Pharmacologic Activation of the Innate Immune System to Prevent Respiratory Viral Infections

Guanjun Cheng*[1], Liang-Chuan S. Wang[1], Zvi G. Fridlender[1], Guang-Shing Cheng[2], Bei Chen[3], Nilam S. Mangalmurti[1], Vassiliki Saloura[1], Zaifang Yu[1], Veena Kapoor[1], Krystyna Mozdzanowska[2], Edmund Moon[1], Jing Sun[1], James L. Kreinder[4], Noam A. Cohen[3], Andrew J. Caton[2], Jan Erikson[2], and Steven M. Albelda[1]

From [1]the Thoracic Oncology Research Laboratory, 1016B ARC, University of Pennsylvania, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318, [2]The Wistar Institute, Philadelphia, Pennsylvania, PA 19104, [3]Department of Otorhinolaryngology-Head and Neck Surgery, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA, [4]Department of Pediatric, Children's Hospital of Philadelphia, 1016D ARC, University of Pennsylvania, 3615 Civic Center Boulevard, Philadelphia, PA 19104.

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*To whom correspondence should be addressed: Guanjun Cheng, Thoracic Oncology Research Laboratory, 1016B ARC, University of Pennsylvania, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318. Phone: 215-573-2593; Fax: 215-573-4469; E-mail: guanjun@mail.med.upenn.edu

ONLINE DATA SUPPLEMENT

Supplemental Methods

Cell culture Normal human bronchial/tracheal epithelial cells (NHBE) were purchased from Lonza/Clonetics (Walkersville, MD) and grown in bronchial epithelial complete growth medium BEGM BulletKit (Lonza/Clonetics). NHBE cells were plated on tissue culture-treated polyester microporous inserts (0.4 μm pore size, Transwell Clear, Costar, Cambridge, MA). Medium was applied both apically and basally until cells reached confluence, the air-liquid interface was created by removing the apical medium and only feeding the cultures from the basal compartment. The culture medium was changed on day one and every other day thereafter. BEAS-2B human bronchial epithelial cells were isolated from normal human bronchial epithelium infected with a adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned (ATCC, Manassas, VA). BEAS-2B cells were cultured in 25-cm^2 tissue culture flasks, precoated with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collegen type I and 0.01 mg/ml bovine serum albumin (BSA) dissolved in base medium for this line, and maintained in ATCC complete growth medium BEGM BulletKit. All cell lines were maintained at 37°C in an atmosphere containing 5% CO_2 and were regularly tested and maintained negative for Mycoplasma spp.

RNA isolation and real time, reverse transcription-PCR. Quantitation of mRNA levels was done as previously described (12, S4). Total RNA was isolated from cells with RNeasy Mini Kit (Qiagen, Valencia, CA). Three micrograms of RNA from each condition was reverse transcribed

using 2 μg random primers (Invitrogen, Carlsbad, CA), 10 mmol/L deoxynucleotide triphosphates (Clontech, Palo Alto, CA), and 1 unit SuperScript III reverse transcriptase in 1x First-Strand Buffer and 10 mmol/L DTT (Clontech) for 60 minutes at 50°C. Equal amounts of cDNA from each condition were pooled. Primers were obtained from the literature or designed using standard protocols. Semi-quantitative analysis of gene expression was done using a Applied Biosystems StepOne Real-Time PCR System following the manufacturer's protocol for Fast SYBR Green Master Mix. cDNA concentrations from each pool were normalized using GAPDH as a control gene. Relative levels of expression of H1N1 influenza A virus NP RNA (fold change versus saline control) were determined. Each sample was run in triplicate. PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide.

Processing and analysis of bronchoalveolar lavage (BAL) fluid and plasma. C57/B6 mice were administered PBS, DMXAA (23mg/kg) or LPS (150ng/kg) intranasally. Mice were euthanized with intraperitoneal injection of ketamine (80mg/kg)/xylazine (10mg/kg). Blood samples were drawn by intracardiac puncture using heparinized 1ml syringes and were immediately centrifuged at 2,400 rpm for 5 minutes at room temperature. Plasma was obtained and frozen in 200ul aliquot at -80°C. The thoracic cavity was opened. The lung was lavaged with 0.6ml of 0.9% NaCl/0.6mM EDTA first, followed by instilled three separate aliquots of 0.5ml of 0.9% NaCl/0.6mM EDTA, total BAL volume 2.1ml of that 90% consistently recovered. The total cells of recovery BAL fluid (BALF) were monitered using a hemacytometer. Cytospins of BALF were prepared as follows. The remaining BAL fluid was centrifuged at 1,500 rpm for 5min at 4°C. Aliquots of the supernatant were stored at −80°C (S1-2).

Cytospins of BALF. Cytospins of BALF from mice collected after PBS, DMXAA or LPS exposure were processed using a Cytospin 3 (Shandon, Pittsburgh, PA). 50 ul BALF was centrifuged at 300 rpm for 3min at room temperature with a cytocentrifuge. The slides were quick differential staining by methanol fixed, eosin stained and methylene blue stained with Hemacolor (Harleco, Gibbstown, NJ) according to the instructions of the manufacturer. Differential cell counts from cytospins were performed with a light microscope. At least 200 cells were counted in random fields using standard morphological criteria in a blinded fashion by two independent investigators. Cell counts were expressed as percentages of total cells (S1-3).

Cytokine/chemokine levels of BALF and plasma. The amount of IFNβ, CXCL1/KC, CXCL2/MIP2, IP10/CXCL10, IL-6 and MCP1/CCL2 in BAL fluid and plasma was quantified using enzyme-linked immunosorbent assay (ELISA) kits to detect murine IFNβ (VeriKine Mouse IFN Beta ELISA Kit, PBL Biomedical Laboratories), CXCL1/KC (DuoSet Mouse CXCL1/KC , R&D Systems,Inc, Minneapolis, MN), CXCL2/MIP2 (DuoSet Mouse CXCL2/MIP2, R&D Systems,Inc, Minneapolis, MN), IP10 (DuoSet Mouse CXCL10/IP10/CRG-2, R&D Systems,Inc, Minneapolis, MN), IL-6 (BD OptEIA Mouse IL-6 ELISA Set, BD Biosciences Pharmingen, San Diego, CA) and MCP1/CCL2 (BD OptEIA Mouse MCP1 ELISA Set, BD Biosciences Pharmingen, San Diego, CA) according to the instructions of the manufacturers.

Supplemental Results

DMXAA inhibits replication of H1N1 influenza A in mouse respiratory epithelial

cells. To assess the inhibitive effect of DMXAA on viral replication of H1N1 influenza A virus in respiratory epithelial cells, mouse C10 bronchial epithelial cells were pre-treated with DMXAA (100ug/ml) for 2 hours or were given DMXAA at the same time, and then exposed to H1N1 influenza A virus (1 MOI) for 48 hours. The mRNA expression profiles were evaluated using real time RT-PCR. H1N1 influenza A nucleoprotein (NP) RNA levels were significantly up-regulated by 83.5 folds at 48 hours versus 0 hour post-infection of 1MOI H1N1 influenza A virus(Fig. S1A) in C10 bronchial epithelial cells ($p<0.01$). When the data is plotted as the cycle number where mRNA was detected, virus infection for 48 hours shows very high enrichment for NP RNA levels (21.9 cycles), with barely detectable at 0 hour post-infection (27 cycles for NP).

H1N1 influenza A virus replicates in mouse C10 bronchial epithelial cells. DMXAA, compared to virus alone ($p<0.01$) were significantly reduced H1N1 influenza A NP RNA levels, suggesting that DMXAA inhibits H1N1 influenza A virus replication in cultured mouse respiratory epithelial cells (Fig. S1B).

DMXAA does not induce strong inflammation. To rule out the possibility that DMXAA alone induces strong inflammation in the lung, mice were administered DMXAA intranasally and the BAL assay was performed at 6 hours and 24 hours post-administration. The total cells and infiltrating neutrophils in BALF were counted using a hemacytometer. Neutrophils were significantly elevated by positive control LPS intranasally ($p<0.01$), but not DMXAA (Fig. S2). More than 90% of BAL cell differentials from mice except for LPS group used in experiments were macrophages. The results indicate that at the dose of 23mg/kg, DMXAA did not significantly induce neutrophil inflammatory cells for 6 to 24 hours. The concentrations of proinflammatory cytokines/chemokines IFNβ, CXCL1/KC, CXCL2/MIP2, IP10/CXCL10, IL-6 and MCP1/CCL2 in BALF and plasma were evaluated by ELISA. IFNβ levels of BALF and plasmawere undetectable in all groups (data not shown). There were no significant differences between DMXAA and control groups in KC, MIP2, IP10, MCP1 and IL6 levels of BALF, and no significant differences between DMXAA and control groups in IP10 levels of plasma. There was a trend for increased level of IL6 in BALF in the DMXAA 24h treated group, and MIP2 in plasma in the DMXAA 6h treated group versus control (Table S1). Marked and significant elevation ($p<0.05$) was found in MCP1 in the DMXAA 6h treated group, and KC, MIP2, MCP1 and IL6 in the DMXAA 24h treated group versus control in plasma (Table S1). DMXAA has less effects on cytokines/chemokines levels in BALF than in plasma circulating levels. Taken together, The data demonstrate that DMXAA has no ability to induce strong inflammation effects, including neutrophils infiltratration and proinflammatory cytokines/chemokines release in the lung.

Effects of DMXAA on human epithelial cells. DMXAA was able to activate the IFNβ signaling pathway in normal human bronchial epithelial cells and to a much lower extent in BEAS-2B cells**.** Effect of DMXAA was tested in both NHBE and BEAS-2B cells.

Cells were exposed to DMXAA and IFNβ mRNA expression was measured using real time RT-PCR, PKR protein levels were detected by immunoblotting. Significant increases in the expression of IFNβ mRNA at 6h after DMXAA challenge was detected in both cell lines (Fig. S3A). Examination of PKR expression from BEAS-2B cells revealed that DMXAA elevated PKR levels, PF1M cells are normal human mesothelial cells as a positive control (Fig. S3B). It thus appears DMXAA can also activate IFNβ signaling pathway in normal human bronchial/tracheal epithelial cells.

Supplemental References

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Supplementary Figure Legends

Figure S1. DMXAA reduces H1N1 influenza A NP RNA levels. A. C10 mouse respiratory epithelial cells were infected with H1N1 influenza A viruse for 48 hours. The results are presented as mean $\pm$ SEM (*$p<0.01$, 0 h verus 48 h of virus post-infection). B. C10 mouse respiratory epithelial cells were pretreated with DMXAA for 2 hours or treated with DMXAA at the same time, and then were infected with H1N1 influenza A viruses. Total RNA was isolated and the relative H1N1 influenza A NP RNA levels were measured by real time RT-PCR post-infection. The results are presented as mean $\pm$ SEM (*$p<0.01$, virus alone group vs DMXAA plus virus groups).

Figure S2. Neutrophils in BAL after DMXAA intranasal administration. C57/B6 mice (3 per group) given DMXAA via the intranasal route and sacrificed at various time points. The percentage of neutrophils in BALF was counted using a hemacytometer (*$p<0.01$, CTR vs LPS groups).

Figure S3. DMXAA induces IFNβ and PKR in human bronchial epithelial cells. (A). NHBE and BEAS-2B cells were incubated with or without DMXAA for 6 hours. The total RNA was isolated and the relative IFNβ mRNA levels were measured by real time RT-PCR. The results are presented as mean ± SEM (*p<0.01, CTR vs DMXAA). (B). BEAS-2B cells were incubated with DMXAA 100ug/ml as indicated time points, lysed and PKR protein levels were analyzed by immunoblot using an anti-OAS1 antibody. Expression levels of β-actin show equal loading of the lanes.

Table S1. Cytokine/chemokine levels in BAL and plasma after DMXAA intranasal administration. C57/B6 mice (3 per group) given DMXAA via the intranasal route and sacrificed at various time points. Induction of CXCL1/KC, CXCL2/MIP2, IP10/CXCL10, IL-6 and MCP1/CCL2 in BAL fluid and plasma was determined from BAL and plasma by ELISA. The results are presented as mean ± SEM (*p<0.01, CTR vs DMXAA groups).