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Perturbations in Cytokine Gene Expression after Inoculation of C57BL/6 Mice with *Pasteurella pneumotropica*

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Pasteurella pneumotropica **can cause inflammation and abscess formation in a variety of tissues. Most commonly, *P. pneumotropica* produces clinical disease in immunodeficient mice or those concurrently infected with other pathogens. Because clinical disease is infrequent in immunocompetent mice harboring *P. pneumotropica*, some scientists consider it an opportunistic pathogen with little clinical relevance to biomedical research. However, other infectious agents, including mouse parvoviruses, mouse rotavirus, and *Helicobacter* spp. alter physiologic or biologic responses without causing clinical signs of illness. We investigated the potential for *P. pneumotropica* to modulate the transcription of cytokine genes in immunocompetent mice. In C57BL/6 mice inoculated oronasally with a minimal colonizing dose of *P. pneumotropica*, modest but statistically significant elevations of *IL1β*, *TNFα*, *CCL3*, *CXCL1*, and *CXCL2* mRNA were detected in mandibular and superficial cervical lymph nodes at 7 d after inoculation, and upregulation of *IL1β* mRNA was detected 28 d after inoculation. These perturbations were not present in C57/BL6 mice inoculated with heat killed-*P. pneumotropica* or the related bacterium *Actinobacillus muris*. Nasal mucosal cytokine transcription did not vary significantly in C57BL/6 mice given a high dose of *P. pneumotropica*. These data indicate that slight and transient experimental perturbations are possible in immunocompetent mice colonized with *P. pneumotropica*. Knowing the full health status of experimental mice is paramount to avoid unwanted experimental variables, especially when using exquisitely sensitive testing methodologies such as those for quantification of gene expression.**

Abbreviation: qPCR, quantitative real-time PCR.

Pasteurella pneumotropica is a gram-negative, nonhemolytic, nonmotile, coccobacillus that commonly colonizes laboratory rodent species including mice, rats, hamsters, and guinea pigs.[20] Potential routes of transmission include direct contact with infected mice and indirect exposure to contaminated bedding or other fomites, with direct contact being the most efficient method.[17] Historically, *P. pneumotropica* infection was associated with pneumonia but only when concurrent infections with other respiratory pathogens such as Sendai virus or *Mycoplasma pulmonis* were present.[6,8] In contemporary colonies, *P. pneumotropica* infection is usually subclinical but may be associated with sporadic disease, including conjunctivitis, periorbital abscess formation, or inflammation of adnexal tissues.[20] These manifestations occur most commonly in immunodeficient or genetically modified mouse strains.[2,11,12,18] Because clinical disease is infrequent in immunocompetent mice colonized with *P. pneumotropica*, this bacterium is perceived as an opportunist pathogen having little to no clinical relevance to most biomedical research studies.[18] However, infections with other agents, including mouse parvoviruses, mouse rotavirus, and *Helicobacter* spp., can alter host physiology or biologic responses without causing clinical signs of illness.[3] These findings raise the questions of whether *P. pneumotropica* induces an immune response in infected rodents, whether this response is sustained, and what effect such a response could have on ongoing research using infected mice. Given the prevalence of *P. pneumotropica* infection in laboratory rodent colonies (approximately 13% in tested mouse colonies in North America), determining whether this agent can induce unwanted variability in data collected from infected mice is critically important.[16] This information will allow investigators and animal care personnel to make informed decisions on whether *P. pneumotropica* should be excluded from certain studies that might otherwise use subclinically colonized rodents.

Communications between various cells of the immune system are carried out by a group of small regulatory proteins called cytokines that are released by a variety of cells usually in response to a stimulus. Cytokines can act on numerous cellular targets through autocrine and paracrine means and are involved in coordinating immune and inflammatory responses.[5] A widely used indirect method to access cytokine levels is to quantify expression of cytokine genes.[15] Microarray analysis and quantitative real-time PCR (qPCR) assays are 2 methods used to detect changes in mRNA levels and gene expression.[9,15] Although results of both qPCR and microarray analyses should be evaluated with regard to protein levels to determine functional levels of gene expression, evaluation of gene mRNA levels remains an invaluable technique in studies elucidating immune processes.[9,15] Because of the inherent sensitivity of techniques such as qPCR and microarray analysis it is imperative that test subjects remain free of unanticipated

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stimuli that may alter gene expression, such as the stimulus of a subclinical bacterial colonization.

The objective of the work reported here was to determine whether *P. pneumotropica* can induce perturbations in the immune response that could confound interpretation of data collected from infected mice. In particular, we examined experimentally infected mice for histologic evidence of inflammation and alterations of cytokine gene expression in nasal turbinates and regional lymph nodes. We tested mice for upregulation of TNFα and IL1β transcripts—both of these cytokines are known to respond to gram-negative bacteria.[1] To further assess potential upregulation of the murine immune system in response to *P. pneumotropica*, mRNA levels of the cytokine genes *CCL3* (whose product is involved in leukocyte recruitment) and *CXCL1* and *CXCL2* (neutrophil chemoattractants) were measured.[5] Moreover, we assessed whether inoculation with killed *P. pneumotropica* or the *Pasteurellaceae*-related bacterium *Actinobacillus muris* altered gene expression. Killed *P. pneumotropica* was used to assess whether responses required viable bacteria or simply bacterial components, such as the cell wall. *Actinobacillus muris* can colonize the oral cavity of mice, is considered a commensal organism in this species, and has not been shown to cause any pathology in colonized rodents.

Materials and Methods

Animals and husbandry. In light of their overwhelming prevalence in biomedical research, we chose to use female C57BL/6 mice (age, 4 to 5 wk) in our studies; the mice were from the National Cancer Institute at Frederick (Frederick, MD), Harlan (Haslett, MI), and Charles River Laboratories (Kingston, NY). Mice were from colonies known to be free of ectoparasites, endoparasites, and known pathogenic and opportunistic bacteria including but not limited to the cilia-associated respiratory bacillus, *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, *Bordetella bronchiseptica*, and *Klebsiella pneumoniae.* Mice were negative by serology for Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Theiler murine encephalomyelitis virus, reovirus 3, ectromelia virus, lymphocytic choriomeningitis virus, and polyoma virus. Mice were confirmed to be negative for *P. pneumotropica* and *A. muris* prior to study by oral culture and PCR-based speciation as described later. Mice were group-housed under a 12:12-h light:dark cycle in static polycarbonate microisolation rodent cages (Allentown Caging, Allentown, PA) on paper chip bedding (Shepherd Specialty Papers, St Charles, MO). Cages, bedding, and acidified water were steam-autoclaved before use. Irradiated feed (PicoLab Mouse Diet 20/ 5058, Purina Mills, Richland, IN) and water were available ad libidum. Room temperature and humidity were maintained at approximately 22 °C (72 °F) and 37%, respectively. Cage changes and animal manipulation were conducted under a class II laminar-flow hood (SterileGuard BioLevel 2, Baker Company, Sanford, ME). The University of Missouri Institutional Animal Care and Use Committee approved all animal experiments in this study by means of an animal use protocol. All animal work was performed in AAALAC-accredited facilities.

Bacteria cultivation, identification, and dosing. *Pasteurella pneumotropica* biotype Heyl and *Actinobacillus muris* isolates were obtained from naturally infected mice.

Bacterial cultivation. The oral cavities of mice were swabbed and inoculated onto trypticase soy agar (Tryptic Soy Agar, Becton Dickinson, Sparks, MD) containing 5% sheep blood (Quad Five, Ryegate, MT) and incubated for 48 h at 35 °C in an atmosphere containing 7% CO_2. Isolated, small, gray to yellow, smooth, nonhemolytic colonies were subcultured, and 1 to 3 colonies with this morphology were touched with a sterile loop, suspended in 0.5 mL sterile PBS, and speciated by using PCR-based techniques.

PCR identification of P. pneumotropica and A. muris. The *P. pneumotropica* PCR assay used in this study tested for both biotypes Jawetz (GenBank accession no. M75083) and Heyl (AF012090) by amplifying a 278-bp or 306-bp region of the respective 16s rRNA gene. PCR amplification of the 16s rRNA gene of *A. muris* was performed by using a generic bacteria forward primer and a *Pasteurella*-family reverse primer for initial identification. Commercial sequence analysis (SeqWright, Houston, TX) was used to confirm that the isolates used in this study were *P. pneumotropica* biotype Heyl (AF012090) and *A. muris* (AY362894). Sequences for all primer sets are presented in Table 1.

Amplifications of *P. pneumotropica* were performed in a 50-µL reaction containing 10× PCR buffer with 20 mM $MgCl_2$ (Roche Diagnostics, Indianapolis, IN), 200 µM each dNTP (Roche), 2 U FastStart *Taq* DNA polymerase (Roche), and 5µL bacterial suspension. PCR amplification was performed in a programmable thermocycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA), with cell wall disruption at 95 °C for 15 min followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s. Amplicons were electrophoresed through a 3% agarose gel containing ethidium bromide (ReadyAgarose Gel, BioRad, Hercules, CA) and visualized under UV light.

PCR amplification of *A. muris* was similar to that for *P. pneumotropica* with the exception of a 30-s denaturing cycle and a 30-s annealing cycle at 53 °C.

Animal inoculations. Heavy lawns of *P. pneumotropica* and *A. muris* were grown on 5% sheep's blood agar plates for 48 h, harvested by using a loop, and suspended in sterile PBS. Heat-killed *P. pneumotropica* was prepared by heating bacterial suspensions at 80 °C for 1 h in a heat block (Fisher Scientific, Pittsburg, PA). Culture of the heat-killed suspension resulted in no growth on BA plates at the previously stated culture conditions. Mice were restrained manually and dosed orally and intranasally (25 µL each site) with bacterial suspensions by using a pipette. *Pasteurella pneumotropica* and *A. muris* colonization status was confirmed by oral swab cultures and PCR-based speciation as described. Bacterial dosages were determined by performing serial dilutions of bacteria on BA plates incubated at the culture conditions previously described.

RNA extraction from lymph nodes and nasal turbinates. Lymph node samples (mandibular and superficial cervical) were collected under dissection microscope after euthanasia of the mice. The samples were frozen in RNAlater (Ambion, Austin, TX) and stored at –80 °C. The lymph nodes subsequently were disrupted and homogenized (TissueLyser, Qiagen, Valencia, CA), and RNA extraction was completed by using a silica-gel membrane (RNeasy, Qiagen) according to the manufacturer's instructions. Extracted RNA was dissolved in 35 µL RNase-free water (Qiagen).

Turbinate samples were collected by sharp dissection of the muzzle just rostral to the eyes. Samples were frozen in RNAlater (Ambion) and stored at –80 °C. Turbinate samples were thawed in Trizol reagent (Invitrogen, Carlsbad, CA) and homogenized (TissueLyser, Qiagen), and isolation of total RNA was completed

Table 1. Sense and antisense primer pairs used for PCR identification of bacteria

	Sense (5′–3′)	Antisense (5′–3′)	Fragment size (bp)
P. pneumotropica (Jawetz)	TAA GGA CAA AAG GGG GCG TA	GGG TAT TAA CCT TAT CAC C	278
P. pneumotropica (Hyel)	ACC GCG TAA AGT CTT TGG AC	AAC GTC AAT CAG CTT GGC TA	306
A. muris	GCT TAA CAC ATG CAA GTC GAA	GTC AGT ACA TTC CCA AGG	703

according to the manufacturer's instructions (Trizol reagent, Invitrogen). Extracted RNA was dissolved in 50 µL RNase-free water (Qiagen). The quantity and quality of all extracted RNA samples was evaluated by spectrophotometry (ND1000, NanoDrop, Wilmington, DE).

RT-PCR. Aliquots (5 µg) of RNA extracted from lymph node or nasal turbinate were reverse-transcribed by using a commercially available kit and oligo-dT primers (SuperScript III, Invitrogen). Transcribed cDNA was diluted with nuclease-free water (Promega, Madison, WI) to a final concentration of 20 ng/µL.

Primer sequences for hypoxanthine–guanine phosphoribosyltransferase (*HPRT*),[17] *CCL3* (*MIP1*α),[18] and *IL1*β[10] were obtained from the literature. Primer sequences for *TNF*α, *CXCL1* (*KC*), and *CXCL2* (*MIP2*α) were designed from published sequence data by using DS Gene (Accelrys, San Diego, CA). To determine the quantity of mRNA in samples, linearized plasmid standards that contained the amplicon of the selected primer set were developed for each assay by use of a commercially available cloning kit (Topo TA Cloning Kit, Invitrogen). Sequences for all primer pairs are shown in Table 2.

Semiquantitative real-time PCR. qPCR analysis for the housekeeping gene *HPRT* and the cytokines and chemokines *IL1*β, *TNF*α, *CCL3*, *CXCL1*, and *CXCL2* were performed in a thermocycler (Light Cycler, Roche Diagnostics, Indianapolis, IN) by using a 20-µL reaction volume in glass capillaries that contained 0.5 µM each primer, 3 mM $MgCl_2$, 2 µL QuantiTect SYBR Green PCR Master Mix (dNTPs, HotStar *Taq* DNA polymerase, reaction buffer, and SYBR green I; Qiagen), and 50 ng cDNA. To quantify mRNA copy number for the various genes, known concentrations (10^1 to 10^6 copies) of the plasmids containing the amplicon of interest were assayed in parallel with the test samples. HPRT levels were used to normalize mRNA copy number for comparison. Thermocycling parameters were: preincubation at 95 °C for 15 min to activate polymerase; 45 cycles (*HPRT*, *CCL3*, *CXCL1*, and *CXCL2*), 42 cycles (*IL1*β), or 40 cycles (*TNF*α) of denaturation at 94 °C for 15 s; annealing for 20 s at 60 °C (HPRT, CXCL1, CXCL2, IL1 β, and TNFα) or 55 °C (CCL3); and extension at 72 °C for 30 s. The ramp rate for annealing was 3 °C/s and 20 °C/s for all other steps. Fluorescence was monitored at the end of each extension phase for all genes except CXCL2, which was monitored at 78 °C after the extension phase. Amplicons underwent melting curve analysis after PCR amplification to verify product purity.

Targeted microarray analysis. Extracted RNA from nasal turbinate samples with increased cytokine expression via qPCR was pooled for gene expression analysis. Pooled samples (4 total) were collected from experimentally inoculated C57BL/6NCr mice (days 2, 4, 8, and 10) and were compared with a pooled, time-matched, control sample (4 total) from sham-inoculated mice. Extracted RNA (3 µg per pooled sample) was purified (ArrayGrade Total RNA Isolation Kit, SuperArray, Frederick, MD). Biotin-labeled antisense RNA was produced through the use of commercially available kits (TrueLabeling-AMP 2.0 and ArrayGrade cRNA Cleanup Kit, SuperArray) and biotin-16-UTP (Roche). A gene expression array (Oligo GEArray Mouse Inflammatory Cytokines and Receptors Microarray, HybTube Format, SuperArray) and Oligo GEArray Starter Kit (SuperArray) were used to profile the expression of 113 genes involved in the inflammatory response. Expression analysis was conducted by using GEArray Expression Analysis Suite (SuperArray). Expression levels were normalized to those of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein S27a, β2 microglobulin, heat-shock protein 1β, and peptidylprolyl isomerase A. A fold increase in mRNA concentration greater than 1.5 between mice inoculated with *P. pneumotropica* and their controls was considered upregulation.

Histology. Samples of nasal turbinate and lung tissue were placed in neutral phosphate-buffered 10% formalin fixative (EVER Scientific, Exton, PA). Nasal turbinate samples were decalcified by using formic acid and sodium citrate. Formalin-fixed turbinate and lung samples from control and experimental mice were embedded in paraffin, cut into 5-µm sections, and processed for staining with hematoxylin and eosin. Tissues were assessed for lesions including inflammation and hyperplasia by using light microscopy.

Experimental studies. ***Kinetics of local cytokine gene perturbations: pilot study.*** To ascertain whether and when an immune response characterized by local cytokine gene expression occurred after inoculation, female C57BL/6NCr mice ($n = 10$) received 1×10^8 *P. pneumotropica* suspended in PBS. This dose was split between the oral and intranasal routes, with 25 µL given at each site by using a pipette. Cohort control mice ($n = 10$) were sham-inoculated with sterile PBS. Mice were euthanized by overdose of CO_2 on days 2, 4, 6, 8, and 10 after inoculation (2 control and 2 experimental mice per time point). Bacterial status was confirmed by using the described culture and PCR methods. Nasal turbinate samples from each mouse were collected and stored in RNAlater (Ambion) according to the manufacturer's instructions. Samples were used for RNA extraction and gene expression analysis by means of qPCR and targeted microarray analysis.

Minimal infectious dose of P. pneumotropica. To optimally mimic natural infections for subsequent studies, we determined the minimal number of bacteria required for colonization. Nine groups of 3 female C57BL/6 NCr mice received log-fold serial diluted inoculums of *P. pneumotropica* ranging from 1×10^0 to 1×10^8 in a volume of 50 µL, with the inoculum divided between the oral and intranasal routes by using a pipette. At 7 d after inoculation, mice were euthanized by overdose of CO_2. Oral samples from all mice were cultured for *P. pneumotropica* to determine the minimal infectious dose that consistently resulted in colonization. Bacterial status was confirmed by using the described culture and PCR methods.

Local and regional cytokine perturbations associated with low-dose exposure. To assess local cytokine perturbations induced with doses of *P. pneumotropica* mimicking those likely to occur in a natural setting and to determine whether perturbations occurred in draining lymph nodes, female C57BL/6NHsd mice

Table 2. Sense and antisense primer pairs used for PCR amplification of mRNA

Gene name	Sense (5′–3′)	Antisense (5′–3′)	Fragment size (bp)
HPRT	GTA ATG ATC AGT CAA CGG GGG AC	CCA GCA AGC TTG CAA CCT TAA CCA	165
CCL3	GCT CAA CAT CAT GAA GGT CTC C	TGC CGG TTT CTC TTA GTC AGG	222
*IL1*β	AGC CCA TCC TCT GTG ACT CAT G	GCT GAT GTA CCA GTT GGG GAA C	420
TNFα	AAG TTC CCA AAT GGC CTC CCT C	TCC ACT TGG TGG TTT GCT ACG	119
CXCL1	GCT GGG ATT CAC CTC AAG AAC	AGC AGT CTG TCT TCT TTC TCC	196
CXCL2	TGT CAA TGC CTG AAG ACC C	CTC TTT GGT TCT TCC GTT GAG	249

Primer sequences for *HPRT* were obtained from reference 14, for *CCL3* from reference 13, and for *IL1*β from reference 10.

were separated into 6 groups of 12 or 13 mice each. Experimental mice received 9×10^3 (low dose) or 1.1×10^8 (high dose) *P. pneumotropica* suspended in PBS, with half of total dose given orally and the other half intranasally by using a pipette. Control mice were sham-inoculated with sterile PBS. Results from the pilot study indicated that cytokine perturbations typically peaked at 6 to 8 d after inoculation (data not shown). Mice were euthanized by overdose of CO_2 at 7 or 28 d after inoculation. Nasal turbinate and lymph node (mandibular and superficial cervical) samples were collected and stored in RNAlater (Ambion) according to the manufacturer's instructions. Extracted RNA underwent RT-PCR and subsequent qPCR analysis. Samples with insufficient RNA and those for which HPRT expression was 2 standard deviations below the mean for all samples were excluded from the study.

Effect of bacterial viability and specificity in cytokine perturbations. To determine whether bacterial viability is important to initiate immune responses to *P. pneumotropica* and to determine whether a genetically related nonpathogenic bacterium could cause a similar response, female C57BL/6NCrl mice were separated into 4 groups of 12 mice each. Experimental mice were dosed orally (25 µL) and intranasally (25 µL) with a total dose of 3.4×10^4 *P. pneumotropica* suspended in PBS, 3.4×10^4 heat-killed *P. pneumotropica* suspended in PBS, or 1.4×10^5 *A. muris* suspended in PBS. *Actinobacillus muris* and *P. pneumotropica* colonization status were determined both before and after inoculation. Control mice were sham-inoculated with sterile PBS. Mice were euthanized by overdose of CO_2 at 7 d after inoculation. Lymph nodes (mandibular and superficial cervical) were collected and stored in RNAlater (Ambion) according to the manufacturer's instructions. RNA extraction followed by RT-PCR and qPCR was conducted. Samples with insufficient quantity of RNA or those for which HPRT expression was 2 standard deviations below the mean for all samples were excluded from the study. Three nasal turbinate samples per group and all lung lobes (right and left) from all mice were collected for histologic evaluation.

Statistical analysis. Because data were distributed nonnormally, nonparametric statistical tests were used. qPCR was used to measure *TNF*α, *CCL3*, *CXCL1*, *CXCL2*, and *IL1*β mRNA levels in nasal turbinate and lymph node samples of mice. For studies involving 2 groups, statistical significance was determined by using the Mann–Whitney rank-sum test. The significance of differences between median values of multiple treatment groups was determined by Kruskal–Wallis one-way ANOVA on ranks. For studies involving variation in dose (high versus low) and time point (7 versus 28 d), a pairwise comparison of treatment groups was performed by using the Dunn method. In studies involving variation in bacterial dosing (*A. muris*, heat-killed-*P. pneumotropica*, or *P. pneumotropica*), the Dunn method with comparisons to an uninfected control group was used. All statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, San Jose, CA) with a P value of less than 0.05 being considered statistically significant.

Results

Kinetics of local cytokine gene perturbations: pilot study. To evaluate the kinetics of the local immune response to *P. pneumotropica*, the nasal turbinates of 2 sham-inoculated control C57BL/6NCr mice and 2 mice inoculated orally and intranasally with 1×10^8 *P. pneumotropica* were collected at 2, 4, 6, 8, and 10 d after inoculation and evaluated by qPCR for *IL1*β, *TNF*α, *CCL3*, *CXCL1*, and *CXCL2* mRNA. These genes were selected based on our present understanding of immune response to a general gram-negative bacterium infection.[4,5,7] All mice inoculated with *P. pneumotropica* were confirmed positive for the bacterium. A subpopulation of 4 of the 10 *P. pneumotropica*-colonized mice (one mouse each from those euthanized on days 2, 4, 8, and 10) had a 5- to 11-fold greater level than the average of control mice at the same time point. For *IL1*β, mRNA levels were highest on day 2, with a gradual decrease to near control levels by day 10. The mRNA levels for *TNF*α and *CCL3* increased steadily, with a peak at day 8, with levels returning to near control levels by day 10. *CXCL1* and *CXCL2* mRNA levels peaked on both days 2 and 8, with levels at day 10 only slightly above control values. Cytokine expression in the remaining 6 colonized mice was similar to that of sham-inoculated mice at all time points (data not shown).

mRNA from nasal turbinates of the 4 mice with robust responses was pooled and compared with a pool of nasal turbinate mRNA from time-matched controls ($n = 4$) by using a targeted microarray that profiles the expression of 113 genes involved in the murine inflammatory response. Eleven genes associated with an acute inflammatory response were upregulated (that is, at least 1.5-fold greater expression than that in controls) in response to *P. pneumotropica* inoculation (Table 3). The remaining 102 genes showed less than 1.5-fold increases in transcript number or were expressed at levels comparable to those of control mice (data not shown).

Minimal infectious dose of *P. pneumotropica*. For subsequent studies, we sought to optimally mimic natural infections, which likely result from *P. pneumotropica* doses much lower than 1×10^8 cfu. To this end, we determined the minimal number of bacteria required for colonization. Groups of mice given log-fold dilutions of *P. pneumotropica* were tested for oral colonization 7 d after inoculation. All *P. pneumotropica* doses of 1.1×10^4 cfu or greater established colonization in all 3 mice per group. In addition, 1.1

Table 3. Upregulated genes from pooled nasal turbinate samples (n = 4) 2 to 10 d after inoculation with *Pasteurella pneumotropica*

Gene name	GenBank no.	Function	Fold change
Complement component 3	NM_009778	Complement activation	1.94
Chemokine (C-C motif) ligand 19	NM_011888	Lymphocyte attraction	1.54
Chemokine (C-C motif) ligand 2	NM_011333	Leukocyte recruitment	1.54
Chemokine (C-X3-C motif) ligand 1	NM_009142	Adhesion; chemotaxis	1.83
Chemokine (C-X-C motif) ligand 2	NM_009140	Neutrophil recruitment	3.25
Fc receptor, IgE, high affinity I, gamma polypeptide	NM_010185	Cell activation (degranulation)	1.74
Fc receptor, IgG, high affinity I	NM_010186	Phagocytic activation	1.70
Interferon alpha family, gene 2	NM_010503	Inhibit viral replication; macrophage activation	2.78
IL12A	NM_008351	Cell-mediated immunity	2.14
IL12B	NM_008352	Cell-mediated immunity	1.80
IL1β	NM_008361	Leukocyte adhesion	3.94

$\times 10^3$ *P. pneumotropica* cfu established colonization in 2 of the 3 mice in that group, and less-concentrated inoculums resulted in no colonization. Given these results, we used dosages of approximately 1×10^4 cfu in experiments evaluating low-dose exposure to *P. pneumotropica*.

Local and regional cytokine perturbations associated with low-dose exposure to *P. pneumotropica*. At 7 d after inoculation, the lymph nodes (mandibular and superficial cervical) of mice inoculated with *P. pneumotropica* at both low dose (9×10^3 cfu; n = 10) and high dose (1.1×10^8 cfu; n = 12) showed modest but significant ($P \leq 0.05$) increases in mRNA for *IL1*β, *TNF*α, *CCL3*, *CXCL1*, and *CXCL2* as compared with levels in sham-inoculated control mice (n = 12; Figure 1 A through E). At 28 d after inoculation, *IL1*β mRNA expression was modestly but significantly ($P \leq 0.05$) increased in lymph nodes from the low-dose group (n = 11) when compared with sham-inoculated controls (n = 13; Figure 1 F). No other significant changes in cytokine mRNA levels were detected in either the low- or high-dose group compared with sham-inoculated controls at the 28-d time point (data not shown).

Cytokine mRNA levels in median nasal turbinates did not differ between any of the groups at either the 7- or 28-d time points. However, gene expression levels of *IL1*β, *TNF*α, *CCL3*, *CXCL1*, and *CXCL2* at 7 d in 4 of the 12 mice inoculated with high-dose *P. pneumotropica* varied from 8 to 52 times the average gene expression of control mice at the same time point (data not shown).

Effect of bacterial viability and specificity in cytokine perturbations. Given that cytokine mRNA levels were upregulated in *P. pneumotropica*-infected C57/BL6NHsd mice receiving a minimal infectious dose of *P. pneumotropica*, we sought to determine whether heat-killed *P. pneumotropica* or a live, closely related, nonpathogenic commensal organism (*A. muris*) could induce a similar response. At 7 d after inoculation, the submandibular and superficial cervical lymph nodes of the *P. pneumotropica*-infected mice showed significant increases in gene expression of *IL1*β, *TNF*α, and *CCL3* compared with those in sham-inoculated mice. In comparison, cytokine gene expression was similar among mice receiving *A. muris* or heat-killed *P. pneumotropica* and sham-inoculated control mice (Figure 2 A through C).

Histopathologic changes associated with *P. pneumotropica* or *A. muris* infection. Light microscopy of sections of all lungs and nasal turbinates from 3 randomly chosen mice from each of the treatment groups revealed no changes compared with samples from sham-inoculated controls (data not shown).

Discussion

Pasteurella pneumotropica is an opportunistic bacterial pathogen that may induce inflammation of ocular, reproductive, subcutaneous, and respiratory tissues in immunodeficient and genetically modified mice.[20] Reports of *P. pneumotropica* as the sole cause of disease in immunocompetent mice are rare, even when a large proportion of the mice in a colony are colonized with the bacterium.[1,2,6,8,19] Although the need to exclude *P. pneumotropica* from immunocompromised colonies is self-evident, our research was undertaken to determine whether *P. pneumotropica* colonization induces alterations in the transcription of immune response genes in immunocompetent mice. To this end, we measured perturbations in local and regional cytokine gene expression in C57BL/6 mice inoculated with high or low infectious doses of *P. pneumotropica*.

In both a pilot study (n = 2 mice per time point) and a second experiment (n = 12) in which mice were inoculated orally and intranasally with a high dose (approximately 10^8 cfu) of *P. pneumotropica*, cytokine mRNA levels detected in the nasal turbinates by qPCR did not differ overall between inoculated and control mice. However, a subset of inoculated mice in both experiments had marked elevations in IL1β, TNFα, CCL3, CXCL1 and CXCL2 mRNA levels between days 2 and 10 after inoculation. This marked response of individual mice was not observed in any high-dose mice at day 28 after inoculation or at 7 or 28 d after inoculation in mice given a minimal infectious dose (approximately 10^4 cfu) of *P. pneumotropica*. Therefore, upregulation of cytokine mRNA in the nasal turbinates was variable and could be induced only briefly in mice given a high dose of *P. pneumotropica* oronasally. To confirm the qPCR findings, a targeted microarray analysis was performed on pooled nasal turbinate samples of 4 C57BL/6NCr mice that had robust response cytokine responses according to qPCR. These mice, which were inoculated with a high dose (approximately 10^8) of *P. pneumotropica*, showed upregulation of 11 of 113 murine genes associated with inflammation compared with those in sham-inoculated controls (Table 3). In 2 instances (IL1β and CXCL2), the microarray results corresponded to findings for qPCR; for both genes, the fold-change was greater than 3.0.

Further evidence of the ability of *P. pneumotropica* to induce alterations in cytokine transcription in mice came from 2 additional experiments, in which mRNA levels of *IL1*β, *TNF*α, and *CCL3* were significantly upregulated at 7 d after inoculation in

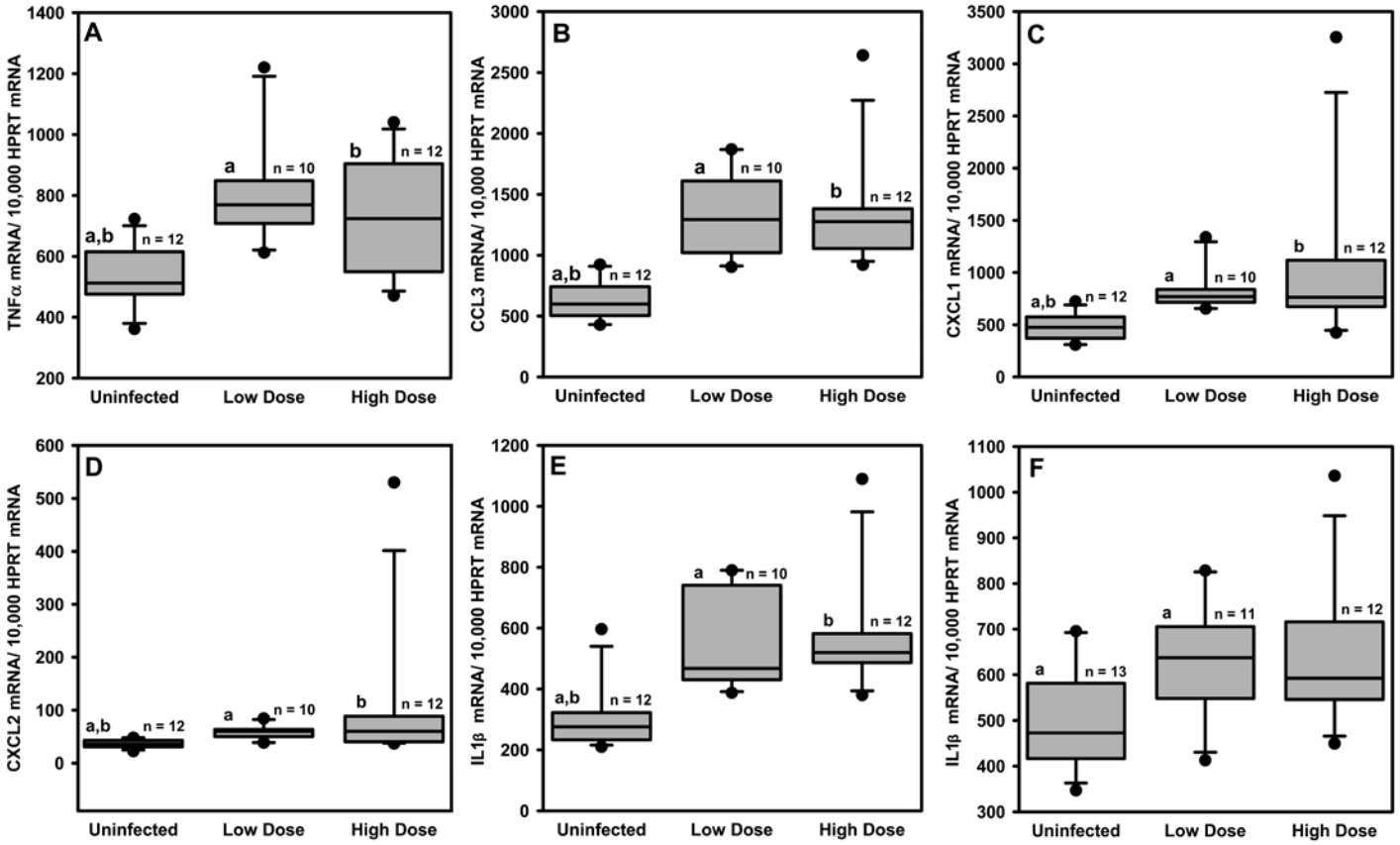

Figure 1. Cytokine mRNA content in the mandibular and superficial cervical lymph nodes of C57BL/6Hsd mice after inoculation with *P. pneumotropica* split between oral and intranasal routes with 9×10^3 bacteria (low dose) or 1.1×10^8 bacteria (high dose). Cytokine mRNA content was determined by use of semiquantitative RT-PCR analysis at (A through E) 7 d and (F) 28 d after inoculation for (A) *TNF*α, (B) *CCL3*, (C) *CXCL1*, (D) *CXCL2*, and (E and F) *IL1*β. Raw data were normalized to the expressed levels of HPRT (no. of cytokine mRNA transcripts per 10,000 HPRT mRNA transcripts). The data are presented as box plots; for each experimental group, the lower boundary of the box indicates the 25th percentile, the upper boundary indicates the 75th percentile, and the line within the box marks the median value. The line below the box marks the 10th percentile; the line above the box marks the 90th percentile, and datum points outside this range are indicated as (•). For each cytokine evaluated, data points that are statistically ($P \leq 0.05$) different from each other are labeled with the same lowercase letter.

the submandibular and superficial cervical lymph nodes of mice given various doses of *P. pneumotropica*. In the experiment evaluating the local and regional cytokine perturbations associated with low-dose exposure to *P. pneumotropica*, mice in both the high- and low-dose groups had significant increases in TNFα, CCL3, *IL1*β, *CXCL1*, and *CXCL2* mRNA at 7 d after inoculation, and IL1β remained upregulated at day 28 after inoculation in mice given a minimally infectious dose of *P. pneumotropica*. Moreover, among groups of C57BL/6 mice inoculated oronasally with a low infectious dose of *P. pneumotropica*, equivalent numbers of heat-killed *P. pneumotropica* or viable *A. muris*, only those that received live *P. pneumotropica* showed significant changes in cytokine mRNA levels (Figure 2). The host cytokine responses induced by *P. pneumotropica* were acute and largely resolved by 28 d after inoculation, even though mice remained colonized.

The principal cytokine in innate immunity that is involved in an acute inflammatory response to gram-negative bacteria is TNFα.[1] This cytokine performs this role through the recruitment and activation of neutrophils and monocytes to areas of infection and by stimulating chemokine secretion from adjacent cells to continue the inflammatory process. Along with IL1β, TNFα also induces the expression of adhesion molecules on the surface of endothelial cells to assist in the extravasation of leukocytes into areas of inflammation.[1] Known functions of CCL3 include the recruitment of various white blood cells, whereas CXCL1 and CXCL2 are known for their chemoattraction of neutrophils.[5] Therefore, upregulation of these cytokine genes in response to viable *P. pneumotropica* was unsurprising.

Although we detected upregulation of selected inflammatory cytokines involved in the immune response to bacteria in the regional lymph nodes that drain the upper respiratory tract, histopathology revealed no inflammatory lesions in the nasal mucosa of mice inoculated with *P. pneumotropica*. Perhaps these responses were insufficient in magnitude or duration to result in inflammation detectable by histology. Indeed, the responses we documented by qPCR were present at 7 d after inoculation and typically had waned by 28 d after inoculation. Regardless, mice inoculated with high or minimally infectious doses of *P. pneumotropica* showed no clinical or histologic evidence of inflammation.

Our data indicate that C57BL/6 mice colonized with *P. pneumotropica* can manifest a slight, transient perturbation in the transcription of inflammatory cytokines. This alteration has the potential to confound experimental studies. Our data further suggest that in immunocompetent mice, *P. pneumotropica* has greater

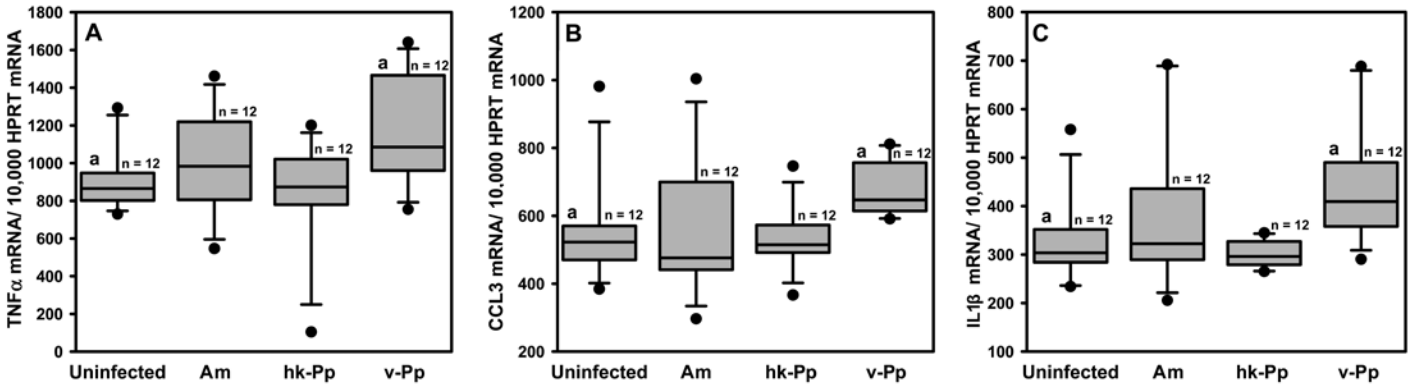

Figure 2. Cytokine mRNA content in the mandibular and superficial cervical lymph nodes of C57BL/6NCrl mice 7 d after oronasal inoculation with 1.4×10^5 cfu *A. muris* (Am), 3.4×10^4 cfu heat-killed *P. pneumotropica* (hk-Pp), or 3.4×10^4 cfu *P. pneumotropica* (v-Pp). Cytokine mRNA content was determined by use of semiquantitative RT-PCR analysis for (A) *TNF*α, (B) *CCL3*, and (C) *IL1*β. Raw data were normalized to the expressed levels of HPRT (no. of cytokine mRNA transcripts per 10,000 HPRT mRNA transcripts). The data are presented as box plots; for each experimental group the lower boundary of the box indicates the 25th percentile, the upper boundary indicates the 75th percentile, and the line within the box marks the median value. The line below the box marks the 10th percentile; the line above the box marks the 90th percentile, and data points outside this range are indicated as (•). Letters on the upper lefthand sides of box plots denote statistical ($P \leq 0.05$) difference between groups.

potential to induce cytokine mRNA alterations in newly infected naïve mice rather than animals from an endemically infected colony. Beyond these transient, localized effects, our data indicated no other biologic significance attributable to *P. pneumotropica* colonization, because mice displayed no clinical or histologic signs of disease. However, with the advent of exquisitely sensitive testing methodologies, such as those used for quantification of gene expression, knowing the full health status of experimental mice becomes paramount to avoid unwanted experimental variables for specific research investigations.

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