Characterization of Macrophage Responses to Lung Microbiome Commensal Bacteria

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Abstract

The human body is host to a variety of microbiomes, communities of microorganisms known to affect human development and function.[1] The lung microbiome,[2] in contrast to the gut microbiome, has been greatly understudied. Differences in lung microbiome diversity have been correlated with the development of cancer,[3] the pathogenesis of chronic obstructive pulmonary disease (COPD),[4] and are thought to contribute to the pathogenesis of asthma,[5] pneumonia,[6] and influenza.[7] The specific bacteria present in the lung have been identified by next-generation sequencing analysis. However, to study the interaction of these bacteria with the cells of the lung, we must be able to isolate and culture them.

Macrophages are phagocytic cells of the innate immune system whose functions range from pro-inflammatory and anti-inflammatory responses to tissue repair.[8] Tissue-resident macrophages perform immune surveillance, respond to infection, and regulate inflammation in the specific microenvironments of the body.[9] In the lung, alveolar macrophages are by far the most common immune cell in the steady state.[10] It is unknown how alveolar macrophages interact with the commensal bacteria of the lung microbiome, particularly how commensal bacteria do not trigger constant immune activation and inflammation. Understanding the response of alveolar macrophages to commensal bacteria from the lung microbiome could give insight as to why changes in the lung microbiome can lead to disease states such as cancer and COPD.

This project sought to isolate the specific bacteria of the mouse lung microbiome, identify the bacteria based on sequencing analysis, culture these bacteria into a library of commensal bacteria, and then use this library to analyze the effects of the commensal bacteria upon macrophages. Completing these steps allowed us to determine the effects these commensal bacteria from the lung have upon the immune system.

1 Background

The human body is host to a variety of microbiomes, microbial communities that vary in composition and area[1]. The gut microbiome, the microbiome that resides within the digestive tract, has been implicated and connected to a growing list of conditions, both as a cause and a treatment. These conditions range from inflammatory bowel diseases[11] to cancer[12] and even autoimmune disorders.[13] Though the gut microbiome is deservedly the focus of a great deal of current scholarship and research, other microbiomes of the human body have been understudied. The lung microbiome, the microbiome present in the lower respiratory tract, is an emerging field of study. Of interest has been the composition and diversity of the lung microbiome. Differences in lung microbiome diversity have been correlated with the development of cancer,[3] the pathogenesis of chronic obstructive pulmonary disease (COPD),[4] and are thought to contribute to the pathogenesis of asthma,[5] pneumonia,[6] and influenza[7] (**Figure 1a**).

Though the exact mechanisms between microbiome dysbiosis and lung cancer have not been elucidated, it is thought that events such as immune malfunction may disturb the composition of the microbiome and result in pathological interactions between the lung microbiome and the immune system; these interactions may then lead to carcinogenesis.[3] Lung microbiome diversity has been shown to decrease steadily from healthy lung tissues to cancerous sites, with increases in abundance of the genus *Streptococcus* and decreases in the genus *Staphylococcus*.[14] Dysbiosis of the microbiome has been shown to modulate many aspects of malignancies, such as increasing genotoxicity and virulence and altering metabolism and immune responses.[3]

Meanwhile, a longitudinal study of the lung microbiome in COPD patients revealed that drastic changes in both composition and diversity in the microbiome were associated with exacerbation events and phenotypes.[4] Overgrowth of distinct bacteria already present in the microbiome is thought to drive respiratory tract dysbiosis, potentially triggering the exacerbation events by eliciting a host inflammatory response.[4]

The same inflammatory responses seen in COPD are also seen during asthma episodes. With the effectiveness of antibiotics and immunosuppressive corticosteroids in treating asthma, dysbiosis of the airway and lung microbiome has been implicated in the pathogenesis of asthma.[15] A study demonstrated that pathogenic Proteobacteria, particularly of the genus *Haemophilus*, were

more frequent in the bronchi of adult and child asthmatics, while healthy controls had a higher prevalence of Bacteroidetes, particularly of the genus *Prevotella*.[16] Another study showed that asthma patients with corticosteroid-resistant asthma had an airway expansion of gram-negative bacteria, which triggered TAK1/MAPK activation and induced corticosteroid resistance.[15]

Lung microbiome dysbiosis does not only affect chronic conditions—microbes frequently characterized as commensals have been shown in studies to have a role in pneumonia pathogenesis.[17–19] *Staphylococcus epidermidis*, a commensal, can cause disease when it overgrows and can transfer gene cassettes containing antibiotic resistance genes to the more pathogenic *Staphylococcus aureus*.[17] Both *Klebsiella pneumoniae* and *Klebsiella oxytoca* are common commensal causes of pneumonia.[19,20]

Even viral infections are affected by the lung microbiome. A study on the role of commensal bacteria in the establishment of adaptive immunity after respiratory infection with influenza A virus found that an intact commensal bacteria community was required for the establishment of Th1, CTL, and IgA responses to the infection.[7]

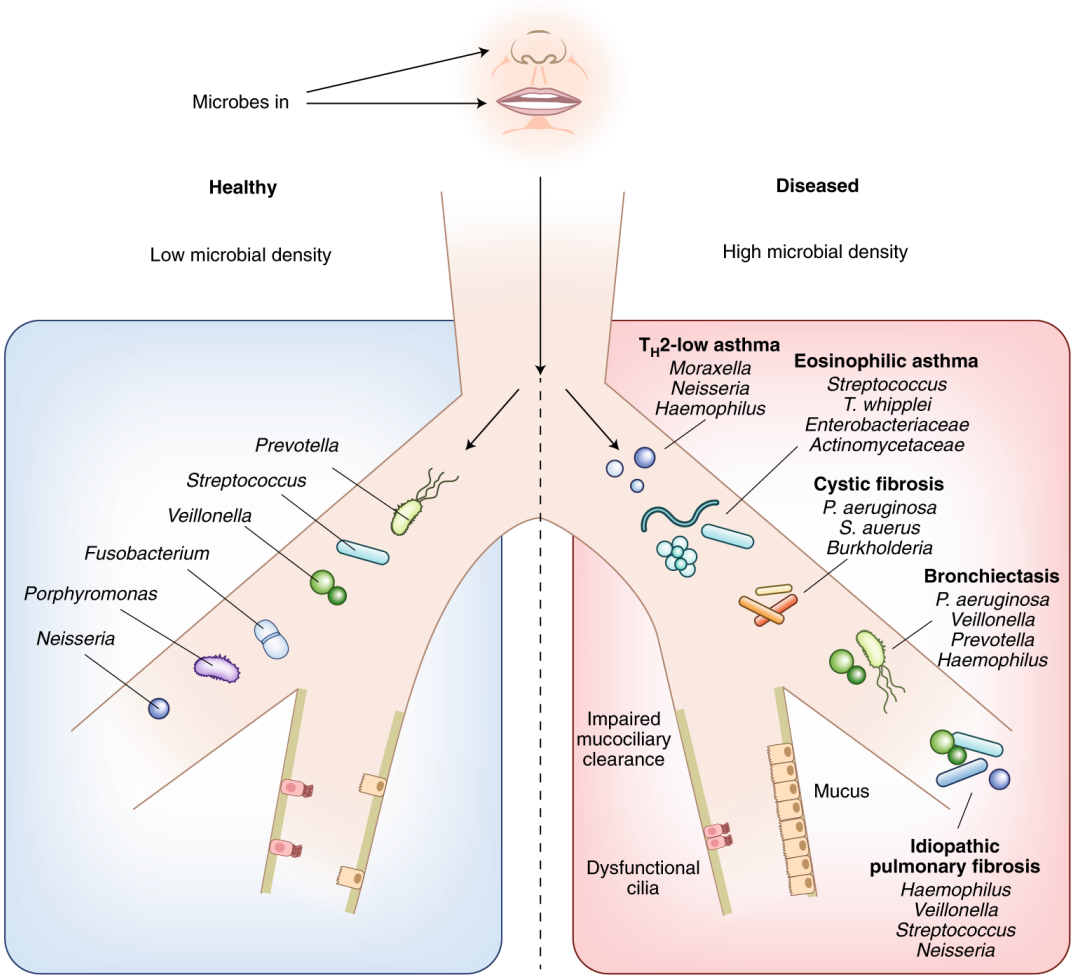


Figure 1a. The lung microbiome in health and disease.[21]

The lung is also host to a variety of immune cells,[8] chief among them the alveolar macrophage. Macrophages are phagocytic effector cells of the innate immune system with functions that range from pro-inflammatory and anti-inflammatory responses to tissue repair.[8] Tissue-resident macrophages are a heterogeneous population of macrophages that reside in such tissues as the liver, peritoneum, and lungs.[10] They perform such functions as immune surveillance, response to infection, and regulation of inflammation.[10] Alveolar macrophages, the tissue-resident macrophages of the alveolar spaces of the lung, are by far the most common immune cell of the lung in steady-state.[10] Alveolar macrophages are known to be integral in maintaining homeostasis within the lung, a process that requires recognizing and clearing pathogens without causing immunopathology that could preclude gas exchange.[22] Alveolar macrophages are known to exist

in a quiescent state, characterized by the low production of cytokines and high tolerance to antigens;[23] however, they are also responsible for early pathogen recognition and the initiation of local immune responses.[22] This delicate balancing act between activation and tolerance underscores the response of alveolar macrophages as an understudied yet key component of lung homeostasis.

Alveolar macrophage dysfunction and disease states are strongly associated. Chronic alcohol use can lead to the depletion of antioxidants critical for alveolar macrophage function, resulting in impaired phagocytosis, and therefore impaired clearance of microbes and dead or dying cells.[24] Therefore, subjects with alcohol use disorders suffer from increased susceptibility to pneumonia and tuberculosis.[24] Alveolar macrophages from COPD patients displayed reduced phagocytic ability in comparison to those from healthy controls,[25] and similar results have been found in children with protracted bacterial bronchitis and bronchiestasis.[26] Reduced alveolar macrophage phagocytic capability may lead to the colonization and overgrowth of usually commensal bacteria, leading to infections and inflammation.[26]

In the gut, the microbiome is kept spatially segregated from the host immune system, a mechanism that prevents immune activation.[27] No such spatial segregation is known to exist in the lung. Therefore, understanding the interactions between the alveolar macrophages and the commensal bacteria of the lung becomes imperative to understand how commensal bacteria do not trigger chronic immune activation, when this immune response might lead to unwanted effects such as inflammation, pleural effusion, and bronchiectasis.[28] Understanding the response of alveolar macrophages to commensal bacteria from the lung microbiome could give insight as to why changes in the lung microbiome can lead to the variety of disease states observed.

The interaction of alveolar macrophages and the lung microbiome has been fairly understudied. However, antibiotic treatment of mice promoted alveolar macrophage polarization and was sufficient to increase allergic airway inflammation.[29] Germ-free mice have decreased numbers of alveolar macrophages, implicating the microbiome in the development of the respiratory innate immune system.[30] Finally, the microbiota in the lung have been found to promote resistance to respiratory infection via granulocyte-macrophage colony-stimulating factor (GM-CSF), stimulating alveolar macrophages to kill and clear opportunistic pathogens such as *Streptococcus pneumoniae* and *Klebsiella pneumoniae*.[31] All of these studies indicate that the

relationship between the lung microbiome and alveolar macrophages is of clinical concern and should be investigated further.

Traditional culture-dependent methods involve the attempted growth of bacteria using samples taken from the site of interest. Microbiota are typically cultured in a variety of environments, ranging from solid to liquid media and from aerobic to anaerobic conditions. However, recent advances in high-throughput sequencing, particularly with the sequencing of the 16S ribosomal RNA gene have allowed for more accurate identification of microbial diversity of the microbiome.[32] These culture-independent methods avoid the biased process of identifying resident commensal bacteria by selecting only for the bacteria whose culture conditions are known and convenient. However, culture-independent methods preclude the future use of the commensal bacteria for assays and analyses. Therefore, for studying alveolar macrophage interaction with commensal bacteria, a culture-dependent method of bacterial identification might be preferred.

A few of the bacteria identified within the context of this study and used subsequently are *Chryseobacterium indologenes, Streptococcus danieliae,* and *Klebsiella oxytoca*. *C. indologenes* is an opportunistic pathogen often present in soil, food, and water sources; infections are known to occur with catheters and other implanted devices.[33] *S. danieliae* is a bacterium first isolated in a mouse cecum, with no previous characterization within the lung microbiome.[34] *Streptococcus* species are normally present in the lung microbiome, but previous studies have shown that *Streptococcus* and *Staphylococcus* species bloom in patients with progressive Idiopathic Pulmonary Fibrosis (IPF).[35] In addition, *Streptococcus pneumoniae* abundance increases during exacerbations of COPD.[35] The apparent commensal status of *Streptococcus danieliae*, along with its familial relations, positions it as a bacterium of interest in studying the host response to lung microbiota. *K. oxytoca* is often characterized as a gut commensal but is increasingly recognized as a pathogen, made dangerous by its multiple drug resistances and its tendency to cause community- and hospital-acquired pneumonia.[18,19] The pathogenic capability of *K. oxytoca* illustrates that many commensal bacteria have the potential to become pathogenic in conditions that cause alveolar macrophage dysfunction. Understanding the alveolar macrophage response to commensal bacteria in steady state could provide insight into how alveolar macrophage dysfunction might lead to microbiome dysbiosis (or even vice versa).

Bone marrow-derived macrophages (BMDMs) are a primary cell line used *in vitro* and derived from bone marrow.[36] BMDMs are naïve cells, having not been exposed to any antigen,[36]

and therefore present a convenient cell line to investigate the responses of naïve macrophages. Alveolar macrophages are exposed to the microbiome in its entirety and their responses are conditioned based upon environmental cues. Given new research on macrophage memory that is distinct from adaptive immune memory,[37] an experimental system re-exposing *ex vivo* alveolar macrophages to bacteria could lead to confounding results. Instead, this study aimed to study the intrinsic responses of macrophages to single bacteria, simplifying the system.

Macrophages can be activated by different intrinsic and extrinsic triggers to perform a variety of tasks, a process called macrophage polarization.[36] Macrophage activation is a spectrum, with the two extremes on either end referred to as M1 and M2, where M1 macrophages display pro-inflammatory functions, while M2 macrophages possess anti-inflammatory functions and perform additional tasks, such as wound healing (**Figure 1b**).[38] Common triggers that drive macrophages to polarize towards an M1 state include cytokines such as IFN-γ and extrinsic antigens such as LPS, whereas cytokines such as IL-4 and IL-13 polarize macrophages towards an M2 state.[38] Though macrophage polarization is an imperfect model that undermines the true spectrum of macrophage activation, it presents convenient criteria for distinguishing macrophage responses and activation.

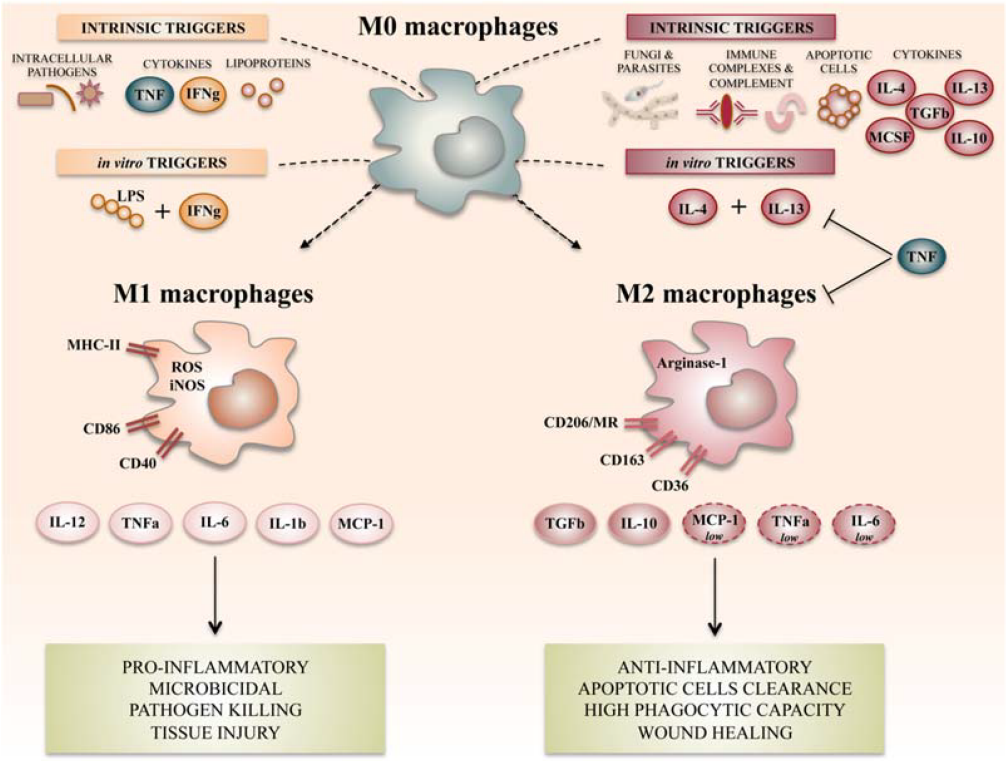


Figure 1b. Macrophage Polarization.[38]

In this study, we sought first to isolate, identify, and culture specific bacteria of the lung microbiome. The second stage of this study attempted to apply these cultured bacteria to BMDMs to characterize differences in the responses to commensal and pathogenic bacteria. Utilizing culture-dependent methods, we identified eleven distinct bacteria and cultured nine. We then used three of these bacteria to infect BMDMs and conducted qPCR to characterize gene expression changes. We found that pro-inflammatory (M1) responses were proportional to the severity of the infection and that *K. oxytoca* evoked a stronger inflammatory response than *S. danieliae* in BMDMs.

2 Completed Experiments

2.1 Methods

Lung harvest and culture

Lungs were harvested from euthanized C57BL/6J mice and homogenized with 1 mL PBS using Miltenyi Biotec gentleMACS M Tubes. Lung homogenate was spread on bacterial culture agar plates (blood agar plates, luria broth (LB) agar plates, ACES-buffered yeast extract (AYE) agar plates, De Man, Rogosa and Sharpe (MRS) agar plates), and cultured at 37°C for 24-120 hours in aerobic or anaerobic conditions. Colonies were selected for further sequencing and culture.

Culturing stocks and plotting standard curves

Identified bacteria were cultured in liquid media (LB, AYE, or Todd Hewitt) at 37°C and frozen down with 20% glycerol at -80 °C. Initial glycerol scrape-stocks were cultured overnight in liquid media at 37°C in shaking and non-shaking incubators and assessed for viability. Aliquots of the overnight cultures were diluted into a day culture and optical density readings were taken every half hour until the desired standard curve was achieved. Immediately before and during the exponential growth ("log") phase, aliquots were taken from the day culture and cultured on an agar plate for CFU counts. The growth methods were then surveilled and optimized. A day culture was used to create aliquots of known concentration, which were frozen down with 20% glycerol at -80 °C. These new stocks were characterized with the same method.

16S rRNA sequencing

Individual colonies of bacteria were lysed in TE buffer at 99°C and then their 16S rRNA V4-V5 regions were amplified with PCR. Gel electrophoresis was used to confirm presence of 16S gene. Bacterial DNA was sent to Eurofins for sequencing. 16S gene sequences were blasted against the NCBI 16S rRNA sequences database and sequences >95% were used to identify the bacteria.

Bone Marrow-Derived Macrophages

Femur and tibia bones were harvested from euthanized C57BL/6J mice and bone marrow cells were isolated. They were then cultured for 10 days, with a split on day 7, in macrophage differentiation media (12% L929 cell supernatant, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 77% RPMI-1640). L929 supernatant was prepared by growing L929 cells in RPMI-1640 media for 10 days and then harvesting and sterilizing the macrophage colony-stimulating factor (M-CSF) secreted.

Infection and harvest

BMDMs were plated in 12-well plates at 350,000 cells per well and incubated overnight to allow them to adhere to the plate. All three bacteria, *C. indologenes, S. danieliae,* and *K. oxytoca*, were grown in an overnight culture in Todd-Hewitt broth at 37°C. *C. indologenes* and *S. danieliae* were cultured exclusively in a shaking incubator. After an overnight culture of 14 hours (*K. oxytoca*) and 16 hours (*C. indologenes* and *S. danieliae*), an aliquot of the overnight culture was diluted into a day culture of 1.5 – 2 hours until a desired optical density was reached. BMBMs were infected with *C. indologenes, S. danieliae,* and *K. oxytoca* bacteria at varying multiplicities of infection (MOI), ranging from 0.025 – 250 MOI for *C. indologenes* and 0.001 – 0.1 MOI for both *S. danieliae* and *K. oxytoca*. After 4 hours, supernatant was collected while cells were lysed for downstream RNA isolation.

qPCR

RNA was isolated using the Promega ReliaPrep™ system according to the manufacturer's instructions. The RNA was then analyzed for quality with a Spectrophotometer. cDNA was synthesized from the mRNA and then cDNA was used as a template for real-time RT quantitative polymerase chain reaction (qPCR). Primer sequences for *Il6, Tnfa, Cxcl10, Nos2, Arg1, Fizz1, Ym1, Ccl17, Vegfc, Mmp2, Mmp9, Hmox1, Il10,* and *Il22* were used to compare gene transcription levels against the housekeeping gene *Hprt*. Primer sequences were as follows:

Hprt F	GTTGGATACAGGCCAGACTTTGTTG
Hprt R	GAGGGTAGGCTGGCCTATTGGCT
Il6 F	TAGTCCTTCCTACCCCAATTTCC

Il6 R	TTGGTCCTTAGCCACTCCTTC
Nos2 F	ACATCGACCCGTCCACAGTAT
Nos2 R	CAGAGGGGTAGGCTTGTCTC
Tnfa F	CCCTCACACTCAGATCATCTTCT
Tnfa R	GCTACGACGTGGGCTACAG
Hmox1 F	CACAGCACTATGTAAAGCGTCT
Hmox1 R	TGTGCAATCTTCTTCAGGACC
Vegfc F	GTTACCTCAGCAAGACGTTGT
Vegfc R	AGGAAGTGTGATTGGCAAAACT
Mmp2 F	GATGTCGCCCCTAAAACAGAC
Mmp2 R	CAGCCATAGAAAGTGTTCAGGT
Mmp9 F	GCAGAGGCATACTTGTACCG
Mmp9 R	TGATGTTATGATGGTCCCACTTG
Il22 F	ATGAGTTTTTCCCTTATGGGGAC
Il22 R	GCTGGAAGTTGGACACCTCAA
Cxcl10 F	CCAAGTGCTGCCGTCATTTTC
Cxcl10 R	GGCTCGCAGGGATGATTTCAA
Ccl17 F	TACCATGAGGTCACTTCAGATGC
Ccl17 R	GCACTCTCGGCCTACATTGG
Il10 F	GCTGGACAACATACTGCTAACC
Il10 R	ATTTCCGATAAGGCTTGGCAA
Arg1 F	CTCCAAGCCAAAGTCCTTAGAG
Arg1 R	GGAGCTGTCATTAGGGACATCA
Ym1 F	CAGGTCTGGCAATTCTTCTGAA
Ym1 R	GTCTTGCTCATGTGTGTAAGTGA
Fizz1 F	CCAATCCAGCTAACTATCCCTCC
Fizz1 R	ACCCAGTAGCAGTCATCCCA

ELISA

Using the supernatants from the *S. danieliae* and *K. oxytoca* infections of BMDMs, a standard ELISA assay was performed with the R&D Systems DuoSet ELISA kit, using rat anti-mouse IL-6 capture antibodies, biotinylated goat anti-mouse IL-6 detection antibodies, and streptavidin-horseradish peroxidase conjugate solution.

2.2 Results

Lungs were harvested from euthanized C57BL/6J mice and homogenized. The lung homogenate was spread on bacterial culture agar plates, and cultured (**Figure 2a**). Bacterial colonies were selected for further sequencing and culture (**Figure 2a**). Because sequencing entire bacterial genomes is difficult and expensive, sequencing was done on the 16S rRNA gene. The 16S rRNA gene is highly conserved among bacterial and archaeal species, and includes nine hypervariable regions (V1-V9) that allow for taxonomic classification of many bacteria.[39] This combination of conservation and hypervariability make 16S rRNA gene sequencing an easy way to identify most bacteria, if not by species then by family. The V1-V6 region specifically was included in our analysis as it represented the most reliable region for providing information on specific taxonomy.[39]

After sequencing, the sequences were blasted with NCBI Blast (**Figure 2b**). This process detected local alignments between the sequences in its 16S rRNA gene database and the unidentified bacterium sequence. The more similar the gene in its database to the unidentified bacterium sequence, the higher its percent identity.

Though some bacterial sequences could not be identified, a majority were identified and catalogued (**Figure 2c**). Of the eleven bacteria identified, only nine could be cultured and frozen with glycerol into a scrape-stock (**Figure 2d**).

Of the original eleven bacteria identified, there were four Staphylococcus species. Of the four, both *Staphylococcus xylosus* and *Staphylococcus cohnii* are usually characterized as commensal bacteria of the skin microbiome,[40] while *Staphylococcus aureus* is usually identified as a pathogen whose presence is commonly found in the upper respiratory microbiome.[35] Additionally, methicillin-resistant *Staphylococcus aureus* (MRSA) is a growing concern, particularly in hospital-acquired infections.[41] *S. aureus* is commonly described as a pathobiont, a potential pathogen that commonly lives as a symbiont within the microbiome, which might be contained by the rest of the commensal bacteria under normal circumstances and grow out of proportion when the microbiome is disturbed.[42] Interestingly, *Staphylococcus lentus* has been shown in a previous study to protect mouse models from asthma,[43] indicating it may provide a protective service as a commensal bacteria of the lung microbiome. *S. lentus* was, however, unable to be cultured and frozen into a glycerol stock, and so we were unable to characterize the macrophage response to infection with this particular bacterium.

Streptococcus danieliae are commensal bacteria first isolated from a mouse cecum, with further isolation from the murine oral microbiome.[34,44] However, *S. danieliae* has had no previous characterization within the lung microbiome.[34] In humans, where respiration can take place through nasal or oral pathways, an oral commensal present in the lung microbiome might suggest a product of respiration. However, as mice are obligate nasal breathers,[45] this pathway is less likely, suggesting that *S. danieliae* is a true member of the lung microbiome. Despite *S. danieliae* being previously unidentified in the lung microbiome, *Streptococcus* species are normally present. Previous studies have shown that *Streptococcus* and *Staphylococcus* species bloom in patients with progressive Idiopathic Pulmonary Fibrosis (IPF),[35] and cancerous lung tissue displays increases in *Streptococcus*.[14] In addition, *Streptococcus pneumoniae* abundance increases during exacerbations of COPD.[35] The apparent commensal status of *Streptococcus danieliae*, along with its opportunistic pathogen relations, positions it as a bacteria of interest in studying the host response to lung microbiota.

Another bacteria identified was *Klebsiella oxytoca*, which is often characterized as a gut commensal, but is increasingly recognized as an opportunistic pathogen, a commensal within the microbiome prone to overgrowth during dysbiosis.[18] *K. oxytoca* is being isolated more frequently from intensive care units and is becoming more of a hazard with multiple drug resistances.[18] *K. oxytoca* is a cause of community- and hospital-acquired pneumonia, but a genetically distinct variant of *K. oxytoca* can cause antibiotic-associated hemorrhagic colitis when it overgrows in the gut microbiome.[19] *K. oxytoca* therefore inhabits a unique space as a commensal bacteria with frequent pathogenic effects.

Like *K. oxytoca*, *Chryseobacterium indologenes* is another opportunistic pathogen.[33] However, in contrast to *K. oxytoca*, it is usually present in soil, food, and water sources and not commonly isolated from microbiomes.[33] *C. indologenes* infections are known to occur with catheters and other implanted devices,[33] particularly in hospital environments due to the ability of *C. indologenes* to survive in chlorine-treated water supplies.[46] Recent studies have indicated a rise in bacteremia and pneumonia caused by *C. indologenes* and note a worrying increase in resistance to a wide spectrum of antibiotics.[47] Though *C. indologenes* has not previously been described in the lung microbiome, its presence might be demonstrative of an opportunistic pathogen and is thus worth exploring further.

The remaining cultured bacteria vary widely. One, *Cutibacterium acnes,* is a skin commensal best known for its role in causing acne;[48] however, it has been identified in lung tissue and has caused rare cases of pleural infection.[49] Another bacteria, *Enterococcus faecalis*, is a component of the gut microbiome. In situations that cause gut microbiome dysbiosis, *E. faecalis* can overgrow to a state where it represents 99% of bacteria in the ileum, cecum, and colon.[50] These infections are often specifically with vancomycin-resistant *Enterococcus* (VRE), a prominent and dangerous hospital-acquired infection that can infect other tissues, including the lung.[50] Also cultured in the lung microbiome survey was *Lactobacillus johnsonii*. A study showed that 90% of mice had the genus *Lactobacillus* in their lung microbiome.[51] *L. johnsonii* in particular has been shown to modulate respiratory adaptive immune responses with respiratory syncytial virus infection.[52] The last of the bacteria, *Sphingomonas roseiflava*, was first identified from a plant of the family Gramineae in Japan.[53] However, *Sphingomonas* species are present in the murine lung microbiome in small, but significant quantities.[54] As the identification step of the sequencing is not exact, it is possible that the criteria allowed for the misidentification of *S. roseiflava* and that the culturing screen caught a different, uncharacterized, species of *Sphingomonas*.

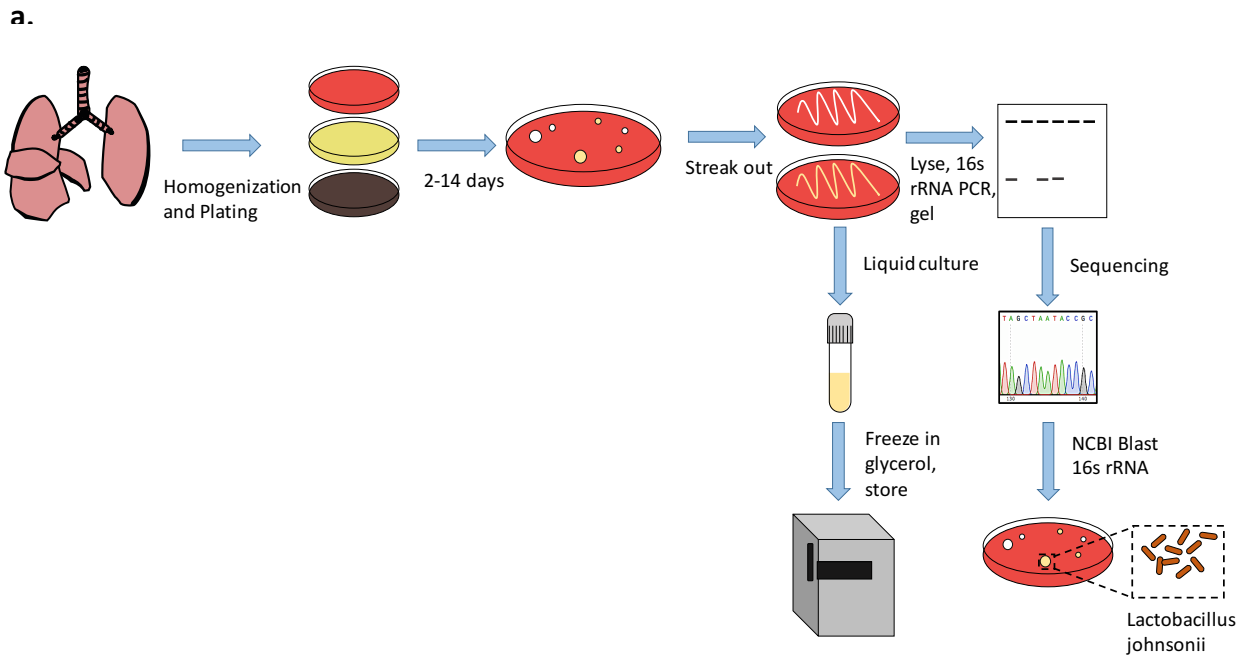
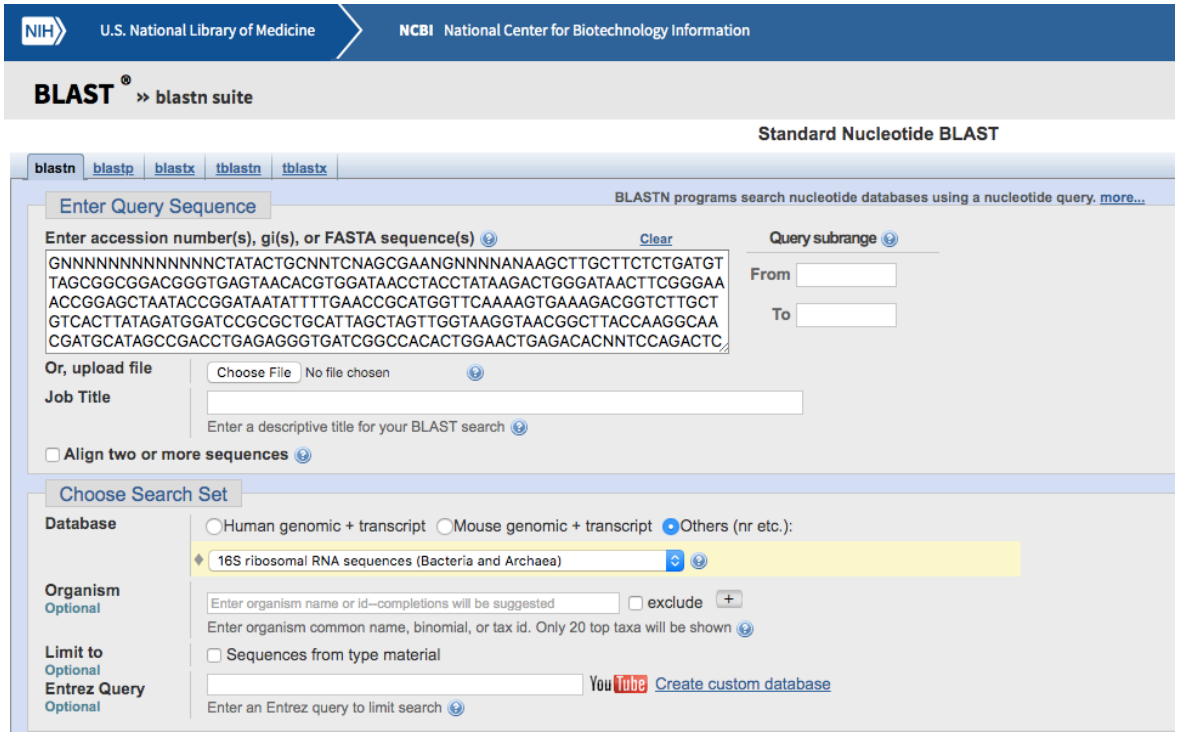
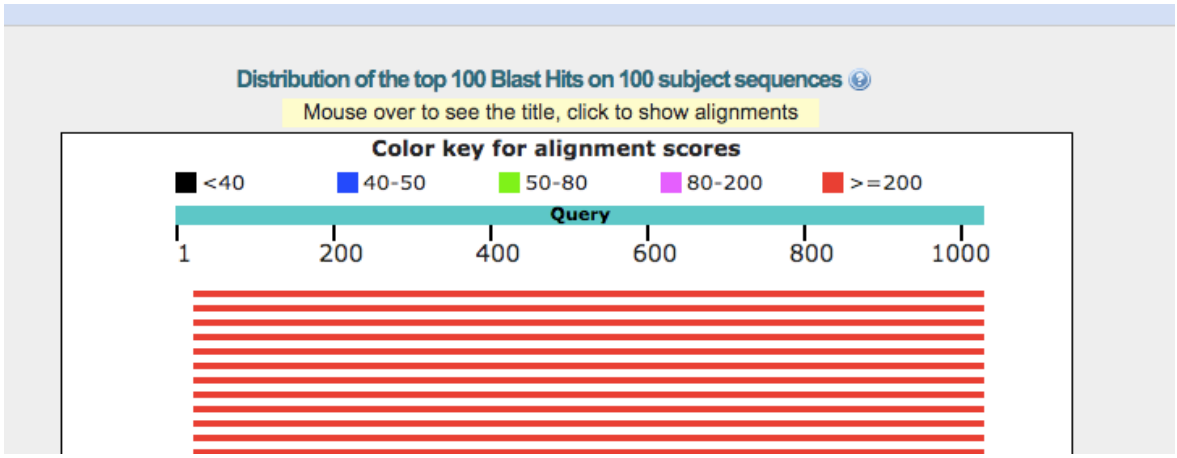


Figure 2a. Method for culturing bacteria

b.





Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Chryseobacterium indologenes NBRC 14944 16S ribosomal RNA, partial sequence	1618	1618	95%	0.0	95.50%	NR_112975.1
☐	Chryseobacterium indologenes NBRC 14944 strain LMG 8337 16S ribosomal RNA, partial sequence	1602	1602	92%	0.0	96.22%	NR_042507.1

c.

Detected and Sequenced
Klebsiella oxytoca
Cutibacterium acnes
Staphylococcus xylosus
Staphylococcus cohnii
Staphylococcus aureus
Staphylococcus lentus
Streptococcus danieliae
Enterococcus faecalis
Lactobacillus johnsonii
Sphingomonas roseiflava
Chryseobacterium indolgenes

d.

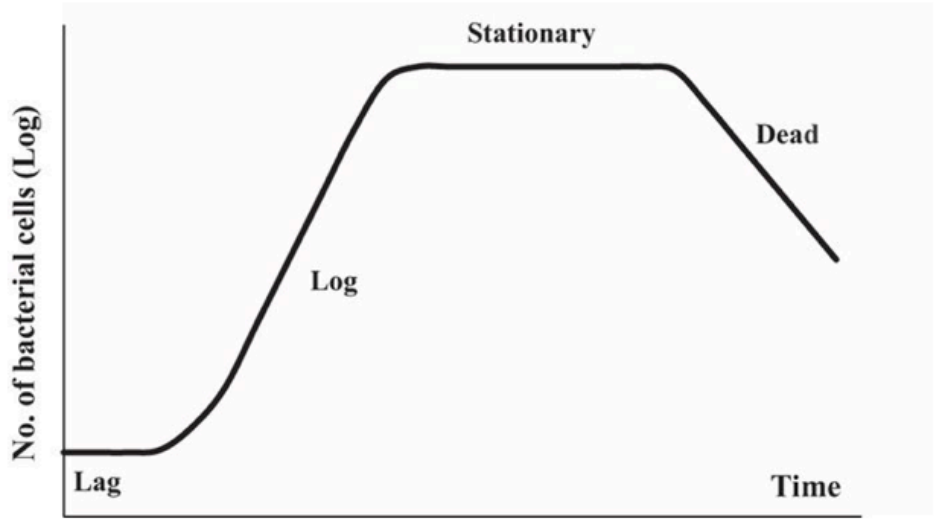
Culturable Bacteria
Klebsiella oxytoca
Cutibacterium acnes
Staphylococcus xylosus
Staphylococcus cohnii
Staphylococcus aureus
Streptococcus danieliae
Enterococcus faecalis
Lactobacillus johnsonii
Chryseobacterium indolgenes

Figure 2. *Identifying and culturing commensal bacteria.* (a) The method used for identifying and culturing bacteria (b) Representative images of the NCBI Blast Sequencing process, from the nucleotides composing the DNA of the 16S rRNA gene to the identification of the bacteria. (c) A

list of unique commensal bacteria identified by sequencing. (d) A list of unique commensal bacteria cultured and frozen down into a stock for later use.

The bacteria that could be used for further study were limited first by the necessity of being cultured and frozen in a glycerol stock for further use (**Figure 2d**). From these frozen stocks, attempts were made to grow the bacteria consistently in standard growth curves (**Figure 3a**), with consistent measurements of optical density (OD) and colony forming units (CFUs). Of the bacteria identified and successfully cultured, *S. danieliae* and *C. indologenes* demonstrated the most consistent growth curves (**Figure 3b, c**). *L. johnsonii* and *S. xylosus* were identified as bacteria that appeared commonly in the microbiome culturing process (**Figure 2a**), but their growth curves were inconsistent and the overnight cultures were prone to overgrowth and bacterial death. Therefore, *S. danieliae* and *C. indologenes* (**Figure 3b, c**), the two bacteria that grew most consistently and easily in culture, were chosen for future experiments. Along with these two bacteria, a pre-existing lab strain of *K. oxytoca* derived from a mouse was also chosen.

a.



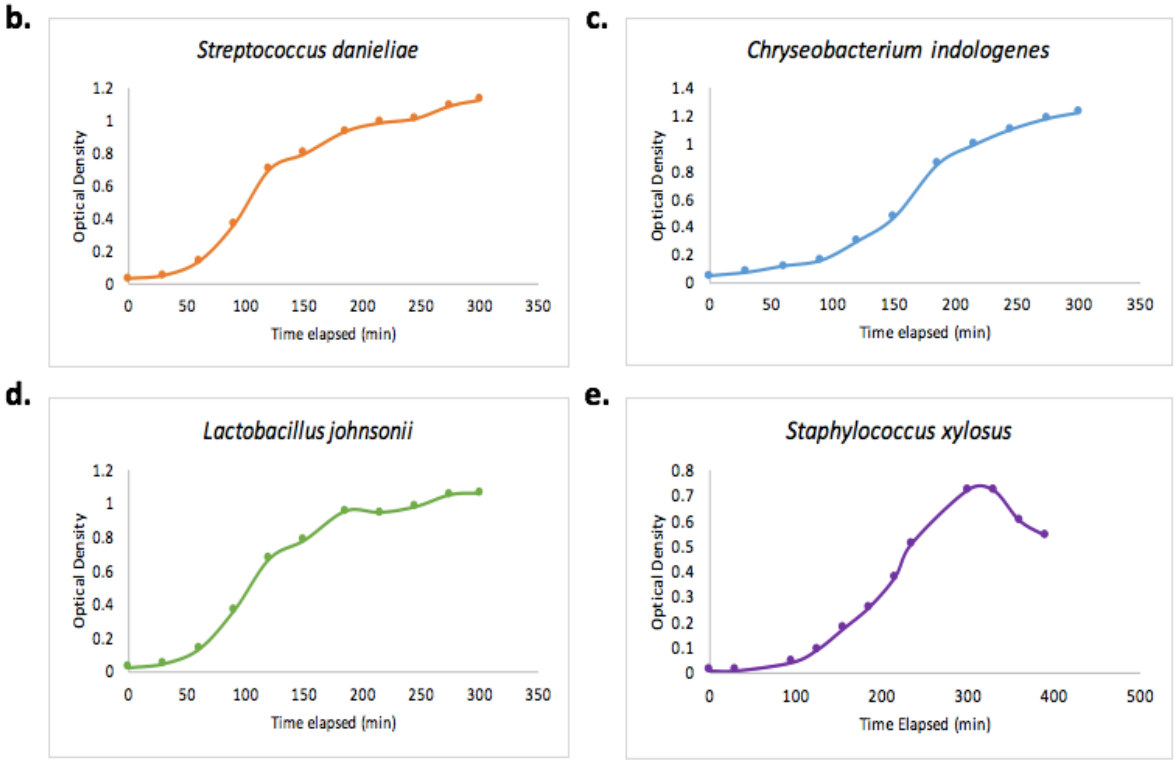


Figure 3. *Standard Curves of Bacteria*. (a) A graphical representation of a typical bacterial growth curve in a culture medium.[55] (b) Standard bacterial growth curve for *Streptococcus danieliae,* (c) *Chryseobacterium indologenes,* (d) *Lactobacillus johnsonii,* and (e) *Staphylococcus xylosus.*

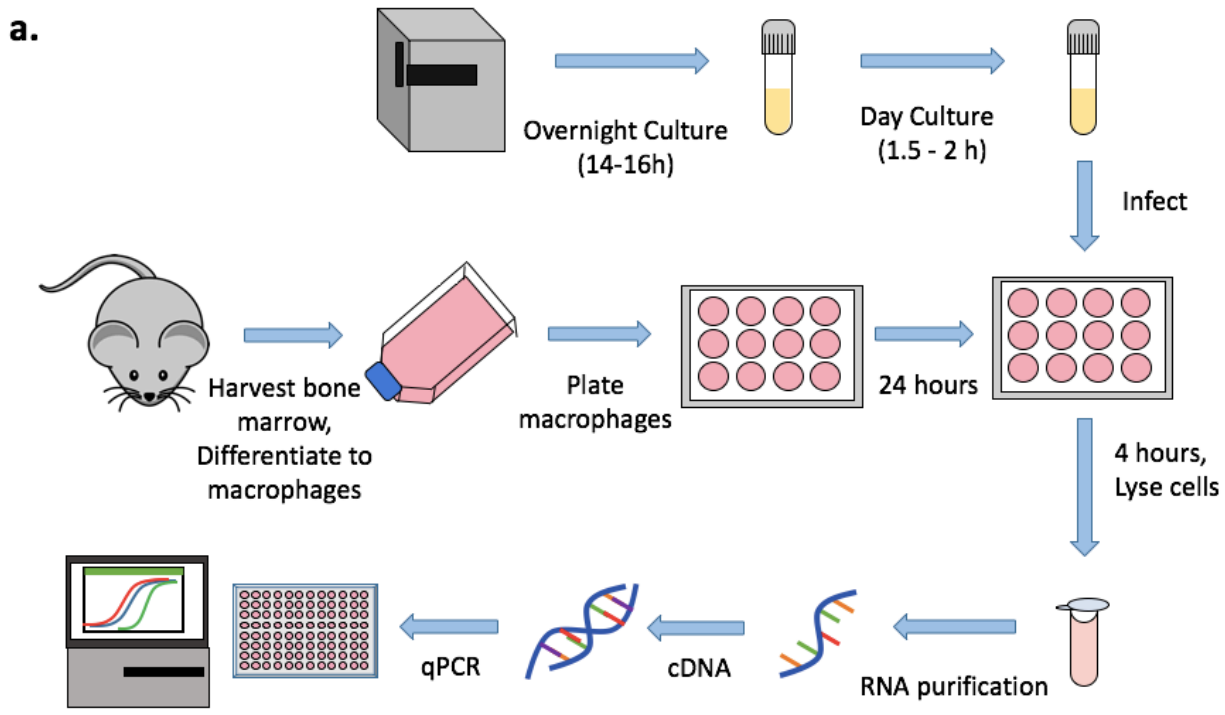
To analyze the influence of *C. indologenes* bacterial infection on macrophage polarization and responses, BMDMs were plated and left to adhere overnight (**Figure 4a**). To simulate healthy versus disease states, we chose both a low MOI (corresponding to a low commensal burden in the steady state environment) and a high MOI (corresponding to the overgrowth that results when commensals become opportunistic pathogens in various disease states). The BMDMs were infected with titrated MOIs, beginning at 25,000 and titrated down to 0.025 for 4 hours (**Figure 4a**). Cell survival was optimal beginning at the MOI of 250 as the BMDMs were overwhelmed by bacteria at higher MOIs. The BMDMs transcription levels of pro-inflammatory, anti-inflammatory, and tissue repair genes were measured with qPCR (**Figure 4 b-d**). Transcription levels of pro-inflammatory genes such as *Il6, Tnfa, Cxcl10,* and *Nos2* all increased exponentially with increasing MOI (**Figure 4b**). *Il6* encodes the pro-inflammatory cytokine IL-6, produced by macrophages in response to bacterial infection.[56] *Tnfa* encodes the pro-inflammatory cytokine

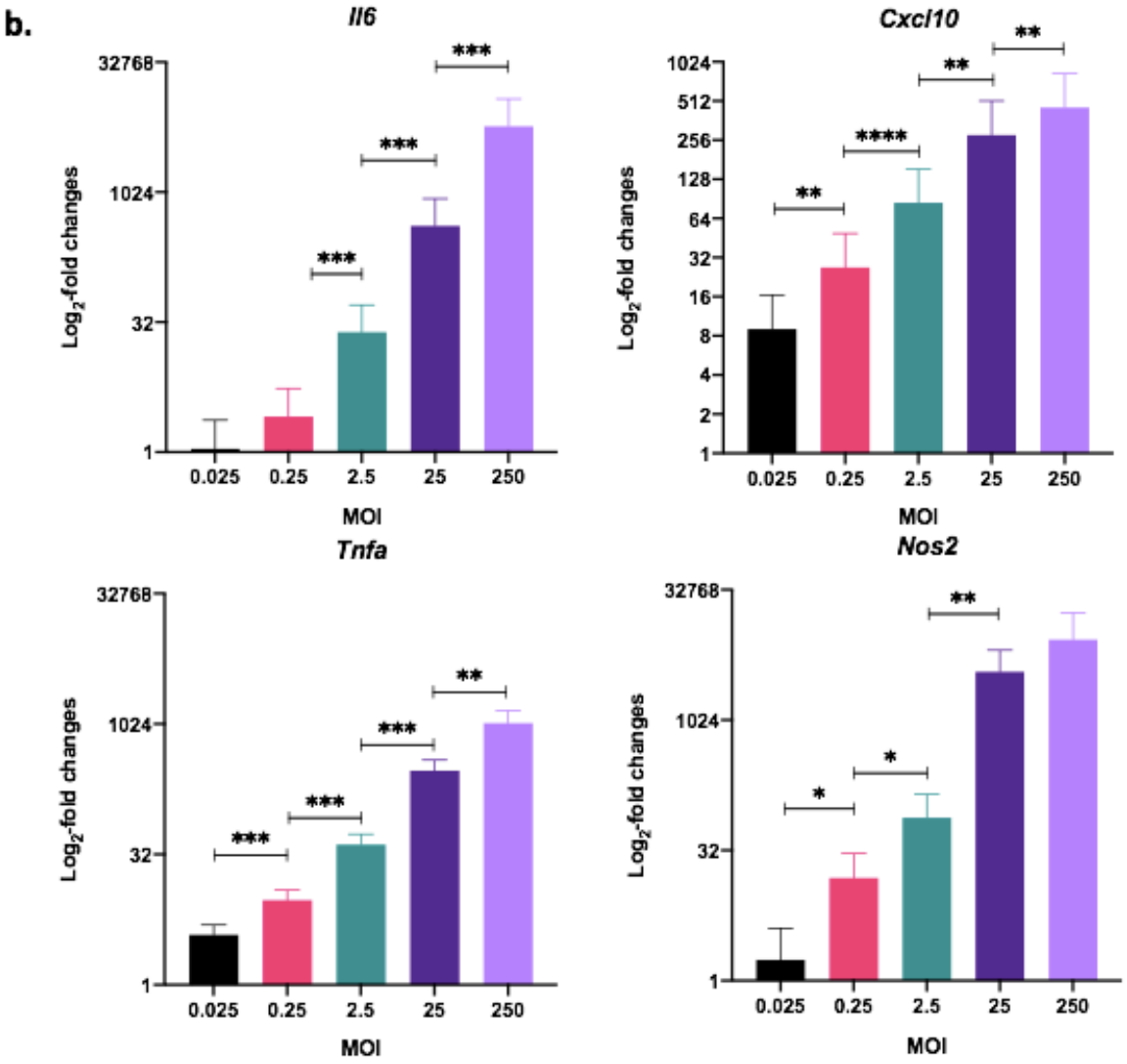
TNF- α, released by macrophages after infection, and playing a role in inflammatory cell activation and recruitment.[57] *Cxcl10* encodes the pro-inflammatory cytokine CXCL10, activated in response to bacterial infection, whose high levels can be an indicator of immunopathology and a poor prognosis of disease.[58] Finally, *Nos2* encodes the enzyme nitric oxide synthase, which produces the reactive oxygen species nitric oxide, which has been shown to be important in controlling different types of bacterial infection. The upregulation of pro-inflammatory genes in response to high bacterial burden demonstrates a protective immune response attempting to clear the bacteria, rather than tolerate the infection. Of particular interest was *Il6*, which showed low background transcription and low transcription with a MOI of 0.025 but increased dramatically to a 5900-fold increase at a MOI of 250 (**Figure 4b**).

Most anti-inflammatory genes measured did not show significant changes in transcription either increasing or decreasing, with the possible exception of *Ym1*, a marker of the M2 phenotype, which demonstrated a slight but insignificant increasing trend with increasing MOI (**Figure 4c**). *Arg1*, the gene for the enzyme arginase 1, and *Fizz1*, the gene for resistin-like molecule alpha or found in inflammatory zone protein (Fizz1) are both markers for M2-like macrophages; neither displayed any significant changes corresponding to increasing MOI. However, transcription of the *Ccl17* gene was extremely low in low MOIs but spiked upward to a 37-fold increase with a MOI of 2.5 and continued increasing up to a 1760-fold increase at a MOI of 250 (**Figure 4c**). *Ccl17* codes for CCL17, a cytokine released by M2 macrophages to inhibit the polarization of macrophages to the M1 phenotype,[59] and may indicate an effort by the infected macrophage population to inhibit excessive pro-inflammatory responses and prevent exorbitant immunopathology. The sudden increase of *Ccl17* gene transcription might indicate no need for this anti-inflammatory measure at low levels of bacterial infection with a sudden need of it when both bacterial presence and pro-inflammatory cytokine levels increase to a critical level.

The transcription level of tissue repair genes showed an increasing trend **(Figure 4d)**. *Vegfc*, the gene that encodes vascular endothelial growth factor c, showed a significant increase from the MOIs 0.25 to 2.5 and then from 2.5 to 25 **(Figure 4d)**. *Mmp2* and *Mmp9*, encoding for the enzymes matrix metallopeptidase 2 and matrix metallopeptidase 9, respectively, are important in such processes as tissue remodeling. Both steadily increased along with increases in MOI, indicating that tissue repair may be promoted alongside pro-inflammatory cytokines, to help mediate immunopathology. *Hmox1* encodes heme oxygenase 1, which has been shown to be a modulator

of chronic inflammation in conditions associated with microbiome dysbiosis, such as inflammatory bowel disease.[60] However, *Hmox1* showed no discernible trend corresponding to MOI and did not significantly increase compared to uninfected controls (**Figure 4d**). Like the anti-inflammatory genes, the overall increasing trend in tissue repair gene transcription may indicate the macrophages fulfilling a need to repair the tissue damage from both the bacteria and the immune response.




b.
Il6
Cxcl10
Tnfa
Nos2
Log2-fold changes
MOI
0.025
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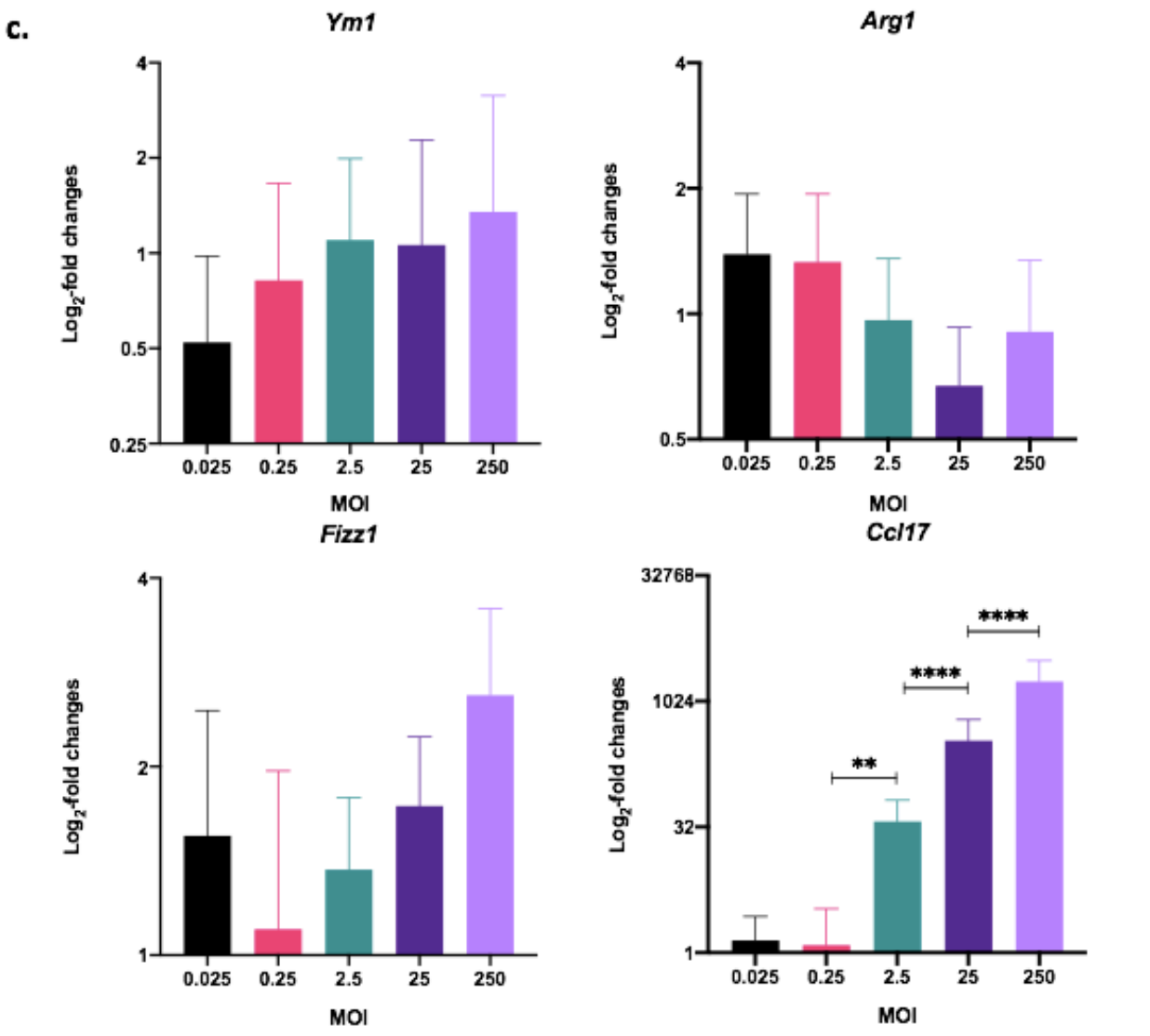
C.
Ym1
Arg1
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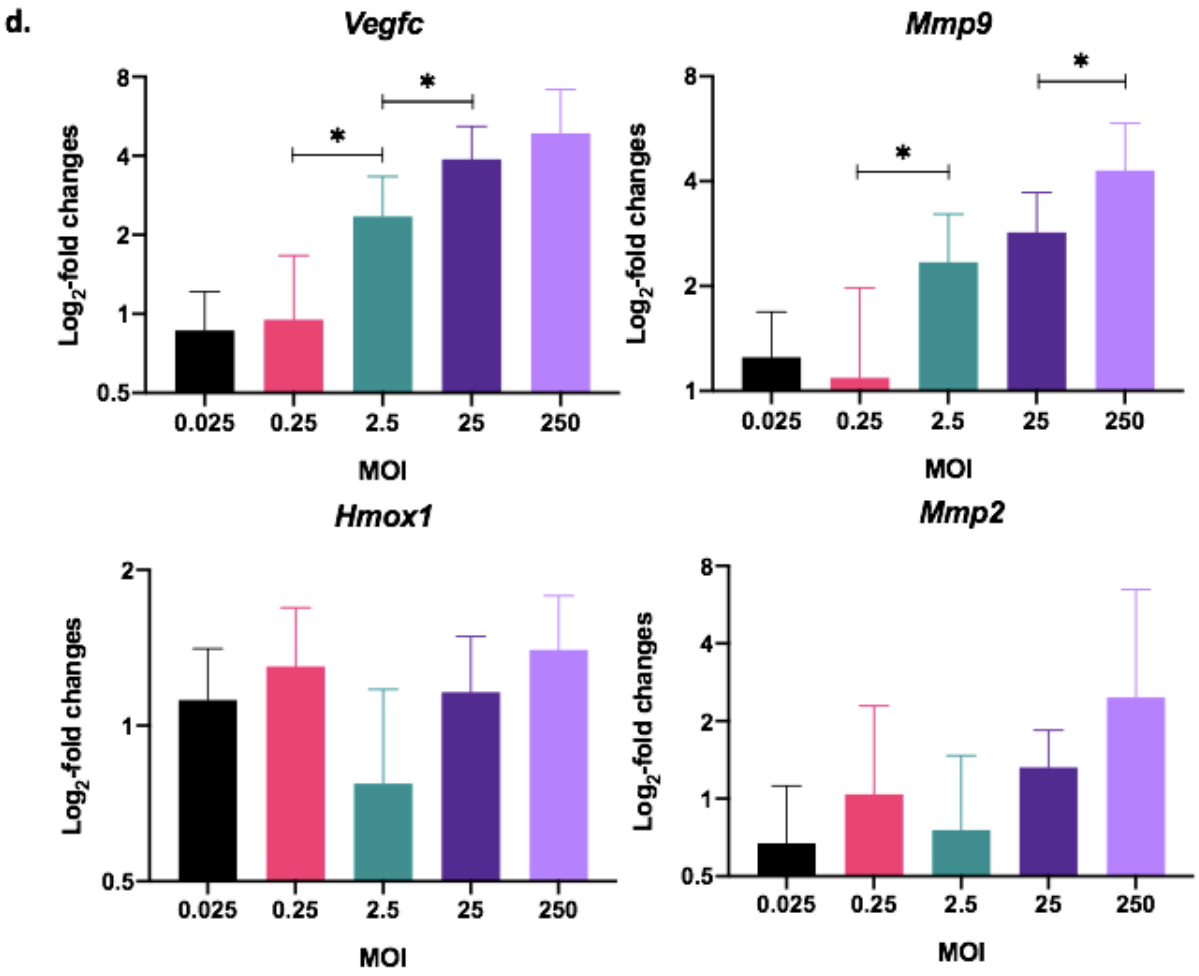


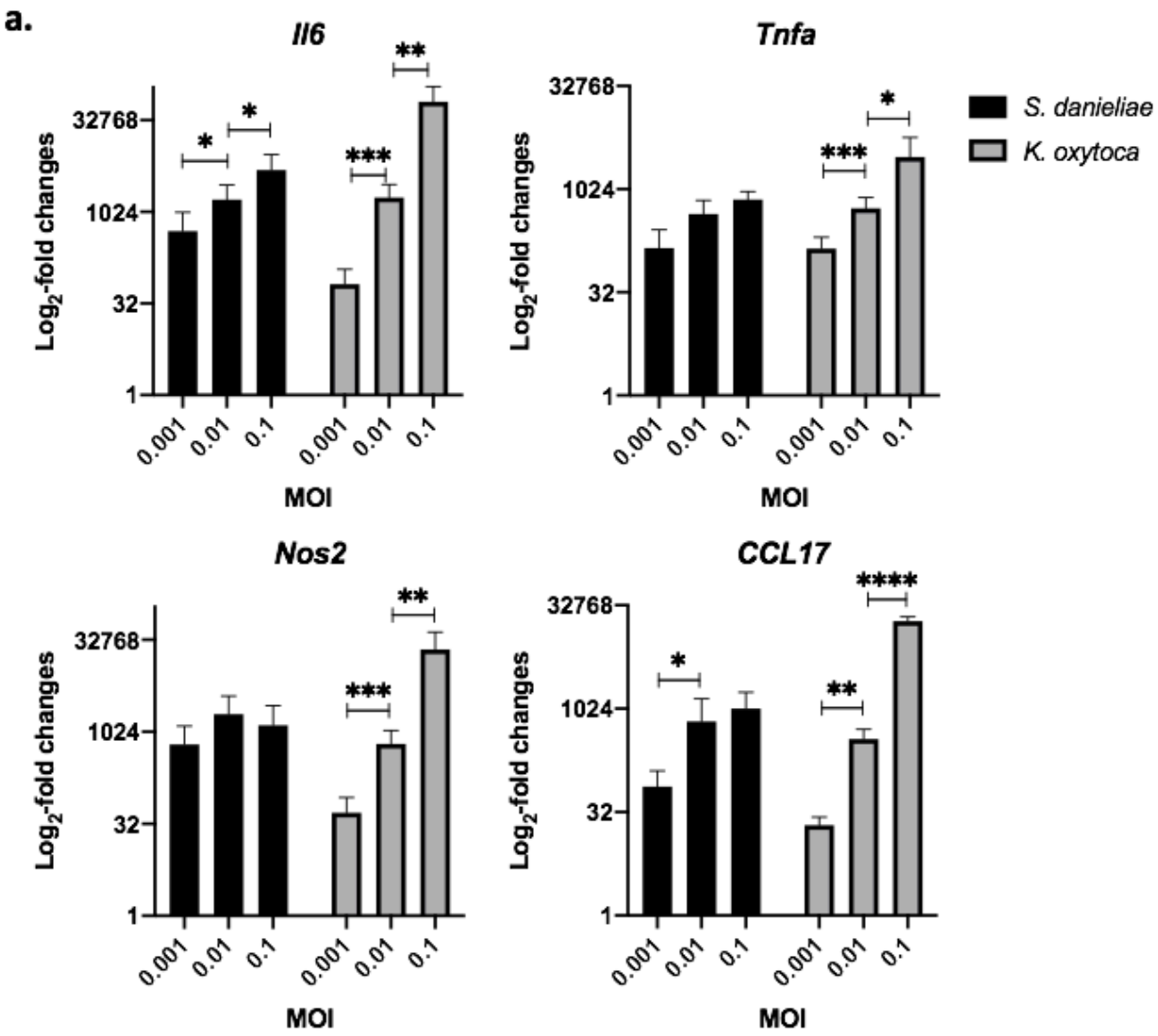
Figure 4. *Pro-inflammatory, Anti-inflammatory, and Tissue Repair Gene Transcription in response to* C. indologenes *infection.* (a) Macrophage studies. Cultured commensal bacteria were expanded and characterized. Harvested bone marrow was treated with L929 supernatant to differentiate macrophages. These macrophages were treated with the commensal bacteria and their gene expression monitored with qPCR. (b) qPCR results of characteristic pro-inflammatory genes, (c) anti-inflammatory genes, and (d) tissue repair genes. Results were normalized to the housekeeping gene *Hprt* and uninfected controls. T-test, *p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.0001.

As with *C. indologenes*, BMDMs were cultured and infected with a titration of MOIs of either *S. danieliae* or *K. oxytoca* (**Figure 3a**). However, in contrast to *C. indologenes,* both *S. danieliae* and *K. oxytoca* induced high levels of cell death, and therefore lower MOIs were used, beginning at a MOI of 0.1 and titrating down to 0.001. Similarly to the *C. indologenes* infections, gene

transcription studies demonstrated that pro-inflammatory gene transcription is upregulated with higher MOIs (**Figure 5a**). The classical pro-inflammatory genes, *Il6*, *Tnfa*, and *Nos2* were all more upregulated for *K. oxytoca* than for *S. danieliae* (**Figure 5a**). Though there might be complicating factors, the trend with this experiment seems to demonstrate that macrophages respond at around the same level to varying MOIs of *S. danieliae*, though this level is a fairly high response (**Figure 5a)**. However, *K. oxytoca* appears to induce a more "dose-dependent" increase in pro-inflammatory genes (**Figure 5a**).

Looking at the anti-inflammatory gene transcription, *Arg1* showed a general trend of decreasing as the MOI increases, with *K. oxytoca* promoting lower transcription at the highest MOI of 0.1 (**Figure 5b**). This M2-like macrophage marker might be decreasing as more macrophages are polarized toward the more pro-inflammatory M1 phenotype in response to higher levels of bacterial infection. However, *K. oxytoca* infection promoted significantly higher *Ccl17* gene transcription at the highest MOI, and somewhat higher *Il10* transcription as well (**Figure 5b**). As indicated above, *Ccl17* codes for CCL17, released by M2 macrophages to inhibit the polarization of macrophages to the M1 phenotype; the increase in both *S. danieliae* and *K. oxytoca* infections is more gradual than the sudden spike seen in *C. indologenes* infection, and may indicate a proportionate anti-inflammatory response to the pro-inflammatory gene transcription described above. It is important to note, however, that the sudden spike seen in *C. indologenes* infection occurred at a high MOI that both *S. danieliae* and *K. oxytoca* were unable to achieve. The trend in increased *Il10*, a gene that codes for IL-10, an anti-inflammatory cytokine that downregulates the bacterial induction of pro-inflammatory cytokines, may indicate a similar anti-inflammatory response in an attempt to prevent immunopathology.

As seen in the *C. indologenes* infections, the tissue repair gene *Vegfc* showed an increase in transcription as the MOI of both *S. danieliae* and *K. oxytoca* increased. The increase in *Vegfc* gene transcription may indicate the macrophages attempting to ameliorate tissue damage caused by both the bacteria and the immune response.


a.
Il6
Tnfa
Nos2
CCL17
Log2-fold changes
32768
1024
32
1
0.001
0.01
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MOI
S. danieliae
K. oxytoca
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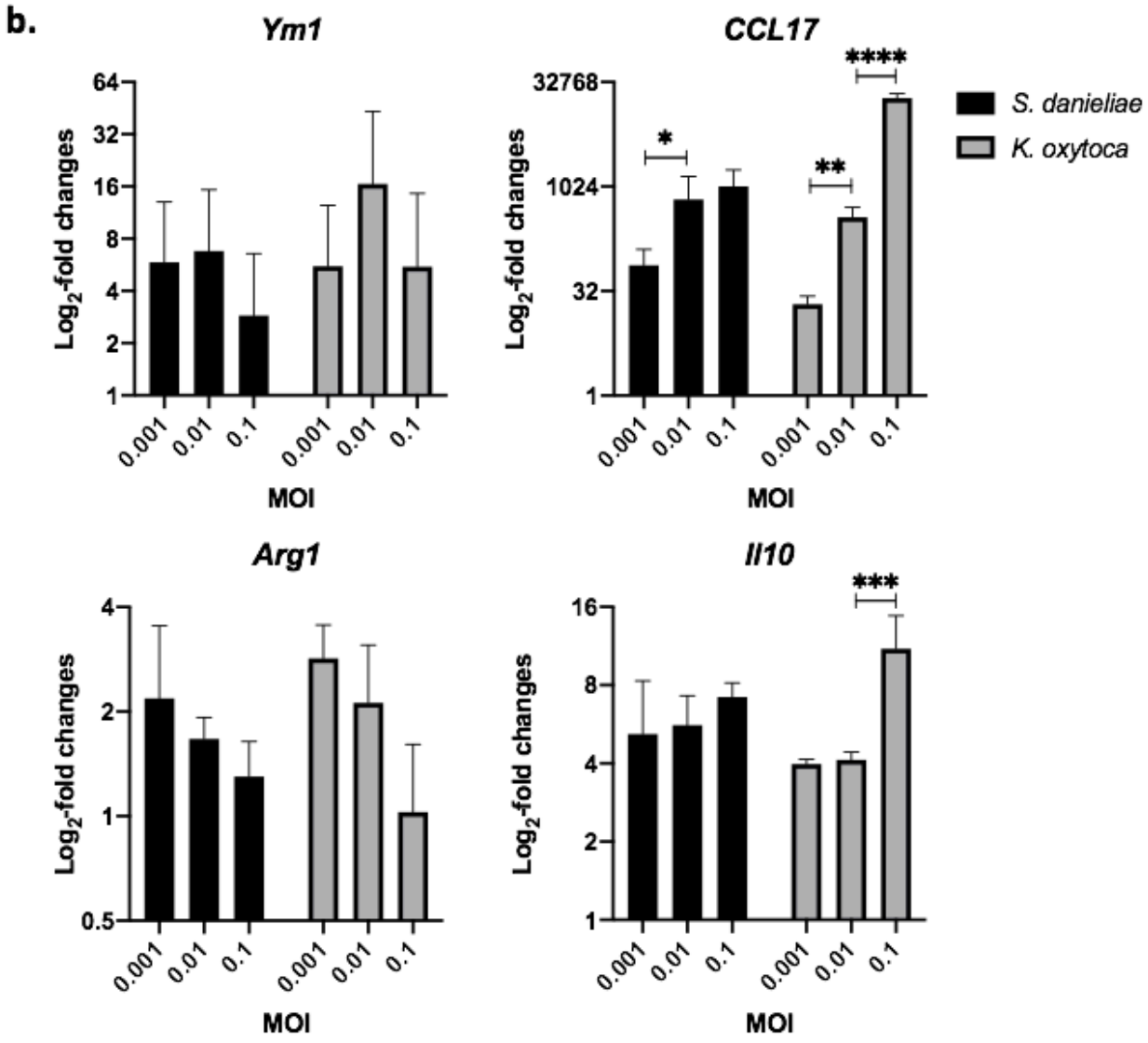
b.
Ym1
CCL17
Arg1
Il10
S. danieliae
K. oxytoca
Log2-fold changes
MOI
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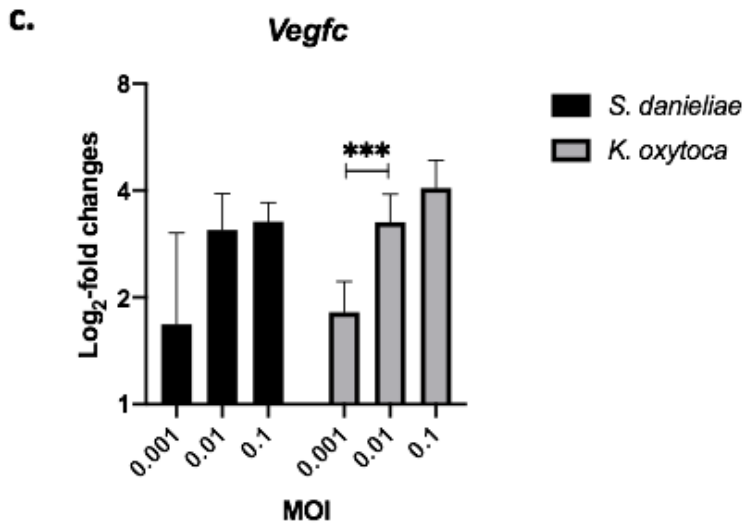

Figure 5. *Pro-inflammatory, Anti-inflammatory, and Tissue Repair Gene Transcription in response to* S. danieliae *and* K. oxytoca *infection.* (a) qPCR results of characteristic pro-inflammatory genes, (b) anti-inflammatory genes, and a (c) tissue repair gene. Results were normalized to the housekeeping gene *Hprt* and uninfected controls. T-test, *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Though gene transcription is a good indicator of what cells are driven to do by changing conditions, it is an incomplete measure of actual cell activity. Gene transcription can be rendered null by various downstream mechanisms—upregulated transcription of a cytokine gene will not always lead to upregulated production of that cytokine. Infection with both *S. danieliae* and *K. oxytoca* increased transcription of *Il6*, a pro-inflammatory cytokine often produced in large quantities during infection; however, *K. oxytoca* promoted transcription at higher levels than *S. danieliae* (**Figure 5a**). To analyze the actual protein levels produced by the BMDMs, the cell supernatants from all *S. danieliae* and *K. oxytoca* infection trials were analyzed with an ELISA assay using anti-mouse-IL-6 antibodies (**Figure 6**). The supernatants from *K. oxytoca*-infected BMDMs contained significantly more IL-6 than the supernatants from *S. danieliae* (**Figure 6**), confirming that the increased *Il6* transcription corresponded to increased IL-6 protein production.

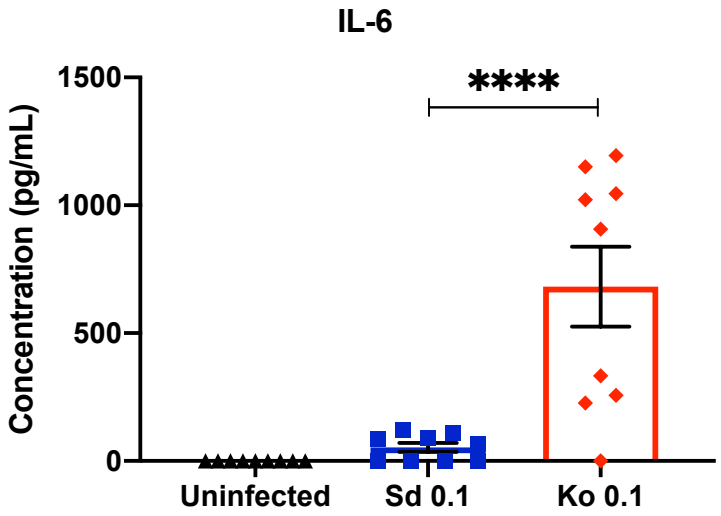

Figure 6. *IL-6 protein production in response to* S. danieliae *and* K. oxytoca *infection.* ELISA results for IL-6 concentration in cell supernatant. T-test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3 Conclusion and Perspectives

The lung microbiome, in contrast to the gut microbiome, has been historically understudied until very recently. With the advent of 16S ribosomal RNA sequencing, the populations of the lung microbiome in health and disease have begun to be characterized, but we lack an understanding of the interaction between the lung microbiome and the innate immune system. Unlike the spatial sequestration of microbes in the gut, the innate immune system and the corresponding microbiome in the lung have no known spatial separation. Though alveolar macrophages are known to be more tolerant and skewed toward the M2 phenotype than other macrophages, their responses to commensal and pathogenic levels of infection are still unknown. Dysfunction of alveolar macrophages leads to dysbiosis, but we lack an understanding of the effects of this dysbiosis on the host or the reasons for alveolar macrophage dysfunction. This study aimed to gain a better understanding of macrophage responses to the same bacteria in commensal and pathogenic conditions, an understanding that could help elucidate the impact of dysbiosis on the host.

We identified eleven distinct species of bacteria present in the murine microbiome, successfully culturing nine of them. We infected naïve BMDMs with *C. indologenes*, *S. danieliae*, and *K. oxytoca* and identified that gene transcription of pro-inflammatory genes increased along with increasing MOI. This result indicates that, as bacterial load increases, so too do the macrophage clearance responses. In *C. indologenes* infection, the transcription of the gene *Ccl17*, which codes for the factor released by M2 macrophages to prevent M1 polarization, increased significantly at higher MOIs, though it was nearly absent at lower MOIs. This result can be compared to the steady increase in *Ccl17* transcription as seen in both *S. danieliae* and *K. oxytoca* infection. When coupled with the pro-inflammatory gene transcription results, these findings indicate that, at high levels of infection, macrophages are simultaneously releasing pro-inflammatory cytokines and anti-inflammatory cytokines, to clear bacteria and to regulate immunopathology, respectively.

Other patterns found were that tissue repair gene transcription, with genes such as *Vegfc*, increased alongside the magnitude of infection. Like the anti-inflammatory gene findings, this may indicate a need for tissue repair at high levels of infection, to repair the damage done by both the pathogen and the immune response.

When infecting BMDMs with equal amounts of *S. danieliae* and *K. oxytoca*, the macrophage responses had subtle differences. Cells infected with *K. oxytoca* displayed higher transcription of pro-inflammatory cytokines than cells infected with *S. danieliae.* Additionally, cells infected with *K. oxytoca* showed higher transcription of *Ccl17.* While macrophages responded to *K. oxytoca* and *C. indologenes* in a dose-dependent manner, the response to *S. danieliae* did not change as much with varying MOIs. Finally, the IL-6 ELISA demonstrated that the BMDMs infected with *K. oxytoca* produced significantly more IL-6 than the BMDMs infected with *S. danieliae*. This may indicate that naïve macrophages might have inherent differences in their responses to various strains of commensal bacteria. These different responses might be explained by different pathogen associated molecular patterns (PAMPs) present on the bacteria, as *K. oxytoca* is gram-negative while both *C. indologenes* and *S. danieliae* are gram-positive. Another possible difference could be the division time of the bacteria, which could promote different magnitudes of immune responses as the bacterial load, identical at the infection time, could change drastically. Even subtle differences in structure or receptors on the bacteria might activate different immune pathways and thus be responsible for these different macrophage responses. Despite the differences between macrophage responses to the various commensal bacteria, the similarities were more apparent. All the commensal bacteria evoked high pro-inflammatory responses at high MOIs, indicating that macrophages tend to respond more or less the same to commensal bacteria—the driving factor for differences in the magnitude of response seems to be the number of bacteria.

In conclusion, the study demonstrated that bacteria of the lung microbiome can be identified and cultured for future experiments. Additionally, the infection studies indicate that naïve macrophages have slightly different responses to various bacteria, though the most important factor in the magnitude of macrophage response was the number of bacteria with which they were infected.

These experiments do not wholly capture and elucidate the intricacies of the innate immune response to commensal and pathogenic bacteria, particularly not the responses of alveolar macrophages nor the other varied parts of the immune system present in the lung. Future studies should include infections of *ex vivo* alveolar macrophages, as well as infections of alveolar macrophage cell-lines, to ascertain the response of activated and naïve alveolar macrophages, respectively. This is of particular importance due to the intrinsic differences between BMDMs and

alveolar macrophage polarization states, where BMDMs are more pro-inflammatory, phagocytic, and lean more towards the M1 phenotype than alveolar macrophages.[61] The BMDMs within this study had upregulated pro-inflammatory responses to even low MOIs, but alveolar macrophages may not share the same phenotype. Further explorations using alveolar macrophage models are necessary to learn more.

Of the cultured bacteria in this study, six out of nine remain an interesting avenue of investigation—do alveolar macrophages have a 'universal commensal' response? Additionally, the roles of various genes within the macrophage response to commensal bacteria should be explored further. In particular, the role of CCL17 in the macrophage response to commensal bacteria needs to be further examined. In this study, *Ccl17* expression was highly upregulated when the commensal bacteria were present at high MOIs. The overexpression of CCL17 has been implicated in the pathogenesis of asthma, and the possibility of CCL17 expression being driven by dysbiosis of the lung microbiome is an interesting possibility that deserves exploring.[62] Another gene of interest might be *Tnfa*, as TNF-α has already been identified as causing the cytokine cascade in a variety of inflammatory diseases.[57] Anti-TNF-α therapeutics are currently prescribed for treating such chronic inflammatory conditions as rheumatoid arthritis and inflammatory bowel disease.[57] If the inflammation that results from lung microbiome dysbiosis can be alleviated with similar drugs, an avenue might exist to treat the chronic inflammation seen in COPD. Similar approaches could be of benefit with IL-6 and nitric oxide synthase. Finally, the gene expression of the anti-inflammatory *Il10* increasing in proportion to increasing MOI might suggest that anti-inflammatory responses could be important in mitigating immunopathology and promoting host tolerance. IL-10-producing macrophages have been identified as preventing neutrophilic asthma.[63] A therapeutic treatment promoting IL-10 production might therefore be effective against inflammatory conditions in much the same way as a treatment targeting pro-inflammatory cytokines such as TNF-α.

Eventually, understanding the interactions between the lung microbiome and the host immune system might allow for the modulation and control of lung immune responses. Host tolerance, the ability of the host to tolerate the damage caused by both the pathogen and the immune system, is increasingly being seen as a key aspect of successful immune response to lung infections.[64] Decreasing pro-inflammatory responses and increasing anti-inflammatory responses might be a potential method to promote host tolerance. However, deepening our understanding of

why and how alveolar macrophages are more tolerant than other macrophages would be of great clinical significance.

Additionally, since many disease states of the lung are characterized by chronic inflammation, studies where macrophages are polarized and activated prior to infection could provide insight into respiratory infections in chronically ill hosts. Other future studies should include treating macrophages with various cocktails of commensal bacteria to see how responses may vary, and then infecting with pathogenic bacteria to see if commensal bacteria presence has an effect on macrophage pathogen response.

Past studies have demonstrated the use of commensal bacteria in preventing colonization of pathogenic bacteria and adverse health outcomes, from skin commensal bacteria protecting against *S. aureus* colonization,[65] to fecal microbiota transplants preventing *Clostridium difficile* infections,[66] and even *S. lentus* preventing mouse models from asthma.[43] The use of commensal bacteria as a therapy for chronic and acute lung illnesses is not unimaginable, but rather only a matter of years away. Understanding the interactions between specific lung microbiota and the immune system of the lung is the first step to achieve this goal.

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