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

Nuria Martinez<sup>a</sup>, Natkunam Ketheesan<sup>a,b</sup>, Gregory W. Martens<sup>a</sup>, Kim West<sup>a</sup>, Egil Lien<sup>c,d</sup>, Hardy Kornfeld<sup>a,\*</sup>

<sup>a</sup>Division of Pulmonary, Allergy and Critical Care, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA

<sup>b</sup>Australian Institute of Tropical Health and Medicine, James Cook University, Queensland 4811, Australia

<sup>c</sup>Program in Innate Immunity, Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA

<sup>d</sup>CEMIR, Department of Cancer Research and Molecular Medicine, NTNU, Trondheim, Norway

### 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-1 $\beta$  and TNF- $\alpha$  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

\*Corresponding author. Department of Medicine, LRB-303, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655 USA Tel: 508-856-2646; Fax: 508-856-7883; Hardy.Kornfeld@umassmed.edu.

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Conflict of interest  
None to declare.

## 1. Introduction

*Klebsiella pneumoniae* is one of the ESKAPE pathogens (also including *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *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]. *Klebsiella 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.

## 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 mg/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<sup>hi</sup>CD11c<sup>-</sup>), resident alveolar macrophages (CD11b<sup>-</sup>CD11c<sup>+</sup>), recruited macrophages (CD11b<sup>+</sup>CD11c<sup>-</sup>) and dendritic cells (CD11b<sup>+</sup>CD11c<sup>+</sup>) 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

*Klebsiella 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<sup>®</sup> Reagent and Purelink<sup>®</sup> 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.

## 2.8. Data analysis

GraphPad Prism 6.0 was used to perform statistical analysis. When normality was confirmed, data were analyzed using a Student's *t*-test. Statistical differences in the survival curves were analyzed by log-rank (Mantel Cox) test. A *p* value of <0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Hyperglycemia increases susceptibility to *K. pneumoniae* infection

Following 12 weeks of STZ-induced hyperglycemia, mice were infected with ~100 CFU *K. pneumoniae* by tracheal instillation. HG mice had a significant reduction in survival compared to control mice (Fig. 1A). Over half of all HG mice succumbed to infection 3 days after challenge. In contrast, the first control mouse succumbed on day 9. Mortality reached 100% for HG mice by day 14 post-infection, at which point survival in the control group was nearly 80%. While there was substantial individual variation in lung bacterial burden measured on day 2 of infection, mean CFU was nearly 1000-fold higher in HG mice compared to euglycemic controls (Fig. 1B).

### 3.2 Reduced granulocyte recruitment to the site of pathology in hyperglycemic mice

The critical initial response to low dose *K. pneumoniae* infection involves the brisk recruitment of myeloid cells to the lung, leading to the clearance of the bacteria. To explore the basis for increased early mortality in HG mice, we analyzed myeloid cell recruitment to the whole lung and to airspace (BAL fluid) in response to tracheal instillation of highly purified *K. pneumoniae* LPS. Total cell numbers in both compartments were significantly reduced in the HG mice at 2 h post-challenge (Fig. 2A). From different populations assessed, only the number and proportion of granulocytes (predominantly neutrophils) were significantly decreased in the HG mice. These results were confirmed by the demonstration of reduced MPO signal, expressed in the granules of polymorphonuclear cells, in lungs from HG mice post LPS instillation (Fig. 2B). The proportion of other cell- populations was not altered, only resident alveolar macrophages show a slight but still significant increased percentage in the BAL (Supplemental Fig. 1).

### 3.3. Hyperglycemia decreases the production and expression of cytokines in the lung

The chemokines CXCL1 and CXCL2, produced mainly by resident alveolar macrophages, are responsible for recruiting neutrophils. Production of these chemokines in the lung after 1 and 2 h of *K. pneumoniae*-LPS instillation was assessed by ELISA. At the 1 h post-instillation time point, HG mice produced significantly less CXCL1 and CXCL2 compared to controls (Fig. 3A, top). Measurement of CXCL1 mRNA expression confirmed the results obtained by ELISA (Fig. 3B). Inflammatory mediators such as IL-1 $\beta$  and TNF- $\alpha$  are induced when bacteria are recognized by pattern recognition molecules such as Toll-like receptors (TLRs) on macrophages. Interestingly, a ~50% reduction in IL-1 $\beta$  protein production was observed in HG mice 1 and 2 h after *K. pneumoniae*-LPS instillation (Fig. 3A). Reduced IL-1 $\beta$  mRNA expression in HG mice was confirmed by qPCR (Fig. 3B). The production of the anti-inflammatory cytokine IL-10 was also significantly reduced in lungs from HG mice 2 h after treatment (Fig. 3A).

To investigate whether the expression of some components of the TLR cascade was altered in HG mice challenged with *K. pneumoniae* LPS, the levels of TLR2, TLR4 and TIRAP mRNA were assessed by qPCR. Expression of TLR2 and TIRAP was significantly reduced in the HG mice compared to controls, whereas there was no difference in TLR4 expression (Fig. 3C).

Changes in the gene expression and production of cytokines were specific for *K. pneumoniae* LPS. There were no significant differences in gene expression or cytokine production between control and HG mice instilled with *E. coli* LPS (Supplemental Fig. 2A). When mice were infected with live *K. pneumoniae* there was significant variability in CFU in the lung at different time points leading to low reproducibility. Although there was a decreased trend in the gene expression for cytokines in HG mice only IL-1 $\beta$  gene expression was significantly reduced 6 h following infection with ~100 CFU *K. pneumoniae* (Supplemental Fig. 2B).

### 3.4 Bactericidal activity in neutrophils and alveolar macrophages

Diabetic susceptibility could be a result of hyperglycemia-induced impairment of elicited neutrophil microbicidal activity. To test this, we infected neutrophils and AM and determine the bacterial growth after 15, 30, 60 and 90 min of infection, relating to the time 0 for each sample (Supplemental Fig. 3A). There was no difference between antibacterial activity between control and HG mice, indicating that the higher bacterial burden in hyperglycemia is probably linked to a defective TLR signaling and late neutrophils recruitment. Also, the analysis of the antibacterial activity of alveolar macrophages was not significantly different between control and HG mice after 30 min of infection (Supplemental Fig. 3B).

## 4. Discussion

Here we demonstrate that hyperglycemia in mice is associated with significantly higher mortality from lower respiratory infection with *K. pneumoniae*. An infecting dose lethal for 100% of HG mice was survived by nearly 80% of euglycemic controls. The early mortality of HG mice can be attributed to poor control of bacterial replication in the lung within the

first 2 days after challenge. Successful defense against this virulent Gram-negative pathogen hinges on the rapid trafficking of neutrophils to the site of infection. That innate immune response is primarily triggered by LPS binding to TLR4 [15,16] with the induction of chemoattractant cytokines, of which CXCL1 may play a dominant regulatory role [17]. Hyperglycemic mice exhibited reduced granulocyte recruitment and a weaker CXCL1 mRNA and protein response to *K. pneumoniae* LPS than controls, demonstrating functional impairment of the TLR4 pathway. The expression of other cytokines linked to successful defense against *K. pneumoniae* and to TLR4 signaling (CXCL2, IL-1 $\beta$  and TNF- $\alpha$ ) was also impaired in HG mice. Other arms of the immune system that contribute to late control of *K. pneumoniae* infection might also be impacted by hyperglycemia but this could not be assessed in our study owing to the early mortality of HG mice.

Jeyaseelan et al. [18] reported that TIRAP<sup>-/-</sup> mice are highly susceptible *K. pneumoniae* infection and have reduced neutrophil accumulation in the lung, whereas TIRAP was not required for defense against *Pseudomonas aeruginosa*. The TIRAP-dependent protection was attributed to downstream induction of IL-1 $\beta$  and TNF- $\alpha$ , both of which were expressed at lower levels by HG compared to control mice in our study. Previous studies have linked the decreased IL-10 gene expression we observed, to disrupted TLR/MAPK signaling [19,20]. The lower expression of TIRAP by HG mice could be a contributing factor to their marked susceptibility to *K. pneumoniae*.

Diabetes is associated with increased susceptibility to *K. pneumoniae* and certain other important pathogens, notably *Burkholderia pseudomallei* and *Mycobacterium tuberculosis* [21,22]. Mice deficient in TLR4 control experimental *B. pseudomallei* infection comparably to wild type mice, while TLR2 deficiency confers enhanced rather than diminished protection [23]. However, impaired early cytokine production at the site of infection was demonstrated in mice with type 2 diabetes mellitus comorbidity [24]. Host defense in tuberculosis hinges on a Th1 biased adaptive immune response but data from the HG model indicate that delayed priming of *M. tuberculosis* antigen-specific T cells in diabetic mice results from a delay in the innate immune response to initial infection of alveolar macrophages by inhaled bacilli [25]. While it is likely that chronic hyperglycemia interferes with a wide range of afferent and efferent immune host defense pathways, defective innate immune sensing is emerging as a common theme for the diabetic complication of susceptibility to infectious diseases.

Our data implicate a deficient cytokine response to the major pathogen-associated molecular pattern of *K. pneumoniae* resulting in delayed neutrophil granulocyte recruitment as a major immunological mechanism of diabetic susceptibility. An appropriate early response occurring within the first hour of infection is critical to prevent an overwhelming expansion of bacterial load. Since this response is impaired by diabetes, intervention strategies based on antibiotics given after *K. pneumoniae* infection in diabetics may not be adequate for a favorable outcome. With the expanding global epidemic of diabetes and the spread of multidrug-resistant *K. pneumoniae*, novel strategies for treatment and prevention are needed. The data presented here suggest that host-directed therapies to improve the early response to *K. pneumoniae* LPS could enhance protection against infection in the vulnerable diabetic population.



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

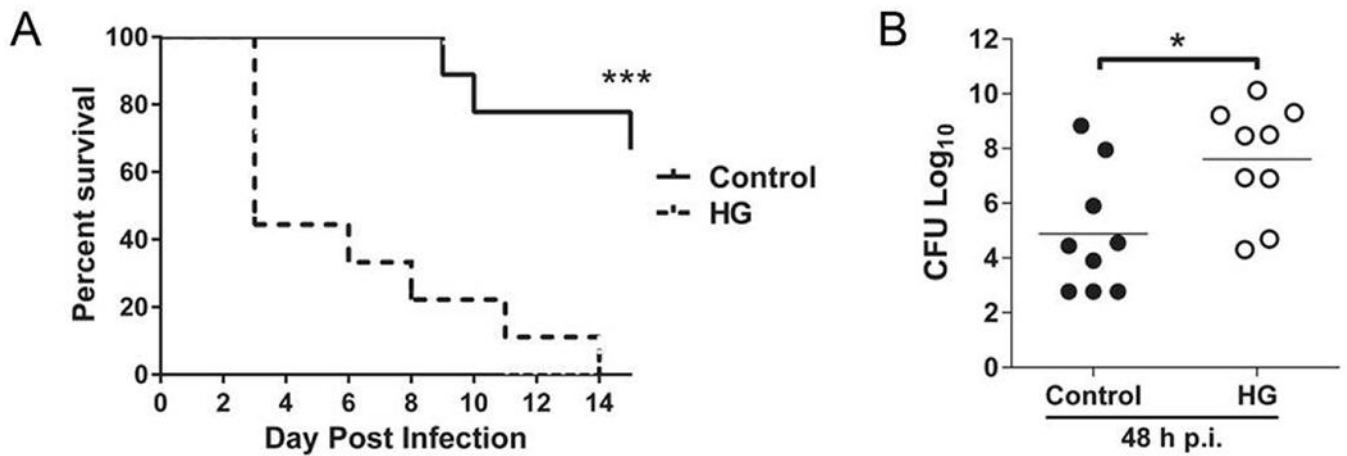
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## References

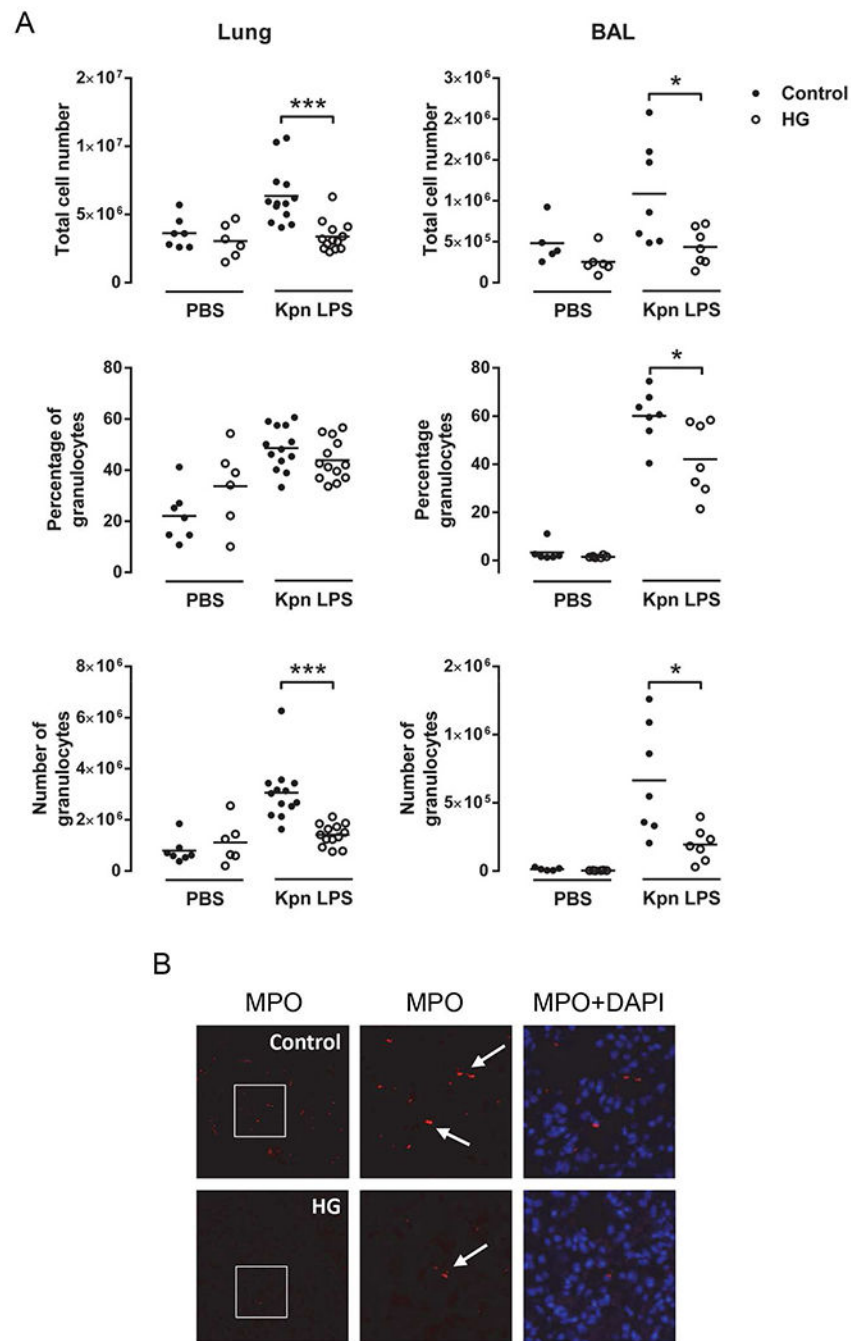
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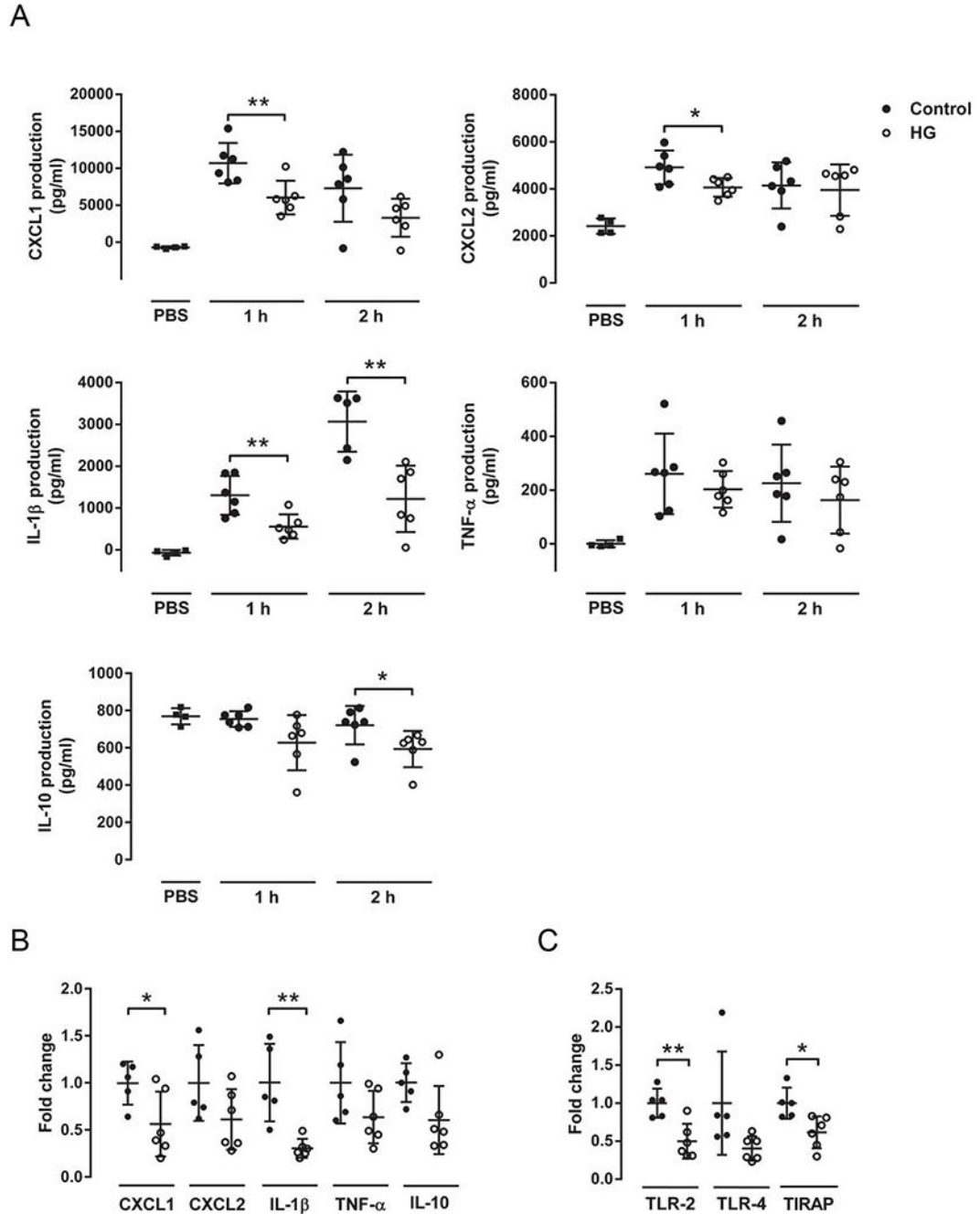




**Fig. 1.** Higher susceptibility of hyperglycemic mice to *K. pneumoniae* infection. Mice were infected with ~100 CFU *K. pneumoniae* by tracheal instillation. (A) Survival curve of control and hyperglycemic (HG) mice demonstrating increased mortality of HG mice compared to controls. (B) Lung bacterial load was significantly higher in HG mice following 48 h of infection ( $n = 9$ ). All experiments were repeated at least three times. Statistical differences were analyzed by Student's *t*-test,  $*p < 0.05$ .



**Fig. 2.** Reduced granulocyte recruitment following instillation of *K. pneumoniae* LPS in hyperglycemic mice. Mice were instilled with 0.05  $\mu\text{g}$  LPS and after 2 h lung tissue was obtained. (A) Flow cytometry of isolated cells from lung homogenate (left) and BAL (right). Total cell number and percentage and cell number of granulocytes were quantified ( $n = 5-13$ ). (B) Representative images of immunofluorescence staining for myeloperoxidase (MPO). All experiments were repeated at least twice. Statistical differences were analyzed by Student's *t*-test, \* $p < 0.05$  \*\*\* $p < 0.001$ .



**Fig. 3.** Reduced production and expression of cytokines in hyperglycemic mice instilled with *K. pneumoniae* LPS. (A) The levels of CXCL1, CXCL2, IL-1 $\beta$ , TNF- $\alpha$  and IL-10 in lung homogenates were lower in HG than control mice after 1 or 2 h instillation with PBS or 0.05  $\mu$ g LPS ( $n = 6$ ). (B) Gene expression of CXCL1, CXCL2, IL-1 $\beta$ , TNF- $\alpha$  and IL-10 in lung homogenates from control and HG mice after 1 h instillation with 0.05  $\mu$ g LPS demonstrates a reduction in expression in HG mice ( $n = 6$ ). (C) Gene expression of TLR2, TLR4 and TIRAP in lung homogenates from control and HG mice after 1 h instillation with

*K. pneumoniae* LPS demonstrates a decrease in HG mice ( $n = 6$ ). All experiments were repeated at least twice. Statistical differences were analyzed by Student's *t*-test, \* $p < 0.05$   
\*\* $p < 0.01$ .

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