Interferon Lambda Signaling in Macrophages Is Necessary for the Antiviral Response to Influenza

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Interferon lambda (IFNλ) signaling is a promising therapeutic target against viral infection in murine models, yet little is known about its molecular regulation and its cognate receptor, interferon lambda receptor 1 (IFNLR1) in human lung. We hypothesized that the IFNλ signaling axis was active in human lung macrophages. In human alveolar macrophages (HAMs), we observed increased IFNLR1 expression and robust increase in interferon-stimulated gene (ISG) expression in response to IFNλ ligand. While human monocytes express minimal IFNLR1, differentiation of monocytes into macrophages with macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) increased IFNLR1 mRNA, IFNLR1 protein expression, and cellular response to IFNλ ligation. Conversely, in mice, M-CSF or GM-CSF stimulated macrophages failed to produce ISGs in response to related ligands, IFNL2 or IFNL3, suggesting that IFNLR1 signaling in macrophages is species-specific. We next hypothesized that IFNλ signaling was critical in influenza antiviral responses. In primary human airway epithelial cells and precision-cut human lung slices, influenza infection substantially increased IFNλ levels. Pretreatment of both HAMs and differentiated human monocytes with IFNLR1 significantly inhibited influenza infection. IFNLR1 knockout in the myeloid cell line, THP-1, exhibited reduced interferon responses to either direct or indirect exposure to influenza infection suggesting the indispensability of IFNLR1 for antiviral responses. These data demonstrate the presence of IFNλ - IFNLR1 signaling axis in human lung macrophages and a critical role of IFNλ signaling in combating influenza infection.

Keywords: IFNLR1, interferon lambda, influenza, macrophage, lung, MCSF, GMCSF

INTRODUCTION

Influenza type A and B virus infections in humans result in nearly 80,000 deaths in the United States and up to 650,000 deaths worldwide annually (1). The US economic burden is staggering; in the range of $47-$150 billion annually (2). Further, opportunistic bacterial pneumonia following influenza infection is a leading cause of death worldwide. Early in infection, interferons are
released and signal through their specific receptors, conferring protection against viral infection (3, 4). IFN-lambda (IFNL) is the most abundant and earliest expressed interferon during influenza infection that via its cognate receptor, interferon lambda receptor 1 (IFNL1), induces a robust antiviral response by upregulating antiviral genes (3, 5).

Influenza, an RNA virus, activates the immune system via multiple mechanisms during infection. One crucial component is the innate immune response, whereby influenza viral RNA is detected by the pattern recognition receptor RIG-I, sequentially enacting MAVS oligomerization, transcriptional activation of IRF and NF-kB, and finally type I (e.g. IFNα, IFNβ) and type III interferon (IFNL) production (6, 7). Interestingly, while both IFNα and IFNβ are antiviral, in some systems, IFNβ exclusively leads to the upregulation of pro-inflammatory genes (5). Bone marrow-derived macrophages, dendritic cells, and neutrophils secrete pro-inflammatory cytokines in response to stimulation with IFNβ but not IFNα (4, 5). In contrast, IFNL inhibits several inflammatory mechanisms, including ROS production, granule mobilization, and the release of neutrophil extracellular traps (NETs) (8). However, cellular tropism of IFNLR1 may mediate this differential response, and it remains to be determined whether these results are reproducible in human studies (9).

In mouse models, Ifnlr1 knockout results in widespread viral dissemination and lethality (5). In addition, mice treated with IFNα after influenza infection exhibit significantly lower mortality, decreased viral burden, with reduced inflammatory cytokines compared to untreated mice (5, 10). Studies have examined interferon production in the lung to varying degrees. In human cell culture, IFNL2, IFNL3, and the human-specific IFNL, isoform, IFNL1, are robustly induced after influenza infection, predicting the transcription of interferon-stimulated genes (ISGs) (11). In murine models, IFNL2 and IFNL3 are produced earlier and more abundantly than type I interferons. In humans, several studies have demonstrated the presence of interferons in the infected lung. Differentiated airway epithelial cells produce IFNL in response to rhinovirus, influenza, and polyIC treatment (12, 13). ATII cells primarily produce IFNα in response to influenza (14). However, there have been no in-depth studies examining IFNL production and the expression of its receptor IFNL1. Differences in the resolution of viral infection may be driven by the tropism of these receptors, underscoring the importance of understanding IFNL1 expression in the human lung.

In this study, we observed that IFNL2, IFNL3, and the human specific IFNL1 were consistently the highest expressed interferons in human airway epithelium. We also found that IFNL1 was enriched in human alveolar macrophages (HAMs) which led to the production of ISGs in response to IFNL treatment. IFNα restricted the infection of HAMs and differentiating primary macrophages. Further, in macrophages, IFNL1 was necessary for a robust ISG response to direct infection and was entirely responsible for ISGs produced in response to secreted interferon. These data suggest that IFNL signaling in macrophages has an important role in the sensing and response to viral infection in the human lung.

**MATERIALS AND METHODS**

**Cells and Tissue**
THP-1 and HEK-293 were purchased from the American Type Culture Collection (ATCC). THP-1 cells were cultured in RPMI supplemented with 10% FBS. HEK-293 cells were cultured in DMEM supplemented with 10% FBS. CD14 monocytes (Lonz) were either untreated for monocytes, or differentiated with M-CSF or GM-CSF (R&D systems) for 7 days to produce macrophages. HAMs were isolated via ex vivo lavage from de-identified lungs rejected for transplant and obtained from Lifeline of Ohio Organ Procurements agency (Columbus, OH) as described (15). All lungs were from subjects with no history of chronic lung disease or cancer and were non-smokers for at least 1 year. After collection, red blood cells were lysed and cells were enumerated and frozen down in FBS and 10% DMSO. Prior to experiments, HAMs were rapidly thawed, added to RPMI (10% FBS, 1% antibiotic/antimycotic). Total concentration and viability of cells were determined with trypan blue staining before seeding. Differentiated human bronchial epithelial (HBE) cultures were supplied by the Cure CF Columbus (C3) Epithelial Cell Core at Nationwide Children’s Hospital as described (16). Briefly, HBE progenitors were isolated from donor airways as described previously, grown for a week to confluence and frozen for later use (17). Progenitor cells were thawed and plated on 0.4 μM pore Transwells (Corning) membranes 12 mm in diameter. Medium in both chambers was replaced with fresh medium every 2–3 days. At 7 days, when the cells were confluent and had formed tight junctions as demonstrated by electrical resistance, the apical medium was removed, and the basal medium was replaced with complete Pneumacult-ALI Medium (STEMCELL Technologies). The medium was replaced with fresh medium and the apical surface was washed with 100 μL of DMEM every 2–3 days for 3 weeks at which time they had become fully differentiated. Murine bone marrow derived macrophage (BMDM) were derived from C57Bl/6 mice. Bone marrow from the femurs and tibias were collected and following RBC lysis cells were resuspended in HEPES-buffered RPMI-1640 containing L-glutamine, penicillin/streptomycin, and 10% HI-FBS with the addition of either recombinant murine GM-CSF or M-CSF. Cells were plated on petri dishes overnight and the non-adherent were passaged to new dishes to allow for expansion and differentiation of macrophages for 7 days. Macrophages were removed from petri dishes, counted, and plated into tissue culture treated wells overnight before experiment initiation. Precision cut lung slices (PCLS) were prepared as described previously (18). Briefly, transplant-rejected lungs were filled with agarose by injecting liquid agar into a lobe via the bronchi. Approximately 1 cm³ of human lung tissue was sliced with a Vibratome into 400μM sections. Tissue was cultured in DMEM containing antibiotic/antimycotic.

**Infection Protocol**
Influenza PR8 and influenza CA09 strains were propagated in MDCK cells (ATCC CCL-34) (19). HAMs, CD14 macrophages,
and THP-1 macrophages were infected at the indicated MOI for 1 hour, and the media was replaced. Differentiated HBECs were infected with CA09 influenza at the indicated MOI on the apical side of the transwell. After 2 h incubation, the apical layer was washed three times with DPBS to remove unbound virus. PCLS were incubated with 1x10^5 pfu PR8 and 8x10^6 pfu CA09 virus for 2 h prior to replacing the media.

**CRISPR/Cas9 IFNLR1 Knockout THP-1 Cell Line**

IFNLR1 knockout THP-1 cells were generated as described previously (20). Briefly, control sgRNA (#1 GTATTACTGATATTGGTGGG; #2 GTTCCGCGTTACATAACTTA) and sgRNA targeted against IFNLR1 (#1 ACAAGTTCAAGGGACGCCGTG; #2 CTCACTTTAGATCCAGTG) were inserted into the backbone plasmid lentCRISPRv2. HEK-293 T cells were transfected with the 3rd generation lentiviral packaging plasmids pMD2.G, pRSV-Rev, pMDLg/pRRE and the lentCRISPRv2 backbone with the targeting sgRNA. Single clones of THP-1 KO monocytes were generated via lentiviral transduction and selection with puromycin.

**Quantitative PCR**

Total cellular RNA was collected from cells using the Qiagen RNeasy Miniprep plus Kit (Qiagen), following the manufacturer’s protocol. The cellular RNA was then used to create cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. qPCR was performed using SYBR Select Master Mix (Applied Biosystems) according to the manufacturer’s protocol with 20 ng cDNA as a template and primer concentration of 200 nM. Each biological replicate was performed in at least technical duplicate; data was analyzed using the ΔΔCq method. qPCR primer sequences are available in Table 1.

**Immunoblotting**

Immunoblotting was performed as described previously (21). Briefly, cells were lysed in RIPA buffer, sonicated and clarified by centrifugation. Lysates were diluted in SDS protein sample buffer. Proteins were separated by electrophoresis and transferred to a nitrocellulose membrane. Blots were blocked in 5% milk, followed by probing overnight with antibodies. Following addition of secondary antibodies (goat anti-mouse, Biorad, 170–6516, and goat anti-rabbit, Biorad, 170–6515; 1:2000 dilution), membranes were developed using a Western Bright Sirius immunoblotting detection kit (Advansta) and imaged using Biorad imaging software. Single band intensity was quantified using ImageJ software. Antibodies used are available in Table 2.

**TABLE 1 | Quantitative PCR primers.**

|                        | Influenza                              | Human      |
|------------------------|----------------------------------------|------------|
|                        | forward                                | reverse    |
| CA09 M                 | GTCTCAQACGAATGTCGG                   | GATGCAATGATTTGCTGCAATG | |
| CA09 NS                | CTTCGCGCTACCTTCTGAG                  | ATTGCTGGCTCTGCGAGAAG  |
| PR8 M                  | CGCTCAAGCATGAAAGACAAATG              | TAACATGCTGCAATGACAGAG  |
| PR8 NS                 | CAGGACATACATGAGAAGTCAG               | GTTGGAGACTGCAACTG    |
| CCL2                   | CACAGTGAATGACATGCG                   | TGCTGGAGATCATGCA     |
| CCL4                   | AGCTGTGATATTCACAACC                  | TCTACAGTACATGACAGAAG |
| CXCL10                 | ACGTGTTGAGATCTGCT                   | AAATCTTGAGGGCTCGGAG  |
| IFIT3                  | AACAGGCGATCAGAAGTCGAG                | AGGTTCAGGTGAAATGCG   |
| IFN1                   | ACATTGGAGGTTACTAACTCTC              | TGAAGTGGACTGCAACAGG   |
| IFN1L2 & 3             | GCAACATGCGCCTGTACAGTG                | GTGACATCTGGGCTTTTAAA |
| IFN8                   | CCTCGCTGCTGCCTTCCACT                | TGTTGAGGAAATGCGAGAAG |
| IFNγ                   | CTTTAAAGATGACAGAAGATCACA            | GGCAGATTTCAAGCCTTCCCA |
| IL-6                   | GATTCATAATGAGAGATCCTGCC             | TGTTCTGGAGATCTGAGAT  |
| IRF7                   | CAAGGGTTGATGTGGGAGTTGG             | ATTTCCACAGATGATGTGA  |
| IRF9                   | TGGCAAGGAGAAATGACAG                 | TCTCAGATGGGATGTGG    |
| ISG15                  | AGCTCTGTGAGAAGAAGACG                 | GACACCTGAAATGGGCG    |
| MX1                    | TAAATAGCCGCAATGCGCA                | TTAGATGAGGAATGCGGGAC |
| OAS1                   | TCAAGGCTGGAATTCAGTGG                | TGAGGAAATGAAACAGGCTC |
| panIFNβx               | GACCTCACTTTGCTGCTGQA                | TATTGCTGCTGGCAGAAAGCT |
| STAT1                  | GATTTAATGAGGGTATCGAGG               | TTTGCTGACTTGTCAAGAGT |
| TNFα                   | CTCTAATACAGCCCTCTGCG                | GAGGCTGGTCAGAACAATGG |
| mIFNLR1                | GAAAGGGGTGGTCTGCTGCC               | CTAAGGGGTCAACGCTACCT |
| mIF27                  | TCAACATGTTGGGACACCTG             | ATCTGGGTGCTGATGGAAG  |
| mIFIT2                 | GTCATGAGTACACAGAGTAAG              | TGCTCAGATGGTCAAGGCTG |
| mIFIT3                 | ATCATGAGTAGGTGCAACAG               | AAATGGTGACCTGTTGAGTC |
| mOAS1                  | AAAGGGATGTTCCGCAAGTG             | TGTCAGGTCTTCTTCTACTG |

![Table 1](image-url)

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**TABLE 2 | Antibodies and reagents.**

| Antibodies | Reagents |
|------------|----------|
| IFIT3 (IFIT3, sc-393512) | Recombinant IL-28a (R&D systems, 1587-IL) |
| IFNLR1 (Sigma Aldrich, HPA017319) | Recombinant IL-28B (R&D systems, 5259-IL) |
| IFNLR1 [IL28RA-PE] (Biolegend, 337804) | Recombinant IL-29 (R&D systems, 1598-IL) |
| CD206-APC [MFR] (Biolegend, 141708) | Recombinant M-CSF (R&D systems, 216-MC) |
| CD36 (Cell Signaling, DB9T) | Recombinant GM-CSF (R&D systems, 215-GM) |
| CD206 (Santa Cruz, sc-376108) | Recombinant murine IL-28A (R&D systems, 4635-ML) |
| CD172a (Millipore, 566510) | Recombinant murine IL-28B (R&D systems, 1789-ML) |
| CD68 (Santa Cruz, sc-17832) | Recombinant M-CSF (R&D systems, 415-ML) |
| β-actin (Sigma, A5441) | Recombinant GM-CSF (R&D systems, 216-MC) |
| STAT1 (Cell Signaling, 9172) | Recombinant M-CSF (R&D systems, 216-MC) |
| pSTAT1 (Cell Signaling, 7649) | Recombinant GM-CSF (R&D systems, 215-GM) |
| matrix-1 (Abcam, ab223986) | Recombinant murine IL-28A (R&D systems, 4635-ML) |
| matrix-2, (Novus Biologicals, 14C2) | Recombinant murine IL-28B (R&D systems, 1789-ML) |
| MHCII [HLA-DR] (Abcam, ab175085) | Recombinant murine IL-28B (R&D systems, 1789-ML) |
| CD14 (Cell Signaling, 56062) | Recombinant murine IL-28B (R&D systems, 1789-ML) |
| GAPDH (Cell Signaling, 56714) | Recombinant murine IL-28B (R&D systems, 1789-ML) |

**Single-Cell RNA Sequencing**

Normal control lungs were obtained under a protocol approved by the University of Pittsburgh, Committee for Oversight of Research and Clinical Training Involving Decedents, following rejection as candidate donors for transplant. The whole lung tissue samples were processed as described previously (22). Briefly, tissue for scRNA-seq was diced and enzymatically digested in DMEM (Thermo Fisher Scientific) containing collagenase A and 30 μg/mL DNAase I and further mechanically dispersed using the Miltenyi gentleMACS Octo Dissociator. Single cell RNA library preparation was performed utilizing the 10X Genomics Chromium instrument and its associated V2 single cell chemistry per the manufacturer’s protocol. In brief, 7000 cells were mixed with reverse transcription reagents, loaded into a Single Cell A chip. Cells were separated into oil micro-droplet partitions by the Chromium instrument, containing a cell and a gel bead scaffold for an oligonucleotide composed of oligo-dT, and 10X transcription reagents, loaded into a Single Cell A chip. Cells were then washed 1x and resuspended in primary antibody and incubated for 30 min at 4°C. After a 2nd wash with FSG buffer, cells were fixed with 4% paraformaldehyde followed by flow cytometry analysis. Antibodies used are available in Table 2.

**RESULTS**

**Influenza Infection Induces IFNL1 in the Airways**

To determine the induction of interferons after influenza infection in human airways, we exposed differentiated human airway epithelial cells to pandemic 2009 influenza virus (CA09). At 24 and 48 h post-infection, the predominant interferons induced in the primary airway cells at the mRNA level were IFNβ, IFNL1, and IFNL2/IFNL3 (qRT-PCR cannot distinguish these isoforms), with IFNL1 and IFNL2/3 being the most upregulated (Figure 1A). Consistent with the gene induction, we primarily detected the secreted interferon, IFNL1, in the supernatant of infected cells (Figure 1B). To further assess human lung responses, we infected human precision cut lung slices (PCLS) with PR8 and CA09 for 24-72h. Lung slices were viable over the course of the experiment as demonstrated by LDH release (Figure 1C). We confirmed that these lung slices were susceptible to infection by measuring the production of viral mRNA (M gene and NS gene) (Figure 1D). Infection of PCLS resulted in the robust secretion of IFNγ, followed by IFNL1, and IFNo2, while secreted IFNβ was unchanged (IFNγ>IFNL1>IFNo2>>IFNβ) (Figures 1E-H and S1A-D). These findings indicate that IFNL1 is highly induced in the infected human lung environment.

**Human Alveolar Macrophages Express Functional IFNLR1**

Because we observed significant IFNL1 induction in the infected lung, we next examined the expression of its receptor, IFNLR1, **via** single-cell RNA sequencing. In human lung tissue, IFNLR1 is
most highly expressed in the CD163 expressing macrophage population and to a lesser degree in the epithelium (Figure 2A). To examine the expression of IFNLR1 in alveolar macrophages in the infected lung, we isolated HAMs from transplant-rejected lungs for in vitro experiments and compared them to undifferentiated CD14 cells and M-CSF differentiated macrophages. We confirmed that HAMs expressed macrophage markers (CD68 and the MHC-II subunit HLA-DR) as well as the alveolar macrophage marker (CD206) (Figure 2B). We also observed IFNLR1 expression in GM-CSF macrophages and HAMs, but not in CD14 monocytes demonstrating that differentiation induced IFNLR1 expression, consistent with prior reports (23). Using an IFNLR1 specific antibody, we observed IFNLR1 expression in HAMs via flow cytometry. To confirm antibody specificity, we measured IFNLR1 expression in two monocyte cell lines THP-1 and HL-60. We did not observe any CD206 or IFNLR1 signal in these cells (Figure S2A, B).

**Differentiation of Monocytes to Macrophages Increases IFNLR1 Expression and Function**

To examine the regulation of IFNLR1 in macrophages, we measured the response of HAMs to exogenous interferons. HAMs displayed a robust ISG induction in response to IFNL1 comparable to IFNβ at 24h, except for ISG15, which was more induced with IFNL1 (Figure 3A). Both M-CSF and GM-CSF drive the differentiation of monocytes to macrophages. GM-CSF is also known to be essential for the differentiation of alveolar macrophages and has been implicated in the expression of IFNLR1 (23–25). We therefore examined the regulation of IFNLR1 expression and signaling in our system by both factors. Compared to nearly undetectable levels of IFNLR1 in monocytes, IFNLR1 was highly upregulated by differentiation with both M-CSF and GM-CSF (Figure 3B). We then treated differentiated macrophages with IFNL1 to measure the induction of ISGs and confirmed that GM-CSF treated macrophages showed a more robust ISG response to IFNL1 than M-CSF macrophages (Figure 3C). Treatment of GM-CSF macrophages...
with either IFNβ or IFNL1 led to a similar induction of ISGs (Figure 3D). We also determined that IFNL1-3 had a similar effect on the induction of ISGs at the same concentration (Figure 3E). To examine the role of viral infection in IFNLR1 expression, we differentiated macrophages with GM-CSF, followed by infection with influenza PR8. IFNLR1 mRNA was unchanged in these cells (Figure S3A). Murine macrophages have been reported to be unresponsive to IFNλ (26). To examine whether M-CSF or GM-CSF differentiated mouse macrophages responded to IFNλ treatment, we isolated bone marrow macrophages and incubated with either M-CSF or GM-CSF. Ifnlr1 expression was relatively similar with either differentiation protocol, and stimulation with interferons did not regulate the receptor (Figure 3F). Although both differentiation procedures led to robust responsiveness to murine IFNβ, there was little to no gene induction after treatment with murine IFNL3 (Figures 3G, H) or murine IFNL2 (Figure S3B) (mice do not express IFNL1).

**Influenza Infection of Human Macrophages Leads to the Production of Lambda Interferon**

We next examined anti-viral responses of M-CSF and GM-CSF differentiated macrophages to infection with influenza. We found that infection of primary macrophages with influenza PR8 (Figure 4A) or CA09 (Figure S4A) led to a robust increase in both IL-6 and TNFα. Other cytokines examined via multiplex ELISA were unchanged (Figure S4B). We next examined interferons produced in response to infection. At the gene level, a wide array of type I and type III interferons were highly induced after PR8 (Figure 4B) and CA09 (Figure S4C). In contrast to the airway epithelium, IFNα was highly upregulated in macrophages. Of note, when we measured interferons in the supernatant, we found that IFNL1 was the most abundant secreted interferon in both GM-CSF and M-CSF macrophages in response to PR8 (Figure 4C) and CA09 (Figure S4D). Interestingly, while M-CSF vs. GM-CSF differentiation did not appear to alter the pattern of the cytokine/chemokine response, the magnitude of induction was reduced in the GM-CSF differentiated macrophages compared to the M-CSF differentiated macrophages. Importantly, the levels of viral mRNAs were not significantly altered by the type of differentiation, suggesting that these cells were infected at a similar level but failed to mount a comparable immune response. This effect held true in experiments performed at the same time in identical cell populations (Figures S5A, B). Finally, we measured the interferon response in infected HAMs. Concordant with the *in vitro* differentiated macrophages, HAMs primarily produced IFNλ in response to influenza infection (Figure 4D). These results suggest that macrophages are susceptible to influenza infection and contribute to the production of lambda interferons in response to infection.
IFNλ Inhibits Macrophage Infection

As both macrophages and epithelial cells produce primarily type III IFN in response to viral infection, we next asked whether IFNλ could inhibit the infection of macrophages. We pre-treated macrophages with IFNL1 for 24 hours prior to infection with influenza PR8 and assayed mRNA copy number of the two viral replication-dependent mRNAs (M and NS gene). Indeed, IFNλ pre-treatment was protective against PR8 (Figure 5A) and CA09 (Figure 5B) infection in GM-CSF differentiated macrophages. We also observed decreased production of the inflammatory cytokines TNFα, MIP1α, and MIP1β in cells pre-treated with IFNL1 (Figures 5C, D). All three of these cytokines have been demonstrated to contribute to the inflammatory response to influenza infection (27–29). To examine the inhibition of influenza infection in HAMs, we pre-treated with IFNL1 and IFNb for 8 or 24 hours prior to influenza infection for 24 hours. As measured by the expression of the influenza viral protein M1 and M2, interferon beta led to a robust inhibition of infection, while IFNL1 led to more modest inhibition (Figure 5E). Due to its antiviral activity and the high amount of circulating interferon lambda in the lung, IFNLR1 likely plays a key role in the inhibition of macrophage infection.

IFNLR1 Is Necessary for the Production of ISGs in Infected and Bystander Cells

The above data suggest a key role for IFNλ in conferring antiviral immunity. We next assessed how the IFNλ receptor alters influenza virus infection. Thus, we knocked out IFNLR1 in the THP-1 myeloid cell line. As with CD14 monocytes, THP-1 monocytes express IFNLR1. However, after PMA-mediated differentiation, IFNLR1 mRNA was significantly increased compared to untreated THP-1 (Figure S6A). Differentiation also increased IFNLR1 protein (Figure S6B).

We generated murine bone marrow derived macrophages (BMDMs) after treatment with either M-CSF or GM-CSF (50 ng/mL) and examined changes in IFNLR1 mRNA expression, ns, not significant (F). We also measured the induction of the ISGs IFIT3, MX1, and IFI77 in response to IFNL3 (100 ng/mL) or IFNb (20 ng/µL) in M-CSF (G) and GM-CSF (H) macrophages. n=3 independent experiments. At least n=5 per group (G–H). Multiple T-test with Holm-Sidak correction (A–H). *p < 0.05 Mono (B) **p < 0.01 vs. M-CSF, #p < 0.05 vs. M-CSF-GM-CSF (C) *p < 0.05, **p < 0.01, ***p < 0.001, vs. Control (Con) (D–H).
measured the phosphorylation of STAT1. Knockout of IFNLR1 inhibited IFNλ signaling as expected (Figure 6B). To examine how IFNLR1 alters the response to influenza infection, we infected IFNLR1-KO macrophages with PR8 and measured the expression of ISGs. Multiple ISGs were significantly reduced in IFNLR1-KO cells vs. control lines despite expressing moderately higher levels of viral mRNA, suggesting an increased susceptibility to infection (Figures 6C, D). The inhibition of the ISG response was also confirmed in a second IFNLR1-KO clonal cell line (Figure S6E). In contrast to the decreased interferon response, and consistent with the increase in viral mRNA, we observed an increased in several secreted cytokines IL1-β, TNF-α, IL-6, and MIP1-α in infected IFNLR1-KO cells. Interestingly, MIP1-β and MCP-1 secretion was reduced in IFNLR1-KO THP-1, potentially because the interferon pathway is required for the transcription of these genes (28, 30) (Figures 6E, F). Alveolar macrophages are both the direct targets of infection, and respond to secreted interferons produced by neighboring airway epithelial cells. To assess cross-talk between human airway epithelia and macrophages, we incubated sgCon and sgIFNLR1 THP-1 cells with supernatants of CA09 infected or non-infected primary differentiated human airway epithelial cells (supernatants characterized in Figure 1B). Treatment of control sgRNA THP-1 cells with virus-infected supernatant that (containing lambda interferons) resulted in the upregulation of a number of ISGs (Figure 6G). However, IFNLR1 depleted THP-1 had a significantly abrogated influenza media-stimulated ISG response compared to sgCon THP-1 treated with the same media. Collectively, these observations strongly suggest that IFNLR1, via engagement of its IFNλ ligands, is crucial for mediating antiviral immunity.

**DISCUSSION**

While both IFNλ and IFNα induce antiviral genes, IFNλ has been suggested to maintain antiviral function without the inflammatory responses observed with type I interferons (5). This has led to significant interest in the type III interferons as potential therapeutics against influenza and other viral infections (10, 31, 32). Compared to type I interferons, murine IFNλ...
signaling alters barrier surfaces, such as the gastrointestinal epithelial layer (33–36). However, data on IFNλ and IFNLR1 in human infection is limited and the murine expression of Ifnlr1 (6, 37, 38) does not appear to match the tropism observed in human cells. In particular, in murine models, macrophages do not respond to secreted IFNλ, suggesting that humans have evolved species-specific adaptive responses through the interferon network for protection against common viral pathogens (5, 26, 39). Here, we have described the expression, regulation, and antiviral activity of the IFNλ-IFNLR1 axis in human lung. We observed abundant IFNLR1 expression in human alveolar macrophages and found that these cells are basally capable of responding to lambda interferons. In addition, IFNα was protective against influenza infection in primary alveolar macrophages. IFNLR1 was also necessary for the production of ISGs in response to secreted factors from infected cells. These data suggest that IFNα signaling in macrophages is integral to the response to influenza infection in the human lung.

In both human and murine models of influenza infection, IFNλ is produced earlier and more abundantly than type I interferons (5, 6). In our studies, polarized airway epithelial cells produced high amounts of IFNλ with lower IFNβ and very little IFNα in response to influenza. In infected lung tissue sections, IFNg was the most upregulated interferon followed by IFNλ. The high amount of IFNg signaling in the lung tissue is likely the result of resident NK cells and lung resident memory T-cells. In ex vivo lung tissue sections macrophages and epithelial cells are directly infected with influenza. Neighboring NK cells in the tissue sections produced high amounts of IFNg in the infected environment (40). In addition, lung-resident memory CD8 T-cells, present in human lung tissue sections, contribute to interferon gamma (IFNg) production in response to influenza (41, 42). When we directly examined infected macrophages, we found that IFNλ was highly induced in response to infection. Although prior studies in murine models suggested monocytes and macrophages do not express IFNLR1 and do not respond to IFNλ (26, 38), more recent observations suggest that human

FIGURE 5 | IFNλ inhibits macrophage infection. CD14 monocytes were differentiated to macrophages with M-CSF or GM-CSF (50 ng/mL, 7 days). GM-CSF macrophages were pretreated with or without IFNL1 (50 ng/mL) for 24 h followed by infection with influenza CA09 (MOI=1) and influenza PR8 (MOI=0.1). Viral replication as determined by measuring the mRNAs of influenza M and NS viral genes by qRT-PCR in PR8 (A) and in CA09 (B) infection. Representative of 3 independent experiments, n=3 samples per group (A, B). We examined the production of inflammatory cytokines in influenza infected macrophages using a custom multiplex from MSD. n=2 independent experiment, n=6 samples per group (C, D). HAMs were pretreated with IFNλ (50 ng/mL) or IFNβ (20 ng/mL) for 24 h followed by infection with influenza PR8. Viral protein production was measured by immunoblotting for influenza proteins matrix-1 (M1) and matrix-2 (M2). Below: Matrix 1 protein levels as determined by densitometry of immunoblots. Representative of 3 independent experiments (E). *p < 0.05 by Students T-test (A, B) ***p < 0.001 by Multiple T-test with Holm-Sidak correction (C, D).
Macrophages possess the necessary machinery to respond to lambda interferons. First, CD14 monocytes differentiated with GM-CSF were responsive to IFN$_l$ treatment (23). More recently, alveolar macrophages derived from bronchoalveolar lavage produced ISGs in response to IFN$_l$ treatment (43). By examining IFNLR1 expression in a scRNA-seq dataset, we found a high degree of IFNLR1 expression in the alveolar macrophages subset. We also detected a significant ISG induction in IFN$_l$ treated HAMs. While IFNLR1 expression and IFN$_l$ signaling was induced by the differentiation of human myeloid cells to macrophages, we did not observe IFN$_l$ signaling in differentiated murine macrophages. Interestingly, porcine alveolar macrophages have also been shown to respond to IFN$_l$ (44). Claims that IFN$_l$ plays an anti-inflammatory role are, in part, based on the lack of IFNLR1 expression in monocyte and macrophages (45). Therefore, the species-dependent expression of macrophage IFNLR1 warrants further exploration and should be considered when studying interferon responses in the murine model.

Experimental depletion of alveolar macrophages with clodronate prior to influenza infection resulted in higher viral titers and mortality in mouse and pig models (46, 47). Genetic depletion of alveolar macrophages prior to influenza infection increases morbidity and mortality and can be rescued with the adoptive transfer of alveolar macrophages (46, 48). Different strains of influenza infect myeloid cells at varying efficacies, with pandemic H5N1 strains showing a higher propensity to infect...
macrophages (49, 50). Lung macrophages are both direct targets of influenza infection and respond to cytokines and chemokines produced by neighboring infected cells. Interestingly, recruited monocytes and resident macrophages are responsive to circulating interferons while viral infection blunts the ISG induction in the epithelium (51). These data strongly implicate a key role of interferons while viral infection blunts the ISG induction in the monocytes and resident macrophages are responsive to circulating produced by neighboring infected cells. Interestingly, recruited in

Overall, these results suggest that IFNLR1 limits viral infection in macrophages. Importantly, this response was completely attenuated after IFNLR1 knockout.

Interestingly, we also found that alternative mechanisms of macrophage differentiation change the response to influenza infection. GM-CSF is necessary for the development of alveolar macrophages in both mice and humans. ATII cells produce high levels of GM-CSF that drives initial development and maintenance of alveolar macrophages (25). Differentiation with GM-CSF also results in a more robust IFNα response vs. M-CSF mediated differentiation (23). Our data suggests that GM-CSF macrophages produce fewer cytokines and interferons in response to influenza infection despite producing higher levels of viral mRNA. Although the magnitude of the chemokine response was reduced in the GM-CSF cells, the overall profile of the response was similar in both groups. Further studies are needed to determine how these factors alter the inflammatory profile of infected macrophages.

In summary, we demonstrate that IFNα is highly induced in the human lung after influenza infection and that resident human alveolar macrophages are responsive to this cytokine. We demonstrate that macrophage IFNLR1 is necessary for the induction of ISGs in response to both direct infection and in response to secreted factors from neighboring infected cells. We also show that murine macrophages are minimally responsive to IFNα, suggesting that IFNLR1 regulation is species-dependent. Due to the high abundance of IFNα production early in infection, the proximity of alveolar macrophages to the site of infection, and the necessity of IFNLR1 to mount an antiviral response to these secreted factors, IFNα signaling in macrophages likely plays an important role in the response to viral infection.

STATISTICS

Statistics were performed using GraphPad Prism. Student t-tests were performed for two samples or multiple T-test with Holm-Sidak correction were performed for three or more samples.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author. ScRNA-seq data can be accessed at the Gene Expression Omnibus: GSE128033.

ETHICS STATEMENT

These experiments were conducted in compliance with biosafety and laboratory biosecurity regulations, guidelines, standards, policies and procedures. The Institutional Animal Care and Use Committee at The Ohio State University reviewed and approved all animal procedures (IACUC protocol #2019A0000019). Human alveolar macrophages and lung slices were derived from de-identified samples and were not subject to IRB oversight.

AUTHOR CONTRIBUTIONS

JL and RM designed the study, performed experiments, analyzed results and wrote the manuscript. JA, AE, DF, IC, ML, MD, and VP performed experiments and analyzed data. RM, ML, JB, AM, and MR reviewed the data and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.735576/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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