MyD88 Functions as a Negative Regulator of TLR3/TRIF-induced Corneal Inflammation by Inhibiting Activation of c-Jun N-terminal Kinase*

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The adaptor molecule MyD88 is necessary for responses to all Toll-like receptors except TLR3 and a subset of TLR4 signaling events, which are mediated by the adaptor molecule TRIF. To determine the role of MyD88 in host inflammatory responses, corneal epithelium of C57BL/6, TLR3−/−, TRIF−/−, and MyD88−/− mice was abraded and stimulated with the synthetic TLR3 ligand poly(I:C). We found that poly(I:C) induced a pronounced cellular infiltration into the corneal stroma, which was TLR3- and TRIF-dependent. Unexpectedly, the inflammatory response was exacerbated in MyD88−/− mice, with enhanced neutrophil and F4/80+ cell infiltration into the corneal stroma and elevated corneal haze, which is an indicator of loss of corneal transparency. To determine whether MyD88-dependent inhibition of TLR3/TRIF responses is a general phenomenon, we examined cytokine production by MyD88−/− bone marrow-derived macrophages; however, no significant difference was observed between MyD88−/+ or MyD88−/− macrophages. In contrast, human corneal epithelial cells (HCECs) transfected with MyD88 small interfering RNA had significantly increased (2.5-fold) CCL5/RANTES production compared with control HCECs, demonstrating a negative regulatory role for MyD88 in TLR3/TRIF responses in these cells. Finally, knockdown of MyD88 in HCECs resulted in increased phosphorylation of c-Jun N-terminal kinase (JNK), but not p38, IRF-3, or NF-κB. Consistent with this finding, the JNK inhibitor SP600125, but not p38 inhibitor SB203580, ablated this response. Taken together, these findings demonstrate a novel JNK-dependent inhibitory role for MyD88 in the TLR3/TRIF activation pathway.

Microorganisms are recognized by pattern recognition receptors, including the innate immune receptors known as Toll-like receptors (TLRs).2 There are 11 human TLRs and 13 mouse TLRs (1, 2), which recognize ligands from bacterial, protozoan, viral, and fungal pathogens (1). Upon recognition of a specific pathogenic ligand, signaling cascades are activated through several adaptor molecules, which are thought to determine the specificity of the response (1–3). The most commonly utilized adaptor is the myeloid differentiation factor 88 (MyD88), which was originally described as part of the IL-1 receptor signaling complex (3). All TLRs, except TLR3 and a subset of TLR4 signaling events, require MyD88 (1–3).

Microarray analysis of lipopolysaccharide-induced murine macrophages found that MyD88-independent genes include CCL5/RANTES, CXCL10/IP-10, and macrophage-colony-stimulating factor (4). Furthermore, the adaptor molecule responsible for MyD88-independent responses is the TIR domain-containing adaptor inducing interferon-β (TRIF) (2, 3), which is a 712-amino acid protein containing the C-terminal TIR domain that is common to many of the TLRs (5). TLR3, which exclusively uses TRIF, is located in endosomes and is the receptor for double-stranded RNAs, either derived from viruses or synthetically produced (6, 7), and TLR3 activation results in anti-viral responses characterized by interferon production (2) as a result of NF-κB, IRF-3, and MAP kinase (p38 and JNK) signaling (8).

TLRs are expressed on both myeloid and nonmyeloid cells, including epithelial cells (1, 2). Corneal epithelial cells are located at the ocular surface, and in addition to providing a physical barrier function, they also mediate the immune response to microbial products through the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides (9, 10). Furthermore, corneal epithelial cells express several TLRs and can respond in vitro to TLR2, TLR3, and TLR5 agonists (11–21). Our previous studies demonstrated that TLR2, TLR4, and TLR9 are expressed in the cornea and can mediate corneal inflammation through the MyD88 adaptor molecule (19, 22). TLR3 is also expressed within the corneal epithelium (14, 16); however, the role of TLR3 in corneal inflammation has yet to be determined.

Therefore in the current study, we examined the role of TLR3, TRIF, and MyD88 in a mouse model of corneal inflam-
mation. Surprisingly, whereas MyD88 is not generally thought to be involved in TLR3 signaling events (1–3), results shown in the current studies clearly demonstrate that MyD88 has a negative regulatory role on TLR3/TRIF-induced corneal inflammation and that JNK mediates this activity in corneal epithelial cells.

EXPERIMENTAL PROCEDURES

Source of Animals—C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). TLR3−/−, TRIF−/−, and MyD88−/− were kindly provided by Shizuo Akira (Research Institute for Microbial Disease, Osaka University, Osaka, Japan). Age-matched wild type littermates were used as controls for MyD88−/− mice. TLR3−/− and TRIF−/− mice were fully backcrossed to C57BL/6 mice; therefore, C57BL/6 mice were used as controls. All of the animals used in these studies were maintained 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 statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Model of Poly(I:C)-induced Keratitis—Six- to ten-week-old mice (described above) were anesthetized via intraperitoneal injection of 0.4 ml of 2,2,2-tribromoethanol (1.2%). 1-mm² area of the central cornea was marked using a sterile trephine (Miltex, Tuttlingen, Germany), and the corneal epithelium within that area was abraded using an Algerbrush II (Alger, Lago Vista, TX) and either poly(I:C) (20 μg; InvivoGen, San Diego, CA) or Pam3Cys (10 μg; EMC Microcollections, Tubingen, Germany) was applied to the surface as described (22). Dose-response studies (not shown) indicated that 20 μg of poly(I:C) induced significant levels of corneal inflammation.

Immunohistochemistry of Corneal Sections—Mouse eyes were enucleated, snap frozen in liquid nitrogen, and sectioned (5 μm). For F4/80 staining, which detects macrophages and dendritic cell subsets (23, 24), the samples were fixed with cold (−20 °C) acetone for 30 min, washed with 0.01 M PBS (pH 7.4) (Sigma), and blocked with 2% fetal calf serum/PBS. Rat anti-mouse F4/80 antibody (AbD Serotec, Raleigh, NC) was then applied for 2 h at a dilution of 1:250 in 1% fetal calf serum/PBS. Sections were incubated for 45 min with fluorescein isothiocyanate-conjugated rabbit anti-rat antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in 1% fetal calf serum/PBS. After washing, the slides were mounted in Vectashield containing 4',6'-diamino-2-phenylindole (Vector Laboratories), which stains cell nuclei, and the number of F4/80+ cells/section was determined by direct counting of fluorescent images (Olympus Optical Co. Ltd., Tokyo, Japan). NIMP-R14, which detects neutrophils (25), was used as previously described (22).

Examination of Stromal Thickness and Haze by in Vivo Confocal Microscopy—Analysis of inflammation in the mouse cornea was assessed using a Nidek Confoscan®, as described previously (22). Mice were euthanized with CO₂ and immobilized on a secure platform. A 40× objective was directed toward the corneal surface, using transparent Gentael gel (Novartis Ophthalmics, Duluth, GA) as a medium between the corneal surface and the objective. NAVIS® software captured images every 5 μm and stored them as a stack for analysis of stromal haze, defined as the stromal thickness × combined light intensity of each image of the corneal stroma. To obtain a numerical value for stromal haze, the series of intensity values for each corneal stroma was saved in Microsoft Excel and imported into Prism (GraphPad Software, San Diego, CA) to generate a curve using the “curves and regression” function. The total area under the curve was then calculated using this software. Base-line measurements in each experiment were determined using naive, untreated mice.

Isolation and Culture of Bone Marrow-derived Macrophages—L929 fibroblast conditioned media, which is enriched in macrophage-colony-stimulating factor, was kindly provided by Fred P. Heinzel, and bone marrow-derived macrophages were derived as previously described (26). Bone marrow cells were harvested from the femurs and tibia of four to six C57BL/6, TLR2−/−, TLR3−/−, TRIF−/−, MyD88+/+, and MyD88−/− mice. The bones were trimmed at each end and centrifuged at 2000 × g for 30 s, and bone marrow cells were suspended in growth medium (Dulbecco’s modified Eagle’s medium, 10% FBS, and 30% L929 conditioned medium) and plated in six-well plates. 24 h later nonadherent cells were collected (leaving behind fibroblasts that were present in the preparation) and placed into new tissue culture dishes. Macrophage precursors were then expanded for 72 h. Finally, nonadherent and semi-adherent cells were transferred into 96-well plates at a density of 50,000 cells/well. The cells were treated with either poly(I:C) or Pam3Cys at the indicated doses for 24 h.

Epithelial Cell Culture and Reagents—The SV-40 immortalized human corneal epithelial cell line (HCEC, 10.014 pRSV-T; American Type Culture Collection, Manassas, VA) was maintained in culture with keratinocyte serum-free medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor (EGF) (Invitrogen) at 37 °C and 5% CO₂. Generally, the cells were placed in keratinocyte serum-free medium lacking EGF 24 h prior to experimental manipulation and once they had reached 70–80% confluency. Cells were then treated with either poly(I:C) or Pam3Cys at specified concentrations in keratinocyte serum-free medium, and culture supernatants were collected 24 h after treatment.

Chemokine Analysis—Bone marrow-derived mouse macrophages and HCECs were treated as described above. At the conclusion of the experiments, the supernatants were collected, and secreted chemokine levels (mCXCL1/KC, mCXCL2/ MIP-2, mCCL5/RANTES, mCXCL10/IP-10, hCXCL8/IL-8, hCCL5/RANTES, and hCXCL10/IP-10) were assayed by sandwich ELISA according to the manufacturer’s directions (R & D Systems, Minneapolis, MN). The limit of detection of the assays was 15 pg/ml.

Western Blot Analysis—HCECs were washed with ice-cold 0.01 M PBS (pH 7.4) (Sigma), and cell lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM SDS, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (Cell Signaling Technology, Boston, MA) was added to the culture dishes. These were then scraped, and the lysates were homogenized using the Mixer Mill 300 (Qiagen). The protein concentration of the samples was then
determined using the BCA protein assay (Pierce). Equivalent amounts of protein samples were then separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk, washed, and incubated in primary antibody overnight at 4 °C with gentle agitation. Primary antibodies were diluted 1:1000 in 5% bovine serum albumin and 5% PBS-Tween 20 and include the following: rabbit anti-human MyD88, rabbit anti-human phospho-IRF-3, rabbit anti-human phospho-IkBα, rabbit anti-human phospho-SAPK/JNK, and mouse anti-human phospho-p38 MAP kinase antibodies (Cell Signaling Technology). The secondary antibody used for these stains was anti-rabbit horseradish peroxidase and anti-mouse horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Loading controls were determined using goat anti-human actin diluted 1:2000 in 5% milk and 5% PBS-Tween 20. The secondary antibody for this control was anti-goat horseradish peroxidase (Santa Cruz Biotechnology). Finally, the blots were visualized with ECL Western blotting detection reagent (GE Healthcare).

RNA Interference Knockdown in Vitro—RNA interference was achieved using siRNAs targeted against human MyD88 or TRIF. Nontargeting scrambled RNA was also included to control for nonspecific effects of the siRNA on the cells. All siRNAs were reconstituted to a stock concentration of 20 μM according to the manufacturer’s directions (Dharmacon RNA Technologies, Lafayette, CO). Cells were washed twice with 0.01M PBS (pH 7.4) (Sigma); 50 nm siRNA was first diluted in Opti-MEM® I reduced serum medium (Invitrogen); and Lipofectamine™ 2000 transfection reagent (Invitrogen) was diluted in Opti-MEM® I medium with reduced serum. The siRNA and transfection reagent were then mixed together to form liposomes containing siRNAs. These complexes were added to the HCECs for 6 h, the transfection reagents were removed, and the cells were placed into normal culture medium (described above).

Inhibition of Cell Signaling Components—Inhibition of JNK and p38 MAP kinase signaling was achieved by using SP600125 and SB203580, respectively (Calbiochem). Briefly, the HCECs were transfected as described above. 30 min prior to 24-h treatment of the cells with TLR agonists, the medium was changed, and either SP600125 (25 μM) or SB203580 (10 μM) was added to the cells. Me2SO was included as a negative control for the inhibitors because they are dissolved in this chemical.

Statistics—Statistical analysis was performed using an unpaired t test (Prism; GraphPad Software). p values less than 0.05 were considered significant.

RESULTS

Poly(I:C)-induced Corneal Inflammation Is Ablated in TLR3−/− and TRIF−/− Mice—Because double-stranded RNA activates receptors other than TLR3 (27–29), we first examined whether poly(I:C)-induced corneal inflammation is mediated by TLR3/TRIF signaling. Therefore, a 1-mm²-diameter area of the corneal epithelium of C57BL/6, TLR3−/−, and TRIF−/− mice was abraded and stimulated with the TLR3 agonist poly(I:C). At set time points, the mice were euthanized, and their eyes were snap frozen. The cellular infiltration in the corneal stroma was determined after F4/80 staining and direct counting of 5 μM corneal sections. Because our preliminary studies indicated that maximal F4/80+ cellular infiltration occurred after 72 h (data not shown), Fig. 1 shows results at this time point. Fig. 1A demonstrates that poly(I:C)-stimulated corneas had elevated F4/80+ cells (which includes macrophages and dendritic cells (23, 24)) in the corneal stroma of C57BL/6 mice. In contrast, there was no significant influx of cells above saline-injected controls (hatched line) in TLR3−/− and TRIF−/− mice. Furthermore, TLR2 activation using Pam3Cys did not result in any significant differences among C57BL/6, TLR3−/−, or TRIF−/− mice, indicating that these gene knockout mice respond normally to other TLR ligands (Fig. 1B). Taken together, these data clearly demonstrate that TLR3 and TRIF are functional in the cornea and that poly(I:C)-induced F4/80+ cellular infiltration to the corneal stroma is dependent on TLR3 and TRIF.

TLR3/TRIF-induced Corneal Inflammation Is Exacerbated in MyD88−/− Mice—Our previous work demonstrated that MyD88 plays a critical role in corneal inflammation induced by TLR2, TLR4, and TLR9 (22). Because TLR3 signaling is MyD88-independent (5, 30), we examined the effect of poly(I:C)-induced inflammation in the absence of MyD88 as an additional control group. MyD88+/+ and MyD88−/− corneas were treated as described above, and after 72 h, the eyes were sectioned and immunostained for either F4/80+ or NIMP+ (neutrophils) cells. Surprisingly, we found that the response in poly(I:C)-treated MyD88−/− corneas was exacerbated compared with littermate controls (Fig. 2). Specifically, MyD88−/− mice had significantly increased F4/80+ cells, and although there were no NIMP+ in TLR3-stimulated MyD88−/+ littermate or C57BL/6 corneas (data not shown),

FIGURE 1. Poly(I:C) induces TLR3/TRIF-specific disease within the mouse cornea. The central 1-mm² region of the corneal epithelium of C57BL/6, TLR3−/−, and TRIF−/− mice was abraded using a trephine and Algerbrush II. Corneas were then exposed to either poly(I:C) (40 μg) (A) or Pam3Cys (10 μg) (B) for 72 h. The eyes were then removed, sectioned, and immunostained for F4/80+ cells. The number of infiltrating cells was determined by counting 5-μm corneal sections. Naive mice had <37 F4/80+ cells/section, and water (trauma) controls, which are denoted by the hatched line, had <81 F4/80+ cells/section. The data presented are the means ± S.E. of the counts of six samples/group. The results represent three repeat experiments.
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**A: Central Corneal Stroma**

**B: TLR3 (Poly(I:C))**

**FIGURE 3. Increased stromal haze in poly(I:C)-treated MyD88<sup>−/−</sup> mice.** The mice were treated as described above with either poly(I:C) (40 μg) or Pam3Cys (10 μg) for 72 h, and corneal inflammation was assessed by Confoscan<sup>TM</sup> microscopy. A, representative Confoscan<sup>TM</sup> images of the central corneal stroma show poly(I:C)-induced cellular infiltrate in MyD88<sup>−/−</sup> mice. B, stromal haze in MyD88<sup>−/−</sup> and MyD88<sup>+/+</sup> mice was quantitated as described under “Experimental Procedures.” Water (trauma) controls are denoted by the hatched lines. The results are representative of three to five repeat experiments.

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**FIGURE 2. Increased cellular infiltration in poly(I:C)-treated MyD88<sup>−/−</sup> mice.** The central corneal epithelium of MyD88<sup>−/−</sup> and MyD88<sup>+/+</sup> mice was treated as described above with either poly(I:C) (20 μg), which was determined by dose-response experiment (not shown) to induce significant levels of inflammation, or Pam3Cys (10 μg) for 72 h. Eyes were abraded, sectioned, and immunostained for F4/80 (macrophages and dendritic cells) and NIMP<sup>+</sup> (neutrophils) cellular infiltrates. The number of infiltrating cells was determined by direct counting of 5-μm corneal sections. Naive corneas did not exhibit significant numbers of infiltrating F4/80<sup>+</sup> or NIMP<sup>+</sup> cells (<60.5 cells/section and <5 cells/section, respectively), and water (trauma) controls did not develop F4/80<sup>+</sup> and NIMP<sup>+</sup> cellular responses (<137.24 cells/section and <41.27 cells/section, respectively). Water controls are denoted by the dotted lines shown on the graphs. Data presented are the means ± S.E. of the counts with four samples/group. Results are representative of five repeat experiments.

poly(I:C)-treated MyD88<sup>−/−</sup> mice had a pronounced neutrophil infiltrate in the corneal stroma compared with littermate controls. As a positive control, MyD88<sup>+/+</sup> and MyD88<sup>−/−</sup> corneas were treated with the TLR2 agonist Pam3Cys, and as previously demonstrated (22), corneal inflammation was ablated in MyD88<sup>−/−</sup> mice. Taken together, we conclude that MyD88 has an inhibitory effect on TLR3/TRIF-induced F4/80<sup>+</sup> cell and neutrophil recruitment to the corneal stroma.

To determine the effect of MyD88 on TLR3/TRIF-induced corneal haze, MyD88<sup>−/−</sup> and littermate corneas were stimulated with poly(I:C), and corneal stromal haze, which is an inverse measure of corneal transparency, was measured by in vivo confocal microscopy. Confoscan<sup>TM</sup> images of the central corneal stroma of wild type and MyD88<sup>−/−</sup> mice 72 h after treatment with either saline control or poly(I:C) are shown in Fig. 3A. An intense cellular infiltrate was visible only in MyD88<sup>−/−</sup> mice treated with poly(I:C). Corneal haze was quantified as described under “Experimental Procedures,” and the results are shown in Fig. 3B. We found that TLR3/TRIF-induced stromal haze was significantly elevated in MyD88<sup>−/−</sup> mice compared with littermate control animals, which is consistent with elevated F4/80<sup>+</sup> and NIMP<sup>+</sup> cellular infiltrates described in Fig. 2. No inflammation was observed in saline-injected controls (hatched lines). These findings demonstrate that MyD88 has a negative regulatory effect on TLR3/TRIF-induced F4/80<sup>+</sup> cell and neutrophil recruitment to the corneal stroma and in the development of corneal haze, which together comprise the inflammatory response in the cornea.

MyD88 Has No Effect on TLR3/TRIF-activated Macrophages—Because there are resident macrophages in the normal corneal stroma (31) and to determine whether the down-regulatory role of MyD88 on TLR3/TRIF responses is a more generalized phenomenon, we examined the response of MyD88<sup>−/−</sup> bone marrow macrophages to poly(I:C).

Bone marrow cells were collected from C57BL/6 and TRIF<sup>−/−</sup> mice and from MyD88<sup>−/−</sup> and wild type littermates, and macrophage precursors were expanded in culture as described (26). Macrophages were then stimulated with either poly(I:C) or Pam3Cys, and cytokine production was measured by ELISA. As shown in Fig. 4A, CCL5/RANTES and CXCL10/IP-10 were increased in C57BL/6 and MyD88<sup>−/−</sup> macrophages stimulated with poly(I:C), with no significant differences between the groups. CXCL1/KC and CXCL2/MIP-2 were not produced in response to TLR3/TRIF stimulation for either group of mice, consistent with previous reports that these are MyD88-dependent chemokines (22). In contrast, TLR2 induced CXCL1/KC, CXCL2/MIP-2, CCL5/RANTES, and CXCL10/IP-10 production, which was completely dependent on MyD88 as reported (1).

As further controls for TLR3 and TLR2 activation of macrophages, bone marrow-derived macrophages from C57BL/6 and TRIF<sup>−/−</sup> mice were stimulated with either poly(I:C) or Pam3Cys, and chemokines were measured. Fig. 4B demonstrates that poly(I:C)-activated TRIF<sup>−/−</sup> macrophages produced significantly less CCL5/RANTES and CXCL10/IP-10, and poly(I:C) did not induce production of either CXCL1/KC or CXCL2/MIP2. Furthermore, Pam3Cys stimulated production of all four chemokines, and no significant differences were observed between C57BL/6 and TRIF<sup>−/−</sup> macrophages. Taken together, these findings demonstrate that macrophages respond to TLR ligands as anticipated and indicate that the MyD88 inhibition of TLR3/TRIF responses in vivo are likely not be due to the response of resident macrophages in the cornea.

MyD88-dependent Inhibition of TLR3/TRIF Responses in Human Corneal Epithelial Cells HCECs—Corneal epithelial cells are the predominant cell type at the ocular surface and can
produce cytokines and β-defensins in response to TLR2 ligands and type 1 interferon in response to TLR3 ligands (9, 10, 12–14, 16, 55). Therefore, we next examined the role of MyD88 on TLR2- and TLR3-stimulated HCECs.

HCECs were transfected with MyD88 siRNA and treated with poly(I:C). To confirm that MyD88 was effectively knocked down by siRNA, the lysates were collected at either 24 or 48 h post-transfection and processed for Western blot analysis using anti-MyD88 antibodies. As shown in Fig. 5A, MyD88 expression was significantly reduced at 24 and 48 h compared with controls that were either mock transfected (Lipofectamine alone) or transfected with scrambled, nontargeting control siRNA. Neither control group inhibited MyD88 protein expression compared with untransfected cells (Fig. 5A).

To determine the effect of MyD88 on TLR3-induced chemokine production by HCECs, cells were either mock transfected or transfected with MyD88 siRNA, TRIF siRNA, or scrambled nontargeting control siRNA. HCECs were then treated with the TLR3 agonist poly(I:C) or the TLR2 agonist Pam3Cys. Fig. 5B demonstrates that poly(I:C)-induced CCL5/RANTES production was inhibited by 76% in TRIF knockdown cells when compared with untransfected, control HCECs, consistent with the reported role of TRIF (2, 3). In marked contrast, CCL5/RANTES production in MyD88 knockdown cells was significantly elevated (2.5-fold) over untransfected cells, indicating that as in the murine model of TLR3/TRIF-induced corneal inflammation, MyD88 has an inhibitory effect on TLR3/TRIF activated corneal epithelial cells.

Interestingly, we found that poly(I:C)-induced CXCL8/IL-8 was also decreased in MyD88 knockdown HCECs when compared with untransfected controls. Although the mechanism has yet to be determined, it is likely that TLR3 induced IL-1 signaling is also impaired by MyD88 knockdown. To determine the effect of MyD88 knockdown on TLR2 responses, HCECs were stimulated with Pam3Cys. As shown in Fig. 5B, TLR2-induced responses were reduced by ∼47% in the absence of MyD88, which is consistent with the known role of MyD88 on TLR2 activation (2, 3). CCL5/RANTES production was not detected in any group of transfected HCECs treated with Pam3Cys. These results clearly demonstrate that knockdown of MyD88 results in significantly elevated CCL5/RANTES production by HCECs, thereby indicating that MyD88 inhibition of TLR3/TRIF-induced corneal inflammation is mediated by epithelial cells.

IRF-3 and NF-κB Do Not Regulate MyD88-dependent Inhibition of TLR3/TRIF-activated HCECs—To identify a molecular basis for the elevated response shown above, we next examined the role of MyD88 on TLR3/TRIF-induced activation of the
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As shown in Fig. 7A, phosphorylated JNK was greatly increased in MyD88 siRNA-transfected HCECs compared with untransfected cells; however, there was no significant difference in the level of p38 MAP kinase activation. Fig. 7A also demonstrates clearly that MyD88 knockdown was effective.

To determine whether JNK signaling is essential for increased production of CCL5/RANTES in the absence of MyD88, HCECs were transfected with MyD88 siRNA as described above and treated with inhibitors for either JNK (SP600125) or p38 (SB203580) prior to stimulation with poly(I:C). As shown in Fig. 7B, SP600125 significantly inhibited CCL5/RANTES production (61% inhibition) compared with untransfected cells; however, there was no significant difference in the level of p38 MAP kinase activation. Taken together, these results demonstrate that TLR3/TRIF-induced phosphorylation of JNK, but not p38, is negatively regulated by MyD88 in HCECs.

DISCUSSION

Results of the current study broaden our general understanding of TLR signaling and regulation by revealing a novel regulatory role for MyD88 within the context of TLR3/TRIF-induced corneal inflammation. Using gene knockout mice, we demonstrated that the mouse cornea responds to poly(I:C) in a TLR3- and TRIF-dependent manner. Surprisingly, this inflammation is not independent of MyD88 as described for macrophages; instead the observed inflammatory response in the cornea is significantly enhanced in the absence of MyD88, both in poly(I:C)-treated MyD88<sup>−/−</sup> corneas and in MyD88 knockdown

transcription factors IRF-3 and NF-κB. TRIF, but not MyD88, activates IRF-3, whereas NF-κB is activated by either the TRIF or MyD88 pathway as shown in other studies (1, 27). Therefore, to determine the role of TRIF and MyD88 in activation of IRF-3 and NF-κB, HCECs were treated with the TLR3 agonist poly(I:C) or the TLR2 agonist Pam3Cys as before. After 0 to 4 h, the cells were lysed, and IRF-3 and 1xBo phosphorylation was examined by Western blot analysis. As shown in Fig. 6A, phosphorylated IRF-3 was induced in a time-dependent manner after stimulation with poly(I:C), but not Pam3Cys. In contrast, phosphorylated 1xBo was induced after incubation with either poly(I:C) or Pam3Cys.

To determine whether MyD88-dependent inhibition of TLR3/TRIF responses is due to regulation of either IRF-3 or NF-κB signaling, HCECs were transfected with MyD88 siRNA and treated with poly(I:C) or Pam3Cys as above. As shown in Fig. 6B, there was no difference in the level of phosphorylated IRF-3 or phosphorylated 1xBo signaling after MyD88 knockdown, indicating that MyD88-dependent inhibition of TLR3/TRIF is not at the level of IRF-3 or NF-κB signaling.

MyD88-dependent Inhibition of TLR3/TRIF in HCECs Is Mediated by JNK, but Not p38—Because TRIF signaling also involves activation of MAP kinases (8), we examined the effect of MyD88 knockdown on TLR3/TRIF-induced MAP kinase activation in HCECs. The cells were transfected with MyD88 siRNA as described above and stimulated with poly(I:C), phosphorylation of JNK, but not p38, was greatly increased in MyD88 siRNA-transfected HCECs compared with untreated cells; however, there was no significant difference in the level of p38 MAP kinase activation. Fig. 7A also demonstrates clearly that MyD88 knockdown was effective.

FIGURE 6. Activation of IRF-3 or NF-κB signaling by TLR3/TRIF activation is not regulated by MyD88. A, HCECs were treated for 0, 0.25, 0.50, 1, 2, 3, 4, and 6 h with either poly(I:C) (5 μg/ml) or Pam3Cys (1 μg/ml). At the indicated times, the cell lysates were collected, and activation of IRF-3 (phosphorylated IRF-3) or NF-κB (phosphorylated IkBα) signaling was determined by Western blot. Actin was included as a loading control. The results presented are representative of five similar experiments. B, HCECs were grown to 40% confluency, EGF-starved, and treated with poly(I:C), but not Pam3Cys. In contrast, CCL5/RANTES production in the absence of MyD88 siRNA as described above. Mock transfected samples were also obtained (not shown) and demonstrated that there was no activation caused by Lipofectamine alone. 48 h after the transfection, HCECs were treated with poly(I:C) (5 μg/ml) for 0, 15, 30, 60, 120, and 240 min. The cell lysates were collected, and activation of IRF-3 (phosphorylated IRF-2) or NF-κB (phosphorylated IkBα) signaling was determined by Western blot. Actin was included as a loading control. The results presented are representative of five similar experiments.

C). JNK and p38 phosphorylation were examined by Western blot analysis.

As shown in Fig. 7A, phosphorylated JNK was greatly increased in MyD88 siRNA-transfected HCECs compared with untransfected cells; however, there was no significant difference in the level of p38 MAP kinase activation. Fig. 7A also demonstrates clearly that MyD88 knockdown was effective.

To determine whether JNK signaling is essential for increased production of CCL5/RANTES in the absence of MyD88, HCECs were transfected with MyD88 siRNA as described above and treated with inhibitors for either JNK (SP600125) or p38 (SB203580) prior to stimulation with poly(I:C). As shown in Fig. 7B, SP600125 significantly inhibited CCL5/RANTES production (61% inhibition) compared with untreated HCECs. In contrast, CCL5/RANTES production in the presence of p38 inhibitor SB203580 was unaffected. Taken together, these results demonstrate that TLR3/TRIF-induced phosphorylation of JNK, but not p38, is negatively regulated by MyD88 in HCECs.

FIGURE 5. Poly(I:C)-induced CCL5/RANTES production is exacerbated in the absence of MyD88. HCECs were grown to ~40% confluency, EGF-starved, and transfected with either scrambled nontargeting control, TRIF siRNA, or MyD88 siRNA as described under “Experimental Procedures.” A, at 48 and 72 h post-transfection the cell lysates were collected and analyzed by Western blot to confirm the knockdown of MyD88. Actin was included as a loading control. The results are representative of four similar experiments. B, additionally, 24 h after transfection, the cells were treated with either poly(I:C) (5 μg/ml) or Pam3Cys (1 μg/ml). The cell culture supernatants were then collected, and the presence of CCL5/RANTES and CXCL8/IL-8 was determined by ELISA (limit of detection < 15 pg/ml). The results presented are representative of six similar experiments with four samples/group (means ± S.E.).
HCECs. Furthermore, we demonstrate that the exacerbated response in HCECs is mediated by JNK activation. Consistent with previous reports (1), we did not observe this response in macrophages, because MyD88\textsuperscript{-/-} macrophages respond normally to poly(I:C).

Given these findings, we propose the following model for MyD88-dependent inhibition of TLR3/TRIF responses in the cornea (Fig. 8): 1) Physical disruption of the corneal epithelium and exposure to microbial products, including lipoproteins (Pam\textsubscript{3}Cys) or double-stranded RNA (poly(I:C)) initiates MyD88-dependent TRIF-dependent signaling. (Lipopolysaccharide/TLR4 activation, which is not addressed in the current study, utilizes both of these signaling pathways (2).) 2) The reported common MyD88 pathway activates MAP kinase (p42/p44, JNK, and p38) and NF-\kappaB, resulting in expression of CXC chemokines, including CXCL8/IL-8. 3) In contrast, the TRIF pathway activates IRF-3, MAP kinases (JNK and p38), and NF-\kappaB, resulting in CCL5/RANTES production. 4) Chemokine production stimulates macrophage and neutrophil infiltration into the cornea, and consequently, normal corneal epithelial, stromal, and endothelial cell function is impaired with the loss of corneal clarity and lack of visual acuity. 5) These observations also extend our understanding of the role of MyD88 in TLR signaling by demonstrating its function as a negative regulator of TLR3/TRIF-induced JNK activation in corneal epithelial cells, because the absence of MyD88 leads to exacerbated responses \textit{in vitro} and \textit{in vivo}.

MyD88-dependent inhibition of TRIF responses does not appear to be a generalized phenomenon, because we did not detect similar regulatory pathways within macrophages. Therefore, we propose that MyD88, either directly or indirectly, negatively regulates TLR3/TRIF-induced CCL5/RANTES production through a JNK-dependent pathway of activation and that MyD88 down-regulates TLR3/TRIF-induced corneal inflammation.

MyD88 was originally described as part of the IL-1 receptor signaling complex (32); however, MyD88 is also essential for the activation of all TLR-mediated signaling pathways except TLR3. Studies presented here clearly demonstrate that MyD88 inhibits TLR3/TRIF responses in corneal inflammation. In addition, a recent study of polymicrobial peritonitis shows that MyD88\textsuperscript{-/-} mice have elevated CXCL10/IP-10 and CCL5/RANTES production (33). Furthermore, microarray studies have identified a low frequency of interferon-inducible genes that are up-regulated in MyD88\textsuperscript{-/-} macrophages stimulated with lipopolysaccharide (4). Both of these studies, therefore, support the concept that MyD88 can suppress innate immune responses associated with the TRIF pathway. Results from the current study extend these findings using an \textit{in vivo} model of inflammation that clearly demonstrates a negative regulatory role of MyD88 on the inflammatory response and attributes...
these effects to epithelial cells. Further studies will determine whether this is an epithelial cell-restricted event.

To determine the molecular basis underlying MyD88-dependent inhibition of TLR3/TRIF responses, we considered several functions associated with MyD88. First, TLR signaling is regulated by several molecules, including an alternatively spliced form of MyD88 known as MyD88 short (MyD88s), which lacks the intermediate domain through an in-frame deletion (34). In the current study, MyD88 and presumably MyD88s are depleted in both gene knockout mice and in MyD88 siRNA-transfected HCECs. Additionally, MyD88s is up-regulated in monocytes from sepsis patients (35), indicating that MyD88s regulates innate immune responses. Although it is possible that MyD88s inhibits TLR3/TRIF responses, MyD88s diminishes NF-κB signaling while leaving AP-1 signaling and JNK activation unaffected (36). Therefore, because MyD88s does not act through a JNK-dependent pathway and our work indicates that JNK is critically important for the effects that result from MyD88 deficiency, it is not likely that MyD88s is involved in this process.

In addition to MyD88s, there are several other regulators that may specifically control TLR3/TRIF activation, including A20, SARM (SAM and ARM-containing protein), phosphoinositide 3-kinase (PI3K), and IRAK1. A20 is a cysteine protease deubiquitinase (37), is produced early after NF-κB activation, and functions by cleaving ubiquitin from TRAF6 (38). TRAF6 is implicated in signaling cascades of all TLRs, including TLR3 (2, 8); therefore, it is possible that A20 regulates TLR3 signaling. Boone et al. (38) recently demonstrated that A20 down-regulates responses generated by TLR2, TLR3, and TLR9. Additionally, SARM functions as a specific negative regulator of the TRIF adaptor molecule by direct physical interactions with TRIF so that the adaptor is no longer available for either TLR3 or TLR4 signaling (39). It is possible that when MyD88 is depleted, A20 and/or SARM are also reduced, although there is no evidence linking MyD88 to either of these directly.

Another possible mechanism underlying MyD88 inhibition of TLR3/TRIF responses is by inhibition of PI3K. PI3K regulates TLR responses by inhibiting p38, JNK, and NF-κB signaling in dendritic cells (8, 37). Furthermore, PI3K inhibition results in TLR3 and TLR4 induced NF-κB activation and interferon-β responses in monocyte-derived dendritic cells (40), indicating a link between PI3K and TRIF. Because MyD88 and PI3K form a complex in mouse macrophages during TLR4 signaling (41), and MyD88 also forms a complex with AKT, which is downstream of PI3K during IL-1 signaling (42), it is possible that the negative activity of MyD88 results from forming a complex with PI3K. The amount of available PI3K for TLR3 activation would then be restricted, resulting in negative regulation of TLR3 signaling. Conversely, when MyD88 is absent from the system, PI3K may become more available to TLR3, and signaling is driven forward, leading to increased inflammatory responses, as our data demonstrate. The role of PI3K in TLR signaling is thought to be very cell type-specific, because it can participate in both negative regulation (37, 40) and positive signaling (8). Future studies will evaluate the role of PI3K in this model.

Another possible mediator in MyD88 inhibition of TLR3/TRIF responses is IRAK-1. Jiang et al. (43) showed increased levels of activated p38 and JNK in the absence of IRAK-1. Furthermore, the MAP kinase kinase kinase upstream of IRAK-1, TAK-1, is also elevated (43). Therefore, it is possible that IRAK-1 is participating in this unique regulatory mechanism that we have observed. Alternatively, IRAK-1 and MyD88 may independently regulate TLR3 signaling. Future studies will also examine the role of TAK-1 and IRAK-1 in MyD88-negative regulation of TLR3/TRIF signaling.

Although we have yet to determine whether MyD88 negative regulation of TLR3/TRIF responses is dependent on ligand binding or is ligand-independent, we propose that it is more likely to be ligand-dependent, with MyD88 and TRIF recruited to the TLR3 receptor complex after ligand binding, and MyD88 inhibits TRIF signaling at this stage.

The role of TLR3 in viral innate immunity has yet to be fully determined, although early studies suggested that TLR3 might not actually contribute to anti-viral responses against lymphocytic choriomeningitis, vesicular stomatitis virus, and reovirus (44). In contrast, TLR3 plays a critical role in the recognition of viruses, such as encephalomyocarditis virus and Semliki Forest virus (45, 46). A broad range of activity for TLR3 against viruses is therefore possible, because these are both positive strand single-stranded RNA viruses, indicating that TLR3 is recognizing double-stranded RNA intermediates in the viral replication cycle (45). Furthermore, TLR3 can partially control mouse cytomegalovirus (47), indicating that TLR3 is involved in recognition of herpesviruses. Because herpes simplex virus type 1, which is an α-herpesvirus (48), is the most frequent cause of corneal viral infection, and TLR3 responds to cytomegalovirus (47), it is possible that TLR3 is also necessary for recognition and response to these viral pathogens in the cornea.

Given that TLR3 has a broad range of reactivity, it is therefore critical that TLR3 responses are very tightly regulated, and because epithelial cells are on the ocular surface, TLR3 activation of these cells is likely to be tightly regulated to prevent or minimize responses that might damage the eye and cause visual impairment. Our data support this concept, because we observed a high level of MyD88-dependent negative regulation within the human corneal epithelial cells, whereas we were unable to demonstrate similar regulation in macrophages. TLRs expressed by epithelial cells are regulated in several ways, including selective expression on the basolateral rather than the apical surface (49), and lower TLR expression levels (50–53). Another regulatory mechanism is the expression of co-receptors such as MD-2 for lipopolysaccharide responses via TLR4. Corneal MD-2 expression is tightly regulated (54), although the source of MD-2 can be either endogenous within the cell or exogenous body fluids such as tears.

In summary, our findings demonstrate a novel regulatory mechanism in epithelial cells, because poly(I:C)-induced corneal disease is exacerbated in the absence of functional MyD88 protein. Activity that is normally tightly controlled by MyD88 and likely includes anti-viral TLR3/TRIF responses and possibly TLR4/TRIF responses.

Further studies will examine the molecular basis underlying this novel MyD88 regulation of TLR3/TRIF signaling within epithelial cells. Furthermore, elucidation of the mechanisms by which MyD88 exerts its negative regulatory effects upon the
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TLR3/TRIF pathway will provide insights necessary for rational drug design for infectious and inflammatory diseases in the cornea and in other tissues that utilize this pathway.

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