Treatment with interferon-α2b and ribavirin improves outcome in MERS-CoV–infected rhesus macaques

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The emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) is of global concern: the virus has caused severe respiratory illness, with 111 confirmed cases and 52 deaths1 at the time of this article’s publication. Therapeutic interventions have not been evaluated in vivo; thus, patient management relies exclusively on supportive care, which, given the high case-fatality rate, is not highly effective. The rhesus macaque is the only known model organism for MERS-CoV infection, developing an acute localized to widespread pneumonia with transient clinical illness2,3 that recapitulates mild to moderate human MERS-CoV cases4,5. The combination of interferon-α2b and ribavirin was effective in reducing MERS-CoV replication in vitro6; therefore, we initiated this treatment 8 h after inoculation of rhesus macaques. In contrast to untreated, infected macaques, treated animals did not develop breathing abnormalities and showed no or very mild radiographic evidence of pneumonia. Moreover, treated animals showed lower levels of systemic (serum) and local (lung) proinflammatory markers, in addition to fewer viral genome copies, distinct gene expression and less severe histopathological changes in the lungs. Taken together, these data suggest that treatment of MERS-CoV infected rhesus macaques with IFN-α2b and ribavirin reduces virus replication, moderates the host response and improves clinical outcome. As these two drugs are already used in combination in the clinic for other infections, IFN-α2b and ribavirin should be considered for the management of MERS-CoV cases.

MERS-CoV is the first lineage-C betacoronavirus known to infect humans2. Similar to SARS (severe acute respiratory syndrome) coronavirus infection, MERS-CoV infection can result in an acute respiratory distress syndrome with multiorgan dysfunction3. The rapid identification of therapeutics for MERS-CoV is a high priority as there is currently no specific therapy or vaccine. A meta-analysis of data from the SARS outbreak in 20039 found that the benefit of different treatment strategies10–18 was inconclusive at best9. We recently demonstrated in vitro that the antiviral effect of interferon-α2b (IFN-α2b) against MERS-CoV is augmented by the concomitant use of ribavirin, and when we combined the treatments, the effective concentrations were such that they could be used in the clinic6. Here we evaluate the effectiveness in vivo of combination IFN-α2b and ribavirin treatment, in an attempt to identify a therapeutic approach that can be immediately implemented in the clinic for MERS-CoV cases.

Two groups of three rhesus macaques were infected with 7 × 10⁶ TCID₅₀ (50% tissue culture infectious dose), of MERS-CoV (hCoV-EMC/2012, a clinical isolate), as previously described2. We initiated treatment 8 h after infection, with subcutaneous delivery of 5 mg international units (MIU)/kg of IFN-α2b and an intravenous loading dose of ribavirin (30 mg per kg body weight). We subsequently treated the rhesus macaques with ribavirin intramuscularly (10 mg per kg body weight) every 8 h and IFN-α2b (5 MIU/kg) subcutaneously every 16 h until 72 h after infection, when all animals were euthanized (Fig. 1a) at the peak of clinical signs and viral loads in this model2,3. Infection with MERS-CoV in the absence of treatment led to similar clinical signs as previously reported, including increased respiration rates, abdominal breathing and piloerection2,3 (Fig. 1b and Supplementary Table 1). The animals’ observed breathing difficulties were supported by evidence of decreased oxygen saturation in untreated animals (2–5% decrease from baseline) (Fig. 1c). We observed significantly increased white blood cell counts in untreated animals 1 d after infection, but they returned to normal levels on subsequent days (Fig. 1d). We attributed the increase in white blood cells mainly to a significant increase in neutrophils (Fig. 1e). In contrast, treated animals did not show evidence of increased respiration, piloerection or decreased oxygen saturation levels, nor did they show significant changes in hematology (Fig. 1d,e). To monitor signs of pneumonia, we X-rayed the animals daily. Radiographs from treated animals remained normal 1 d after infection and showed, at most, light

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infiltration in a single lobe 2 or 3 d after infection in one or two of three animals, respectively (Supplementary Table 2). In contrast, untreated animals showed evidence of increasing amounts of interstitial infiltration between 1 and 3 d after infection, ranging from localized (single lobe) to diffuse severe pulmonary interstitial infiltration throughout the lungs (Fig. 2 and Supplementary Table 2).

Upon necropsy, gross pathology revealed lungs that were normal in appearance in all treated animals (Fig. 3a). In contrast, lungs from untreated animals were firm and edematous, failed to collapse (Fig. 3a) and contained gross lesions with a mean affected area of 14.3% (Fig. 3e) and represented 14.3% (Fig. 3e) and contained gross lesions with a mean affected area of 14.3% (Fig. 3e) and contained gross lesions with a mean affected area of 14.3% (Fig. 3e) and contained gross lesions with a mean affected area of 14.3% (Fig. 3e). Multifocal consolidation and dark red discoloration, consistent with interstitial pneumonia, was most prevalent on the dorsal surface of the lungs of untreated animals. We did not observe any other pathological changes at necropsy.

We used quantitative real-time RT-PCR (qRT-PCR) to determine viral loads as TCID$_{50}$ equivalents in tissues, fluids and nasal and throat swabs. There was no detectable viral RNA in the blood at any time point or in urine taken at necropsy. Daily bronchial alveolar lavage samples showed similar viral loads between treated and untreated groups (Supplementary Fig. 1). With the exception of the oronasopharynx in treated animals, all respiratory tract tissues collected at necropsy were positive for MERS-CoV by qRT-PCR (Fig. 3b). Viral RNA was also absent from the tonsils, spleen and kidney of treated animals, but it was present in these tissues in two of three untreated animals. In all of the tissues analyzed, with the exception of left middle lobe lung specimens, the viral loads were lower in specimens from treated animals as compared to untreated animals (Fig. 3b). When we combined the values from all lung specimens, the mean viral load in treated animals was 0.81 log lower than in untreated animals ($P = 0.0428$, unpaired $t$-test, one tail), demonstrating a statistically significant reduction in virus replication throughout the lung (Fig. 3b, inset).

All animals developed some degree of pulmonary pathology with mild to marked multifocal bronchointerstitial pneumonia, predominantly centered on the terminal bronchioles (Fig. 3c,d). However, untreated rhesus macaques 4 and 5 showed moderate to marked lesions with abundant alveolar edema, fibrin with formation of hyaline membranes and low to moderate numbers of macrophages (Fig. 3d), a lesion severity not observed in the treated animals (Fig. 3c and Supplementary Table 3). Only untreated animals showed significant scoring for perivascular infiltrates within and adjacent to affected areas of the lung (Fig. 3d) ($P < 0.001$, Mann-Whitney). We observed higher numbers of neutrophils in lungs of untreated animals compared to treated ones (9.3% compared to 6.1%, respectively) (Fig. 3e).
We monitored serum IFN-α levels to assess the delivery of IFN-α2b. Treated animals had at least 2.3 times the level of IFN-α measured in untreated animals (1 and 3 d after infection), whereas 2 d after infection, treated animals had 37 times the level of untreated animals (Supplementary Fig. 2), which probably reflects the timing of IFN-α2b administration in relation to blood collection. Untreated animals showed evidence of an IFN response 1 and 2 d after infection, with means of 190 and 103 pg/ml, respectively; however, IFN-α was undetectable by 3 d after infection. Treated animals did not show alterations in systemic (serum) interleukin-2 (IL-2), monocyte chemotactic protein-1 (MCP-1), IL-1 receptor antagonist (IL-1RA), IL-6, IL-15 and IFN-γ levels compared to those observed in untreated animals (Supplementary Fig. 3). We observed elevated local levels of IL-6, IFN-γ and MCP-1 in homogenate from lung tissue collected 3 d after infection of untreated animals compared to treated animals (Supplementary Fig. 3), which indicates a tissue-specific host response to infection that is moderated by treatment.

We analyzed the effect of treatment on the lung transcriptome by microarray of RNA extracted from the right lower lobe (RLL). We performed a singular value decomposition–coupled multidimensional scaling (SVD-MDS) analysis, providing an overall view of the transcriptome, on samples from infected and uninfected animals in addition to control lung tissue samples from naive rhesus macaques (Fig. 4a). Comparison of group means revealed a clear separation between the treated and untreated lung RLL samples; treated and untreated samples were also separated from naive control samples, indicating that treatment induces unique transcriptional changes. Using a relaxed P value cutoff ($P < 0.05$), we observed 966 differentially expressed genes (DEGs) when treated, infected and untreated, infected animals were compared to naive animals (Supplementary Table 4), many associated with innate antiviral processes induced by IFN-α2b and ribavirin$^{20,21}$. These included genes encoding type 1 and 3 IFN, pattern recognition receptors and IFN-induced with helicase C domain 1 (IFIH1, or MDA5), effector molecules and associated signal transducers. Comparison of uninfected, untreated animals to infected, treated and infected, untreated animals revealed lower expression of inflammatory genes in treated animals suggestive of milder disease (Supplementary Fig. 5).

To elucidate the mechanism by which treatment reduces inflammation and disease during MERS-CoV infection, we sought to identify DEGs that are not associated with known IFN-stimulated gene induction programs. By examining the 205 DEGs identified using a more stringent P value cutoff ($P < 0.01$) (Fig. 4b and Supplementary Table 5) and Ingenuity Pathway Analysis, we identified many genes that were downregulated in untreated animals. Many of these were associated with hedgehog signaling (Fig. 4c), a pathway involved in respiratory morphogenesis and vascularization$^{22-27}$ and potentially involved in inflammatory injury$^{28}$. In treated animals, we observed upregulation of sonic hedgehog (SHH) and subsequent downregulation of genes associated with cell proliferation (Supplementary Table 5). SHH, the priming protein of the hedgehog pathway, and the associated molecule GLI family zinc finger 1 have been shown to block injurious responses in the pulmonary vasculature induced by multiple types of inflammatory and stress stimuli$^{29-32}$. Consistent with the reduced inflammation, reduction in cellular infiltrates and lack of perivasculitis in the lungs of treated animals, the upregulation of SHH suggests that the hedgehog pathway may regulate the host response to protect the lung from potentially injurious inflammation.

Rhesus macaques treated with IFN-α2b and ribavirin 8 h after MERS-CoV infection showed improved clinical parameters with no
Methods

Methods and any associated references are available in the online version of the paper.
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ONLINE METHODS

Biosafety statement. All infectious work with MERS-CoV was approved by the Rocky Mountain Laboratories (RML) Institutional Biosafety Committee (IBC) and performed in a high-containment facility at the RML, Division of Intramural Research (DIR), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). IBC-approved standard operating procedures were applied for all infectious work.

Virus and cells. Vero cells (African green monkey kidney) were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (FBS), 50 U/ml penicillin and 50 µg/ml of streptomycin. MERS-CoV (isolate hCoV-EMC/2012)8 was subsequently propagated on Vero cells using DMEM as above with 2% FBS (complete DMEM).

Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Rocky Mountain Laboratories and performed following the NIH Guide for the Care and Use of Laboratory Animals and the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) by certified staff in an AAALAC-approved facility. We inoculated six healthy rhesus macaques (Macaca mulatta), aged 4–6 years, with a total of 7 × 106 TCID50 of MERS-CoV by combined intratracheal, intranasal, oral and ocular routes as previously established12,35. Animals were randomly assigned to either the treated (two males, one female) or untreated (two females, one male) group in a nonblinded manner.

Treatment. Treatment was initiated at 8 h after infection. The initial ribavirin (MP Bioscience) loading dose (30 mg per kg body weight) was delivered intravenously; subsequent doses (10 mg per kg body weight) were delivered every 8 h intramuscularly. Human interferon-α2b, as Intron A (Merck) (5 MIU/kg) was delivered subcutaneously every 16 h. Three animals received IFN-α2b and ribavirin (treated), whereas three animals received sham treatment (untreated) by the same routes with a comparable volume per kg body weight of sterile saline. The animals were monitored three times daily through clinical scoring and/or examinations as described previously35,36. Clinical examinations included daily ventral-dorsal and lateral X-rays, evaluations of body temperature, blood pressure, heart rate and respiration rate, pulse oximetry, venous bleeding, bronchoalveolar lavage and collection of swabs from nasal and oral mucosa. A board-certified veterinarian interpreted the radiographic data. On day 3 after infection, all animals were necropsied, and conjunctiva, nasal mucosa, tonsil, oronasopharynx, mandibular lymph node, salivary gland, trachea, right bronchus, left bronchus, all six lung lobes, mediastinal lymph node, inguinal lymph node, axillary lymph node, mesenteric lymph node heart, liver, spleen, kidney and urinary bladder were collected for virological and histopathological assays.

Hematology assays. Hematological analysis was carried out following standard protocols. The total white blood cell, lymphocyte, platelet, reticulocyte and red blood cell counts, hemoglobin and hematocrit values, mean cell volume, mean corpuscular volume, and mean corpuscular hemoglobin concentrations were determined from EDTA blood with the HemaVet 950FS+ laser-based hematology analyzer (Drew Scientific).

Serum and lung homogenate cytokine and chemokine analysis. Samples from all lung lobes were weighed and subsequently homogenized in 400 µl of Biotect PCR buffer (BioRad) with a stainless steel bead on the Qiashredder (Qiagen). Debris were pelleted and the supernatant removed. Both serum and tissue samples were inactivated with 5 MRads γ-radiation according to standard operating procedures. The total protein concentration in the tissue homogenate was determined with the DC Protein Assay (BioRad) and used to normalize the input. Concentrations of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23 (p40), IL-13, IL-15, IL-17, MCP-1 and macrophage inflammatory protein-1α (MIP-1α), MIP-1β, soluble CD40 ligand (sCD40L), transforming growth factor-α (TGF-α), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF) and IL-18 were measured on a Bio-Plex 200 instrument (Bio-Rad) using the Non-Human Primate Cytokine MILLIXPLEX map 23-plex kit (Millipore) according to the manufacturer’s instructions. Levels of IFN-α in the serum were determined with the VeriKine human IFN-α ELISA kit (PBL, Piscataway, NJ) according to the manufacturer’s instructions.

Histopathology. Tissues were placed in cassettes and fixed in 10% neutral buffered formalin for 7 d. Subsequently, tissues were processed with a Sakura VIP-5 Tissue Tek, on a 12-h automated schedule, using a graded series of ethanol, xylene and ParaPlast Extra. Embedded tissues were sectioned at 5 µm and dried overnight at 42 °C before staining. Tissue sections were stained with H&E. Specific anti-CoV immunoreactivity was detected using a polyclonal rabbit antibody generated against hCoV-EMC/2012 at a 1:1,000 dilution. Tissues were also stained with a myeloperoxidase-specific antibody (1:450 dilution; 760–159, R&D Systems, Minneapolis, MN). The tissues were then processed for immunohistochemistry using the Discovery XT automated processor (Ventana Medical Systems) with a DAPMap (Ventana Medical Systems) kit, scanned with the Aperio ScanScope XT (Aperio Technologies, Inc.) and the entire section analyzed with the ImageScope Positive Pixel Count algorithm (version 9.1).

Genome quantification and microarray sample preparation. RNA from AVL-treated blood and RLT-treated tissues were extracted with the QiaAmp Viral RNA and the RNeasy kits, respectively (Qiagen). qRT-PCR using primers and probe previously described19 were performed on the RotorGene Q (Qiagen). A tenfold dilution series of viral RNA based on TCID50 equivalents was used as a standard. For microarray analysis, lung homogenates were stored at ~80 °C in equal volumes RLT buffer (Qiagen) and 70% ethanol until preparation. Samples were thawed, and two additional volumes of RLT buffer with 0.01 volumes of 2-mercaptoethanol were added, followed by an additional two volumes of 70% ethanol. RNA was then extracted using RNeasy spin columns per the manufacturer’s protocol. Low-yield samples were concentrated using the RNA Clean and Concentrator (Zymo Research). Probe labeling was carried out using the Agilent Low Input processing protocol, and probes were hybridized to Agilent Rhesus macaque 4×44K microarrays (Agilent Technologies) using the manufacturer’s one-color analysis protocol.

Microarray data and functional analysis. For comparisons of differentially expressed genes (DEGs) in infected lungs of both IFN-α2b– and ribavirin-treated and untreated animals, raw array data were uploaded to Genedata Analyst 7.6 (Genedata). Data were normalized using the quantile normalization method, and the log2 ratio expression was calculated relative to the mean probe values of the three untreated animals. Statistically significant DEGs were determined using Welch’s t-test (P < 0.01, fold change ≥ 1.5). Hierarchical clustering of DEGs was performed by the unweighted average method (unweighted pair group with arithmetic mean; UPGMA) using Spotfire DecisionSite 9.1.1 (Tibco). Analysis of functional enrichment was performed using Ingenuity Pathway Analysis software (Ingenuity Systems).

For measures of gene expression dynamics, the array data were normalized using NeONORM36 and subjected to SVD-MS analysis37. In parallel, the data for each individual were merged using weighted averages to generate combined profiles. These were visualized using SVD-MS.

Statistical analyses. With the exception of the data from the microarray experiment, data were analyzed in Prism (GraphPad Software) using the unpaired t-test, two-way ANOVA with Bonferroni’s post hoc test and the Mann-Whitney test as indicated in the figure legends.

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