Supplementary Materials for

Role of Tissue Protection in Lethal Respiratory Viral-Bacterial Coinfection

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Mice

C57Bl/6 (B6) mice were obtained from National Cancer Institute and Jackson Laboratory and were between 6 and 12 weeks of age. Rag2\(^{-/-}\) Tlr2\(^{-/-}\)/Tlr4\(^{-/-}\), Myd88\(^{-/-}\), Nos2\(^{-/-}\), and IFN\(\alpha\)R1\(^{-/-}\) were bred in the Yale School of Medicine animal facility or provided by biomodels Austria in specific pathogen free conditions. All experiments were performed in accordance with the relevant institutional animal care and use guidelines.

Bacterial and Viral Strains

The A/WSN/33 (H1N1) strain of influenza A virus was obtained from the laboratory of Dr. Akiko Iwasaki and was propagated using MDCK cell as described in [32]. Unless otherwise indicated mice were infected with the JR32 ΔflaA strain 3 days after infection with influenza virus. The LP01ΔflaA, LP01ΔdotA, and thymidine auxotroph strain LP02 L. pneumophila strains were obtained from Dr. Craig Roy and the LP02 ΔflaAΔdotA L. pneumophila strain used were provided by Dr. Russell Vance, and were grown as described in [33].

Infectious Models

For intranasal infections of influenza and Legionella mice were anaesthetized with a ketamine/xylazine mixture and the pathogen was administered dropwise. Mice were infected with a sublethal dose of 300
PFU of influenza in a volume of 30 µl intranasally, and 1X10⁶ L. pneumophila was administered in a volume of 40 µl. For AREG experiments mice were injected I.P. daily with 10 µg of AREG (Peprotech).

**Quantification of Viral and Bacterial Loads**

Viral titers in the lungs were determined by titration of organ homogenate on MDCK cells and plaque forming units (PFUs) were quantified as described in (32). L pneumophila levels were determined by plating titrated amounts of organ homogenate on CYE plates as described in (21). Organs were homogenized using the Polytron PT100 in 1ml of appropriate buffer for PFUs or 10ml of sterile water for L. pneumophila for CFUs.

**BAL Collection, Cytospin Analysis, and BALF Albumin Measurement**

Bronchalveolar lavages were performed as described elsewhere (21). Briefly mice were euthanized and the trachea was exposed. Using plastic tubing threaded over a 26 gauge needle attached to a 1ml syringe .5ml of PBS was slowly injected intratracheally into the lung, and slowly removed into the syringe. Tubes containing the fluid were centrifuged at 1300 rpm to separate the supernatant from the cells. The supernatant was used to measure cytokine levels as described below and albumin levels. Albumin levels were determined using the mouse albumin ELISA kit (Immunology Consultants Laboratory Inc.) according to manufacturers instructions. The cells were suspended at a concentration of 1X10⁶ cells/ml, and 100 µl were spun onto slides at 700 rpm for 7 minutes, followed by autofix at 700 rpm for 3 minutes. After desiccation for 30 minutes the cells were stained using the diff-quick staining method according to manufacturer’s instructions, and differential cell counts were performed.
Measurement of Cytokines and Chemokines Expression Levels

Cytokine and chemokine levels were determined by quantitative PCR according to manufacturers’ instructions. Briefly, RNA was isolated from organs using the RNA-bee reagent and cDNA was produced using the superscript III reagent after treatment with DNase. This cDNA was used with the Stratagene qPCR machine. Serum and BALF cytokines and chemokine levels were determined using the Luminex (Millipore) system according to manufacturer’s instructions.

Histology

Organs used for histology were fixed overnight in FormaldeFresh™ or paraformaldehyde, and embedded in paraffin for sectioning. Pathology scoring was done in a blind randomized manner. The scoring system was as follows: 0= no airway necrosis, 1= focal necrosis, 2= some confluence of necrosis in larger airways, 3= confluent airway necrosis in most airways.

Statistical Analysis

Statistical analysis was general done with either prism graphpad or Microsoft excel. The logrank test, student t-test, or ANOVA was performed where appropriate.
Supplemental Figures

**Fig. S1. Systemic bacterial and viral load.** CFU levels in the (A) liver and (B) spleen are below the limit of detection. PFU levels in the (C) kidney and (B) spleen are below the limit of detection.

**Fig. S2. Growth of bacterial strains in vivo.** CFUs in the lung of JR32ΔflaA, and LP01ΔdotA 1 hour, 3 days, and 4 days after infection.

**Fig. S3.** (A) Survival of mice with NAi treatment. (B) Temperature loss after NAi treatment (C) Weight loss of mice treated with NAi (D) Viral PFUs days post influenza infection following treatment with NAi.

**Fig. S4.** Expression of immune response genes in lungs from mice infected with influenza virus, JR32ΔflaA alone, or JR32ΔflaA 3 days after influenza virus infection 1 day and 3 days after bacterial infection.

**Fig. S5.** Survival of (A) Rag2−/− mice and (B) Ifnar1−/− mice singly infected or coinfect ed with JR32ΔflaA 3 days after influenza virus infection. (C) Survival of singly and coinfect ed mice treated with dexamethasone 2 days after bacterial infection. (D) Survival of coinfect ed mice treated with NAC.

**Fig. S6.** Expression of tissue repair genes in lungs from mice infected with influenza virus, JR32ΔflaA alone, or JR32ΔflaA 3 days after influenza virus infection 1 day and 3 days after bacterial infection.
Days after L.p. infection

<table>
<thead>
<tr>
<th>Days</th>
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<th>Coinfected</th>
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CCL7
CXCL13
CXCL10
CXCL1
CXCL2
CCL3

CCL8
CCL4
CCL2
Nos2
G-CSF
TNFα

Influenza
Legionella
Coinfected

fold increase

days after L.p. infection

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References and Notes


