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ORIGINAL ARTICLE

Characterization of the antiviral effects of interferon-α against a SARS-like coronoavirus infection *in vitro*

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Interferon (IFN)-αs bind to and activate their cognate cell surface receptor to invoke an antiviral response in target cells. Well-described receptor-mediated signaling events result in transcriptional regulation of IFN sensitive genes, effectors of this antiviral response. Results from a pilot study to evaluate the clinical efficacy of IFN-α treatment of SARS patients provided evidence for IFN-inducible resolution of disease. In this report we examined the contribution of IFN-inducible phosphorylation-activation of specific signaling effectors to protection from infection by a SARS-related murine coronavirus, MHV-1. As anticipated, the earliest receptor-activation event, Jak1 phosphorylation, is critical for IFN-inducible protection from MHV-1 infection. Additionally, we provide evidence for the contribution of two kinases, the MAP kinase p38MAPK, and protein kinase C (PKC) δ to antiviral protection from MHV-1 infection. Notably, our data suggest that MHV-1 infection, as for the Urbani SARS coronoavirus, inhibits an IFN response, inferred from the lack of activation of *pkr* and *2'5'-oas*, genes associated with mediating the antiviral activities of IFN-αs. To identify potential target genes that are activated downstream of the IFN-inducible signaling effectors we identified, and that mediate protection from coronavirus infection, we examined the gene expression profiles in the peripheral blood mononuclear cells of SARS patients who received IFN treatment. A subset of differentially regulated genes were distinguished with functional properties associated with antimicrobial activities.

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Introduction

In March 2003, a number of laboratories independently reported the isolation and characterization of a novel coronavirus (CoV) from specimens of patients with severe acute respiratory syndrome (SARS) [1, 2]. Although an effective therapeutic strategy against SARS has yet to be developed, interferons (IFN) are recognized to play critical roles in host resistance to viral infection [3-6], thereby implicating them as potential candidates for SARS CoV treatment. IFNs inhibit viral infection by directly interfering with viral replication and by inducing both an innate and adaptive immune response to infection. IFN-αs are effective in the treatment of hepatitis B and C [7, 8] and respiratory coronavirus infections unrelated to the Urbani strain of SARS-CoV [9-12]. IFN-αs and IFN-β [13-17], and combinations of IFN-α/β and IFN-γ [15,18] are effective in inhibiting SARS-CoV replication *in vitro*. Moreover, it was reported that macacque monkeys were protected from infection with SARS CoV by treatment with IFN-α [19].

IFN alfacon-1 (Infergen, Intermune Corp, Brisbane, California), a synthetic IFN-α designed to represent a consensus IFN-α [20], has been shown in both cell culture systems [21] and comparative clinical trials [22] to inhibit viral replication more potently than other IFN-αs. Based on these findings, a pilot study to evaluate the potential clinical benefit and safety of IFN alfacon-1 in SARS treatment was conducted by our laboratory. Patients treated with

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IFN alfacon-1 and corticosteroids had a shorter time to 50% resolution of lung radiographic abnormalities, better oxygen saturation, and resolved their need for supplemental oxygen more rapidly, when compared with a group receiving corticosteroids alone [23].

IFN-αs and IFN-β bind to and activate the Type I IFN receptor, IFNAR [24]. High affinity binding of IFNs to IFNAR induces association of the two receptor chains and subsequent reciprocal transphosphorylation and activation of the associated Janus kinases (Jaks). The Jak-STAT (signal transducer and activator transcription) pathway induces IFN-mediated signal transduction and transcriptional activation, and is required for antiviral responses [25]. In recent years it has become apparent that activation of the IFNR-associated Jaks also regulates multiple downstream signal transducing molecules, such as the p38 mitogen activated protein kinase (MAPK) [26], CrkL [27], phosphatidylinositol 3 kinase [28], the protein kinase C family (PKC) [29], and the Vav proto-oncogene [30]. Such signaling diversity is consistent with the pleiotropic biological effects of the IFNs in target cells and tissues [31].

Mouse Hepatitis Viruses (MHV) are type 2 coronaviruses that typically cause pneumonitis, encephalitis, entercolitis and hepatitis. [32-34]. Phylogenetic data suggest that the SARS-CoV is a distinct group 2 coronavirus [35, 36]. Previous studies have shown that IFNs-α/β provide protection from MHV-A59 and MHV-2 infections *in vivo* [37, 38]. In ongoing experiments, we have evidence that MHV-1, when injected intra-nasally into mice, will induce a lung infection indistinguishable from that of the SARS-CoV infection in humans (G Levy *et al*., UHN, unpublished). Specifically, A/J mice are highly susceptible to intranasal infection with MHV-1, develop pulomonary disease characterized by marked pulmonary congestion, hyaline membranes, interstitial thickening and inflammation and alveolar exudates similar to SARS lung disease in humans. Accordingly, to understand the mechanism of action of IFN alfacon-1 in limiting SARS-CoV infection in humans, we developed an *in vitro* model system, using C3H murine L2 lung fibroblast cells, permissive for MHV-1 infection. In this report we provide evidence for the direct antiviral effects of IFN-α against MHV-1 and identify several signaling effectors which mediate these effects. Additionally, by examining the changes in gene expression profiles in the PBMC of SARS patients who received IFN alfacon-1 treatment, we identify a subset of IFN-responsive genes whose differential expression may influence resolution of disease.

Materials and Methods

Cells, virus and reagents

L2, C3H murine lung fibroblast cells [39] were maintained in DMEM/ 10% heat inactivated fetal calf serum with 100U/ml penicillin, 100mg/ml streptomycin, 4.5 g/L L-glutamine. Murine IFN-α4 was provided by Dirk Gewert (Wellcome Research Lab, Kent, UK). The pharmacological inhibitors SB203580, Rottlerin, Jak inhibitor 1, and AG490 were obtained from Calbiochem. Polyclonal Abs against phosphorylated STAT1 (Tyr701), p38 MAPK (Thr180/Tyr182), PKCδ (Thr505), and Jak1 (Tyr1022/1023) were obtained from Cell Signaling Technology, polyclonal Abs against STAT1-α p91, p38 MAPK, and PKCd were obtained from Santa Cruz Biotechnology, while the polyclonal Ab against Jak1 was from Upstate Biotechnology, and the monoclonal Ab against STAT3 was obtained from Zymed Laboratories. Stock MHV-1 at a titer of 2.9×10^5 PFU/ml was used for all experiments.

28S rRNA degradation assay

10^6 L2 cells were either left untreated or treated with prescribed doses of murine IFN-α4 for 14 h, then infected with MHV-1 for 36 h. Cytoplasmic RNA was isolated using the Qiagen RNeasy mini kit according to the manufacturer's protocol, and then resolved by electrophoresis in a 1% denaturing agarose-formaldehyde gel. Northern blot analysis was performed as previously described [40]. A [γ-^{32}P]ATP-labeled oligonucleotide probe (5'-CTA ATC ATT CGC TTT ACC GG-3'), which specifically binds to nucleotides -1532 to -1551 from the 5' end of murine 28S rRNA, was used for the detection of 28S rRNA and its cleavage products.

Antiviral assays

The assay for IFN-induced antiviral activity in monolayer cells was described as previously [41-43]. Briefly, 10^4 cells were seeded in 96-well tissue culture plates in DMEM containing 2% FCS. After 24 h, appropriate dilutions of IFN-α4 were added and cells were incubated for an additional 24 h. Then, medium containing IFN was aspirated and the appropriate dilution of MHV-1 in a volume of 200μl was added to the cells. After a further 24 h, cells were fixed in 95% ethanol, stained with crystal violet solution (0.1% in 2% ethanol) and destained in 0.5M NaCl, 50% ethanol. IFN-induced inhibition of viral replication was assessed by reading the absorbance at 570 nm using a Microplate reader (Molecular Devices) and SOFTmax® 2.32 software relative to infected, untreated cells.

Cell lysis and Western blot

For immunoblotting, L2 cells were stimulated with IFN-α4 (10^4 U/ml) for the indicated times and lysed in phosphorylation lysis buffer. Immunoprecipitations and immunoblotting using an ECL (enhanced chemiluminescence) method were performed as described previously [45]. In experiments in which pharmacological inhibitors were used, the cells were pre-treated for 60 min with indicated concentrations of the inhibitors and subsequently treated with IFN-α4 prior to lysis in phosphorylation lysis buffer.

RNA extraction, cDNA synthesis and real-time PCR

To harvest RNA for Real Time PCR, 10^6 L2 cells were either left untreated, treated with MHV-1 or IFN-α4 (10^4 U/ml) for the indicated times, or treated with pharmacological inhibitors for 1 h prior to treatment with 10^4 U/ml IFN-α4 for 12 h. Cells were lysed and homogenized using Qiagen QIA-shredder columns and RNA isolation was performed using the Qiagen RNeasy mini kit according to the manufacturer's protocol. cDNA was synthesized using 1 mg RNA in the presence of random primers and AMV Reverse Tanscriptase for 1 h at 42°C (Promega). Reaction components were

obtained from the LightCycler® FastStart DNA Master SYBR Green^{PLUS} I Kit (Roche). The LightCycler® instrument (Roche) and corresponding software were used for all reactions. The PCR was performed in a final volume of 20 μl, 0.5 μM of each primer and 5 μl template cDNA (concentration 100 ng/μl). Primer sets were as follows, *gapdh* forward primer 5'-CCT GCA CCA CCA ACT GCT TA-3' and the reverse primer 5'-TCA TGA GCC CTT CCA CAA TG-3', *pkr* forward 5'-GGC TCC TGT GTG GGA AGT CA-3' and the reverse primer 5'-TAT GCC AAA AGC CAG AGT CCT T-3', *2'5'oas* forward primer 5'-TGA GCG CCC CCC ATC T-3' and the reverse primer 5'-CAT GAC CCA GGA CAT CAA AGG-3'. Standard curves were established for each primer set and both reference and target reactions were performed for each sample.

Affymetrix analysis of ISG expression in PBMC of SARS patients

Serial peripheral blood mononuclear cells (PBMC) from 3 of the 22 SARS patients included in the IFN alfacon-1 pilot study [44] were analyzed for gene expression profiles using the Affymetrix U113A 2.0 plus gene chips. As described in Table 1 and 2, 2 patients received subcutaneous IFN alfacon-1 in addition to the standard of care, corticosteroid treatment. RNA was extracted from PBMC. PBMC were lysed, homogenized using Qiagen QIA-shredder columns and RNA isolation was performed using the Qiagen RNeasy mini kit according to the manufacturer's protocol. To ensure effective inactivation of RNases, lysis reagents contained denaturing guanidine isothiocyanate. A high-salt buffer system allowed RNA to selectively bind the silica-gel membrane of the spin column, while contaminants were washed away. Total cellular RNA was eluted in water. Total RNA was amplified using the Ambion MessageAmp™ II aRNA Amplification Kit. Amplification was performed according to the manufacturer's protocol. Briefly, during the first round of amplification, 10 μl (1000 ng) of total RNA was used, and converted to aRNA. aRNA concentrations were calculated, and 2 μg of amplified RNA (aRNA) was used in the second round of amplification. During second round amplification, biotin labeled NTPs (10mM biotin-11-CTP and 10mM biotin-16-UTP) were incorporated into the cRNA. 30 μg of biotinylated cRNA was sent for Affymetrix gene expression analysis to the Centre for Applied Genomics Microarray Facility at The Hospital for Sick Children, Toronto Ontario. An additional 5 μl (200 ng/μl) was sent for Bioanalyzer analysis.

Gene expression analysis was performed by hybridization of cDNA to the Affymetrix® gene chip - U133A 2.0 plus. This chip codes for over 50 000 probes. The specific effect of IFN alfacon-1 treatment on gene expression was analyzed using the microarray analysis software, Array Assist®. Each gene expression profile was analyzed using GCRMA analysis. This method uses a basic algorithm in order to identify genes whose expression is altered/regulated in the sample array when compared to the basal levels expressed on the Affymetrix® chip. From this list of genes, using the gene expression levels measured on day 6 relative to onset of symptoms, designated as 'basal' for each SARS patient, a new list of genes whose expression was regulated greater than three fold by IFN treatment was obtained.

Table 1 IFN alfacon-1 treatment regimen

Patient	IFN alfacon-1 dose (μg/d)	Start date for IFN alfacon-1	Total days receiving IFN alfacon-1	Total dose IFN alfacon-1 (μg)
Patient 1	0	-	-	-
Patient 2	9	d 6	10	90
Patient 3	9	d 6	11	99

Table 2 Corticosteroid treatment regimen

Patient	Drug	Day of disease	Dose (mg)	Frequency (/h)	Route	Total disease days	Total dose (mg)
Patient 1	0	6 – 11	200	12	oral	5	1000
	0	11-13	50	24	oral	2	100
	0	13-15	20	24	oral	2	40
Patient 2	1	6-10	80	12	intravenous	4	320
	0	10-12	100	12	intravenous	2	200
	0	12-15	50	12	oral	3	150
	0	15-16	40	12	oral	2	80
Patient 3	0	6	100	12	oral	1	100
	1	6-8	500	24	intravenous	2	1000
	1	8-12	250	12	intravenous	4	1000
	1	13-14	50	24	intravenous	2	100
	1	14-15	25	24	intravenous	2	50

Drug 0 –prednisone, Drug 1 - methylprednisolone

Results

The data in panels A and B in Figure 1 show IFN-α4-dependent protection of L2 cells from MHV-1-inducible 28S RNA cleavage and cytopathic effects (CPE). To examine the influence of specific IFN activated signaling effectors in protection from MHV-1 infection, we studied the effects of a number of pharmacological inhibitors targeted against specific signaling molecules. High affinity binding of IFN to IFNAR leads to the phosphorylation-activation of the receptor-associated Jaks, Jak1 and Tyk2. L2 cells treated with the Jak1 inhibitor, Jak inhibitor 1, led to a reduction in IFN-α4 induced tyrosine phosphorylation of Jak1 (Figure 1C). Additionally, we observed a reduction in IFN-inducible tyrosine phosphorylation of STAT1, STAT2 and STAT3 (Figure 1D). When L2 cells were treated with the Jak 1 inhibitor prior to IFN treatment, then infected with MHV-1, we observed a reduction in IFN-inducible protection from MHV-1 infection (Figure 1B). As a negative control, we examined the effect of the inhibitor AG490, which specifically inhibits Jak2 at 100 μM [45]. As anticipated, treating cells with 100 μM AG490 did not influence the anti-MHV-1 activity of IFN-α4 (Figure 1B).

The p38 MAPK pathway is essential for induction of antiviral responses by IFNs against hepatitis C virus [46], vesicular stomatitis virus and encephalomyocarditis virus infections [47, 48]. To examine the importance of IFN-inducible p38 MAPK signaling in protection against MHV-1 infection, L2 cells were treated with SB203580 prior to IFN-α4. 10 μM SB203580 reduced the IFN-α4-induced phosphorylation of p38 MAPK (Figure 1C) and IFN-inducible protection from MHV-1 infection (Figure 1B). In subsequent experiments, using the PKCδ inhibitor Rottlerin, we studied the influence of PKCδ on IFN-inducible anti-MHV-1 activity. 1 μM Rottlerin decreased the IFN-α4 induced tyrosine phosphorylation of PKCδ, (Figure 1C). Additionally, Rottlerin reduced the anti-MHV-1 activity of IFN-α4 (Figure 1B). As expected, inhibition of PKCδ did not decrease the IFN-α4 induced tyrosine phosphorylation of STAT1 (Figure 1D), since inhibition of PKCδ activity blocks Ser727 STAT1 phosphorylation [29].

IFN-inducible control of viral replication is effectively regulated by the transcriptional activation of key interferon stimulated genes (ISG) such as the RNA-dependent protein kinase (PKR) and 2'5'- oligoadenylate synthetase (OAS)/endoribonuclease L (RNase L) [49, 50]. In murine cells, virus-inducible activation of Toll like receptors leads to the transcriptional activation of IFN-β, and IFN-α4 [51, 52]. Therefore, MHV-1 infection per se should induce transcriptional activation of IFNs, and lead to the subsequent transcriptional activation of *pkr* and *2'5'-oas*. Accordingly, L2 cells were infected with MHV-1, and RNA harvested at different time points. The extent of gene expression was analyzed using real time PCR. In time course studies we observed minimal transcriptional activation for both *pkr* and *2'5'-oas* by MHV-1 (Figure 2A). In subsequent experiments we examined the effect of IFN-α4 treatment on their transcriptional activation. In a similar time course study, when L2 cells were treated with 10^4 U/ml IFN-α4, transcriptional activation of both *2'5'-oas* and *pkr* were observed (Figure 2B).

In subsequent experiments we examined the effects of inhibiting IFN-α4 inducible activation of Jak1, p38 MAPK and PKCδ on IFN-α4 inducible transcriptional activation of *pkr* and *2'5'-oas* gene expression. L2 cells were treated with 10 μM Jak inhibitor 1, 10 μM SB203580 or 1 μM Rottlerin, for 1 h, then with 10^4 U/ml IFN-α4 for 12 h. RNA was harvested, and gene expression for *pkr* and *2'5'-oas* examined. This 12 h time point was selected, based on the data from Figure 2B, namely that maximal IFN-inducible transcriptional activation of these genes is attained at 12h. Notably, inhibition of IFN-inducible activation of Jak1, p38 MAPK or PKCδ effectively reduced the IFN-inducible gene expression for *pkr* and *2'5'-oas* (Figure 2C).

In the absence of any clear cause and effect relationship between *pkr* and *2'5'-oas* gene expression and IFN-inducible anti-MHV-1 activity, we subsequently examined the signature patterns of gene expression in the PBMC from 2 of the SARS study patients we had treated with IFN alfacon-1, during the Toronto 2003 outbreak [44]. For comparison we measured gene expression in the PBMC of a SARS patient that was treated with corticosteroids alone (see Materials and Methods). PBMC were sampled on days 6, 8, 10 and 13 post-onset of symptoms. For the IFN-treated SARS patients, the day 6 PBMC sample represented a baseline in the absence of IFN treatment, whereas days 8, 10 and 13 corresponded to days 2, 4 and 7 relative to the start of IFN treatment. The data in Table 3 reveal 2 lists of genes expressed differentially between the 2 IFN-treated SARS patients and the 1 non-IFN treated patient, using a cut-off of $\geq$ (A) or $\leq$ (B) 3-fold change in expression, relative to basal expression levels on day 6 post-onset of symptoms. In Figure 3, differentially expressed genes are clustered to indicate high and low gene expression, using a red-green colour scale to show the relative range. Notably, our analyses identified 11 genes that were upregulated and 21 genes that were downregulated in the IFN-treated patients, according to these criteria. Upregulated genes included interleukin 8 (IL-8) and defensins alpha 1 and alpha 4. IL-8 is a chemoattractant for neutrophils, induces neutrophil degranulation and enhances defensin release from neutrophils. Defensins are potent antimicrobial peptides that have been shown to exhibit antiviral activity against enveloped viruses and HIV [53, 54]. Downregulated genes

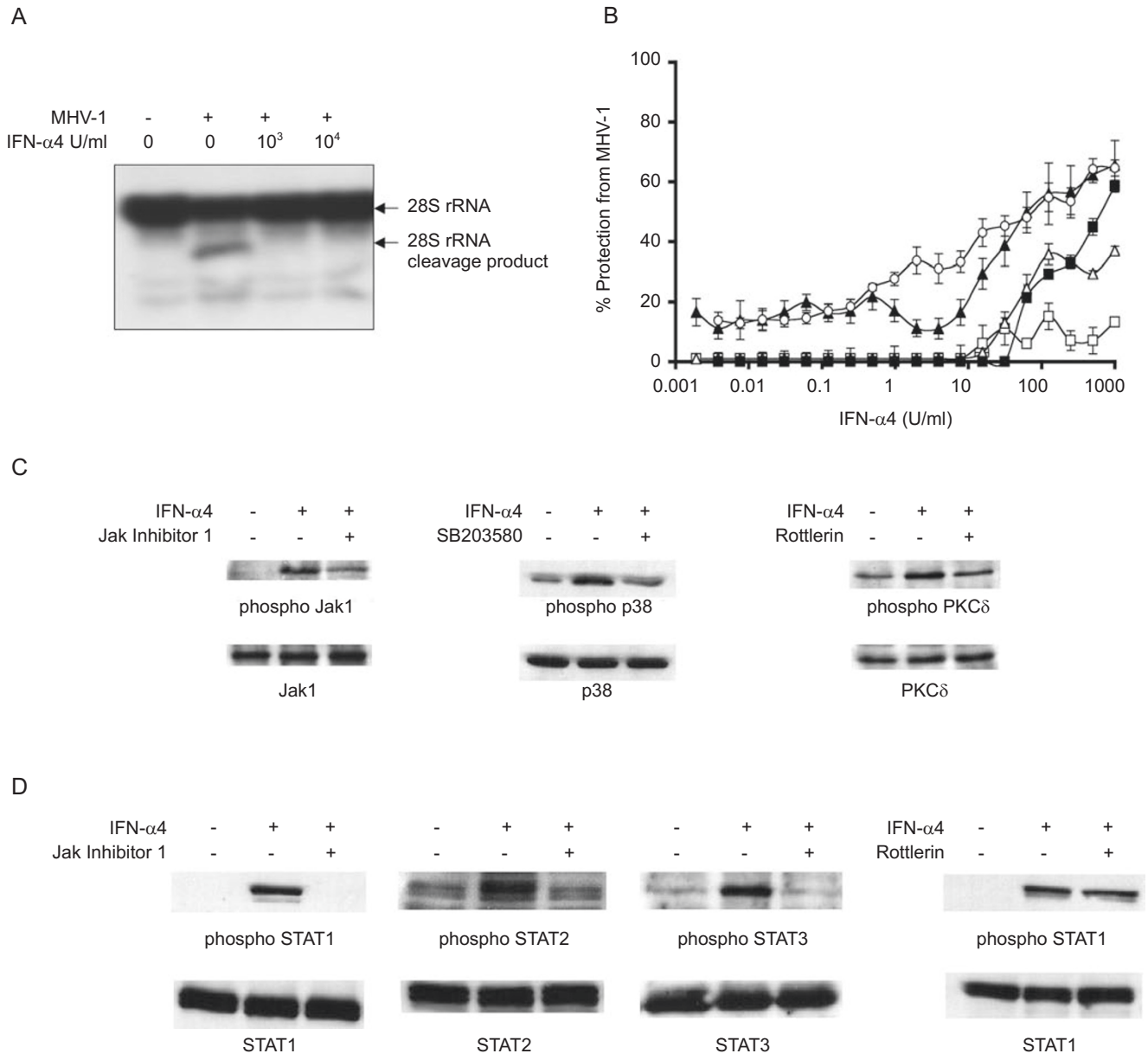

Figure 1 The pharmacological inhibitors: Jak inhibitor 1, Rottlerin and SB203580 inhibit IFN-inducible anti-MHV-1 activity. **(A)** 10^6 L2 cells were either left untreated, or treated with the indicated doses of IFN-α4 for 14 h, then challenged with MHV-1 for 36 h. Cells were lysed, RNA extracted and resolved by electrophoresis in agarose and Northern analyses of 28S RNA and cleavage products performed. Data are representative of 2 independent experiments. **(B)** 10^4 L2 cells were either left untreated (▲) or treated with 10μM Jak inhibitor 1(■), 1μM Rottlerin (❑), 10μM SB203580 (△), or 100μM AG490 (O) for 1 h. Cells were then incubated, in triplicate, with the indicated dose of IFN-α4 for 16 h, then challenged with MHV-1. After 24 h CPE were quantitated using a colorimetric assay (Materials and Methods). Data are expressed as percent protection from CPE. Mean values ± SE of three independent experiments are shown. **(C)** 10^7 L2 cells were either left untreated, or treated with 10μM Jak Inhibitor 1, 10 μM SB203580 or 1μM Rottlerin for 1 h prior to a 30 min IFN-α4 (10^4 U/ml) treatment. Cells were lysed and equal amounts of whole cell lysates were resolved by SDS-PAGE and examined for Jak1, p38 MAPK or PKCδ phosphorylation by immunoblotting with anti-phospho-Jak1,-p38 MAPK or -PKCδ antibodies. Membranes were stripped and re-probed with anti-Jak1, anti-p38 MAPK, or anti-PKCδ antibodies to control for loading. Blots shown are representative of 3 independent experiments. **(D)** 10^7 L2 cells were either left untreated, or treated with 10 μM Jak Inhibitor 1 or 1 μM Rottlerin for 1 h prior to a 30 min IFN-α4 (10^4 U/ml) treatment. Cells were lysed and equal amounts of whole cell lysates were resolved by SDS-PAGE and examined for STAT tyrosine phosphorylation by immunoblotting with anti-phospho-STAT antibodies. Membranes were stripped and re-probed with anti-STAT antibodies to control for loading. Blots shown are representative of 3 independent experiments.

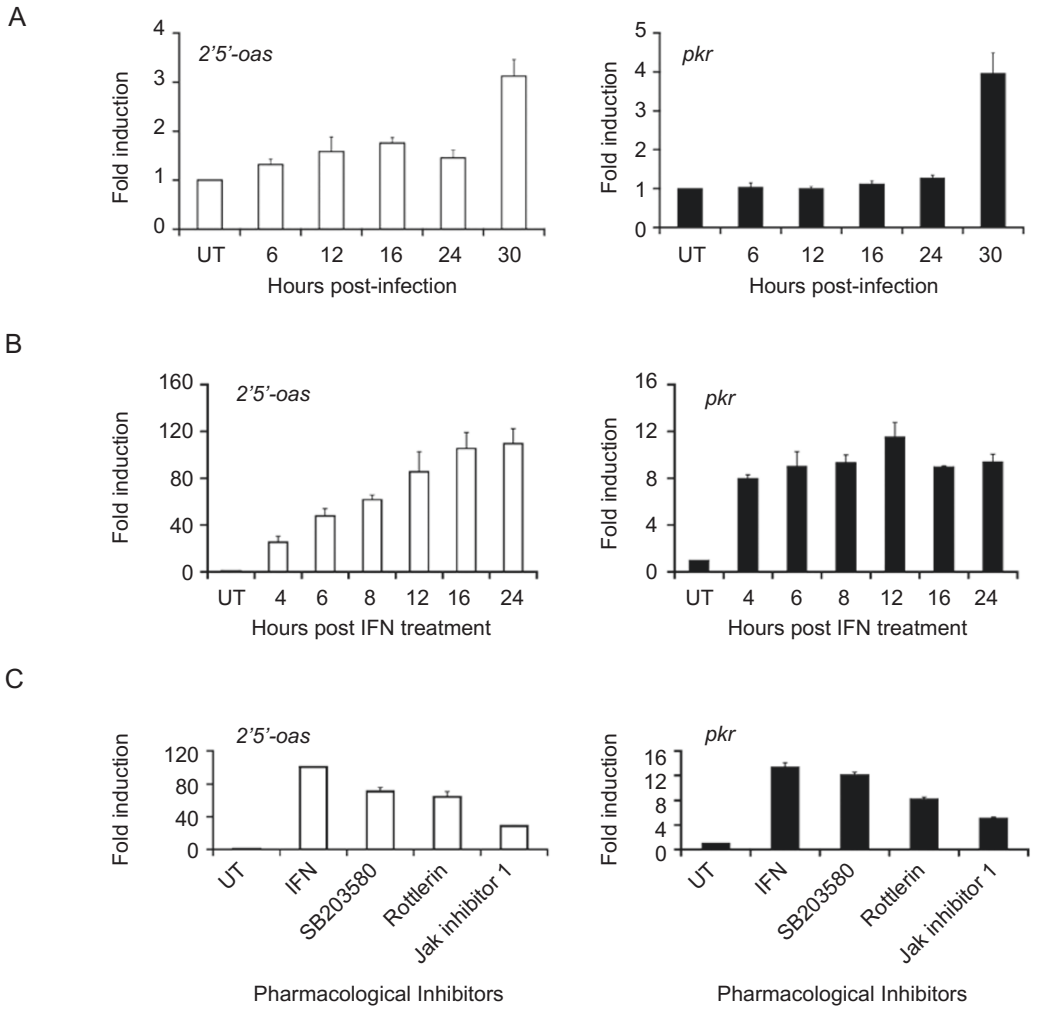

Figure 2 MHV-1 infection leads to negligible transcriptional activation of *2'5'-oas* and *pkr*. 10^6 L2 cells were either left untreated (UT), infected with MHV-1 **(A)**, treated with 10^4U/ml IFN-α4 for the times indicated **(B)**, or treated with 10 μM SB203580, 1 μM Rottlerin or 10μM Jak 1 inhibitor for 1h then 10^4U/ml IFN-α4 for 12h **(C)**. RNA was extracted, cDNA synthesized and quantitative real-time PCR analyses performed for reference *GAPDH* and target genes *2'5'-oas* and *pkr* expression. Data are presented as the fold-change in expression compared to untreated L2 cells. Values ± SE were calculated using Relative Quantification software (Roche) and are the mean of three separate reactions, each performed in triplicate.

included protein-kinase repressor (PRKRIR), which acts to prevent PKR synthesis [55]. Expression for exportin 1 (CRM1) was also downregulated in the PBMC of the IFN-treated SARS patients. CRM1 is a transport receptor that mediates nuclear export of proteins. CRM1 interacts with the Rev protein of HIV [56], suggestive of a potential for inhibition of nuclear activities associated with viral replication. Additionally, the expression of genes encoding RNA binding proteins and helicases including ELAV1, RNU22, DDX1 and DHX15 was also downregulated, in further support of inhibitory effects on viral replication intermediates. Interestingly, DDX1, a DEAD box protein, has been implicated in facilitating HIV replication[57, 58]. Viewed altogether, the implications are that IFN alfacon-1 treatment affected gene expression in the PBMC of SARS patients to promote direct antiviral effects and inhibit viral replication.

Discussion

Using murine IFN-α4 and an *in vitro* model for SARS, namely MHV-1 infection of murine lung fibroblast cells, we investigated the influence of various signaling molecules during IFN-α4 mediated antiviral protection. Our findings suggest that Jak1, PKCδ, and p38 MAPK play important roles in IFN-α-mediated anti-MHV-1 activity.

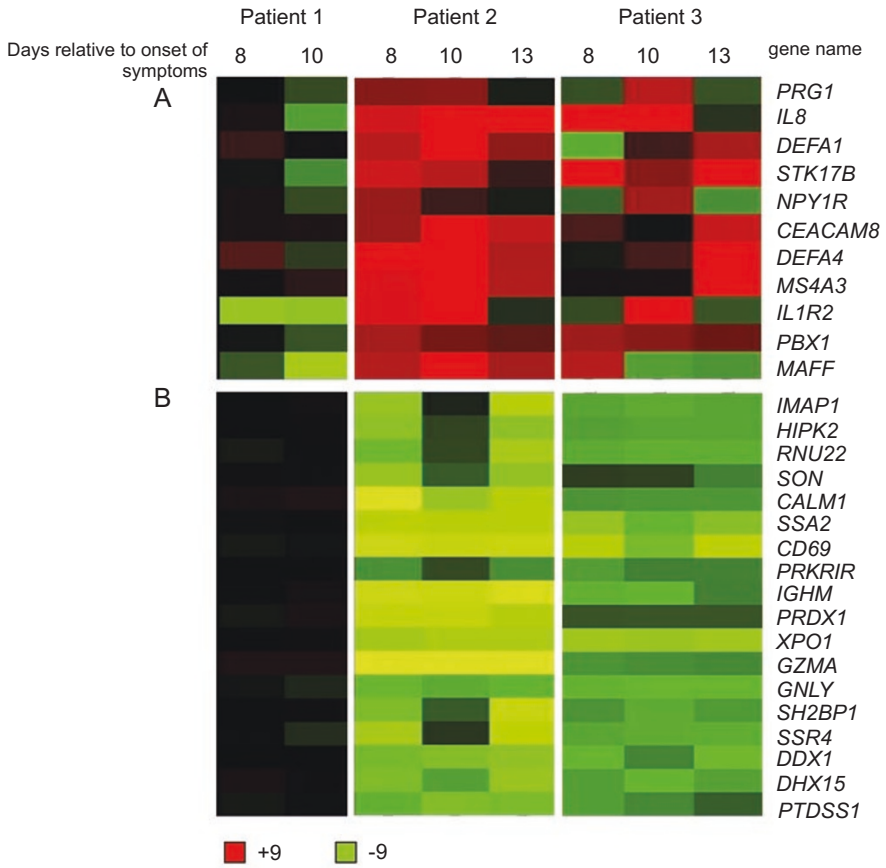

Figure 3 Heat map of genes regulated by IFN alfacon-1 in PBMC of SARS patients. The relative expression levels of the genes identified in Table 3 are described. Upregulated genes **(A)** are shown in red and downregulated genes **(B)** are shown in green. Maximum intensity values +/- 9 are shown. Patient 1, SARS patient treated with corticosteroids alone; Patients 2 and 3, SARS patients treated with IFN alfacon-1 (as per Tables 1 and 2).

At the lower doses of IFN-α4, pre-treating the cells with Jak 1 inhibitor completely abrogated the protective effects of IFN-α4. Since Jak1 activation is an early post-receptor activation event, it is not surprising that abrogating this upstream effector in the IFN-receptor signaling cascade has a profound effect on subsequent downstream signaling events and biological responses. By contrast, at the higher doses of IFN-α4, inhibition of PKCδ inhibited IFN-induced antiviral activity to a greater extent, implying that STAT1-sensitive ISG activation is necessary for IFN-inducible anti-MHV-1 activity. Notably, IFN-α4-induced antiviral activity is unlikely to be exclusively attributable to the activities of Jak1, p38 MAPK, and PKCδ.

Further, we demonstrate that L2 cells treated with IFN-α4 induce the transcriptional activation of *pkr* and *2'5'-oas*, in stark contrast to the negligible activation observed when cells were infected with MHV-1. We infer from these data that MHV-1 infection per se may prevent the activation of the IFN system. Indeed, many different viruses evade an immune response by interfering with transcriptional activation of IFN-α/β genes [59]. Notably, in order to escape activation of the IFN system, the SARS-CoV appears to block a step after the early nuclear transport of IRF-3, the transcription factor essential for IFN-β and IFN-α4 promoter activity [60].

Inhibition of IFN-induced activation of Jak1, PKCδ and p38 MAPK was shown to also influence the transcriptional activation of *pkr* and *2'5'-oas*. The anti-MHV-1 effects of IFN-α4 are unlikely to be wholly attributable to the inhibitory activities of PKR and 2'5'OAS. Indeed, many other

Table 3 note:
Genes whose expression was upregulated (A) or downregulated (B) by ≥ 3-fold following IFN alfacon-1 treatment are indicated.
Values denote expression levels relative to the basal gene expression (1) for each gene for that patient on day 6 post-onset of symptoms.
Day in () is relative to start of IFN treatment.

Table 3 Genes regulated by IFN-alfacon-1 in PBMC of SARS patients.

Gene name	Gene symbol	Patient 1		Patient 2			Patient 3		
		d 8	d 10	d 8(2)	d 10 (4)	d 13 (7)	d 8 (2)	d 10 (4)	d 13 (7)
A									
defensin, alpha 4, corticostatin	*DEFA4*	1.974	0.633	0.888	1.749	16.925	8.571	23.211	4.559
membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	*MS4A3*	1.045	1.347	1.096	1.116	8.299	7.151	34.289	4.699
interleukin 1 receptor, type II	*IL1R2*	0.121	0.135	0.560	9.913	0.505	7.033	11.503	0.720
interleukin 8	*IL8*	1.122	0.251	10.544	9.135	0.681	6.368	31.185	23.271
serine/threonine kinase 17b	*STK17B*	0.941	0.306	0.438	3.996	0.291	6.121	4.978	1.544
defensin, alpha 1, myeloid-related sequence	*DEFA1*	1.574	1.043	0.218	1.669	4.226	4.945	29.850	3.366
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	*MAFF*	0.503	0.085	4.885	0.250	0.279	4.819	21.063	4.149
pre-B-cell leukemia transcription factor 1	*PBX1*	0.930	0.521	3.849	3.085	2.388	4.103	2.608	2.170
carcinoembryonic antigen-related cell adhesion molecule 8	*CEACAM8*	1.107	1.143	1.851	0.952	5.775	3.664	54.562	5.462
neuropeptide Y receptor Y1	*NPY1R*	1.112	0.564	44.139	3.039	20.327	3.600	1.645	0.884
proteoglycan 1, secretory granule	*PRG1*	1.002	0.556	0.528	4.993	0.521	3.041	3.228	0.868
B									
CD69 antigen	*CD69*	1.178	1.195	0.061	0.174	0.054	0.055	0.060	0.060
homeodomain interacting protein kinase 2	*HIPK2*	1.125	1.019	0.244	0.238	0.235	0.129	0.809	0.061
SH2 domain binding protein 1	*SH2BP1*	1.108	1.189	0.280	0.221	0.257	0.192	0.218	0.202
ADP-ribosylation factor-like 7	*ARL7*	1.050	1.017	0.178	0.222	0.175	0.032	0.256	0.030
SON DNA binding protein	*SON*	1.045	1.031	0.486	0.560	0.560	0.192	0.235	0.223
CD2 antigen (p50), sheep red blood cell receptor	*CD2*	1.043	1.038	0.580	0.281	0.300	0.098	0.078	0.081
phosphatidylserine synthase 1	*PTDSS1*	1.017	1.029	0.247	0.307	0.467	0.163	0.480	0.041
calmodulin 1	*CALM1*	1.012	1.023	0.278	0.266	0.271	0.126	0.479	0.135
signal sequence receptor, delta	*SSR4*	1.010	1.045	0.231	0.218	0.216	0.163	0.246	0.123
poly (ADP-ribose) polymerase family, member 14	*PARP14*	1.007	0.988	0.162	0.396	0.243	0.283	0.473	0.226
pericentrin 2 (kendrin)	*PCNT2*	1.006	1.035	0.232	0.208	0.230	0.258	0.340	0.409
DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	*DDX1*	1.002	1.023	0.200	0.320	0.185	0.177	0.154	0.137
peroxiredoxin 1	*PRDX1*	1.001	1.014	0.513	0.502	0.506	0.047	0.047	0.064
immunoglobulin heavy constant mu	*IGHM*	1.000	1.022	0.209	0.203	0.336	0.040	0.044	0.042
RNA, U22 small nucleolar	*RNU22*	0.995	1.011	0.225	0.213	0.215	0.139	0.590	0.141
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Huantigen R)	*ELAVL1*	0.993	1.052	0.251	0.308	0.125	0.173	0.228	0.141
exportin 1 (CRM1 homolog, yeast)	*XPO1*	0.986	1.066	0.107	0.121	0.102	0.040	0.041	0.029
granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	*GZMA*	0.984	0.995	0.274	0.291	0.305	0.025	0.025	0.026
Sjogren syndrome antigen A2 (60kDa, ribonucleoprotein autoantigen SS-A/Ro)	*SSA2*	0.958	0.855	0.125	0.199	0.148	0.026	0.120	0.061
protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (P58 repressor)	*PRKRIR*	0.936	1.107	0.253	0.334	0.328	0.284	0.567	0.293
immunity associated protein 1	*IMAP1*	0.936	0.975	0.236	0.222	0.233	0.207	0.160	0.171
granulysin	*GNLY*	0.933	0.965	0.205	0.197	0.194	0.094	0.077	0.077
DEAH (Asp-Glu-Ala-His) box polypeptide 15	*DHX15*	0.926	0.987	0.248	0.198	0.241	0.087	0.659	0.052
nucleoporin 205 kDa	*NUP205*	0.904	0.916	0.184	0.179	0.182	0.293	0.273	0.298

IFN-inducible genes have been identified which encode factors implicated in IFN-induced antiviral responses [50, 61-63]. Interestingly, when we analyzed the signature patterns of gene expression in the PBMC of 2 SARS patients that received IFN alfacon-1, compared with a patient that did not, we observed a trend of differential gene expression in the IFN-treated patients that was suggestive of antimicrobial activities. In ongoing studies we are investigating the contribution of their specific gene products to antiviral activity.

In conclusion, our data suggest that activation of Jak1, p38 MAPK and PKCδ are involved in the generation of IFN-α-mediated anti-MHV-1 activity, and in the control of IFN-induced transcriptional activation of, minimally, *pkr* and *2'5'-oas*. The implications are that IFN-inducible activation of specific signaling molecules leads to the transcriptional regulation of a subset of ISGs that determine the direct antiviral activity of IFN-α against coronavirus infections.

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