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Short communication

Defects in early cell recruitment contribute to the increased susceptibility to respiratory *Klebsiella pneumoniae* infection in diabetic mice

Nuria Martinez [a], Natkunam Ketheesan [a,b], Gregory W. Martens [a], Kim West [a], Egil Lien [c,d], Hardy Kornfeld [a,*]

[a] *Division of Pulmonary, Allergy and Critical Care, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA*
[b] *Australian Institute of Tropical Health and Medicine, James Cook University, Queensland 4811, Australia*
[c] *Program in Innate Immunity, Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA*
[d] *CEMIR, Department of Cancer Research and Molecular Medicine, NTNU, Trondheim, Norway*

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Abstract

Diabetes is associated with increased susceptibility to *Klebsiella pneumoniae* and poor prognosis with infection. We demonstrate accelerated mortality in mice with streptozotocin-induced diabetes following tracheal instillation of *K. pneumoniae*. Diabetic mice recruited fewer granulocytes to the alveolar airspace and had reduced early production of CXCL1, CXCL2, IL-1β and TNF-α following tracheal instillation of *K. pneumoniae*-lipopolysaccharide. Additionally, TLR2 and TIRAP expression following *K. pneumoniae*-lipopolysaccharide exposure was decreased in hyperglycemic mice. These findings indicate that impaired innate sensing and failure to rapidly recruit granulocytes to the site of infection is a mechanism for diabetic susceptibility to respiratory *K. pneumoniae* infection.

Keywords: Diabetes; *Klebsiella pneumoniae*; Lung; Pneumonia

1. Introduction

Klebsiella pneumoniae is one of the ESKAPE pathogens (also including *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) responsible for the majority of hospital infections in the United States [1]. Resistance of *K. pneumoniae* to carbapenem antibiotics has spread to all regions of the world and in some countries carbapenem resistance is present in more than half of the patients treated for *K. pneumoniae* infections [2]. *K. pneumoniae* is also emerging as an agent of severe community-acquired infection including bacteremic pneumonia [3].

Comorbid diabetes mellitus is associated with increased risk for and severity of liver, urinary tract, head and neck, bloodstream and lung infections with *K. pneumoniae* [4–8]. The global prevalence of diabetes is increasing and will reach to over 590 million in 20 years. Approximately 80% of people with diabetes currently live in low- and middle-income countries with the greatest increases predicted to occur in these regions [9]. With an aging population and the rising prevalence of diabetes, particularly in Asian countries which have also become a source of antibiotic resistant *K. pneumoniae* strains [10], a better understanding of the diabetic host-*K. pneumoniae* interaction is essential. To that end, we investigated the impact of streptozotocin (STZ)-induced diabetes in mice on susceptibility to respiratory *K. pneumoniae* infection. Results suggest that diabetic vulnerability to *K. pneumoniae* stems from a reduced cytokine response to bacterial lipopolysaccharide (LPS) resulting in delayed granulocyte trafficking to the airspace.

* Corresponding author. Department of Medicine, LRB-303, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, USA. Tel.: +1 508 856 2646; fax: +1 508 856 7883.
E-mail address: Hardy.Kornfeld@umassmed.edu (H. Kornfeld).

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2. Materials and methods

2.1. Animals and induction of hyperglycemia

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used for studies conducted under protocols approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the University of Massachusetts Medical School. Hyperglycemia was induced by intraperitoneal injection of 150 m/kg body weight STZ (Sigma-Aldrich, St Louis, MO). At regular intervals, blood glucose levels were assessed in control and STZ-treated mice using a BD Logic glucometer (BD Biosciences, Franklin Lakes, NJ). Mice were considered hyperglycemic if their blood glucose was >300 mg/dL and euglycemic or control when blood glucose was <200 mg/dL.

2.2. Infection and bacterial burden

One hundred colony-forming units (CFU) of *K. pneumoniae* (strain 43816; ATCC, Manassas, VA) was delivered by tracheal instillation. Hyperglycemic (HG) mice were challenged 12 weeks after STZ treatment. After 48 h of infection, lungs were homogenized in PBS and serial dilutions were plated on nutrient agar plates. Colonies were counted after 4−6 h of incubation at 37 °C.

2.3. Flow cytometry

Bronchoalveolar lavage (BAL) cells and lung homogenates were obtained as previously described [11] and analyzed by flow cytometry. Cells were treated with Fc blocking mAb then stained with anti-CD11b, anti-CD11c (BD Bioscience Pharmingen, San Diego, CA) and F4/80-APC (eBioscience, San Diego, CA). Cells were gated based on forward scatter/side scatter of light for different cell populations and the total and percentages of leukocytes and granulocytes ($CD11b^{hi}$ $CD11c^{-}$), resident alveolar macrophages ($CD11b^{-}CD11c^{+}$), recruited macrophages ($CD11b^{+}CD11c^{-}$) and dendritic cells ($CD11b^{+}CD11c^{+}$) were enumerated. Results were analyzed using FlowJo version 7 (Tree Star, Ashland, OR).

2.4. Histopathology

Lungs were fixed with 4% paraformaldehyde and prepared for histology and stained with hematoxylin and eosin or myeloperoxidase (MPO; anti-MPO rabbit IgG, LifeSpan Biosciences, Seattle, WA) and DAPI for nucleus.

2.5. Response to lipopolysaccharide

K. pneumoniae LPS was purchased from Sigma-Aldrich (L1519, source strain ATCC 15380) and subjected to re-purification with two phenol−water extractions to remove contaminating lipoproteins [12]. Quality control was performed by verifying IL-8 inducing activity towards HEK293-huTLR4/MD-2 cells and lack of IL-8 induction in HEK293-huTLR2 cells [13]. *Escherichia coli* LPS was also purchased from Sigma-Aldrich (L2630, serotype O111:B4) and it was purified by phenol extraction. Following anesthesia with isoflurane, 0.05 µg of *K. pneumoniae* LPS or 2 mg/kg *E. coli* LPS in 50 µl of PBS was delivered to the lungs via the trachea. At 1 h and 2 h post LPS instillation, mice were sacrificed and lungs removed for histology and tissue homogenates prepared for ELISA or RNA purification.

2.6. Assessment of cytokine levels

Production of selected chemokines and cytokines (CXCL1, CXCL2, IL-1β TNF-α and IL-10) in control and hyperglycemic (HG) mice: 1 and 2 h after *K. pneumoniae* LPS challenge, 1 h after *E. coli* LPS challenge and 6 h after *K. pneumoniae* infection (~100 CFU) was determined by multiplex ELISA (R&D Systems, Minneapolis, MN). Absorbance was measured with a Multiskan Ascent microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the concentrations of the selected chemokines and cytokines in each sample were extrapolated using a standard curve.

2.7. Gene expression

Total RNA was isolated from lung tissue by using TRIzol® Reagent and Purelink® RNA mini kit from Ambion (Life Technologies, Grand Island, NY). Equal amounts of RNA were used and retro-transcribed with High Capacity Reverse Transcription Kit (Applied Biosystems, Life Technologies). Quantitative real-time polymerase chain reaction (qPCR) for CXCL1, CXCL2, IL-1β, TNF-α, IL-10, TLR2, TLR4 and TIRAP was performed at 60 °C of annealing temperature using SYBR Green PCR mix (Applied Biosystems, Life Technologies) according to the manufacturer's guidelines. TBP and GADPH were used as housekeeping genes and primer sequences were designed by Primer3 input (Supplemental information). Results were calculated as fold change to the control group and relative to the reference genes of mice by the [delta] [delta] Ct method of Livak [14].

2.8. Bactericidal activity in neutrophils and alveolar macrophages

Neutrophils were isolated from the peritoneum 24 h after thioglycollate i.p. injection and using anti-Ly-6G microbeads from Miltenyi Biotec Inc. (San Diego, CA). Alveolar macrophages (AM) were obtained by bronchoalveolar lavage. One hundred thousand neutrophils or AM were seeded in a flat-bottom 96-well plate and infected with *K. pneumoniae* (MOI 1:10). After 30 min, media was washed and cells were lysed with 1% Triton-X at different time points: 0, 15, 30, 60 and 90 min (only 0 and 30 min for AM). Results were calculated as fold change in bacterial counts compared to time 0 for each mouse sample.

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