Interferon-γ-induced MD-2 Protein Expression and Lipopolysaccharide (LPS) Responsiveness in Corneal Epithelial Cells Is Mediated by Janus Tyrosine Kinase-2 Activation and Direct Binding of STAT1 Protein to the MD-2 Promoter

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The inability of epithelial cells from the cornea and other tissues to respond to LPS is reportedly due to low expression of the TLR4 co-receptor MD-2. We generated MD-2−/− bone marrow chimeras, and showed that MD-2 expression on non-myeloid cells was sufficient to mediate LPS-induced corneal inflammation. As IFN-γ is produced during Pseudomonas aeruginosa corneal infection, we examined the role of this cytokine on MD-2 expression by primary human corneal epithelial (HCE) cells and HCE cell lines. Exogenous IFN-γ was found to induce MD-2 mRNA, MD-2 cell surface expression, and LPS responsiveness as determined by p65 translocation to the nucleus and production of IL-6, CXCL1, and CXCL8/IL-8. Incubation with either the AG490 JAK2 inhibitor or with STAT1 siRNA blocked STAT1 phosphorylation and MD-2 transcription. Furthermore, EMSA analysis demonstrated that STAT1 binds to the MD-2 promoter, indicating that STAT1 is an MD-2 transcription factor. Together, these findings demonstrate that IFN-γ induces MD-2 expression and LPS responsiveness in HCE cells by JAK2-dependent STAT1 activation and direct binding to the MD-2 promoter. Furthermore, given our findings on LPS-induced corneal inflammation, it is likely that IFN-γ-induced MD-2 expression by corneal epithelial cells contributes to the host response in vivo, determining the extent of tissue damage and bacterial clearance.

MD-2 is a ~25-kDa LPS-binding protein that forms a heterodimer with TLR4 (1–3). The crystal structure of the TLR4-MD-2 complex shows that when five of the six acyl chains of lipid A bind MD-2 and one chain binds to TLR4, it undergoes a structural change that facilitates dimerization and cell activation (4). The canonical signaling pathway involves recruitment of MyD88 and Mal/TIRAP adaptor molecules to the TIR domain of TLR4, which initiates the canonical signaling pathway leading to NFκB translocation to the nucleus and production of proinflammatory and chemotactic cytokines (5, 6). The TLR4-MD-2 complex is also internalized (7), leading to recruitment of adaptor molecules TRIF-related adaptor molecule (TRAM) and TRIF and to nuclear translocation of NFκB and IRF3 and production of type 1 IFNs (5, 6).

In macrophages and dendritic cells, MD-2 is expressed constitutively, thereby mediating LPS responsiveness (1, 8). In contrast, epithelial cells do not constitutively express MD-2 and do not respond to LPS unless MD-2 is provided exogenously (9–11). MD-2 expression in intestinal, airway, oral, and conjunctival epithelial cells is induced by IFN-γ (12–16), although neither the source of IFN-γ or the mechanism of induction has been fully elucidated.

In the current study we examine the role of MD-2 and expression of IFN-γ in the context of corneal infection and inflammation and determine the molecular basis for LPS hypo-responsiveness and MD-2 expression in the presence of IFN-γ in human corneal epithelial cells.

EXPERIMENTAL PROCEDURES

Source of Reagents—Keratinocyte serum-free medium, trypsin, Hanks’ balanced salt solution, and gentamycin were purchased from Invitrogen. The Ultrapure LPS was purchased from Invivogen (SanDiego). ELISA detection kits for IL-6, CXCL8/IL-8, and CXCL1 were purchased from R&D Systems Inc. (Minneapolis, MN). Peroxidase-conjugated goat antimouse IgG and goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against asialo ganglio-N-tetraosylceramide (asialoGM1) was purchased from Wako (Richmond, VA). Collagenase IV was from Sigma. Antibody to NK1.1 was obtained from ebiosciences (San Diego, CA). The AG490 JAK2 inhibitor was purchased from Tocris Bioscience, Ellisville, MO.
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Animals—For bone marrow chimera experiments, 6–8-week-old eGFP/C57BL/6TgN (ACTbEGFP) 10sb mice were used (The Jackson Laboratory). MD-2−/− mice were obtained with permission from Dr. K. Miyake, University of Tokyo. All animals were housed in specific pathogen-free conditions in microisolator cages and treated in accordance with the guidelines provided in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Chimeric Mice—Bone marrow cells from C57BL/6TgN (ACTbEGFP) 10sb (eGFP) mice or MD-2−/− were isolated as described (17). Recipient mice received 2 × 600 gray doses of whole body irradiation 3 h apart. Immediately after the second dose, mice were injected intravenously with 5 × 10^6 cells in 200 μl of DMEM. Mice were used in experiments 2 weeks after bone marrow transplantation, when eGFP + cells were detected in the cornea (17).

Mouse Models of Corneal Inflammation and Infection—Mice were anesthetized and the corneal epithelium was abraded with three parallel 1-mm scratches using a 26-gauge needle. For corneal inflammation, 20 μg of UltraPure TLR4-specific Escherichia coli LPS (strain K12; Invivogen) in 2 μl of sterile endotoxin-free water were placed on the ocular surface as described (9, 18, 19). After 24 h, mice were euthanized, corneal haze was measured by in vivo confocal microscopy using the Nidek ConfoscanTM, and neutrophil recruitment to the cornea was examined by immunohistochemistry using rat anti-mouse neutrophil antibody (NIMP-R14; Abcam, Cambridge, MA). Corneal haze was calculated from stromal thickness and light intensity of each image of the corneal stroma using Prism (GraphPad Software, San Diego, CA) as described (9, 19). Corneal infection studies were performed as recently described (19). Briefly, corneas were abraded as before, and 1 × 10^5 Pseudomonas aeruginosa strain 19660 (ATCC, Manassas, VA), 19660Δflic, PAO1 and PAO1Δflic (generated by Dr. Arne Rietzsch, Case Western Reserve University) were placed on the ocular surface. A 2-mm diameter punch from a contact lens was used to keep the bacterial suspension in place for 2 h, whereas the mice were under anesthesia. After 24 h, mice were euthanized, corneas were dissected and homogenized, and IFN-γ was measured by ELISA according to the manufacturer’s directions (R&D systems, Minneapolis, MN).

Human Corneal Epithelial Cell Lines—The SV40-immortalized human corneal epithelial cell line 10.014 pRSV-T (HCEC) was obtained from the American Type Culture Collection (Manassas, VA) and maintained by culturing in keratinocyte serum-free medium without EGF. The hTCEpi culture was at 70–80% confluency, cells were incubated overnight according to the manufacturer’s directions (R&D systems), 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 70% confluent (21).

Flow Cytometric Analysis—HCEC and HCET and primary human corneal epithelial cells treated or untreated with IFN-γ were incubated with human IgG (20 μg/ml) for 15 min followed by incubation with anti-TLR2, anti-TLR4, or anti-INFγR2 (eBiosciences) and anti-MD-2 (Abcam) or isotype control antibodies (BD Biosciences). For evaluation of natural killer (NK) cells in cornea of C57BL/6 mice, corneas were digested with collagenase IV as described (19), and cells were incubated with FITC-labeled rabbit anti-NK1.1.

Cytokine ELISA—Human corneal cells were grown overnight in 12-well plates (4 × 10^5 cells/well), supernatants were collected, and IL-6, IL-8, and CXCL1 were determined by sandwich ELISA according to the manufacturer’s directions (R&D systems). Absorption was measured at 450 nm on a Versa Max microplate reader using SoftMaxPro software 5.2 (Molecular Devices, Sunnyvale, CA).

Western Blot Analysis—Human corneal epithelial cells were incubated with IFN-γ and/or AG490 for 24 h, washed in PBS, and lysed using cold 1× lysis buffer (Cell Signaling Technology, Beverly, MA). Total protein was quantified using standard BCA assay, and 10 μg of protein were separated by 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with phosphorylated (p)-STAT1, total STAT1 (R&D Systems), and β-actin (Cell Signaling Technology). Proteins were detected using HRP-conjugated secondary antibodies and developed with Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

RNA Isolation and Quantitative PCR—Quantitative real-time PCR was used to evaluate of MD-2 mRNA expression in human corneal epithelial cells. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), and 1 μg of RNA was reverse-transcribed using Superscript II (Invitrogen) according to the manufacturer’s protocol. β-Actin was used as reference gene. Real-time PCR was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the SYBR Green PCR Master Mix for 40 cycles. The primers used for human MD-2 gene were based on GenBankTM (accession number NT_008183) for human MD-2 and designed using PrimerBLAST (NCBI). Primer sequences are shown in Table 1.
Relative quantities of MD-2 mRNA expression were normalized using the $2^{-\Delta\Delta Ct}$ method with $\beta$-actin as the housekeeping gene.

$p65$ Nuclear Translocation—Corneal epithelial cells were cultured as $5 \times 10^4$ cells/well in 12-well plates on sterile coverslips and treated with IFN-γ alone or followed by LPS (100 ng/ml) for 60 min. Translocation of the NFκB p65 subunit was determined as described for bone marrow-derived cells (19). Briefly, corneal epithelial cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100, and incubated with rabbit anti-mouse p65 (1:100; eBioscience Ltd). p65 binding was detected using Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (Molecular Probes Inc.).

Electrophoretic Mobility Shift Assay (EMSA)—Double-stranded biotin-labeled oligonucleotide primers were synthesized based on DNA sequence for the human MD-2 promoter (accession number NT_008183), and sequences are shown in Table 1. Nuclear extracts (6 μg of protein) from cells stimulated with or without IFN-γ were incubated in presence of poly(dI-dC) and wild type biotinylated probes for 30 min at room temperature. For competition assays, nuclear extracts were preincubated with unlabeled wild type probes for 10 min. And for supershift assays, nuclear extracts were preincubated with anti-STAT1 and p-STAT1 polyclonal antibody for 1 h on ice. The samples were analyzed by 5% non-denaturing PAGE followed by transfer to Biodyne B Nylon membranes (PALL Life Sciences).

RNA Interference Knockdown in Vitro—RNA interference was achieved using siRNAs targeted against human STAT1. Non-targeting 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 1× PBS (pH 7.4) (Sigma); 50 nM siRNA was first diluted in Opti-MEM I-reduced serum medium (Invitrogen), and Lipofectamine TM 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 corneal epithelial cells for 6 h, the transfection reagents were removed, and the cells were placed into normal culture medium.

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

### RESULTS

**LPS Responsiveness in Corneal Inflammation Is Mediated by MD-2 Expressed on Non-myeloid Cells**—To examine the role of MD-2 in myeloid and non-myeloid cell responses in LPS-induced corneal inflammation, we generated bone marrow chimeras with MD-2−/− and C57BL/6 recipient mice using donor cells from MD-2−/− or C57BL/6 mice expressing eGFP under a $\beta$-actin promoter. After 2 weeks, which is sufficient time for reconstitution (17, 19), corneas were abraded, and LPS was placed on the cornea as described (18). Trauma control corneas were abraded, and endotoxin-free water was added. After 24 h, eyes were sectioned and stained for neutrophils, and the number of neutrophils per 5-μm section is shown in Fig. 1A. Naïve and H2O-treated (trauma control) C57BL/6 and MD-2−/− corneas had <20 neutrophils/section. LPS-treated C57BL/6/C57BL/6 (donor/recipient) corneas had significantly higher neutrophil numbers (111.5 ± 7.4) compared with LPS-treated MD-2−/−/MD-2−/− chimera (26.7 ± 5.8), which is in agreement with our previous study showing impaired LPS responses in MD-2−/− mice (9). Neutrophil numbers in corneas of C57BL/6/MD-2−/− and MD-2−/−/C57BL/6 bone marrow chimeras were significantly higher than MD-2−/−/MD-2−/− corneas, indicating that MD-2 expression non-myeloid cells is sufficient to induce neutrophil recruitment to the cornea.

To determine the effect on light passage through the cornea and disruption of corneal transparency, we measured corneal stromal reflectivity by in vivo confocal microscopy as described (9, 19). Fig. 1B shows that as with neutrophils, reflectivity was significantly lower in the absence of MD-2 but that MD-2 expression on either myeloid or non-myeloid cells increased compared with MD-2−/−/MD-2−/− corneas. Together, these findings demonstrate that LPS responsiveness in the cornea can be mediated by MD-2 expressed on resident, non-myeloid cells including epithelial cells.

**Elevated IFN-γ in the Corneal Stroma and MD-2 Expression in the Corneal Epithelium after Infection with P. aeruginosa**—Given that (i) MD-2 expression on non-myeloid cells regulates corneal inflammation (Fig. 1), (ii) MD-2 regulates the outcome of P. aeruginosa corneal infection (19), and (iii) that IFN-γ regulates MD-2 mRNA expression in gut, oral, and conjunctival epithelial cells (14–16), we next examined if IFN-γ is produced in the cornea at this time point after infection. The corneal epithelium of C57BL/6 mice was abraded and infected with $1 \times 10^3$ P. aeruginosa strain 19960 or $1 \times 10^3$ PAO1 or with aflagellar (ΔflIC) mutants as described (19). After 24 h, corneas were excised.

### TABLE 1

| Oligonucleotide sequences | Forward primer | Reverse primer | Description |
|-------------------------|----------------|----------------|-------------|
| MD-2                    | 5′-AGACGCAAGTTGGTTCTGCAA-3′ | 5′-TTGGAAGATTCATGGTGTTGACA-3′ | STAT1 binding sequence is underlined. |
| β-Actin                 | 5′-AGACGCAAGTTGGTTCTGCAA-3′ | 5′-TTGGAAGATTCATGGTGTTGACA-3′ | STAT1 binding sequence is underlined. |
| Wild type STAT1         | 5′-AGACGCAAGTTGGTTCTGCAA-3′ | 5′-TTGGAAGATTCATGGTGTTGACA-3′ | STAT1 binding sequence is underlined. |
| Mutated STAT1           | 5′-AGACGCAAGTTGGTTCTGCAA-3′ | 5′-TTGGAAGATTCATGGTGTTGACA-3′ | STAT1 binding sequence is underlined. |
| MD-2 promoter           | 5′-AGACGCAAGTTGGTTCTGCAA-3′ | 5′-TTGGAAGATTCATGGTGTTGACA-3′ | STAT1 binding sequence is underlined. |

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and homogenized, and IFN-γ was measured by ELISA. Fig. 2A shows that infection with each strain of *P. aeruginosa* stimulated more IFN-γ production than trauma controls (PBS-treated); however, Δ*fliC* mutants induced significantly less IFN-γ than the flagellate parent strains, indicating that flagellin may also stimulate IFN-γ production.

IFN-γ is produced by CD4+ Th1 cells, CD8+ cells, and NK, and NK-T cells (23); however, given that 24-h post-infection is
early for development of adaptive immunity, we determined if NK cells are a source of IFN-γ at this time point. To deplete NK and NK-T cells, mice were injected intraperitoneally with asialoGM1 antibody 2 days and 2 h before infection with P. aeruginosa strain 19960. A control group of infected mice was injected intraperitoneally with normal rabbit IgG. As shown in Fig. 2B, IFN-γ production was ablated in asialoGM1-treated compared with control mice. Consistent with this finding, flow cytometric analysis showed that NK1.1+ cells were depleted in asialoGM1-treated mice (Fig. 2C). Although this population was too small to detect intracellular IFN-γ by flow cytometry, depletion studies clearly demonstrate that NK1.1+ cells are essential for production of IFN-γ at this time point.

**IFN-γ Up-regulates Expression of MD-2, but Not TLR4, TLR2, or IFN-γR2, in Human Corneal Epithelial Cells—**Our findings that IFN-γ is produced during corneal infection in mice and that inflammation is regulated by MD-2 on non-myeloid cells are consistent with the reported role of IFN-γ on MD-2 expression in epithelial cells (14–16). As mouse primary corneal epithelial cells are difficult to culture and cell lines are not available, we examined the effect of IFN-γ on MD-2 mRNA and protein expression in primary human corneal epithelial (HCE) cells in an SV40-transfected HCE cell line (HCEC) (9, 21, 26) and in a telomerase immortalized HCE cell line (HCET) (20).

HCE cells were stimulated with 40 ng/ml IFN-γ, total RNA was isolated, and MD-2 gene expression was measured by real time PCR relative to β-actin, which was used as the reference gene. PCR products were also run in an agarose gel. Fig. 3A shows that in two cell lines and primary cells from two donors, MD-2 gene expression increases within 6 h and further after 24 h of incubation with IFN-γ.

Cell surface expression of MD-2 was examined by flow cytometry after 6 h of incubation with IFN-γ. Unstimulated cells showed a similar profile to the isotype control, indicating no constitutive MD-2 expression. However, IFN-γ incubation induced 51.4, 59.9, and 60.2% surface MD-2 expression of primary cells and HCEC and HCET cells respectively (Fig. 3B, Table 2).

In marked contrast to MD-2, TLR4 expression was elevated compared with the isotype control in unstimulated primary, HCEC, and HCET cells, indicating constitutive surface expression, and incubation with IFN-γ had a minimal effect (Table 2). Similar to TLR4, TLR2 and IFN-γR2 expression was constitutive, with no effect of incubation with IFN-γ (Fig. 3B). These findings demonstrate that IFN-γ selectively activates MD-2 and also indicates that LPS unresponsiveness of these cells is not due to the absence of TLR4 expression on the cell surface as reported by others (11, 27, 28).

**IFN-γ Confers LPS Responsiveness in Human Corneal Epithelial Cells—**As IFN-γ stimulates MD-2 expression on the surface of HCE cells, we next examined if increased surface expression leads to LPS responsiveness in these cells. HCEC, HCET, and primary human corneal epithelial cells were incubated with IFN-γ for 2 h before the addition of LPS for 1 h, and p65 translocation to the nucleus was examined by fluorescence microscopy. Fig. 4A shows that p65 translocation does not occur in human corneal epithelial cells treated with IFN-γ or LPS alone, but only after incubation with IFN-γ and LPS.

To measure the effect of IFN-γ on HCE cell cytokine production, primary HCE cells and the HCE cell lines were incubated for 2 h with IFN-γ before the addition of LPS. After 24 h, cytokine production in cell supernatants was measured by ELISA. As shown in Fig. 4B, primary HCE cells and both HCE cell lines produced IL-6, IL-8, and CXCL1 only when incubated with both LPS and IFN-γ. Together, these data clearly demonstrate that in addition to increasing MD-2 RNA and cell surface expression, IFN-γ confers LPS responsiveness in HCE cells.

**IFN-γ-induced MD-2 Expression Is Regulated by the JAK/STAT Pathway—**IFN-γ activates JAK2 through IFN-γR1/2, resulting in phosphorylation and subsequent nuclear translocation of the DNA-binding protein STAT1 (29, 30). To determine whether STAT1 signaling plays a role in IFN-γ-induced MD-2 transcriptional up-regulation in human corneal epithelial cells, HCEC and HCET cells were incubated with IFN-γ in the presence or absence of the AG490 JAK2 inhibitor (29), and MD-2 expression was determined by RT-PCR.

Fig. 5A shows STAT1 phosphorylation only after incubation with IFN-γ and that phosphorylation was completely inhibited in the presence of AG490. In marked contrast, no phosphorylation was observed in cells treated with AG490 alone. Furthermore, MD-2 transcription in HCEC and HCET cells was completely inhibited by AG490 as shown by real time PCR (Fig. 5B), indicating that IFN-γ-induced MD-2 expression is dependent on the JAK/STAT pathway.

As an additional approach to determine the role of STAT1 in MD-2 expression, HCEC and HCET cells were transfected with STAT1 siRNA before stimulation with IFN-γ. Fig. 5C shows diminished STAT1 expression in STAT1, but not control siRNA-treated cells. Furthermore, MD-2 expression was significantly inhibited in STAT1 knockdown cells, thereby demonstrating an essential role for STAT1 in MD-2 expression.

To determine whether STAT1 binds directly to the MD-2 promoter region, biotin-labeled oligonucleotides corresponding to a putative STAT1 binding site of the MD-2 promoter were incubated with nuclear extracts of unstimulated or IFN-γ-stimulated HCEC and HCET cells, and the resulting protein-DNA complex was examined by EMSA.

Fig. 5, D and E, show that whereas there is no detectable protein-DNA complex in unstimulated cells (lanes 1), there is a prominent band when labeled probes were incubated with nuclear extracts of IFN-γ-stimulated HCEC and HCET cells (lanes 2). To determine the specificity of this reaction, nuclear extracts were either preincubated with a 10-fold higher concentration of unlabeled probe or were incubated with biotinylated primers that have base substitutions in the γ-IFN activation site (GAS) (Table 1). As shown in Fig. 5D, (lanes 3 and 4), the protein-DNA complex was absent in both of these conditions. We also examined STAT1 binding to the MD-2 promoter site in supershift assays using antibodies to p-STAT1 or unphosphorylated STAT1. As shown in Fig. 5E, a higher molecular weight band was detected by both antibodies in lanes 3 and 4. Taken together these findings clearly demonstrate that STAT1 binds to the promoter region of MD-2.
FIGURE 3. Expression of MD-2 on human primary corneal epithelial cells and corneal epithelial cell lines. Human corneal epithelial cells (primary cells from two donor corneas and the HCEC and HCET cell lines) were incubated with IFN-γ (40 ng/ml) for indicated time points. A, expression of MD-2 was analyzed by real time PCR, and -fold change was measured compared with non-stimulated cells relative to β-actin. Agarose gel electrophoresis of the PCR-amplified products is shown below. The mean and S.D. of duplicate wells is shown, which is representative of three repeat experiments. B, flow cytometric analysis of surface expression of MD-2, TLR4, IFN-γR2, and TLR2 is shown. The experiment was repeated three times with similar results.
DISCUSSION

*P. aeruginosa* infections of the cornea are a major cause of visual impairment and blindness worldwide. Although the tight junctions between epithelial cells in the external layer of the cornea function as a barrier to prevent microbial invasion to the underlying stroma, corneal abrasion from traumatic injury or long term contact lens wear can cause loss of barrier function and lead to corneal infection. In addition, stimulation of corneal epithelial cells by bacterial products induces production of cytokines and anti-microbial peptides.

Using murine models of corneal inflammation and infection, we showed that specific activation of TLR2, TLR3, TLR4, TLR5, and TLR9 induce corneal inflammation (18, 21, 26) and that *P. aeruginosa* infection is regulated by TLR4-MD-2 and TLR5 (19). Bone marrow derived cells are present in the normal cornea (24, 25), and we showed that TLR4 expression on resident bone marrow-derived cells can mediate the host response to LPS and *P. aeruginosa* (17, 19); however, results from the current study demonstrate that MD-2 expression on either bone marrow-derived or non-myeloid cells is sufficient to mediate an inflammatory response, indicating that in addition to bone marrow cells, there is also a role for corneal epithelial cells in vivo.

Human corneal epithelial cells respond to TLR2 (21, 28), TLR3 (26, 32), and TLR5 ligands (33); however, they do not respond to LPS (9, 10, 27), indicating that the TLR4-MD-2 response is tightly regulated. Given that (i) recombinant MD-2 confers LPS responsiveness in HCE cell lines (9, 10), (ii) MD-2 transcripts are not detected in these cells as shown in the current study, and (iii) MD-2 RNA expression is low or undetectable in intestinal, lung, and conjunctival epithelial cells but can be induced by IFN-γ (12, 13, 15, 16), we determined if IFN-γ is produced early after *P. aeruginosa* corneal infection in a murine model and examined the role of IFN-γ and MD-2 in LPS responsiveness in primary HCE and HCE cell lines.

We found that IFN-γ is produced within 24 h of *P. aeruginosa* corneal infection, that NK1.1+ cells are readily detected at this time point, and that these cells are essential for IFN-γ production at this time point. These observations are consistent with an earlier study showing that depletion of NK and NK-T cells in *P. aeruginosa* results in decreased IFN-γ RNA expression, impaired bacterial killing, and accelerated corneal perforation (34).

Having shown in murine models of corneal inflammation that MD-2 expression in the non-myeloid cell population regulates LPS responses and that IFN-γ is produced early after *P. aeruginosa* infection, we next examined the role of IFN-γ on MD-2 and LPS responsiveness in primary human corneal epithelial cells and in two well characterized HCE cell lines. Our findings clearly demonstrate that IFN-γ induces MD-2 mRNA and cell surface expression and LPS responsiveness. In contrast to earlier reports indicating that TLR4 is intracellular (11, 27, 28), we found that TLR4 is constitutively expressed on the surface of primary HCE cells and in two HCE cell lines and that IFN-γ has no regulatory role. Taken together, results from the current study indicate that LPS responsiveness in HCE cells is regulated by IFN-γ induced surface expression of MD-2.

**TABLE 2**

Percent MD-2 and TLR4 expressing human corneal epithelial cells after stimulation with IFN-γ

|       | MD-2 |       | TLR4 |       |
|-------|------|-------|------|-------|
|       | Unstimulated | +IFN-γ | Unstimulated | +IFN-γ |
| Primary | 7.4 | 58.8 | 72.5 | 81.9 |
| HCEC   | 2.7 | 62.6 | 82.5 | 84.2 |
| HCET   | 4.8 | 65   | 87.8 | 89.1 |

**FIGURE 4.** IFN-γ induced LPS responsiveness in human corneal epithelial cells. HCEC, HCET, and primary human corneal epithelial cells were incubated with LPS and IFN-γ, and responses were measured by translocation of the NF-κB p65 subunit to the cell nucleus (A), or CXCL1, CXCL8/IL-8, and IL-6 production was measured by ELISA (B). A, representative wells show translocation of p65 in HCEC and HCET cells after 2 h of incubation with LPS in the presence or absence of IFN-γ. B, cytokine production of the mean ± S.D. of three wells for each condition is shown. Data are indicative of three repeat experiments with similar results.
The IFN-γ receptor, which is also constitutively expressed on these cells, activates the JAK/STAT pathway, inducing phosphorylation and dimerization of STAT1, which binds to the GAS of the promoter region of target genes (35). In the current study we show that IFN-γ R2 is constitutively expressed on HCE cells, that STAT1 is phosphorylated in response to IFN-γ, but not in the presence of the AG490 JAK2 inhibitor, demonstrating that this pathway is activated in HCE cells. Importantly, we now demonstrate that STAT1 binds to the GAS site of the MD-2 promoter in HCE cells, thereby demonstrating that STAT1 is a transcriptional activator of this gene. Although STAT1-mediated transcription generally occurs after nuclear translocation of phosphorylated STAT1 dimers, Cheon and Stark (22) showed that unphosphorylated STAT1 also translocates to the nucleus and can initiate transcription of selected genes. We found that both phosphorylated and unphosphorylated forms of STAT1 were detected by supershift assay, suggesting that both forms translocate to the nucleus. However, it is also possible that the presence of the unphosphorylated form in the nucleus is due to dephosphorylation rather than translocation. Future studies will examine the relative contribution of both forms of STAT1.

Taken together, our findings are consistent with the sequence of events shown in Fig. 6. 1) IFN-γ produced by NK1.1+ cells in the corneal stroma binds to IFN-γ R2, which is constitutively expressed on the cell surface. 2) JAK2 is auto-phosphorylated and mediates STAT1 phosphorylation and
dimerization. 3) STAT1 translocates to the nucleus and binds to the MD-2 promoter γ-IFN activating site, 4) leading to MD-2 gene transcription. 5) The MD-2 protein is transported to the plasma membrane, where it can bind LPS and interact with TLR4 to form the LPS receptor. 6) TLR4-MD-2 homodimerization recruits the MyD88 and Mal/TIRAP adaptor molecules (HCE cells express TRIF and MyD88 proteins (26), although it has yet to be determined if they also express TRAM to activate the TLR4/TRIF pathway). 7) The TLR/IL-1R canonical signaling pathway leads to NF-κB translocated to the nucleus and transcription of proinflammatory and chemotactic cytokines. 8) CXC chemokines recruit neutrophils to the corneal stroma, and IL-6 and other proinflammatory cytokines stimulate neutrophil degranulation, causing localized tissue damage resulting in loss of corneal transparency and visual impairment. As this sequence of events is driven by the presence of LPS at the site of infection and inflammation, it is likely that IFN-γ production and MD-2 expression returns to normal levels after bacterial killing and LPS clearance.

Given that MD-2 expression in intestinal epithelial cells is blocked by a STAT inhibitor (15), it seems likely that STAT1 transcriptional activation of MD-2 is a common regulatory mechanism of LPS responsiveness in epithelial cells. HCE and other epithelial cells are located at the interface of the host and the external environment and given that the TLR4-MD-2 complex can respond to picomolar levels of lipid A (36), it is critical that LPS responses are tightly regulated to prevent or minimize inflammatory responses that can cause tissue damage. Tight regulation of MD-2 expression is clearly one of these mechanisms, but other regulatory mechanisms shown in the cornea and other mucosal surfaces include TLR expression on underlying rather than surface epithelial cell layers (18, 33, 37). An additional regulatory mechanism is maintaining TLR4 within the cell rather than at the cell surface, although as noted earlier, this is controversial in HCE cells but has also been proposed for lung and intestinal epithelial cells (13, 15).

Cytosine methylation plays an important role in gene regulation and occurs in the MD-2 promoter region of human colonic and intestinal epithelial cells (38). In that study, IFN-γ dependent activation was found by deletion studies to be dependent on a region of the promoter between −728 and −1000. However, in human corneal epithelial cells, we found that of nine methylation sites in this region, IFN-γ stimulation altered the −769 site only (supplemental Fig. S1). This observation together with the STAT1 binding site being further upstream, indicates that in contrast to intestinal epithelial cells, methylation is unlikely to have a major regulatory role of STAT1 dependent transcription of MD-2.

In conclusion, results from the current study further our understanding of the regulation of host responses under conditions of disease pathogenesis by demonstrating a regulatory mechanism for MD-2 expression and LPS responsiveness in human corneal epithelial cells. MD-2 is an attractive target for anti-inflammatory therapy as lipid A antagonists such as Lipid IVa or eritoran tetrasodium (E5564) can block lipid A binding and cell activation, thereby inhibiting potential tissue damage (9, 40, 41). In addition, crystal structure analysis of the TLR4-MD-2 complexed with eritoran or Lipid IVa shows the precise binding sites of these antagonists, with all four acyl chains in the MD-2 hydrophobic pocket and none interacting with TLR4 (39, 42). The data presented here indicate additional targets for
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therapeutic intervention that will also inhibit tissue damage caused by endotoxin or Gram-negative bacteria.

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