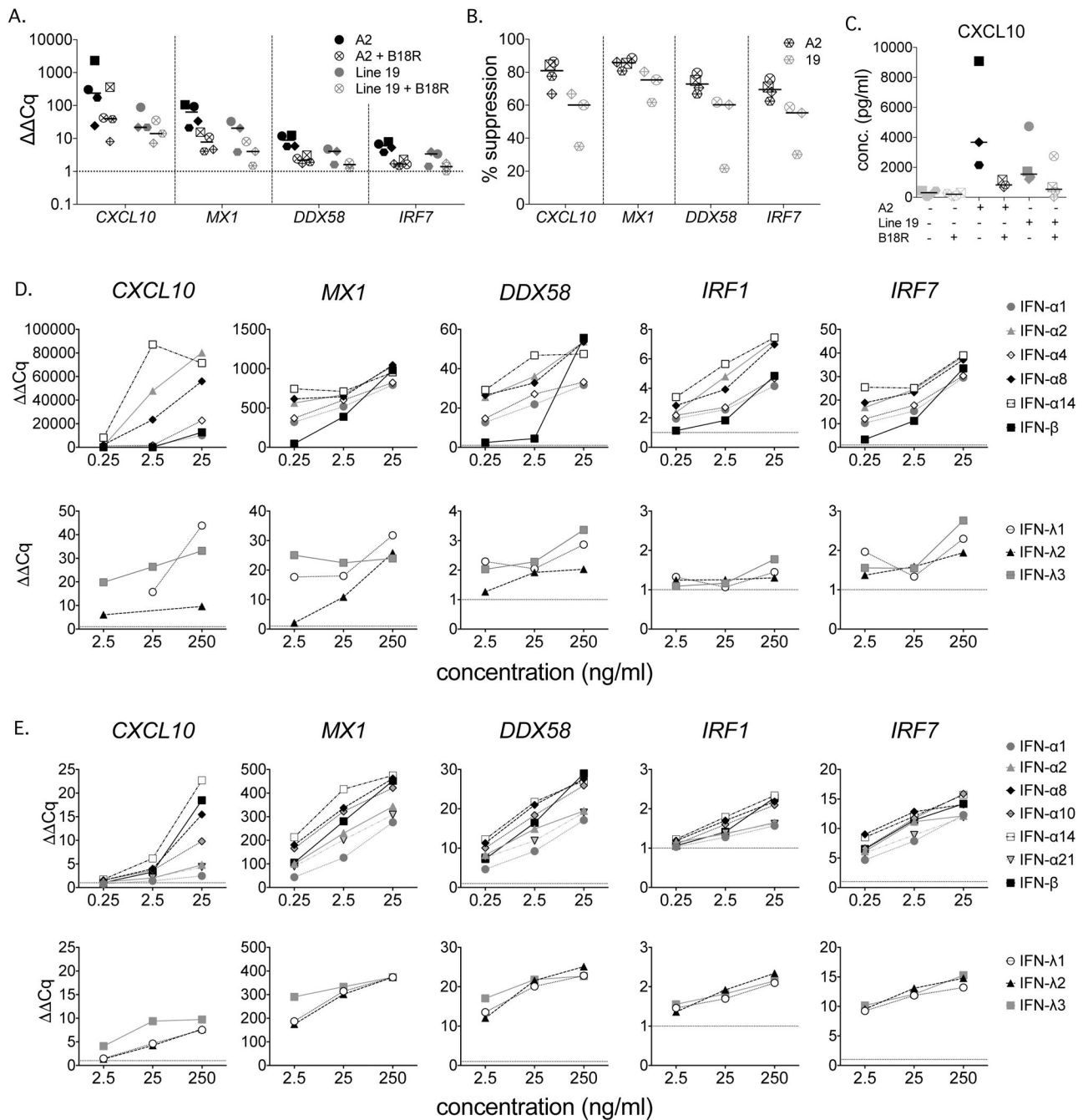


Figure 5. Type I IFNs are the major inducers of ISGs by MDDCs

A – C. MDDCs from four donors were stimulated with live RSV of each strain (MOI = 3) for 24 hours in the presence or absence of B18R, a vaccinia virus encoded soluble type I IFN receptor. Gene expression was measured by qRT-PCR. Data were normalized to *SDHA* and expressed as $\Delta\Delta Cq$ (fold change) compared to unstimulated MDDCs (A), or as percent suppression in the presence of B18R compared to RSV alone (B). A2 n=4, Line 19, n=3. CXCL10 protein expression was quantified by multiplex bead assay (C). RSV A2 n=3, Line 19 n=4. D-E. MDDCs and BEAS-2B cells were stimulated with the indicated levels of each

IFN for 24 hours and gene expression measured by qRT-PCR. The dotted horizontal line indicates expression in unstimulated cultures. D. MDDCs, Data were normalized to *SDHA* and expressed as Cq (fold change) compared to unstimulated MDDCs. Data shown is one representative donor of four analyzed. E. BEAS-2B cells, data were normalized to *GAPDH* and expressed as Cq (fold change) compared to unstimulated BEAS-2B cells. Data shown is one representative experiment of two analyzed.

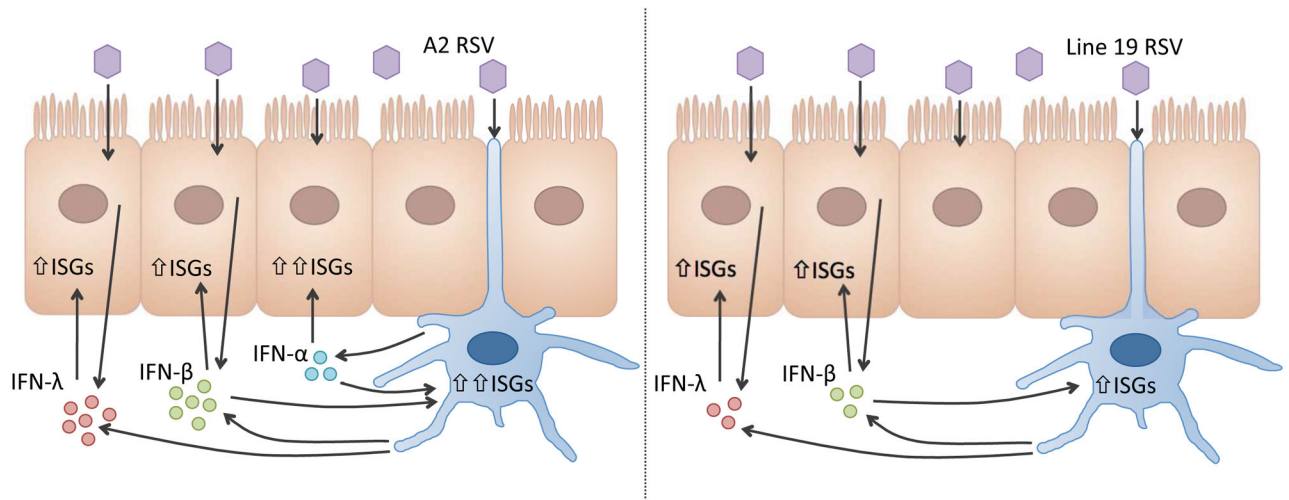


Figure 6. Strain dependent IFN expression by DCs

RSV A2 (left panel) induces DCs to express high levels of IFN- β and IFN- λ 1, and to express three IFN- α subtypes: IFN- α 2, - α 8, and - α 14. These IFNs induce high expression of ISGs by DCs with a generalized hierarchy of IFN- α 14 > - α 2 > - α 8 > - β and by bronchial epithelial cells with a hierarchy of IFN- α 14 > - α 8 > - β > - λ 3 > - λ 1, - λ 2 and - α 2. In contrast, RSV line 19 induces lower expression of IFN- β and - λ 1, and IFN- α 1, which may not be optimal for inducing an antiviral response in the epithelial cells, or for the DCs to mediate an appropriate adaptive response.