Human and Mouse Eosinophils Have Antiviral Activity Against Parainfluenza Virus

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ONLINE DATA SUPPLEMENT

Supplemental Methods

Virus Propagation

Parainfluenza virus-type 1 (Sendai, ATCC, Manassas, VA) was propagated in rhesus monkey kidney cells (RMKs; Viromed, Minneapolis, MN) as previously described (1). Briefly, RMK cell monolayers were infected with parainfluenza and grown for 1 week at 34^0 C/5% CO_2 and harvested following freeze-thaw fractionation. Virus stocks were clarified by centrifugation at 10,000xg for 10 minutes. For *in vitro* experiments, stocks were further purified by centrifugation at 65,000xg at 4^0C for 90 minutes over a discontinuous 15%/60% (w/v) sucrose gradient. Virus was collected from the 15%/60% sucrose interface, sedimented by centrifugation at 40,000xg for 45 minutes, and resuspended in RPMI1640 media (Life Technologies, Grand Island, NY).

Virus Titer Determination by Hemadsorption Assay

Virus stocks were titered by hemadsorption assay using RMK cell monolayers as previously described (2). Briefly, confluent monolayers of RMK cells were infected with serial 10-fold dilutions of parainfluenza-containing culture supernatants in triplicate. After incubating for 7 days, infectious titers was quantified per the Reed and Muench method (2). One $TCID_{50}$ (tissue culture infectious dose) of virus was defined as the amount of stock required to infect 50% of RMK monolayers.

Real-time RT-PCR

RNA was isolated from homogenized lung using an RNeasy Mini Kit (Qiagen, Germantown, MD). Eosinophil RNA was isolated using Ambion SYBR Green Cells-to-CT kit (Life Technologies). Viral RNA was isolated from culture supernatants using High Pure Viral RNA kit per the manufacturer's protocol (Roche, Basel, Switzerland).

Sample cDNA was generated use Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and PCR was performed on a 7500 Fast RT-PCR system (Applied Biosystems, Carlsbad, CA). Parainfluenza RNA was transformed to $TCID_{50}$ units using a parainfluenza standard curve quantified by RMK cell titration. Primers were custom ordered as follows: parainfluenza matrix protein 5'-ATGCGGCTGATCTTCTCACT-3' and 5'-CTTTGCCACGACATTAGGGT-3'; parainfluenza fusion protein 5'-GAGTGGCAACATCAGCACAG-3' and 5'-CTTATCGCGGGTTTGATCTC-3'; parainfluenza nucleocapsid protein 5'-GAGCTATGCAATGGGAGTCG-3' and 5'-TTGCCAAATGATGTCTGAGC-3'; TLR7 5'-TCTGCCCTGTGATGTCACTC-3' and 5'-GGTTAATGGTGAGGGTGAGG-3' (Integrated DNA Technologies, Coralville, IA).

Eosinophil Isolation

Granulocytes were isolated from the blood of healthy human volunteers by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) per the manufacturers protocol. Red blood cells were removed by hypotonic lysis, and eosinophils were purified from other granulocytes by negative selection using antiCD16-linked magnetic beads (Miltenyi, Auburn, CA). Eosinophils were collected, and purity (>99%) and viability (>99%) were determined by Hemacolor staining and trypan blue exclusion, respectively. Eosinophils were cultured in RPMI1640, 5% human serum (Invitrogen, Carlsbad, CA), and penicillin-streptomycin on human placental collagen-type IV (Sigma, St. Louis, MO) coated dishes at a density of $1x10^6$ cells/mL.

Immunohistochemistry

Primary human eosinophils were cultured with or without IFNγ (300 units/mL) overnight, then blocked with 4% Normal Goat Serum, 1% Triton X-100, 5% powdered milk in Tris-

buffered Saline (TBS), pH 7.4. Cells were treated with rabbit polyclonal antibody against TLR7 (Imgenex, San Diego, CA) at 4^0C. Cells were then washed with TBS followed by immersion in goat anti-rabbit 555 ($F(ab')_2$-conjugated secondary antibody (Life Technologies, Grand Island, NY). Cell were washed, then treated with 4',6-Diamidino-2-Phenylindole (DAPI) nuclear stain (Life Technologies). Control experiments consisted of a no primary control that excluded the primary antibody step from the staining protocol. Cells were imaged with a laser-scanning confocal microscope (LSM 780).

Bronchoalveolar Lavage

On day 31 of the treatment protocol, mice were euthanized with a lethal dose of pentobarbital (150 mg/kg intraperitoneal). The lungs were lavaged with sterile PBS (0.5 mL aliquots x 3). Lavage samples were centrifuged for 10 minutes at 300xg. Cells were resuspended in 10 mL of PBS and total leukocytes were quantified using a hemocytometer. Aliquots of the cell suspension were cytospun onto glass slides, stained with Hemacolor (EMD Chemicals, Philadelphia, PA) and counted to obtain differential cell counts. Following lavage, lungs were flash frozen in liquid nitrogen to preserve viral RNA for quantification.

Nitric Oxide Synthase Inhibition

Primary human eosinophils were incubated with IFNγ (300 units/mL) overnight in 96-well plates then treated with the nitric oxide synthase inhibitor L-NG-Nitroarginine Methyl Ester *(*L-NAME; 100 μM; Sigma, St. Louis, MO*)* or vehicle for 30 minutes prior to infection with parainfluenza (1 x 10^5 $TCID_{50}$ units/mL). Parainfluenza infection was quantified by hemadsorption assay after 2 hours of inoculation. In separate

experiments, eosinophil viability following L-NAME treatment was assessed by trypan blue exclusion.

Intracellular Nitric Oxide Detection

Intracellular nitric oxide production was quantified 1 hour after infection using a fluorescent copper-conjugated probe CuFL (NO-ON, Strem, Newburyport, MA) (3). Eosinophils were loaded with CuFL for 1 hour followed by 1 hour of infection with parainfluenza virus or treatment with synthetic TLR7 agonist R837 (Invivogen, San Diego, CA). Some cultures also contained L-NAME (100 μM), the oligonucleotide TLR7 antagonist IRS661 (Integrated DNA Technologies, Coralville, IA) or control oligonucleotide (Integrated DNA Technologies). Fluorescence was measured by plate reader and representative images were obtained on a spinning disc confocal microscope (100X, 1.4 NA). Nitric oxide production was represented as fluorescence intensity (fluorescence intensity of sample minus background fluorescence).

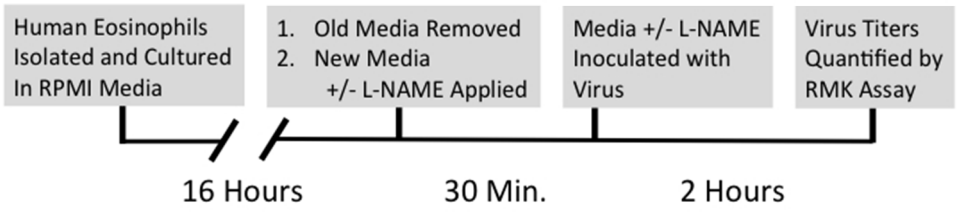
Supplemental References

1. Adamko DJ, Yost BL, Gleich GJ, Fryer AD, Jacoby DB. Ovalbumin sensitization changes the inflammatory response to subsequent parainfluenza infection. Eosinophils mediate airway hyperresponsiveness, m(2) muscarinic receptor dysfunction, and antiviral effects. *J Exp Med* 1999; 190: 1465-1478.
2. Reed LJ MH. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 1938; 27: 493-497.
3. Lim MH. Preparation of a copper-based fluorescent probe for nitric oxide and its use in mammalian cultured cells. *Nat Protoc* 2007; 2: 408-415.

Supplemental Figure

Supplemental Figure S1. Experimental protocol for eosinophils treated with L-NAME followed by parainfluenza infection. Eosinophils were cultured overnight in media containing 300 U/mL IFNγ. Supernatants were then removed and fresh media containing L-NAME (100 μM) was added for 30 minutes prior to inoculation with parainfluenza virus. Parainfluenza infection was quantified by hemadsorption assay 2 hours after inoculation. Viral content was expressed as viral $TCID_{50}$ Units/mL.

Figure S1



Supplemental Figure S1.
Experimental protocol for eosinophils treated with L-NAME followed by parainfluenza infection. Eosinophils were cultured overnight in media containing 300 U/mL IFNγ. Supernatants were then removed and fresh media containing L-NAME (100 μM) was added for 30 minutes prior to inoculation with parainfluenza virus. Parainfluenza infection was quantified by hemadsorption assay 2 hours after inoculation. Viral content was expressed as viral $TCID_{50}$ Units/mL.
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