HHS Public Access

Author manuscript

Curr Eye Res. Author manuscript; available in PMC 2022 July 29.

Published in final edited form as:

Curr Eye Res. 2013 October ; 38(10): 1036–1048. doi:10.3109/02713683.2013.804094.

Modulation of Immune Signaling, Bacterial Clearance, and Corneal Integrity by Toll-like Receptors during *Streptococcus pneumoniae* Keratitis

Nathan A. Tullos[1], **Hilary W. Thompson**[2,3], **Sidney D. Taylor**[1], **Melissa Sanders**[1], **Erin W. Norcross**[4], **Isaiah Tolo**[1], **Quincy Moore**[5], **Mary E. Marquart**[1]

[1]Department of Microbiology, University of Mississippi Medical Center, Jackson, MS, USA

[2]Department of Biostatistics, Section of Biostatistics, Louisiana State University Health Sciences Center School of Public Health, New Orleans, LA, USA

[3]Department of Ophthalmology, LSU Eye Center, New Orleans, LA, USA

[4]Department of Biology, Mississippi College, Clinton, MS, USA

[5]Department of Biology, Prairie View A&M University, Prairie View, TX, USA

Abstract

Purpose: Bacterial keratitis, without effective antimicrobial treatment, leads to poor patient prognosis. Even after bacterial clearance, the host inflammatory response can contribute to corneal damage. Though *Streptococcus pneumoniae* (pneumococcus) is a common cause of bacterial keratitis, the role of host innate immunity during pneumococcal keratitis is not well characterized. This study investigated the role of Toll-like receptors (TLRs) during pneumococcal keratitis.

Materials and Methods: C57BL/6, as well as TLR2$^{-/-}$ and TLR4$^{-/-}$ mice, were infected with *S. pneumoniae*, and infected corneas were examined for 21 days. Quantitative real-time reverse-transcriptase polymerase chain reaction was performed using primers for genes involved in the inflammatory response and TLR signaling. Bacterial survival and leukocyte invasion were examined over a 72-h period.

Results: The corneal expression of TLR2, TLR4, and other inflammatory genes was increased at 72 h post-infection (p.i.) compared to uninfected C57BL/6 scratch controls. TLR2$^{-/-}$ mice showed a significant increase in bacterial survival at 24 h p.i. likely due to decreased neutrophil infiltration; however, after Day 5 p.i. observed clinical scores of TLR2$^{-/-}$ and C57BL/6 mice were not significantly different. In contrast, permanent corneal damage was observed for TLR4$^{-/-}$ mice over 21 days. Initially, both TLR$^{-/-}$ mouse strains exhibited lower expression levels in many immune genes, but returned to similar or elevated levels compared to C57BL/6 mice by 72 h p.i.

Conclusions: TLR2 and TLR4 are involved in the response to pneumococcal keratitis and TLR2 may aid in bacterial clearance by recruitment of neutrophils to the cornea, whereas TLR4 may be necessary to modulate the immune response to limit cellular damage.

Correspondence: Mary E. Marquart, Department of Microbiology, University of Mississippi Medical Center, Jackson, MS 39216, USA. Tel: +601-815-6934. Fax: +601-984-1708. mmarquart@umc.edu.

Keywords

Keratitis; mouse; *Streptococcus pneumoniae* ; Toll-like receptors

INTRODUCTION

Bacterial keratitis, if left untreated, may cause serious damage to the human cornea and permanent vision loss for the patient.[1] The gram-positive diplococcus, *Streptococcus pneumoniae*, has been shown to be a major etiologic agent of bacterial keratitis[2,3] and is often reported as one of the top three causes along with *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[2,4–8] Ocular surgery, contact lens use, and injury have all been implicated as predisposing factors for bacterial keratitis.[2,9]

Our understanding of the ocular immune response to pneumococcal keratitis, particularly the innate response, is limited. Molecules such as Toll-like receptors (TLRs) have important roles in other models of bacterial keratitis, such as in *Pseudomonas* infections.[10,11] TLRs are members of the pattern-recognition receptor (PRR) family of proteins. Their role in the innate immune response is to survey the extracellular environment and detect fundamental pathogen-associated molecular patterns (PAMPs). Once activated, these TLRs initiate a signaling cascade that leads to the rapid production of cytokines which, in turn, stimulate the infiltration of neutrophils, phagocytosis of bacteria, presentation of antigen to T and B cells, and additional downstream immunological events. TLR surveillance is prevalent in the murine eye, with mRNA and proteins of almost all the TLRs found at the various levels of the ocular structure including the corneal epithelium, stromal fibroblasts and retinal pigment epithelial cells.[12]

TLR2 has been observed to become activated in the presence of a variety of structurally non-related agonists. These include glycosylphosphatidylinositol anchors of *Trypanosoma cruzi*,[13] lipoteichoic acid from *S. aureus*,[14] lipoarabinomannan from *Mycobacterium tuberculosis*,[15] zymosan from *Saccharomyces cerevisiae*,[16] and viral proteins from herpes simplex,[17] varicella zoster[18] and cytomegalovirus.[19] TLR2, unlike other TLRs, does not form a signal inducing homodimer but instead heterodimerizes with either TLR1 or TLR6.[20]

TLR4 has been reported to become activated in the presence of LPS.[21] TLR4 forms homodimers and has been shown to require the presence and action of accessory molecules such as CD14, MD-2 and LPS-binding protein[21,22] to fully produce a functional cytokine response to LPS. The interaction between TLR4 and the pneumococcal toxin, pneumolysin, has been studied. Pneumolysin-induced apoptosis and cytokine production has been shown to be TLR4 mediated.[23,24] $TLR4^{-/-}$ mice exhibit enhanced bacterial growth in a pneumococcal pneumonia model,[25] and pneumolysin-induced, TLR4-mediated protection was critical for preventing invasive disease in a nasopharyngeal carriage model.[23]

Currently, nothing has been reported regarding the possible functions of TLRs during *S. pneumoniae* keratitis. Pneumococcal peptidoglycan has been shown to activate TLR2 in fibroblast cell lines as detected by increased translocation of NFκβ.[26] Similarly, pneumococcal pneumolysin has been shown to activate TLR4 in nasopharyngeal[23] and

upper respiratory mouse infection models.[24] Based on these studies we sought to determine the possible roles of TLR2 and TLR4 during pneumococcal keratitis.

METHODS

Bacterial Growth Conditions

Clinical keratitis isolate K1263 was kindly provided by Regis Kowalski (Charles T. Campbell Eye Microbiology Laboratory, Pittsburgh, PA). K1263 was grown overnight in Todd-Hewitt broth enriched with 0.5% yeast extract (THY) at 37 °C supplemented with 5% CO_2. The overnight culture was sub-cultured into 10 mL THY using a 1:100 dilution. This culture was grown to a concentration of 10^9 colony-forming units (CFU)/mL as determined by optical density. From this culture, 1 mL aliquots were centrifuged and re-suspended in 100 μL of THY. To ensure the correct CFU/mL concentration was achieved, the inoculum was quantified by culturing aliquots of dilutions on blood agar.

Mouse Strains and Corneal Infection Model

C57BL/6 mice as well as the isogenic TLR2 knockout B6.129-TLR2tm1Kir/J and TLR4 knockout B6.B10ScN-Tlr4lps-del/JthJ were obtained from The Jackson Laboratory (Bar Harbor, ME) and animal studies adhered to the tenets of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. A homozygote × homozygote breeding colony for both knockout systems was established according to the guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi Medical Center (UMMC). The corneal infection model was performed on 6-week-old mice of either sex as follows: each mouse was anesthetized by a subcutaneous injection of a ketamine hydrochloride and xylazine solution, which was determined to not cause any noticeable changes in ocular clarity or integrity in mice. The corneas of each mouse were scarified with a 31-gauge needle in a cross-hatch pattern and 10 μL of the bacterial inoculum (corresponding to 10^8 total CFU) was topically applied to the surface of each cornea. Each anesthetized mouse was allowed to rest for a minimum of 10 min in the prone position to ensure proper inoculation of the bacteria.

Determination of Bacterial Viability

To determine the effect of TLR signaling on bacterial viability during a pneumococcal keratitis infection, a bacterial viability assay was performed as previously described by Girgis et al.[27] Briefly whole mouse eyes were removed and homogenized at 24 and 72 h post-infection (p.i). Each eye was suspended and homogenized in 1 mL of sterile phosphate-buffered saline (PBS). This homogenate was serially diluted and cultured in triplicate on blood agar. Following a 24-h incubation at 37 °C with 5% CO_2 colonies were counted and averaged. These averages were then analyzed for statistical significance using a Student's *t*-test.

Determination of Gross Ocular Damage

At the appropriate 24-h time point, from Day 1 to Day 21 p.i., each mouse cornea was examined and scored according to a clinical score rubric described previously.[28] Briefly,

each mouse was anesthetized and its corneas were examined under 25 × magnification using a TOPCON slit-lamp biomicroscope (Tokyo, Japan). Each cornea was given a score from 0 to 4 based on cellular infiltrate, opacity, presence of corneal erosion, hemorrhage and corneal edema. A score of 0 was used to describe a clear cornea while a score of 4 was used to denote the maximum opacity, corneal hemorrhage or the presence of corneal erosion. These scores were averaged together for each infection group and analyzed for statistical significance using the Student's *t*-test.

Determination of Immune Response Modulation due to Loss of TLR Signaling

To determine the effect of TLR signaling on immune response during pneumococcal keratitis, mRNA concentrations of key innate immune response molecules were examined via quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR). To examine the downstream genes of the TLR signal cascade as well as related cytokines and immune regulators, TLR signaling RT^2 PCR arrays from SABiosciences (Valencia, CA) were used according to the manufacturer's instructions. These arrays examine 84 genes involved in TLR signaling and immune regulation.

At 24 and 72 h p.i. mice were anesthetized, sacrificed and their corneas were extracted. Each qRT-PCR sample was created by pooling four mouse corneas in order to obtain sufficient quantities of mRNA, as single corneas did not provide sufficient template for qRT-PCR. These corneas were suspended in 100 μL of RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) to stabilize their mRNA. Extracted corneas were immediately placed in liquid nitrogen for transport. Each pool of four corneas was homogenized and the mRNA was isolated using a protocol developed by Huang et al.[11] Briefly, an acidified chloroform RNA isolation was performed and the resulting mRNA was re-suspended in nuclease free H_2O. Following isolation, the mRNA samples were treated with DNase according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO) to eliminate the possibility of mouse genomic DNA (gDNA) contamination.

The gDNA-free mRNA was converted to cDNA using the SABiosciences (Valencia, CA) RT^2 first-strand cDNA Synthesis Kit per manufacturer instructions. Due to the relatively low concentration of mRNA isolated, each gDNA-free mRNA sample was normalized to 135.2 ng/μL before cDNA synthesis was performed.

Following cDNA synthesis the samples were loaded into the 96 wells of the qRT-PCR array. Each plate contained 84 TLR signaling genes and 12 controls including gDNA contamination, RT efficiency and mouse housekeeping controls. Real-time PCR of these arrays was performed with a Bio-Rad iCycler Machine (Hercules, CA) with a PCR protocol provided by SABiosciences (Valencia, CA). At least three plates from three separate infections and isolations were performed. Each gene's average C_t value was normalized by subtraction from the mean of the housekeeping gene GAPDH. Results were displayed as gene fold up or down regulation compared to controls. Fold change was calculated using the $\Delta\Delta C_t$ method. All C_t values >30 were amended as 30 for $\Delta\Delta C_t$ calculations. The controls used for infected C57BL/6 mouse corneas were uninfected, scratched corneas. Infected C57BL/6 corneas served as the controls for the $TLR2^{-/-}$ and $TLR4^{-/-}$ infected corneas.

Values of the normalized gene expression levels were analyzed using a mixed model in the analysis of variance.[29] In this model, the genetic strain of animal was considered a random effect and the time after infection (0, 24 and 72 h) was treated as a fixed effect. Estimation of model parameters was via restricted maximum likelihood.[30] Pairwise comparisons of mouse strain by time after infection interaction means were conducted using protected *t*-tests with alpha level adjustment done using a simulation method.[31] These mixed models were conducted separately on each gene. All data manipulation and analysis were carried out using procedures and programs in the Statistical Analysis System, (SAS Institute, Cary, NC).

Determination of Gross Clinical Damage, Immune Cell Recruitment and TLR Expression

At each time point, and for each variable, representative corneas were extracted for histologic sectioning, which was done by Excalibur Pathology (Moore, OK). Each sample was stained with rabbit polyclonal antibodies to TLR2 (ab24192; Abcam, Cambridge, MA) and TLR4 (M-300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to determine TLR expression in the tissues of the cornea. Hematoxylin–eosin (H/E) stain was used to quantify neutrophils by limbus-to-limbus counts. These neutrophil populations were then verified using an anti-neutrophil elastase rabbit polyclonal antibody (ab21595; Abcam, Cambridge, MA). Optimization of histologic staining was performed by Excalibur Pathology (Moore, OK). The dilutions of the primary antibodies were as follows: anti-TLR2 1:200, anti-TLR4 1:400, anti-neutrophil elastase 1:100 and rabbit IgG 1:100. All rabbit polyclonal antibodies used in this study were cross-reactive with mouse proteins. Isotype controls were done to ensure the absence of non-specific background staining using polyclonal antibody to rabbit IgG (ab-105-c; Abcam, Cambridge, MA). Neutrophils were counted limbus to limbus from three histology cross sections, averaged and analyzed by the Student's *t*-test.

Cytometric Bead Array Analysis of Cytokine Concentrations

A cytometric bead array (CBA) was performed utilizing a kit available from BD Biosciences (San Jose, CA) according to manufacturer's instructions. Following topical scratch inoculation, C57BL/6 corneas were harvested at 24 and 72 h p.i. and homogenized in 1 mL PBS. Fifty microliters of this homogenate was incubated with beads conjugated with IFN-γ, IL-2, IL-6, and IL-10 and phycoerythrin-conjugated secondary antibodies provided by BD Biosciences (San Jose, CA). Protein concentrations were determined using a Gallios™ Flow Cytometer (Beckman Coulter, Inc., Brea, CA) according to manufaturer's instructions. A positive protein curve of phycoerythrin fluorescence using 10 dilutions ranging from 2500 to 10 pg/mL was compared to samples and protein concentrations were calculated and analyzed for significance by the Student's *t*-test.

RESULTS

TLR2 and TLR4 Expression in C57BL/6 Mice

The presence of TLR2 and TLR4 in infected C57BL/6 corneas was determined by histology. Infected and scratched C57BL/6 corneas harvested at 24 and 72 h p.i. were stained with anti-TLR2 (Figure 1A and C) and anti-TLR4 (Figure 1B and D) antibodies. Both TLR2 and TLR4 were detected at the 24 (Figure 1A and B) and 72-h (Figure 1C and D) time points.

TLR expression appeared to be increased in infected corneal sections compared to scratch controls, indicating an increase in expression following infection.

Pneumococcal Keratitis Pathogenesis in C57BL/6 Mice

Clinical Severity—C57BL/6 mice were monitored for corneal damage over the course of 21 days (Figure 2). Moderate levels of damage were detected at 24 h p.i. (mean slit lamp examination [SLE] = 1.82, $n = 63$ corneas) which steadily increased to the maximum score observed at Day 4 p.i. (mean SLE = 2.63, $n = 32$ corneas) and then decreased over the course of 3 weeks (Day 7: mean SLE = 1.54, $n = 32$ corneas; Day 14 SLE = 1.32, $n = 17$ corneas). After 21 days corneal damage and scarring was still present (Day 21: mean SLE = 1.26, $n = 17$ corneas).

CFU Recovery—Viable bacteria were observed at 24 h p.i. in the whole eye homogenates of C57BL/6 mice [1.28 ± 0.65 $\log_{10}$CFU/mL ± standard error of mean (SEM), $n = 8$ eyes]. Viable bacteria were not detected at 72 h p.i. ($n = 8$ eyes).

Neutrophil Recruitment—Neutrophil invasion of the C57BL/6 corneas was quantified at 24 and 72 h p.i. (Figure 3). Neutrophils were quantified by counting observed neutrophils from limbus to limbus of histologic sections using H/E (not shown) and an anti-neutrophil elastase antibody (see representative pictures in Figure 4A). Significantly higher amounts of neutrophils were detected in infected C57BL/6 corneas (mean = 14.5 ± 1.0, $n = 3$ cornea sections) compared to scratch control C57BL/6 corneas at 24 (mean = 2.33 ± 0.88, $n = 3$ cornea sections, $p < 0.001$) and 72 h p.i. (mean = 0.667 ± 0.33, $n = 3$ cornea sections, $p < 0.001$).

C57BL/6 Immune and TLR Signaling Gene Expression—Immune signaling genes were upregulated in infected C57BL/6 corneas compared to C57BL/6 scratch control corneas at both 24 and 72 h p.i. (Figure 5). At 24 h p.i., granulocyte maturation cytokines CSF2 and CSF3 were higher (≥6.54-fold) in infected C57BL/6 mice compared to scratch controls (Figure 5). The chemokine CXCL10 was also upregulated (88.05-fold) as well as cytokines IL-2 and IL-6 (6.11- and 8.52-fold, respectively). TLR signaling pathway genes were also upregulated (myeloid differentiation primary response gene 88 [MyD88] = 20.36-fold, Toll-interleukin 1 receptor-domain-containing adapter-inducing interferon-beta [TRIF] = 4.08-fold, TRIF-related adapter molecule [TRAM] = 4.34-fold).

The same upregulation trend continued at the 72-h time point (Figure 5). CXCL10 expression remained high at 83.68-fold, as did MyD88 which dropped at the 24-h time point to 16.12-fold. While there was no measurable increase in either TLR2 or TLR4 at the 24-h time point, at 72 h p.i., expression of both increased >2-fold (TLR2 = +8.26-fold) and (TLR4 = +3.50-fold). While these genes exhibited an upregulation trend at both 24 and 72 h p.i., they were not found to be statistically significant compared to the scratch controls.

Pneumococcal Keratitis Pathogenesis in TLR2$^{-/-}$ BL6 Mice

Clinical Severity—The mean clinical scores of infected TLR2$^{-/-}$ mouse corneas over the course of 21 days were not significantly different than those of the C57BL/6 control corneas

($p > 0.05$, $n \geq 8$ corneas per time point; Figure 2). However, the clinical scores for five points (Days 2, 6, 17, 18 and 21 p.i.) were significantly higher in TLR2$^{-/-}$ mouse corneas ($p \leq 0.003$, $n \geq 8$ corneas per time point; Figure 2). Despite these time points (Days 2, 6, 17, 18 and 21), the progression of keratitis in the TLR2$^{-/-}$ mice was similar to the C57BL/6 mice.

CFU Recovery—At 24 h p.i. significantly higher numbers of CFUs were recovered from the corneas of TLR2$^{-/-}$ mice (3.78 ± 0.38 $\log_{10}$CFU/mL) as compared to C57BL/6 mice (1.28 ± 0.65 mean $\log_{10}$ CFU/mL ± SEM, $p = 0.005$; $n = 8$ eyes per group). As previously determined for the C57BL/6 whole eye homogenates, the homogenates of TLR2$^{-/-}$ mice were sterile at 72 h p.i.

Neutrophil Recruitment—At 24 and 72 h p.i. limbus-to-limbus counts of corneal neutrophils of *S. pneumoniae* infected TLR2$^{-/-}$ mice were compared to infected C57BL/6 controls (counts, Figure 3; anti-neutrophil elastase stained, Figure 4B). Significantly lower counts of neutrophils were observed in TLR2$^{-/-}$ corneas ($p < 0.05$, mean = 18, $n = 3$ cornea sections) compared to C57BL/6 corneas at 24 h p.i. (mean = 43, $n = 3$ cornea sections). This trend was also observed at 72 h p.i. (TLR2$^{-/-}$: mean = 1.66, $n = 3$ cornea sections; C57BL/6: mean = 14.5, $n = 3$ cornea sections; $p < 0.05$).

TLR2$^{-/-}$ Immune and TLR Signaling Gene Expression—A decrease in inflammatory cytokines was observed in TLR2$^{-/-}$ corneas as compared to C57BL/6 controls at 24 h p.i. (Figure 6). IL-2 was shown to be significantly decreased in TLR2$^{-/-}$ infected mice at 24 h compared to C57BL/6 mice ($p < 0.0075$). TLR signaling genes were also downregulated (MyD88 = −16.19-fold, TRIF = −18.42, TRAM = −20.73) though this difference was not significant. At 72 h p.i., TLR signaling genes such as MyD88 and TRAM became upregulated at least 2-fold (Figure 6) though not significantly.

Pneumococcal Keratitis Pathogenesis in TLR4$^{-/-}$ BL6 Mice

Clinical Severity—Over the course of 21 days TLR4$^{-/-}$ mice were monitored for corneal damage (Figure 2). At 24 h p.i., a significantly lower SLE score was observed in TLR4$^{-/-}$ corneas compared to C57BL/6 corneas (TLR4$^{-/-}$: mean SLE = 1.02 ± 0.26, $n = 22$ corneas versus C57BL/6: mean SLE = 1.82 ± 0.17, $n = 63$ corneas; $p = 0.02$). However, by 48 h, the amount of corneal damage significantly increased to a level higher than the observed maximum score of C57BL/6 mice (48 h p.i. TLR4$^{-/-}$: mean SLE = 3.71 ± 0.15, $n = 14$ corneas versus C57BL/6: mean SLE = 2.17 ± 0.23, $n = 43$; $p = 0.003$). Over 21 days, a significantly higher SLE score was observed in TLR4$^{-/-}$ corneas compared to C57BL/6 controls at all time points except Day 4 ($p < 0.04$; $n = 12$ per time point; Figure 2). From Day 2 to Day 21 p.i., the SLE scores observed in TLR4$^{-/-}$ mice exceeded the maximum SLE score determined for C57BL/6 corneas.

CFU Recovery—In contrast to TLR2$^{-/-}$ whole eye homogenates, no significant difference in bacterial load was recovered from the whole eyes of TLR4$^{-/-}$ mice compared to wild-type mice at 24 h p.i. (1.45 0.71 $\log_{10}$CFU/mL±SEM; $n = 8$ eyes per group). As with the TLR2$^{-/-}$ and C57BL/6 mice, all TLR4$^{-/-}$ homogenates were sterile at 72 h p.i.

Neutrophil Recruitment—The limbus-to-limbus neutrophil counts observed in *S. pneumoniae* infected TLR4$^{-/-}$ mouse corneas at 24 h p.i. were significantly higher than counts observed in C57BL/6 corneas (counts, Figure 3; anti-neutrophil elastase stained, Figure 4C), with TLR4$^{-/-}$ doubling C57BL/6 counts (TLR4$^{-/-}$: mean = 86, $n = 3$ cornea sections, $p = 0.001$). At 72 h p.i., the neutrophil counts in TLR4$^{-/-}$ corneas dropped to a level significantly lower than C57BL/6 corneas (TLR4$^{-/-}$: mean = 4.66, $n = 3$ cornea sections, $p = 0.002$). However, these counts were significantly higher than counts observed in uninfected C57BL/6 scratch controls at 72 h p.i. (data not shown, $p = 0.013$).

TLR4$^{-/-}$ Immune and TLR Signaling Gene Expression—A decrease in expression of cytokines and immune signaling genes was observed in TLR4$^{-/-}$ corneas at 24 h p.i. (Figure 7). IFN-γ was significantly decreased in TLR4$^{-/-}$ mice at 24 h p.i. compared to C57BL/6 mice ($p < 0.0394$) while cytokines IFN-β1, IL-2, CSF2, CSF3 and CXCL10 were also downregulated. The expression of TLR signaling molecules including MyD88, TRIF and TRAM were similarly decreased (MyD88 = −8.49-fold, TRIF = −8.96-fold, TRAM = −8.44-fold). At 72 h, the TLR4$^{-/-}$ corneas were similar to C57BL/6 controls except in the case of CSF3 (+15.43-fold change) and TIRAP (+2.80-fold change) (Figure 7), though these differences were not found to be statistically significant.

CBA Analysis of Protein Concentrations—To determine the protein levels of the significantly downregulated cytokine genes (IFN-γ and IL-2) and compare the Th1 and Th2 response, a CBA was performed. IFN-γ and IL-2, representing the Th1 response, were detected in K1263 infected C57BL/6 corneas at 24 and 72 h p.i. (Figure 8A and B) though their concentration was low (<5 pg/mL). In contrast, Th2 response associated genes, IL-6 and IL-10, were detected at higher concentrations, especially IL-10 at both time points. Though higher concentrations of IL-6 and IL-10 were detected in the infected corneas these differences were not significant.

DISCUSSION

The current treatment for bacterial keratitis is typically limited to topical antibiotics. However, this study showed that pneumococcal pathogenesis during keratitis involves more than the bacterial load. The bacterial load decreased to ~1 $\log_{10}$CFU per cornea from an initial inoculum of 8 $\log_{10}$ CFU by 24 h in wild-type mice. Though corneas were sterile at 72 h p.i., corneal damage and opacity persisted until the mice were euthanized at 21 days p.i.

The importance of TLR surveillance has been documented in several ocular surface diseases,[32] including viral,[33] fungal[34] and bacterial keratitis.[10,11] Despite these and other studies, the specifics of TLR surface expression in corneal epithelial cells is unclear. Song et al.[35] first detected surface expressed TLR2 and TLR4 in primary and immortalized human corneal epithelial cells (HCECs). Ueta et al.[36] followed this study with evidence that TLR2 and TLR4 were not surface associated in HCECs but instead were found intracellularly and inactive. Indeed our own preliminary work with an immortalized HCEC line found it to express TLR2 but not TLR4 (unpublished data). These studies highlight the need for caution when working with immortalized cell lines. In light of these studies, we elected to detect TLR expression in the murine cornea with immunohistochemistry. TLR2 and TLR4 were

both detected in infected and uninfected scratched C57BL/6 corneas (Figure 1). This present study follows a study by Huang et al.[11] which demonstrated that loss of TLR4 signaling in BALB/c mice increased polymorphonuclear cells (PMN) infiltration and decreased pro-inflammatory cytokines during *Pseudomonas* keratitis. Although the TLR4 ligands for *P. aeruginosa* and *S. pneumoniae* are different (LPS versus pneumolysin), deletion of the TLR4-mediated response appears to result in similar consequences for these two species.

In the current study, *S. pneumoniae* causes an upregulation of TLR2 and TLR4 expression in the cornea after 72 h p.i., and both of these TLRs play important but different roles in the host innate immune response.[11,21,37–40] Loss of TLR2 resulted in significantly higher bacterial recovery but did not affect overall clinical severity as measured by SLE score, compared to wild-type mice. In contrast, loss of TLR4 resulted in no change in bacterial recovery but significantly increased clinical scores. TLR signaling also impacted neutrophil invasion as evidenced by the loss of TLR2 causing significantly less neutrophil involvement, whereas the absence of TLR4 caused an increase in initial neutrophil levels during pneumococcal infection.

Despite an initial elevated population of neutrophils in the corneas of infected $TLR4^{-/-}$ mice (at 24 h), lower neutrophil counts were observed at 72 h p.i., the time at which the clinical scores peaked. These data could indicate two possibilities. The first is that the initial surge of neutrophils in $TLR4^{-/-}$ corneas leads to the increased damage in these corneas at a later time point (72 h), and that damage is not repaired despite the clearing of neutrophils. This scenario suggests a role for TLR4 in controlling neutrophil recruitment during pneumococcal keratitis. The finding that bacterial recovery in $TLR4^{-/-}$ corneas was not different from that of wild-type corneas also suggests that bacterial load may not always necessarily be a factor in neutrophil recruitment. The second possibility is that neutrophil presence may not be related to the damage in the $TLR4^{-/-}$ corneas, indicating a possible role for other as-yet-unidentified factors that are influenced by TLR4 signaling. Since few neutrophils were quantified in $TLR4^{-/-}$ corneas at 72 h p.i., perhaps the TLR4 signaling cascade has an effect on corneal integrity that does not involve leukocytes.

The role of TLR2 in pneumococcal keratitis appears to involve bacterial clearance mediated by neutrophils. Significantly higher bacterial loads, and significantly lower numbers of neutrophils, were quantified in the corneas of $TLR2^{-/-}$ mice at 24 h. These findings indicate that TLR2 could be necessary for neutrophil recruitment and phagocytosis of the bacteria.

An increase was observed in the mRNA expression of at least 12 cytokines and TLR signaling genes in C57BL/6 mice at 24 h p.i. These genes included neutrophil growth and maturation genes CSF2 and CSF3, intracellular pathogen response genes IFN-β1, IFN-γ and lymphotoxin A (LTA) and TLR signaling genes MyD88, TRIF and TRAM. The increase in interferons and LTA suggest the possibility that *S. pneumoniae* may be invading corneal epithelial cells. These data suggest a possible role for TLR related genes and cytokines during pneumococcal keratitis. Whereas TLR2 and TLR4 genes were not upregulated at 24 h p.i., they were upregulated at 72 h p.i. during the height of clinical damage. This delay is similar to data from Jin et al.[41] who detected a delayed rise of innate genes in patients with *Pseudomonas* keratitis 12.5 days p.i.

Reduced mRNA concentrations of several innate immunity genes were observed for both TLR2$^{-/-}$ and TLR4$^{-/-}$ mice early (24 h) in pneumococcal keratitis, though this reduction was more severe in TLR2$^{-/-}$ corneas than TLR4$^{-/-}$ corneas. An interesting observation to note is the overall "recovery" of gene expression in both TLR2$^{-/-}$ and TLR4$^{-/-}$ corneas from the 24-h time point to the 72-h time point compared to wild-type corneas (Figures 6 and 7). This return to expression levels that more closely resemble or surpass wild-type levels indicates that a redundancy of function may be present in the host corneal immune surveillance system, that is, other unidentified immune factors may be compensating for the loss of TLR2 or TLR4. Nucleotide-binding oligomerization domain (Nod) receptors, also known as Nod-like receptors (NLRs), are a class of intracellular PRRs. NLRs Nod1 and Nod2 detect bacterial cell wall components such as meso-diaminopimelic acid[42] and muramyl dipeptide,[43] respectively. Activation of Nod1/2 induces chemokine production and neutrophil invasion in a mouse model.[44] Interestingly some PAMPs, such as flagellin, can be recognized by both NLRs and TLRs.[45,46] Of particular interest, Nod1/2 have been documented in the cornea[47] and have been implicated in pneumococcal disease and bacterial keratitis. NOD1$^{-/-}$ mice were shown to be more susceptible to *S. pneumoniae* sepsis.[48] Additionally, a recent study demonstrated that *S. aureus*-derived cell wall component muramyl dipeptide activated Nod1 in limbal fibroblasts which induced vascular endothelial growth factor-A expression.[49] The relationship of *S. pneumoniae* and other PRRs such as NLRs in the cornea has not been examined and may be acting as compensating factors in the absence of TLR2 or TLR4.

Also of interest is the increased expression of intracellular pathogen immune response genes. IFN-γ, IFNβ−1 and LTA mRNA levels were higher with infection than scratch controls in the C57BL/6 mice at 24 h p.i. and downregulated in both TLR deficient mutants. This trend could indicate invasion of corneal epithelial cells during pneumococcal keratitis.

To verify the qRT-PCR data, we attempted to correlate the mRNA expression levels of IFN-γ, IL-2, IL-6 and IL-10 with their protein concentrations. IFN-γ mRNA was significantly downregulated in TLR4$^{-/-}$ mice while IL-2 was significantly downregulated in the TLR2$^{-/-}$ infected mice compared to C57BL/6 mice (Figures 6 and 7). IFN-γ, IL-6 and IL-10 protein concentrations were all higher in infected C57BL/6 mice compared to scratch controls at 24 h p.i. (Figure 8) though this increase was not significant. The difficulty in correlating mRNA levels with protein concentrations has been well documented.[50] Recent large scale studies examining bacterial proteomes have also verified the difficulty.[51,52] While the mRNA expression levels presented in this study cannot be definitively linked to increased protein concentrations, the genes implicated by the qRT-PCR data offer new leads for the elucidation of TLR mediated innate response to *S. pneumoniae* keratitis. The trends (>2-fold) observed in gene expression in infected C57BL/6 corneas (Figure 5) prompted an analysis of the roles of TLR2 and TLR4 *in vivo*. Increases in expression of other TLRs were also detected (not shown), but we chose to focus on TLR2 and TLR4 for this study based on previous findings documenting the importance of these TLRs in bacterial and fungal keratitis[53–56] and invasive pneumococcal diseases.[37,38,40] The regulation trends in infected wild-type corneas were verified by the downregulation of the same genes in the TLR-negative mice at 24 h p.i., which accompanied lower clinical scores in these knockout mice at 24 h.

The overall findings of this study clarify the role of the host innate immune system in pneumococcal keratitis. To date, the only knowledge regarding host inflammation in pneumococcal ocular disease was that neutrophils contributed to the damage and opacity in the cornea.[57–59] The current study identified TLR-related genes that were upregulated during pneumococcal keratitis and a possible redundancy of function among the TLRs. Moreover, these results indicate that other factors in addition to neutrophils are involved in the host response to this disease as evidenced by the irreversible damage in TLR4$^{-/-}$ corneas later in the time course despite lack of neutrophils. Analysis of both unique functions of each TLR as well as their redundant functions in the absence of a fully intact immune system will help identify possible targets for immune modulation during this disease.

DECLARATION OF INTEREST

This work was supported by Public Health Services Grant (R01EY016195 to M.E.M.), National Eye Institute, National Institutes of Health, Bethesda, MD. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES

1. Holden BA, Sweeney DF, Sankaridurg PR, Carnt N, Edwards K, Stretton S, et al. Microbial keratitis and vision loss with contact lenses. Eye Contact Lens 2003;29: S131–S134. [PubMed: 12772749]
2. Bharathi MJ, Ramakrishnan R, Vasu S, Meenakshi R, Shivkumar C, Palaniappan R. Epidemiology of bacterial keratitis in a referral centre in south India. Indian J Med Microbiol 2003;21:239–245. [PubMed: 17643035]
3. Ormerod LD. Causation and management of microbial keratitis in subtropical Africa. Ophthalmology 1987;94: 1662–1668. [PubMed: 3501559]
4. Bharathi MJ, Ramakrishnan R, Shivakumar C, Meenakshi R, Lionalraj D. Etiology and antibacterial susceptibility pattern of community-acquired bacterial ocular infections in a tertiary eye care hospital in south India. Indian J Ophthalmol 2010;58:497–507. [PubMed: 20952834]
5. Green M, Apel A, Stapleton F. A longitudinal study of trends in keratitis in Australia. Cornea 2008;27:33–39. [PubMed: 18245964]
6. Cheung J, Slomovic AR. Microbial etiology and predisposing factors among patients hospitalized for corneal ulceration. Can J Ophthalmol 1995;30:251–255. [PubMed: 8529159]
7. Asbell P, Stenson S. Ulcerative keratitis: survey of 30 years' laboratory experience. Arch Ophthalmol 1982;100: 77–80. [PubMed: 6173033]
8. Varaprasathan G, Miller K, Lietman T, Whitcher JP, Cevallos V, Okumoto M, et al. Trends in the etiology of infectious corneal ulcers at the F. I. Proctor Foundation. Cornea 2004;23:360–364. [PubMed: 15097130]
9. Dethorey G, Daruich A, Hay A, Renard G, Bourges JL. Severe bacterial keratitis referred to ophthalmology emergency departments: a retrospective study of 268 cases. J Fr Ophtalmol 2013;36:129–137. [PubMed: 23017327]
10. Huang X, Barrett RP, McClellan SA, Hazlett LD. Silencing Toll-like receptor-9 in *Pseudomonas aeruginosa* keratitis. Invest Ophthalmol Vis Sci 2005;46:4209–4216. [PubMed: 16249500]
11. Huang X, Du W, McClellan SA, Barrett RP, Hazlett LD. TLR4 is required for host resistance in *Pseudomonas aeruginosa* keratitis. Invest Ophthalmol Vis Sci 2006;47: 4910–4916. [PubMed: 17065506]
12. Kumar A, Yu FS. Toll-like receptors and corneal innate immunity. Curr Mol Med 2006;6:327–337. [PubMed: 16712478]
13. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. J Immunol 2006;177: 3515–3519. [PubMed: 16951309]

14. Mullaly SC, Kubes P. The role of TLR2 *in vivo* following challenge with *Staphylococcus aureus* and prototypic ligands. J Immunol 2006;177:8154–8163. [PubMed: 17114491]
15. Means TK, Jones BW, Schromm AB, Shurtleff BA, Smith JA, Keane J, et al. Differential effects of a Toll-like receptor antagonist on *Mycobacterium tuberculosis*-induced macrophage responses. J Immunol 2001;166:4074–4082. [PubMed: 11238656]
16. Frasnelli ME, Tarussio D, Chobaz-Peclat V, Busso N, So A. TLR2 modulates inflammation in zymosan-induced arthritis in mice. Arthritis Res Ther 2005;7:R370–R379. [PubMed: 15743485]
17. Aravalli RN, Hu S, Rowen TN, Palmquist JM, Lokensgard JR. Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus. J Immunol 2005;175: 4189–4193. [PubMed: 16177057]
18. Wang JP, Kurt-Jones EA, Shin OS, Manchak MD, Levin MJ, Finberg RW. Varicella-zoster virus activates inflammatory cytokines in human monocytes and macrophages via Toll-like receptor 2. J Virol 2005;79:12658–12666. [PubMed: 16188968]
19. Compton T, Kurt-Jones EA, Boehme KW, Belko J, Latz E, Golenbock DT, et al. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. J Virol 2003;77:4588–4596. [PubMed: 12663765]
20. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc Natl Acad Sci USA 2000;97:13766–13771. [PubMed: 11095740]
21. Beutler B Tlr4: central component of the sole mammalian LPS sensor. Curr Opin Immunol 2000;12:20–26. [PubMed: 10679411]
22. Yang H, Young DW, Gusovsky F, Chow JC. Cellular events mediated by lipopolysaccharide-stimulated Toll-like receptor 4. MD-2 is required for activation of mitogen-activated protein kinases and Elk-1. J Biol Chem 2000;275: 20861–20866. [PubMed: 10877845]
23. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, et al. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci USA 2003;100:1966–1971. [PubMed: 12569171]
24. Srivastava A, Henneke P, Visintin A, Morse SC, Martin V, Watkins C, et al. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. Infect Immun 2005;73:6479–6487. [PubMed: 16177320]
25. Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, et al. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun 2004;72:788–794. [PubMed: 14742522]
26. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999;163: 1–5. [PubMed: 10384090]
27. Girgis DO, Sloop GD, Reed JM, O'Callaghan RJ. Susceptibility of aged mice to *Staphylococcus aureus* keratitis. Curr Eye Res 2004;29:269–275. [PubMed: 15590472]
28. Moore III QC, McCormick CC, Norcross EW, Onwubiko C, Sanders ME, Fratkin J, et al. Development of a *Streptococcus pneumoniae* keratitis model in mice. Ophthalmic Res 2009; 42:141–146. [PubMed: 19628954]
29. McCulloch CE, Searle SR, Neuhaus JM. Generalized, linear, and mixed models. 2nd ed. Hoboken (NJ): Wiley; 2008.
30. Swallow WH, Monahan JF. Monte Carlo comparison of ANOVA, MIVQUE, REML, and ML estimators of variance components. Technometrics. 1984;26:47–57.
31. Edwards D, Berry JJ. The efficiency of simulation-based multiple comparisons. Biometrics 1987;43:913–928. [PubMed: 3427176]
32. Redfern RL, McDermott AM. Toll-like receptors in ocular surface disease. Exp Eye Res 2010;90:679–687. [PubMed: 20346359]
33. Sarangi PP, Kim B, Kurt-Jones E, Rouse BT. Innate recognition network driving herpes simplex virus-induced corneal immunopathology: role of the toll pathway in early inflammatory events in stromal keratitis. J Virol 2007;81: 11128–11138. [PubMed: 17686871]

34. Sun Y, Chandra J, Mukherjee P, Szczotka-Flynn L, Ghannoum MA, Pearlman E. A murine model of contact lens-associated *Fusarium* keratitis. Invest Ophthalmol Vis Sci 2010;51:1511–1516. [PubMed: 19875664]
35. Song PI, Abraham TA, Park Y, Zivony AS, Harten B, Edelhauser HF, et al. The expression of functional LPS receptor proteins CD14 and Toll-like receptor 4 in human corneal cells. Invest Ophthalmol Vis Sci 2001;42:2867–2877. [PubMed: 11687531]
36. Ueta M, Nochi T, Jang MH, Park EJ, Igarashi O, Hino A, et al. Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium. J Immunol 2004;173:3337–3347. [PubMed: 15322197]
37. Dessing MC, Florquin S, Paton JC, Van Der Poll T. Toll-like receptor 2 contributes to antibacterial defence against pneumolysin-deficient pneumococci. Cell Microbiol 2008; 10:237–246. [PubMed: 17711480]
38. Echchannaoui H, Frei K, Schnell C, Leib SL, Zimmerli W, Landmann R. Toll-like receptor 2 deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis 2002;186:798–806. [PubMed: 12198614]
39. Han F, Yu H, Tian C, Li S, Jacobs MR, Benedict-Alderfer C, et al. Role for Toll-like receptor 2 in the immune response to *Streptococcus pneumoniae* infection in mouse otitis media. Infect Immun 2009;77:3100–3108. [PubMed: 19414550]
40. Klein M, Obermaier B, Angele B, Pfister HW, Wagner H, Koedel U, et al. Innate immunity to pneumococcal infection of the central nervous system depends on Toll-like receptor (TLR) 2 and TLR4. J Infect Dis 2008;198:1028–1036. [PubMed: 18700834]
41. Jin X, Lin Z, Xie X. The delayed response of Toll-like receptors may relate to *Pseudomonas aeruginosa* keratitis exacerbating rapidly at the early stages of infection. Eur J Clin Microbiol Infect Dis 2010;29:231–238. [PubMed: 20012880]
42. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, et al. An essential role for Nod1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol 2003;4:702–707. [PubMed: 12796777]
43. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869–8872. [PubMed: 12527755]
44. Masumoto J, Yang K, Varambally S, Hasegawa M, Tomlins SA, Qiu S, et al. Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment *in vivo*. J Exp Med 2006;203:203–213. [PubMed: 16418393]
45. Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozoren N, Jagirdar R, et al. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in *Salmonella*-infected macrophages. Nat Immunol 2006;7: 576–582. [PubMed: 16648852]
46. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410:1099–1103. [PubMed: 11323673]
47. Benko S, Tozser J, Miklossy G, Varga A, Kadas J, Csutak A, et al. Constitutive and UV-B modulated transcription of Nod-like receptors and their functional partners in human corneal epithelial cells. Mol Vis 2008;14:1575–1583. [PubMed: 18769647]
48. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med 2010; 16:228–231. [PubMed: 20081863]
49. Juarez-Verdayes MA, Rodriguez-Martinez S, Cancino-Diaz ME, Cancino-Diaz JC. Peptidoglycan and muramyl dipeptide from *Staphylococcus aureus* induce the expression of VEGF-A in human limbal fibroblasts with the participation of TLR2-NFkappaB and NOD2-EGFR. Graefes Arch Clin Exp Ophthalmol 2013;251:53–62. [PubMed: 22890731]
50. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 2012;13:227–232. [PubMed: 22411467]

51. Jayapal KP, Philp RJ, Kok YJ, Yap MG, Sherman DH, Griffin TJ, et al. Uncovering genes with divergent mRNA-protein dynamics in *Streptomyces coelicolor*. PLoS One 2008; 3:e2097. [PubMed: 18461186]
52. Maier T, Schmidt A, Guell M, Kuhner S, Gavin AC, Aebersold R, et al. Quantification of mRNA and protein and integration with protein turnover in a bacterium. Mol Syst Biol 2011;7:511. [PubMed: 21772259]
53. Kumar A, Zhang J, Yu FS. Innate immune response of corneal epithelial cells to *Staphylococcus aureus* infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. Invest Ophthalmol Vis Sci 2004;45: 3513–3522. [PubMed: 15452057]
54. Chinnery HR, Carlson EC, Sun Y, Lin M, Burnett SH, Perez VL, et al. Bone marrow chimeras and c-fms conditional ablation (Mafia) mice reveal an essential role for resident myeloid cells in lipopolysaccharide/TLR4-induced corneal inflammation. J Immunol 2009;182:2738–2744. [PubMed: 19234168]
55. Karthikeyan RS, Leal SM Jr, Prajna NV, Dharmalingam K, Geiser DM, Pearlman E, et al. Expression of innate and adaptive immune mediators in human corneal tissue infected with *Aspergillus* or *Fusarium*. J Infect Dis 2011; 204:942–950. [PubMed: 21828275]
56. Leal SM Jr, Cowden S, Hsia YC, Ghannoum MA, Momany M, Pearlman E. Distinct roles for Dectin-1 and TLR4 in the pathogenesis of *Aspergillus fumigatus* keratitis. PLoS Pathog 2010;6:e1000976. [PubMed: 20617171]
57. Harrison JC, Karcioglu ZA, Johnson MK. Response of leukopenic rabbits to pneumococcal toxin. Curr Eye Res 1982;2:705–710. [PubMed: 7186437]
58. Norcross EW, Sanders ME, Moore III QC, Marquart ME. Pathogenesis of a clinical ocular strain of *Streptococcus pneumoniae* and the interaction of pneumolysin with corneal cells. J Bacteriol Parasitol 2011;2:108. [PubMed: 22229113]
59. Norcross EW, Sanders ME, Moore III QC, Taylor SD, Tullos NA, Caston RR, et al. Active immunization with pneumolysin versus 23-valent polysaccharide vaccine for *Streptococcus pneumoniae* keratitis. Invest Ophthalmol Vis Sci 2011;52:9232–9243. [PubMed: 22039231]

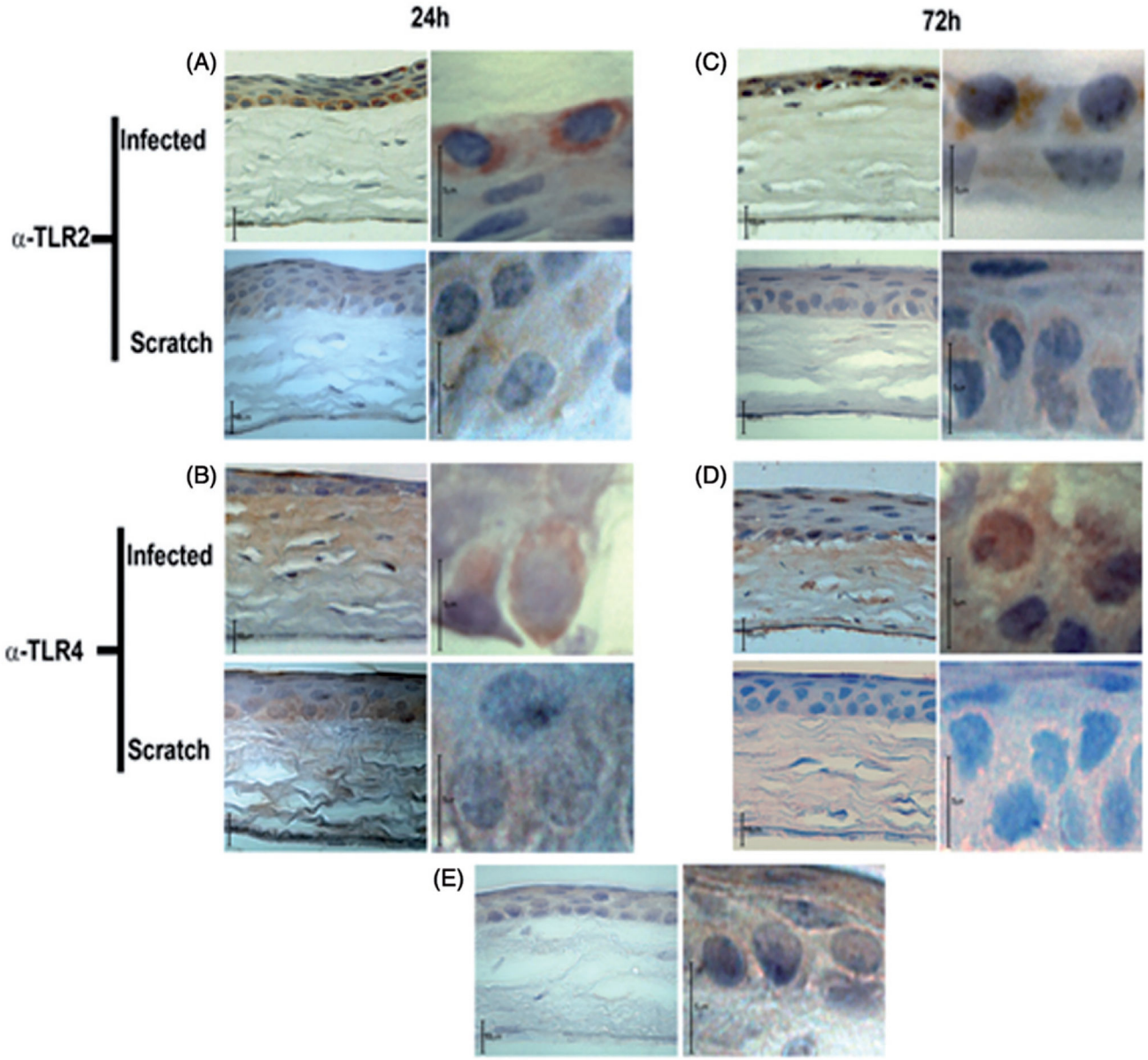

FIGURE 1.

TLR expression in infected C57BL/6 corneas. Infected and scratched C57BL/6 corneas were harvested at 24 h p.i. (A and B) and 72 h p.i. (C and D). Harvested corneas were stained with anti-TLR2 (A and C) or anti-TLR4 (B and D) antibodies. Representative corneal sections (left panel) and individual epithelial cells (right panel) for infected and scratched corneas stained with either anti-TLR2 or anti-TLR4 are provided. Expression of both TLRs was detected at both 24 and 72 h p.i. The anti-TLR antibodies stained more strongly in infected corneas than in scratched corneas at both time points (compare top panel pair to bottom panel pair in each group). The antibody isotype control corneal section and individual epithelial cells shows diffuse, non-specific staining (E). Scale bar for corneal sections represents 10 μm and for individual epithelial cells represents 5 μm.

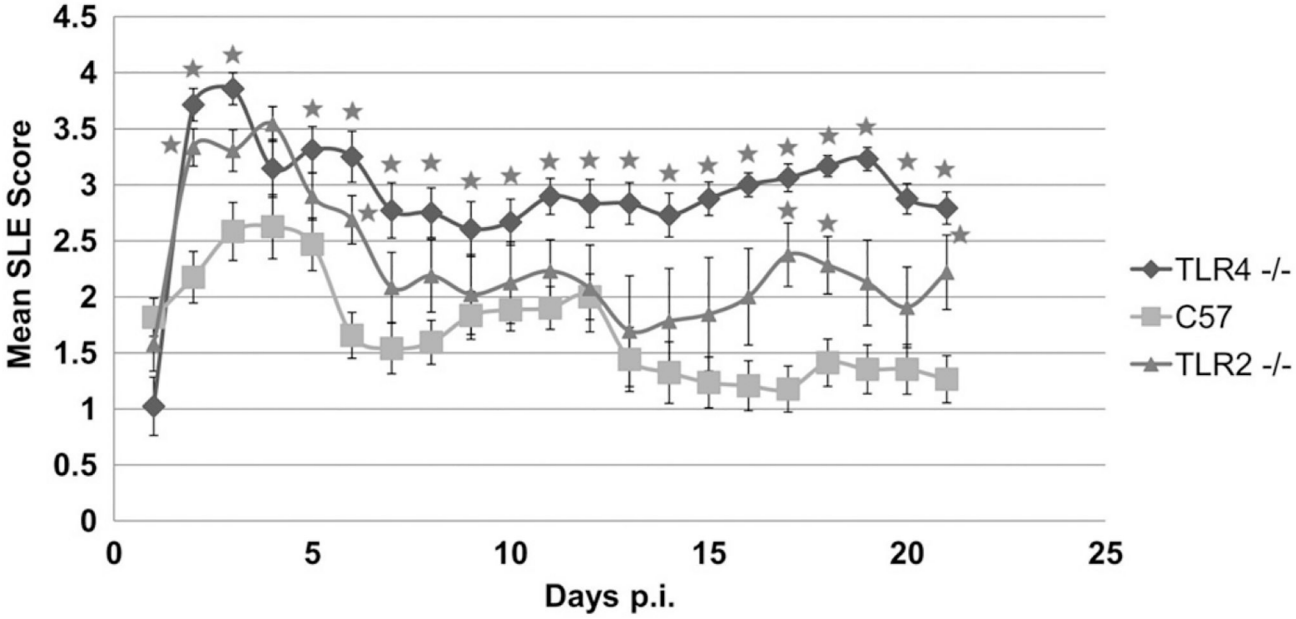

FIGURE 2.

Mean clinical scores. C57BL/6, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were inoculated with 10^8 total CFU of pneumococci and gross corneal damage was measured using the clinical score rubric from Day 1 to Day 21 p.i. TLR2-deficient mutants showed decreased damage at 24 h and increased damage at 48, 72 and 96 h p.i. compared to C57BL/6 mice. Damage observed in TLR2-deficient mice was similar to C57BL/6 mice over the 21-day period. TLR4-deficient mutants showed increased damage compared to C57BL/6 mice over the 21-day period. Error bars represent SEM at each time point. Stars indicate significance ($p < 0.05$).

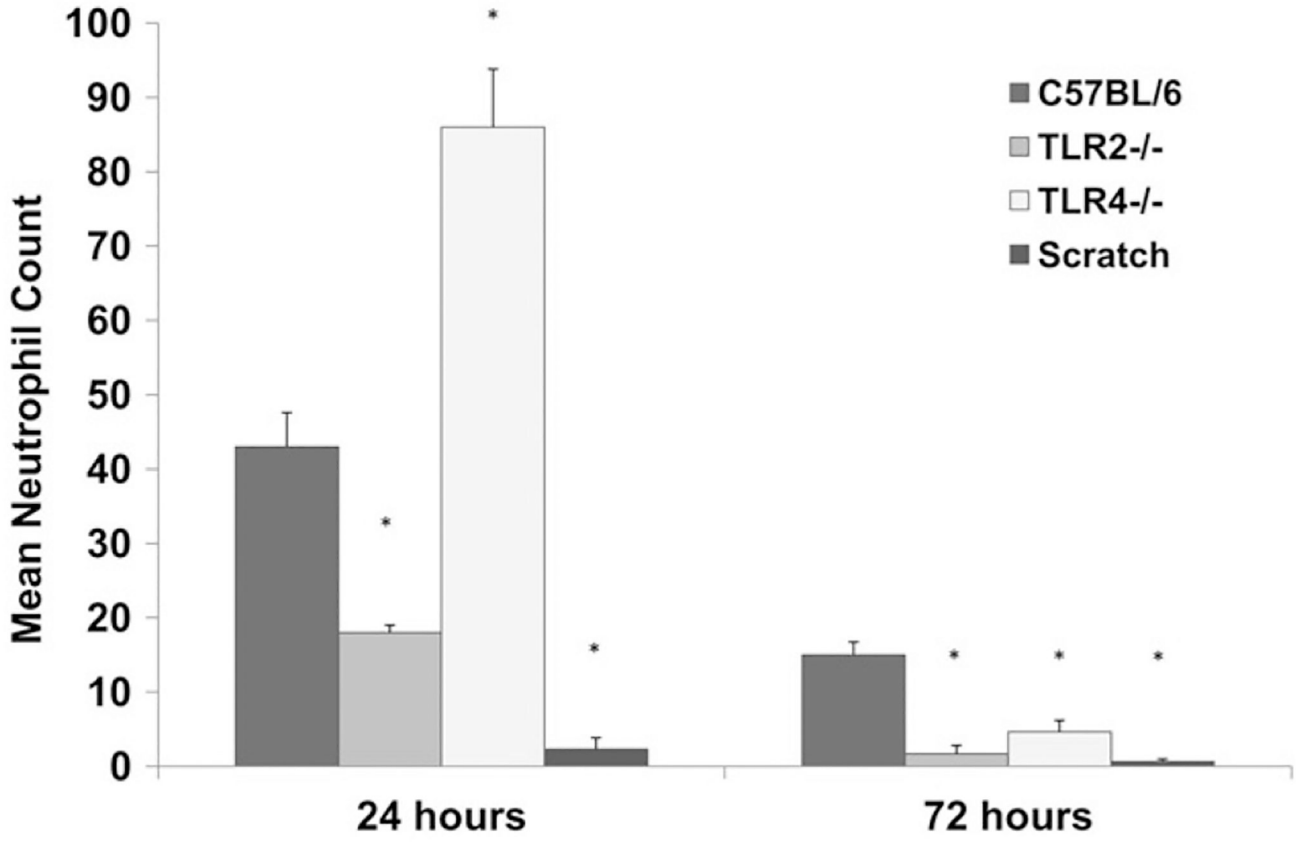

FIGURE 3.

Mean neutrophil counts of corneal histology sections. Limbus-to-limbus counts were obtained from three sections each for each mouse strain and time point. “Scratch” represents C57BL/6 corneal scratch controls. Asterisks denote statistical significance compared to C57BL/6 corneas ($p < 0.05$). Error bars represent SEM at each time point.

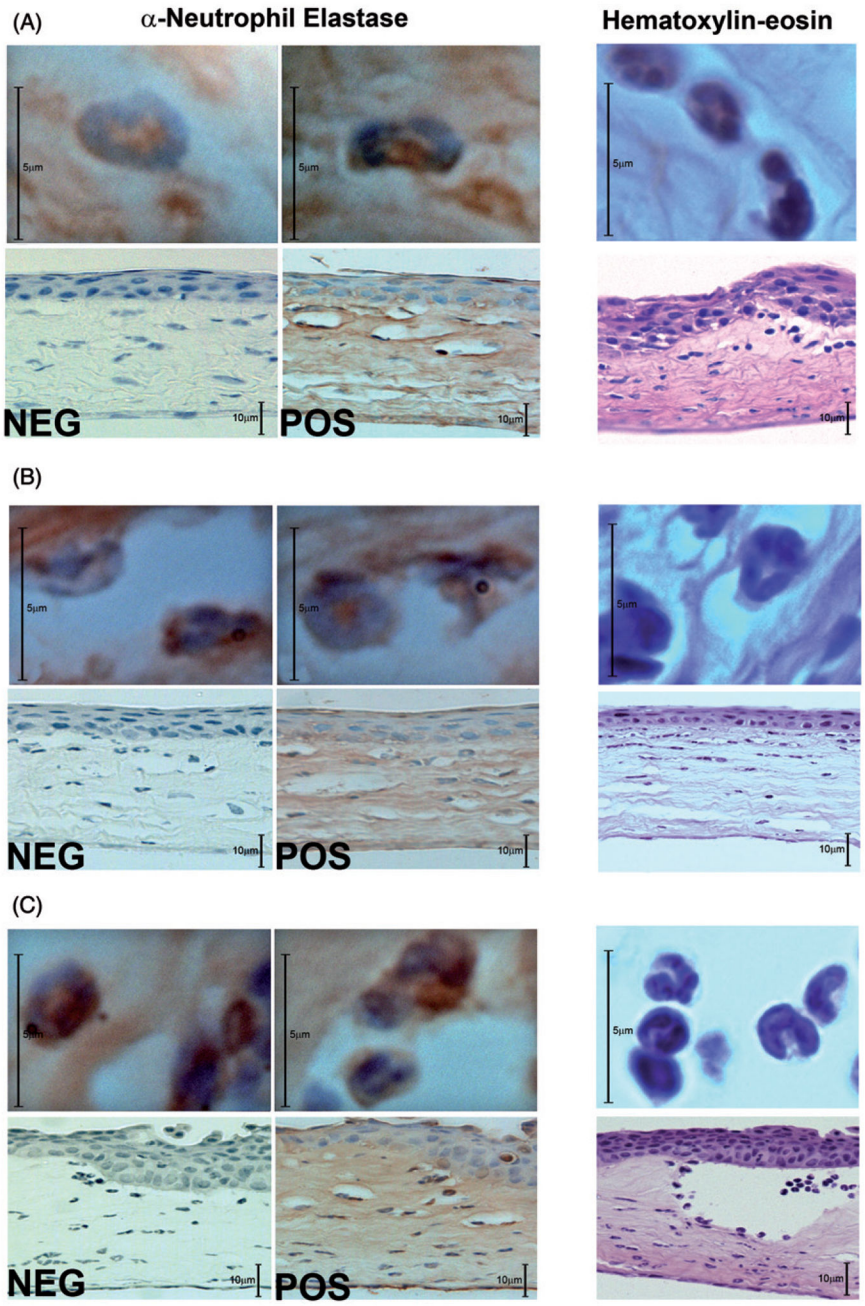

FIGURE 4.

Histology of C57BL/6, TLR2$^{-/-}$ and TLR4$^{-/-}$ mouse corneas. Corneas were stained with anti-neutrophil elastase antibody. Representative histology slides showing neutrophils stained with anti-neutrophil elastase as well as isotype controls and whole cornea sections at 24 h p.i. H/E-stained corneas for each group are also shown. Panel A: C57BL/6 corneas at 24 h p.i. Panel B: TLR2$^{-/-}$ corneas at 24 h p.i. Panel C: TLR4$^{-/-}$ corneas at 24 h p.i. Neg: isotype control antibody for each group. Pos: anti-elastase antibody stained corneas for each group. Corneal cross-section scale bar represents 10 μm. Individual neutrophil panel scale bar represents 5 μm.

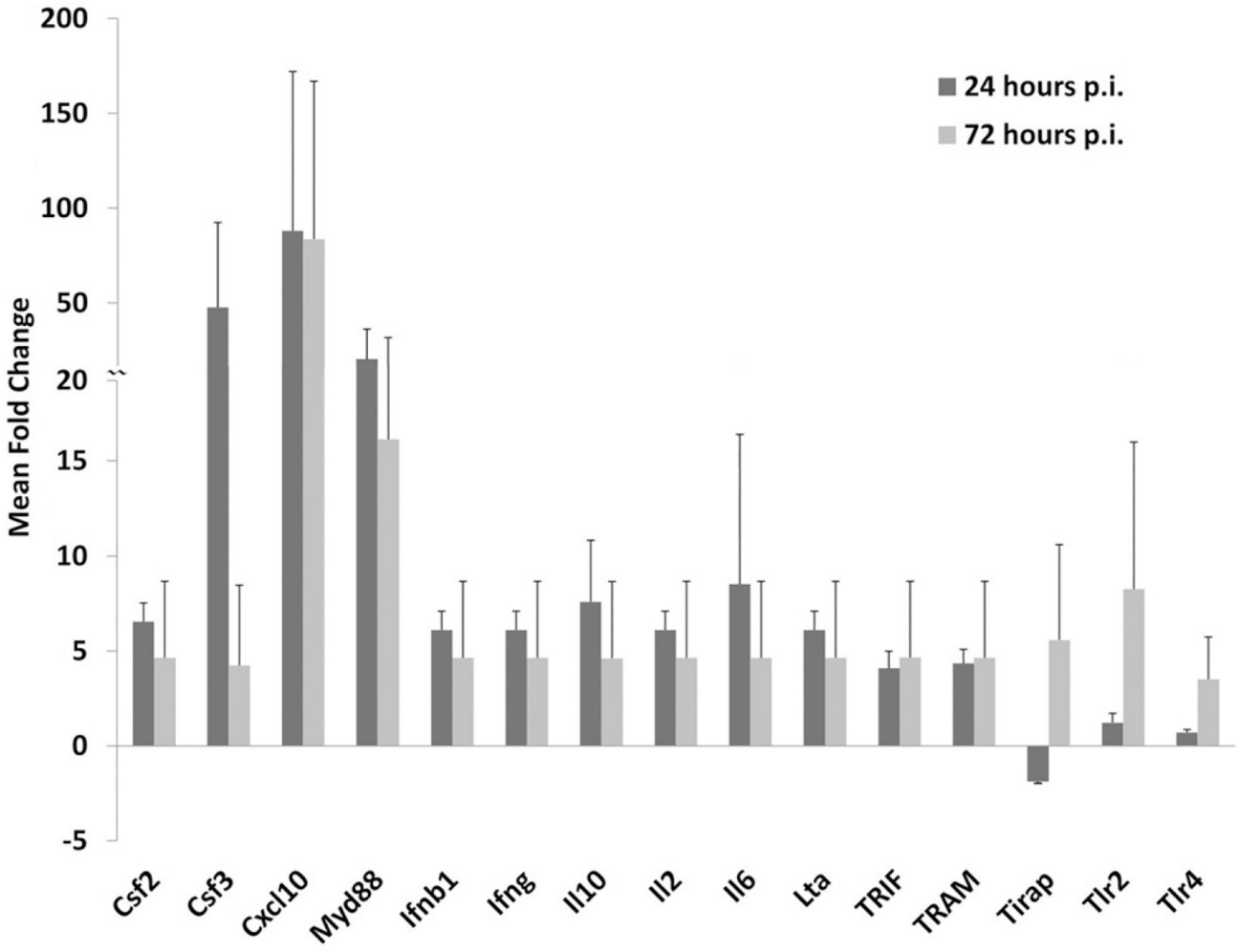

FIGURE 5.

Immune gene expression in infected C57BL/6 corneas. C57BL/6 corneas were harvested at 24 and 72 h p.i. and qRT-PCR performed on 84 immune genes with error bars representing SEM for each gene. Granulocyte maturation genes and chemokines (CSF2, CSF3 and CXCL10) were upregulated as well as cytokines (IL-10, IL-2 and IL-6) and TLR signaling genes (MyD88, TRIF and TRAM). Upregulation decreased but was still >2-fold at 72 h.

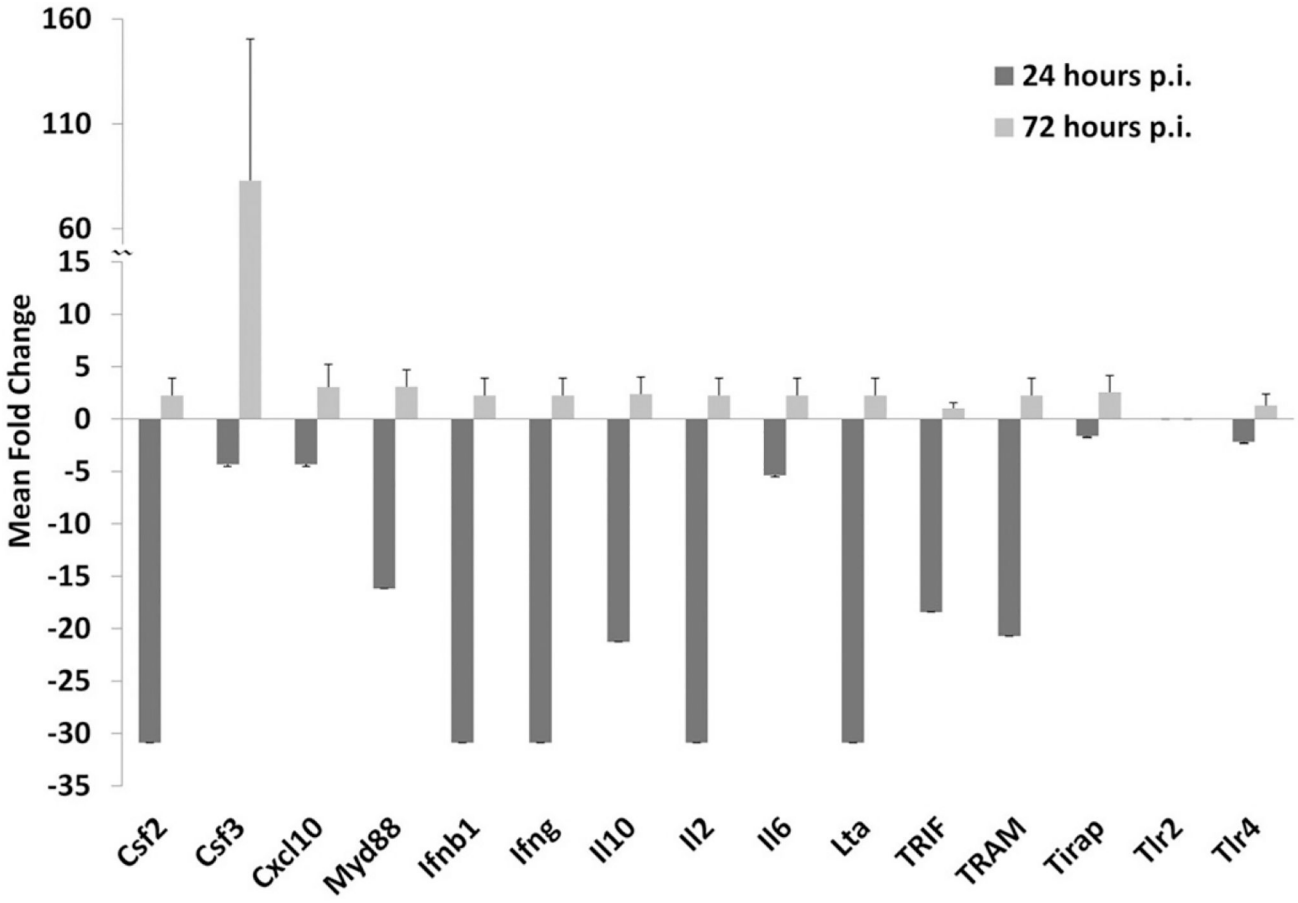

FIGURE 6.

Immune gene expression in TLR2$^{-/-}$ corneas. TLR2$^{-/-}$ corneas were harvested at 24 and 72 h p.i. and qRT-PCR performed using primers for 84 immune genes with error bars representing SEM for each gene. Granulocyte maturation genes and chemokines (CSF2, CSF3 and CXCL10) were all downregulated as well as cytokines (IL-10, IL-2 and IL-6) and TLR signaling genes (MyD88, TRIF, TRAM and TIRAP). Upregulated expression levels were observed at 72 h p.i.

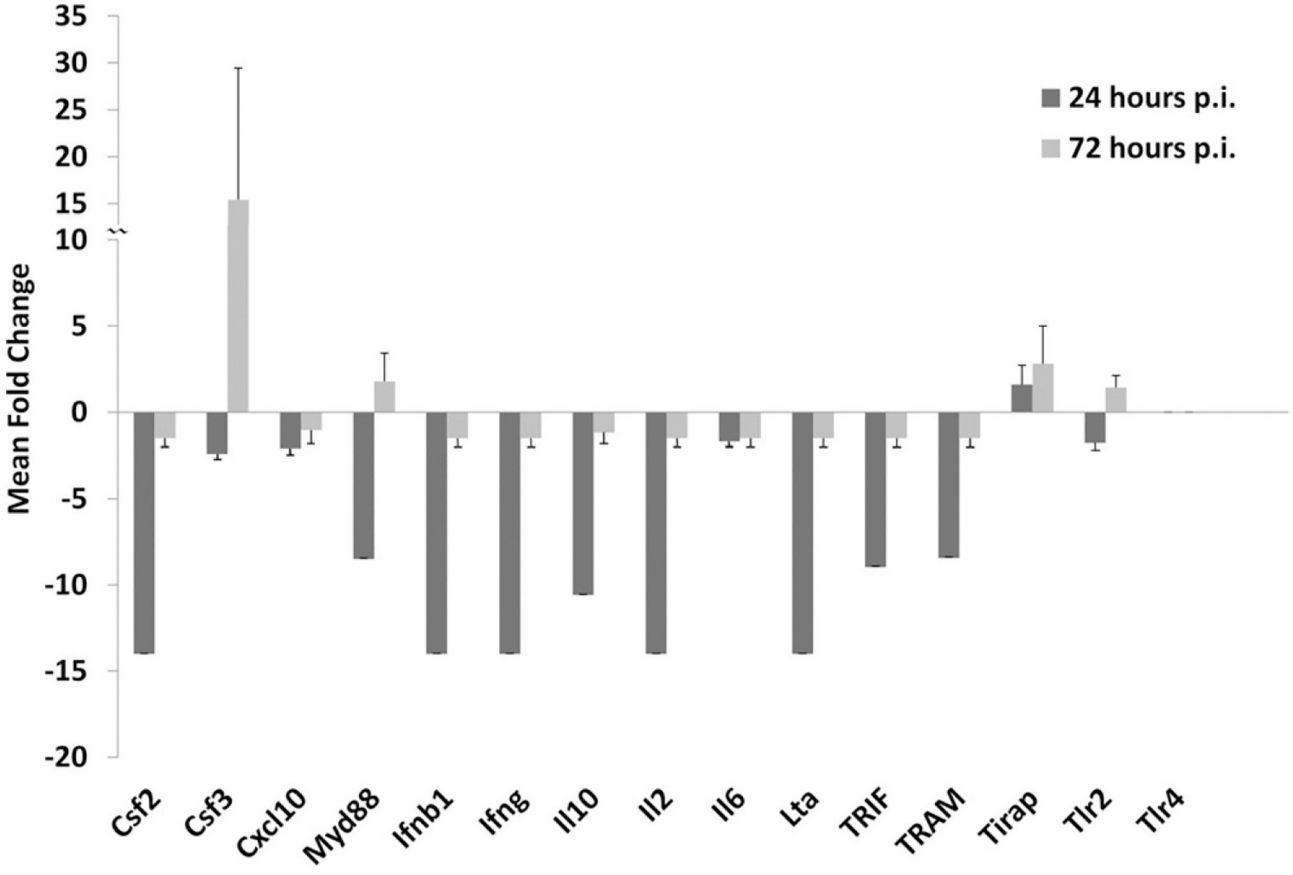

FIGURE 7.

Immune gene expression in TLR4$^{-/-}$ corneas. TLR4$^{-/-}$ corneas were harvested at 24 and 72 h p.i. and qRT-PCR performed using primers for 84 immune genes with error bars representing SEM for each gene. Granulocyte maturation genes and chemokines (CSF2, CSF3 and CXCL10) were all downregulated as well as cytokines (IL-10, IL-2 and IL-6) and TLR signaling genes (MyD88, TRIF and TRAM) except for TIRAP. Slightly downregulated expression of these genes was observed at 72 h p.i. with the exception of CSF3 and TIRAP.

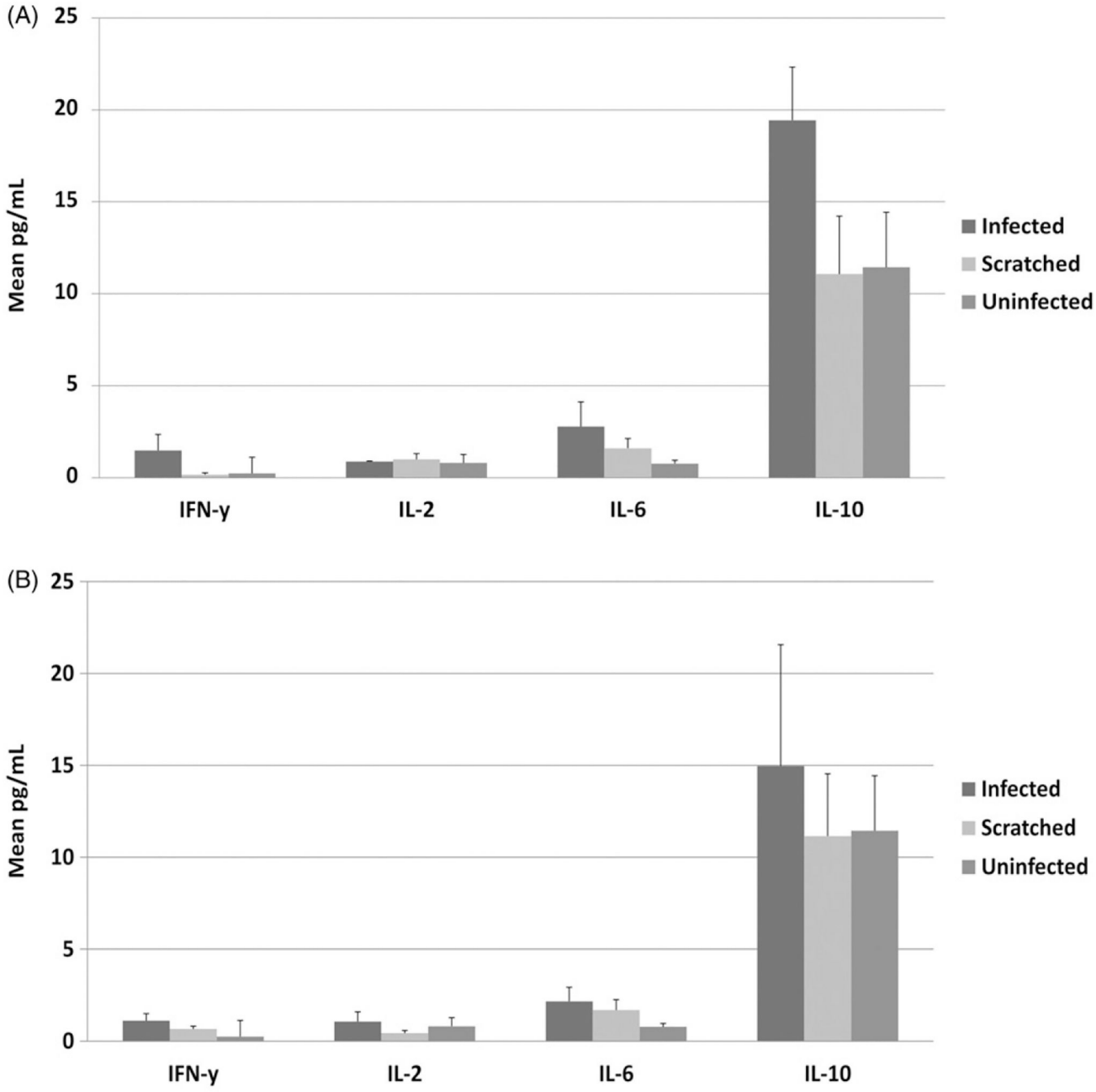

FIGURE 8.

Detection of Th1 and Th2-associated cytokines. C57BL/6 mice were infected with 10^8 total CFU and their corneas were harvested at 24 and 72 h p.i. Cytokine protein concentrations were determined using a CBA. Low concentrations (<5 pg/mL) of the Th1-associated IFN-y and IL-2 were detected for infected, scratched and uninfected C57BL/6 corneas at 24 (A) and 72 h p.i. (B). Higher concentrations of the Th2-associated IL-6 and IL-10 were detected at both time points though not significantly higher than scratch or uninfected controls. Error bars represent SEM for each cytokine.