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14. ABSTRACT
Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of life threatening infections, with many traditional antimicrobial agents becoming ineffective. An additional potential threat is the use of biological agents and genetically modified pathogens against military personnel during combat operations or against a civilian population. Our main hypothesis is that predatory bacteria are able to serve as a novel therapeutic agent in controlling intractable bacterial infections. By co-culturing Select Agents in the presence of predator bacteria, we have confirmed that Bdellovibrio bacteriovorus and Micavibrio aeruginosavorus are able to

15. SUBJECT TERMS
Predatory bacteria, Bdellovibrio bacteriovorus, Micavibrio aeruginosavorus, Select agent, Upper respiratory infections

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Daniel Kadouri
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 973-972-7401

Report Title

Final Report: The Use of Predatory Bacteria to Control Select Pathogens and Treat Respiratory Infections

ABSTRACT

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of life threatening infections, with many traditional antimicrobial agents becoming ineffective. An additional potential threat is the use of biological agents and genetically modified pathogens against military personnel during combat operations or against a civilian population. Our main hypothesis is that predatory bacteria are able to serve as a novel therapeutic agent in controlling intractable bacterial infections. By co-culturing Select Agents in the presence of predator bacteria, we have confirmed that Bdellovibrio bacteriovorus and Micavibrio aeruginosavorus are able to prey on Yersinia pestis, Burkholderia mallei and Francisella tularensis. However, no measurable predation was observed when Burkholderia pseudomallei and Brucella melitensis were used as host. In order to measure the in-vivo effect of exposure to predatory bacteria, a respiratory and systemic mouse infection model was used. No reduction in mouse viability was seen after intranasal or intravenous inoculation of high doses of predatory bacteria. Furthermore, introducing predatory bacteria into healthy mice did not provoke a sustainable inflammatory response post-exposure.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

1 abstract presented in meeting:
Shatzkes, K., Chae, R., Tang, C., Ramirez, G., Mukherjee, S., Connell, N., and Kadouri, E. D. Evaluating the in vivo effect of predatory bacteria in a mouse infection model. 5th ASM Conference on Beneficial Microbes. Washington DC. September 2014.

1 talk presented in meeting, no abstract presented:
Kadouri, D. The use of predatory bacteria to control lung infections, Chemical & Biological Terrorism Defense, Gordon Research Conference, Ventura, CA. March 2015.

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

None

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Kenneth Shatzkes	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
None	0.00
FTE Equivalent:	**0.00**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Daniel Kadouri PI	0.25	
Nancy Connell CO-PI	0.25	
FTE Equivalent:	**0.50**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Matt Dudek	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 2.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 2.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 2.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 1.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:..... 0.00

Names of Personnel receiving masters degrees

NAME	
Gregory Ramirez	
Total Number:	**1**

Names of personnel receiving PHDs

NAME	
None	
Total Number:	**1**

Names of other research staff

NAME	PERCENT_SUPPORTED
Kamlesh Bhatt	0.50
Chi Tang	1.00
Richard Chae	1.00
Riccardo Russo	0.50
FTE Equivalent:	**3.00**
Total Number:	**4**

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment W911NF1420016 Final Report

Technology Transfer

None

Final Progress Report

The use of predatory bacteria to control select pathogens and treat respiratory infections

Period of performance: December 16th 2013 to January 15th 2015

Table of Contents

Introduction

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of life threatening infections, with many traditional antimicrobial agents becoming ineffective. Resistance can be considered a natural response to the selective pressure of a drug and can develop in both free-floating bacteria, and in surface-attached bacteria or biofilms. An additional potential threat is the use of biological agents and genetically modified pathogens against military personnel during combat operations or against a civilian population. The problem of multidrug-resistant (MDR)-bacterial infections, biofilm related infections and the risk of weaponized biological agents has driven researchers to examine other potential anti-bacterial strategies. Among these alternative therapies is the use of biological control agents. One biological based microbial control strategy is the use of predatory bacteria. *Bdellovibrio* and *Micavibrio* are both Gram-negative bacteria ubiquitous to many natural environments. Unlike most bacteria, these organisms are obligatory predators that survive by feeding exclusively on other Gram-negative bacteria. Furthermore, it is now believed that predatory bacteria are a part of the human gut commensal microbial population and might play a role in maintaining healthy intestinal microflora.

We hypothesize that predatory bacteria are able to serve as a novel therapeutic agent in controlling non-treatable bacterial infections.

In a previous study, we confirmed that predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to prey on a wide range of pathogens. *Bdellovibrio* was also found to be active against numerous MDR *Acinetobacter* strains isolated from Wounded Warriors as well as additional clinically relevant MDR pathogens.

The ability of these predators to attack Gram-negative bacteria and their capability to feed and proliferate on an array of human pathogens and biofilms, make these organisms ideal candidates for use as biological control agents. We believe that these unique organisms could be used not only against human pathogens that are already posing a threat in the field, but also against natural and man-made pathogens that we might encounter in future conflicts.

The objectives of this proposal were: (I) To evaluate the ability of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* to attack Gram-negative pathogens that might be weaponized and used in future conflicts or acts of terror. (II) To assess the potential use of predatory bacteria to be used as a novel biological-therapeutic agent to infections in vivo.

Summary Aim I, Task 1 and 2

Aim I. *The use of predatory bacteria to control select agents.*

Task-1. *Measuring the ability of B. bacteriovorus to prey on select pathogens.*

Task-2. *Measuring the ability of M. aeruginosavorus to prey on select pathogens.*

Aim I, Task 1 and 2 Experiments conducted
Select agent experiments using cell turbidity to measure predation were conducted for *Y. pestis , B. mallei , F. tularensis, B. pseudomallei* and *B. melitensis.*
Select agent experiments using direct cell enumeration were conducted for *Y. pestis* and *B. mallei* .

Summary Aim I, Task 1 and 2

Aim I. *The use of predatory bacteria to control select agents.*
Evaluate the ability of predatory bacteria to prey on a selection of Gram-negative pathogens grown in liquid suspension.

The aim was divided into two subtasks.

Task-1. *Measuring the ability of B. bacteriovorus to prey on select pathogens.*
Task-2. *Measuring the ability of M. aeruginosavorus to prey on select pathogens.*

Accomplishment:

Select agent experiments using cell turbidity to measure predation were conducted for *Y. pestis , B. mallei , F. tularensis, B. pseudomallei* and *B. melitensis.*

Summary Aim-I, Task 1 and 2

Select agent experiments using cell turbidity to measure predation. We have measured predation by the reduction in prey turbidity, a well established technique to measure predation.

A. *Yersinia pestis* NR-20 125 B. plague Bombay was used as host and treated with the three predators.
B. *Yersinia pestis* NR-641 CO92 was used as prey and treated with the three predators.
C. *Burkholderia mallei* NR-21 China 5 was used as prey and treated with the three predators.
D. *Burkholderia mallei* NR-21 China 7 was used as prey and treated with the three predators.
E. *Burkholderia pseudomallei* NR-4073 K96243 was used as prey and treated with the three predators.
F. *Burkholderia pseudomallei* NR-8071 1710a was used as prey and treated with the three predators.
G. *Burkholderia pseudomallei* OH, was used as prey and treated with the three predators.
H. *Francisella tularensis* NR-644. WY96-3418 was used as prey and treated with the three predators.
I. *Francisella tularensis* SCHU S4 (FSC237) was used as prey and treated with the three predators.
J. *Brucella melitensis* NR-256 16M was used as prey and treated with the three predators.

- All experiments were conducted in BSL-3.
- Predation was measured by change in culture turbidity.

Summary Aim-I, Task 1 and 2

Results.
Change (%) in prey cell turbidity following predation.

	Predator		
	***B. bacteriovorus* strains 109J**	***B. bacteriovorus* HD100**	***M. aeruginosavorus***
Prey			
Y. pestis plague Bombay	+ (45 ± 13%) (55± 15%)*	+ (39 ± 7%) (54 ± 6%)*	+ (8± 2%) (42 ± 17%)*
Y. pestis NR-641 CO92	+ (39 ± 14%) (56± 14%)*	+ (16 ± 5%) (49± 17%)*	+ (17± 10%) (44 ± 15%)*
B. mallei NR-21 China 5	+ (66 ± 4%) (72 ± 6%)*	+ (65 ± 3%) (66 ± 6%)*	+ (7 ± 2%) (27±5%)*
B. mallei NR-21 China 7	+ (66 ± 9%) (80 ± 5%)*	+ (67 ± 5%) (69 ± 2%)*	+ (5 ± 2%) (6 ±2%)*
B. pseudomallei NR-8071 1710a	-	-	-

Co-cultures were prepared by adding prey cells to harvested predator cells or predator free control. Data represent the % reduction in culture turbidity as compared to the predator free control, following 24 and 48 (*) hrs of incubation. Each experiments was conducted in triplicate. Value representing mean and standard error.
+ Positive predation (reduction in culture turbidity).
- Negative predation (no reduction in culture turbidity).

Summary Aim-I, Task 1 and 2

Results.
Change (%) in prey cell turbidity following predation.

	Predator		
	***B. bacteriovorus* strains 109J**	***B. bacteriovorus* HD100**	***M. aeruginosavorus***
Prey			
B. pseudomallei NR-4073 K96243	-	-	-
B. pseudomallei OH	-	-	-
F. tularensis NR-644. WY96-3418	+ (10 ± 3%) (21 ± 3%)*	+ (6 ± 1%) (7 ± 0.5%)*	-
F. tularensis SCHU S4	+ (18 ± 4%) (29 ± 4%)*	+ (2 ± 1%) (9 ± 2%)*	-
B. melitensis NR-256 16M	-	-	-

Co-cultures were prepared by adding prey cells to harvested predator cells or predator free control. Data represent the % reduction in culture turbidity as compared to the predator free control, following 24 and 48 (*) hrs of incubation. Each experiments was conducted in triplicate. Value representing mean and standard error.
+ Positive predation (reduction in culture turbidity).
- Negative predation (no reduction in culture turbidity).

Summary Aim-I, Task 1 and 2

Select agent experiments using cell enumeration to measure predation.

A. *Yersinia pestis* NR-641 CO92 was used as prey and treated with the predators.
B. *Burkholderia mallei* NR-21 China 5 was used as prey and treated with the predators.
C. *Burkholderia mallei* NR-21 China 7 was used as prey and treated with the predators.

- All experiments were conducted in BSL-3.
-Predation was measured by change in host cell viability.

Summary Aim-I, Task 1 and 2

Results.

Change in prey viability following predation.

	Predator		
	B. bacteriovorus 109J (Log_{10} change)	*B. bacteriovorus* HD100 (Log_{10} change)	*M. aeruginosavorus* ARL-13 (Log_{10} change)
Prey			
Y. pestis NR-641 CO92	4 ± 0.3	1.5+0.5	1.9+0.2
B. mallei NR-21 China 5	4.6 ± 0.5	4.1± 0.4	0.8± 0.2
B. mallei NR-21 China 7	5.3 ± 0.5	4.7 ± 0.5	1.6 ± 1.1

Select pathogens were co-cultured by adding prey cells to harvested predator or predator free control. Values represent the Log_{10} reduction measured following 48 hrs of incubation as compared to the predator free control. Each experiments was conducted in triplicate. Value representing mean and standard error.

Summary, Aim II, Task 1A

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. Measuring the in-vivo effect of exposure to predatory bacteria
Task-1A. *Initial model optimization*

Aim-II, Task 1 **Experiments conducted**
Task-1A. *Initial model optimization.*

Summary, Aim II, Task 1A

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. Measuring the in-vivo effect of exposure to predatory bacteria.

Task-1A. *Initial model optimization.*
A pilot experiment was conducted in order to verify that the predators are not hyper toxic to the host.

Mice, 5 mice per group, were divided into 6 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $1x10^9$ PFU/mouse *B. bacteriovorus* strain 109J.
(3) mice inoculated with $1x10^9$ PFU/mouse heat killed* *B. bacteriovorus* strain 109J.
(4) mice inoculated with $1x10^9$ PFU/mouse *B. bacteriovorus* strain HD100.
(5) mice inoculated with $1x10^9$ PFU/mouse heat killed* *B. bacteriovorus* strain HD100.
(6) mice inoculated with $1x10^6$ PFU/mouse *M. aeruginosavorus* ARL-13.

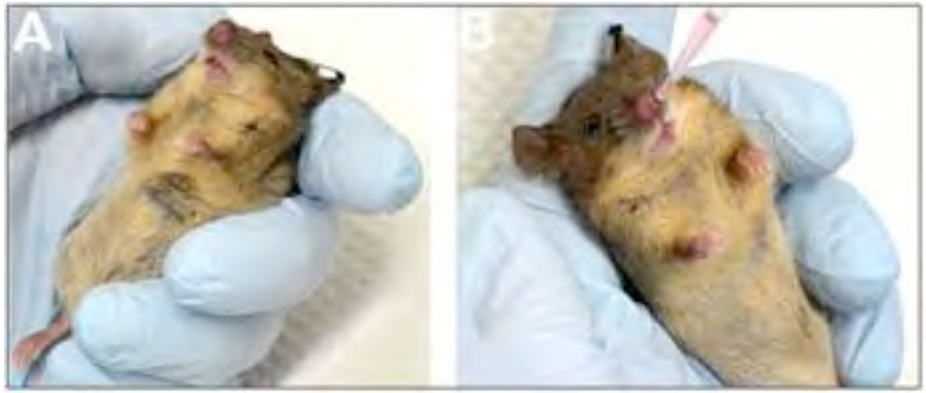
*20min at 90^oC

Mice were kept for 5 days and visually assessed for signs of infection, illness and discomfort.
After 5 days 3 mice from each group were euthanized and lung, and spleen samples were collected.
Two mice from each group are kept for an additional 50 days.

Summary, Aim II, Task 1A

Intranasal infection of mice:

1. Animals were lightly anaesthetized using a isoflurane vaporizer.
2. Treatment is 3% isoflurane in oxygen for four minutes.
3. A total of 25 µl of bacterial suspension are gently applied to the nostrils.
4. Animals are returned to cage and monitored for behavior.
5. After recovery (2 minutes) no adverse effects were noted.



http://biomedfrontiers.org/alzheimer-2013-11-6/

Summary, Aim II, Task 1A

Results.
As seen in below, inoculation of high doses of predatory bacteria did not cause any reduction in mice viability. No visual signs of infection, illness and discomfort were observed in any of the treatment groups.

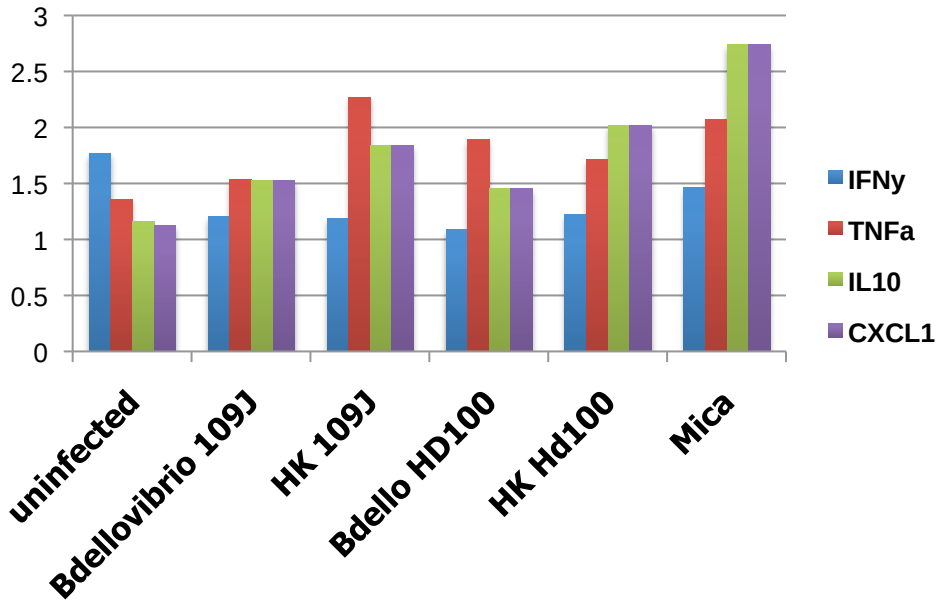
Effect of predatory bacteria on mice survival.

Treatment	Number of mice used	% Viability on day 5
PBS control	5	100%
B. bacteriovorus 109J	5	100%
B. bacteriovorus 109J heat killed	5	100%
B. bacteriovorus HD100	5	100%
B. bacteriovorus HD100 heat killed	5	100%
M. aeruginosavorus ARL-13	5	100%

For the remaining two mice, no reduction in mice viability was seen at day 50 for any of the treatments.

Conclusion: Based on our data it seems that when inhaled predatory bacteria are non-toxic to mice at high concentration.

Summary, Aim II, Task 1A



Measuring inflammatory responses 5 days post-exposure:
Three mice from each group were euthanized and lungs were removed. Lung tissues were homogenized in Trizol, RNA extracted and reversed transcribed. cDNAs were amplified and fold induction in gene expression relative to uninfected tissue was calculated by the ΔΔCT method; B-actin was used as normalizer. Primer sets were purchased from Invitrogen.

Conclusion: None of the exposed mice exhibited significant increases in inflammatory marker gene expression (10-fold increase is considered a positive response). The data are the average of three animals. Based on our data it appears that predatory bacteria when inhaled do not provoke a sustained inflammatory response.

Summary, Aim II, Task 1B

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. Measuring the in-vivo effect of exposure to predatory bacteria
Task-1B. *Predatory bacteria exposure experiment*

Aim-II, Task 1B **Experiments conducted**
Task-1B. *Predatory bacteria exposure experiment*. Two experiments were completed using *Bdellovibrio* and *Micavibrio.*

Summary, Aim II, Task 1B

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. Measuring the in-vivo effect of exposure to predatory bacteria.

Task-1B. *Predatory bacteria exposure experiment*.
Two separate experiments aimed at evaluation the effect of *Bdellovibrio* on mice were conducted. 12 mice per group, were divided into 3 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $1.1x10^9$ PFU/mouse *B. bacteriovorus* strain 109J.
(3) mice inoculated with $1.1x10^9$ PFU/mouse *B. bacteriovorus* strain HD100.

Mice were kept for 24 or 48 hrs post exposure and visually assessed for signs of infection, illness and discomfort. 24 and 48 hrs post inoculation, animals (6 from each group) were euthanized and lung, serum liver, and spleen samples were collected. Samples were used for histology, host immune response profile and predator bacteria dissemination experiments.

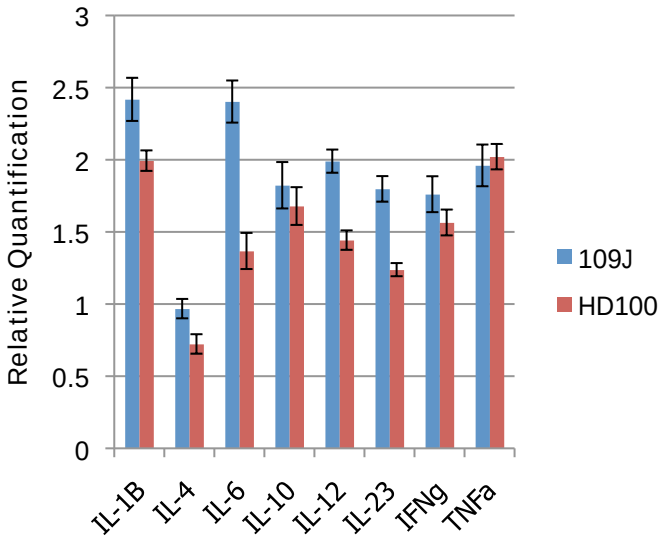
Experiments were conducted twice. See results for Exp I and II.

Summary Aim-II, Task 1B, Exp I

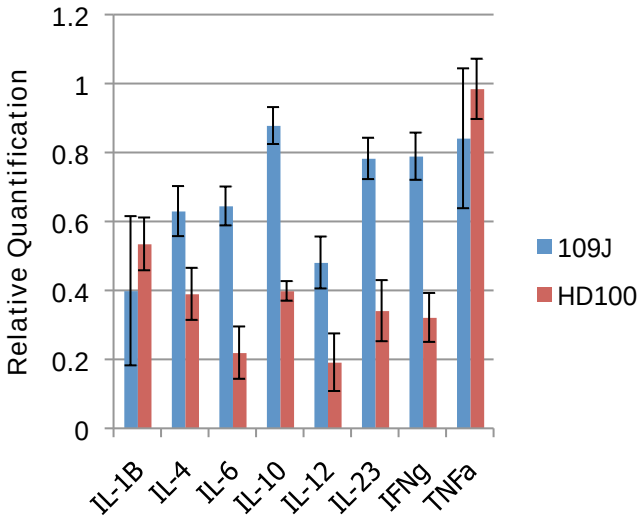
Lung

Inflammatory responses 24 and 48 hours post-exposure:
qPCR / N=6

24 hrs



48 hrs

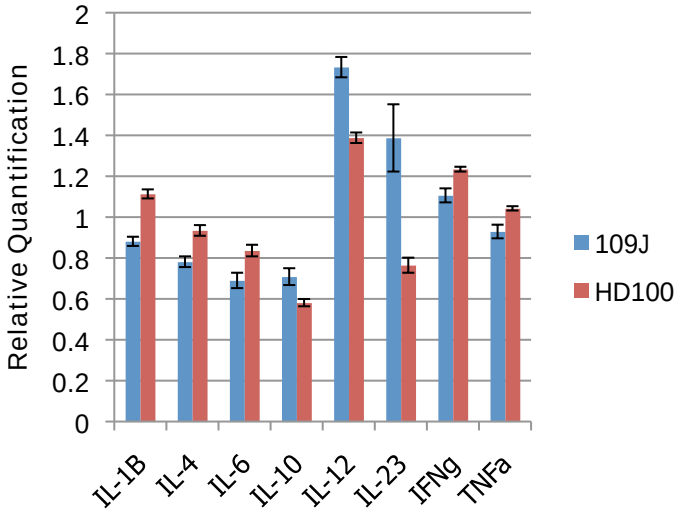


Summary Aim-II, Task 1B, Exp I

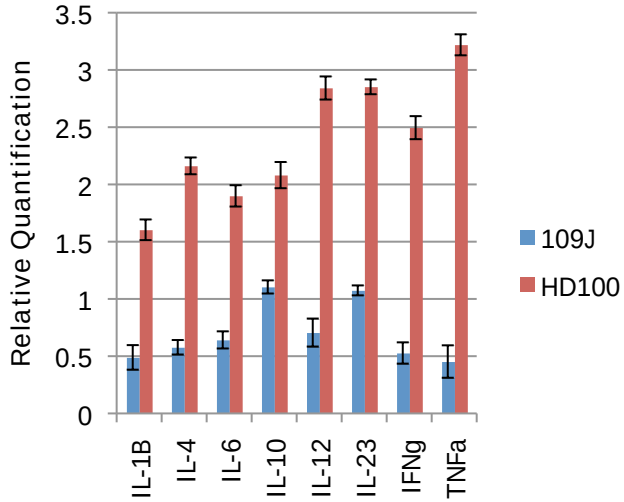
Spleen

Inflammatory responses 24 and 48 hours post-exposure:
qPCR / N=6

24 hrs



48 hrs



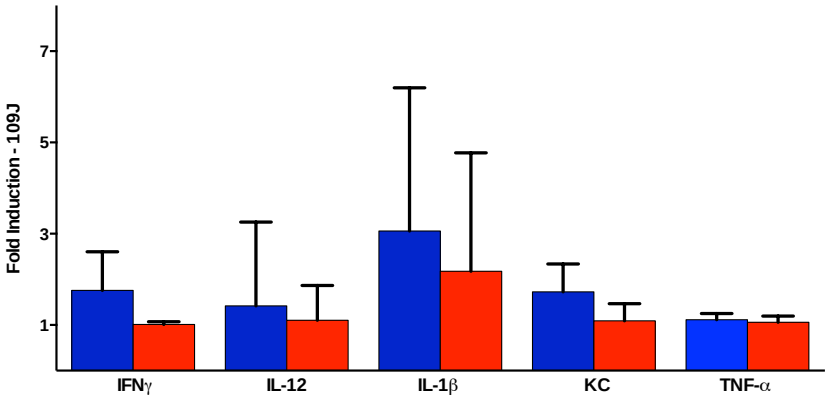
Summary Aim-II, Task 1B, Exp I

Lung

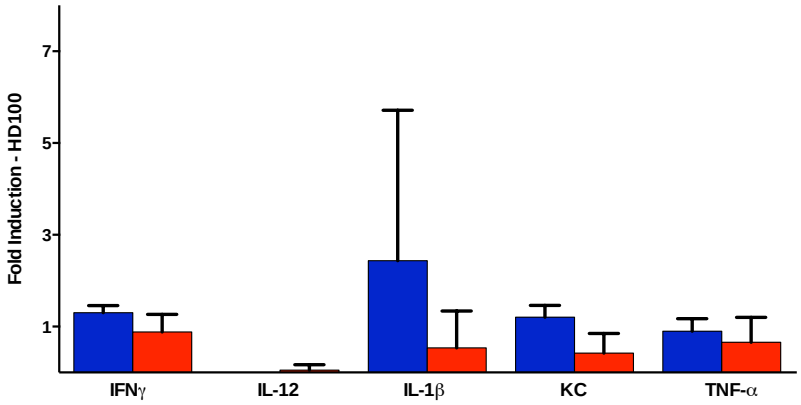
Inflammatory responses 24 and 48 hours post-exposure

ELISA test / N=6

109J



HD100



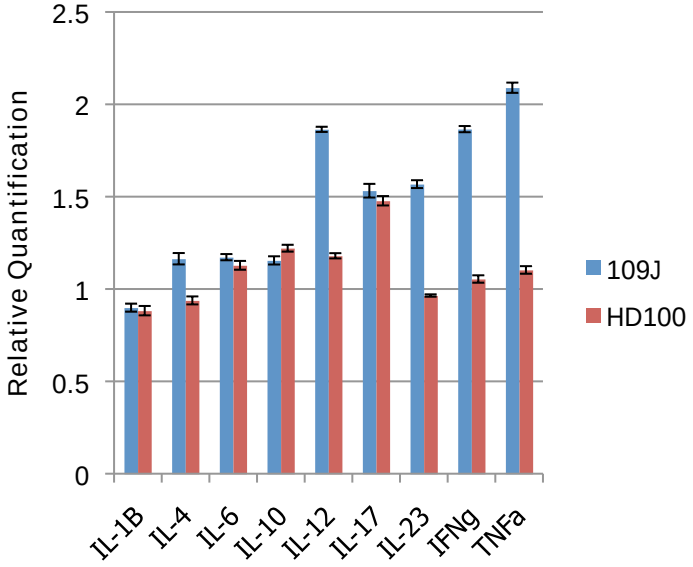
Conclusion: None of the exposed mice exhibited significant increases in inflammatory marker gene or protein expression (10-fold increase is considered a positive response). The data are the average of six animals. Our data indicate that predatory bacteria when inhaled do not provoke a significant inflammatory response.

Summary Aim-II, Task 1B, Exp II

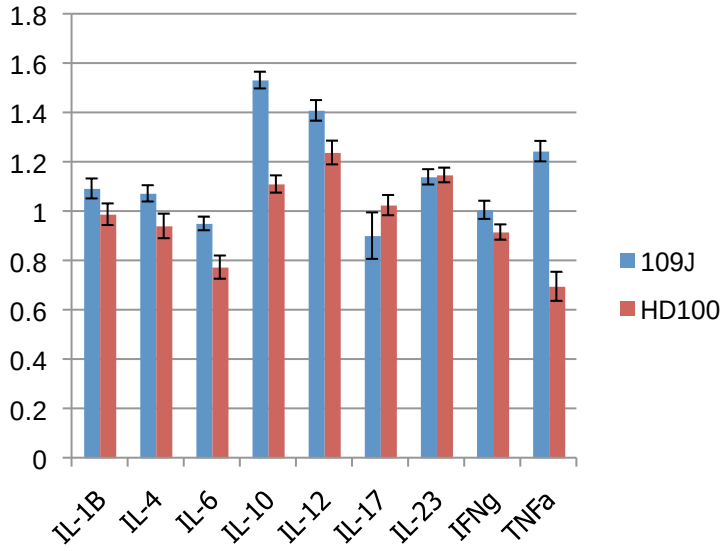
Lung

Inflammatory responses 24 and 48 hours post-exposure:
qPCR / N=6

24 hrs



48 hrs

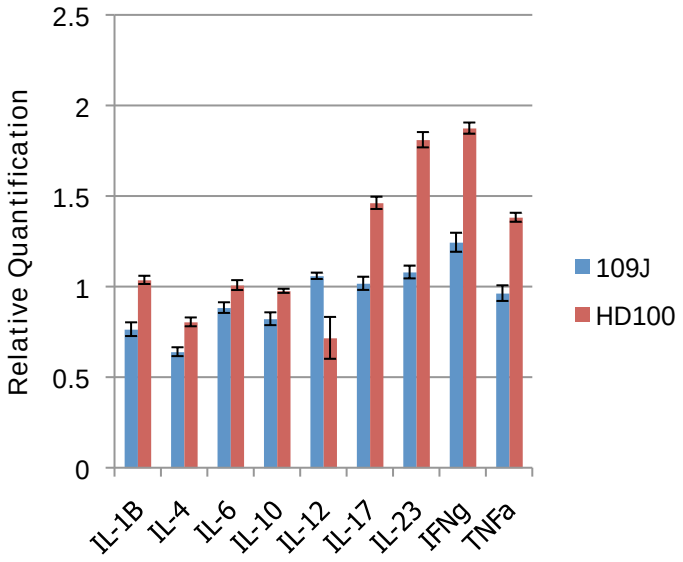


Summary Aim-II, Task 1B, Exp II

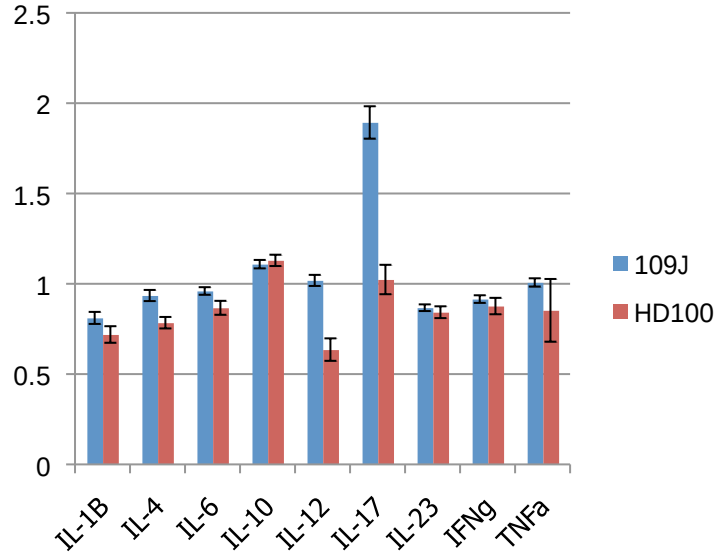
Spleen

Inflammatory responses 24 and 48 hours post-exposure:
qPCR / N=6

24 hrs



48 hrs



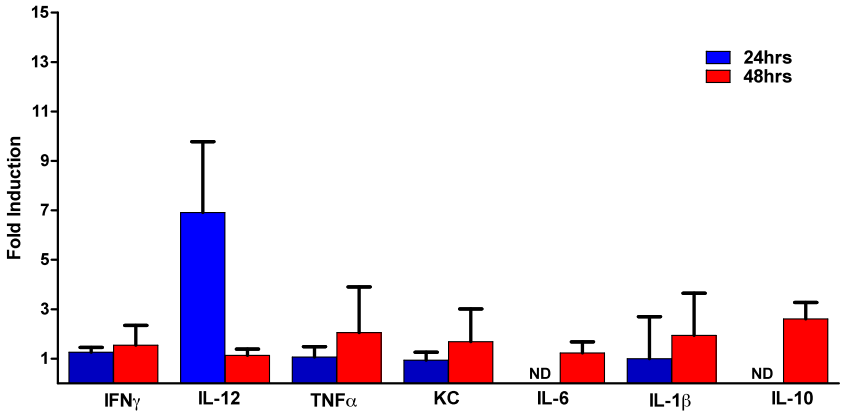
Summary Aim-II, Task 1B, Exp II

Lung

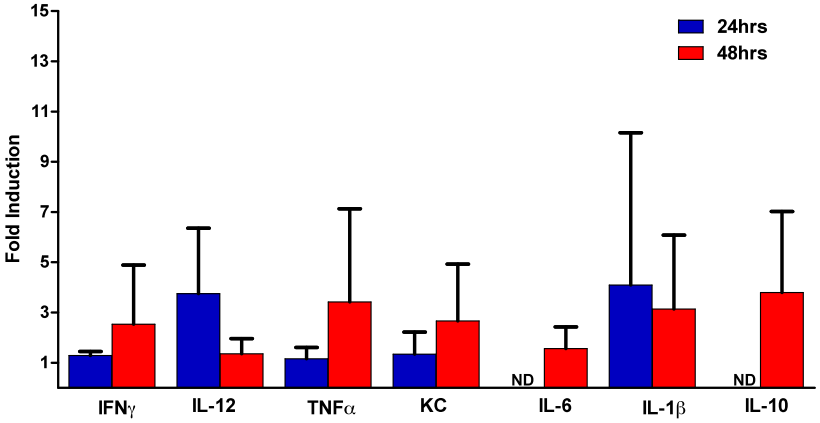
Inflammatory responses 24 and 48 hours post-exposure

ELISA test / N=6

109J



HD100

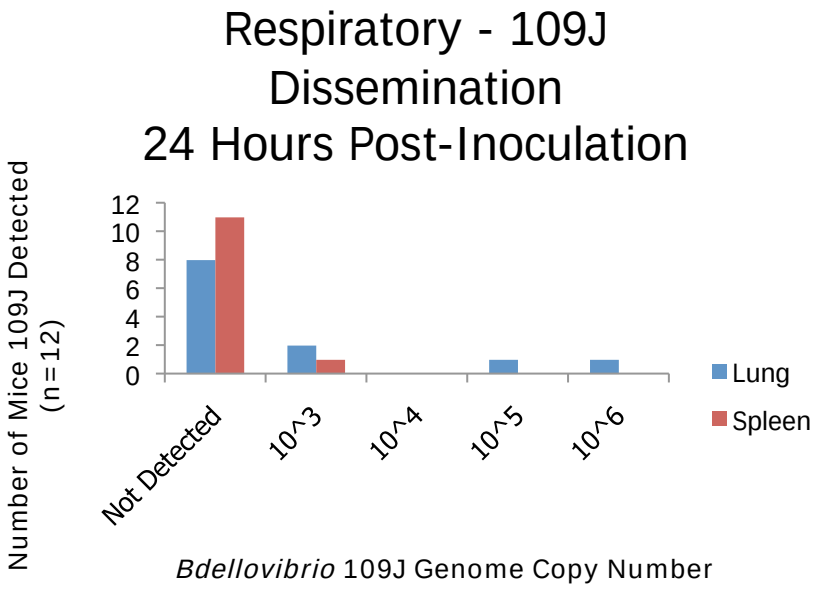


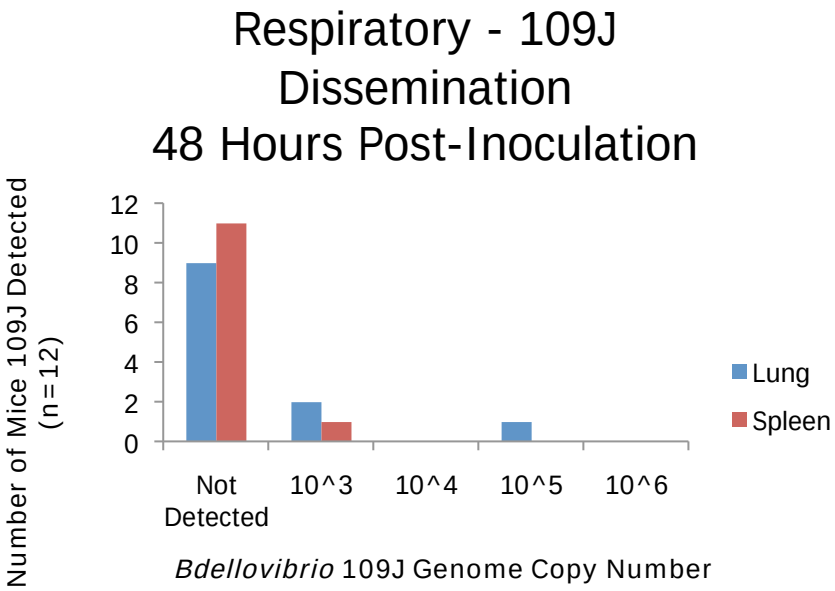
Conclusion: None of the exposed mice exhibited significant increases in inflammatory marker gene or protein expression (10-fold increase is considered a positive response). The data are the average of six animals. Our data indicate that predatory bacteria when inhaled do not provoke a significant inflammatory response.

Summary Aim-II, Task 1B

Task-1B. *Predatory bacteria exposure experiment*.

Bdellovibrio 109J DNA detected using RT-PCR in lungs and spleens of mice infected via nasal inoculation 24 and 48 hr post infection

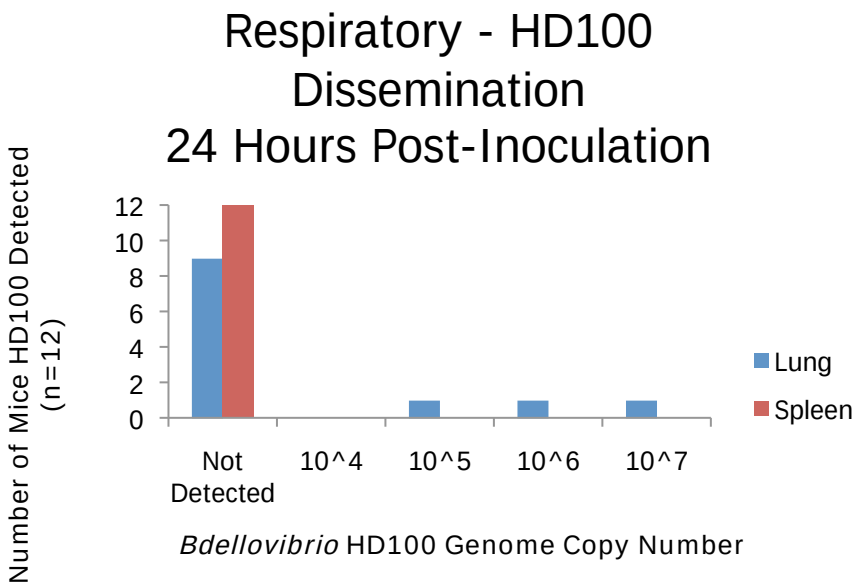


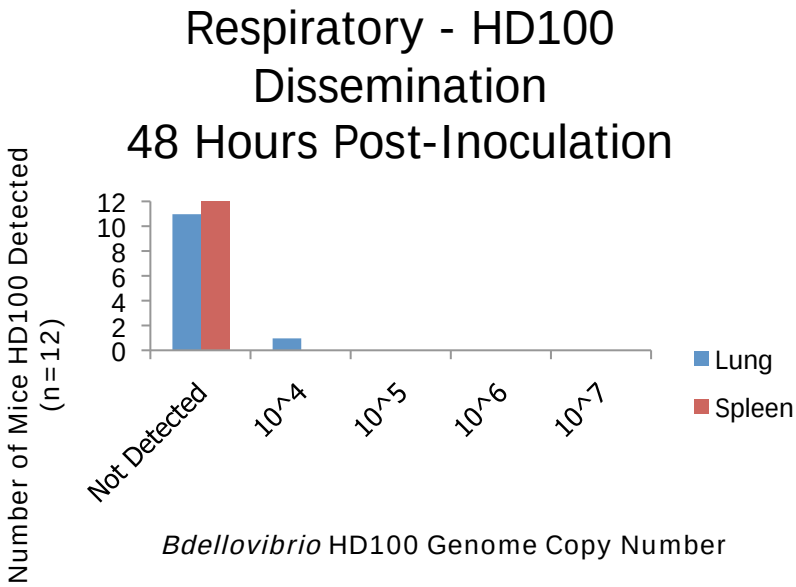


Summary Aim-II, Task 1B

Task-1B. *Predatory bacteria exposure experiment*.

Bdellovibrio HD100 DNA detected using RT-PCR in lungs and spleens of mice infected via nasal inoculation 24 and 48 hr post infection





Summary Aim-II, Task 1B

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. Measuring the in-vivo effect of exposure to predatory bacteria.

Task-1B. *Predatory bacteria exposure experiment*.
Two separate experiments aimed at evaluation the effect of *Micavibrio* on mice were conducted. 12 mice per group, were divided into 2 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) Mice inoculated with $1.1x10^{11}$ PFU/mouse *M. aeruginosavorus* strain ARL-13.

Mice were kept for 24 or 48 hrs post exposure and visually assessed for signs of infection, illness and discomfort. 24 and 48 hrs post inoculation, animals (6 from each group) were euthanized and lung, serum liver, and spleen samples were collected. Samples were used for histology, host immune response profile and predator bacteria dissemination experiments.

Experiments were conducted twice. See results for Exp III and IV.

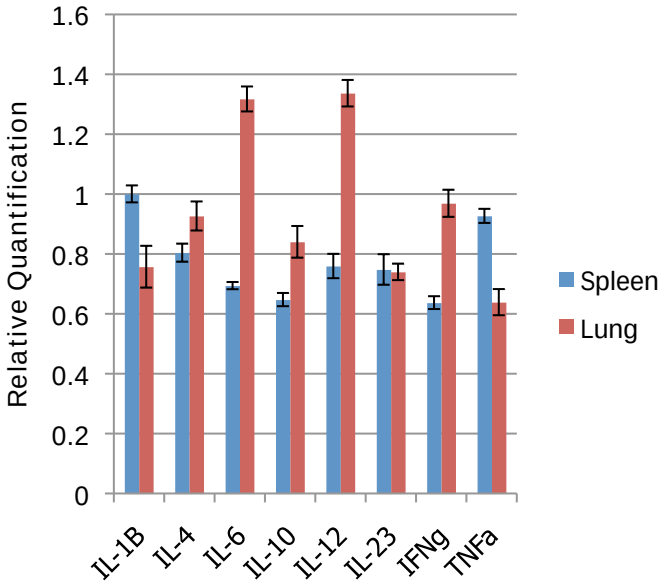
Summary Aim-II, Task 1B, Exp III

Lung and spleen

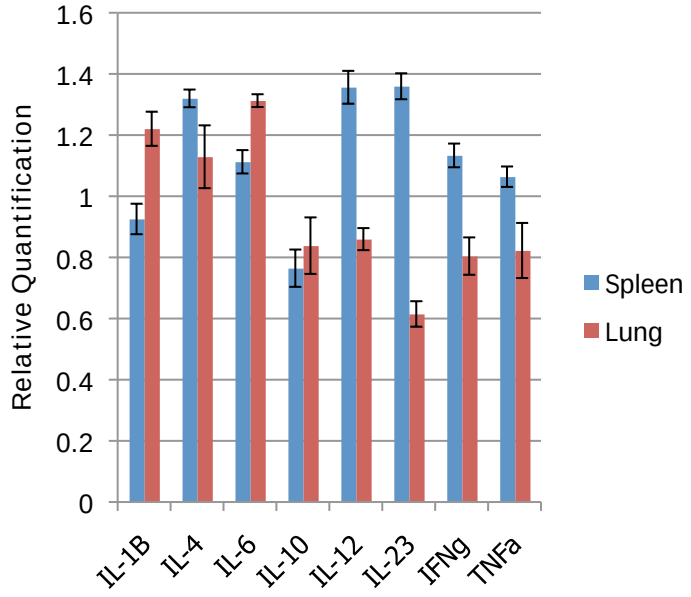
Inflammatory responses 24 and 48 hours post-exposure to *Micavibrio*:

qPCR / N=6

24 hrs



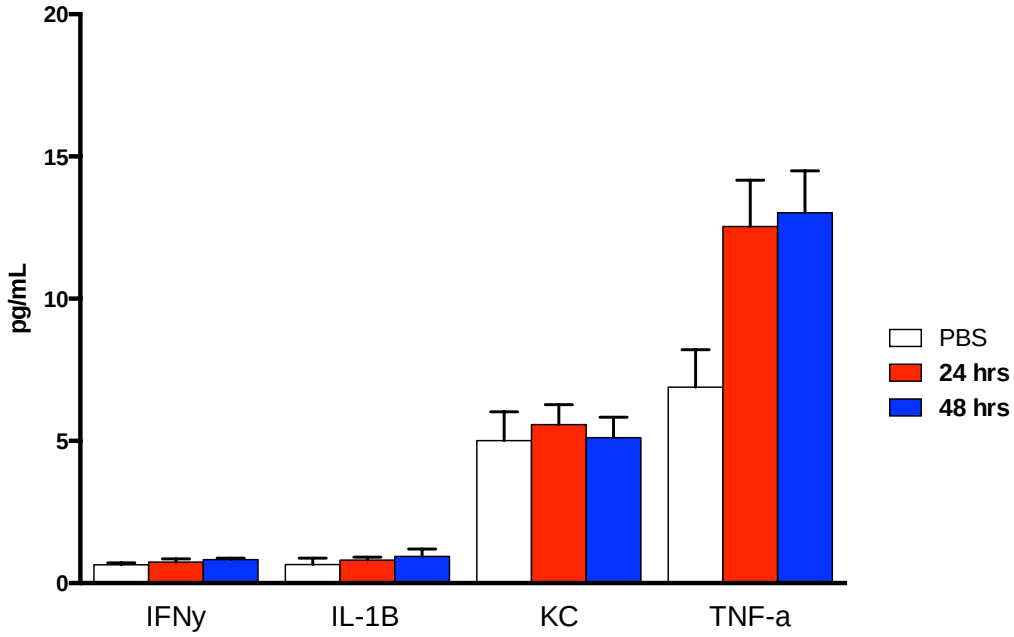
48 hrs



Summary Aim-II, Task 1B, Exp III

Lung

Inflammatory responses 24 and 48 hours
post-exposure to *Micavibrio*
ELISA test / N=6

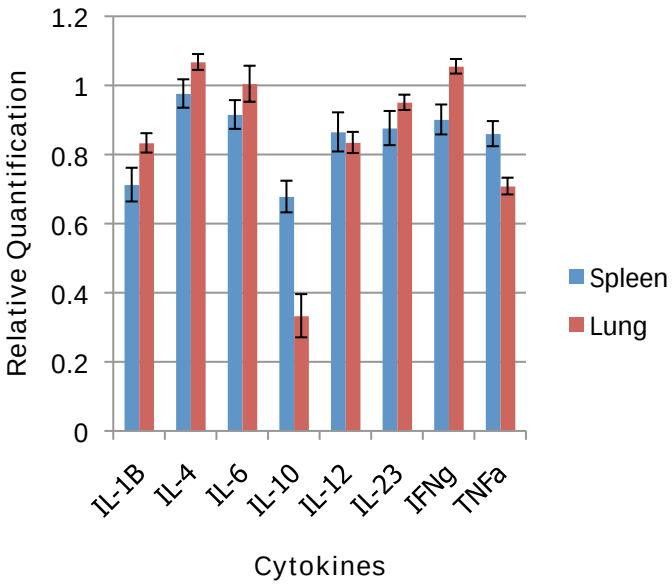


Conclusion: None of the exposed mice exhibited significant increases in inflammatory marker gene or protein expression (10-fold increase is considered a positive response). The data are the average of six animals. Our data indicate that predatory bacteria when inhaled do not provoke a significant inflammatory response.

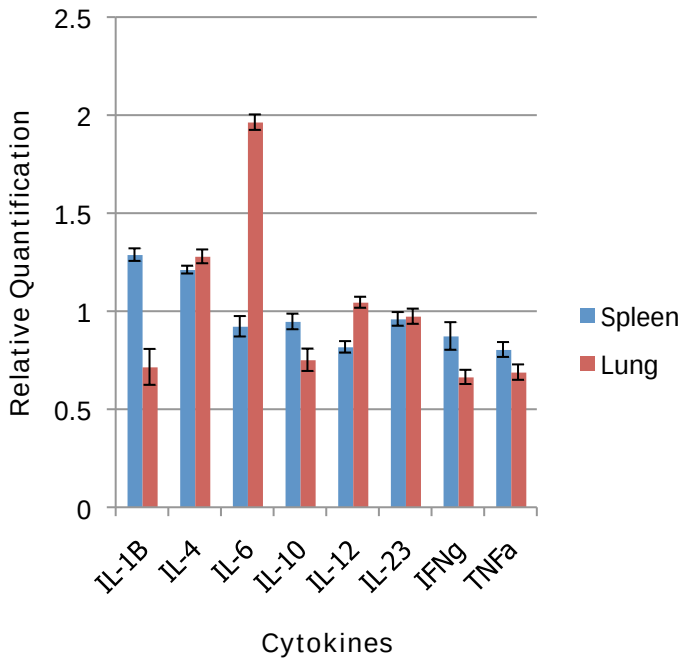
Summary Aim-II, Task 1B, Exp IV

Lung and spleen
Inflammatory responses 24 and 48 hours post-exposure to *Micavibrio*:
qPCR / N=6

24 hrs



48 hrs

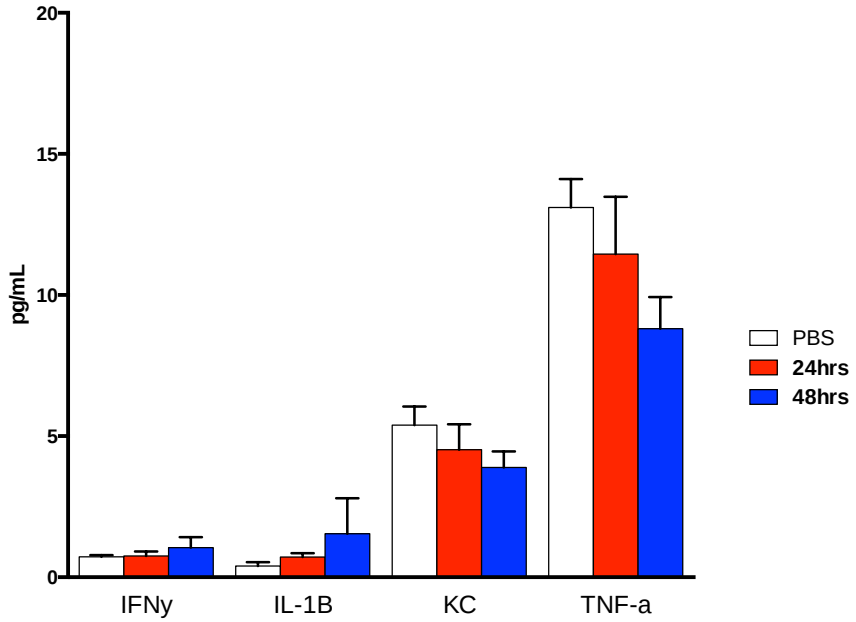


Summary Aim-II, Task 1B, Exp IV

Lung

Inflammatory responses 24 and 48 hours post-exposure to *Micavibrio*

ELISA test / N=6

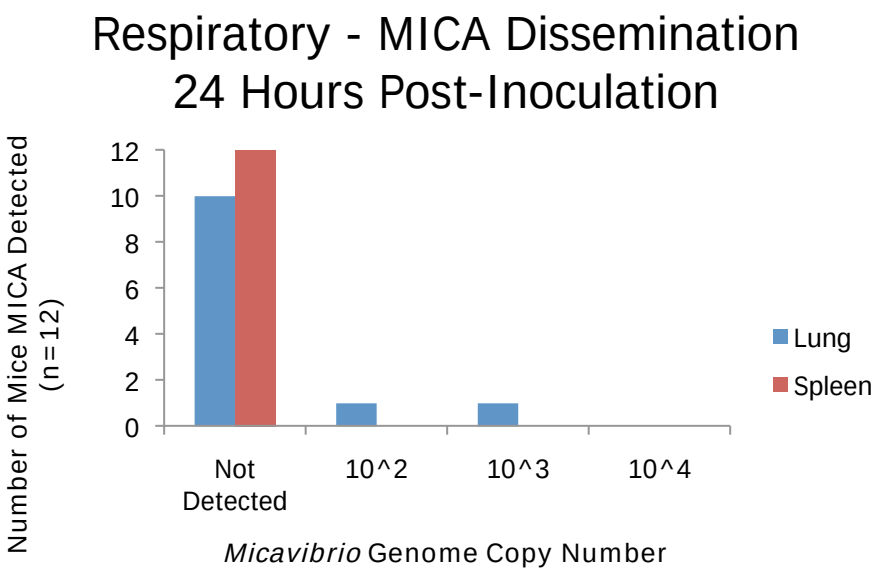


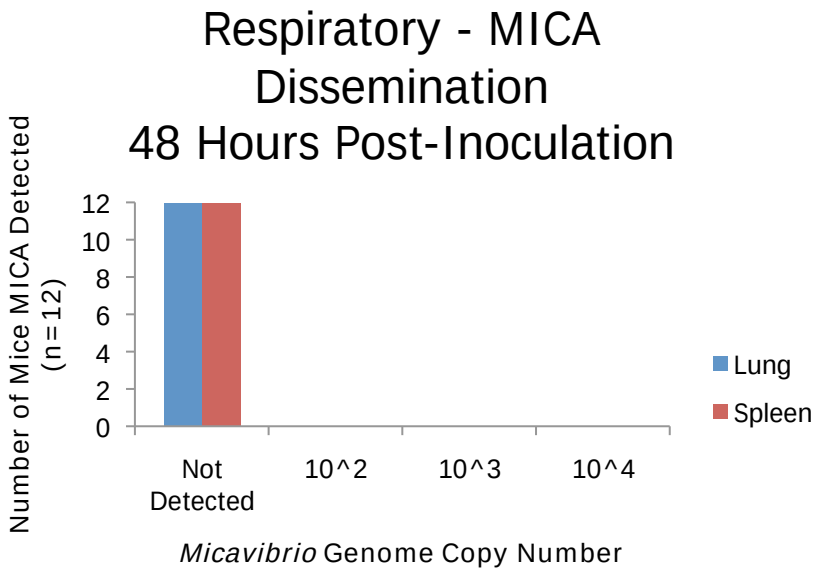
Conclusion: None of the exposed mice exhibited significant increases in inflammatory marker gene or protein expression (10-fold increase is considered a positive response). The data are the average of six animals. Our data indicate that predatory bacteria when inhaled do not provoke a significant inflammatory response.

Summary Aim-II, Task 1B

Task-1B. *Predatory bacteria exposure experiment*.

Micavibrio DNA detected using RT-PCR in lungs and spleens of mice infected via nasal inoculation 24 and 48 hr post infection





Summary Aim-II, Task 1B

Histological report:

Histological examination of the lungs and spleens of mice infected with *Bdellovibrio* and *Micavibrio* revealed no pathology compared to the control mice, treated with PBS. Lung parenchyma was with normal appearance and well preserved in most of the sections. Some of the sections from both groups (experimental and control) in certain areas showed increased cellularity, predominantly mononuclear cells (lymphocytes and macrophages) but no neutrophils. Most likely these changes result from removing and processing the tissue. No evidence of inflammation.

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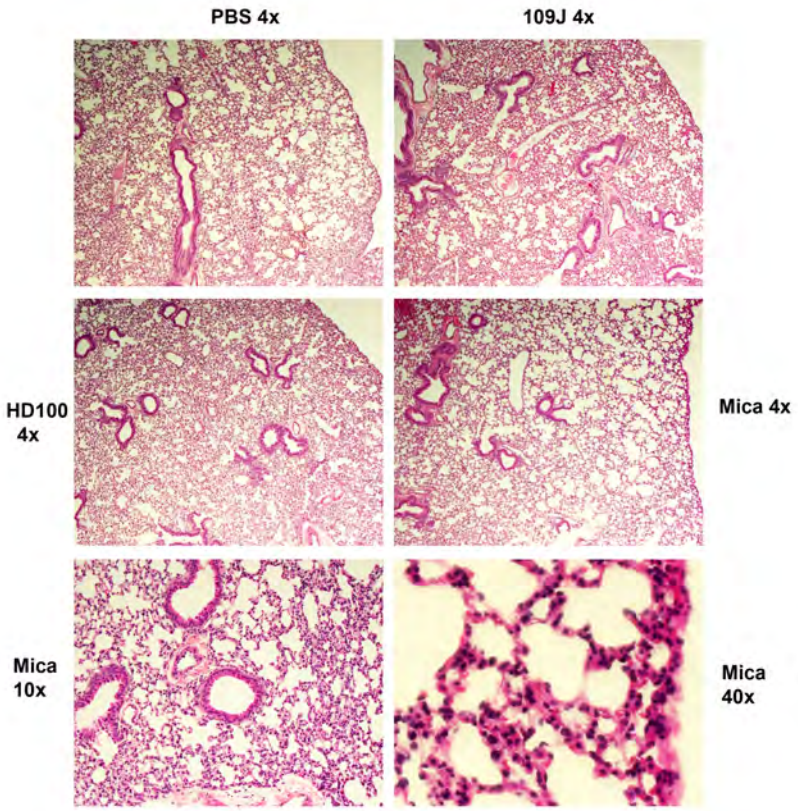
Laboratory of Mycobacterial Immunity and Pathogenesis
Public Health Research Institute (PHRI)
Rutgers, New Jersey Medical School

Summary Aim-II, Task 1B

Histological report:

Histological examination of the lungs with *Bdellovibrio* and *Micavibrio* revealed no pathology compared to the control mice treated with PBS. Images taken 48h post infection.



Summary Aim-II, Task 1B

Summary of histological analysis of lung and spleen in mice
exposed to predatory bacteria after 24 and 48 hrs:
No differences were found between PBS controls and infected samples

Predatory strain	Date of infection	Organs	Time, post inf'n	Samples
B. Bacteriovorus 109J	2/18/14	Lung, spleen	T=24, 48	N=6
	3/3/14	Lung, spleen	T=24, 48	N=6
B. Bacteriovorus HD100	2/18/14	Lung, spleen	T=24, 48	N=6
	3/3/14	Lung, spleen	T=24, 48	N=6
M. aeruginosavorus	3/17/14	Lung, spleen	T=24, 48	N=6
	3/19/14	Lung, spleen	T=24, 48	N=6

Summary Aim-II, Task 1B, addition

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. Measuring the in-vivo effect 60 minutes after exposure to predatory bacteria.

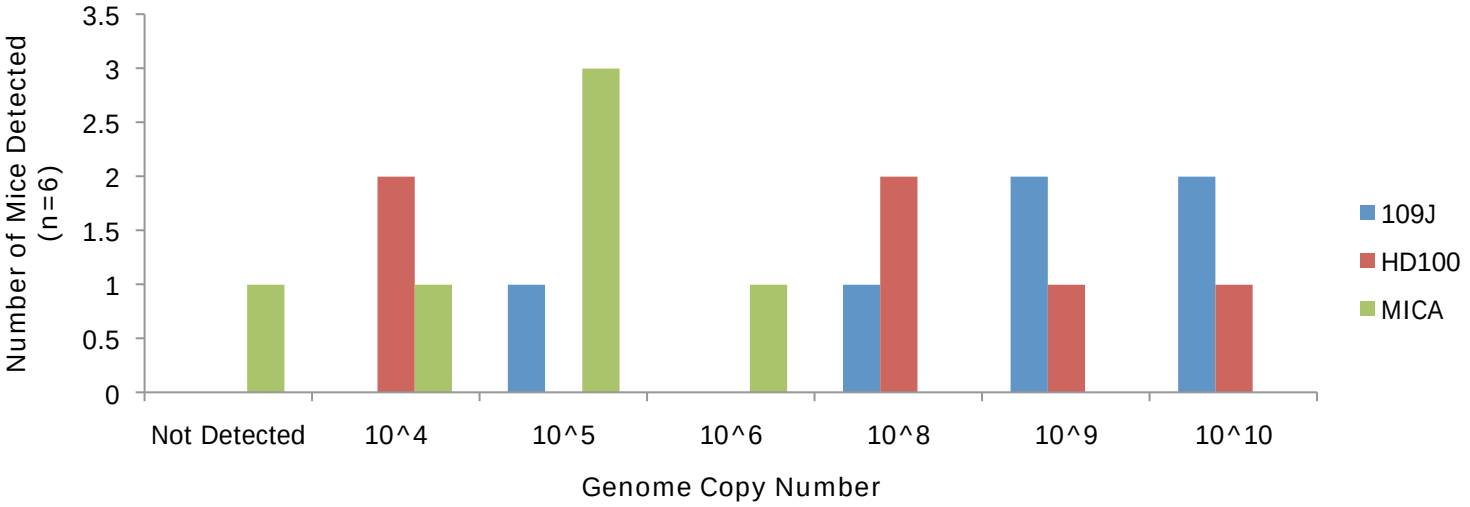
Task-1B. *Predatory bacteria exposure experiment*.
6 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $3.7x10^9$ PFU/mouse *B. bacteriovorus* strain 109J.
(3) mice inoculated with $7.5x10^9$ PFU/mouse *B. bacteriovorus* strain HD100.
(4) mice inoculated with$4.2x10^8$PFU/mouse *M. aeruginosavorus* strain ARL-13.
(5) mice inoculated with $1.2x10^9$ CFU /mouse *K. pneumoniae* ATCC 43816 .

Mice were kept for 60 min post exposure and visually assessed for signs of infection, animals (6 from each group) were euthanized and lung samples were collected. Samples were used for host immune response profile and predator bacteria dissemination experiments.

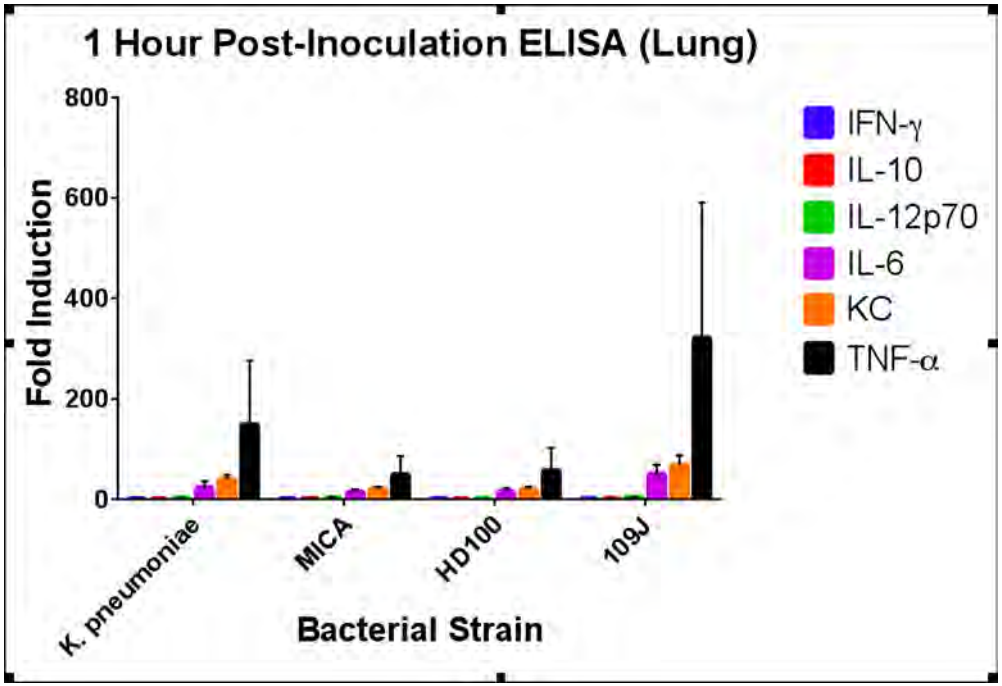
Summary Aim-II, Task 1B, addition

Detection of predatory bacterial DNA in the lungs of mice
60 minutes after exposure to predatory bacteria



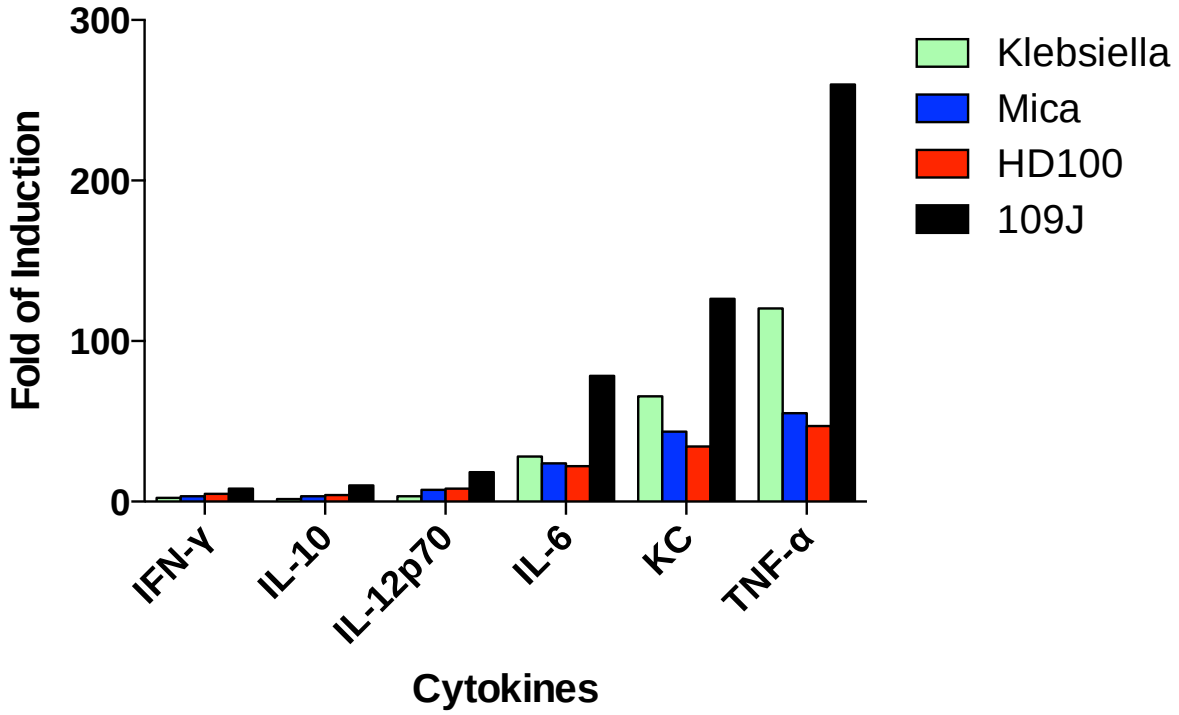
Summary Aim-II, Task 1B, addition

Inflammatory cytokine RNA expression in the lungs of mice 60 minutes after intranasal exposure to predatory bacteria



Summary Aim-II, Task 1B, addition

Inflammatory cytokine expression (measured by ELISA) in the lungs of mice 60 minutes after intranasal exposure to predatory bacteria



Summary Aim-II, Task 1C

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. Measuring the in-vivo effect of exposure to predatory bacteria
Task-1C. *Systemic exposure to predatory bacteria.*

Aim-II, Task 1	Experiments connected
Task-1C, 1. *Short exposure experiment*	Completed 1 experiments using *Bdellovibrio*
Task-1C, 2, *Long exposure experiment*	Completed 2 experiments using *Bdellovibrio*

Summary Aim-II, Task 1C, 1.

Task-1C, 1. *Short exposure experiment*.
The aim of this task was to evaluate the immediate effect of an acute bacteremia event on mice viability and predator clearance.

5 mice per group, were divided into 4 groups:

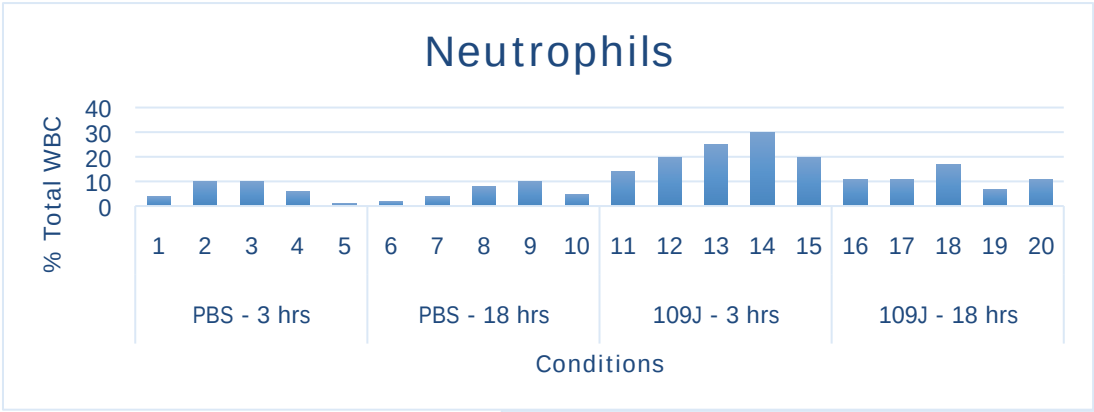
(1) Control, injected intravenously with the carrier PBS and euthanized 3 hrs post inoculation.
(2) Mice inoculated intravenously with 10^8 PFU/mouse *B. bacteriovorus* strain 109J and euthanized 3 hrs post inoculation.
(3) Control, injected intravenously with the carrier PBS and euthanized 18 hrs post inoculation.
(4) Mice inoculated intravenously with 10^8 PFU/mouse *B. bacteriovorus* strain 109J and euthanized 18 hrs post inoculation.

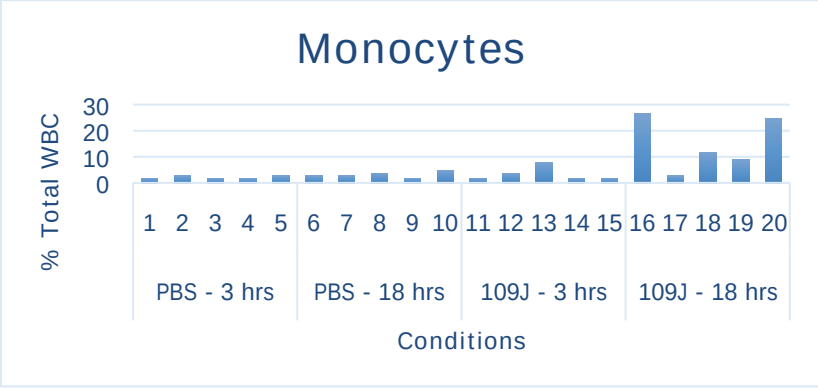
Three and 18 hrs post inoculation, for group 1-2 and 3-4 respectively, animals were euthanized and blood, liver, kidney and spleen samples were collected.

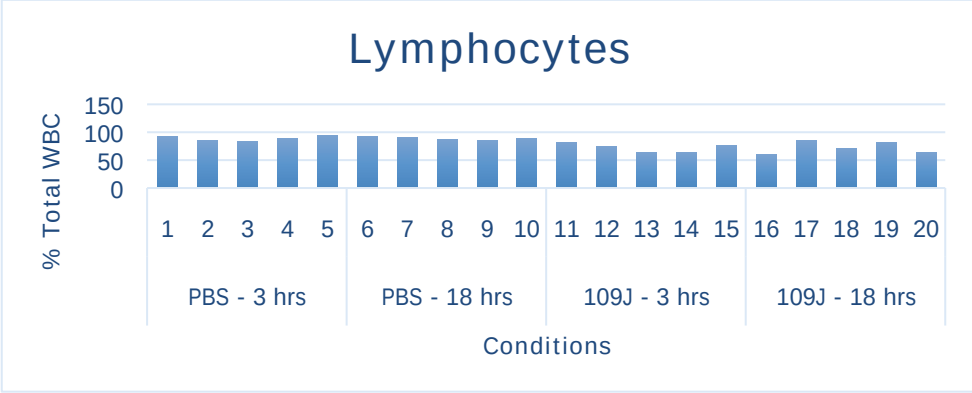
Summary Aim-II, Task 1C, 1.

Task-1C, 1. *Short exposure experiment*.

Results: No visual signs of infection were seen in any of the mice 3 and 18 hrs post-infection. White blood cell counts were performed and the levels of individual cell types shown are summarized below. At 3 hrs post-injection, a 2-3 fold increase in neutrophil counts was seen in the mice injected with *Bdellovibrio* 109J. However, this increase was resolved by 18 hrs and the counts returned to levels comparable to the PBS-injected control animals.

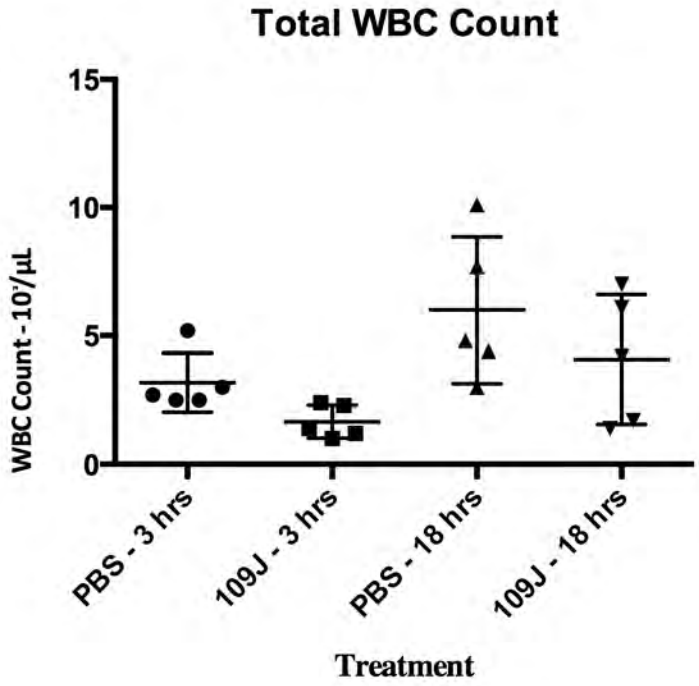






Summary Aim-II, Task 1C, 1.

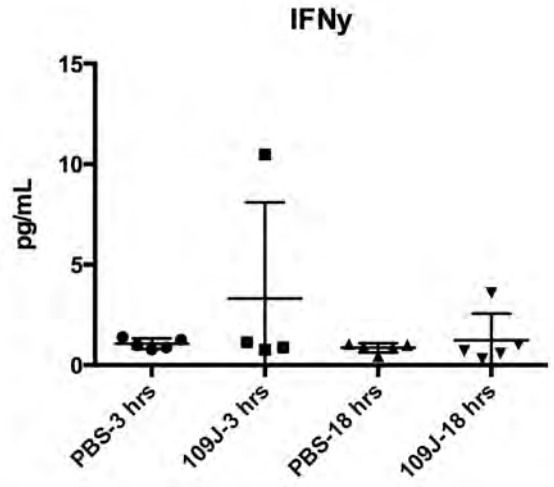
Task-1C, 1. *Short exposure experiment*.

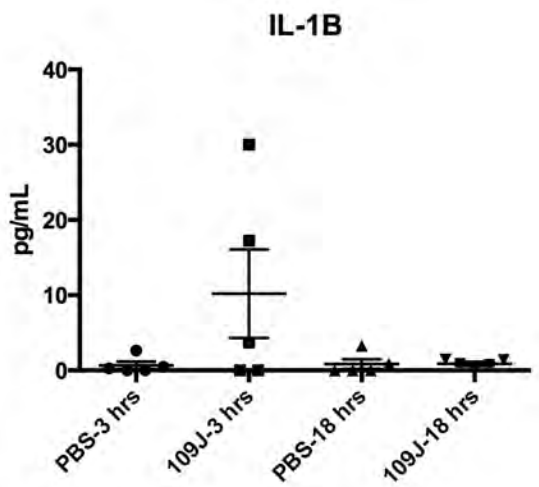


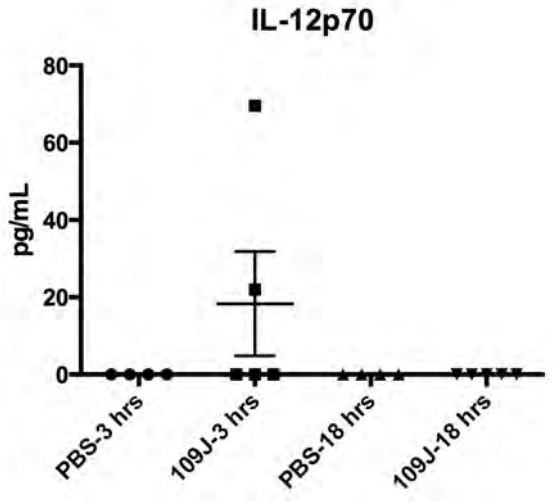
No statistical significant difference (P>0.05) in total WBC levels was found between 109J and PBS control 3hr and 18 hr post-inoculation. Graphpad Prism 5 software was used to perform statistical analysis.
This analysis consisted of One-way ANOVA with Tukey's multiple comparison test.

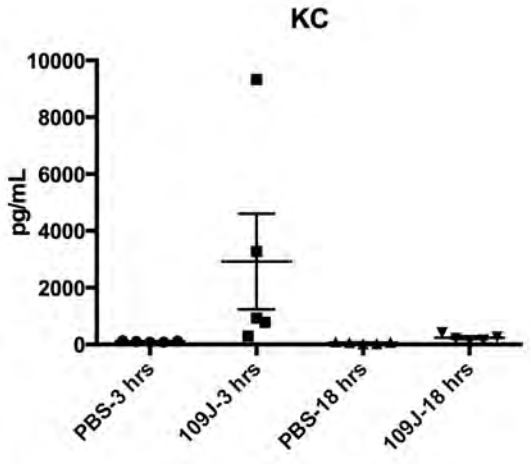
Summary Aim-II, Task 1C, 1.

Inflammatory cytokine levels in blood, 3 and 18 hr post-injection of *Bdellovibrio*.



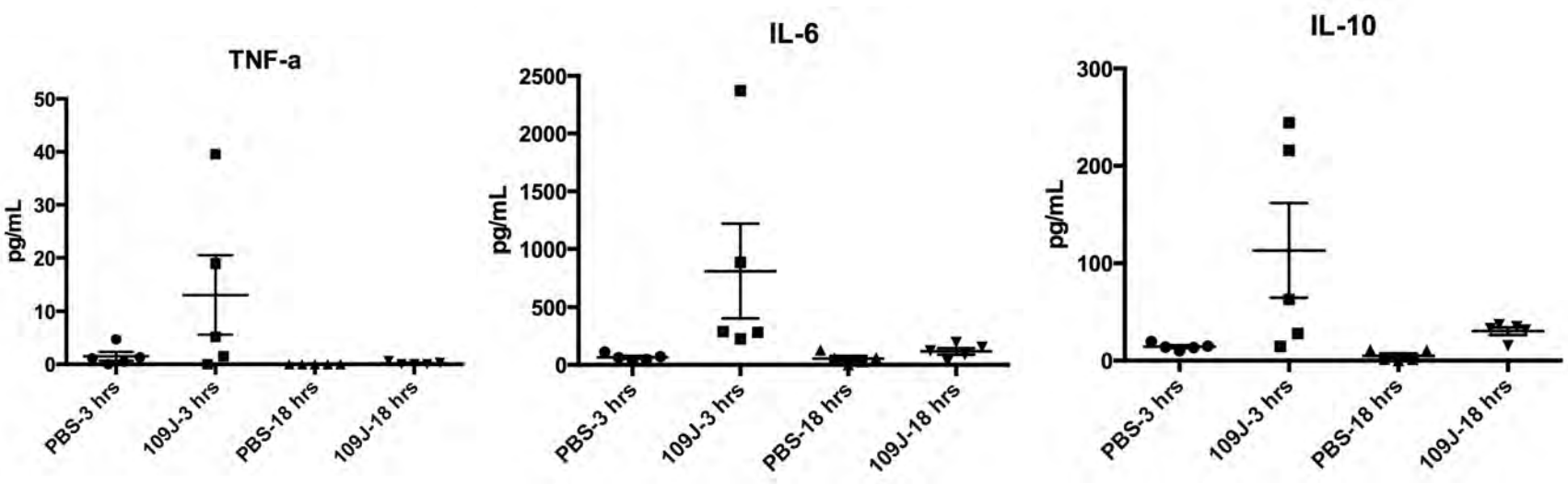






Summary Aim-II, Task 1C, 1.

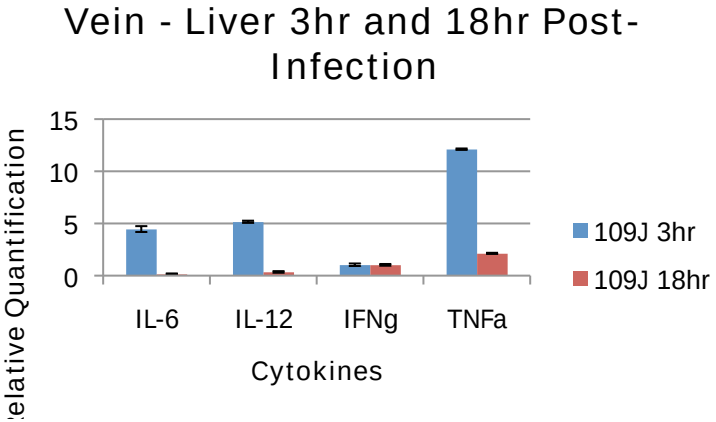
Inflammatory cytokine levels in blood, 3 and 18 hr post-injection of *Bdellovibrio*.

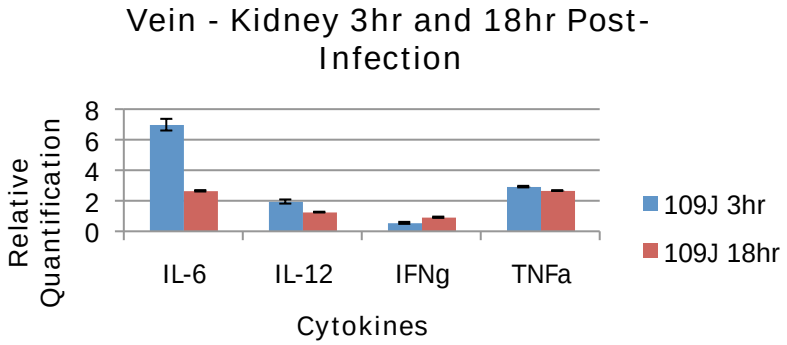


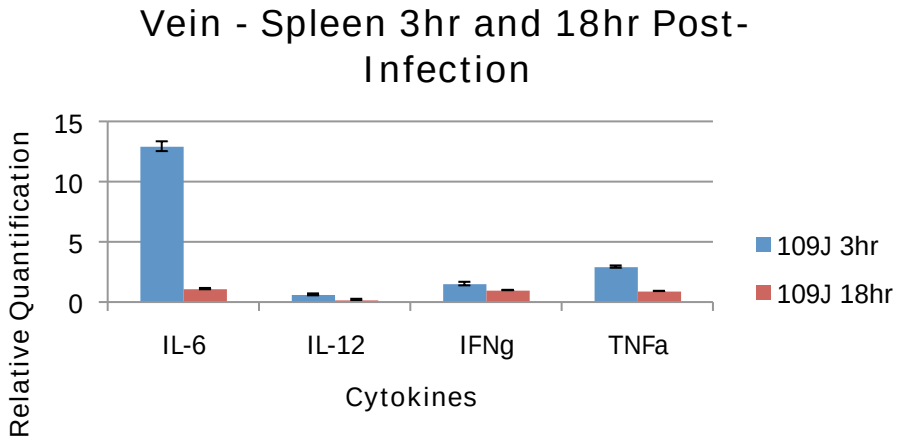
No statistical significant difference (P>0.05) in cytokine levels was found between 109J and PBS control 3hr and 18 hr post-inoculation. Graphpad Prism 5 software was used to perform statistical analysis.
This analysis consisted of One-way ANOVA with Tukey's multiple comparison test.

Summary Aim-II, Task 1C, 1.

Task-1C, 1. *Short exposure experiment*.
Cytokine profiles of inflammatory cytokines measured by qPCR in liver, kidney and spleen from animals injected with *Bdellovibrio* 109J. Increased levels of expression TNF (liver), IL-6 (kidney and spleen) were noted at 3 hours post-injection, but returned to normal levels by 18 hrs. This result reflects the host's response to and efficient clearance of invading organism.



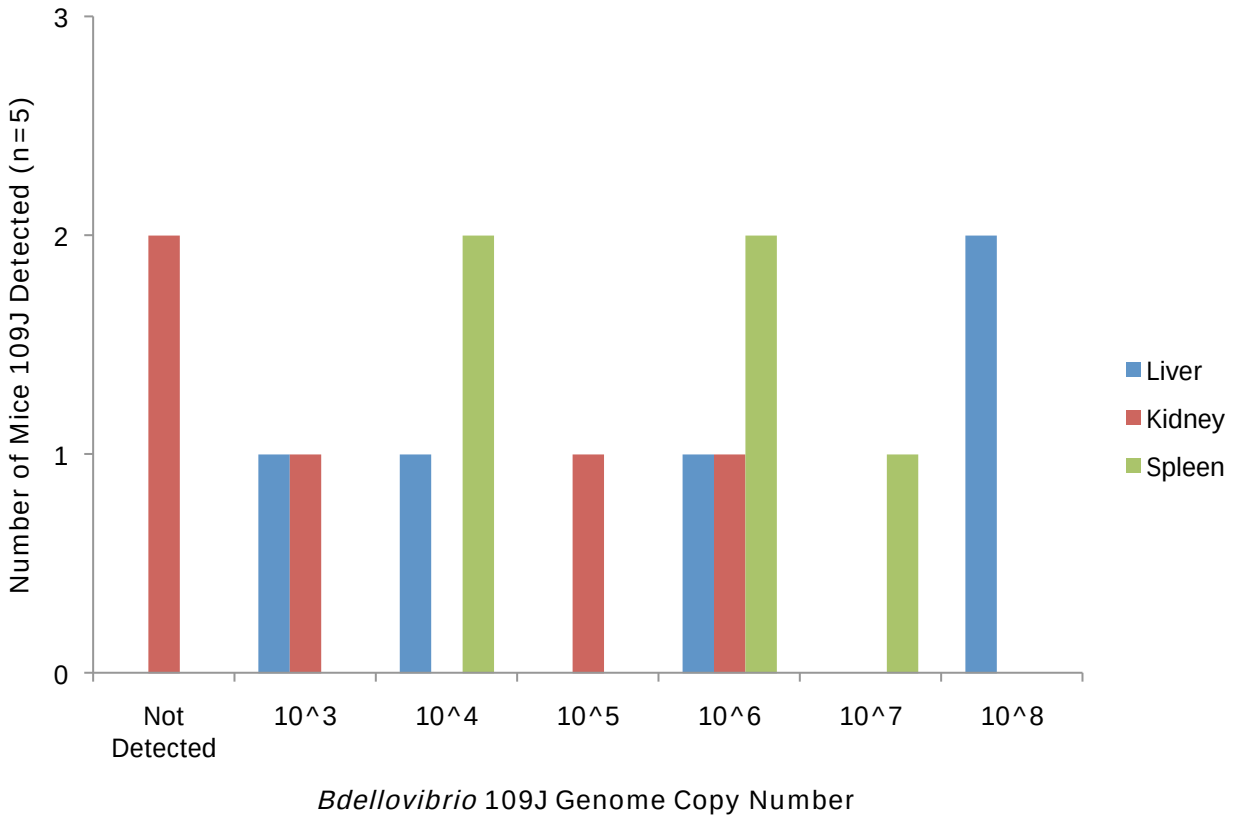




Summary Aim-II, Task 1C, 1.

Task-1C, 1. *Short exposure experiment*.

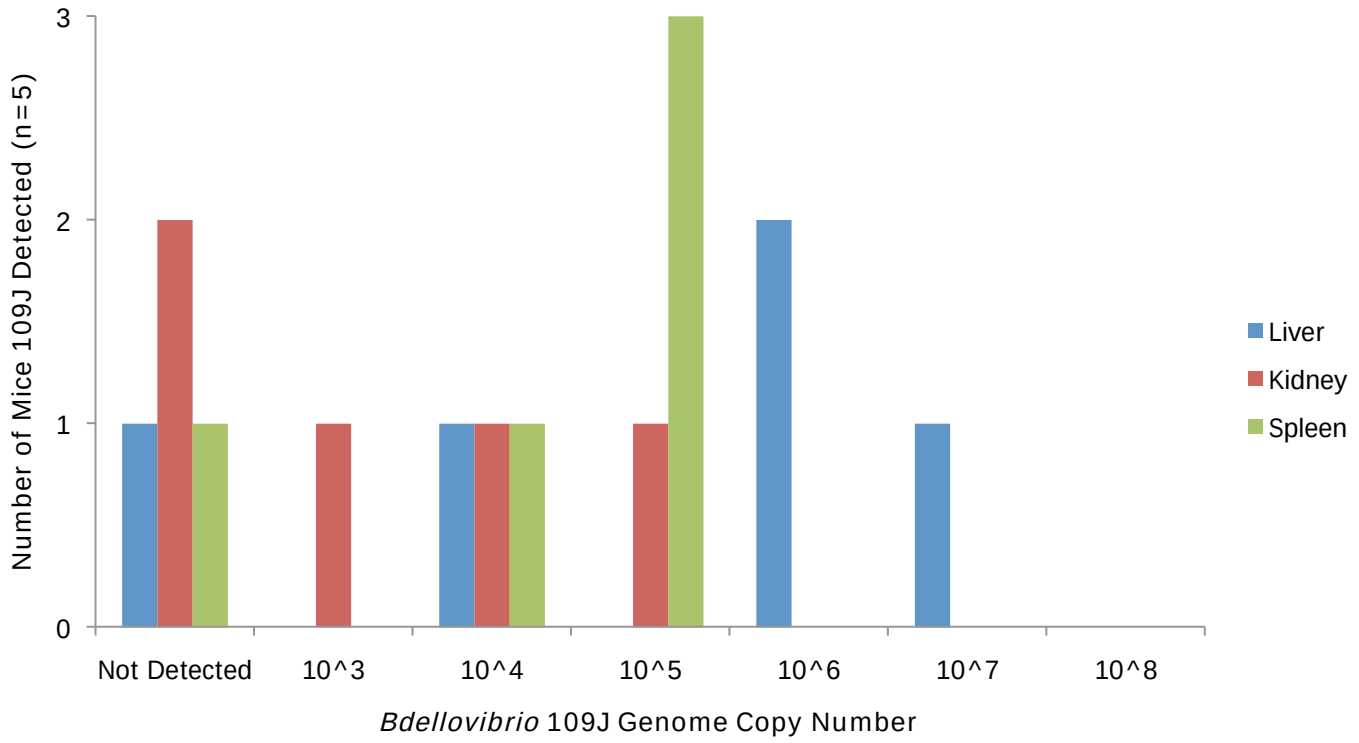
B. Bacteriovorus 109J DNA detected using RT-PCR
in liver, kidney and spleen of mice infected via tail vein, 3 hr post infection



Summary Aim-II, Task 1C, 1.

Task-1C, 1. *Short exposure experiment*.

B. Bacteriovorus 109J DNA detected using RT-PCR
in liver, kidney and spleen of mice infected via tail vein, 18 hr post infection



Summary Aim-II, Task 1C, 2A.

Task-1C, 2A*, Long exposure experiment*.
The aim of this task was to evaluate the effect of a single bacteremia event on mice viability.

5 mice per group, were divided into 2 groups:

(1) Control, injected intravenously with the carrier PBS and euthanized 20 days post inoculation.
(2) Mice inoculated intravenously with 10^8 PFU/mouse *B. bacteriovorus* strain 109J and euthanized 20 days post inoculation.

Twenty days post initial inoculation, animals were euthanized and blood, liver, kidney and spleen samples will be collected.

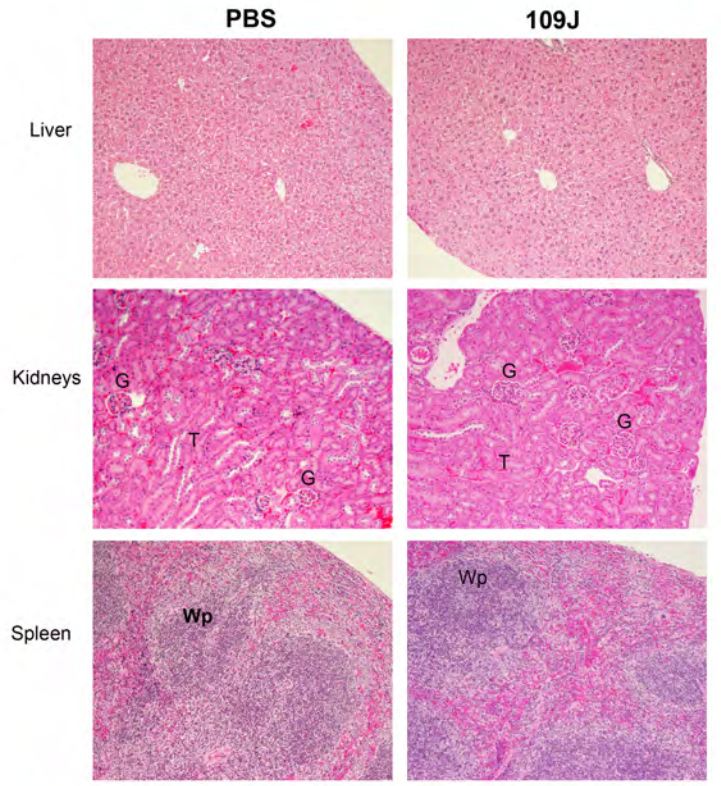
Results: No visual signs of infection were seen in any of the mice 20 days post-injection.

Summary Aim-II, Task 1C, 2A.

Task-1C, 2A*, Long exposure experiment*.

Histological report:

Histological examination of the slides from mice infected by the tail vein revealed no pathology in any of the organs (liver, kidneys and spleen). Micrographs of the liver show normal hepatic cells in both groups of mice, control and 109J-infected. The kidneys also have normal structure with glomeruli and tubules. No pathology is detected in the spleen, with well preserved red (presence of erythrocytes) and white pulp (tightly packed lymphocytes). No signs of inflammation at this time point.



Total magnification of all micrographs is 100x
G - glomeruli, T - tubules, Wp - white pulp

Summary Aim-II, Task 1C, 2B.

Task-1C, 2B*, Long exposure experiment*.
The aim of this task was to evaluate the effect of multiple bacteremia event on mice viability.

5 mice per group, were divided into 2 groups:

(1) Control, injected intravenously with the carrier PBS on day 1 and again on day 10. Animals will be euthanized 20 days post initial inoculation.
(2) Mice inoculated intravenously with 10^8 PFU/mouse *B. bacteriovorus* strain 109J on day 1 and again on day 10. Animals will be euthanized 20 days post initial inoculation.

Twenty days post initial inoculation, animals will be euthanized and blood, liver, kidney and spleen samples will be collected.

Results: No visual signs of infection were seen in any of the mice 10 days post-initial injection and 10 days post second injection.

The histological examination and detection of *B. bacteriovorus* strain 109J in the tissues are in progress.

Summary Aim-II, Task 1C, 2A-B.

Long exposure experiment.

20-Day Dissemination:

There was no detectable *Bdellovibrio* DNA at 20 days,
with and without re-injection after 10 days.

20-Day Tail Vein (109J)

KIDNEY	PBS	Not Detected	Total # Detected
20 Days	0/4	4	0/4
10 Day Re-inject	0/4	4	0/4

LIVER	PBS	Not Detected	Total # Detected
20 Days	0/4	4	0/4
10 Day Re-inject	0/4	4	0/4

SPLEEN	PBS	Not Detected	Total # Detected
20 Days	0/4	4	0/4
10 Day Re-inject	0/4	4	0/4

Summary Aim-II, Task 2.

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-2. Measuring the ability of predatory bacteria to control and clear an upper respiratory infection.
Task-2A. *Initial model optimization.*
Task-2B. *Predatory bacteria efficacy.*

Task 2	Experiments connected
Task-2A. *Initial model optimization.*	Conducted 4 times
Task-2B. *Predatory bacteria efficacy.*	Completed 4 experiments using *B. bacteriovorus* 109J Completed 1 experiments using *B. bacteriovorus* HD100 Completed 1 experiments using *M. aeruginosavorus*

Summary Aim-II, Task 2A

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-2. Measuring the ability of predatory bacteria to control and clear an upper respiratory infection.

Task-2A. *Initial model optimization.*
A pilot experiment will be conducted in order to verify that the *K. pneumoniae* BAA-1706 will infect the mice without being hyper virulent.

Mice, 4 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2-5) mice inoculated with $1x10^2$ to $1x10^5$ CFU/mouse *K. pneumoniae* ATCC BAA-1706.

Mice were kept for 2 days and visually assessed for signs of infection, illness and discomfort.
After 2 days 1 mouse from each group was euthanized, lung, liver, and spleen samples were collected and homogenized. The presence of the pathogen in the tissue was evaluated by direct cell enumeration.

Results: No visual signs of infection were seen in any of the mice 7 days post-infection. We were not able to recover viable *K. pneumoniae* cells form the collected tissue.

Conclusion: *K. pneumoniae* strain BAA-1706 is not virulent at $1x10^5$ CFU/mouse.

Summary Aim-II, Task 2A

Task-2A. *Initial model optimization.*
A pilot experiment was conducted in order to verify that the *K. pneumoniae* ATCC 43816 can infect the mice without being hyper virulent.

Mice, 2-4 mice per group, were divided into 8 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2-8) mice inoculated with $1x10^3$ to $1x10^9$ CFU /mouse *K. pneumoniae* ATCC 43816 .

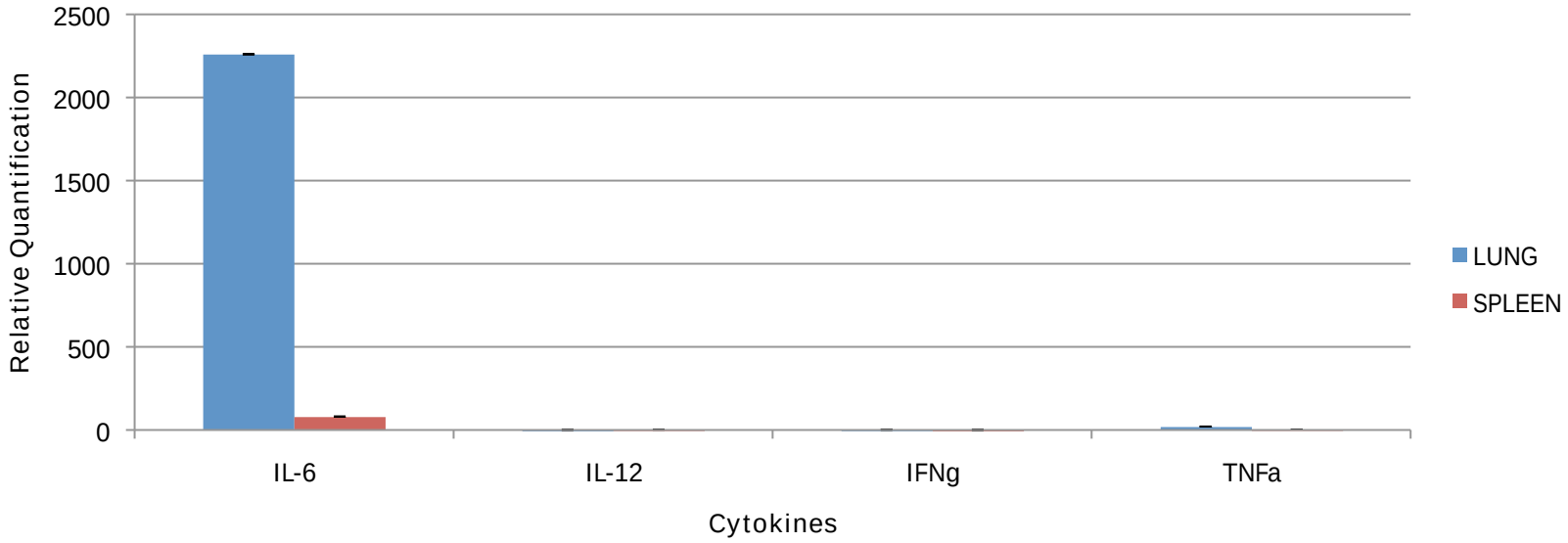
Mice were kept for 2 days and visually assessed for signs of infection, illness and discomfort.
After 1-2 days mice which show visual signs of infection were euthanized and lung samples were collected. The presence of the pathogen in the tissue was evaluated by direct cell enumeration.

Results: Visual signs of infection were seen 24 hrs post infection in mice inoculated with $1x10^9$ CFU /mouse *K. pneumoniae* ATCC 43816.

Conclusion: *K. pneumoniae* strain 43816 is virulent at $1x10^9$ CFU/mouse.

Summary Aim-II, Task 2A

Cytokine profiles of inflammatory cytokines measured by qPCR in lung and and spleen of mice infected with *K. pneumoniae.* Two mice were euthanized at 24 hrs post-infection. IL-6 levels were increased in the lungs, reflecting a strong response to *K. pneumoniae*.



Summary Aim-II, Task 2A

Task-2A. *Initial model optimization.*
A second pilot experiment was conducted in order to verify that the *K. pneumoniae* HV1 can infect the mice without being hyper virulent.

Mice, 2-4 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2-5) mice inoculated with $1x10^4$ to $1x10^7$ CFU /mouse *K. pneumoniae* HV1.

Mice were kept for 1 days and visually assessed for signs of infection, illness and discomfort.

Results: No visual signs of infection were seen 48 hrs post infection in mice inoculated with $1x10^7$ CFU / mouse *K. pneumoniae* HV1.

Conclusion: *K. pneumoniae* strain HV1 is not virulent at $1x10^7$ CFU/mouse.

Summary Aim-II, Task 2A

Task-2A. *Initial model optimization.*
A second pilot experiment was conducted in order to verify that the *K. pneumoniae* HV2 can infect the mice without being hyper virulent.

Mice, 2-4 mice per group, were divided into 6 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2-6) mice inoculated with $1x10^3$ to $1x10^7$ CFU /mouse *K. pneumoniae* HV2.

Mice were kept for 1 days and visually assessed for signs of infection, illness and discomfort.

Results: Visual signs of infection were seen 24 hrs post infection in mice inoculated with $1x10^6$ CFU /mouse *K. pneumoniae* HV2.

Conclusion: *K. pneumoniae* strain HV2 is virulent at $1x10^6$ CFU/mouse.

Summary Aim-II, Task 2B, Exp-1

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-2. Measuring the ability of predatory bacteria to control and clear an upper respiratory infection.

Task-2B. *Predatory bacteria efficacy.*
The aim of this task was to evaluate the ability of *Bdellovibrio* to control and clear an upper respiratory infection of *K. pneumoniae.*

5 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $1x10^8$ CFU /mouse *K. pneumoniae* ATCC 43816 .
(3) mice inoculated with $2x10^9$ PFU/mouse *B. bacteriovorus* strain 109J.
(4) experimental groups inoculated with $1x10^8$ CFU/ml *K. pneumoniae*, together with $2x10^9$ PFU/ml of *B. bacteriovorus* strain 109J.
(5) experimental groups inoculated with $1x10^8$ CFU/ml *K. pneumoniae*, followed by $2x10^9$ PFU/ml of *B. bacteriovorus* strain 109J inserted 60 min after the initial *K. pneumoniae* inoculation.

Twenty hrs post inoculation, animals were euthanized and lung, serum liver, and spleen samples were collected.

Summary Aim-II, Task 2B, Exp-1

Task-2B. *Predatory bacteria efficacy.*

To evaluate the effect of the inoculation on the mice, mice were visually assessed for signs of infection. Physiological observations, including body, mobility, skin ruffling, and the general condition of the animals was conducted and recorded.

Group	Total no of mice	Mice showing no visual signs of infection	Mice showing visual signs of infection
PBS control	5	5 (100%)	0 (0%)
K. pneumoniae	5	3 (60%)	2 (40%)
B. bacteriovorus	5	5 (100%)	0 (0%)
K. pneumoniae + *B. bacteriovorus*	5	5 (100%)	0 (0%)
K. pneumoniae + *B. bacteriovorus* after 1 hr	5	4 (80%)	1 (20%)

Summary Aim-II, Task 2B, Exp-1

Task-2B. *Predatory bacteria efficacy.*
The efficacy of predatory bacteria in reducing the pathogen concentration was evaluated by direct cell enumeration. Lung tissue was homogenized in PBS and total CFU per lung was determined by plating.

K. pneumoniae load measured in lung 24 hr post infection (CFU/ml).

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
Group					
PBS control	-	-	-	-	-
K. pneumoniae	$6x10^4$	$6.5x10^4$	0	$6.5x10^8$	$9.5x10^8$
B. bacteriovorus	-	-	-	-	-
K. pneumoniae + *B. bacteriovorus*	$2.5x10^3$	$9x10^3$	$1.5x10^3$	0	$3x10^4$
K. pneumoniae + *B. bacteriovorus* after 1 hr	0	$3.5x10^4$	$5x10^2$	0	$6x10^8$

Summary Aim-II, Task 2B, Exp-2

5 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $1x10^9$ CFU /mouse *K. pneumoniae* ATCC 43816 .
(3) mice inoculated with $3.7x10^9$ PFU/mouse *B. bacteriovorus* strain 109J.
(4) experimental groups inoculated with $1x10^9$ CFU/ml *K. pneumoniae*, together with $3.7x10^9$ PFU/ml of *B. bacteriovorus* strain 109J.
(5) experimental groups inoculated with $1x10^9$ CFU/ml *K. pneumoniae*, followed by $3.7x10^9$ PFU/ml of *B. bacteriovorus* strain 109J inserted 60 min after the initial *K. pneumoniae* inoculation.

Twenty hrs post inoculation, animals were euthanized and lung, serum liver, and spleen samples were collected.

Summary Aim-II, Task 2B, Exp-2

Task-2B. *Predatory bacteria efficacy.*

To evaluate the effect of the inoculation on the mice, mice were visually assessed for signs of infection. Physiological observations, including body, mobility, skin ruffling, and the general condition of the animals was conducted and recorded.

Group	Total no of mice	Mice showing no visual signs of infection	Mice showing visual signs of infection
PBS control	5	5 (100%)	0 (0%)
K. pneumoniae	5	2 (40%)	3 (60%)
B. bacteriovorus	5	5 (100%)	0 (0%)
K. pneumoniae + *B. bacteriovorus*	5	3 (60%)	2 (40%)
K. pneumoniae + *B. bacteriovorus* after 1 hr	5	3 (60%)	2 (40%)

Summary Aim-II, Task 2B, Exp-2

Task-2B. *Predatory bacteria efficacy.*
The efficacy of predatory bacteria in reducing the pathogen concentration was evaluated by direct cell enumeration. Lung tissue was homogenized in PBS and total CFU per lung was determined by plating.

K. pneumoniae load measured in lung 24 hr post infection (CFU/ml).

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
Group					
PBS control	-	-	-	-	-
K. pneumoniae	$5x10^6$	$2x10^5$	$4.5x10^8$	$6.45x10^8$	$8x10^8$
B. bacteriovorus	-	-	-	-	-
K. pneumoniae + *B. bacteriovorus*	$5.5x10^5$	$4x10^4$	$3.5x10^8$	$6.25x10^5$	$1.5x10^6$
K. pneumoniae + *B. bacteriovorus* after 1 hr	$9x10^4$	$1.25x10^6$	$1x10^4$	$8.5x10^8$	$3.5x10^9$

Summary Aim-II, Task 2B, Exp-3

5 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $6.6x10^{10}$ CFU /mouse *K. pneumoniae* ATCC 43816 .
(3) mice inoculated with $1x10^{11}$ PFU/mouse *B. bacteriovorus* strain 109J.
(4) experimental groups inoculated with $6.6x10^{10}$ CFU/ml *K. pneumoniae,* together with $1x10^{11}$ PFU/ml of *B. bacteriovorus* strain 109J.
(5) experimental groups inoculated with $6.6x10^{10}$ CFU/ml *K. pneumoniae,* followed by $1x10^{11}$ PFU/ml of *B. bacteriovorus* strain 109J inserted 30 min after the initial *K. pneumoniae* inoculation.

Twenty hrs post inoculation, animals were euthanized and lung, serum liver, and spleen samples were collected.

Summary Aim-II, Task 2B, Exp-3

Task-2B. *Predatory bacteria efficacy.*

To evaluate the effect of the inoculation on the mice, mice were visually assessed for signs of infection. Physiological observations, including body, mobility, skin ruffling, and the general condition of the animals was conducted and recorded.

Group	Total no of mice	Mice showing no visual signs of infection	Mice showing visual signs of infection
PBS control	5	5 (100%)	0 (0%)
K. pneumoniae	5	1 (20%)	4 (80%)
B. bacteriovorus	5	5 (100%)	0 (0%)
K. pneumoniae + *B. bacteriovorus*	5	3 (60%)	2 (40%)
K. pneumoniae + *B. bacteriovorus* after 30 min	5	0 (0%)	5 (100%)

Summary Aim-II, Task 2B, Exp-3

Task-2B. *Predatory bacteria efficacy.*
The efficacy of predatory bacteria in reducing the pathogen concentration was evaluated by direct cell enumeration. Lung tissue was homogenized in PBS and total CFU per lung was determined by plating.

K. pneumoniae load measured in lung 24 hr post infection (CFU/ml).

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
Group					
PBS control	-	-	-	-	-
K. pneumoniae	$3x10^8$	$5.5x10^8$	$6x10^8$	$1.25x10^8$	$1.05x10^5$
B. bacteriovorus	-	-	-	-	-
K. pneumoniae + *B. bacteriovorus*	$2x10^6$	$1.5x10^4$	0	$8x10^8$	$1x10^9$
K. pneumoniae + *B. bacteriovorus* after 1 hr	$3.25x10^6$	$8x10^4$	$6.5x10^8$	$1.1x10^9$	$3.5x10^9$

Summary Aim-II, Task 2B, Exp-4

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-2. Measuring the ability of predatory bacteria to control and clear an upper respiratory infection.

Task-2B. *Predatory bacteria efficacy.*
The aim of this task was to evaluate the ability of *Bdellovibrio* to control and clear an upper respiratory infection of *K. pneumoniae.*

Mice were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $1.7x10^9$ CFU /mouse *K. pneumoniae* ATCC 43816 .
(3) mice inoculated with $8.5x10^8$ PFU/mouse *B. bacteriovorus* strain 109J.
(4) experimental groups inoculated with $1.7x10^9$ CFU/ml *K. pneumoniae*, together with $8.5x10^8$ PFU/ml of *B. bacteriovorus* strain 109J.
(5) experimental groups inoculated with $1.7x10^9$ CFU/ml *K. pneumoniae*, followed by $8.5x10^8$ PFU/ml of *B. bacteriovorus* strain 109J inserted 30 min after the initial *K. pneumoniae* inoculation.

Twenty hrs post inoculation, animals were euthanized and lung, serum liver, and spleen samples were collected.

Summary Aim-II, Task 2B, Exp-4

Task-2B. *Predatory bacteria efficacy.*

To evaluate the effect of the inoculation on the mice, mice were visually assessed for signs of infection. Physiological observations, including body, mobility, skin ruffling, and the general condition of the animals was conducted and recorded.

Group	Total no of mice	Mice showing no visual signs of infection	Mice showing visual signs of infection
PBS control	5	5 (100%)	0 (0%)
K. pneumoniae	15	0 (0)	15 (100)
B. bacteriovorus	5	5 (100%)	0 (0%)
K. pneumoniae + *B. bacteriovorus*	15	5 (33.3%)	10 (76.6%)
K. pneumoniae + *B. bacteriovorus* after 30 min	15	0 (0%)	15 (100%)

Summary Aim-II, Task 2B, Exp-4

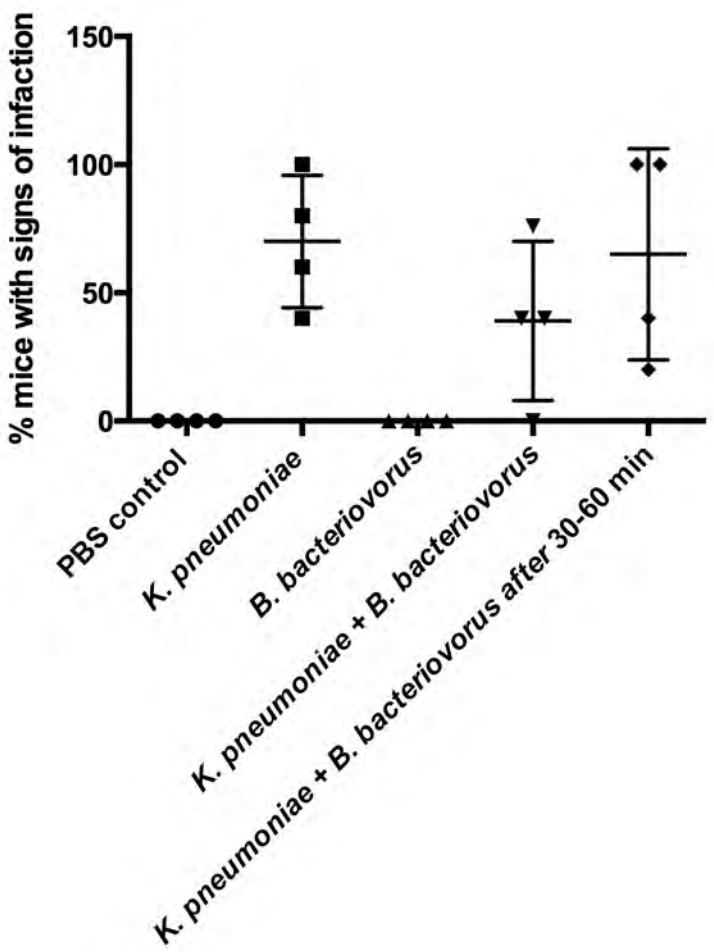
Task-2B. *Predatory bacteria efficacy.*

To evaluate the effect of the inoculation on the mice, mice were visually assessed for signs of infection. Physiological observations, including body, mobility, skin ruffling, and the general condition of the animals was conducted and recorded.

Group	Total no of mice	Mice showing no visual signs of infection	Mice showing visual signs of infection
PBS control	20	20 (100%)	0 (0%)
K. pneumoniae	30	6 (20%)	24 (80%)
B. bacteriovorus	30	30 (100%)	0 (0%)
K. pneumoniae + *B. bacteriovorus*	30	16 (53.3%)	14 (46.6%)
K. pneumoniae + *B. bacteriovorus* after 30-60 min	30	7 (23.3%)	23 (76.6%)

Summary Aim-II, Task 2B, average Exp 1-4

Task-2B. *Predatory bacteria efficacy.*



Summary Aim-II, Task 2B, Exp-5

5 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $1.5x10^9$ CFU /mouse *K. pneumoniae* ATCC 43816 .
(3) mice inoculated with $1x10^{11}$ PFU/mouse *B. bacteriovorus* strain HD100.
(4) experimental groups inoculated with $1.5x10^9$ CFU/ml *K. pneumoniae*, together with $1x10^{11}$ PFU/ml of *B. bacteriovorus* strain HD100.
(5) experimental groups inoculated with $1.5x10^9$ CFU/ml *K. pneumoniae*, followed by $1x10^{11}$ PFU/ml of *B. bacteriovorus* strain HD100 inserted 30 min after the initial *K. pneumoniae* inoculation.

Twenty hrs post inoculation, animals were euthanized and lung, serum liver, and spleen samples were collected.

Summary Aim-II, Task 2B, Exp-5

Task-2B. *Predatory bacteria efficacy.*

To evaluate the effect of the inoculation on the mice, mice were visually assessed for signs of infection. Physiological observations, including body, mobility, skin ruffling, and the general condition of the animals was conducted and recorded.

Group	Total no of mice	Mice showing no visual signs of infection	Mice showing visual signs of infection
PBS control	5	5 (100%)	0 (0%)
K. pneumoniae	5	0 (0%)	5 (100%)
B. bacteriovorus	5	5 (100%)	0 (0%)
K. pneumoniae + *B. bacteriovorus*	5	2 (40%)	3 (60%)
K. pneumoniae + *B. bacteriovorus* after 30 min	5	2 (40%)	3 (60%)

Summary Aim-II, Task 2B, Exp-6

5 mice per group, were divided into 5 groups:

(1) control non-infected, which was inoculated with the carrier, Phosphate buffered saline (PBS).
(2) mice inoculated with $6x10^9$ CFU /mouse *K. pneumoniae* ATCC 43816 .
(3) mice inoculated with $6.2x10^8$ PFU/mouse *M. aeruginosavorus*.
(4) experimental groups inoculated with $6x10^9$ CFU/ml *K. pneumoniae*, together with $6.2x10^8$ PFU/ml of *M. aeruginosavorus*.
(5) experimental groups inoculated with $6x10^9$ CFU/ml *K. pneumoniae*, followed by $6.2x10^8$ PFU/ml of *M. aeruginosavorus* inserted 30 min after the initial *K. pneumoniae* inoculation.

Twenty hrs post inoculation, animals were euthanized and lung, serum liver, and spleen samples were collected.

Summary Aim-II, Task 2B, Exp-6

Task-2B. *Predatory bacteria efficacy.*

To evaluate the effect of the inoculation on the mice, mice were visually assessed for signs of infection. Physiological observations, including body, mobility, skin ruffling, and the general condition of the animals was conducted and recorded.

Group	Total no of mice	Mice showing no visual signs of infection	Mice showing visual signs of infection
PBS control	5	5 (100%)	0 (0%)
K. pneumoniae	5	0 (0%)	5 (100%)
M. aeruginosavorus.	5	5 (100%)	0 (0%)
K. pneumoniae + *M. aeruginosavorus*.	5	4 (80%)	1 (20%)
K. pneumoniae M. aeruginosavorus after 30 min	5	0 (0%)	5 (100%)

Summary of the most important results

Aim I. The use of predatory bacteria to control select agents.

- *B. bacteriovorus* and *M. aeruginosavorus* are able to prey on *Yersinia pestis* and *Burkholderia mallei*.
- Modest predation was also measured in co-cultures of *B. bacteriovorus* and *Francisella tularensis.*
- *B. bacteriovorus* and *M. aeruginosavorus* are unable to prey on *Burkholderia pseudomallei* and *Brucella melitensis*.

Aim II. The use of predatory bacteria as a novel biological-therapeutic agent to treat upper respiratory infections.

Task-1. *Measuring the in-vivo effect of exposure to predatory bacteria*

- Intranasal and intravenous inoculations of high concentrations of predatory bacteria are non-toxic to mice and provoke only a transient immune response.
- In both models, none of the exposed mice exhibited a sustained increases in inflammatory marker gene or protein expression indicating that predatory bacteria do not provoke a significant immune response.
- A bacteremia model using serial intravenous inoculations showed no effect on mouse viability after repeat exposure to *B. bacteriovorus* 109J.
- Histological examination of tissue in both models revealed no pathology in any of the organs tested, suggesting predatory bacteria have no visible negative effects on the overall health of the mice.
- Bacterial dissemination experiments showed predatory bacteria were efficiently cleared from mice in both models.

Task-2. Measuring the ability of predatory bacteria to control and clear an upper respiratory infection.

- Administering *K. pneumoniae* simultaneously with *B. bacteriovorus* or *M. aeruginosavorus* saw measurable, but not statistical significant, decrease in the number of mice showing visual signs of infection.
- Administering *B. bacteriovorus* or *M. aeruginosavorus* 30-60 minutes after *K. pneumoniae* infection did not produce a measurable decreasing in the number of mice showing visual signs of infection.

Examining the effect of respiratory and intravenous inoculation of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* in a mouse model

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ABSTRACT

Bdellovibrio spp. and *Micavibrio* spp. are Gram-negative predators that feed on other Gram-negative bacteria, making predatory bacteria potential alternatives to antibiotics for treating multi-drug resistant infections. While the ability of predatory bacteria to control bacterial infections *in vitro* is well documented, the *in vivo* effect of predators on a living host has yet to be examined extensively. In this study, respiratory and intravenous inoculations were used to determine the effects of predatory bacteria in mice. We found no reduction in mouse viability after intranasal or intravenous inoculation of *B. bacteriovorus* 109J, HD100 and *M. aeruginosavorus.* Introducing predators into the respiratory tract of mice provoked a modest inflammatory response at 1 hour post-exposure, but was not sustained at 24 hours, as measured by RT-qPCR and ELISA. Intravenous injection caused an increase of IL-6 in the kidney and spleen, TNF in the liver and CXCL-1/KC in the blood at 3 hours post-exposure, regressing to baseline levels by 18 hours. Histological analysis of tissue showed no pathological effect due to predatory bacteria. Furthermore, qPCR detected predators were cleared from the host quickly and efficiently. This work addresses some of the safety concerns regarding the potential use of predatory bacteria as a live antibiotic.

INTRODUCTION

Bdellovibrio bacteriovorus and *Micavibrio aeruginosavorus* are small, highly motile, uniflagellate Gram-negative bacteria that naturally prey on other Gram-negative bacteria[1,2]. Recently, predatory bacteria have been viewed as potential alternatives to traditional antibiotics for treating multi-drug resistant (MDR) Gram-negative bacterial infections. *B. bacteriovorus* undergo a predatory lifestyle where they attach to and enter the prey periplasm, multiply by exhausting the nutrients, lyse the cell, and then continue to seek out more prey to invade[1,3,4]. In contrast, *Micavibrio* attach to, grow and kill prey at the surface of the prey cell in a 'vampire'-like fashion[2,5,6].

Bdellovibrio-and-like organisms (BALOs) are a promising potential novel agent against mammalian pathogens and present several advantages for their use for controlling infection[7]. Previous studies have confirmed the ability of predatory bacteria to control a broad range of important human pathogens *in vitro*, including MDR bacteria[8], grown both planktonically and in biofilms[9-11]. In addition, BALOs appear to have no negative effect on human cells when challenged *in vitro*[12]. Recent studies have presented evidence that BALOs might be native commensals of the human gut and might even play a role in maintaining healthy gut flora[13]. In addition, development of genetically stable predation-resistance in a normally susceptible species has yet to be confirmed[14], a major advantage over current available antibiotic therapies. To date, the majority of the studies dealing with predatory bacteria have been performed *in vitro*, and as such, the *in vivo* effect of predatory bacteria on a living host is still not well understood.

Early animal studies found *Bdellovibrio* to be non-pathogenic when injected into mice, rabbits and guinea pigs[7,15], while another study demonstrated that *Bdellovibrio* could not survive

in the gastrointestinal tracts of fish, mice and frogs[16]. A more recent study showed predatory bacteria are non-toxic when fed to young chicks[17]. Nevertheless, studies aiming to understand the effects of predatory bacteria in an animal host are still few and limited with no study, to our knowledge, focusing on the host immunological response to predatory bacteria inoculation *in vivo*.

Before predatory bacteria can be used clinically, their safety in a mammalian host must be confirmed. In this study, respiratory and intravenous inoculation models were used to demonstrate the effect of predatory bacteria in a mouse. The work presented here highlights the potential use of predatory bacteria as a future biological-based agent for controlling infection.

RESULTS

Effect of Respiratory Introduction of Predatory Bacteria

***Host viability and histology*.** To examine the effect of respiratory exposure of predatory bacteria on host survival, we administered intranasally 1 x 10^9 PFU/mouse of *B. bacteriovorus* 109J, HD100 or 1 x 10^6 PFU/mouse *M. aeruginosavorus* ARL-13 to three groups of C57BL/6 mice (5 mice per group) and monitored animals for any signs of infection, illness or discomfort. To measure the effect of predatory bacteria cell particles, 1 x 10^9 PFU/mouse of non-viable heat-killed *B. bacteriovorus* 109J and HD100 were also administered to two other groups of mice. Phosphate buffered saline (PBS) was used as a negative control. At five days post-inoculation, all 25 mice inoculated with viable or heat-killed predatory bacteria were healthy with no visual adverse effects or change in behavior (Table 1). At this point, three mice were sacrificed for further evaluation and two mice from each group were kept and visually assessed for up to 50

days. As before, no visual signs of infection were seen, with all inoculated mice remaining viable and healthy 50 days post-inoculation (Table 1).

Forty-eight hours post-inoculation, histological examination of the lungs and spleens of mice inoculated with *Bdellovibrio* and *Micavibrio* revealed no pathology compared to the control mice, treated with PBS (Fig. 1). Lung parenchyma was found with normal appearance and well preserved in most of the sections. Some of the sections from both groups (inoculated and control) showed increased cellularity in some areas, predominantly mononuclear cells (lymphocytes and macrophages), but no neutrophils. It is most likely these changes resulted from removing and processing the tissue.

Host immune response to predatory bacteria. To examine the effects of respiratory introduction of predatory bacteria on the host immune response, we introduced each predator through intranasal inoculation into the respiratory tract of mice. Mice were visually monitored for signs of illness or discomfort, and euthanized at 1, 24 or 48 hours post-inoculation when organs and blood were harvested.

For the one hour time point experiment, 6 mice per predatory bacterial strain were exposed to 4 x 10^9 PFU/mouse of *B. bacteriovorus* 109J, 7 x 10^9 PFU/mouse of *B. bacteriovorus* HD100 and 5 x 10^8 PFU/mouse of *M. aeruginosavorus* ARL-13. At the 24 and 48 hour time points, 12 mice per strain were exposed to an inoculation dose of 1 x 10^9 PFU/mouse of both *B. bacteriovorus* 109J, HD100 and 1 x 10^{11} PFU/mouse of *M. aeruginosavorus* ARL-13. Total RNA was extracted from the lungs and spleen, and inflammatory cytokines were measured using RT-qPCR.

As before, none of the total 90 mice that were inoculated with predatory bacteria exhibited any visual adverse effects and all were healthy at the time when sacrificed. At one hour

post-inoculation, we observed an increase of IL-1β (9.0- and 12.3-fold), IL-23 (6.3- and 12.6-fold) and TNF (5.0- and 7.9-fold) in the lungs of mice exposed to *B. bacteriovorus* 109J and *M. aeruginosavorus*, respectively (Fig. 2A). However, this increased expression was not sustained at 24 or 48 hours post-inoculation (Fig. 2B-C). Conversely, none of the mice exposed to *B. bacteriovorus* HD100 exhibited a substantial (5-fold or higher) increase in expression of any inflammatory marker gene in the lung or spleen at any time point (Fig. 2). Furthermore, no inflammatory response was detected in the spleens of mice inoculated with either *B. bacteriovorus* 109J or *M. aeruginosavorus* at 24 or 48 hours (Fig. 2D-E). Inflammatory proteins in the lungs of inoculated mice were measured with ELISA to confirm the results obtained from qPCR. We did observe a 4.68-fold increase in CXCL-1/KC protein expression due to *B. bacteriovorus* 109J at 24 hours. However, no other inflammatory protein showed a substantial fold induction (5-fold or higher) due to the inoculation with any of the predatory bacteria (Fig. 3). Additionally, mice examined at five days post-inoculation still exhibited no substantial increases in proinflammatory marker gene expression (data not shown). As before, all mice inoculated with the predators were healthy with no visual adverse effects at any of the examined time points.

To assess the change in cytokine levels in the host's response to a known respiratory bacterial pathogen, we introduced, through intranasal inoculation, 1.2 x 10^9 CFU/mouse of *K. pneumoniae* to mice (n=2). As expected, we observed a 2260.3- and 80.7-fold induction of IL-6 in the lungs and spleen, respectively, as well as a 20.8-fold induction of TNF in the lungs of the host at 24 hours post-infection. In comparison, mice exposed to *B. bacteriovorus* 109J and *M. aeruginosavorus* showed only a 1.9- and 4.7-fold induction of IL-6, respectively at 1 hour post-inoculation (Fig. 2A), reflecting a much stronger immune response to *K. pneumoniae*.

Collectively, our data indicate that when inhaled, predatory bacteria do not provoke an elevated, sustained immune response.

Effect of Intravenous Introduction of Predatory Bacteria

Host viability and histology. The effect of predatory bacteria introduced through the intravenous route was also investigated. The tail veins of mice (5 mice per group) were injected with 1 x 10^8 PFU/mouse of *B. bacteriovorus* 109J or PBS control. At 20-days post-inoculation, all mice injected with predatory bacteria were viable and healthy (Table 2). To model a multiple bacteremia event, a group of 5 mice were re-injected with 1 x 10^8 PFU/mouse of *B. bacteriovorus* 109J at 10-days post-initial injection. Again, we did not observe any reduction in mouse viability due to re-injection of predatory bacteria (Table 2). Histological examination, taken 20 days following injection, of the liver, kidney, and spleen revealed no pathology or signs of inflammation compared to the control mice inoculated with PBS (Fig. 4). Micrographs of the liver showed normal hepatic cells in both predator- and control-infected mice. Kidneys also showed normal structure with glomeruli and tubules. No pathology was detected in the spleen, with well preserved red (presence of erythrocytes) and white pulp (tightly packed lymphocytes).

Blood profiling. To determine the effect of intravenous inoculation of predatory bacteria on host blood cell profile, 100 µl of blood was removed from each mouse at 3 and 18 hours post-exposure. White blood cell (WBC) counts were performed and the levels of individual cell types determined (Fig. 5). Surprisingly, total WBC counts decreased compared to the control at 3 and 18 hours post-injection. At 3 hours post-injection, a 3.51- fold increase in the percentage of neutrophils in the blood was seen in mice injected with *B. bacteriovorus* 109J. However, by 18 hours post-exposure, the level of neutrophils in the blood returned to that comparable to the PBS-

injected control animals. In contrast, the percentage of monocytes in the blood remained elevated by 4.7-fold at 18 hours post-injection. Decreased percentages of lymphocytes in the blood were seen in conjunction with the observed increases of neutrophils and monocytes resulting from inoculation with predatory bacteria. Eosinophils were found at comparably low levels in both the control and treated mice.

***Host immune response to B. bacteriovorus* 109J.** To examine the effects of intravenous introduction of predatory bacteria on the host immune system, we injected 1 x 10^8 PFU/mouse of *B. bacteriovorus* 109J into the tail vein of mice (5 mice per treatment group). Mice were once more visually monitored for signs of illness or discomfort, and euthanized at either 3, 18 hours or 20 days post-injection when organs and blood were harvested. To model a multiple bacteremia event, a group of mice were re-injected with 1 x 10^8 PFU/mouse of *B. bacteriovorus* 109J at 10 days post- initial injection. The kidney, liver and spleen were harvested to measure inflammatory cytokines (Fig. 6).

As we observed in the respiratory inoculation model, none of the 20 mice that were injected with predatory bacteria exhibited any visual adverse effects. At 3 hours post injection, we detected an increase in inflammatory cytokines TNF (9.0-fold) in the liver (Fig. 6B) and IL-6 (18.0- and 12.9-fold) in the kidney and spleen (Fig. 6A and 6C, respectively) relative to control. However, as with our results obtained from respiratory introduction, this increased expression was not sustained by 18 hours post-injection (Fig. 6). An ELISA using whole blood from inoculated mice revealed increases in inflammatory proteins, including IL-1β (12.9-fold), IL-6 (18.1-fold), IL-10 (12.5-fold), CXCL-1/KC (52.7-fold), IFNγ (27.4-fold) and TNF (8.7-fold), at 3 hours post-injection, but returned to baseline levels by 18 hours post-injection (Fig. 6D). Taken together, the data suggest that intravenous injection of *B. bacteriovorus* 109J does not provoke a

sustained inflammatory response. Our data reflect the host's response to and efficient clearance of the invading organism.

Bacterial Dissemination within the Host

In order to examine predatory bacterial dissemination and migration following inoculation, we utilized primers targeting the 16S ribosomal RNA region for each of *B. bacteriovorus* 109J, HD100, or *M. aeruginosavorus*. Total RNA from organ samples collected from the previously described respiratory and intravenous mouse experiments were probed for detectable levels of predators using RT-qPCR (Fig. 7). As previously stated, for the one hour time point experiment, an inoculation dose of 4×10^9 PFU/mouse of *B. bacteriovorus* 109J, 7×10^9 PFU/mouse of *B. bacteriovorus* HD100 and 5×10^8 PFU/mouse of *M. aeruginosavorus* ARL-13 was used. For each of the 24 and 48 hour time points, an inoculation dose of 1×10^9 PFU/mouse of both *B. bacteriovorus* 109J and HD100, and 1×10^{11} PFU/mouse of *M. aeruginosavorus* ARL-13 was introduced.

In the respiratory model, at one hour post-inoculation, *B. bacteriovorus* 109J was detected in the lungs in 6 out of the 6 mice examined (ranging from 10^5 to 10^{10} gene copy numbers), HD100 in 6/6 mice (10^4-10^{10}), and *M. aeruginosavorus* in 5/6 mice (10^4-10^6), (Fig. 7A). However, detectable predatory bacteria in the lungs dropped substantially by 24 and 48 hours post-inoculation with all tested strains. No predators were detected in the spleens of mice at either 24 or 48 hours post-inoculation (Figure 7B).

In the intravenous model, *B. bacteriovorus* 109J was detected at high levels (10^3-10^8) in 5 out of the 5 injected mice at 3 hours post-injection (Figure 7C). A modest drop in detectable 109J was seen at 18 hours post-injection, with complete clearance of the predators in all mice

observed at 20 days post-injection. In addition, no predators were detected 10 days post-second injection in the multiple bacteremia model, suggesting complete clearance of the predators by 10 days post-injection (Figure 7C). Altogether, our data indicate that *B. bacteriovorus* 109J bacteria are quickly and efficiently cleared from the tissue of mice exposed either intranasally or intravenously.

DISCUSSION

The antibiotic-resistance crisis has inspired researchers in recent years to look for new approaches to treat life-threatening bacterial infections. One biologically-based microbial control strategy is the use of predatory bacteria[7]. The ability of predatory bacteria to efficiently prey on Gram-negative bacteria suggests a promising, novel way to combat infection. However, while efficacy has been shown *in vitro*, the effects of predatory bacteria *in vivo* have not been examined extensively.

In this study, we aimed to assess the effect of predatory bacteria in a mouse model. To verify that the obtained results are not strain specific, we used two strains of *B. bacteriovorus.* Unfortunately, we are not aware of additional *M. aeruginosavorus* strains other than the ARL-13 strain. We administered high doses of predatory bacteria to mice via respiratory and intravenous routes and examined the effect on viability and host immune response. Across the entire study, 105 mice were inoculated intranasally and 20 mice were intravenously injected with predatory bacteria. In both models, we observed neither reduction in host viability nor any adverse effects when administering high concentrations of predatory bacteria. A multiple bacteremia model also showed no effect on mouse viability after repeat exposure to predators. Furthermore, histological

examination of tissue revealed no pathology in any of the organs tested, suggesting predatory bacteria have no visible negative effects on the overall health of the mice. To reduce the number of animals being sacrificed in the study, only *B. bacteriovorus* 109J was used in the intravenous model. Future studies should involve additional isolates to confirm the results.

Our results align with the findings reported in other animal models which found predatory bacteria non-toxic[15-17]. One such study evaluated the effects of *B. bacteriovorus* HD100 when orally administered to young chicks[17]. Surprisingly, *B. bacteriovorus* HD100 was found to be adaptable and was able to survive in the anaerobic conditions and higher body temperatures of the chick gut. While oral administration of the predators altered the chick's normal gut microbiota, there were no other visual adverse effects on their well-being. However, the study did not assess the chick's immune response to predatory bacteria, and combined with the lack of adverse effects on the host, this left questions as to the immunogenicity of predatory bacteria in a living host.

We next looked to profile the host immune response to predatory bacteria introduction. Interestingly, we detected only a modest immune response to predatory bacteria in both the respiratory and intravenous models. An increase in specific proinflammatory cytokines and chemokines was detected (namely IL-1β, IL-6, IL-23, CXCL-1/KC, IFNγ, and TNF). However, the increase paled in comparison to the response caused by a known respiratory bacterial pathogen, *K. pneumoniae*. The increase in proinflammatory cytokines we observed was not sustained, and cytokine levels were back to baseline levels by 24 and 18 hours post-inoculation, for the respiratory and intravenous models, respectively. Furthermore, bacterial dissemination experiments showed predatory bacteria were efficiently cleared from the host in both models. In the respiratory model, we observed efficient clearance of the predators over time. Although all

mice were initially inoculated intranasally with 4 x 10^9 PFU/mouse, qPCR was able to detect 10^{10} gene copies of *Bdellovibrio* in three of the mice. qPCR has been found to slightly overestimate quantities of bacteria compared to standard microbiological plating methods, as there is no discrimination in amplification between viable and dead cells[18]. This could account for the slight difference in the numbers we observed. We did not detect any predators in the spleen at either 24 or 48 hours, possibly due to the predators being cleared from the host before reaching the spleen. The intravenous model showed complete clearance of the predators by 20 days post-injection and also determined that *B. bacteriovorus* 109J bacteria inoculated by repeated injections were just as efficiently cleared.

The proinflammatory cytokines that were found to be induced due to predatory bacteria are hallmarks of activation of the innate immune response. Furthermore, profiling of mice exposed to *B. bacteriovorus* 109J saw a 3.51-fold increase in the percentage of neutrophils present in the blood at 3 hours post-injection. Neutrophils are key players in the innate immune response and constitute the first line of defense against invading pathogens[19]. The increase in neutrophils in the blood corresponds to the large increase of CXCL-1/KC (52.7-fold) and IFNγ (27.4-fold) expression in the blood at 3 hours, as analyzed through ELISA. CXCL-1 is expressed by macrophages, neutrophils and epithelial cells. Both CXCL-1 and IFNγ have been found to have neutrophil chemoattractant activity[20,21]. Thus, we suspect that *B. bacteriovorus* 109J is being cleared from the blood by neutrophils recruited to the site through a chemotactic gradient of expressed cytokines and chemokines.

While the observed immune response to predatory bacteria may surprise some, it is important to note that predatory bacteria may be inherently non-pathogenic to mammalian hosts. A study looking at the effects of non-pathogenic Gram-negative bacteria on the immune

response in the gut found similar patterns in cytokine expression levels when challenging with non-pathogenic strains of *Escherichia coli*, as well as an increase in TLR-4 expression[22]. Toll like receptors are a family of pattern recognition receptors that play a key role in innate immunity. It has been reported that *B. bacteriovorus* contains a unique lipopolysaccharide (LPS) possessing neutral lipid As containing α-D-mannoses that replace the usual negatively-charged phosphate residues found in the LPS on pathogenic bacteria[23]. The same study showed that this unique LPS provokes a weak immunogenic response from human mononuclear cells *in vitro*. Similar to what we observed in our *in vivo* model, they detected smaller inductions of TNF and IL-6 as compared to that induced by pathogenic *E. coli*. As TLR-4 is responsible for detecting LPS expressed on or released from the surface of Gram-negative bacteria to activate the innate immune response, the neutral-charged LPS on *B. bacteriovorus* prevents a more robust response and thus results in less inflammation. This may also explain the slightly larger induction of cytokines observed in the lungs when inoculating the host with *M. aeruginosavorus* as compared to the two *B. bacteriovorus* strains. The LPS of *M. aeruginosavorus* has not been characterized, however, a previous study focusing on the use of predatory bacteria to control ocular infections found that both *B. bacteriovorus* and *M. aeruginosavorus* induced similarly weak expression of IL-8 and TNF in human corneal-limbal epithelial cells *in vitro*[12], signaling that *M. aeruginosavorus* may contain an altered LPS as well. Further analysis of the LPS of *M. aeruginosavorus* must be done to confirm these results.

In conclusion, our results demonstrate that predatory bacteria are non-pathogenic in a mammalian host, do not induce a robust or sustained immune response, and are efficiently cleared from the host. Future studies should focus on assessing the efficacy of predatory bacteria to prey on Gram-negative pathogens *in vivo*. The non-toxic nature of predatory bacteria to the

host may provide an advantageous strategy to combat infection effectively without adverse effects on the host.

METHODS

Bacteria, strains and growth conditions. The predatory bacteria used in this study were *Bdellovibrio bacteriovorus* 109J (ATCC 43826), *B. bacteriovorus* HD100[3] and *Micavibrio aeruginosavorus* strain ARL-13[6]. *Klebsiella pneumoniae* ATCC 43816 was used and grown in LB media. Predatory bacteria were cultured and processed as previously described[9,12]. Predator stock-lysates were prepared by co-culturing the predators with host cells in diluted nutrient broth (DNB) (a 1:10 dilution of nutrient broth supplemented with 3 mM $MgCl_2$ and 2 mM $CaCl_2$). The co-cultures were incubated at 30°C until the culture cleared (stock-lysates). To cultivate high concentrations of *Bdellovibrio* for inoculation experiments, 10 ml of washed overnight culture host cells (~1 x 10^9 CFU/ml) were re-suspended in 90 ml of DNB containing 10 ml of predatory bacteria from the stock-lysates. *Micavibrio* was initially cultured in the same manner. To obtain higher predator concentrations, *Micavibrio* co-cultures were prepared in 200 ml of DNB containing 20 ml of the host and 20 ml of *Micavibrio* stock-lysates. *Bdellovibrio* were incubated on a rotary shaker for 24 hours. *Micavibrio* were incubated on a rotary shaker for 48 hours for the initial experiment. After the initial experiment, a 72 hour incubation period was used to reach higher concentrations of the predator. To purify and concentrate the predators, co-cultures were passed three times through a 0.45-µm Millex pore-size filter (Millipore) to remove residual prey and cell debris (filtered lysate). To further purify and concentrate predator samples, filtered lysate was pelleted three times by centrifugation at 29,000 g for 45 min using a Sorvall LYNX 4000 centrifuge (Thermo Fisher Scientific Inc). Each time, the pellet was washed and re-

suspended in 50 ml of phosphate buffered saline (PBS). For the last wash, the predator pellet was re-suspended in 1-2 ml of PBS solution to reach a final optical absorbance of ~0.3-0.4 at 600 nm. Predator cell concentrations were determined each time using the standard double-layered agar method[24]. To confirm that the samples were free of host cells, 50 µl aliquots of the predator samples were removed and plated on LB agar TSB-blood plates. Since the predatory bacteria were used directly after isolation, the actual viable predator dose was known only a few days after each experiment, as the plaque-forming unit (PFU) appeared. Therefore, in some experiments, mainly involving *M. aeruginosavorus,* the inoculation size varied somewhat. Actual predator inoculation doses are specified for each experiment.

Mice. Wild type C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD, USA). The mice were housed under pathogen-free conditions at the Rutgers New Jersey Medical School animal facility. Guidelines from the Rutgers New Jersey Medical School Institutional Animal Care and Use Committee were followed in handling the animals (protocol #13112A1).

Respiratory inoculation model. Predatory bacteria were introduced by intranasal inoculation of C57BL/6 mice to model a respiratory infection. Animals were lightly anaesthetized with 3% isoflurane in oxygen for four minutes using an isoflurane vaporizer. Twenty-five µl of purified bacterial suspension were gently applied at both nostrils. Mice were inoculated with either PBS, *B. bacteriovorus* strain 109J, *B. bacteriovorus* strain HD100, or *M. aeruginosavorus* strain ARL-13. After initial inoculation, animals were observed for the following five days and visually assessed for signs of infection, illness and discomfort. Two mice from each treatment group were kept and visually assessed for up to 50 days. To assess the immune response, lung, serum, liver, and spleen samples were collected at 1, 24, and 48 hours post-exposure. Organs for RNA

extraction were stored in 1.0 ml of Trizol at -80°C. Organs for ELISA were stored at -80°C in 1.0 ml of PBS containing protease inhibitor. Samples for histology were stored in 1.0 ml of paraformaldehyde at 4°C.

Intravenous inoculation model. Twenty-five µl of purified *B. bacteriovorus* strain 109J were introduced by tail vein injection to evaluate the effects of an acute bacteremia event on mouse viability and predator clearance. C57BL/6 mice were injected with either PBS or 1 x 10^8 PFU/mouse *B. bacteriovorus* strain 109J. After initial inoculation, animals were observed for up to 20 days and visually assessed for signs of infection, illness and discomfort. To model a multiple bacteremia event, groups of mice were re-injected with either PBS or 1 x 10^8 PFU/mouse *B. bacteriovorus* strain 109J at 10 days post-initial injection. To assess the host immune response, mice were kept for 3 or 18 hours post-exposure, when they were euthanized and lung, serum, liver, kidney and spleen samples collected.

RNA extraction. Samples were prepared as previously described[25]. Organs were homogenized in bead beater tubes. To extract total RNA, liquefied samples were centrifuged at 13,200 RPM for 20 minutes at 4°C to remove tissue debris, and the supernatants were transferred to a new tube. Two-hundred µl of chloroform were added, and centrifuged again at 11,600 RPM for 15 minutes. The aqueous phase was transferred to a new tube and combined with equal volume of isopropanol to precipitate the RNA. Samples were then centrifuged at 11,600 RPM for 15 minutes, and remaining isopropanol removed. Pellets were washed twice with 500 ml of ice-cold 70% ethanol, removing the ethanol from the sample after centrifugation. The pellets were then re-suspended in 30 µl of nuclease-free water. Total RNA was then purified using the 'RNA Cleanup' protocol in the RNeasy® Mini Kit (Qiagen), and stored at -80°C.

Host immune response profiling (qPCR). Samples were prepared as previously described[25]. cDNA synthesis was performed on total RNA isolated using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) according to manufacturer's instructions. To profile the host immune response, TaqMan® probes targeting selected cytokines and an endogenous calibrator (β-actin) were utilized for qPCR. Samples were tested in duplicate, with each reaction containing: template (2.0 µl of synthesized cDNA), TaqMan® Gene Expression Master Mix (Applied Biosystems), TaqMan® probe for selected cytokine (Applied Biosystems), and nuclease-free water to 10 µl. A 7900HT Fast Real-Time PCR System (Applied Biosystems) was used with the following protocol: 50°C for 2 min (1X), 95°C for 10 min (1X), 95°C for 15sec/60°C for 1 min (40X). Relative quantification of cytokines was performed using the $\Delta\Delta C_t$ by approximation method (As described in Reference [26] and [27]). Relative fold expression compared to control was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta C_t = C_t$ (gene of interest) – C_t (normalizer = β-actin) and the $\Delta\Delta C_t = \Delta C_t$ (sample) – ΔC_t (calibrator). Calibrator was total RNA from mice inoculated with PBS.

ELISA. Organ samples were homogenized in bead beater tubes. Liquefied tissues were spun down at 12,000 RPM for 10 minutes at 4°C. Resulting supernatant was filtered through a 0.22 µm filter at 12 x g RCF for 4 minutes. Cytokines were measured using a V-Plex Proinflammatory Panel1 (mouse) Kit (K15048D-1; Meso Scale Discovery) according to manufacturer's instructions, and read on a SECTOR Imager 2400 (Meso Scale Discovery).

Blood profiling. One hundred µl of blood samples were removed from mice at 3 and 18 hours post-injection with *B. bacteriovorus* 109J and sent to ANTECH Diagnostics (New Hyde Park, NY, USA) for blood cell profiling.

Bacterial dissemination. To detect predatory bacterial dissemination within the host, primers targeting the 16S rRNA gene of each predatory bacterial strain were synthesized: *B. bacteriovorus* HD100 (Forward): 5'-GGAGGCAGCAGTAGGGAATA-3', (Reverse): 5'-GCTAGGATCCCTCGTCTTACC-3'[28]; 109J (Forward): 5'-ACACGGTCCAGACTCCTACG-3', (Reverse): 5'-ACGCTAGGATCCCTCGTCTT-3'; *M. aeruginosavorus* strain ARL-13 (Forward): 5'-GGCTTCACTTTGTCCAGAGC-3'; (Reverse): 5'-CAGAAAAACGCGAAATCCTC-3'. Samples were tested in triplicates, with each reaction containing: template (1.0 μl of cDNA synthesized above), SYBR Green PCR Master Mix (Applied Biosystems), and 500 nM (for 109J and *Micavibrio*) or 900 nM (for HD100) of each primer (synthesized at the Rutgers New Jersey Medical School Molecular Resource Facility). A 7900HT Fast Real-Time PCR System (Applied Biosystems) was used: 50°C for 2 min (1X), 95°C for 10 min (1X), 95°C for 15 sec/60°C for 1 min (40X), 95°C for 15 sec/60°C for 15 sec/95°C for 15 sec (1X). For each qPCR run, a 10-fold dilution series of the standard (purified DNA from each predatory strain) was assessed in triplicate to validate qPCR performance and facilitate quantification. In addition, each qPCR run included negative controls (no template). Gene copy number was calculated using the 'Calculator for determining the number of copies of a template,' by URI Genomics & Sequencing Center (http://cels.uri.edu/gsc/cndna.html)[29].

Histology. Paraformaldehyde-fixed organ segments from infected mice were paraffin-embedded and stained with hematoxylin and eosin (H&E) for cellular composition as previously described[30]. Stained sections were analyzed and photographed using a Nikon Microphot-FX photomicrographic system with NIS-Elements F3.0 software (Nikon Instruments Inc, Melville, NY).

Statistical analysis. qPCR data are presented as mean ± standard deviation. ELISA data are presented as mean ± standard error of the mean. Significant differences between treated samples compared to control were examined using independent-samples student's *t*-tests. A *P* value of <0.05 was considered significant.

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AUTHOR CONTRIBUTIONS STATEMENT

N.D.C., D.E.K and K.S conceived and designed the experiments. K.S., R.C., C.T., G.C.R. and S.M. performed the experiments. K.S., R.C., and L.T. analyzed the data. K.S. drafted the manuscript. N.D.C. and D.E.K. supervised the study and revised the manuscript. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

Competing financial interests: The authors declare no competing financial interests.

Figure Legends

FIG 1. Histological examination of mouse lungs after respiratory introduction of predatory bacteria. Mice were inoculated intranasally with *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus* ARL-13. Histological examination of lungs exposed to *B. bacteriovorus* and *M. aeruginosavorus* revealed no pathology compared to the control mice treated with PBS. All images are representative micrographs that were taken at 48 hours post-intranasal inoculation and at 40X total magnification.

FIG 2. **Inflammatory cytokine profile in response to respiratory introduction of predatory bacteria.** qPCR analysis of IL-1β, IL-4, IL-6, IL-10, IL-12, IL-23, IFNγ, and TNF in response to intranasal inoculation of predatory bacteria relative to PBS control. Mice were inoculated intranasally with *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus* ARL-13. Cytokines were assessed in the lung at (A) 1, (B) 24, and (C) 48 hours post-inoculation. Cytokines were assessed in the spleen at (D) 24 and (E) 48 hours post-inoculation. Fold induction was calculated using the $\Delta\Delta C_t$ by approximation method using an endogenous calibrator (β-actin). For the one hour experiment, 6 mice per predatory bacterial strain (and PBS) were used. Twelve mice per strain (and PBS) were used at each of the 24 and 48 hour time points, with the exception of the Lung/24 hour experiment, where 6 mice were used. Data for the one hour time point is from one experiment; data for the 24 and 48 hour time points are from two independent experiments. Data represent mean ± standard deviation. Student's *t*-test, **P*<0.05; ***P*<0.01.

FIG 3. Inflammatory protein profile of the lung in response to intranasal inoculation of predatory bacteria. ELISA analysis of IL-1β, IL-12, CXCL-1/KC, IFNγ, and TNF in response to intranasal inoculation of predatory bacteria relative to PBS control. Mice were inoculated intranasally with *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus* ARL-13. Inflammatory proteins were assessed in the lung at 24 and 48 hours post-inoculation of (A) *B. bacteriovorus* 109J, (B) HD100, and (C) *M. aeruginosavorus* strain ARL-13. 12 mice per treatment group were used at each time point. Data from two independent experiments. Data represent mean ± standard error of the mean. Student's *t*-test, **P*<0.05; ***P*<0.01.

FIG 4. Histological examination of mice after intravenous injection of *B. bacteriovorus* 109J. Histological examination of mice injected through the tail vein with *B. bacteriovorus* 109J revealed no pathology compared to the control mice treated with PBS. All images are representative micrographs that were taken at 20 days post-tail vein injection and at 100X total magnification. G – glomeruli; T – tubules; Wp – white pulp.

FIG 5. Inflammatory cell response to intravenous injection of *B. bacteriovorus* 109J. To profile the host cell response in the blood, mice were injected through the tail vein with *B. bacteriovorus* 109J. Profile of (A) total white blood cell (WBC) counts and (B) inflammatory cells after tail vein injection of *B. bacteriovorus* 109J. Blood was assessed at 3 and 18 hours post-injection. Blood profiles were performed by ANTECH Diagnostics (New Hyde Park, NY, USA). Data represent mean ± standard error of the mean. Student's *t*-test, **P*<0.05; ***P*<0.01.

FIG 6. Inflammatory cytokine profile in response to intravenous injection of *B. bacteriovorus* 109J. (A-C) qPCR analysis of IL-6, IL-12, IFNγ, and TNF in response to tail vein injection of *B. bacteriovorus* 109J relative to PBS control. Cytokines were assessed in the (A) kidney, (B) liver and (C) spleen at 3 and 18 hours post-injection. Fold induction was calculated using the $\Delta\Delta C_t$ by approximation method using an endogenous calibrator (β-actin). Five mice per treatment group were used at each time point. Data represent mean ± standard deviation. (D) ELISA analysis of IL-1β, IL-6, IL-10, IL-12, CXCL-1/KC, IFNγ, and TNF in response to tail vein injection of *B. bacteriovorus* 109J relative to PBS control. Inflammatory proteins were assessed in the blood at 3 and 18 hours post-injection. Five mice per treatment group were used at each time point. Data represent mean ± standard error of the mean. Student's *t*-test, **P*<0.05; ***P*<0.01; ****P*<0.001.

FIG 7. Predatory bacterial dissemination within host. qPCR detection of predatory bacteria within the host. For the respiratory model, the (A) lung and (B) spleen were probed for *B. bacteriovorus* 109J, HD100, and *M. aeruginosavorus*. In the intravenous model (C), the liver, kidney and spleen were probed for only *B. bacteriovorus* 109J. In the respiratory model, 6 mice per predatory bacterial strain (and PBS) were used at the one hour time point; 12 mice per treatment group were used at the 24 and 48 hour time points. Five mice per treatment group at each time point were used in the intravenous model. Each data point represents a single mouse's bacterial load. 20d* - mice re-injected with *B. bacteriovorus* 109J or PBS control at 10 days post-initial-injection (to model multiple bacteremia event).

TABLE 1. Host viability of intranasal inoculation of predatory bacteria.

Treatment	**# of Mice**	**% Viable on Day 5**	**% Viable on Day 50***
Control (PBS)	5	100%	100%
B. bacteriovorus 109J	5	100%	100%
B. bacteriovorus 109J (HK)	5	100%	100%
B. bacteriovorus HD100	5	100%	100%
B. bacteriovorus HD100 (HK)	5	100%	100%
M. aeruginosavorus ARL-13	5	100%	100%

*: 2 mice from each treatment group from the ‘5 Day’ experiment were visually assessed for up to 50 days.

HK: heat-killed.

TABLE 2. Host viability after intravenous injection of predatory bacteria.

Treatment	**# of Mice**	**% Viable on Day 20**
Control (PBS)	5	100%
– Re-inject at 10 days	5	100%
B. bacteriovorus 109J	5	100%
– Re-inject at 10 days	5	100%

Figure 1

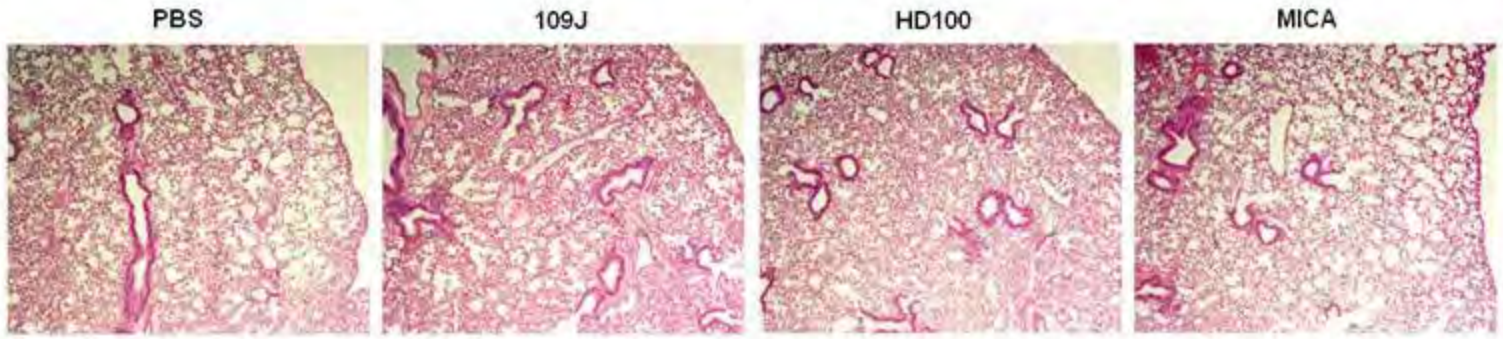


Figure 2

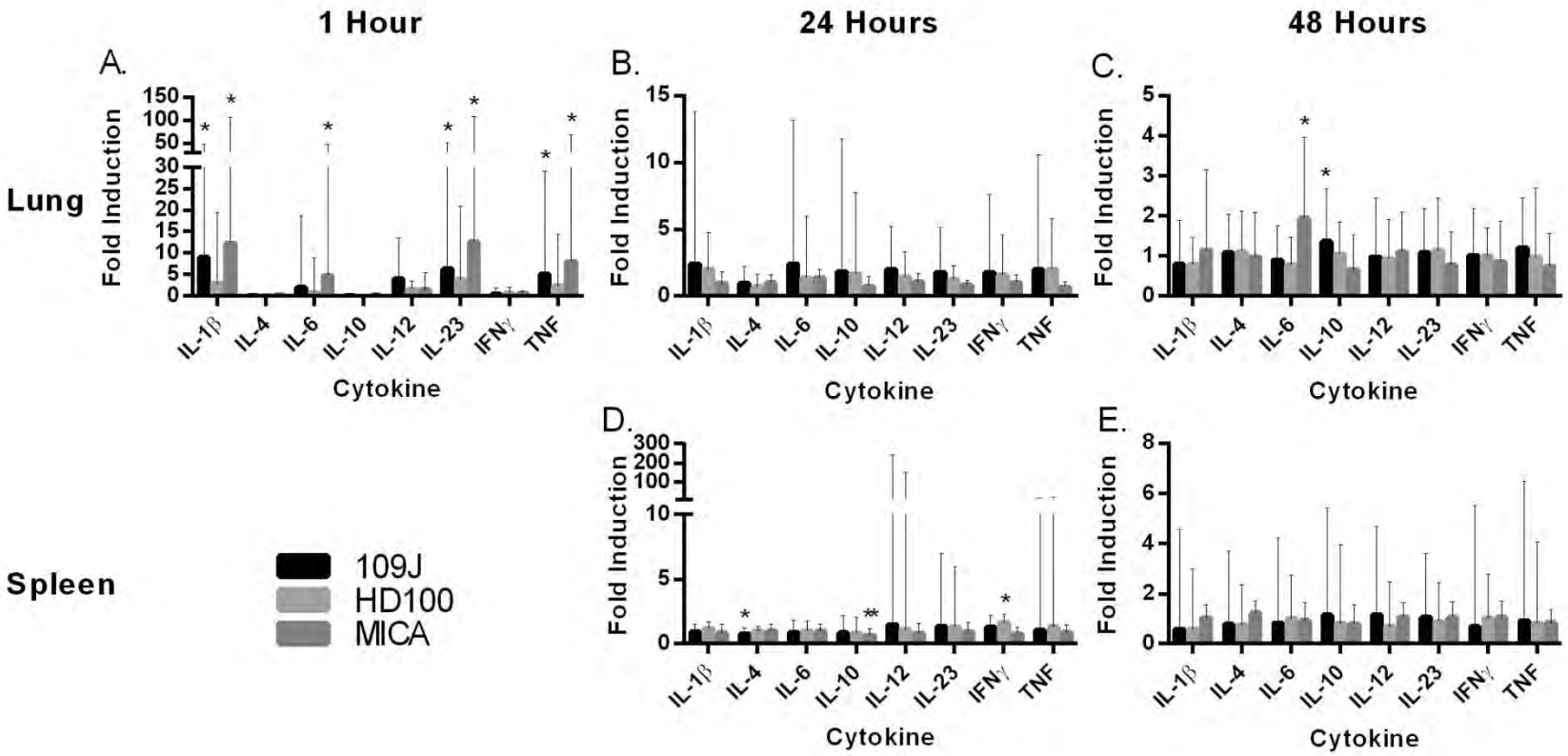

1 Hour
24 Hours
48 Hours
A.
B.
C.
D.
E.
Lung
Spleen
Fold Induction
Cytokine
IL-1β
IL-4
IL-6
IL-10
IL-12
IL-23
IFNγ
TNF
109J
HD100
MICA

Figure 3

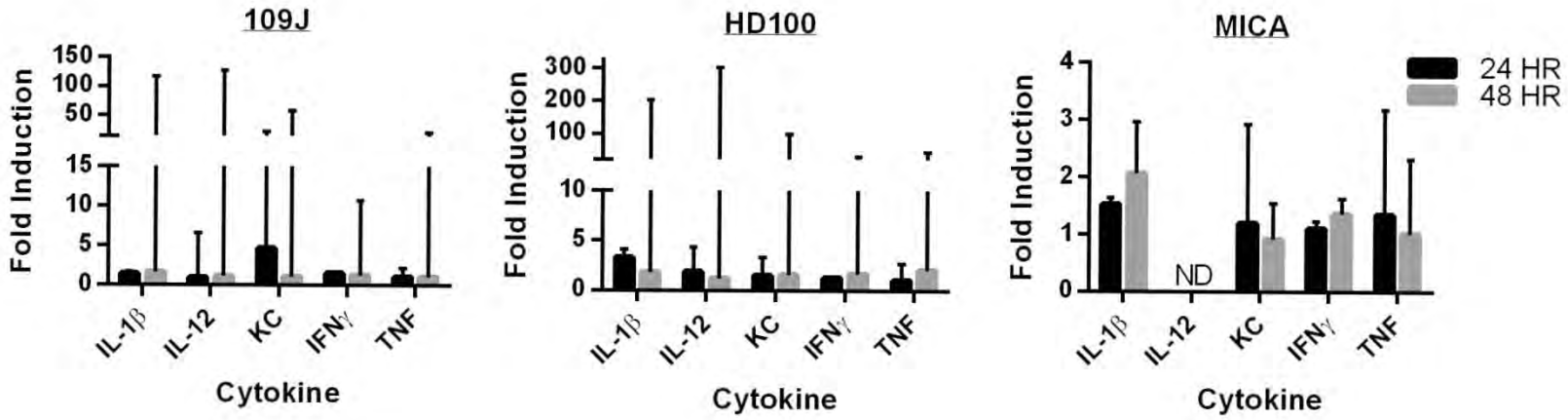

109J
HD100
MICA
Fold Induction
150
100
50
15
10
5
0
300
200
100
10
5
0
4
3
2
1
0
ND
24 HR
48 HR
IL-1β
IL-12
KC
IFNγ
TNF
Cytokine

Figure 4

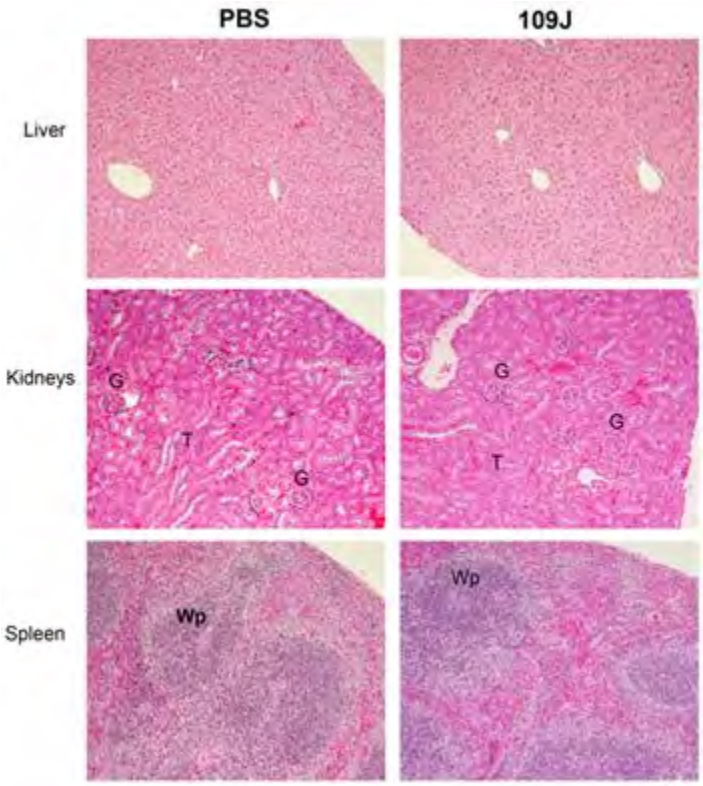


Figure 5

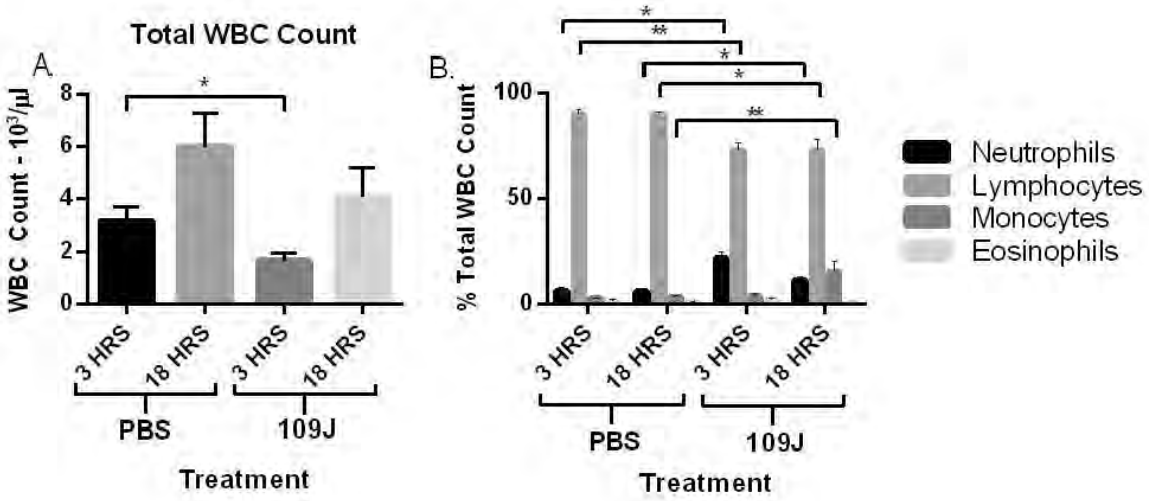

A.
Total WBC Count
WBC Count - 10³/µl
8
6
4
2
0
*
3 HRS
18 HRS
3 HRS
18 HRS
PBS
109J
Treatment
B.
% Total WBC Count
100
50
0
*
**
*
*
**
3 HRS
18 HRS
3 HRS
18 HRS
PBS
109J
Treatment
Neutrophils
Lymphocytes
Monocytes
Eosinophils

Figure 6

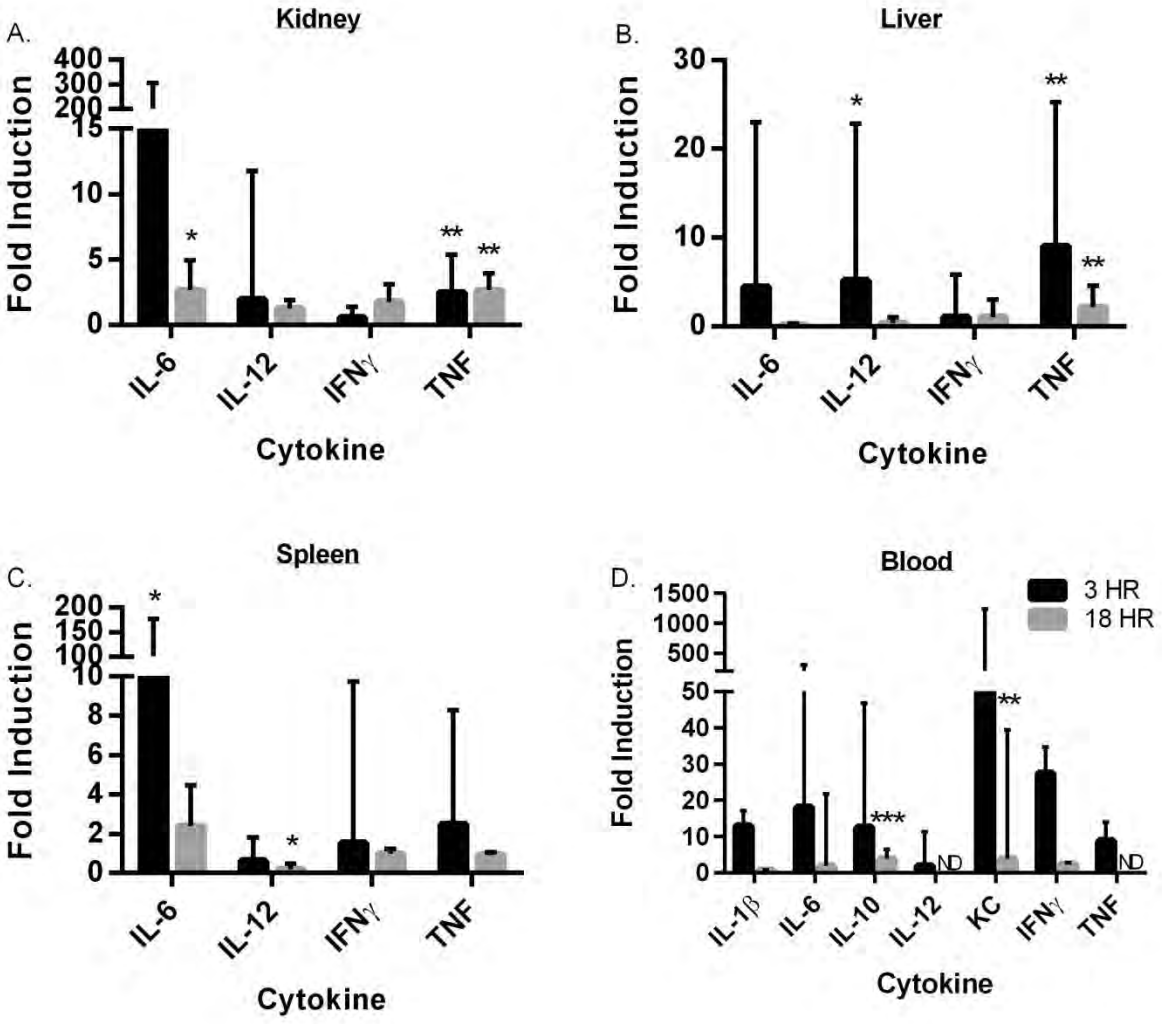
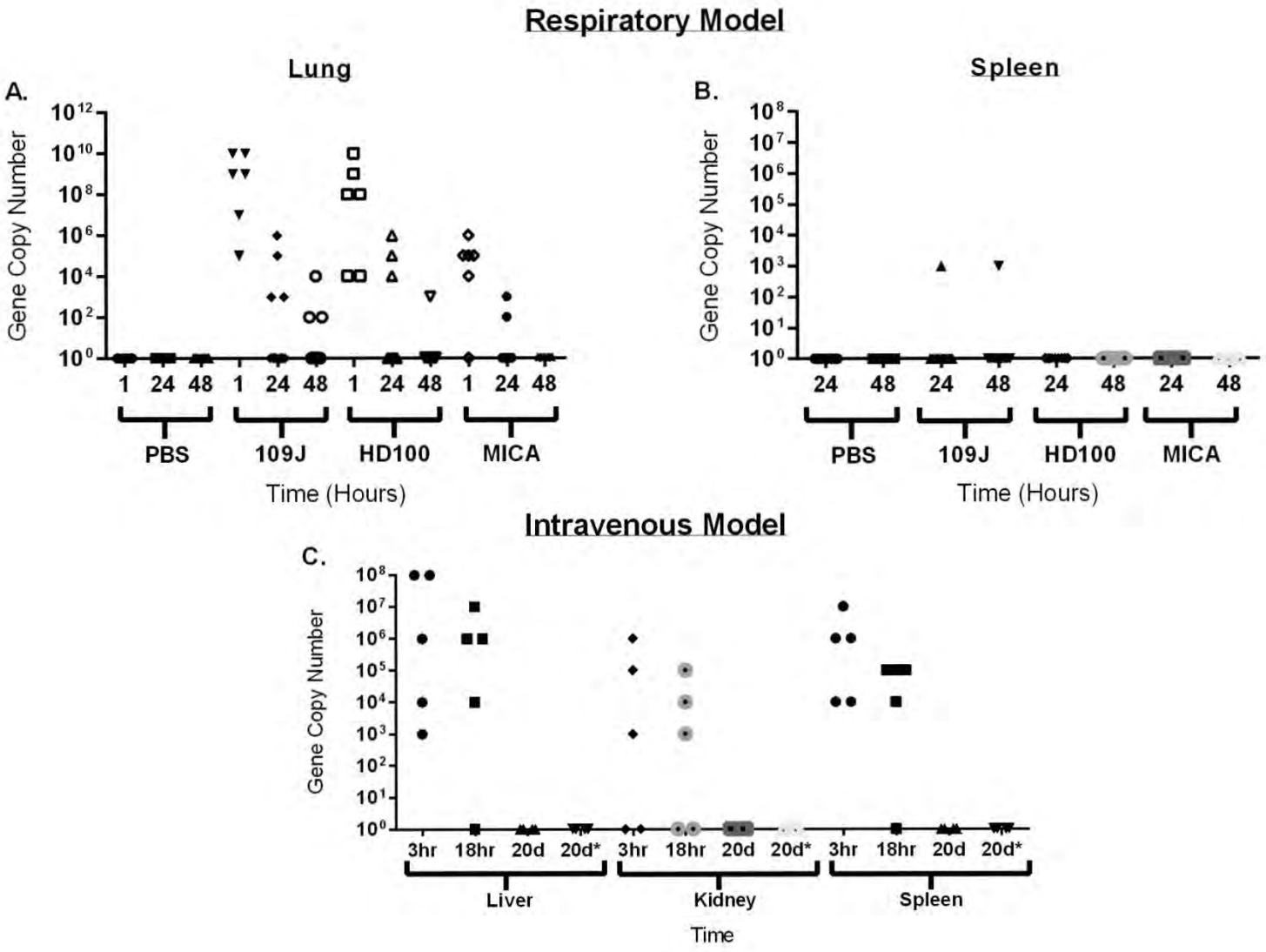

A.
Kidney
Fold Induction
400
300
200
15
10
5
0
*
**
**
IL-6
IL-12
IFNγ
TNF
Cytokine
B.
Liver
Fold Induction
30
20
10
0
*
**
**
IL-6
IL-12
IFNγ
TNF
Cytokine
C.
Spleen
Fold Induction
200
150
100
10
8
6
4
2
0
*
*
IL-6
IL-12
IFNγ
TNF
Cytokine
D.
Blood
3 HR
18 HR
Fold Induction
1500
1000
500
50
40
30
20
10
0
**
***
ND
ND
IL-1β
IL-6
IL-10
IL-12
KC
IFNγ
TNF
Cytokine

Figure 7


Respiratory Model
Lung
A.
Gene Copy Number
10^{12}
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
1 24 48 1 24 48 1 24 48 1 24 48
PBS
109J
HD100
MICA
Time (Hours)
Spleen
B.
Gene Copy Number
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
24 48 24 48 24 48 24 48
PBS
109J
HD100
MICA
Time (Hours)
Intravenous Model
C.
Gene Copy Number
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
3hr 18hr 20d 20d* 3hr 18hr 20d 20d* 3hr 18hr 20d 20d*
Liver
Kidney
Spleen
Time