Role of macrophage-derived versican in the innate immune response to LPS

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Abstract

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Versican, a chondroitin sulfate proteoglycan (CSPG), is an integral component of the extracellular matrix (ECM) necessary for embryonic development, but thereafter is expressed in very low levels in healthy adult lungs. As a key component of the extracellular matrix (ECM), versican modulates the innate immune response through interactions with other extracellular matrix molecules, regulating cell migration and influencing the accumulation and release of cytokines and growth factors. An essential feature of versican is that its impact is highly contextual. Among other factors, the influence of versican depends on the cellular source, specific agonist, and phase of the inflammatory response. Although previous work from our group shows versican has both pro and anti-inflammatory effects, the influence on pulmonary function, inflammation, and injury is not well understood. Here, we investigated the role of macrophage-derived versican in a mouse model of LPS-induced lung injury. We demonstrate that bone marrow-derived macrophages (BMDMs) and mice deficient in macrophage-derived versican show an altered acute immune response to Toll-Like Receptor-4(TLR4) activation compared to wild-type (WT) controls. The significance of these findings is furthered by histologic evidence that a lack of macrophage-derived versican contributes to increased leukocyte recruitment at 48 hours post-challenge. This study demonstrates that versican is an immunomodulatory molecule and controls the innate immune system.

Keywords: Versican, extracellular matrix, LysM, inflammation, lipopolysaccharide

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Acknowledgments

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As well as a special thanks to my mentor Chuck Frevert, without whom none of this research would have been possible.

Dedication

This work is dedicated to my fiancé, Mike McNamee, and my parents Karen and Vincent Mosca, who supported me endlessly across the last 11 years of my higher education.

This work is also dedicated to the mice in this project and beyond who have been used to make great scientific and medical advances.

Introduction

Respiratory tract infections are among the top three causes of death in humans globally. The early innate immune response against respiratory pathogens consists of physical and cellular defenses and leukocyte recruitment (Fig. 1). It is recognized that components of the extracellular matrix (ECM), including proteoglycans (PG) and glycosaminoglycans (GAGs), provide fine control of the innate immune response to lung infections. [1-3, 8-11] The ECM is a multifaceted, complex meshwork that facilitates cellular activity, such as proliferation and migration, through mechanical and biochemical cues. Versican, a chondroitin sulfate proteoglycan (CSPG), is an immunomodulatory molecule whose expression increases during lung infection. It is required for embryonic development, but its expression is down-regulated and minimal in in healthy adult lungs. However, it is highly expressed and accumulates in response to lung injury.[10] Versican interacts with cells by binding non-integrin and integrin receptors, and it has been shown that versican interacts with myeloid and lymphoid cells.

Work performed by Chang et al. demonstrated that versican mRNA is increased in the lungs of LPS-treated mice and is expressed at the highest levels 6 hours post instillation. By utilizing immunohistochemistry of the glycosaminoglycan-β domain of versican in the lungs of LPS-treated mice, it was also determined that one of the cells responsible for versican production in the lungs is the macrophage (Fig. 2). This observation raised questions about what other cells are responsible for versican synthesis and the role the cellular source has in the innate immune response. It was found that various cells produce versican, including fibroblasts, macrophages, dendritic cells, and epithelial cells. Through the development of the *Vcan*$^{fl/fl}$ mouse, our group investigated the contextual role of versican and the importance of the cellular source of versican. These studies

showed that versican has anti- or pro-inflammatory effects depending on the agonist, cellular source, and timing within the immune response. [2-3, 6-9, 11]

Alveolar, interstitial, and recruited macrophages are crucial for eliminating bacterial and viral pathogens from the pulmonary space, and restriction of this activity has been shown to worsen disease outcomes. [11] A better understanding of the mechanisms regulating versican expression and its properties in the context of TLR4 activation is required to understand the immunomodulatory properties of versican. Although previous work from our group established that versican has both pro and anti-inflammatory effects, the influence of macrophage-derived versican on pulmonary function, inflammation, and injury is not well understood. Described as an interferon-stimulated gene, versican (gene = *Vcan*) expression in macrophages has anti-inflammatory properties in acute pulmonary inflammation. The development of the LysM$^{+/+}$/*Vcan*$^{-/-}$ mouse strain, in which myeloid lineage cells have a conditional deletion of the versican gene, helped us further define the role of macrophage-derived versican. LysM$^{+/+}$/*Vcan*$^{-/-}$ BMDMs and lung tissue *in vivo* showed a significant decrease in the production of IFNβ and IL10 protein in response to poly(I:C) 1 day post instillation (dpi). [6] There was also a significant decrease in *Cxcl2* and *Ccl2* gene expression *in vitro*. A similar effect was subsequently seen during *in vivo* studies, in which LysM$^{+/+}$/*Vcan*$^{-/-}$ mice exhibited decreased accumulation of IL6 and TNFα in the BAL compared to control mice in response to poly(I:C).[6] In this report, we follow up on our previous findings to determine the role of versican in the innate immune response to LPS using bone marrow-derived macrophages (BMDMs) and *in vivo* studies. We also investigate the effects of a lack of macrophage-derived versican on the clinical course of lung injury.

In utilizing LPS rather than poly[I:C], we increase the level of complexity of these studies because LPS activates Trif and MyD88 signaling pathways. Our previous data showed that TLR4 is essential for versican production in response to LPS, which in turn activates two distinct signaling pathways: Trif-signaling and MyD88. The latter promotes the initial inflammatory response to LPS with proinflammatory cytokines such as IL6, which distinguishes it from the TLR3 activation of poly(I:C). We also compare mRNA expression to measure the relative expression of pro-inflammatory or M1 markers such as iNos and anti-inflammatory or M2 markers such as Arg1. Versican expression and synthesis identified in macrophages are defined as pro-inflammatory (M1), and it has not been detected in macrophages of other phenotypes.[8] Given that our previous studies suggest that macrophage-derived versican limits inflammatory recruitment and promotes the production of anti-inflammatory cytokines, we speculate that LysM$^{+/+}$/*Vcan*$^{-/-}$ mice would be more susceptible to LPS-induced lung injury. We hypothesize that versican-deficient macrophages treated with LPS would demonstrate a significant increase in the amount of pro-inflammatory M1-like gene expression compared to wild-type macrophages. We also hypothesize that LysM$^{+/+}$/*Vcan*$^{-/-}$ mice will accumulate less versican in the lungs and consequentially have increased pulmonary inflammation and injury caused by a decrease in the synthesis of anti-inflammatory cytokines such as IL10. Herein, we show that a lack of macrophage-derived versican in the LysM$^{+/+}$/*Vcan*$^{-/-}$ contributes to an increase in pro-inflammatory cytokine expression. Although the LysM$^{+/+}$/*Vcan*$^{-/-}$ mice did not have a significantly worse clinical response to the LPS challenge than WT mice, histological differences are seen between the two strains at 48 hours post-instillation and characterized by an increase in neutrophilic infiltration. Thus, our studies are important as they further add to our understanding of the role of versican in

LPS-induced lung injury and help to characterize the mechanisms of macrophage-derived versican and its role in the innate immune system.

Materials and Methods

LPS Administration

LPS from *E. coli* serotype 0111:B4 was purchased from List Biological Laboratories (Campbell, CA). Oropharyngeal instillation of LPS (2.0 μg/g) was performed in 8-12 week-old mice anesthetized with 3-4% isoflurane. After instillation, mice were returned to cages and allowed free access to food and water for the remainder of the study. Mice were euthanized by an excess of isoflurane followed by exsanguination at 6 hours, 48 hours, 96 hours, and 6 days. Lungs were collected for histology or lung homogenization and RNA purification.

Reagents

Gene-specific TaqMan primer-probe mixes used for quantitative real-time polymerase chain reaction (PCR) of versican were from ThermoFisher Scientific (Grand Island, NY). Primers and TaqMan probes for *Vcan 3-4* (Mm01283063_m1), *Il-6* (Mm00446190_m1), *Tnfα* (Mm00443258_m1), *Cxcl1 (*Mm04207460_m1) , *Cxcl2 (*Mm00436450_m1*), Il-1b (*Mm00434228_m1), *Il-6 (*Mm00446190_m1) *Ifnβ* (Mm00439552_s1), *Nos2* (Mm00440502_m1), *Il-10* (m01288386_m1), and *Arg1* (Mm00475988_m1) were used with cDNA as described below.

Animals

The University of Washington's animal facilities are AAALAC-accredited and all animal studies were approved by the University of Washington's IACUC. Studies were performed with female LysM$^{+/+}$/*Vcan*$^{-/-}$, *Vcan*$^{fl/fl}$, and LysM$^{-/-}$ mice. Mice were fed standard irradiated rodent chow ad libitum (Purina Lab Diet 5053, Brentwood, MO), housed in autoclaved, individually ventilated cages (Allentown, Allentown, NJ) with corncob bedding (The Andersons, Maumee, OH), and provided acidified, reverse-osmosis purified, autoclaved water in bottles. Mice were maintained specific pathogen-free via a rodent health monitoring program and were certified by the

university's rodent health monitoring program to be free of specific rodent pathogens, including ectoparasites, endoparasites, known enteric and respiratory bacterial pathogens, and mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler's murine encephalomyelitis virus, ectromelia virus, polyoma virus, lymphocytic choriomeningitis virus, mouse adenovirus, minute virus of mice, mouse parvovirus, mouse rotavirus, mouse cytomegalovirus, mouse thymic virus, Hanta virus, K virus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme.*

Generation of LysM$^{+/+}$/*Vcan*$^{-/-}$ mouse model

Work was performed in a Class II Type A2 biosafety cabinet (Labgard ES NU-540, Plymouth, MN) disinfected with chlorine dioxide (dilution 1:18:1, Clidox S, Pharmacal Research Laboratories, Naugatuck, CT). Mice were utilized in which the endogenous lysozyme 2 promoter elements constitutively express Cre-recombinase. This work resulted in a myeloid cell and alveolar type II cell versican-deficient mouse strain (LysM$^{+/+}$/*Vcan*$^{-/-}$) that is homozygous at the *Vcan*$^{fl/fl}$ and Lyz-Cre alleles. *Vcan*$^{fl/fl}$ mice that were initially bred to LysMCre$^{+/+}$ mice in the creation of a targeted mutation of the *Vcan* gene will act as wild-type (WT) controls. It has been previously shown that the deletion of LysM and Cre-recombinase expression does not modulate the inflammatory response to LPS-induced acute lung injury. [14] During the *in vitro* and *in vivo* studies, LysMCre$^{+/+}$ mice were used as controls to rule out the potential that the lack of endogenous *Lyz2* gene function modulates the response to LPS injury that would otherwise be attributed to the lack of versican in the targeted cells. Our initial studies detected a possible phenotypic effect of the targeted mutation on white blood cell function and proliferation in the male mice that were not observed in the females. Thus, all subsequent studies were performed in female LysM$^{+/+}$/*Vcan*$^{-/-}$ mice.

Isolation of RNA from lung tissue and qPCR

For lungs examined by qPCR, n = 15 mice in the PBS-treated groups— 6 LysM$^{+/+}$/*Vcan*$^{-/-}$, 6 *Vcan*$^{fl/fl}$, 3 LysMCre$^{+/+}$ mice and n = 19 in the LPS-treated groups—8 LysM$^{+/+}$/*Vcan*$^{-/-}$, 8 *Vcan*$^{fl/fl}$, and 3 LysMCre$^{+/+}$ mice. This was performed over three separate experiments. The method of euthanasia involved exsanguination secondary to overdose of isoflurane anesthesia in order to decrease the amount of blood in the lung compartment and potential effects of the blood on RNA analysis. After exsanguination, the thoracic cavity was opened via a midline incision and the lungs were removed and placed into RNAlater. Lungs were stored overnight at 4°C. RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed with an ABI PRISM 7000 Sequence Detector (Applied Biosystems). Real-time PCR was carried out in a total volume of 25 µl with a master mixture including all reagents required for PCR and gene-specific TaqMan primer-probe mixes. Relative mRNA expression was expressed as a fold increase over the values obtained from RNA collected from untreated mice (i.e., normal lungs) of the appropriate genotype. Quantitative PCR analyses were performed with two technical replicates.

In vitro cell culture

BMDMs were isolated from both left and right femurs and tibias of mice. Bone marrow was cultured in RPMI, FCS, L929 cell supernatant, L-glutamine, and penicillin/streptomycin. After eight days, macrophages were replated in macrophage media in six-well tissue culture dishes at a density of 1 x 10^6 cells/well for 24-48 hours before LPS treatment at 10 ng/ml or 100 ng/ml in RPMI for 2 or 6 hours. All treatment wells were duplicated for each mouse at the 10 and 100 ng/ml LPS doses and the 2 and 6 hour time points.

Isolation of RNA from cell cultures and qPCR

RNA was obtained from cell culture with RNeasy Plus Mini Kits (Qiagen, Valencia, CA). The quantity of RNA was determined with a NanoDrop spectrophotometer (NanoDrop Inc., Wilmington, DE). Copy number estimates were generated from standard curves created by using selected reference cDNA templates and TaqMan probes.

Histology

Lungs for histology experiments were collected at 48 hours, 96 hours, and 6 days and each timepoint had an n = 4 mice in the PBS-treated groups and n = 6 in the LPS-treated groups. Lungs were inflated with 10% neutral buffered formalin (NBF) at a pressure of 21 cm H_2O. The lungs and mediastinum were placed in 10% NBF for 24 hours and then transferred to 70% ethanol. The tissue was embedded in paraffin and trimmed into 4 mm sagittal sections. Histologic changes were examined in consultation with a veterinary pathologist for the extent of lung edema, infiltration of inflammatory cells, and alveolar hemorrhage. The lung injury was graded as mild, moderate, or severe.

Pulse Oximetry

In some cohorts, following oropharyngeal instillations, the hair around the neck of each mouse was shaved and then fully removed with a depilatory cream for placement of the infrared measuring device around the neck. Every 24 hours, beginning on the day of the challenge, the arterial saturation, heart rate, and respiratory rate were measured by MouseOx Plus (Starr Life Sciences Corp, USA). Measurements were recorded for 10 consecutive minutes to collect error-free data. An average of 10 minutes was used for all parameters.

Body Weight Measurements

Body weights were measured once daily in the morning. Initial body weight was defined as the weight on the day of the LPS challenge.

Statistics

All statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA). For gene expression, the fold increase over the average value from untreated cells or PBS-treated mice was calculated. PCR analyses were performed in two technical replicates. Comparisons for qPCR were analyzed by one-way ANOVA with multiple comparisons. A p-value <0.05 was considered statistically significant.

Results

A lack of macrophage-derived versican does not alter the clinical course of oropharyngeal LPS-challenge.

Mice challenged with LPS and the PBS-treated control mice were monitored daily. Body weight data is presented in Figure 4A. The LysM$^{+/+}$/*Vcan*$^{-/-}$ *and Vcan*$^{fl/fl}$ mouse strains treated with LPS began to lose weight 1 day post instillation (dpi). *Vcan*$^{fl/fl}$ mice reached their maximum weight loss between 3-4 dpi, but LysM$^{+/+}$/*Vcan*$^{-/-}$ mice reached their maximum weight loss between 4-5 dpi as measured by the percentage of initial weight on day 0. In mice instilled with PBS, the weight did not change significantly through 6 dpi. No significant differences in mean weights between LPS groups were detected in multiple comparisons posttests. Mice treated with LPS lost significantly more weight than the PBS control mice for both mouse strains.

In mice administered LPS, a significant reduction in arterial O_2 saturation measured with pulse oximetry was observed by 48h post instillation (Figure 4B). Peak oxygen saturation decline was seen 72h post-instillation. By 6 dpi, oxygen saturation was nearly recovered to normal levels. Respiration rate (Figure 5A) showed a modest decrease 24h post-instillation but did not change significantly in any of the treatment groups throughout the study. In both groups of mice administered LPS, there was a decline in heart rate 24h post-instillation which normalized by 48h post-instillation (Figure 5B). The oxygen saturation, respiratory rate, and heart rate of mice treated with PBS did not change significantly throughout the study. *Vcan*$^{fl/fl}$ *and* LysM$^{+/+}$/*Vcan*$^{-/-}$ groups exhibited similar patterns of change in cardiopulmonary parameters in response to the LPS stimulus and there was not a significant difference between the two groups in any of the clinical parameters.

Lung inflammation induced by LPS is mildly enhanced in the absence of macrophage-derived versican 6 dpi.

LPS-induced lung injury is characterized by interstitial and intra-alveolar accumulations of neutrophils and fibrin with increased alveolar macrophages and intra-alveolar hemorrhage. At 48 hours post-instillation, both *Vcan*$^{fl/fl}$ *and* LysM$^{+/+}$/*Vcan*$^{-/-}$ strains had lung changes consistent with moderate lung injury characterized by neutrophilic inflammation, alveolar thickening, edema, and focal areas of hemorrhage and the injuries (Fig. 11). At 96 hours post-instillation, both mouse strains had decreased amounts of inflammation and areas of hemorrhage, and the severity of changes was not significantly different between them. At 6 days post-instillation, the *Vcan*$^{fl/fl}$ lungs had minimal to mild increase in intra-alveolar inflammation; however a majority of the LysM$^{+/+}$/*Vcan*$^{-/-}$ LPS-treated lungs contained focally extensive, moderate, regions of histiocytic and neutrophilic inflammation which possibly represented delayed resolution of inflammation in the lung tissue in these mice (Figure 13).

Macrophage-derived versican contributes to cytokine production and acute inflammation in the lungs *in vivo*.

We previously reported that macrophage-derived versican produced in response to poly(I:C)-mediated activation is largely responsible for IFNβ and IL10 production in mouse lungs. Previous work also supports the role of versican in the expression of poly(I:C)-responsive cytokines and chemokines, and that production of these cytokines is dependent on the accumulation of versican in the lung.

To further characterize the acute inflammatory response, LPS was dosed at 2.0 μg/g and whole lung tissue was collected 6h post-instillation. We then measured the gene expression of several cytokines and chemokines in whole lung tissue (Figures 6-7). Overall, versican expression measured in the whole lung homogenate sample was modestly but not significantly attenuated in the LysM$^{+/+}$/*Vcan*$^{-/-}$ mice treated with LPS compared to WT controls.

A significant decrease in *Nos2* expression was seen in the LysM$^{+/+}$/*Vcan*$^{-/-}$ mice. Expression of *Il*-6, *Cxcl1*, *Cxcl2*, and *Il-1b* were significantly increased in LPS-treated LysM$^{+/+}$/*Vcan*$^{-/-}$ mice. The upregulation of these genes suggests a shift towards a more pro-inflammatory environment in the lungs during the first 6h after LPS administration when there is a lack of macrophage-derived versican. Expression of *Arg1*, *Tnfα,* and *Ifnβ* did not differ in the lungs of LPS-treated WT and LysM$^{+/+}$/*Vcan*$^{-/-}$ mice.

Macrophage-derived versican contributes to cytokine expression in BMDMs.

Our group has previously shown that BMDMs from the LysM$^{+/0}$/*Vcan*$^{-/-}$ mice hemizygous for Lysozyme 2-Cre and BMDMs from LysM$^{+/+}$/*Vcan*$^{-/-}$ mice have decreased expression of versican and that the ability of LPS to induce versican is attenuated in macrophages from LysM$^{+/+}$/*Vcan*$^{-/-}$ mice. In that study BMDMs from the LysM$^{+/+}$/*Vcan*$^{-/-}$ strain exhibited a 97% decrease in the copy number of versican per 10^5 copies 18S. [8] In mice used for the current study we demonstrate a similar effect in which untreated WT, LysM$^{+/+}$/*Vcan*$^{-/-,}$ and LysMCre$^{+/+}$ BMDMs constitutively express very low levels of versican. When stimulated at 10 and 100 ng/ml LPS there was an 86% and 91% decrease in versican expression respectively at 2 hours and a similar effect seen at 6 hours in LysM$^{+/+}$/*Vcan*$^{-/-,}$ BMDMs (Figure 11). Thus, BMDMs were used as a tool to investigate pro-inflammatory gene expression. For these studies, BMDMs were treated for 2 and 6 hours with LPS at doses of 10 ng/ml and 100 ng/ml. For the untreated cells, both LPS doses, and PBS treatments, wells were duplicated for each mouse for both the 2 and 6 hour time treatments.

We initially predicted that a lack of versican derived from macrophages would increase the activation state on BMDMs and result in the BMDMs being more consistent with M1 activation, or a pro-inflammatory response to LPS stimulation. This was based on past observations that macrophage-derived versican has demonstrated anti-inflammatory properties. With the

understanding that M1 and M2 macrophages are polarized extremes defined by treatments of macrophages *in vitro*, we predicted that we would observe a spectrum of macrophage activation between these two extremes in this study.

Similar to our previous work with poly(I:C), the expression of *Il-10* was attenuated at 2 hours and significantly attenuated in the high-dose treatment group at 6 hours in the LysM$^{+/+}$/*Vcan*$^{-/-}$ macrophages as compared to LPS-treated WT control BMDMs (Figure 12). *Nos2* production was also significantly decreased (Figure 13). Differences in gene expression of *Arg1* and *Il-6* were not observed between LysM$^{+/+}$/*Vcan*$^{-/-}$ and *Vcan*$^{fl/fl}$ macrophages at either dose or time point. The decreased expression of *Nos2* and *Il-10* suggests that the expression of versican by macrophages is an important component in an appropriate cellular response to the TLR4 activation. The explanation for these changes likely pertains to the fact that there could be a mix of M1 and M2 macrophages and that versican is altering the function of both. As determined in our *in vivo* studies, the precise mechanism of these changes is unknown but provides further evidence that versican exerts control of the innate immune system.

Discussion

In this study, we evaluated how the lack of macrophage-derived versican would alter the innate immune response to LPS, since we previously have shown that it appears to limit inflammatory cell recruitment and promote the synthesis of critical anti-inflammatory cytokines in response to poly[I:C].[3] We examined the production of cytokines both *in vitro* and *in vivo* and examined the lungs histologically to assess the differences between LysM$^{+/+}$/*Vcan*$^{-/-}$ and *Vcan*$^{fl/fl}$ mice. We also assessed the clinical course of acute lung injury as measured by the percent of total body weight loss and cardiopulmonary parameters in awake mice. We found that LysM$^{+/+}$/*Vcan*$^{-/-}$ BMDMs exhibited less of an inflammatory phenotype than WT macrophages and that mice lacking macrophage-derived versican had a more pro-inflammatory environment in the lungs in the acute phase of lung injury.

Given that the peak versican expression in BMDMs was determined to be around 6 hours post-treatment, we evaluated the cytokine response at two times. At 2 hours, we found a significant decrease in the pro-inflammatory *Tnfα* expression by the LysM$^{+/+}$/*Vcan*$^{-/-}$ BMDMs, and a significant reduction in the anti-inflammatory *Il-10*. At 6 hours, *Vcan*, *Nos2*, and *Il-10* expression was significantly diminished in the LysM$^{+/+}$/*Vcan*$^{-/-}$ BMDMs. These findings may not provide a definitive explanation for how versican influences acute inflammation; however, the data show a general trend that LysM$^{+/+}$/*Vcan*$^{-/-}$ BMDMs express lower relative levels of these cytokines. In contrast, *Il-6* expression was increased 2 h after LPS administration. The differences in the expression of iNOs and the various cytokines makes it difficult to classify the macrophages studied as either M1 or M2 macrophages. These findings do suggest however that versican is integral to the expression of these cytokines in response to LPS and perhaps critical to mounting an appropriate response to gram-negative bacteria. These findings also do demonstrate that the

versican-deficient macrophages do not properly respond to TLR4 activation. Due to the way BMDMs are cultured *in vitro*, the activation states and cellular responses may not necessarily represent the overall complexity of what is happening *in vivo*. However, we can conclude from these *in vitro* data that versican is an immunomodulatory molecule influencing innate immunity in macrophages.

Also, in this study, we evaluated the innate immune response to LPS *in vivo*. The respiratory epithelium and macrophages are a crucial part of the cellular defenses against respiratory pathogens and contribute to the dynamic interactions between the outside environment and the body's innate immunity to bacterial infection via cytokine secretion. Some of our findings, such as the significant decrease in *Nos2*, may be related to the effects of the Cre recombinase insertion in off-target cells, such as neutrophils, and deletion of versican in other off-target cells that contain the lysozyme 2 gene, such as a fraction of type II alveolar epithelial cells. Therefore, we must be careful not to overinterpret the differences between the LysM$^{+/+}$/*Vcan*$^{-/-}$ mice and control mice as it pertains to myeloid cells specifically due to the overall complexity in activation states and cellular responses *in vivo*. With the understanding that the lack of macrophage-derived versican may have altered cell-to-cell and cell-to-ECM interactions, we can conclude that macrophage-derived versican likely promotes an anti-inflammatory ECM in the acute response to LPS.

Macrophage phenotype varies depending on the origin of the cell, prior exposure to inflammatory mediators, and the microenvironment. One of the major differences between this study and previous work is that we examined a more acute phase of the immune response. Based on previous findings, measuring cytokine expression at 2 and 6 hours seemed most appropriate as versican expression is highest 6 hours post-instillation. Although the amount of versican expression was not significantly decreased in the whole lungs of the LysM$^{+/+}$/*Vcan*$^{-/-}$ mice, this

result aligned with expected findings due to the fact that macrophages are not the primary producers of versican in the lungs. A drastic reduction of versican in the LysM$^{+/+}$/*Vcan*$^{-/-}$ mice was not predicted because other cells, such as fibroblasts and epithelial cells, express versican in response to the strong TLR4 stimulus. Chang et al. showed a significant decrease in CXCL2 and no difference in the recovery of CXCL1 protein in LysM$^{+/+}$/*Vcan*$^{-/-}$ bronchoalveolar fluid in response to poly(I:C) after 24h. [8] The differences in time points studied likely plays a major role in the differences observed. Based on our observations it will be important to conduct future studies to better characterize how differences in agonist (TLR3 versus TLR4) may influence the ability of versican to modulate the immune response.

The present study further supports our findings that the influence of versican modulating the innate immune response is contextual and depends on the agonist, pathway, cells producing versican, timing of the inflammatory response, and the tissue region in which the versican is present. Another consideration is that although we eliminated as much of the blood from the lung tissue as possible, we cannot rule out the possibility that scant amounts of blood in the tissue influenced our cytokine measurements. Our methods were standardized to maintain consistency between cohorts; however, future work may include flushing the lung vasculature with sterile saline before tissue collection.

Lax et al. showed that measuring cardiopulmonary parameters in awake mice can be used to assess lung function in mice following LPS administration.[41] To follow the course of lung injury over time, we assessed both the clinical status and the degree of lung inflammation and injury on histology to determine how LysM$^{+/+}$/*Vcan*$^{-/-}$ mice differ from WT controls. Our data showed that, based on arterial saturation, clinical lung dysfunction peaks between 75-96h. An important finding of this study is that arterial O_2 saturation correlated with weight loss for the first 72-96 h but had a

lag of several days to return to normal, whereas mice tended to regain body weight by 4-6 dpi. Even by day 6, the arterial O2 saturation of LPS-treated mice had not completely returned to normal. We selected 48h, 96h, and 6 d to examine lungs histologically based on this information. These findings may indicate that the lungs have recovered their ability to oxygenate the blood but that the presence of inflammatory cells and perhaps damage to the alveoli as a result of the LPS challenge persisted.

In conclusion, our goal was to further define the role of macrophage-derived versican in the innate immune response to TLR4 activation. We further demonstrate that macrophage-derived versican has anti-inflammatory properties and provides fine control of the innate immune system. It is critical to continue to investigate various agonists in different temporal phases of the innate immune response due to the highly contextual nature of versican and the gap in our knowledge regarding the precise signaling pathway downstream of TLR4 that regulates the versican promoter. The data presented in this study provide a foundation for future studies to be performed *in vivo* in perhaps other models of macrophage-derived versican-deficient mouse models and expand upon our current knowledge of the immunomodulatory properties of versican.

Figures

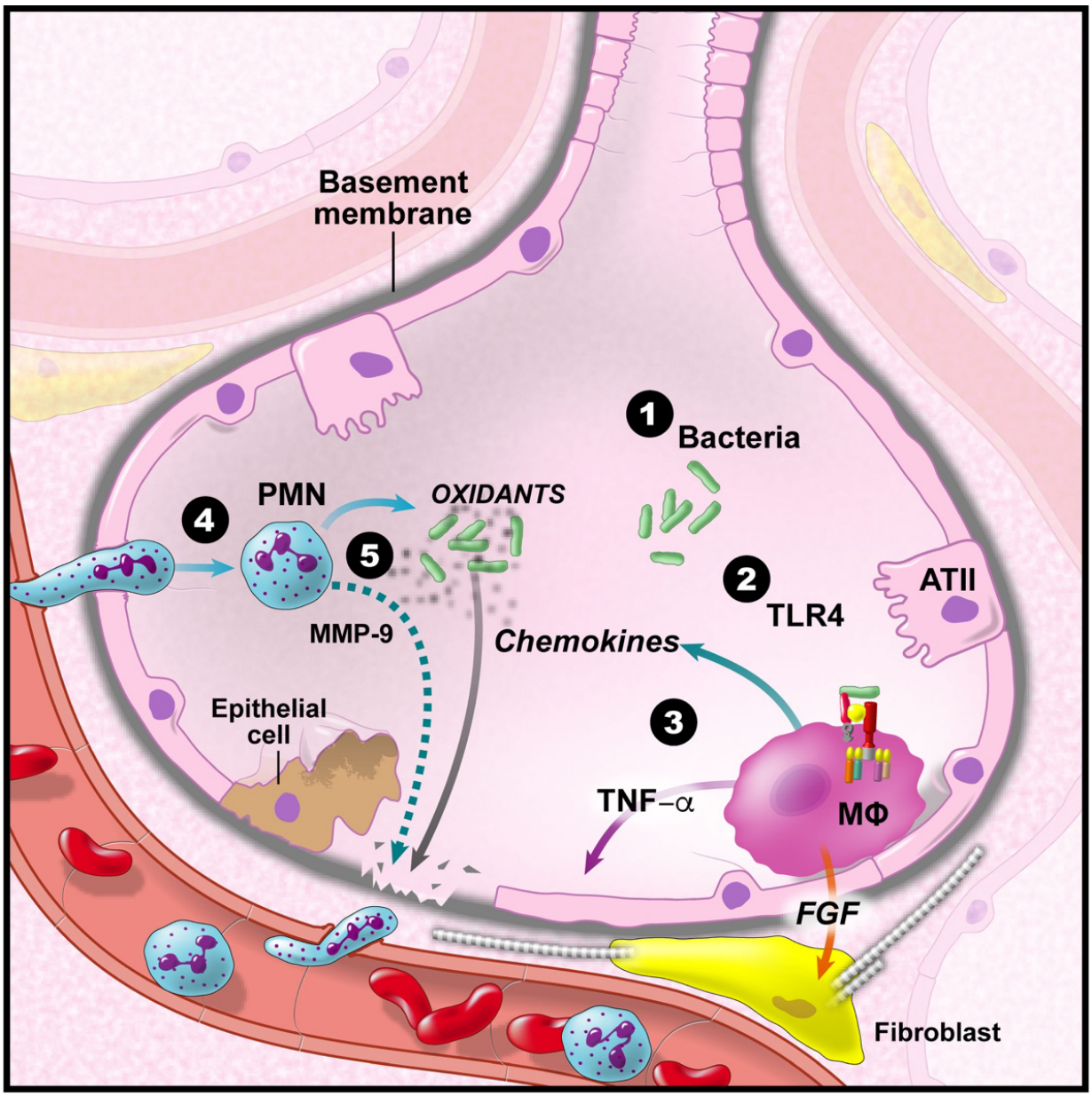


Figure 1. The respiratory epithelium and macrophages comprise the dynamic interface between the environment and the host's first line of defense to pathogens such as gram-negative bacteria. LPS induced-inflammation via TLR4 activation markedly increases the expression of cytokines such as IL6, TNFα, and other components of the innate immune system leading to changes to the ECM and the pulmonary recruitment of leukocytes. (Adapted from Gill et al., 2010 and Tang et al., 2022).

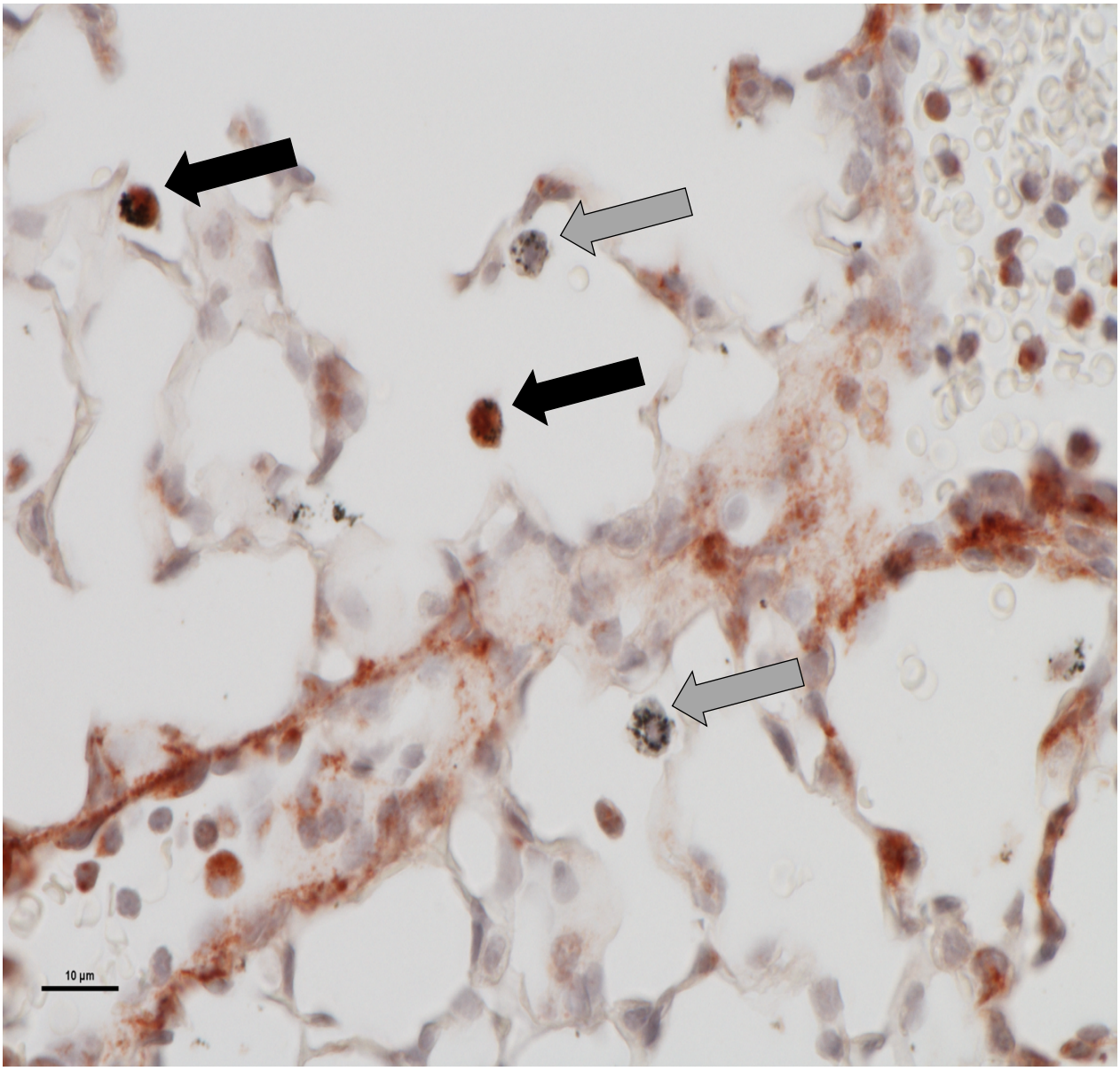


Figure 2. Positive immunostaining for versican is increased in a subset of macrophages in the lungs of LPS-treated mice. Immunohistochemistry for versican in lung tissue obtained from an LPS-treated mouse. This mouse was treated for 6h with 1 mg/kg LPS, and was stained with an antibody specific for the glycosaminoglycan-β domain of versican. Gray arrows indicate alveolar macrophages. The black arrows indicate alveolar macrophages that show positive staining for versican. (Adapted from Chang et al., 2014).

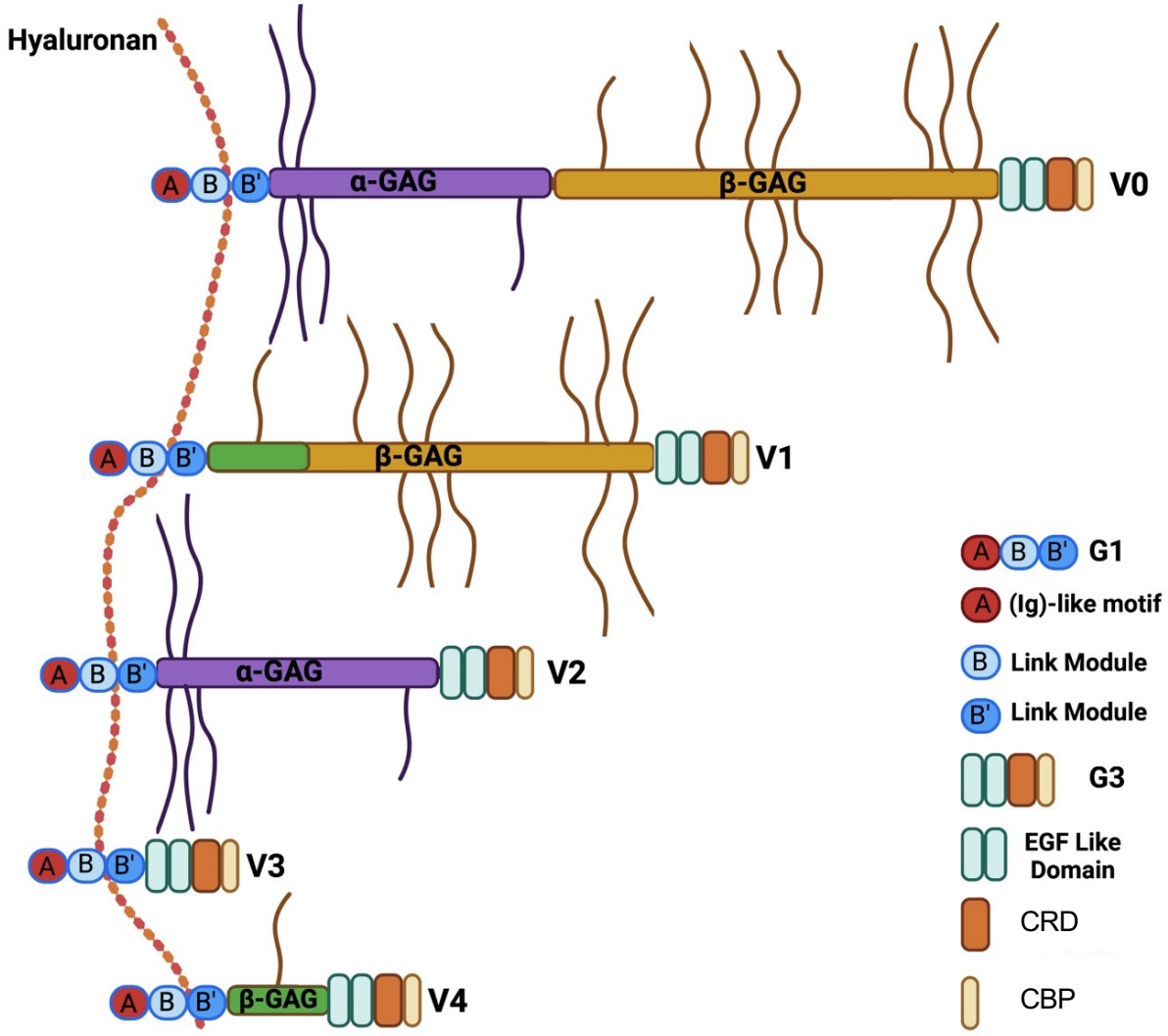
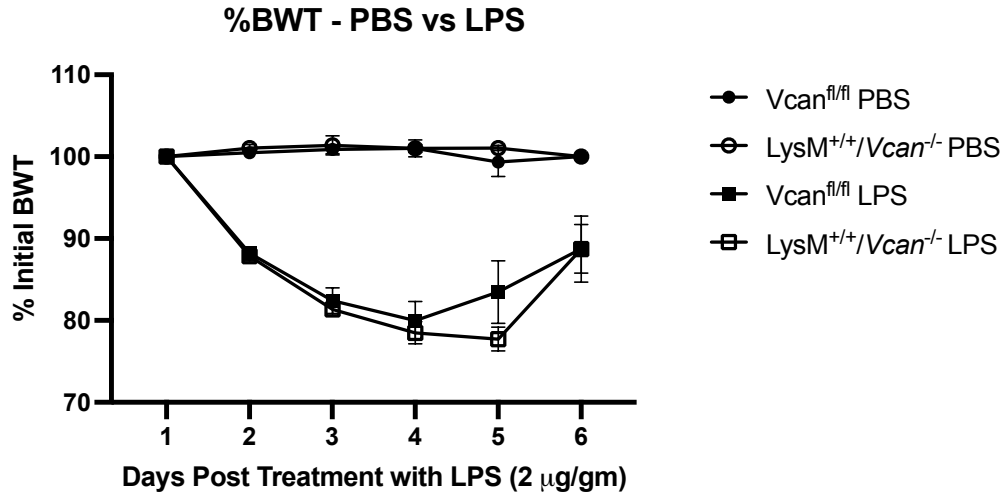


Figure 3. Domain structures of the 5 versican isoforms. The structures provide locations for the binding of various cell surface receptors and molecules to versican. The chondroitin sulfate side chains and multiple forms of versican make it a dynamic molecule with the ability to bind to numerous factors that make up the versican interactome. (Adapted from Tang et al., The Versican Interactome).

A



B

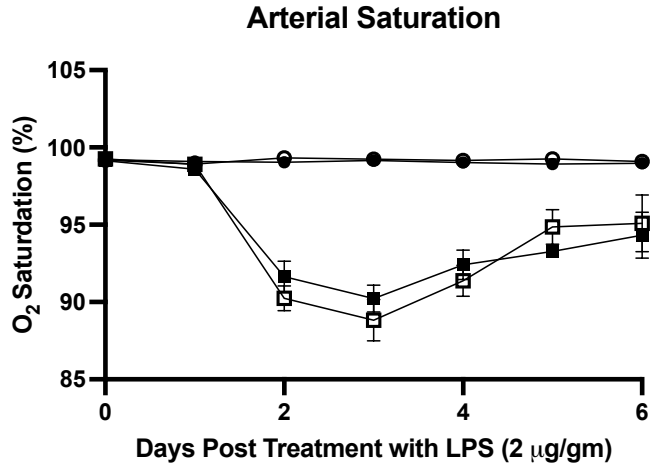


Figure 4. Data looking at strain-related differences in clinical data collected in LysM$^{+/+}$/Vcan$^{-/-}$ and Vcan$^{fl/fl}$ mice after oropharyngeal LPS administration. Data above shows A) change in % body weight in LysM$^{+/+}$/Vcan$^{-/-}$ mice (empty symbols) and Vcan$^{fl/fl}$ mice (solid symbols) instilled via an oropharyngeal route with 2 ug/g LPS (squares). Control animals were treated with PBS vehicle (circles). B) Arterial saturation was monitored using infrared pulse oximetry in awake mice instilled with LPS. Values are the mean ± SEM. Data shown is combined from 2 independent experiments, each experiment with 4-5 mice per group.

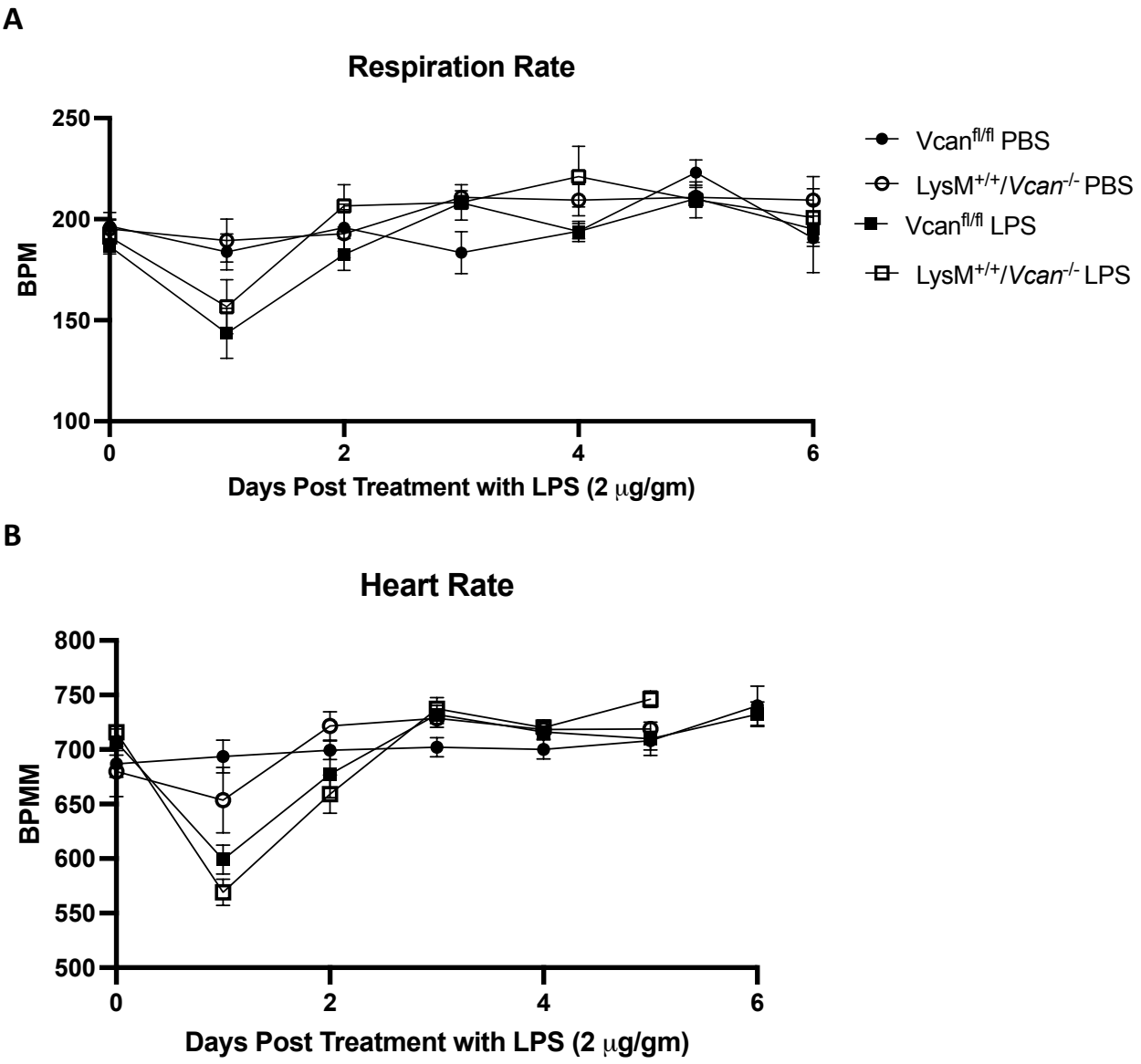


Figure 5. Cardiopulmonary parameters measured in awake animals utilizing MouseOx Plus Data above show A) change in the respiratory rate in LysM$^{+/+}$/*Vcan*$^{-/-}$ mice (empty symbols) and Vcan$^{fl/fl}$ mice (solid symbols) instilled via an oropharyngeal route with 2 ug/g LPS (squares). B) Heart rate with a pronounced decrease at 24h. Values are the mean ± SEM. Data shown is combined from 2 independent experiments, each experiment with 4-5 mice per group.

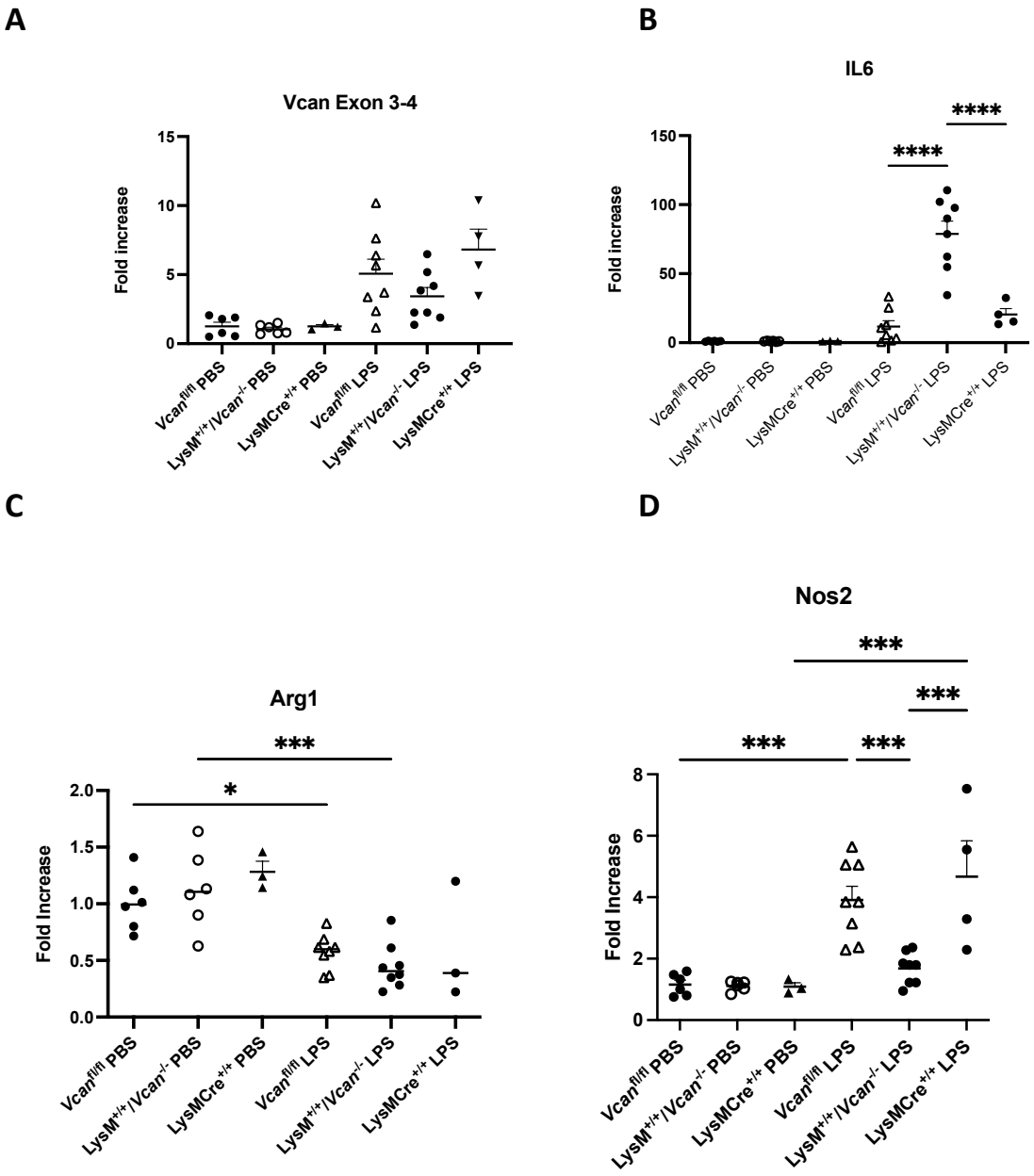


Figure 6. Cytokine and chemokine production by LysM$^{+/+}$/*Vcan*$^{-/-}$ mice. WT and LysM$^{+/+}$/*Vcan*$^{-/-}$ mice were exposed to oropharyngeal treatment with LPS (2 mg/kg) for 6 hours. LysMCre$^{+/+}$ mice did not show differences from Vcanfl/fl controls. A) Fold changes in the mRNA from homogenized lung tissue in *Vcan* expression B) *IL6* was significantly increased in LysM$^{+/+}$/*Vcan*$^{-/-}$ mice C) *Arg1* D) *Nos2* expression was significantly diminished in mice lacking macrophage-derived versican Bars represent mean ± SE, ** = $P < 0.05$, *** = $P < 0.01$, ****= $P < 0.001$.

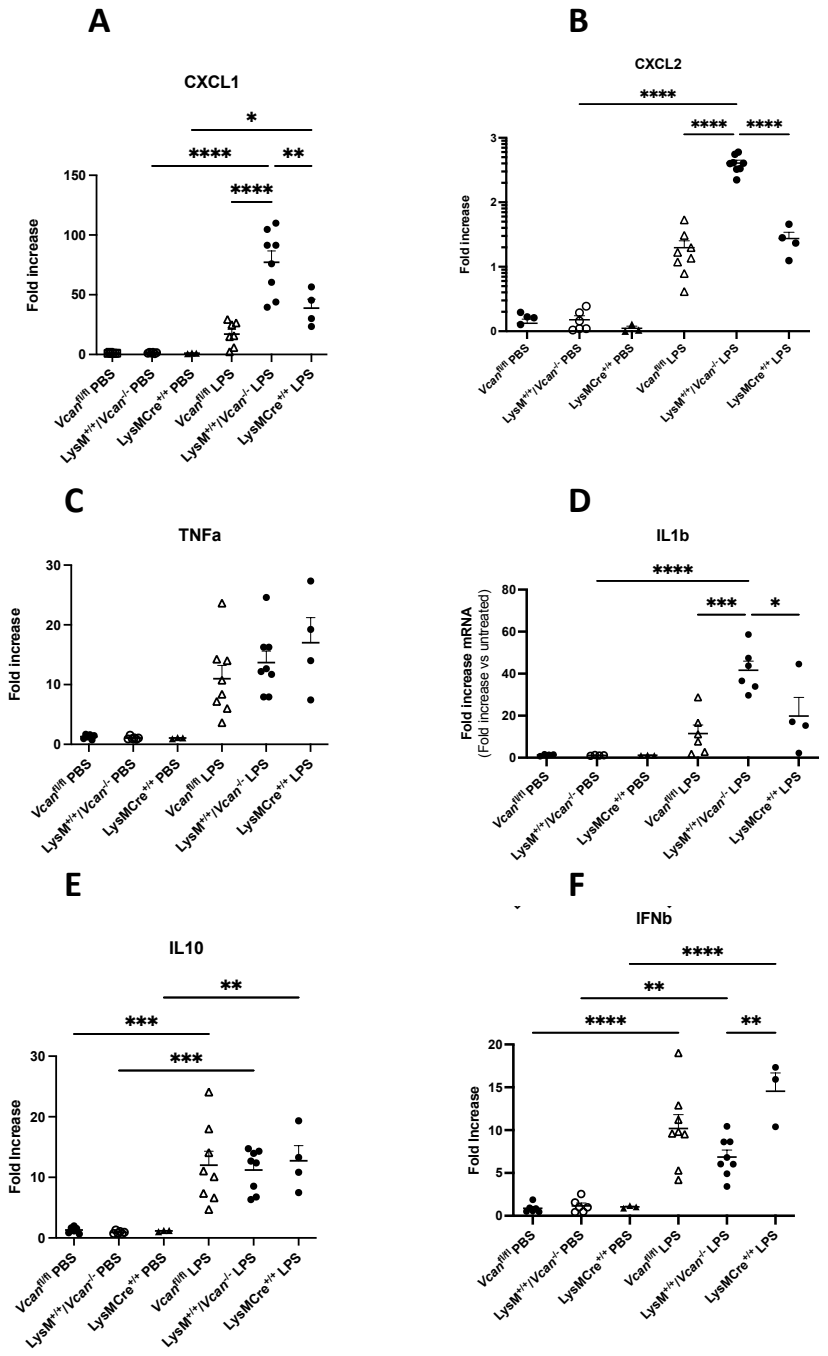


Figure 7. Cytokine and chemokine production by LysM$^{+/+}$/*Vcan*$^{-/-}$ mice. WT, LysM$^{+/+}$/*Vcan*$^{-/-}$, and LysMCre$^{+/+}$ mice were exposed to oropharyngeal treatment with LPS (2 mg/kg). Fold changes in the mRNA from homogenized lung tissue. A,B) CXCL1 and CXCL2 were significantly increased in mice lacking macrophage-derived versican. C) TNFα expression D) IL1b was significantly increased in the lungs of LysM$^{+/+}$/*Vcan*$^{-/-}$ mice E,F) IL10 and IFNβ expression. Bars represent mean ± SE, ** = $P < 0.05$, *** = $P < 0.01$, ****= $P < 0.001$.

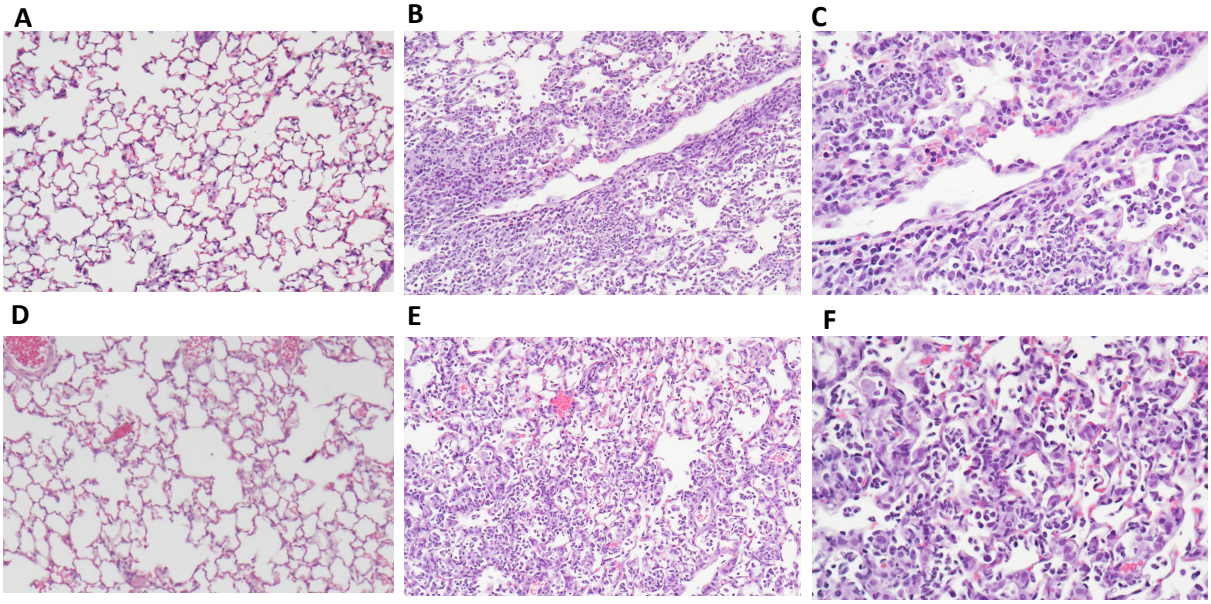


Figure 8. Lung histopathology of PBS control treated lung (panels A and D), and LPS-treated lungs (B, C, E, F) 48 hours after instillation, H&E staining. A) Lungs from LysM$^{+/+}$/*Vcan*$^{-/-}$ mice treated with PBS (left) at 20x magnification B,C) Lungs from LysM$^{+/+}$/*Vcan*$^{-/-}$ mice treated with 2 mg/kg LPS 48 hours after instillation at 20x and 40x magnification D) Lungs from control *Vcan*$^{fl/fl}$ E,F) LPS-treated lungs from *Vcan*$^{fl/fl}$ control mice at 20x and 40x magnification. Both LPS-treated groups had lung lesions characterized by polymorphonuclear cells, alveolar thickening, and focal areas of hemorrhage. Both mouse strains demonstrate moderate lung injury at 48h. PBS controls have thin alveolar septa and no significant inflammation.

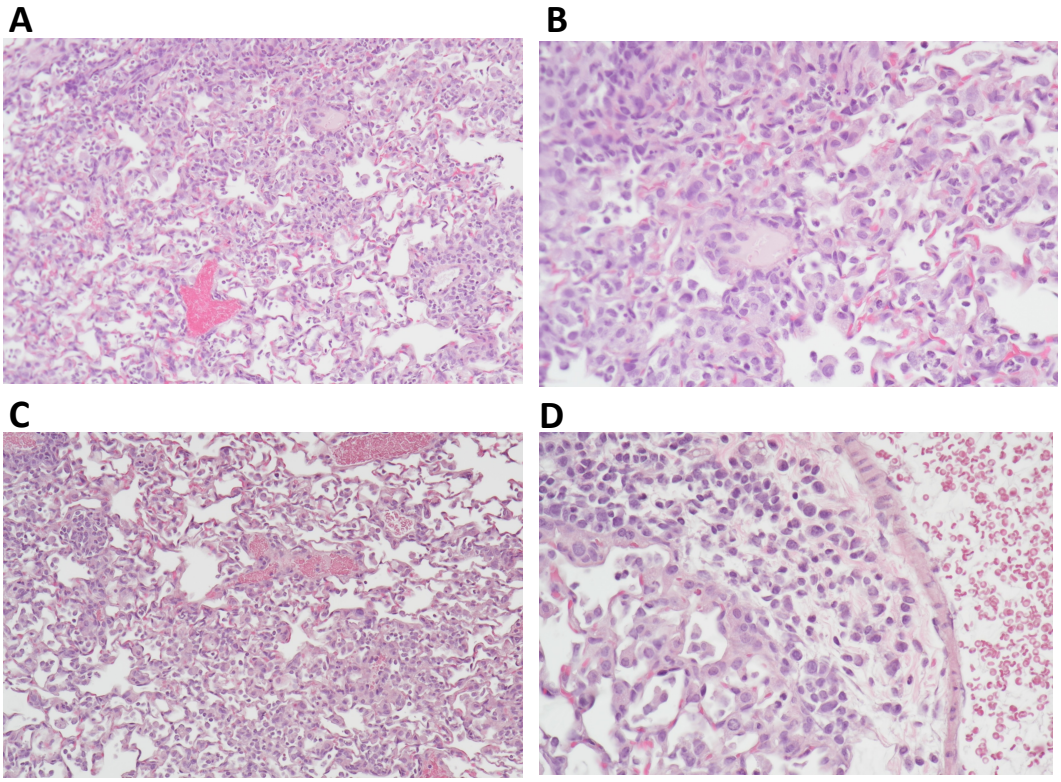


Figure 9. Representative lung histopathology with H&E staining. A,B) LysM$^{+/+}$/*Vcan*$^{-/-}$ mice at 20x and 40x magnification and C,D) *Vcan*$^{fl/fl}$ mice at 20x and 40x magnification. Mice were administered LPS via oropharyngeal instillation and lungs were collected 4 dpi. Both LPS-treated groups had lung tissue characterized by the presence of neutrophils, alveolar thickening, edema, and occasional focal areas of hemorrhage. Both mouse strains developed a similar extent and severity of lesions at 4 dpi.

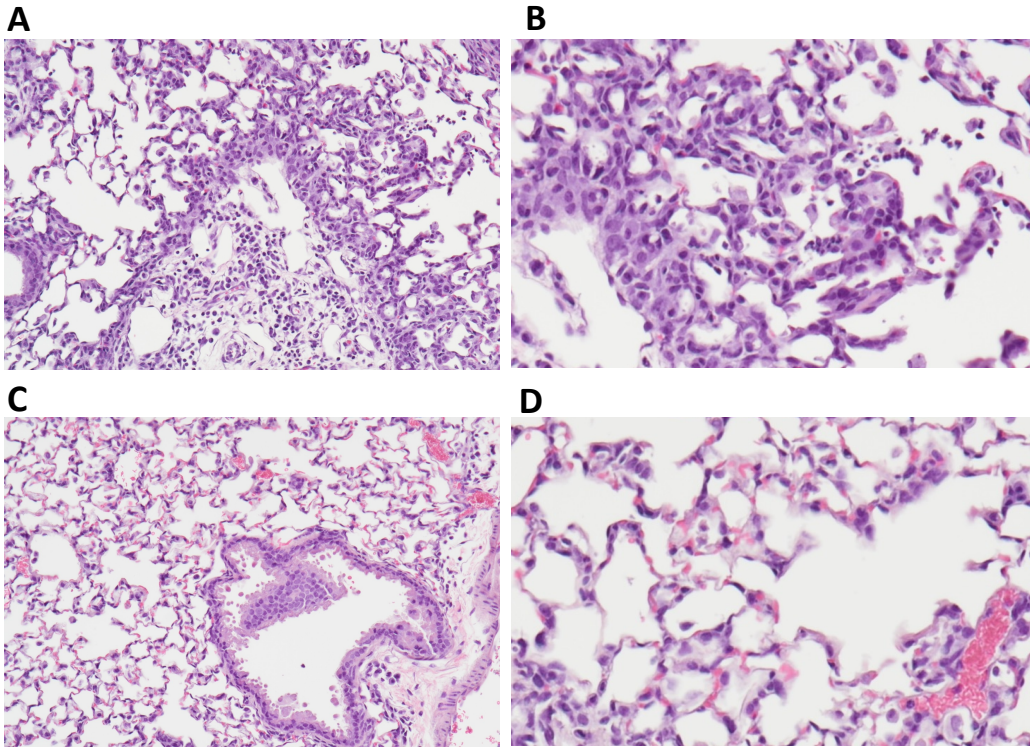


Figure 10. Representative lung histopathology with H&E staining 6 dpi. A,B) LysM$^{+/+}$/*Vcan*$^{-/-}$ mice at 20x and 40x magnification and C,D) *Vcan*$^{fl/fl}$ mice at 20x and 40x magnification. Mice were administered LPS via oropharyngeal instillation and lungs were collected 6 dpi. LysM$^{+/+}$/*Vcan*$^{-/-}$ lungs had occasional, mild, focally-extensive regions of neutrophilic and histiocytic inflammation. *Vcan*$^{fl/fl}$ mice we observed to have fewer aggregates of inflammatory lesions.

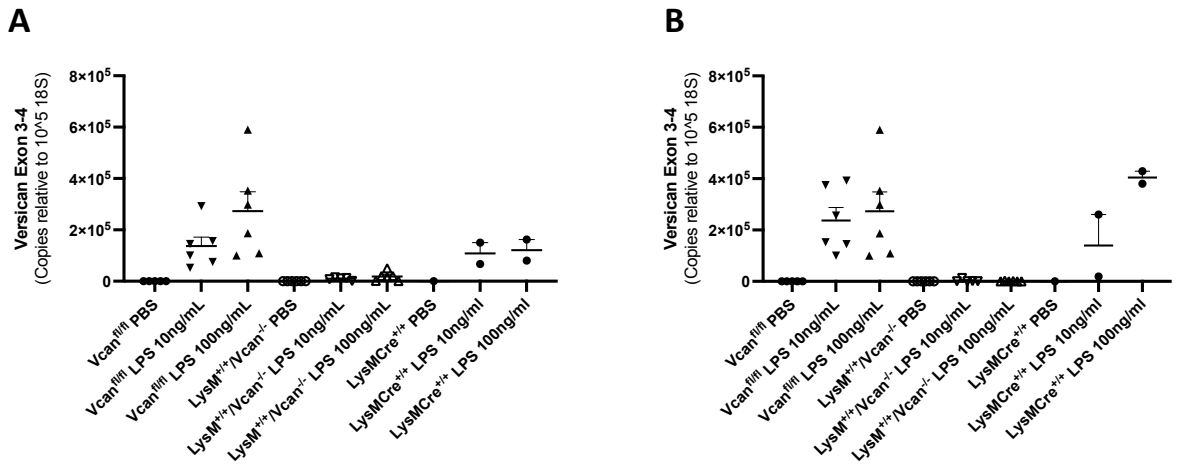


Figure 11. Versican copy numbers. BMDMS from WT (*Vcan*$^{fl/fl}$), LysM$^{+/+}$/*Vcan*$^{-/-}$, and LysMCre$^{+/+}$ mice 2 and 6 hours post-treatment. A) BMDMs were treated with PBS or LPS (10 and 100 ng/ml) for 2 hours B) BMDMs were treated for 6 hours. In both studies the production of versican copy numbers per 10^5 18S was decreased by >85% in LysM$^{+/+}$/*Vcan*$^{-/-}$ cells.

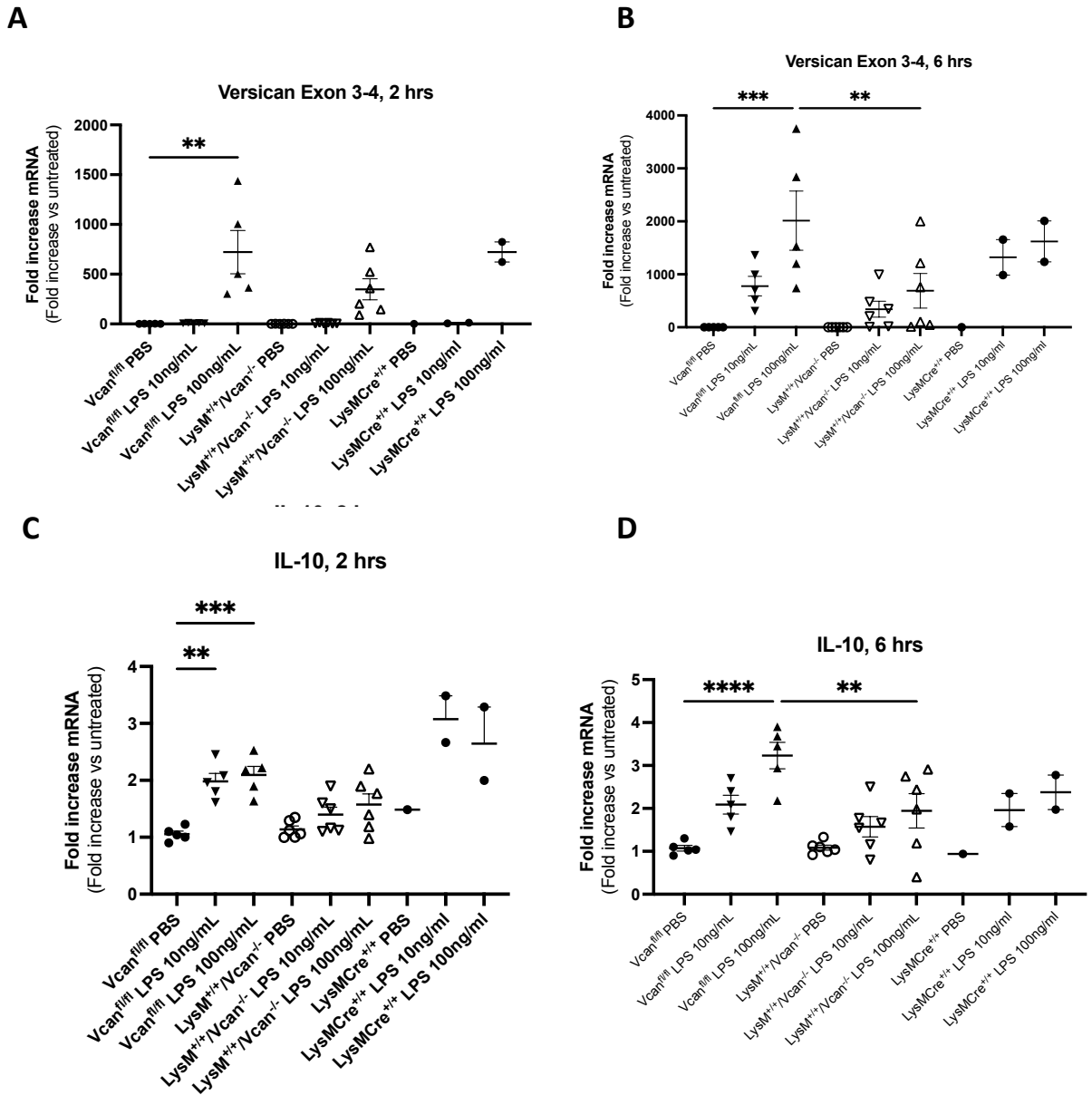


Figure 12. Cytokine and chemokine production by LysM$^{+/+}$/*Vcan*$^{-/-}$ macrophages. Fold changes in the mRNA from cell lysates of BMDMs from control (*Vcan*$^{fl/fl}$ and LysMCre$^{+/+}$) and LysM$^{+/+}$/*Vcan*$^{-/-}$ mice 2 and 6h after treatment with LPS (10 and 100 ng/ml). A,B) *Vcan* expression 2 and 6 hours post-treatment. C,D) In the higher dose group IL10 production was significantly decreased at 6 hours in LysM$^{+/+}$/*Vcan*$^{-/-}$ BMDMs. Data shown is combined from 3 independent experiments, each point representing one mouse. $^{**}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

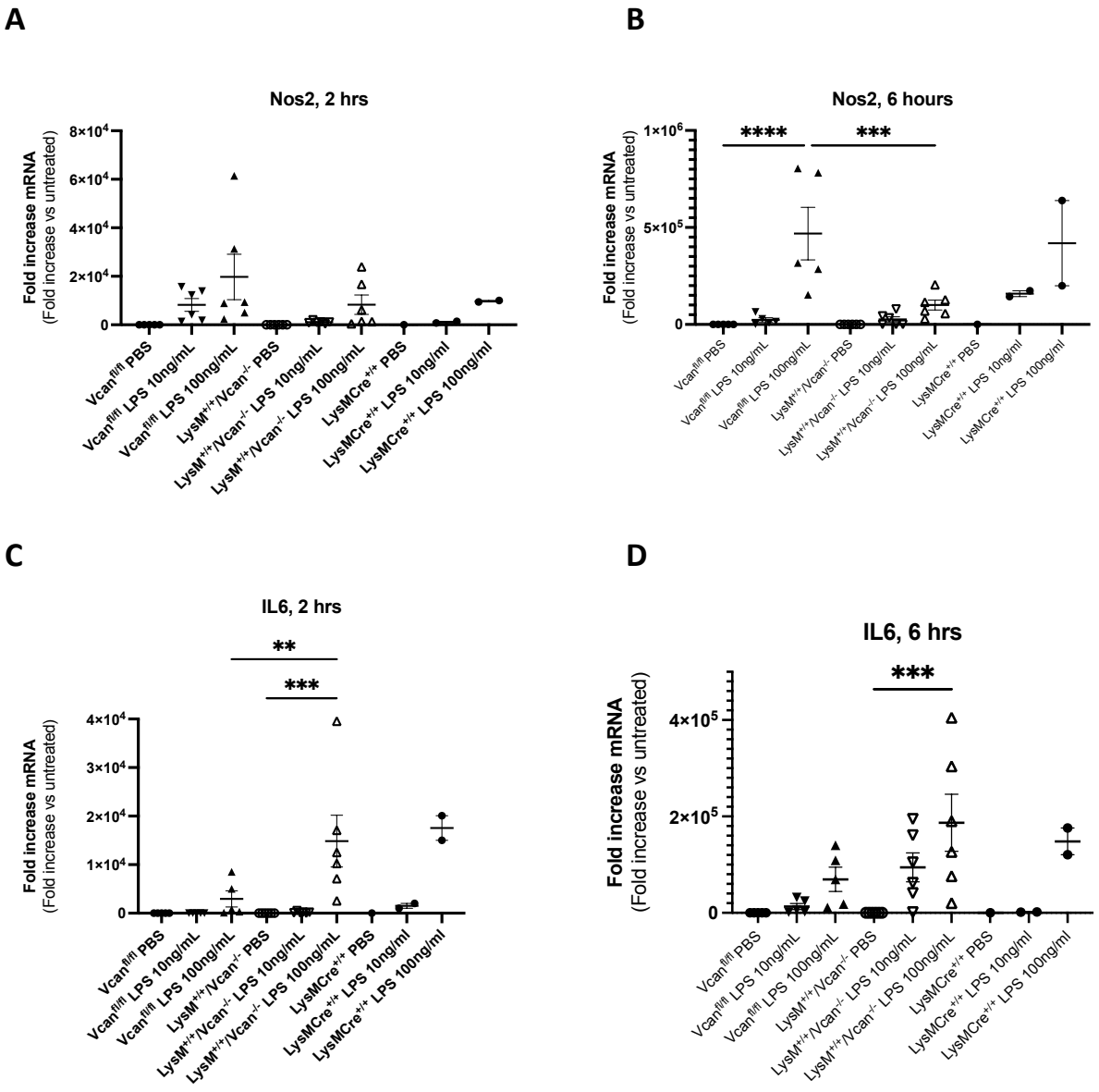


Figure 13. Cytokine and chemokine production by LysM$^{+/+}$/*Vcan*$^{-/-}$ macrophages. Fold changes in the mRNA from cell lysates of BMDMs from control (*Vcan*$^{fl/fl}$ and LysMCre$^{+/+}$) and LysM$^{+/+}$/*Vcan*$^{-/-}$ mice 2 and 6h after treatment with LPS (10 and 100 ng/ml). A,B) *Nos2* expression 2 and 6 hours post-treatment. At 6 hours *Nos2* expression was significantly decreased in the higher dose group. C,D) *IL6* expression at 2 and 6 hours. Data shown is combined from 3 independent experiments, each point representing one mouse. $^{**}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

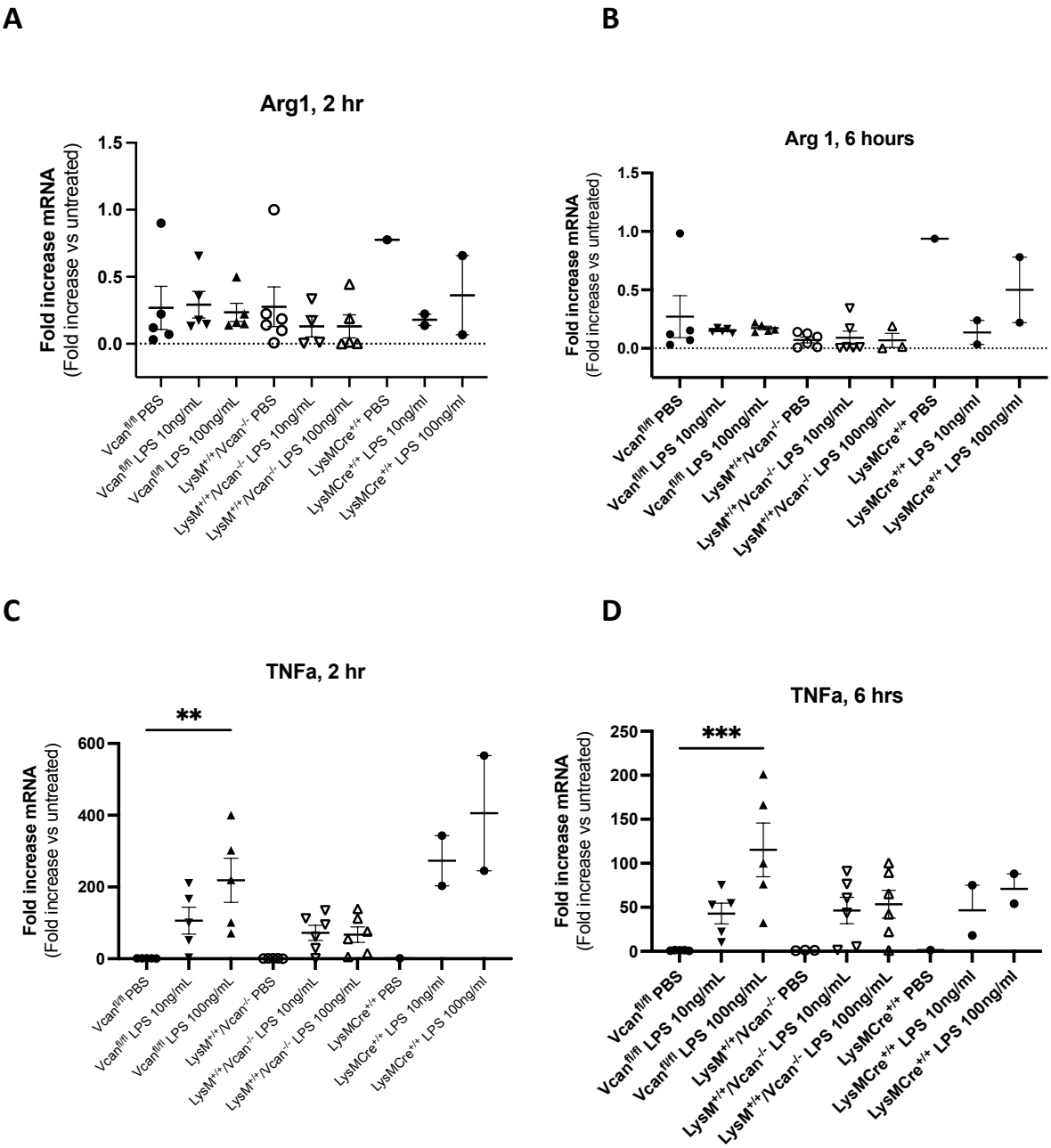


Figure 14. Cytokine and chemokine production by LysM$^{+/+}$/*Vcan*$^{-/-}$ macrophages. Fold changes in the mRNA from cell lysates of BMDMs from control (*Vcan*$^{fl/fl}$ and LysMCre$^{+/+}$) and LysM$^{+/+}$/*Vcan*$^{-/-}$ mice 2 and 6h after treatment with LPS (10 and 100 ng/ml). A,B) *Arg1* expression C,D) TNFα expression. Data shown is combined from 3 independent experiments, each point representing one mouse. $^{**}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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