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C-Jun NH₂ terminal kinase (JNK) is an essential mediator of Toll-like receptor 2-induced corneal inflammation

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Abstract

TLRs play an important role in the host inflammatory response to bacteria and bacterial products by activating a cascade of intracellular events leading to production of proinflammatory and chemotactic cytokines. To determine the role of MAPKs in TLR- induced corneal inflammation, we stimulated human corneal epithelial (HCE) cells with TLR2 ligands, tripalmitoyl-S-glycero-Cys-(Lys)4 (Pam3Cys) or inactivated *Staphylococcus aureus*, and examined the time course of expression of MAPKs and the effect of MAPK inhibition on IκBα degradation and CXC chemokine production. We found that *S. aureus* and Pam3Cys stimulate phosphorylation of JNK, p38 MAPK, and ERK within 4 h and that blockade of JNK, but not p38 or ERK phosphorylation, had an inhibitory effect on IκBα degradation and CXC chemokine production. To determine if JNK is also important in TLR2-induced corneal inflammation in vivo, we examined JNK1^{-/-} mice and pharmacological inhibitors in a murine model of TLR2-induced corneal inflammation which is characterized by neutrophil recruitment to the corneal stroma and development of corneal haze. We found that corneal inflammation was significantly impaired in JNK1^{-/-} mice compared with control mice, and in mice treated with the JNK inhibitor compared with vehicle control. Taken together with results from HCE cells, these findings demonstrate that JNK has an essential role in TLR2-induced corneal inflammation.

Keywords

TLR2; *S. aureus*; corneal epithelial cells; inflammation; MAP kinase; neutrophil; chemokines; innate immunity

INTRODUCTION

The ocular surface is continuously exposed to commensal and potentially pathogenic microbes, including *Staphylococcus aureus*, which can cause a severe keratitis in infected individuals and in animal models [1–3]. Although the intact corneal epithelium is normally resistant to microbial infection, disruption of the epithelial layer by trauma or chemical injury facilitates bacterial invasion of the underlying stroma, inducing an intense neutrophil infiltration and resulting in corneal opacification and loss of vision [1–5]. However, antimicrobial products in the tears can kill and lyse bacteria at the surface, and if the otherwise intact corneal epithelium is breached as a result of trauma or chemical injury, dead bacteria and bacterial cell wall products can activate resident cells and induce an inflammatory response that is characterized by neutrophil recruitment to the corneal stroma

and disruption of normal corneal clarity [6–9]. Individuals with sterile corneal inflammation have severe pain, red eye, photophobia, and impaired vision [10, 11], and neutrophils appear to be the predominant infiltrating cells [12]. Although the underlying mediators have yet to be fully identified, several studies have focused on the role of TLRs as initiators of inflammation [13, 14].

There are conflicting reports about expression and function of TLR2 and TLR4 on cultured human corneal epithelial (HCE) cells [15–17]; however we demonstrated that highly specific activation of TLR2, TLR4, and TLR9 in the mouse corneal epithelium induces an inflammatory response characterized by neutrophil recruitment from peripheral, limbal vessels to the corneal stroma, and increased corneal thickness and haze [6–8]. We also showed that corneal inflammation induced by inactivated *S. aureus* is dependent on TLR2 [9] and that TLR2, TLR4, and TLR9 activation is dependent on the common TLR adaptor protein MyD88 [6, 9, 18].

Recruitment of MyD88 from the cytoplasm to the intracellular Toll/IL-1R (TIR) region of TLRs leads to phosphorylation of downstream kinases, including IL-1R-associated kinase (IRAK)1 and IRAK4, TGF- β -associated kinase (TAK)1, and the MAPKs p38MAPK, JNK, and ERK [19]. MAPKs mediate several cell functions, including phosphorylation of transcription factors NF- κ B and AP-1 and transcription of proinflammatory and chemotactic cytokines [19–22]. Furthermore, this pathway can be blocked by small molecule, pharmacological MAPK inhibitors [23–25].

The current study examined the role of MAPK in HCE cell activation by killed *S. aureus* and by the synthetic TLR2 ligand tripalmitoyl-S-glycero-Cys-(Lys)4 (Pam3Cys). We also examined the role of MAPKs in our murine model of TLR2-induced keratitis [6]. Results of these studies demonstrate a selective role for JNK in TLR2-induced chemokine production by HCE cells and a role for JNK in TLR2-induced corneal inflammation using JNK^{-/-} mice and the anthrapyrazolone JNK inhibitor SP600125. JNK is therefore a potential target for anti-inflammatory therapy in TLR2/MyD88 inflammatory responses.

MATERIALS AND METHODS

Source of reagents

Keratinocyte serum-free medium (KSFM), trypsin, HBSS, and gentamicin were purchased from Invitrogen (Carlsbad, CA, USA). The synthetic TLR2 ligand Pam3Cys was purchased from EMC Microcollections (Tubingen, Germany). Antibody specific for total and phosphor-I κ B α , JNK, p38MAPK, and ERK was purchased from Cell Signaling Technology (Beverly, MA, USA). ELISA detection kits for CXCL8/IL-8, CXCL1/growth-related oncogene- α (GRO α), and CXCL5/epithelial-derived neutrophil-activating factor 78 (ENA-78) were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Antibody specific for β -actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Inhibitors SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), and SB203580 (p38 MAPK inhibitor) were purchased from Calbiochem (San Diego, CA, USA). Antibody specific for β -actin was purchased from Sigma-Aldrich, and peroxidase-conjugated goat anti-mouse IgG (SC-2005) and goat anti-rabbit IgG (SC-2004) were obtained from Santa Cruz Biotechnology.

S. aureus

S. aureus (strain 8325-4) was obtained from Dr. Richard O'Callaghan at the University of Mississippi Medical Center (Jackson, MS, USA) and incubated in 10 ml tryptic soy broth

(TSB; Difco, Detroit, MI, USA) at 37°C overnight, subcultured 1:100 in fresh TSB, and grown to an OD of 0.3 at 650 nm to obtain a log-phase culture ($\sim 10^8$ CFU/ml). Bacteria were washed three times with PBS (BioWhittaker, Walkersville, MD, USA) and suspended at 3×10^{10} /ml in PBS. A UV Stratalinker (Stratagene, La Jolla, CA, USA) at a setting of 2400 for 15 min was used to kill *S. aureus* (confirmed by the absence of growth in TSB).

Primary HCE cells

Human eyes, which were unsuitable for corneal transplantation, from donors 40–75 years of age, were obtained from Cleveland Eye Bank at University Hospitals (Cleveland, OH, USA). Tissue procurement was approved by the Case Western Reserve University (Cleveland, OH, USA)/University Hospitals of Cleveland Institutional Review Board. The globes were transferred to the laboratory in the KSFM. Bulbar conjunctival tissue was removed from the corneal epithelium surface using a sterile scalpel. The cornea was then excised and placed in 4 mL sterile HBSS containing 10 mg/mL dispase and 5 mg/mL gentamicin for 5 h at 4°C. The corneal epithelium was then collected by gentle scraping and by incubation with 5 mL 0.25% trypsin, 5 min at 37°C. The epithelial cell suspension was transferred to medium containing 2.5 mL DMEM containing 10% FCS to block further enzyme activity. Epithelial cells from each cornea were then collected by centrifugation and resuspended in KSFM containing epithelial growth factor and bovine pituitary extract (BPE) with antibiotics and distributed in two 9.5 cm² surface-area dishes in 4 ml culture medium. After Passage 1, antibiotics were omitted from the culture medium, and cells were harvested with 0.25% trypsin and transferred to a 50-cm² flask. The medium was changed every 4 days, and cells from Passages 2–5 were used for experiments when cells were 50–70% confluent.

HCE cell line

The SV40-immortalized HCE cell line 10.014 pRSV-T (HCE-T) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained by culturing in KSFM with BPE and recombinant human epidermal growth factor (EGF; Invitrogen) at 37°C and 5% CO₂. For TLR stimulation experiments, when the culture was at 70–80% confluency, cells were incubated overnight in KSFM without EGF.

SDS-PAGE and Western blot analysis

For immunoblot analysis, equivalent amounts of protein were electrophoresed on denaturing and reducing 8% polyacrylamide gels and transferred to nitrocellulose membrane, which was blocked by 5% nonfat dry milk and then incubated with antibody specific for total or phosphor forms of MAPKs or with anti- β -actin at a dilution of 1:3000. Peroxidase-conjugated goat anti-mouse and anti-rabbit IgG (SC-2004) was used at a dilution of 1:5000. Secondary antibody binding was visualized using a commercial chemiluminescence detection kit (Amersham Bioscience, Piscataway, NJ, USA). The intensity of the bands in X-ray film was quantified using “ImageJ” software [National Institutes of Health (NIH), Bethesda, MD, USA], and the ratio of phosphorylated: nonphosphorylated was determined by densitometry.

For immunoblot analysis, equivalent amounts of protein were separated by 8% SDS-PAGE under denaturing and reducing conditions and transferred to a nitrocellulose membrane, which was blocked with 5% nonfat dry milk and then incubated with primary and secondary antibodies. Secondary antibody binding was visualized using chemiluminescence detection technology (Amersham Bioscience).

Mouse models of TLR2 keratitis

Six- to 10-week-old C57BL/6 mice or JNK1^{-/-} mice (on a C57BL/6 background) obtained from Jackson Laboratory (Bar Harbor, ME, USA) were anesthetized by i.p. injection of 0.4 ml 2,2,2-tribromoethanol (1.2%), and the central corneal epithelium was abraded with three contiguous scratches using a 26-gauge needle as shown previously [6, 7]. Pam₃Cys (20 µg/2 µl) was added topically to induce an inflammatory response [6], and histological examination of the abraded cornea showed that the wound was limited to the epithelial layer. Animals used in these studies were raised in specific pathogen-free conditions in microisolator cages and were treated in accordance with the guidelines provided in the Association for Research in Vision and Ophthalmology (Rockville, MD, USA) statement for the use of Animals in Ophthalmic and Vision Research.

Quantification of corneal inflammation

Analysis of cellular infiltration was accomplished by in vivo confocal microscopy (Confoscan, Nidek Technologies America, New Orleans, LA, USA), as described [6]. Briefly, mice were anesthetized and immobilized on a secure platform. A 40× objective was maneuvered into place on the corneal surface, using transparent gel (Genteel, Novartis Ophthalmic, Duluth, GA, USA) as a medium between the corneal surface and the objective, and use of the Navis software (Lucent Technologies, Murry Hill, NJ, USA) allowed us to capture images with numerical values for light intensity every 0.5 µm and store them as a stack for analysis of corneal thickness and haze.

Stromal thickness was defined as the area between the basal epithelium and corneal endothelium, and stromal haze was calculated from the light intensity of each image of the corneal stroma. To obtain these values, a series of intensity values for each image of the corneal stroma was saved to a computer spreadsheet (Excel, Microsoft, Redmond, WA, USA) and exported into Prism (Graph-Pad Software, San Diego, CA, USA) to generate a curve using the “curve and regression” function. The total area under the curve, representing total thickness and light intensity, was calculated and used as a measure of corneal haze.

Detection of neutrophils in the cornea by immunohistochemistry

Eyes were enucleated and snap-frozen in liquid nitrogen, and 5 µm cryosections from the central region of the cornea were fixed in 4% formaldehyde for 30 min. Slides were then washed with PBS, and sections were incubated 2 h with antineutrophil antibody NIMP-R14 hybridoma sups in 1% FCS/PBS. Sections were then incubated 45 min with FITC-conjugated rabbit anti-rat antibody (Vector Laboratories, Burlingame, CA, USA), and the total number of neutrophils per 5 µm section was counted by fluorescence microscopy.

Statistics

Prism (GraphPad Software) was used to calculate Student's *t*-test, and a value of <0.05 between test groups was considered statistically significant.

RESULTS

TLR2 agonists *S. aureus* and Pam₃Cys induce MAPK phosphorylation and NF-κB signaling in human corneal epithelial cells

Our previous studies showed that corneal inflammation induced by Pam₃Cys or killed *S. aureus* is completely dependent on expression of TLR2 and MyD88 [6, 9]. To determine the effect of TLR2 activation on MAPK and IκBα phosphorylation in the cornea, primary HCE cells and the HCE-T cells were stimulated with UV-killed *S. aureus*, and phosphorylation of

JNK, p38, ERK, and I κ B α was examined by Western blot analysis using total or phospho-specific antibodies.

As shown in Figure 1, left panel, incubation with *S. aureus* induced phosphorylation of JNK, p38, and ERK in primary HCE cells within 3 h and was maximal after 6 h. Similar results were seen in the SV40-transformed HCE-T cell line stimulated with *S. aureus* (Fig. 1, middle panel) or with the synthetic lipopeptide Pam₃Cys (Fig. 1, right panel). The time course of I κ B α phosphorylation was similar to that of MAPKs, with maximal activation detected 6 h after stimulation. Together, these findings demonstrate that TLR2 stimulation of HCE cells induces phosphorylation of MAPKs and I κ B α within a similar time-frame.

TLR2-induced I κ B α phosphorylation is dependent on JNK

Activation of the transcription factor NF- κ B involves signaling through TAK1, phosphorylation of precursor IKK β , and subsequent phosphorylation of I κ B α . The latter event targets this subunit for ubiquitination and degradation and frees the NF- κ B complex (p50/REL-A) to translocate into the nucleus and initiate gene transcription [26]. As I κ B α phosphorylation is induced in TLR2-stimulated HCE cells, we next examined if there is a selective role for MAPKs in I κ B α activation.

HCE-T cells were incubated with *S. aureus* or Pam₃Cys in the presence of specific anthrapyrazolone inhibitors for JNK, p38, or ERK. After 6 h, cells were harvested, and I κ B α phosphorylation was determined by Western blot analysis. As shown in Figure 2, *S. aureus* and Pam₃Cys stimulated I κ B α phosphorylation in these cells as before; however, preincubation with JNK inhibitor SP600125 completely inhibited this reaction, whereas p38 inhibitor SB203580 and ERK inhibitor PD98059 had only a minor inhibitory effect. These findings demonstrate a selective role for JNK in TLR2-induced I κ B α phosphorylation.

JNK inhibits *S. aureus*-induced cytokine production by HCE cells

As CXC chemokine transcription is induced by NF- κ B, and CXC chemokines are important in TLR- induced corneal inflammation [13], we next examined if JNK inhibitor SP600125 blocks *S. aureus*-induced CXC chemokine production by primary corneal epithelial cells. As shown in Figure 3A, *S. aureus* induces production of CXCL1/GRO α , CXCL5/ENA-78, and CXCL8/IL-8 by HCE cells. However, JNK inhibitor SP600125 completely blocked CXCL1 and CXCL5 production and significantly inhibited CXCL8 production (Fig. 3B). These findings indicate that JNK regulates CXC chemokine production and are consistent with the dominant role for JNK in NF- κ B activation shown in Figure 2.

MAPKs and I κ B α are phosphorylated in vivo in a murine model of TLR2-induced corneal inflammation

Our previous studies demonstrated that topical application of Pam₃Cys to an abraded corneal surface induces TLR2-dependent corneal inflammation that is characterized by increased corneal thickness and haze and neutrophil infiltration to the corneal stroma [6]. To determine if this process involves activation of MAPKs and I κ B α , we scarified the corneal epithelium of C57BL/6 mice using a 26-gauge needle and added Pam₃Cys or endotoxin-free water (trauma control) as before [6]. Whole corneas were dissected at several time-points thereafter, and were processed for SDS-PAGE and immunoblot analysis.

The trauma, control corneas (treated with H₂O, Fig. 4, left panel) show constitutive expression of total, but not phosphorylated JNK and p38, thereby demonstrating that trauma alone does not activate these MAPKs in the corneal epithelium. In marked contrast, Pam₃Cys-treated corneas showed activation of these kinases and I κ B α within 1 h (Fig. 4, right panel). P-JNK levels peaked at 2–4 h, P-p38 at 1–2 h, and P-I κ B α remained elevated

from 1 to 8 h. In addition, P-ERK was induced by trauma alone, but expression was greatly increased after stimulation with Pam₃Cys. Together, these findings demonstrate that TLR2 activation of the cornea induces phosphorylation of JNK, p38, ERK, and IκBα, although the time course of activation differs for each.

SP600125 inhibits TLR2-induced corneal inflammation

As we showed above that JNK inhibitor SP600125 selectively inhibits phosphorylation of IκBα in HCE cells and that JNK is phosphorylated in Pam₃Cys-treated corneas, we next determined if SP600125 inhibits TLR2-induced corneal inflammation, characterized by neutrophil recruitment to the corneal stroma and development of increased corneal thickness and haze.

Corneas were scarified as before, and 2 μl 10 mM SP600125 or vehicle control was added topically three times: 1 h prior to and at the time of exposure to Pam₃Cys and 6 h after stimulation. After 24 h, which is the peak of corneal inflammation in this model [6], corneal thickness and haze were measured by in vivo confocal microscopy, and neutrophil infiltration to the cornea was determined by immunohistochemistry, as described previously [6, 9].

As shown in Figure 5A, neutrophil infiltration was elevated in Pam₃Cys-treated corneas given vehicle control compared with naïve mice or trauma controls treated with vehicle alone. In contrast, corneas pretreated with SP600125 had significantly fewer neutrophils, and corneal thickness and haze were significantly lower (Fig. 5, B and C). These findings clearly indicate that TLR2-induced corneal inflammation is mediated by JNK.

JNK1^{-/-} mice have impaired TLR2-induced corneal inflammation

Isoforms of JNK, including JNK1 and JNK2, are differentially expressed and have distinct roles in stimulated cells (predominantly JNK1) compared with unstimulated cells (predominantly JNK2) [27–29]. Furthermore, JNK1 is the predominant isoform involved in macrophage proliferation and LPS stimulation [30]. We therefore hypothesized that the JNK1 isoform mediates TLR2-induced corneal inflammation. To determine the effect of JNK1 on Pam₃Cys-induced responses and to support the findings with SP600125, C57BL/6 and JNK1^{-/-} corneas were scarified and treated with Pam₃Cys as described above. Corneal thickness and haze and number of neutrophils in the corneal stroma were assessed as before.

We found that JNK1^{-/-} mice had significantly fewer neutrophils in the corneal stroma compared with C57BL/6 mice (Fig. 6A) and that TLR2-induced increases in corneal thickness and haze were significantly impaired (Fig. 6A). Taken together with results from Figure 5, these findings demonstrate that JNK, especially the JNK1 isoform, is an essential mediator of TLR2-induced corneal inflammation.

DISCUSSION

Our previous studies using murine models of corneal inflammation demonstrated that: 1) TLR2 is expressed in the corneal epithelium; 2) TLR2 activation of the corneal epithelium induces CXC chemokine production; TLR2 induces neutrophil recruitment to the corneal stroma and development of corneal haze; 3) TLR2-induced corneal inflammation is ablated in TLR2^{-/-} mice and MyD88^{-/-} mice; and 4) corneal epithelium exposed to killed *S. aureus* results in corneal inflammation that is TLR2- and MyD88-dependent [6, 9].

In the current study, we extend these observations to show that TLR2 induces phosphorylation of p38, JNK, and ERK; however, JNK is the key MAPK in TLR2-induced corneal inflammation, as blockade of JNK using the anthrapyrazolone SP600125 inhibits

I κ B α phosphorylation in the cornea and blocks neutrophil recruitment and development of corneal haze in response to TLR2 activation. We confirmed the critical role of JNK using JNK1^{-/-} mice, which also showed diminished TLR2-induced corneal inflammation, and given that SP600125 inhibits all three JNK isoforms, the use of JNK1^{-/-} mice demonstrated that JNK1 is an essential mediator in corneal inflammation. This observation is consistent with studies showing that JNK1 is involved in inflammatory responses and T cell immunity [30–33]. Although we have yet to examine other cell types in the cornea, TLR2-induced JNK activation occurs in corneal epithelial cells, as JNK inhibition using SP600125 selectively blocked I κ B α phosphorylation and production of CXC chemokines in primary and in HCE cells, which are the predominant cells at the ocular surface. We also showed recently that the TLR adaptor molecule MyD88 has an inhibitory role in TLR3/TIR domain-containing adaptor-inducing IFN- β -induced cytokine production by HCE cells and that JNK is an essential mediator of this activity [18]. JNK therefore appears to have a central position in multiple TLR signaling pathways.

In addition to cytokines, Kumar et al. [16, 34] showed that human β -defensin-2 RNA expression was partially blocked by JNK or p38 inhibitors, although it is not clear if there is a selective role for JNK compared with p38 in cytokine production compared with production of antimicrobial peptides. In contrast to our findings, these investigators also reported that heat-killed *S. aureus* stimulates HCE cells [35]. The basis for this difference has yet to be determined, although it likely relates to differences in cell lines and bacterial strains.

MAPKs are elevated in the stress responses in human limbal epithelial cells [36], and MAPK inhibitors have been used in murine models of a broad range of disorders, including inflammation associated with arthritis [23, 31, 37]. Furthermore, preliminary clinical trials with some of these inhibitors show that they are nontoxic (reviewed in refs. [24, 25]). Of particular interest for infectious disease is the selective use of MAPK inhibitors in anti-inflammatory versus antimicrobial activity. In a murine model of Group B Streptococcal sepsis, SP600125 blocked cytokine production with no effect on phagocytosis or formation of antibacterial oxidative species [38].

In summary, the current findings extend our understanding of TLR-induced inflammatory responses in the cornea by identifying JNK as the predominant MAPK that mediates NF- κ B activation and production of CXC chemokines in HCE cells. The consequence of these JNK-dependent events is recruitment of neutrophils to the tissue and disruption of normal corneal structure. Future studies will examine the role of other isoforms of JNK and other intracellular mediators in TLR-induced corneal inflammation.

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Fig. 1. *S. aureus*- and Pam₃Cys-induced activation of MAPKs and NF-κB in HCE cells. (Left) Human primary corneal epithelial cells were isolated from donor eyes obtained from the Cleveland Eye Bank and incubated with 1×10^8 UV-killed *S. aureus* strain 8325. At indicate times, cells were harvested, lysed, and processed for SDS-PAGE and Western blot analysis using antibody specific for total or phosphorylated (P) JNK, p38, ERK, and IκBα. Actin was used as a loading control. The SV40-immortalized HCE cell line (HCE-T) was stimulated with *S. aureus* (Middle) or the synthetic TLR2 ligand Pam₃Cys (Right). At indicated times, cells were examined as described for the left panel. The experiment is representative of four repeat experiments.

Fig. 2.

IκBα phosphorylation is selectively blocked by the JNK inhibitor SP600125. HCE-T cells were stimulated with *S. aureus* or Pam₃Cys, together with inhibitors for ERK, p38, or JNK. After 6 h, cells were harvested and processed for SDS-PAGE/Western blot analysis, as described in the legend to Figure 1. Densitometry shows inhibition of IκBα phosphorylation by the JNK inhibitor SP600125 but not by other MAPK inhibitors. This experiment was repeated three times with similar results.

Fig. 3.

CXC chemokine production in HCE-T cells is blocked by SP600125. (A) HCE-T cells were incubated with *S. aureus* for 6 h or 24 h, and supernatants were examined for production of CXCL1, CXCL5, and CXCL8 by ELISA. (B) HCE cells were incubated 24 h with *S. aureus* in the presence of the JNK inhibitor SP600125, and supernatants were examined as before. Note that cells treated with SP600125 had significantly impaired CXC chemokine production compared with cells incubated with vehicle alone (*, $P < 0.05$). This experiment was repeated twice with similar results. Cont, Control.

Fig. 4.

Phosphorylation of MAPKs and IκBo in TLR2-induced corneal inflammation. Corneas of C57BL/6 mice were gently scarified by three parallel scratches and treated with H₂O (trauma control) or with the TLR2 ligand Pam₃Cys. At indicated times, corneas were dissected and processed for SDS-PAGE and Western blot analysis. Each sample represents a pool of two corneas and is representative of two repeat experiments.

Fig. 5.

SP600125 inhibits TLR2-induced corneal inflammation. Corneal epithelium of C57BL/6 mice was scarified using three parallel scratches and treated topically with SP600125 or with vehicle alone 1 h prior to and at the same time as and 6 h after corneas were treated topically with Pam₃Cys. After 24 h, neutrophils in the corneal stroma were detected by immunohistochemistry (A), and corneal thickness and haze were measured by in vivo confocal microscopy (B, C). Data are mean $\pm$ SEM for five corneas per group, and the experiment was repeated twice.

Fig. 6. TLR2-induced corneal inflammation in JNK1^{-/-} mice. Corneas of JNK1^{-/-} and C57BL/6 mice were scarified as described above and stimulated by topical application of Pam₃Cys (P3C). After 24 h, corneal inflammation was measured as described above. Data are mean ± SEM for five corneas per group and are representative of two repeat studies.