A Novel Role for the Nuclear Receptor, NR4A1, in *Klebsiella pneumoniae* Lung Infection

Jessica Partyka^{a,b}, Matthew Henkel^{a,b}, Brian T. Campfield^{a,b,c#}

^aDivision of Pediatric Infectious Diseases, Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA

^bUPMC Children's Hospital of Pittsburgh, Pittsburgh, PA

^cRichard K. Mellon Institute for Pediatric Research, Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA

Running Head: NR4A1 in *K. pneumoniae* Lung Infection

#Address correspondence to:

Brian T. Campfield, M.D.

4401 Penn Ave.

8125 Rangos Research Building

Pittsburgh, PA 15224

brian.campfield@chp.edu

KEYWORDS

pneumonia, *K. pneumoniae*, NR4A1, Nur77, neutrophils

NR4A1 in *K. pneumoniae* Lung Infection

ABSTRACT

Klebsiella pneumoniae is a Gram-negative bacterial pathogen and common cause of pneumonia and bacteremia. Increasingly, *K. pneumoniae* has become a public health concern due to its rate of nosocomial infection and emerging, broad-spectrum antibiotic resistance. The nuclear receptor NR4A1 exhibits functionality in a multitude of organ systems and is implicated as having a role in the immune response to bacterial infection, though its role in *K. pneumoniae* infection is unknown. To determine if *Nr4a1* functions in response to *K. pneumoniae* pulmonary disease, we infected wild-type and *Nr4a1*^{-/-} mice with *K. pneumoniae* and assessed bacterial growth, immune cell recruitment and function, and cytokine production. We found that *Nr4a1*^{-/-} mice had increased bacterial burden in the lungs and spleen, though no differences in cell recruitment. Pro-inflammatory cytokines, *Il1β* and *Il6*, as well as chemokine, *Cxcl2*, were significantly decreased in the BAL fluid cells of *Nr4a1*^{-/-} mice 5 hours post-infection. Additionally, *Nr4a1*^{-/-} mice had reduced IL-1β and myeloperoxidase protein production. We then examined the bactericidal function of macrophages and neutrophils from WT and *Nr4a1*^{-/-} mice. We identified that *Nr4a1*^{-/-} neutrophils had decreased bactericidal function compared to wild-type neutrophils, which was associated with reduced expression of *Il1β*, *Lcn2*, *Mpo*, and *Lyz2*. These data suggest *Nr4a1* plays a novel and essential role in neutrophil function during the host immune response to *K. pneumoniae* pulmonary infection.

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative bacterium of the *Enterobacteriaceae* family. Recognized as an intestinal commensal in healthy individuals, *K. pneumoniae* is a common cause of disease in immunocompromised patients and is the third most common healthcare

NR4A1 in *K. pneumoniae* Lung Infection

associated infection, manifesting as urinary tract infections, bacteremia, and pneumonia (1). In addition, *K. pneumoniae* strains are becoming increasingly resistant to antibiotic treatments, including carbapenems and extended-spectrum β -lactams (ESBL); thus, *K. pneumoniae* constitutes a public health threat for which new treatment modalities are urgently needed (2, 3).

The orphan nuclear receptor subfamily 4 group A member 1 (NR4A1) is a steroid-thyroid receptor and intracellular transcription factor encoded by the *Nr4a1* gene, also known as Nur77. NR4A1 is a molecule with diverse biologic functions, including regulation of inflammation in the central nervous system, heart, and lung (4–11). Specifically, NR4A1 is a factor in macrophage development and inflammatory response through polarization and transcriptional regulation (12–16). Within macrophages, NR4A1 exhibits a direct effect on the NF- κ B pathway by directly blocking p65 from binding to the κ B element, and prior work has shown deletion of the *Nr4a1* gene increases p65 phosphorylation, thus activating NF- κ B mediated transcription, in macrophages (17, 18).

Due to its varied impact in immunological pathways, the role of NR4A1 in bacterial infection has been explored but remains poorly understood. In *Escherichia coli* induced peritonitis, NR4A1 has a limited role in the clearance of the bacteria (19). However, Hamers *et al.* later showed the deletion of the *Nr4a1* gene aggravated *E. coli* induced peritonitis due to pro-inflammatory macrophage polarization (20). In addition to showing NR4A1 directly interacts with the NF- κ B pathway, Li *et al.* also showed NR4A1 is a vital protein for inflammation reduction and, ultimately, survival in a sepsis model (17). The sole study of NR4A1 in bacterial lung infection examined *E. coli*, an uncommon cause of pneumonia, and showed that *Nr4a1* was

NR4A1 in *K. pneumoniae* Lung Infection

rapidly induced during infection, and that *Nr4a1*^{-/-} mice had decreased bacterial burden *in vivo*, though the mechanisms underlying these observations remain unclear (21).

The current study examined the role of NR4A1 in *K. pneumoniae* pneumonia. Here, we found that *Nr4a1* deficiency led to an increase of *K. pneumoniae* bacterial lung burden and dissemination. Mechanistically, phagocyte recruitment was unaffected, though inflammatory cytokine/chemokine production was decreased in *Nr4a1*^{-/-} mice, identifying that *Nr4a1*^{-/-} neutrophils had defective bactericidal function and attenuated inflammatory gene expression, suggesting that *Nr4a1* is a novel, critical mediator of neutrophil function.

RESULTS

***Nr4a1*^{-/-} mice are more susceptible to *Klebsiella pneumoniae* pulmonary infection**

To explore the impact of *Nr4a1* on control of *K. pneumoniae* pulmonary infection, WT and *Nr4a1*^{-/-} mice had bacterial burden determined by colony-forming units (CFU) at 24 and 48 hours after infection in the lung, as a measure of local control, and in the spleen as a measure of bacterial dissemination. Compared to WT mice, *Nr4a1*^{-/-} mice had significantly increased *K. pneumoniae* lung burden and spleen burden 48 hours post-infection (Fig. 1c-d). These differences were not present at 24 hours post-infection (Fig. 1a-b). These data identify that *Nr4a1*^{-/-} mice controlled *K. pneumoniae* acute lung infection poorly, suggesting that *Nr4a1* may play an important role in the immune response to acute *K. pneumoniae* lung infection.

***Nr4a1*^{-/-} mice have reduced pro-inflammatory cytokine production in BAL fluid cells and whole lung early following *K. pneumoniae* lung infection**

NR4A1 in *K. pneumoniae* Lung Infection

Next, to determine if immediate phagocyte function was impaired in the absence of *Nr4a1*, we analyzed cytokine/chemokine gene expression and protein production in the bronchoalveolar lavage fluid (BALF) and lung from WT and *Nr4a1*^{-/-} mice 5 hours post *K. pneumoniae* pulmonary infection. Pro-inflammatory cytokines, *Il1β* and *Il6*, and the neutrophil chemokine, *Cxcl2*, were significantly decreased in the BALF of *Nr4a1*^{-/-} mice (Fig. 2a, b, e). However, *Tnfa* and the chemokines *Cxcl1* and *Cxcl5* were not different (Fig. 2c, d, f), suggesting the NR4A1-dependent gene regulation was reflective of a specific cell population or pathway mediating the phenotype and this was observable within 5 hours post-infection.

Whole lung protein from WT and *Nr4a1*^{-/-} mice was also assessed at 5 hours and 24 hours post *K. pneumoniae* infection. Consistent with early gene expression, IL-1β protein was significantly reduced in *Nr4a1*^{-/-} compared to WT (Fig. 3a) at 5 hours post-infection. This reduction in IL-1β protein was sustained through 24 hours post-infection (Fig. 3c). We then sought to determine whether the *Nr4a1*-dependent phenotype was associated with reduced production of the effector molecule myeloperoxidase (MPO), an enzyme critical for phagocyte-mediated bacterial killing of *K. pneumoniae* (22). In the *Nr4a1*^{-/-} strain, MPO production trended down within 5 hours post-infection ($P = 0.0689$) (Fig. 3b). By the 24 hours post-infection timepoint, *Nr4a1*^{-/-} mice had significantly decreased MPO protein in the lungs compared to WT mice (Fig. 3d). These data suggest that early immune gene transcription reflects protein production during *K. pneumoniae* lung infection, and that *Nr4a1*-deficient susceptibility to infection is associated with reduced production of phagocyte markers.

NR4A1 in *K. pneumoniae* Lung Infection

***Nr4a1*^{-/-} and WT mice have similar immune cell abundance and cytokine gene expression 24 hours following *K. pneumoniae* lung infection**

To examine a potential mechanism by which *Nr4a1* protects against lung infection, we performed flow cytometry on the lungs of WT and *Nr4a1*^{-/-} mice at 24 hours post *K. pneumoniae* infection, prior to differences in bacterial burden, to determine if *Nr4a1* deletion resulted in a change in immune cell recruitment. No significant differences were observed in inflammatory macrophages (CD45⁺/CD11b⁺/CD11c⁺), alveolar macrophages (CD45⁺/CD11c⁺/SiglecF⁺), or neutrophils (CD45⁺/CD11b⁺/Ly6G⁺) (Fig. S1b-d). Interestingly, no differences in transcript were found between pro-inflammatory cytokines *Il1β*, *Tnfa*, or *Il6*, or the neutrophil-specific gene *Lcn2* (Fig. S2a-d). These data suggest the role *Nr4a1* plays in the immune response to *K. pneumoniae* acute lung infection is not due to cell recruitment and is not reflected in gene transcription through 24 hours post-infection.

Nr4a1*^{-/-} macrophage and neutrophil bactericidal function *in vitro

Bone marrow macrophage bacteria killing assays were analyzed to determine bacterial growth as a measure of *in vitro* bactericidal function from WT and *Nr4a1*^{-/-} mice. No differences in bacterial growth were observed between the two strains at 30 minutes or 60 minutes co-culture with *K. pneumoniae* (Fig. S4a), identifying that macrophages of both genotypes have similar bactericidal function. Similarly, the gene transcript levels for *Il1β*, *Il6*, and *Cxcl2* were not different, suggesting that the differences seen in the BALF cells and lung homogenate *in vivo* does not likely reflect *Nr4a1*-mediated function in macrophages (Fig. S4b-d).

NR4A1 in *K. pneumoniae* Lung Infection

Neutrophil bacteria killing assays were similarly analyzed to determine bacterial growth as a measure of *in vitro* bactericidal function from WT and *Nr4a1*^{-/-} mice. Notably, in WT neutrophils co-cultured with *K. pneumoniae*, *Nr4a1* gene transcription is induced compared to naïve WT neutrophils (Fig. 4a). Supernatants from *Nr4a1*^{-/-} neutrophils had significantly higher *K. pneumoniae* growth compared to WT neutrophils within 60 minutes, though at 30 minutes no differences were observed between genotypes (Fig. 4b-c). These data suggest that *Nr4a1*^{-/-} neutrophils *K. pneumoniae* killing is time dependent. Taken together, these data suggest *Nr4a1* transcription is induced in neutrophils by *K. pneumoniae* infection and is critical for the bactericidal activity.

***Nr4a1*^{-/-} neutrophils have decreased cytokine transcript compared to WT neutrophils**

To assess *Nr4a1*-dependent transcriptional changes in neutrophils, *Nr4a1*^{-/-} neutrophil RNA was isolated 30 minutes after *K. pneumoniae* co-culture (when the bacterial burdens are equivalent). Here, *Nr4a1*^{-/-} neutrophils had decreased *Il1β* transcript compared to WT neutrophils, as was seen in BALF cells 5 hours post-infection (Fig. 5a). *Nr4a1*^{-/-} neutrophils also had significantly decreased expression of neutrophil antibacterial transcripts *Lcn2*, *Mpo*, and *Lyz2* (Fig. 5b, c, d), further suggesting an important role for *Nr4a1* in neutrophil response to *K. pneumoniae* infection.

DISCUSSION

Due to its nosocomial infection rate and developing antibiotic resistance, *Klebsiella pneumoniae* is a growing public health concern (1–3). Developing new approaches to treat *K. pneumoniae* and other antibiotic resistant infections is an increasingly urgent task. One of the methods

NR4A1 in *K. pneumoniae* Lung Infection

through which this may be done is targeting the host immune response to stimulate better clearance of bacterial burden from the infection site. This can be done by targeting different immune cells, including T-cells, macrophages, and neutrophils, to enhance response to infection (23–25). For macrophages and neutrophils, this can be done by augmenting phagocytosis of bacteria (24–27). However, modulating host immunity is an understudied approach for the treatment of bacterial infection. More study of host immune responses and host-pathogen interaction in bacterial infection, including *K. pneumoniae*, is required if harnessing host immunity as a potential treatment is to be successful.

The orphan nuclear receptor NR4A1 is expressed in many immune cells, such as T-cells and myeloid cells. NR4A1 is necessary for proper T-cell function and development (28, 29). It has likewise been shown to be important for the development and function of several subsets of macrophages, and as an important regulator of macrophage polarization (12–16). Little is known about the role of NR4A1 in neutrophils, though human and murine neutrophils express all three of the transcripts for Nur nuclear receptors, *Nr4a1*/Nur77, *Nr4a2*/Nurr1, and *Nr4a3*/Nor1, in response to inflammatory stimuli (30, 31). Highly expressed in numerous immune cell types, we sought to identify cellular mechanisms for NR4A1 in order to elucidate its potential as a target for immunomodulatory therapies.

The current study aimed to determine the role of NR4A1 in *K. pneumoniae* pulmonary infection. Prior studies have described a limited or detrimental role for NR4A1 in bacterial infections (19, 21). To date, the only study of bacterial lung infection employed an *E. coli* model, in which *Nr4a1*^{-/-} mice had improved bacterial clearance and survival (21). However, the clinical

NR4A1 in *K. pneumoniae* Lung Infection

relevance is limited, as *E. coli* is not a cause of community or hospital acquired pneumonia, and little mechanistic information was ascertained from this work. Conversely, our study determined that *Nr4a1* loss was detrimental in the *K. pneumoniae* pneumonia model. Mechanistically, the observation that NR4A1 deficiency did not impair macrophage or neutrophil cell recruitment to the lung suggested that NR4A1 may be important for innate antibacterial cell-intrinsic function. While some genes were unchanged, reduced lung expression of the innate pro-inflammatory cytokines, *Il1 β* and *Il6*, as well as the neutrophil recruitment chemokine, *Cxcl2*, 5 hours after infection suggested an innate phagocyte response pathway defect (32). Several disease models have shown that macrophage or monocyte expression of NR4A1 has significant impact on inflammation in models of cardiovascular disease, LPS-induced sepsis, and fibrosis (6, 7, 17, 18, 33, 34). However, in each of these models, NR4A1 modulated macrophage inflammatory genes in response to insult or the deletion of *Nr4a1* resulted in an *exaggerated* macrophage inflammatory phenotype, while we observed *reduced* pro-inflammatory cytokines in *K. pneumoniae* infected *Nr4a1*^{-/-} lung tissue, which prompted further study into cell-specific responses to *K. pneumoniae* infection in the context of NR4A1.

Given that our work and others indicated *Nr4a1*^{-/-} macrophages had a dysregulated tolerization phenotype, it was unexpected that *Nr4a1*^{-/-} macrophages retained *K. pneumoniae* killing ability (18). Despite genetic profiles noting that *Nr4a1* is induced in neutrophils in response to inflammatory stimuli, no prior work has determined what role *Nr4a1* has in the neutrophil response, and the finding that *Nr4a1* is critically important in neutrophils, and not macrophages, has not been previously reported (30, 31). Specifically, reduced *Nr4a1*^{-/-} neutrophil *Mpo*, *Lcn2*, *Lyz2*, and *Il1 β* gene expression suggests that *Nr4a1* deletion impairs antimicrobial responses by

NR4A1 in *K. pneumoniae* Lung Infection

regulating cell-intrinsic early inflammatory gene expression. Alternatively, it may be that deletion of *Nr4a1* leads to a dysfunctional metabolic response in neutrophils following *K. pneumoniae* infection, as *Nr4a1* has profound impacts on T-cell and macrophage immunometabolism in several disease models (28, 35, 36). *Nr4a1*, as well as its family members *Nr4a2* and *Nr4a3*, are also known to be important in the stimulation of lipolysis and glucose utilization, and all are induced in neutrophils among other lipolysis-related genes in response to inflammatory stimuli (31, 37). Regardless, further study is necessary to dissect the molecular, cell-specific mechanism of NR4A1.

This work is not without limitations. Most prominently, all data represented here were performed in mouse models, and thus remains to be determined what role *Nr4a1* possesses in human neutrophil function. Though prior work suggests that *Nr4a1* is induced in human neutrophils responding to *E. coli*, human and murine neutrophils differ in numerous ways, including abundance and protein makeup (30, 38). Further study is required to discern what role, if any, NR4A1 has in the inflammatory response of human neutrophils. In addition, confirming the role of *Nr4a1* neutrophil-specific function *in vivo* remains undetermined and requires experimental approaches not currently available. We must also acknowledge that *in vitro* study of neutrophils is limited by the short, hours long half-life the cells have *ex vivo* without manipulation, complicating classical *in vitro* molecular and genetic experimental approaches (39).

Further study into the use of NR4A1 as a potential therapeutic target for host-immunity responses is also warranted. While the present study shows the deletion of *Nr4a1* to be

NR4A1 in *K. pneumoniae* Lung Infection

detrimental to murine clearance of *K. pneumoniae* pneumonia through loss of neutrophil function, further study of the ability to rescue or enhance NR4A1 function is arranged in order to determine this potential, including the use of the NR4A1 agonist, cytosporone B, or the NR4A1 ligand-binding chemical, PDNPA, in the context of *K. pneumoniae* (11, 17, 40).

The current study also only looked at the role of NR4A1 in *K. pneumoniae* pneumonia. While *K. pneumoniae* is a prominent cause of pneumonia, it is not the only bacteria to do so, nor does *K. pneumoniae* only infect the lungs. More work should be done to determine if NR4A1 has a role in the response to other infectious bacteria (e.g. *S. pneumoniae*, *H. influenzae*) and viruses (coronavirus, influenza). Similarly, future studies should also be conducted to determine if the role of NR4A1 in host immune response is specific to the lungs, or if NR4A1 immune responses are important for other organ-specific infections.

The current study illustrates that *Nr4a1* is an important component in host immune response to *K. pneumoniae* pulmonary infection, specifically in neutrophils, marking NR4A1 as a potential target for future immunotherapies. Finding potential immunotherapy targets against bacterial infections is an increasing concern for the public health community as antibiotic-resistant strains continue to increase. Demonstrating the role NR4A1 has in host immune response to *K. pneumoniae* provides a rational biologic target around which to develop immunotherapies. Additionally, the novel finding that *Nr4a1* is an essential factor in neutrophil-specific response to *K. pneumoniae* infection can lead to future understanding of neutrophil-specific mechanisms in response to infection and how to modulate them.

NR4A1 in *K. pneumoniae* Lung Infection

METHODS

Klebsiella pneumoniae strain and inoculation

Klebsiella pneumoniae strain 396, a K1 serotype, was used for all experiments with a desired inoculation of 5×10^3 colony forming units (CFU) per mouse for *in vivo* experiments and 2.5×10^6 CFU per well for *in vitro* experiments. *K. pneumoniae* isolates were stored at -80°C in Luria-Bertani broth (LB; Thermo Fisher Scientific, Waltham, MA) supplemented with 15% glycerol.

Working cultures were grown overnight (18 hours) in 2mL of Trypticase soy broth (TSB; Sigma Aldrich, St. Louis, MO) at 37°C and 250 RPM. The next day, 20 μL *K. pneumoniae* was sub-cultured into 2mL TSB and grown for ~2 hours at 37°C and 250 RPM to reach the bacterial growth log phase and a concentration of 1×10^9 CFU mL^{-1} . The sub-cultured *K. pneumoniae* was then spun down at 5000G for 5 minutes and resuspended in 1mL 1XPBS (Gibco, Gaithersburg, MD). Sub-cultured *K. pneumoniae* was then diluted 1:10000 (1×10^5 concentration) in 1XPBS for *in vivo* experiments or 1:20 (5×10^7 concentration) for *in vitro* experiments. 50 μL of 1×10^5 inoculum was administered intratracheally to mice under 1:1 isoflurane for *in vivo* experiments. For *in vitro* experiments, 5mL of the 1:20 dilution was added to 5mL 1XPBS and 100 μL of that solution was added to the well. Inoculum concentrations were confirmed by dilution series and LB agar plate CFU calculation.

Animal models

Wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and *Nr4a1* global knockout mice (*Nr4a1*^{tm1Jmi/J}; Jackson Laboratory, Bar Harbor, ME) were housed in a pathogen-free environment with free access to autoclaved water and irradiated pellet food. All experiments

NR4A1 in *K. pneumoniae* Lung Infection

were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh School of Medicine.

All *in vivo* experiments were performed on sex-matched mice between 8-10 weeks old. At the start of the experiment, mice were weighed and inoculated with 5×10^3 *Klebsiella pneumoniae* CFUs. After 24 or 48 hours, mice were weighed again and euthanized under isoflurane and a secondary terminal bleed. The left lobe of the lung and the spleen was extracted into 1mL 1XPBS and homogenized for serial dilution and LB agar plate CFU calculation. The middle lobe of the right lung was extracted and placed into RNeasy lysis buffer (Qiagen by Thermo Fisher Scientific, Carlsbad, CA) for RNA purification and the top and bottom lobes of the right lung were harvested for flow cytometry.

For BALF, 9-week-old male mice were inoculated with 5×10^3 *Klebsiella pneumoniae* CFUs. After 5 hours, the mice were euthanized under isoflurane and a secondary terminal bleed and BALF was acquired by flushing the lungs with 1mL sterile 1XPBS. The 5-hour timepoint was selected based on prior LPS-induced acute lung injury (ALI) data suggesting neutrophil influx to the lungs starts as early as 2 hours after insult and as early as 3 hours after a gram-negative bacterial insult (41–43).

Flow cytometry

To obtain single lung cell suspensions, lung lobe was collected and digested in DMEM containing 4 mg mL^{-1} Collagenase (Sigma Aldrich, St. Louis, MO) and 0.2 mg mL^{-1} Dnase (Sigma Aldrich, St. Louis, MO) at 37°C with agitation for 1 hour, followed by straining digest

NR4A1 in *K. pneumoniae* Lung Infection

through a 70µm filter. Strained cell pellets were collected by centrifugation at 500xg for 5 minutes and red blood cells were lysed using ACK Lysing Buffer (Gibco, Carlsbad, CA) according to manufacturer recommendations. Total lung cells were enumerated with 0.2% trypan blue solution and an Invitrogen Countess automated cell counter.

Cells were then subjected to Fc blockade with anti-CD16/CD32 (Thermo Fisher Scientific, Waltham, MA), washed with 1XPBS, then stained with surface and/or intracellular flow cytometry antibodies specific to antigens of interest using eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA) according to manufacturer protocol. Cells were labeled for detection with surface antibodies to CD11c (HL3, BD Biosciences, San Jose, CA), SiglecF (E50-2440, BD Biosciences, San Jose, CA), CD11b (M1/70, Fisher Scientific, Waltham, MA) and Ly6G (1A8, BioLegend, San Diego, CA). Data acquisition was performed on an LSR Fortessa (SORP, BD Biosciences, San Jose, CA) using FACSDiva software version 8.0.1. Data was analyzed using FlowJo software version 10.1 (TreeStar, Ashland, OR). Cells were enumerated with 0.2% trypan blue solution and an Invitrogen Countess automated cell counter. Absolute cell numbers per mouse lung were enumerated using the total lung cell digest count and the flow cytometry percentage of their respective cell type.

Bone marrow macrophage and neutrophil isolation

Macrophages.

Age matched mice between 8-10 weeks old were euthanized under isoflurane and a secondary terminal bleed. Both femurs of each mouse were extracted and flushed with DMEM media

NR4A1 in *K. pneumoniae* Lung Infection

(Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin-Glutamine through a 100µm filter into a 50mL conical tube. Cells were spun down at 500xg for 5 minutes, red blood cell lysed with ACK lysis buffer (Gibco, Carlsbad, CA) for 1 minute, then spun down again at 500g for 5 minutes.

Again, cells were enumerated with 0.2% trypan blue solution and an Invitrogen Countess automated cell counter. 1×10^7 cells were plated in 10cm dishes in 10mL of DMEM media supplemented with 10% FBS, Penicillin-Streptomycin-Glutamine, and M-CSF (Life Technologies, Carlsbad, CA) brought to a concentration of 20 ng mL^{-1} . Cells were incubated at 37°C and 5.0% CO_2 for 7 days before being harvested in unsupplemented DMEM and used in the bacterial killing assay.

Neutrophils.

Age matched mice between 8-10 weeks old were euthanized under isoflurane and a secondary terminal bleed. The isolation protocol was modified based on the neutrophil isolation protocol developed by Swamydas and Lionakis (44). Both femurs of each mouse were extracted and flushed with RPMI 1640 media (Fisher Scientific, Waltham, MA) supplemented with 10% FBS and 1mM EDTA (Gibco, Carlsbad, CA) through a 100µm filter into a 50mL conical tube. Cells were centrifuged for 7 minutes at 427xg and 4°C . Cells were then red blood cell lysed by adding 2mL 0.2% NaCl for 20 seconds and 2mL 1.6% NaCl. Cells were spun down again at the previous settings, washed with the supplemented RPMI media, and spun again. Cells were resuspended in 3mL PBS and counted using 0.2% trypan blue solution and an Invitrogen Countess automated cell counter.

NR4A1 in *K. pneumoniae* Lung Infection

Once counted, cells were overlaid on a 40%/70% Percoll (Fisher Scientific, Waltham, MA) gradient and centrifuged for 30 minutes at 427xg and 28°C. After 30 minutes, neutrophils were collected from the interface of the 40% and 70% Percoll layers, washed twice with unsupplemented RPMI 1640 and spun at 427xg and 4°C for 7 minutes, and resuspended in 1mL PBS. Neutrophils were counted and then plated in a 96-well plate at 2.5×10^5 neutrophils/well. Neutrophils were incubated at 37°C and 5.0% CO₂ for a 1-hour recovery period before being used in a bacterial killing assay.

Bacterial killing assay

Both bone marrow macrophages and bone marrow neutrophils were individually plated in a 96-well plate at 2.5×10^5 cells per well in their respective media. *K. pneumoniae* was added at a concentration of 2.5×10^6 CFU per well for a multiplicity of infection (MOI) of 10. The plates were then incubated at 37°C and 5.0% CO₂ on an orbital shaker set to 250 RPM.

Supernatants were collected at 30 and 60 minutes (for neutrophils) or 30 and 60 minutes (for macrophages) and the bacterial burdens of each well were calculated using serial dilutions of the supernatants and LB agar plate CFU calculations. In addition, cells were lysed using RNA lysis buffer (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) and stored for RNA extraction and qPCR analysis.

RNA extraction and qPCR

NR4A1 in *K. pneumoniae* Lung Infection

For lung gene expression, the middle lobe of the right lung lobe was homogenized in 1mL of RNA lysis buffer (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) and RNA was purified per the manufacturer's procedures. Cellular RNA was extracted by aspirating media and plating 300μL of the same RNA lysis buffer onto the cells directly after the killing assay. RNA was purified per the manufacturer's procedures. RNA was quantified using the Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) and 40ng of RNA for lung or 5-10ng of RNA for cells was converted to cDNA with iScript (Biorad, Hercules, CA). Real-time PCR was done on the cDNA with SYBR green master mix (Biorad, Hercules, CA) or Taqman master mix (Applied Biosystems, Foster City, CA) depending on primer probe. Primer probe assays were from ordered from Applied Biosciences or IDT. All lung gene expression was compared to the housekeeper gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) and all cellular gene expression was compared to the housekeeper gene beta-2-microglobulin (B2M).

ELISA protein analysis

For protein analysis of the whole lung, the left lung lobe was homogenized in 1mL of PBS and then diluted 1:10 to use in the IL1-β (Thermo Fisher Scientific, Waltham, MA) and MPO (R&D Systems, Minneapolis, MN) ELISAs. After collection of BALF, the BALF was spun at 500xg for 5 minutes to separate lung cells from the fluid. Undiluted BALF was used in the IL1-β and MPO ELISAs. All ELISAs were conducted per the manufacturer's procedures. Plates were read at 450nm with a correctional wavelength of 540nm (Synergy H1, BioTek, Winooski, VT).

Statistical analysis. Investigators were not blinded to treatment but were blinded to individual/group during data analysis. All *in vivo* and *in vitro* statistical analyses were

NR4A1 in *K. pneumoniae* Lung Infection

389 performed using Prism 7 (GraphPad, San Diego, CA). Briefly, all data are presented with mean $\pm$
 390 SEM. All studies comparing two groups were analyzed by two-sided student's *t*-test or by the
 391 Mann-Whitney *U* test when the *F*-value was significant. All statistical analyses considered *P* <
 392 0.05 significant.

393

394 **ACKNOWLEDGEMENTS**

395 This work was supported by funding from the National Institutes of Health K08HL128809
 396 (B.T.C.) and in part by UPMC Children's Hospital of Pittsburgh. Special thanks to Dr. Taylor
 397 Eddens for insightful discussions of this work.

398

399 **CONFLICT OF INTEREST**

400 The authors declare no conflict of interest.

NR4A1 in *K. pneumoniae* Lung Infection

REFERENCES

1. Magill SS, Edwards JR, Bamberg W, Beldavs ZG, Dumyati G, Kainer MA, Lynfield R, Maloney M, McAllister-Hollod L, Nadle J, Ray SM, Thompson DL, Wilson LE, Fridkin SK, Emerging Infections Program Healthcare-Associated Infections and Antimicrobial Use Prevalence Survey Team. 2014. Multistate point-prevalence survey of health care-associated infections. N Engl J Med 370:1198–1208.
2. Sakkas H, Bozidis P, Ilia A, Mpekoulis G, Papadopoulou C. 2019. Antimicrobial Resistance in Bacterial Pathogens and Detection of Carbapenemases in Klebsiella pneumoniae Isolates from Hospital Wastewater. 3. Antibiotics 8:85.
3. Paterson DL, Bonomo RA. 2005. Extended-Spectrum β -Lactamases: a Clinical Update. CMR 18:657–686.
4. Estrada SM, Thagard AS, Dehart MJ, Damicis JR, Dornisch EM, Ippolito DL, Burd I, Napolitano PG, Ieronimakis N. 2020. The orphan nuclear receptor Nr4a1 mediates perinatal neuroinflammation in a murine model of preterm labor. Cell death & disease 11:11.
5. Rothe T, Ipseiz N, Faas M, Lang S, Perez-Branguli F, Metzger D, Ichinose H, Winner B, Schett G, Krönke G. 2017. The Nuclear Receptor Nr4a1 Acts as a Microglia Rheostat and Serves as a Therapeutic Target in Autoimmune-Driven Central Nervous System Inflammation. J Immunol 198:3878–3885.
6. Hamers AAJ, Vos M, Rassam F, Marinković G, Marincovic G, Kurakula K, van Gorp PJ, de Winther MPJ, Gijbels MJJ, de Waard V, de Vries CJM. 2012. Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. Circ Res 110:428–438.

NR4A1 in *K. pneumoniae* Lung Infection

- 422 7. Hamers AAJ, Hanna RN, Nowyhed H, Hedrick CC, de Vries CJM. 2013. NR4A Nuclear
423 Receptors in Immunity and Atherosclerosis. *Curr Opin Lipidol* 24:381–385.
- 424 8. Reddy AT, Lakshmi SP, Banno A, Jadhav SK, Pulikkal Kadamberi I, Kim SC, Reddy RC.
425 2020. Cigarette smoke downregulates Nur77 to exacerbate inflammation in chronic
426 obstructive pulmonary disease (COPD). *PLoS One* 15.
- 427 9. Kurakula K, Vos M, Logiantara A, Roelofs JJ, Nieuwenhuis MA, Koppelman GH, Postma
428 DS, Rijk LS van, Vries CJM de. 2015. Nuclear Receptor Nur77 Attenuates Airway
429 Inflammation in Mice by Suppressing NF- κ B Activity in Lung Epithelial Cells. *The Journal*
430 *of Immunology* 195:1388–1398.
- 431 10. Banno A, Lakshmi SP, Reddy AT, Kim SC, Reddy RC. 2019. Key Functions and
432 Therapeutic Prospects of Nur77 in Inflammation Related Lung Diseases. *Am J Pathol*
433 189:482–491.
- 434 11. Egarnes B, Blanchet M-R, Gosselin J. 2017. Treatment with the NR4A1 agonist
435 cytosporone B controls influenza virus infection and improves pulmonary function in
436 infected mice. *PLOS ONE* 12:e0186639.
- 437 12. Tacke R, Hilgendorf I, Garner H, Waterborg C, Park K, Nowyhed H, Hanna RN, Wu R,
438 Swirski FK, Geissmann F, Hedrick CC. 2015. The transcription factor NR4A1 is essential
439 for the development of a novel macrophage subset in the thymus. *Sci Rep* 5:10055.
- 440 13. Hanna RN, Carlin LM, Hubbeling HG, Nackiewicz D, Green AM, Punt JA, Geissmann F,
441 Hedrick CC. 2011. The transcription factor NR4A1 (Nur77) controls bone marrow
442 differentiation and the survival of Ly6C – monocytes. 8. *Nature Immunology* 12:778–785.

NR4A1 in *K. pneumoniae* Lung Infection

- 443 14. Pei L, Castrillo A, Tontonoz P. 2006. Regulation of macrophage inflammatory gene
444 expression by the orphan nuclear receptor Nur77. *Mol Endocrinol* 20:786–794.
- 445 15. Hanna RN, Shaked I, Hubbeling HG, Punt JA, Wu R, Herrley E, Zaugg C, Pei H,
446 Geissmann F, Ley K, Hedrick CC. 2012. NR4A1 (Nur77) deletion polarizes macrophages
447 toward an inflammatory phenotype and increases atherosclerosis. *Circ Res* 110:416–427.
- 448 16. Shaked I, Hanna RN, Shaked H, Chodaczek G, Nowyhed HN, Tweet G, Tacke R, Basat
449 AB, Mikulski Z, Togher S, Miller J, Blatchley A, Salek-Ardakani S, Darvas M, Kaikkonen
450 MU, Thomas G, Lai-Wing-Sun S, Rezk A, Bar-Or A, Glass CK, Bandukwala H, Hedrick
451 CC. 2015. The orphan nuclear receptor Nr4a1 couples sympathetic and inflammatory cues
452 in CNS-recruited macrophages to limit neuroinflammation. *Nat Immunol* 16:1228–1234.
- 453 17. Li L, Liu Y, Chen H, Li F, Wu J, Zhang H, He J, Xing Y, Chen Y, Wang W, Tian X, Li A,
454 Zhang Q, Huang P, Han J, Lin T, Wu Q. 2015. Impeding the interaction between Nur77 and
455 p38 reduces LPS-induced inflammation. *Nat Chem Biol* 11:339–346.
- 456 18. Henkel M, Partyka J, Gregory AD, Forno E, Cho MH, Eddens T, Tout AR, Salamacha N,
457 Horne W, Rao KS, Wu Y, Alcorn JF, Kostka D, Hirsch R, Celedón JC, Shapiro SD, Kolls
458 JK, Campfield BT. 2020. FSTL-1 Attenuation Causes Spontaneous Smoke-Resistant
459 Pulmonary Emphysema. *Am J Respir Crit Care Med* 201:934–945.
- 460 19. Hamers AAJ, Uleman S, van Tiel CM, Kruijswijk D, van Stalborch A-M, Huveneers S, de
461 Vries CJM, van 't Veer C. 2014. Limited Role of Nuclear Receptor Nur77 in *Escherichia*
462 *coli*-Induced Peritonitis. *Infect Immun* 82:253–264.

NR4A1 in *K. pneumoniae* Lung Infection

- 463 20. Hamers AAJ, Dam L van, Duarte JMT, Vos M, Marinković G, Tiel CM van, Meijer SL,
464 Stalborch A-M van, Huveneers S, Velde AA te, Jonge WJ de, Vries CJM de. 2015.
465 Deficiency of Nuclear Receptor Nur77 Aggravates Mouse Experimental Colitis by
466 Increased NFκB Activity in Macrophages. PLOS ONE 10:e0133598.
- 467 21. Cui P, Wu S, Xu X, Ye H, Hou J, Liu X, Wang H, Fang X. 2019. Deficiency of the
468 Transcription Factor NR4A1 Enhances Bacterial Clearance and Prevents Lung Injury
469 During Escherichia Coli Pneumonia. Shock 51:787–794.
- 470 22. Hirche TO, Gaut JP, Heinecke JW, Belaouaj A. 2005. Myeloperoxidase plays critical roles
471 in killing Klebsiella pneumoniae and inactivating neutrophil elastase: effects on host
472 defense. J Immunol 174:1557–1565.
- 473 23. Bengoechea JA, Sa Pessoa J. 2019. Klebsiella pneumoniae infection biology: living to
474 counteract host defences. FEMS Microbiol Rev 43:123–144.
- 475 24. Broug-Holub E, Toews GB, van Iwaarden JF, Strieter RM, Kunkel SL, Paine R, Standiford
476 TJ. 1997. Alveolar macrophages are required for protective pulmonary defenses in murine
477 Klebsiella pneumonia: elimination of alveolar macrophages increases neutrophil
478 recruitment but decreases bacterial clearance and survival. Infect Immun 65:1139–1146.
- 479 25. Kovach MA, Standiford TJ. 2012. The function of neutrophils in sepsis. Curr Opin Infect
480 Dis 25:321–327.
- 481 26. Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR, Schwarzenberger P, Shellito
482 JE, Kolls JK. 2001. Interleukin-17 and Lung Host Defense against Klebsiella pneumoniae
483 Infection. Am J Respir Cell Mol Biol 25:335–340.

NR4A1 in *K. pneumoniae* Lung Infection

27. Moore TA, Perry ML, Getsoian AG, Newstead MW, Standiford TJ. 2002. Divergent Role of Gamma Interferon in a Murine Model of Pulmonary versus Systemic *Klebsiella pneumoniae* Infection. *Infect Immun* 70:6310–6318.
28. Liu X, Wang Y, Lu H, Li J, Yan X, Xiao M, Hao J, Alekseev A, Khong H, Chen T, Huang R, Wu J, Zhao Q, Wu Q, Xu S, Wang X, Jin W, Yu S, Wang Y, Wei L, Wang A, Zhong B, Ni L, Liu X, Nurieva R, Ye L, Tian Q, Bian X-W, Dong C. 2019. Genome-wide analysis identifies NR4A1 as a key mediator of T cell dysfunction. *Nature* 567:525–529.
29. Sekiya T, Kashiwagi I, Yoshida R, Fukaya T, Morita R, Kimura A, Ichinose H, Metzger D, Chambon P, Yoshimura A. 2013. Nr4a receptors are essential for thymic regulatory T cell development and immune homeostasis. *Nat Immunol* 14:230–237.
30. Zhang X, Kluger Y, Nakayama Y, Poddar R, Whitney C, DeTora A, Weissman SM, Newburger PE. 2004. Gene expression in mature neutrophils: early responses to inflammatory stimuli. *Journal of Leukocyte Biology* 75:358–372.
31. Ericson JA, Duffau P, Yasuda K, Ortiz-Lopez A, Rothamel K, Rifkin IR, Monach PA, ImmGen Consortium. 2014. Gene expression during the generation and activation of mouse neutrophils: implication of novel functional and regulatory pathways. *PLoS ONE* 9:e108553.
32. Schaum N, Karkanias J, Neff NF, May AP, Quake SR, Wyss-Coray T, Darmanis S, Batson J, Botvinnik O, Chen MB, Chen S, Green F, Jones RC, Maynard A, Penland L, Pisco AO, Sit RV, Stanley GM, Webber JT, Zanini F, Baghel AS, Bakerman I, Bansal I, Berdnik D, Bilen B, Brownfield D, Cain C, Chen MB, Chen S, Cho M, Cirolia G, Conley SD,

NR4A1 in *K. pneumoniae* Lung Infection

505 Darmanis S, Demers A, Demir K, de Morree A, Divita T, du Bois H, Dulgeroff LBT, Ebadi
506 H, Espinoza FH, Fish M, Gan Q, George BM, Gillich A, Green F, Genetiano G, Gu X,
507 Gulati GS, Hang Y, Hosseinzadeh S, Huang A, Iram T, Isobe T, Ives F, Jones RC, Kao KS,
508 Karnam G, Kershner AM, Kiss BM, Kong W, Kumar ME, Lam JY, Lee DP, Lee SE, Li G,
509 Li Q, Liu L, Lo A, Lu W-J, Manjunath A, May AP, May KL, May OL, Maynard A, McKay
510 M, Metzger RJ, Mignardi M, Min D, Nabhan AN, Neff NF, Ng KM, Noh J, Patkar R, Peng
511 WC, Penland L, Puccinelli R, Rulifson EJ, Schaum N, Sikandar SS, Sinha R, Sit RV, Szade
512 K, Tan W, Tato C, Tellez K, Travaglini KJ, Tropini C, Waldburger L, van Weele LJ,
513 Wosczyzna MN, Xiang J, Xue S, Youngyunpipatkul J, Zanini F, Zardeneta ME, Zhang F,
514 Zhou L, Bansal I, Chen S, Cho M, Cirolia G, Darmanis S, Demers A, Divita T, Ebadi H,
515 Genetiano G, Green F, Hosseinzadeh S, Ives F, Lo A, May AP, Maynard A, McKay M,
516 Neff NF, Penland L, Sit RV, Tan W, Waldburger L, Youngyunpipatkul J, Batson J,
517 Botvinnik O, Castro P, Croote D, Darmanis S, DeRisi JL, Karkanias J, Pisco AO, Stanley
518 GM, Webber JT, Zanini F, Baghel AS, Bakerman I, Batson J, Bilen B, Botvinnik O,
519 Brownfield D, Chen MB, Darmanis S, Demir K, de Morree A, Ebadi H, Espinoza FH, Fish
520 M, Gan Q, George BM, Gillich A, Gu X, Gulati GS, Hang Y, Huang A, Iram T, Isobe T,
521 Karnam G, Kershner AM, Kiss BM, Kong W, Kuo CS, Lam JY, Lehallier B, Li G, Li Q,
522 Liu L, Lu W-J, Min D, Nabhan AN, Ng KM, Nguyen PK, Patkar R, Peng WC, Penland L,
523 Rulifson EJ, Schaum N, Sikandar SS, Sinha R, Szade K, Tan SY, Tellez K, Travaglini KJ,
524 Tropini C, van Weele LJ, Wang BM, Wosczyzna MN, Xiang J, Yousef H, Zhou L, Batson J,
525 Botvinnik O, Chen S, Darmanis S, Green F, May AP, Maynard A, Pisco AO, Quake SR,
526 Schaum N, Stanley GM, Webber JT, Wyss-Coray T, Zanini F, Beachy PA, Chan CKF, de
527 Morree A, George BM, Gulati GS, Hang Y, Huang KC, Iram T, Isobe T, Kershner AM,

NR4A1 in *K. pneumoniae* Lung Infection

- 528 Kiss BM, Kong W, Li G, Li Q, Liu L, Lu W-J, Nabhan AN, Ng KM, Nguyen PK, Peng
529 WC, Rulifson EJ, Schaum N, Sikandar SS, Sinha R, Szade K, Travaglini KJ, Tropini C,
530 Wang BM, Weinberg K, Wosczyzna MN, Wu SM, Yousef H, Barres BA, Beachy PA, Chan
531 CKF, Clarke MF, Darmanis S, Huang KC, Karkanias J, Kim SK, Krasnow MA, Kumar
532 ME, Kuo CS, May AP, Metzger RJ, Neff NF, Nusse R, Nguyen PK, Rando TA,
533 Sonnenburg J, Wang BM, Weinberg K, Weissman IL, Wu SM, Quake SR, Wyss-Coray T,
534 The Tabula Muris Consortium, Overall coordination, Logistical coordination, Organ
535 collection and processing, Library preparation and sequencing, Computational data
536 analysis, Cell type annotation, Writing group, Supplemental text writing group, Principal
537 investigators. 2018. Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris.
538 7727. Nature 562:367–372.
- 539 33. Li X-M, Zhang S, He X-S, Guo P-D, Lu X-X, Wang J-R, Li J-M, Wu H. 2016. Nur77-
540 mediated TRAF6 signalling protects against LPS-induced sepsis in mice. J Inflamm (Lond)
541 13.
- 542 34. Hamers AAJ, Argmann C, Moerland PD, Koenis DS, Marinković G, Sokolović M, de Vos
543 AF, de Vries CJM, van Tiel CM. 2016. Nur77-deficiency in bone marrow-derived
544 macrophages modulates inflammatory responses, extracellular matrix homeostasis,
545 phagocytosis and tolerance. BMC Genomics 17:162.
- 546 35. Liebmann M, Hucke S, Koch K, Eschborn M, Ghelman J, Chasan AI, Glander S, Schädlich
547 M, Kuhlencord M, Daber NM, Eveslage M, Beyer M, Dietrich M, Albrecht P, Stoll M,
548 Busch KB, Wiendl H, Roth J, Kuhlmann T, Klotz L. 2018. Nur77 serves as a molecular

NR4A1 in *K. pneumoniae* Lung Infection

- 549 brake of the metabolic switch during T cell activation to restrict autoimmunity. PNAS
- 550 115:E8017–E8026.
- 551 36. Koenis DS, Medzikovic L, van Loenen PB, van Weeghel M, Huveneers S, Vos M, Evers-
- 552 van Gogh IJ, Van den Bossche J, Speijer D, Kim Y, Wessels L, Zelcer N, Zwart W,
- 553 Kalkhoven E, de Vries CJ. 2018. Nuclear Receptor Nur77 Limits the Macrophage
- 554 Inflammatory Response through Transcriptional Reprogramming of Mitochondrial
- 555 Metabolism. Cell Rep 24:2127-2140.e7.
- 556 37. Zhao Y, Bruemmer D. 2010. NR4A orphan nuclear receptors: transcriptional regulators of
- 557 gene expression in metabolism and vascular biology. Arterioscler Thromb Vasc Biol
- 558 30:1535–1541.
- 559 38. Mestas J, Hughes CCW. 2004. Of mice and not men: differences between mouse and
- 560 human immunology. J Immunol 172:2731–2738.
- 561 39. Boxio R, Bossenmeyer-Pourié C, Steinckwich N, Dournon C, Nüße O. 2004. Mouse bone
- 562 marrow contains large numbers of functionally competent neutrophils. Journal of
- 563 Leukocyte Biology 75:604–611.
- 564 40. Zhan Y, Du X, Chen H, Liu J, Zhao B, Huang D, Li G, Xu Q, Zhang M, Weimer BC, Chen
- 565 D, Cheng Z, Zhang L, Li Q, Li S, Zheng Z, Song S, Huang Y, Ye Z, Su W, Lin S-C, Shen
- 566 Y, Wu Q. 2008. Cyclosporine B is an agonist for nuclear orphan receptor Nur77. Nat Chem
- 567 Biol 4:548–556.

NR4A1 in *K. pneumoniae* Lung Infection

- 568 41. Szarka RJ, Wang N, Gordon L, Nation PN, Smith RH. 1997. A murine model of pulmonary
569 damage induced by lipopolysaccharide via intranasal instillation. *Journal of Immunological*
570 *Methods* 202:49–57.
- 571 42. Dubin PJ, Martz A, Eisenstatt JR, Fox MD, Logar A, Kolls JK. 2012. Interleukin-23-
572 Mediated Inflammation in *Pseudomonas aeruginosa* Pulmonary Infection. *Infect Immun*
573 80:398–409.
- 574 43. Greenberger MJ, Strieter RM, Kunkel SL, Danforth JM, Laichalk LL, McGillicuddy DC,
575 Standiford TJ. 1996. Neutralization of Macrophage Inflammatory Protein-2 Attenuates
576 Neutrophil Recruitment and Bacterial Clearance in Murine *Klebsiella* Pneumonia. *J Infect*
577 *Dis* 173:159–165.
- 578 44. Swamydas M, Lionakis MS. 2013. Isolation, purification and labeling of mouse bone
579 marrow neutrophils for functional studies and adoptive transfer experiments. *J Vis Exp*
580 e50586.

581

NR4A1 in *K. pneumoniae* Lung Infection

Figure 1. *Nr4a1*^{-/-} mice are more susceptible to *Klebsiella pneumoniae* pulmonary infection

The (a) lung burden and (b) spleen burden of Wild-type (WT, *n*=6) and *Nr4a1* knockout (*Nr4a1*^{-/-}, *n*=8) mice 24 hours post pulmonary *K. pneumoniae* infection. The (c) *K. pneumoniae* lung burden and (d) spleen burden in WT (*n*=7) and *Nr4a1*^{-/-} (*n*=7) mice 48 hours post pulmonary infection. Panels (a) and (b) for the 24-hour lung and spleen burdens are representative of two experiments. Panels (c) and (d) for the 48-hour lung and spleen burdens are pooled from two experiments. Significance was determined by the Student's *t*-test. **P* < 0.05, ***P* < 0.01

Figure 2. *Nr4a1*^{-/-} mice have selectively reduced transcription of pro-inflammatory cytokines and chemokines in BAL cells early post-infection

Transcript abundance for pro-inflammatory cytokines (a) *Il1β*, (b) *Il6*, and (c) *Tnfa*, as well as chemokines (d) *Cxcl1*, (e) *Cxcl2*, and (f) *Cxcl5* from the BAL fluid cells of WT (*n*=5) and *Nr4a1*^{-/-} (*n*=5) mice 5 hours after *K. pneumoniae* infection. These data are representative of two experiments. All gene transcripts were compared to the housekeeper beta-2-microglobulin (*B2M*). Significance was determined by the Student's *t*-test. **P* < 0.05, ***P* < 0.01

Figure 3. *Nr4a1*^{-/-} mice have reduced IL1β and MPO lung protein after *K. pneumoniae* lung infection

Nr4a1^{-/-} mice (*n*=6) have reduced (a) IL1β 5 hours post-infection with *K. pneumoniae* compared to WT controls (*n*=5), though (b) MPO is not significantly reduced. By 24 hours post infection, *Nr4a1*^{-/-} mice (*n*=6) have significantly reduced (c) IL1β and (d) MPO protein compared to WT mice controls (*n*=5). These data are all representative of two experiments. Significance was determined by the Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.005

NR4A1 in *K. pneumoniae* Lung Infection

Figure 4. *Nr4a1* is essential for neutrophil anti-bactericidal function

(a) *Nr4a1* gene expression of unstimulated ($n=5$) and *K. pneumoniae* co-cultured ($n=5$) WT neutrophils. *Nr4a1* gene expression is significantly increased in WT neutrophils co-cultured with *K. pneumoniae*. The (b) *K. pneumoniae* burden from the supernatants of WT ($n=9$) and *Nr4a1*^{-/-} ($n=9$) neutrophils at 30 minutes and 60 minutes (WT $n=9$, *Nr4a1*^{-/-} $n=8$) after co-culture with *K. pneumoniae*. (c) *Nr4a1* gene expression of WT ($n=5$) and *Nr4a1*^{-/-} ($n=5$) neutrophils 60 minutes after *K. pneumoniae* co-culture. WT neutrophils have significantly more *Nr4a1* gene transcript than *Nr4a1*^{-/-} neutrophils. All gene transcripts were normalized to *B2M*. The burden results in panel (b) are pooled from two separate experiments and representative of five experiments total. Panels (a) and (c) are representative of three experiments. Significance was determined by the Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$

Figure 5. *Nr4a1*^{-/-} neutrophils have decreased inflammatory gene expression following *K. pneumoniae* infection

Nr4a1^{-/-} neutrophils ($n=3$) have decreased genetic expression of (a) *Il1 β* and neutrophil-specific genes (b) *Lcn2*, (c) *Mpo*, and (d) *Lyz2* compared to WT controls ($n=5$). These data are all representative of five experiments. All gene transcripts were normalized to *B2M*. Significance was determined by the Mann-Whitney *U*-test. * $P < 0.05$

Supplemental Figure 1. *Nr4a1*^{-/-} and WT mice have similar immune cell abundance following *K. pneumoniae* lung infection

NR4A1 in *K. pneumoniae* Lung Infection

(a) The total amount of lung cells from WT ($n=5$) and *Nr4a1*^{-/-} ($n=6$) mice 24 hours post infection. The (b) total CD45⁺/CD11b⁺/CD11c⁺ (inflammatory macrophages), (c) CD45⁺/CD11c⁺/SiglecF⁺ (alveolar macrophages), and (d) CD45⁺/CD11b⁺/Ly6G⁺ (neutrophils) 24 hours after infection. Data are representative of two individual experiments.

Supplemental Figure 2. *Nr4a1*^{-/-} and WT mice have similar cytokine expression after 24 hours of *K. pneumoniae* lung infection

Transcript abundance for cytokines (a) *Il1β*, (b) *Tnfa*, and (c) *Il6*, as well as the neutrophil-specific gene (d) *Lcn2* from WT ($n=6$) and *Nr4a1*^{-/-} ($n=8$) whole lung. No significant differences were observed between genotypes 24 hours after *K. pneumoniae* pulmonary infection. All gene transcripts were normalized to *HPRT*.

Supplemental Figure 3. FACS gating strategy for myeloid cells

Supplemental Figure 4. *Nr4a1*^{-/-} macrophages retain bactericidal function *in vitro*

(a) *K pneumoniae* burden from the supernatants of WT ($n=5$) and *Nr4a1*^{-/-} ($n=4$) bone marrow macrophages 30 minutes and 60 minutes after co-culture with *K. pneumoniae*. The gene transcript for (b) *Il1β*, (c) *Cxcl2*, and (d) *Il6* at 60 minutes exhibits no difference between WT and *Nr4a1*^{-/-} mice. All gene transcripts were normalized to *B2M*.

a

b

c

d

Figure 1. *Nr4a1*^{-/-} mice are more susceptible to *Klebsiella pneumoniae* pulmonary infection

The (a) lung burden and (b) spleen burden of Wild-type (WT, n=6) and *Nr4a1* knockout (*Nr4a1*^{-/-}, n=8) mice 24 hours post pulmonary *K. pneumoniae* infection. The (c) *K. pneumoniae* lung burden and (d) spleen burden in WT (n=7) and *Nr4a1*^{-/-} (n=7) mice 48 hours post pulmonary infection. Panels (a) and (b) for the 24-hour lung and spleen burdens are representative of two experiments. Panels (c) and (d) for the 48-hour lung and spleen burdens are pooled from two experiments. Significance was determined by the Student's *t*-test. **P* < 0.05, ***P* < 0.01

Figure 2. *Nr4a1*^{-/-} mice have selectively reduced transcription of pro-inflammatory cytokines and chemokines in BAL cells early post-infection
Transcript abundance for pro-inflammatory cytokines (a) *Il1β*, (b) *Il6*, and (c) *Tnfa*, as well as chemokines (d) *Cxcl1*, (e) *Cxcl2*, and (f) *Cxcl5* from the BAL fluid cells of WT (n=5) and *Nr4a1*^{-/-} (n=5) mice 5 hours after *K. pneumoniae* infection. These data are representative of two experiments. All gene transcripts were compared to the housekeeper beta-2-microglobulin (*B2M*). Significance was determined by the Student's *t*-test. * $P < 0.05$, ** $P < 0.01$

Figure 3. *Nr4a1*^{-/-} mice have reduced IL1 β and MPO lung protein after *K. pneumoniae* lung infection

Nr4a1^{-/-} mice (n=6) have reduced (a) IL1 β 5 hours post-infection with *K. pneumoniae* compared to WT controls (n=5), though (b) MPO is not significantly reduced.

By 24 hours post infection, *Nr4a1*^{-/-} mice (n=6) have significantly reduced (c) IL1 β and (d) MPO protein compared to WT mice controls (n=5).

These data are all representative of two experiments. Significance was determined by the Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.005

Figure 4. *Nr4a1* is essential for neutrophil anti-bactericidal function

(a) *Nr4a1* gene expression of unstimulated (n=5) and *K. pneumoniae* co-cultured (n=5) WT neutrophils. *Nr4a1* gene expression is significantly increased in WT neutrophils co-cultured with *K. pneumoniae*. The (b) *K. pneumoniae* burden from the supernatants of WT (n=9) and *Nr4a1*^{-/-} (n=9) neutrophils at 30 minutes and 60 minutes (WT n=9, *Nr4a1*^{-/-} n=8) after co-culture with *K. pneumoniae*. (c) *Nr4a1* gene expression of WT (n=5) and *Nr4a1*^{-/-} (n=5) neutrophils 60 minutes after *K. pneumoniae* co-culture. WT neutrophils have significantly more *Nr4a1* gene transcript than *Nr4a1*^{-/-} neutrophils. All gene transcripts were normalized to *B2M*. The burden results in panel (b) are pooled from two separate experiments and representative of five experiments total. Panels (a) and (c) are representative of three experiments. Significance was determined by the Student's *t*-test. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001

a

b

c

d

Figure 5. *Nr4a1*^{-/-} neutrophils have decreased inflammatory gene expression following *K. pneumoniae* infection

Nr4a1^{-/-} neutrophils (n=3) have decreased genetic expression of (a) *Il1β* and neutrophil-specific genes (b) *Lcn2*, (c) *Mpo*, and (d) *Lyz2* compared to WT controls (n=5). These data are all representative of five experiments. All gene transcripts were normalized to *B2M*. Significance was determined by the Mann-Whitney *U*-test. **P* < 0.05