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SIGIRR Promotes Resistance against *Pseudomonas aeruginosa* Keratitis by Down-Regulating Type-1 Immunity and IL-1R1 and TLR4 Signaling[1]

Xi Huang,[2] Linda D. Hazlett, Wenjin Du, and Ronald P. Barrett

***Pseudomonas aeruginosa* keratitis destroys the cornea in susceptible Th1 responder C57BL/6 (B6), but not resistant Th2 responder (BALB/c) mice. To determine whether single Ig IL-1R-related molecule (SIGIRR) played a role in resistance, mRNA and protein expression levels were tested. Both were constitutively expressed in the cornea of the two mouse groups. A disparate mRNA and protein expression pattern was detected in the cornea of BALB/c vs B6 mice after infection. SIGIRR protein decreased significantly in BALB/c over B6 mice at 1 day postinfection. Thus, BALB/c mice were injected with an anti-SIGIRR Ab or IgG control. Anti-SIGIRR Ab over control-treated mice showed increased corneal opacity, stromal damage, and bacterial load. Corneal mRNA levels for IL-1β, MIP-2, IL-1R1, TLR4, IL-18, and IFN-γ and protein levels for IL-1β and MIP-2 also were significantly upregulated in anti-SIGIRR Ab over control mice, while no changes in polymorphonuclear cell number, IL-4, or IL-10 mRNA expression were detected. To further define the role of SIGIRR, RAW264.7 macrophage-like cells were transiently transfected with SIGIRR and stimulated with heat-killed *P. aeruginosa* or LPS. SIGIRR transfection significantly decreased mRNA levels for IL-1R1, TLR4, and type 1 immune response-associated cytokines (IL-12, IL-18, and IFN-γ) as well as proinflammatory cytokines IL-1β and MIP-2 protein expression. SIGIRR also negatively regulated IL-1 and LPS, but not poly(I:C)-mediated signaling and NF-κB activation. These data provide evidence that SIGIRR is critical in resistance to *P. aeruginosa* corneal infection by down-regulating type 1 immunity, and that it negatively regulates IL-1 and TLR4 signaling. *The Journal of Immunology,* 2006, 177: 548–556.**

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that causes sight-threatening corneal infections, especially in extended wear contact lens users and in immunocompromised patients (1, 2). Both bacterial (e.g., LPS) and host factors released from infiltrating cells during infection are thought to contribute to a rapidly progressing liquefactive stromal necrosis (3–5). In addition, compelling evidence suggests that the initial phase of host defense against many invading microbes also involves a family of proteins called TLRs, which activate signaling cascades and trigger innate immune responses that lead to expression of various proinflammatory cytokines and chemokines (6, 7).

Little is known about the role of TLRs in *P. aeruginosa* keratitis (8), and despite extensive experimental animal studies, the precise mechanisms involved in its development remain incompletely defined (9). Studies have shown that dominant Th1 responder mouse strains such as C57BL/6 (B6) are susceptible (cornea perforates), whereas dominant Th2 responder strains (e.g., BALB/c) are resistant (cornea heals) after similar bacterial challenge (10, 11). Studies using these models have provided information regarding the role of inflammatory cells (e.g., polymorphonuclear cells (PMN),[3] T cells, NK cells, macrophages, and Langerhans cells) as well as cytokines and chemokines (e.g., IFN-γ, TNF-α, IL-4, IL-10, IL-12, and IL-18) that modulate innate and Th1 vs Th2 immune responses to *P. aeruginosa* keratitis (11, 12). Therefore, these well-defined animal models provided a unique opportunity to test whether corneal expression levels of members of the TLR/IL-1R superfamily are important in mediating innate and directing the divergent adaptive immune response, resulting in phenotype differences (susceptibility vs resistance) after *P. aeruginosa* ocular challenge.

In this regard, TLR activation is a double-edged sword, and negative regulation for TLR signaling may be required to avoid detrimental and inappropriate inflammatory responses (13), as the immune system needs to constantly strike a balance between activation by TLR signaling and inhibition by negative regulators. Several negative regulators of TLR signaling, including single Ig IL-1R-related molecule (SIGIRR), have been identified over the past several years. The negative regulatory response is achieved at multiple levels (14). The first level is production of soluble TLRs such as soluble TLR2 and soluble TLR4, acting as decoy receptors for TLR2 and TLR4 signaling, respectively. The second level involves the transmembrane proteins IL-1RII, ST2, and SIGIRR that sequester recruitment of adaptor molecules such as MyD88 and IL-1R-associated kinase (15). SIGIRR also sequesters the formation of TLR signaling complexes and tunes the action of inflammatory cytokines/chemokines by inhibiting IL-1R and TLR4 signaling (13, 16). Once TLR and ligand interaction has occurred, the

Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, MI 48201

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[2] Address correspondence and reprint requests to Dr. Xi Huang, Department of Anatomy and Cell Biology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201. E-mail address: xhuang@med.wayne.edu

[3] Abbreviations used in this paper: PMN, polymorphonuclear cell; HK-PA, heat-killed *P. aeruginosa*; MPO, myeloperoxidase; p.i., postinfection; SIGIRR, single Ig IL-1R-related molecule; TIR, Toll/IL-1R.

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third level of negative regulation controls TLR signaling by intracellular regulators such as MyD88s, suppressor of cytokine signaling 1, and IL-1R-associated kinase-M. The fourth level of negative regulation reduces TLR expression or increases degradation of TLRs. TLR-induced apoptosis, the final level of negative regulation, may also be activated to control pathogenesis and sepsis caused by bacterial infection.

In this study, we investigated the expression and function of SIGIRR in the cornea of susceptible (B6) vs resistant (BALB/c) mice before and after bacterial infection, testing the hypothesis that SIGIRR may be an important negative regulator of TLR signaling in *P. aeruginosa* keratitis. Our data provide direct evidence that SIGIRR is disparately expressed in the cornea of BALB/c vs B6 mice after *P. aeruginosa* infection. In addition, we present direct evidence that SIGIRR is required for host resistance against bacterial infection and functions to negatively regulate type 1, but not type 2 cytokine/chemokine production, reducing IL-1R and TLR4 expression and inhibiting IL-1 and TLR4 (LPS) signaling.

Materials and Methods

Infection of mice

Eight-week-old female B6 and BALB/c mice (The Jackson Laboratory) were anesthetized with ether and placed beneath a stereoscopic microscope at ×40 magnification, and the cornea of the left eye was wounded with three 1-mm incisions using a sterile 25-gauge needle. A bacterial suspension (5 μl) containing 1×10^6 CFU/μl *P. aeruginosa* American Type Culture Collection (ATCC) strain 19660, prepared as described before (11), was topically applied onto the scarified cornea. Eyes were examined and graded for disease at 1–7 days postinfection (p.i.) in the rat anti-SIGIRR Ab (endotoxin <0.1 EU/μg; R&D Systems) and in rat IgG (Sigma-Aldrich) control-treated mice. Results with the control Ab were similar to use of a rat IgG1 Ab (R&D Systems; endotoxin <0.1 EU/μg; data not shown). Mice were treated humanely and in compliance with the Association for Research in Vision and Ophthalmology Resolution on Usage and Treatment of Animals in Research.

Ocular response to infection

Corneal disease was graded, as described before (17): 0 = clear or slight opacity, partially or fully covering the pupil; +1 = slight opacity, fully covering the anterior segment; +2 = dense opacity, partially or fully covering the pupil; +3 = dense opacity, covering the entire anterior segment; and +4 = corneal perforation or phthisis. A clinical score was calculated for each group of mice (n = 5/group/treatment) to express disease severity, and slit lamp photography was used to illustrate the disease response.

Ab treatment

Resistant BALB/c mice (n = 5/group/treatment) were injected subconjunctivally with 10 μg/mouse anti-SIGIRR Ab (R&D Systems) or control (rat IgG; Sigma-Aldrich) 1 day before infection. At 1 and 3 days p.i., each mouse was injected i.p. with an additional 150 μl (150 μg) of anti-SIGIRR Ab diluted in PBS. Control mice similarly received an equal volume and amount of rat IgG.

Real-time PCR

Methods for real-time RT-PCR were described before (12, 18). In this study, mouse corneas or RAW264.7 macrophage-like cells were homogenized in RNA STAT-60 (Tel-Test), and total RNA was isolated per the manufacturer's instruction to produce a cDNA template for PCR. Primers used were 5′-GTG GCT GAA AGA TGG TCT GGC ATT G-3′ (sense) and 5′-CAG GTG AAG GTT CCA TAG TCC TCT GC-3′ (antisense) for mouse SIGIRR; 5′-CGC AGC AGC ACA TCA ACA AGA GC-3′ (sense) and 5′-TGT CCT CAT CCT GGA AGG TCC ACG-3′ (antisense) for mouse IL-1β; 5′-TGT CAA TGC CTG AAG ACC CTG CC-3′ (sense) and 5′-AAC TTT TTG ACC GCC CTT GAG AGT GG-3′ (antisense) for mouse MIP-2; 5′-CTC TGC TTC TTG ACA ACG TGA GCT TC-3′ (sense) and 5′-TAT AGT CCC CTC TGT GCT CTT CAG CC-3′ (antisense) for mouse IL-1R1; 5′-CGC TTT CAC CTC TGC CTT CAC TAC AG-3′ (sense) and 5′-ACA CTA CCA CAA TAA CCT TCC GGC TC-3′ (antisense) for mouse TLR4; 5′-AGT GAG CAA GGG AGA ATG AGC AAG-3′ (sense) and 5′-TCA CGG GAT TGG TGA GTC TGA AG-3′ (antisense) for mouse TLR3; 5′-TGT CAT CCT GCT CTT CTT TCT CG-3′ (sense) and 5′-GTT TGG CAC ATC CAT CTC CG-3′ (antisense) for mouse IL-4; 5′-AAA GAG AAG TGT GGC GAG GAG AGA C-3′ (sense) and 5′-CCT TCC ATT GCC CAC TCT GTA CTC ATC-3′ (antisense) for mouse IL-5; 5′-TGC TAA CCG ACT CCT TAA TGC AGG AC-3′ (sense) and 5′-CCT TGA TTT CTG GGC CAT GCT TCT C-3′ (antisense) for mouse IL-10; 5′-GGT CAC ACT GGA CCA AAG GGA CTA TG-3′ (sense) and 5′-ATT CTG CTG CCG TGC TTC CAA C-3′ (antisense) for mouse IL-12p40; 5′-GCC ATG TCA GAA GAC TCT TGC GTC-3′ (sense) and 5′-GTA CAG TGA AGT CGG CCA AAG TTG TC-3′ (antisense) for mouse IL-18; 5′-CAG AGC CAG ATT ATC TCT TTC TAC CTC AGA C-3′ (sense) and 5′-CTT TTT CGC CTT GCT GTT GCT GAA G-3′ (antisense) for mouse IFN-γ; and 5′-GAT TAC TGC TCT GGC TCC TAG C-3′ (sense) and 5′-GAC TCA TCG TAC TCC TGC TTG C-3′ (antisense) for mouse β-actin. For PCR amplification, 1 μl of each cDNA sample was used per 25 μl of PCR. Real-time PCR measurements were analyzed in duplicate in three independent runs using a Cepheid Smart Cycler System (Cepheid). Relative mRNA levels were calculated after normalizing to β-actin (12).

Cell culture and SIGIRR expression

Murine monocyte/macrophage-like RAW264.7 (ATCC; TIB-71) cells, originally derived from BALB/c mice, were cultured in DMEM containing 10% heat-inactivated FBS (Invitrogen Life Technologies), 4 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml; Invitrogen Life Technologies) at 37°C and 5% CO_2. Following the manufacturer's protocol, a six-well plate of RAW cells (5×10^5 cells/well) were transiently transfected with a mouse SIGIRR expression plasmid (InvivoGen) or empty plasmid (InvivoGen) control using transfectamine-2000 (Invitrogen Life Technologies). Cells were lysed in a lysis buffer 18 h after transfection, and SIGIRR protein expression in transfected cells was detected by Western blot.

Western blot analysis

Corneas (n = 10/group/time) were collected from normal uninfected BALB/c and B6 mice, and at 1, 3, and 5 days p.i. Pooled corneas (10/group) were lysed and homogenized using a 1-ml glass tissue homogenizer in ice-cold lysis buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 1 mM PMSF) for 30 min. Murine SIGIRR-transfected RAW cells were harvested, washed twice in cold PBS buffer, and lysed in lysis buffer for 20 min. Cell debris was pelleted by centrifugation for 10 min at 12,000 rpm, and protein concentration of the supernatant was determined by bicinchoninic acid (Bio-Rad) protein assay. Supernatants were separated on 10% SDS-PAGE, and 100 μg of corneal protein sample or 20 μg of protein from SIGIRR-transfected cells was added to each lane. The electrophoretically separated material was transferred to a supported nitrocellulose membrane (Millipore), and blocked in a 5% solution of nonfat dry milk prepared in 1× PBS and 0.05% Tween 20. Blots were incubated with primary goat anti-mouse SIGIRR Ab (R&D Systems) diluted in PBS overnight at 4°C, washed three times for 10 min each with PBS, detected with HRP-conjugated secondary Ab (R&D Systems) diluted 1/2000 in PBS + 5% nonfat milk, and developed using the ECL method (ECL Plus; Amersham Biosciences) following the manufacturer's protocol.

ELISA analysis of cytokines

Protein levels for proinflammatory cytokines/chemokines were quantitated using ELISA kits (R&D Systems). After anti-SIGIRR Ab or control IgG treatment, individual corneas (n = 5/group/time) were harvested from infected mice at 1, 3, and 5 days p.i., individually homogenized in 1.0 ml of PBS with 0.5% hexadecyltrimethylammonium bromide with a glass microtissue grinder (Fisher Scientific), and centrifuged at 13,000 rpm for 10 min. Sample supernatants were diluted (1/10 for IL-1β; 1/20 for MIP-2), and a 50-μl aliquot was assayed for IL-1β and MIP-2 protein levels. Supernatants from SIGIRR-transfected RAW cells treated with anti-SIGIRR Ab or murine rSIGIRR (see below) were assayed similarly for MIP-2 protein following stimulation with heat-killed *P. aeruginosa* (HK-PA) (56°C for 60 min, 1×10^5 CFU). The reported sensitivity of these assays is <3.0 pg/ml for IL-1β and 1.5 pg/ml for MIP-2.

Histopathology

Whole infected eyes (n = 3/group) were enucleated from anti-SIGIRR Ab- vs rat-IgG-treated BALB/c mice at 5 days p.i.; immersed immediately in PBS; rinsed; and fixed in 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorenson's phosphate buffer (pH 7.4) (1:1:1) at 4°C for 3 h. Eyes were rinsed with 0.1 M phosphate buffer, dehydrated in graded ethanols and propylene oxide, and then infiltrated and embedded in Epon-araldite. Thick sections (1.5 μm) were cut, stained with Richardson's stain, observed, and photographed, as described before (10, 11). Representative

sections were photographed with a Zeiss Axiophot photomicroscope (Carl Zeiss, Morgan Instruments).

Quantitation of viable bacteria in cornea

Bacteria were quantitated at 3 and 5 days p.i. in individual infected corneas of BALB/c mice after anti-SIGIRR Ab or rat IgG (n = 5/group/time) treatment. Each cornea was homogenized in sterile 0.9% saline containing 0.25% BSA. To quantitate viable bacteria per cornea, a 0.1-ml aliquot of the corneal homogenate was serially diluted 1/10 in sterile PBS-BSA. Serial 10-fold dilutions of the samples were plated on *Pseudomonas* isolation agar (Difco) in triplicate, and plates were incubated overnight at 37°C. The number of viable bacteria in an individual cornea was determined by counting individual colonies on plates from the various dilutions. Results are reported as $\log_{10}$ number of CFU/cornea ± SEM.

Quantitation of PMN

Samples were assayed for myeloperoxidase (MPO) activity, as described before (19). Corneas from anti-SIGIRR Ab- or rat IgG-treated mice (n = 5/group/time) were collected at 1 and 5 days p.i. and homogenized in 1.0 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. Samples were freeze thawed three times and centrifuged at 13,000 × g for 10 min. Supernatant (0.1 ml) was added to 2.9 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml) and hydrogen peroxide (0.0005%). The change in absorbance at 460 nm was monitored for 5 min using a Helios-α spectrophotometer (Thermo Spectronics), and the results were expressed as units of MPO/cornea. One unit of MPO activity = ~2.0×10^5 PMN (20, 21).

Transient transfection

The 293T/NF-κB-luc cell line (Panomics) is a human 293T embryonic kidney cell line stably transfected with pNF-κB-luciferase plasmid. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml hygromycin-B, and 2 mM L-glutamine (all obtained from Invitrogen Life Technologies). Stable transfected cell lines 293-hTLR4/MD2-CD14 and 293-hTLR3 (InvivoGen) were cultured in DMEM with 10% FBS and 10 μg/ml Blasticidin-S (InvivoGen). Cells (5×10^5 cells/well of 6-well plate) were transiently transfected using transfectamine-2000 (Invitrogen Life Technologies), following the manufacturer's protocol. Cells were cotransfected with various concentrations (0, 10, 100, and 1000 ng) of SIGIRR plasmid (InvivoGen) plus 100 ng of NF-κB luciferase reporter plasmid pNF-κB-Luc (Panomics) and 20 ng of internal control plasmid phRL-TK (Promega). In all cases, the same amount of empty vector (InvivoGen) was similarly transfected to control the various concentrations of SIGIRR plasmid. To ensure each sample received equal amounts of DNA, transfection efficiency was normalized in all experiments by cotransfection of cells with 20 ng of internal control reporter phRL-TK (Promega) encoding renilla luciferase. After 18 h of transfection, cells were stimulated with IL-1α (20 ng/ml), LPS (5 μg/ml), or poly(I:C) (50 μg/ml) for 6 h. Cells were lysed using Reporter Lysis Buffer (Promega), and luciferase activity was assessed using Dual-Glo Luciferase Assay Reagent (Promega).

In another series of experiments, SIGIRR-transfected RAW cells were treated with anti-SIGIRR Ab (R&D Systems) or rat IgG1 (R&D Systems) control Ab for 18 h, or with murine rSIGIRR (R&D Systems) or PBS (control), followed by stimulation with HK-PA for 6 h. Cells were collected and processed for ELISA analysis. All results reported represent duplicate experiments with at least three independent transfections.

Statistical analysis

An unpaired, two-tailed Student's t test was used to determine statistical significance of real-time PCR, ELISA, clinical score, bacterial plate counts, and MPO assays. Data were considered significantly different at $p < 0.05$. All experiments were repeated at least once to ensure reproducibility, and data from both experiments are shown.

Results

SIGIRR in cornea

To determine whether SIGIRR was present in the cornea of BALB/c and B6 mice before and after infection with *P. aeruginosa*, we tested SIGIRR mRNA and protein expression levels in uninfected and infected corneas using real-time PCR and Western blot, respectively. Data from a representative experiment are shown in Fig. 1. Although SIGIRR was constitutively similarly expressed (mRNA and protein) in the uninfected, normal cornea of both mouse groups, its expression pattern was disparate after bacterial infection. In the infected cornea of BALB/c and B6 mice, SIGIRR mRNA levels (Fig. 1*A*) were down-regulated at 12 h p.i. At 1 day p.i., SIGIRR mRNA expression in BALB/c over B6 mice was significantly up-regulated ($p < 0.001$), peaked at 3 days p.i. ($p < 0.001$), and maintained at a higher level at 5 days p.i. ($p < 0.01$). SIGIRR protein (Fig. 1, *B* and *C*) was constitutively expressed in uninfected BALB/c and B6 cornea. In BALB/c over B6 cornea, at 1 day p.i., protein expression was significantly decreased ($p < 0.01$), but was significantly increased at both 3 ($p < 0.01$) and 5 ($p < 0.01$) days p.i. in BALB/c over B6 cornea.

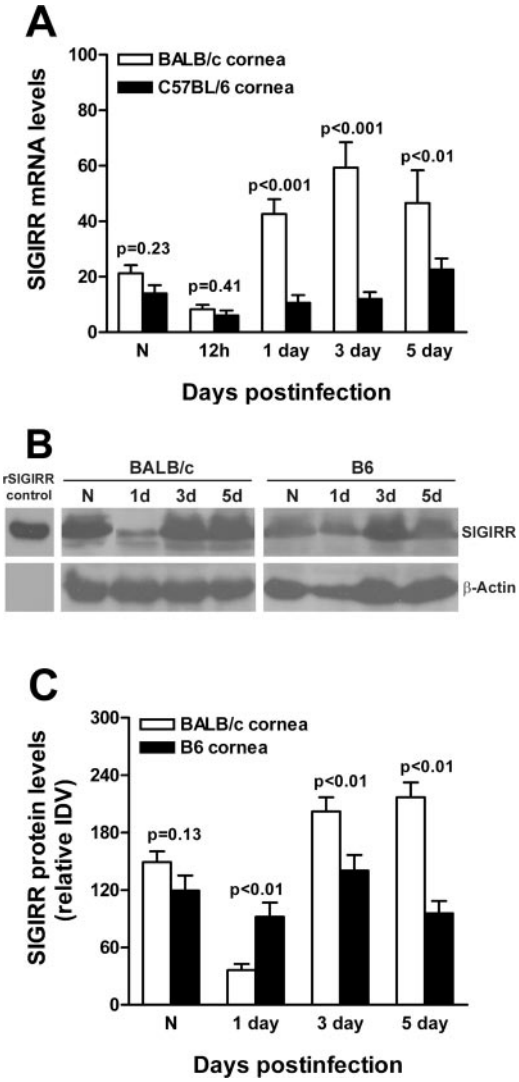

FIGURE 1. SIGIRR expression in the cornea of BALB/c and B6 mice. *A*, SIGIRR mRNA levels (normalized to β-actin) in BALB/c and B6 normal (N) and infected cornea at 12 h to 5 days p.i. by real-time PCR. *B*, Western blot of SIGIRR protein levels in the cornea of BALB/c and B6 normal (N) and infected mice at 1, 3, and 5 days p.i. Equivalent protein loaded (100 μg each in *lanes 2–9*). Lanes: *1*, murine rSIGIRR (60 ng); *2*, BALB/c normal cornea; *3*, BALB/c 1 day p.i.; *4*, BALB/c 3 days p.i.; *5*, BALB/c 5 days p.i.; *6*, B6 normal cornea; *7*, B6 1 day p.i.; *8*, B6 3 days p.i.; *9*, B6 5 days p.i. *C*, The intensity of bands was quantitated and normalized to the β-actin control. Data, expressed as the mean ± SEM integrated density values (IDV) at each time point, are significant ($p < 0.01$, $p < 0.01$, $p < 0.01$) at 1, 3, and 5 days p.i., respectively.

SIGIRR is required in host resistance against infection

Because SIGIRR mRNA and protein levels were detected and differentially expressed in infected cornea of BALB/c and B6 mice,

the next series of in vivo studies tested whether SIGIRR was protective in bacterial keratitis. First, BALB/c mice were injected subconjunctivally and i.p. with an anti-SIGIRR Ab to determine whether this would impair host resistance. Clinical score data showed that BALB/c mice injected with an anti-SIGIRR Ab over rat IgG exhibited significantly increased disease (Fig. 2*A*, $p < 0.01$, $p < 0.001$, $p < 0.05$, and $p < 0.001$, at 1, 3, 5, and 7 days p.i., respectively). A representative slit lamp photograph at 5 days p.i. revealed more corneal opacity/disease in the anti-SIGIRR Ab (Fig. 2*B*)- vs control (Fig. 2*C*)-treated group. To confirm these data, eyes from both mouse groups were enucleated for histopathology at 5 days p.i. The anti-SIGIRR Ab-treated BALB/c mice exhibited corneal swelling, infiltrating cells between the iris and the corneal endothelium, and infiltrating cells in the anterior chamber (Fig. 2*D*). In contrast, the corneas of rat IgG-treated mice were less swollen, with no detectable inflammatory cells in the anterior chamber or associated with the iris or corneal endothelium and exhibited decreased stromal damage and more normal cytoarchitecture (Fig. 2*E*). In addition, bacterial load (Fig. 2*F*) was significantly increased ($p < 0.01$ and $p < 0.001$ at 3 and 5 days p.i., respectively) in the cornea of anti-SIGIRR Ab over control-treated mice. We also tested the effect of SIGIRR Ab treatment on PMN infiltration by quantitation of MPO activity in the cornea. There was no significant difference in the number of PMN in the anti-SIGIRR Ab- over control-treated corneas at 1 and 5 days p.i. (Fig. 2*G*; $p = 0.23$ and 0.91, respectively). Nonetheless, in the infected cornea, treatment with anti-SIGIRR Ab led to a significant increase in mRNA levels of IL-1β (Fig. 3*A*; $p < 0.01$, $p < 0.01$, and $p = 0.03$ at 1, 3, and 5 days p.i., respectively) and MIP-2 (Fig. 3*B*; $p < 0.01$, $p < 0.001$, and $p = 0.02$ at 1, 3, and 5 days p.i., respectively) when compared with levels of these cytokines in the infected cornea of rat IgG-treated mice. ELISA analysis showed that in the infected cornea, protein levels of IL-1β (Fig. 3*C*; $p < 0.001$ and $p < 0.01$ at 3 and 5 days p.i., respectively) and MIP-2 (Fig. 3*D*; $p < 0.001$ and $p < 0.001$ at 3 and 5 days p.i., respectively) also were significantly up-regulated in BALB/c mice treated with anti-SIGIRR Ab over controls, confirming the mRNA data.

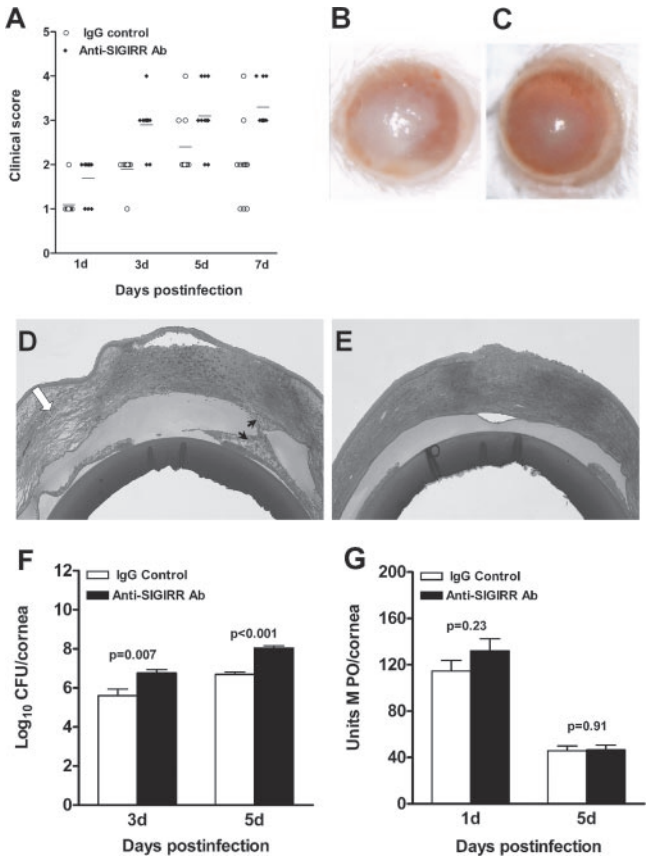

FIGURE 2. In vivo studies of SIGIRR in host resistance. Clinical score (*A*) shows that more corneas perforated in the anti-SIGIRR Ab over rat IgG-treated group ($p < 0.01$, $p < 0.001$, $p < 0.05$, and $p < 0.001$ at 1, 3, 5, and 7 days p.i., respectively). Slit lamp at 5 days p.i. (*B* and *C*) shows more opacity/disease in the Ab (*B*)- vs rat IgG (*C*)-treated cornea. Histopathology at 5 days p.i. shows that the cornea of anti-SIGIRR Ab-treated mice (*D*) was swollen (block arrow) and exhibited inflammatory cells not only in the stroma, but in the anterior chamber associated with the iris and corneal endothelium (arrows), whereas the cornea of rat IgG-treated mice (*E*) was less swollen, more intact, and had no cellular infiltrate in the anterior chamber, associated with the iris or with the corneal endothelium. Bacterial load (*F*) in individual corneas at 3 and 5 days p.i. was reported as $\log_{10}$ number viable bacteria per cornea ± SEM. Significantly increased bacterial counts ($p < 0.01$ and $p < 0.001$ at 3 and 5 days p.i., respectively) were observed, but no differences were detected in PMN recruitment (*G*, MPO activity) between Ab- and rat IgG-treated corneas.

SIGIRR differentially regulates TLR expression and type 1/type 2 immune responses

Because SIGIRR Ab treatment in BALB/c mice enhanced proinflammatory cytokine production in the cornea and worsened disease, the next series of studies were initiated to investigate the mechanisms involved. mRNA expression levels for IL-1R1 and TLR4 in the infected cornea of BALB/c mice were tested after treatment with anti-SIGIRR Ab. Data showed that treatment with anti-SIGIRR Ab over rat IgG led to a significant increase in mRNA for IL-1R1 (Fig. 4*A*; $p < 0.001$ and $p < 0.01$ at 1 and 3 days p.i., respectively) and TLR4 (Fig. 4*B*; $p < 0.05$ and $p = 0.01$ at 1 and 3 days p.i., respectively). We then tested whether anti-SIGIRR Ab exacerbated type 1- and/or type 2-associated cytokine production in vivo. Gene expression of type 1 immune response-associated cytokines such as IL-18 (Fig. 4*C*; $p < 0.01$, $p < 0.01$,

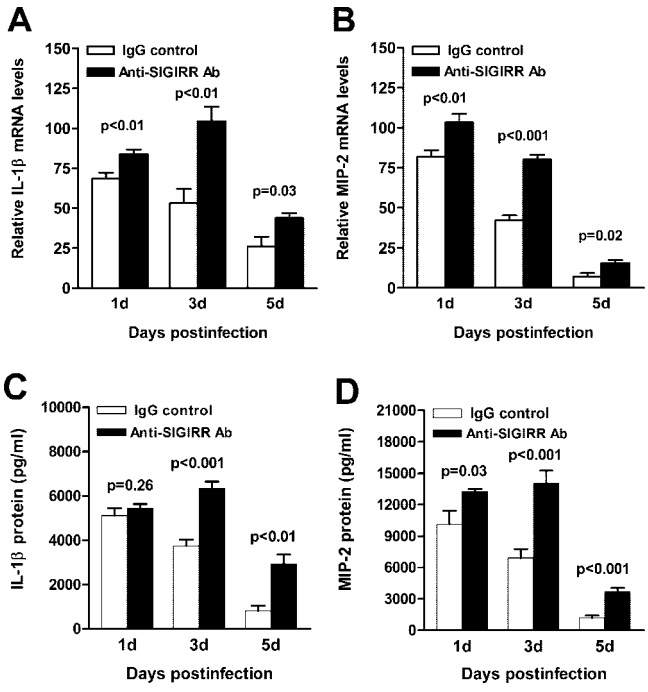

FIGURE 3. SIGIRR Ab treatment augments proinflammatory cytokine production. mRNA expression levels for IL-1β (*A*) and MIP-2 (*B*) as well as protein levels for each (*C* and *D*) were significantly up-regulated in the cornea of anti-SIGIRR Ab- vs rat IgG-treated mice.

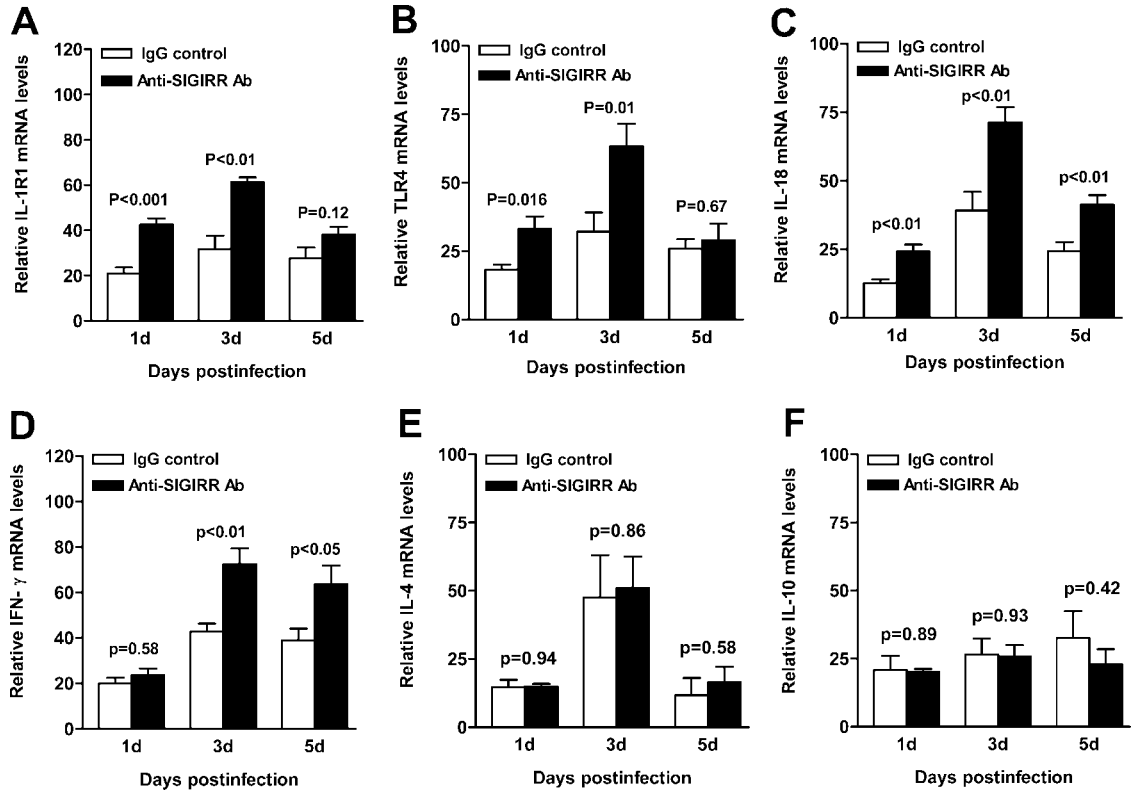

FIGURE 4. SIGIRR differentially regulates TLR expression and type 1/type 2 cytokine production. mRNA levels for IL-1R1 (*A*) and TLR4 (*B*) were significantly elevated at 1 and 3, but not at 5 days p.i. in the corneas of BALB/c mice treated with anti-SIGIRR Ab over rat IgG. Gene expression for IL-18 (*C*) and IFN-γ (*D*) mRNA levels was also significantly enhanced, whereas IL-4 (*E*) and IL-10 (*F*) mRNA levels were unchanged at 1, 3, and 5 days p.i.

and $p < 0.01$ at 1, 3, and 5 days p.i., respectively) and IFN-γ (Fig. 4*D*; $p < 0.01$ and $p < 0.05$ at 3 and 5 days p.i., respectively) was significantly up-regulated in Ab over IgG-treated cornea in BALB/c mice. In contrast, no significant difference in mRNA expression levels was detected for type 2 immune response-associated cytokines such as IL-4 (Fig. 4*E*) and IL-10 (Fig. 4*F*).

SIGIRR expression and its regulatory role in vitro

To further stringently test the immunoregulatory role of SIGIRR, RAW cells were transiently transfected with the SIGIRR gene. Before transfection, low mRNA levels of endogenous SIGIRR were detected in RAW cells by real-time PCR. mRNA expression levels of SIGIRR were down-regulated in a time-dependent manner after LPS (1 μg/ml) or HK-PA (1×10^5 CFU/μl) treatment (Fig. 5*A*). Next, the SIGIRR vector was transiently transfected into RAW cells, and its mRNA and protein expression levels were confirmed by real-time PCR (Fig. 5*B*) and Western blot (Fig. 5*C*), respectively. After stimulating with HK-PA, in SIGIRR vector- over empty vector control-transfected RAW cells, mRNA levels for IL-1R1 and TLR4, but not TLR3, were significantly reduced (Fig. 5*D*; $p < 0.001$, $p < 0.001$, and $p = 0.83$ for IL-1R1, TLR4, and TLR3, respectively). Similarly, overexpression of SIGIRR vs control in HK-PA-stimulated RAW cells significantly inhibited the mRNA expression of type 1 immune response-associated cytokines, including IL-12, IL-18, and IFN-γ (Fig. 5*E*; $p < 0.01$, $p < 0.01$, and $p < 0.001$ for IL-12, IL-18, and IFN-γ, respectively). In contrast, there was no significant difference in mRNA levels for type 2-associated cytokines such as IL-5 and IL-10 (Fig. 5*F*; $p = 0.64$ and 0.78 for IL-5 and IL-10, respectively) after similar stimulation with HK-PA. SIGIRR vector- vs empty vector-transfected and HK-PA-stimulated cells also were tested for mRNA and protein expression levels of proinflammatory cytokines IL-1β and MIP-2 using real-time PCR (Fig. 5*G*) and ELISA (Fig. 5*H*). IL-1β and MIP-2 mRNA and protein expression levels were significantly decreased in SIGIRR vector- over empty vector-transfected and HK-PA-stimulated RAW cells.

To further test whether anti-SIGIRR Ab or rSIGIRR protein affected proinflammatory cytokine production, SIGIRR-transfected RAW cells were treated with each of the above in separate experiments and then stimulated with HK-PA. Supernatants were tested for protein expression for MIP-2, and in each case, MIP-2 protein expression was elevated (Fig. 5, *I* and *J*), putatively indicating that signaling via SIGIRR can be blocked through its receptor or by consuming its ligand.

SIGIRR inhibits IL-1 and LPS, but not poly(I:C) signaling

We then assessed the effect of SIGIRR on IL-1, LPS, and poly(I:C) signaling using various cell lines and an NF-κB-dependent luciferase reporter assay. Overexpression of SIGIRR by cotransfection of increasing amounts of SIGIRR plasmid and IL-1R1 (10 ng) in 293T/NF-κB-Luc cells (HEK293T cell line stably transfected with pNF-κB-luc plasmid; Fig. 6*A*) and RAW cells (Fig. 6*B*) substantially reduced IL-1-mediated NF-κB activation in a dose-dependent manner. Cotransfection of empty vector with IL-1R1 in each of the above cell lines was used as a control for IL-1-mediated NF-κB activation. A dose-dependent inhibitory effect of SIGIRR on LPS-induced NF-κB activation was seen after similar testing using 293-hTLR4A/MD2-CD14 cells (HEK293 cell line stably transfected with hTLR4A/MD2-CD14, a TLR4 signaling complex; Fig. 6*C*). In contrast, SIGIRR expression after transfecting SIGIRR plasmid into 293-hTLR3 cells (HEK293 cell line stably transfected with plasmid encoding TLR3 gene; Fig. 6*D*) did not inhibit poly(I:C)-induced NF-κB activation. These results indicate

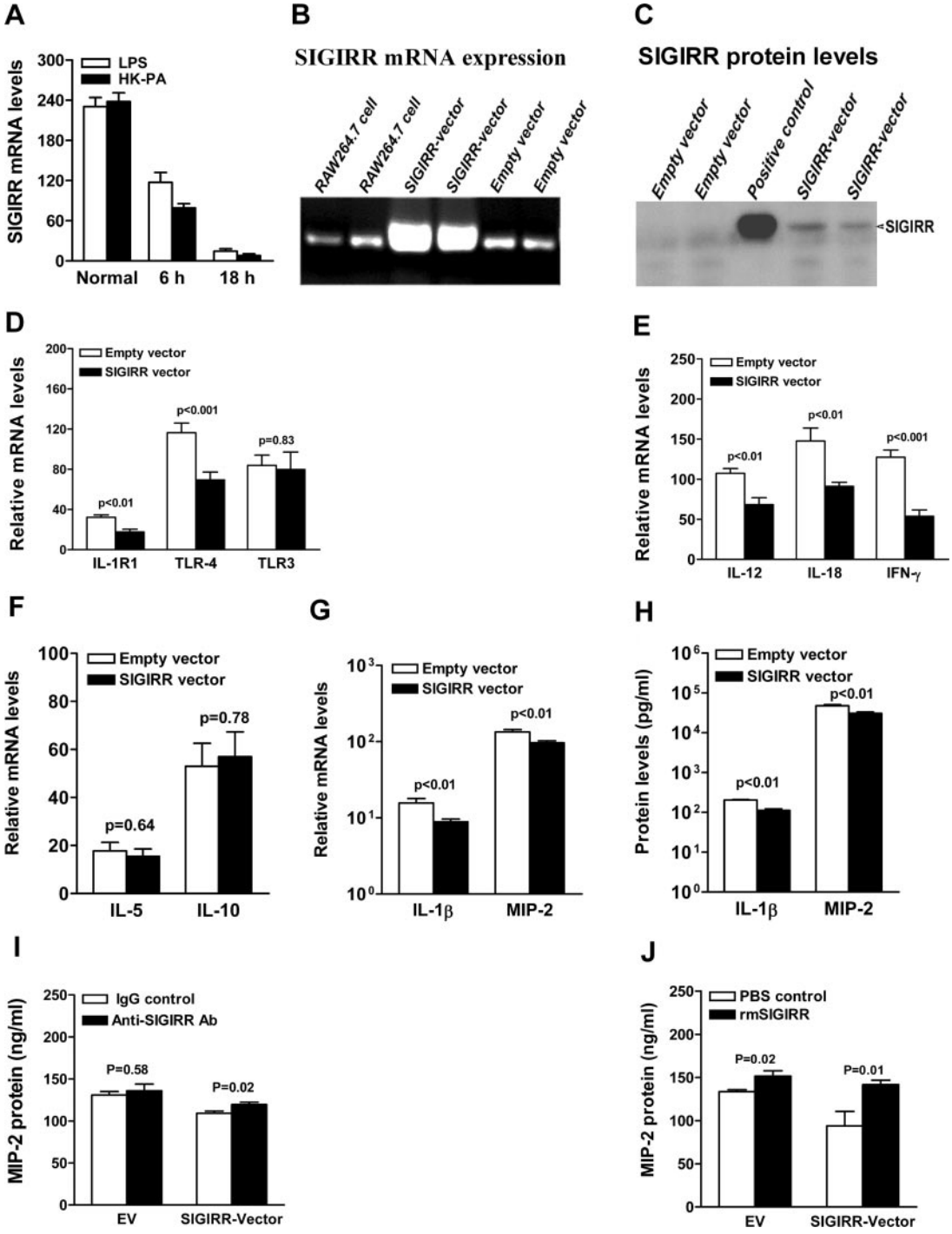

FIGURE 5. SIGIRR expression and its regulatory role in vitro. Low SIGIRR mRNA expression was detected in macrophage-like RAW cells by real-time PCR. Endogenous SIGIRR mRNA levels were down-regulated in a time-dependent manner after LPS or HK-PA treatment (*A*). After transient transfection of SIGIRR into RAW cells, SIGIRR mRNA and protein expression were confirmed by real-time PCR (*B*) and Western blots (*C*), respectively. After stimulating SIGIRR- vs vector-transfected RAW cells with HK-PA, mRNA levels for IL-1R1 and TLR4, but not TLR3, were significantly reduced (*D*), and mRNA levels for type 1-associated cytokines (*E*, IL-12, IL-18, and IFN-γ) and proinflammatory cytokines (*F*, IL-1β and MIP-2) were down-regulated significantly, while there was no significant mRNA expression difference for type 2 cytokines (*G*, IL-5 and IL-10). Protein expression (*H*) for IL-1β and MIP-2 was significantly reduced in culture supernatant of similarly treated RAW cells. Protein expression (*I* and *J*) for MIP-2 was significantly increased in SIGIRR-transfected RAW cells after treatment with anti-SIGIRR Ab ($p = 0.02$) or rSIGIRR ($p = 0.01$) over control-treated cells.

that SIGIRR negatively regulates IL-1/IL-1R1 and LPS/TLR4, but not poly(I:C)/TLR3 signaling.

Discussion

TLRs function as primary sensors of microbial products and initiate innate immunity through activation of the transcription factor NF-κB and stress-activated protein kinases, leading to the expression of immune and proinflammatory genes (22). Among the best characterized TLRs are TLR3, TLR4, TLR5, and TLR9, which sense dsRNA (23), LPS (24), flagellin (25), and CpG-DNA (8), respectively. In contrast, SIGIRR is a novel member of the TLR/IL-1R superfamily with unique anti-inflammatory properties (26,

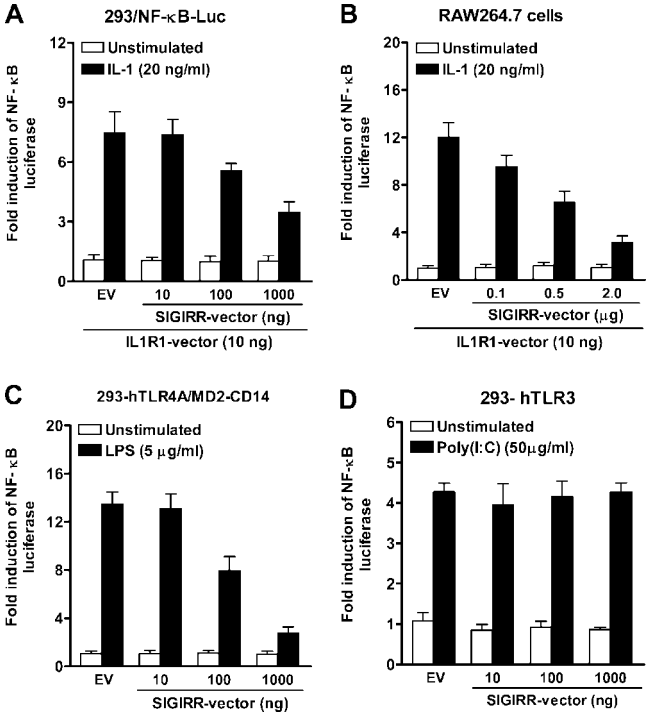

FIGURE 6. SIGIRR inhibits IL-1- and LPS- but poly(I:C)-induced NF-κB signaling. Overexpression of SIGIRR by cotransfecting SIGIRR (amounts shown on horizontal axes) and IL-1R1 (10 ng) into 293/NF-κB-luc (*A*) or RAW (*B*) cells impaired IL-1-induced NF-κB activation in a dose-dependent manner. A dose-dependent inhibitory effect of SIGIRR on LPS-mediated NF-κB activation was also seen in 293/TLR4-MD2-CD14 cells (*C*), but not seen in 293/TLR3 cells (*D*) on poly(I:C)-mediated NF-κB activation.

27). Although SIGIRR is normally expressed in various human (28) and murine (16, 27) tissues, with high mRNA levels in kidney, gut, liver, and lung (16), this is the first study to show the expression pattern and function of SIGIRR in cornea before and after infection. Results presented in this study revealed that SIGIRR is constitutively expressed (mRNA and protein) in the normal cornea of both B6 and BALB/c mice. After infection, SIGIRR mRNA levels were down-regulated at 12 h p.i. in the cornea of both mouse groups, but expression levels were significantly increased after 1 day p.i. in BALB/c cornea only. In contrast, protein expression of SIGIRR was significantly decreased at 1 day p.i., but remained elevated at 3 and 5 days p.i. in BALB/c over B6 mice. These findings at the mRNA level are consistent with a previous study showing that SIGIRR mRNA expression (protein levels not tested) was down-regulated at 6 and 12 h after i.p. injection of LPS, and returned to baseline 24 h later (16). From this study, we concluded that this pattern of SIGIRR consumption related to its functional involvement in ameliorating inflammation. This is an attractive hypothesis, and, in our study, it appears that protein expression is inversely related to mRNA levels at 1 day after infection, but is consistent with the mRNA pattern of expression at later times. We suggest that the decreased protein expression at 1 day p.i. may be due to consumption of SIGIRR, with the end result being amelioration of disease. These data also are similar to data obtained for IL-1RII, another negative regulator of the TLR/IL-1R superfamily, showing that IL-1RII was down-regulated in monocytes after bacterial challenge or LPS treatment (29, 30).

Because mRNA expression levels were markedly increased in the cornea of resistant BALB/c mice at 1 day p.i., SIGIRR was neutralized in BALB/c mice to test whether SIGIRR is required for host resistance against *P. aeruginosa* infection. Data from clinical score, slit lamp, and histopathology showed that BALB/c mice treated with anti-SIGIRR Ab over IgG treatment exhibited significantly increased corneal disease with more opacity and more severe stromal swelling and destruction. These findings are consistent with a previous study showing that SIGIRR$^{-/-}$ mice are more susceptible to inflammatory bowel disease induced by a noninfectious stimulus, dextran sulfate sodium, than wild-type mice (27). In addition, we found that expression of proinflammatory cytokines/chemokines such as IL-1β and MIP-2 and bacterial load in the cornea of BALB/c mice also were significantly increased in anti-SIGIRR Ab over control treatment, which further confirmed that SIGIRR is critical for host resistance against infection. However, PMN recruitment, indicated by detecting MPO activity in cornea, did not show a marked difference between anti-SIGIRR Ab- vs control-treated mice. This was surprising to us, because we detected elevated levels of both IL-1β and MIP-2 protein, both of which are chemoattractants for PMN in the cornea (19). Nonetheless, other proinflammatory cytokines, such as IL-18 and IFN-γ, for instance, were also elevated, and as we have shown in other studies (31), elevation of the latter cytokine will lead to increased NO levels, which can cause detrimental side effects, one of which is the breakdown of proteins of the corneal stroma. This in turn could contribute to an enhanced food source for the bacteria and actually increase the number of bacteria in the cornea, as we have shown in this study. In addition, after SIGIRR neutralization, histopathology revealed that corneal edema was prominent with PMN attached to the corneal endothelium, suggesting hypothetically that the capacity to maintain corneal hydration by the corneal endothelium also could be compromised. In line with our data, Garlanda et al. (27) showed that no significant difference in PMN recruitment (MPO activity) was detectable between SIGIRR$^{+/+}$ and SIGIRR$^{-/-}$ mice following i.p. injection of LPS.

Because members of TLR/IL-1R superfamily are involved not only in innate, but also in Th1/Th2 adaptive immune responses after induction of inflammation (32–34), we also focused our attention on defining whether SIGIRR-modulated cytokines associated with the adaptive immune response. In this regard, pathogenesis studies, including those from this laboratory, have shown that type 1/type 2 immune responses control resistance and susceptibility to many microorganisms (35), including *P. aeruginosa* (35, 36). Th1 cells promote type 1 immune responses by secreting cytokines such as IFN-γ, IL-2, and TNF-α, and Th2 cells mediate type 2 immunity by secreting the cytokines IL-4, IL-5, IL-10, and IL-13 (37). In fact, past gene array and RT-PCR studies from this laboratory have confirmed that susceptible B6 mice are dominant type 1 responders and resistant BALB/c mice are dominant type 2 responders to *P. aeruginosa* Ags (12). In the current study, we provide evidence that type 1-associated cytokines such as IL-18 and IFN-γ, both of importance in disease resolution in BALB/c mice (9), were significantly up-regulated in the cornea of anti-SIGIRR- over IgG-treated mice. Type 2 cytokines such as IL-4 and IL-10 were not affected after similar treatment. These data suggest that SIGIRR may participate in regulation and balance between Th1 and Th2 cytokines in the infected BALB/c cornea, and when this balance is disrupted, increased pathology occurs. Specifically, we hypothesize that in the infected cornea of the resistant mouse, a delicate balance between cytokines such as IFN-γ and IL-10 may exist. Therefore, by increasing the amount of IFN-γ and not changing the level of IL-10 (as shown in the current study), the balance would tip toward a Th1-like vs Th2-like response with enhanced pathology. In contrast, IL-33 (ligand for ST2) activation of ST2, an inhibitory molecule similar to SIGIRR (38), significantly increased

the expression of type 2-associated cytokines such as IL-4, IL-5, and IL-13, but had no effects on type 1 immune response-associated cytokines (IL-1β, IL-2, IL-12, TNF-α, and IFN-γ) (39). These data suggest that SIGIRR and ST2 differentially regulate the immunological homeostasis of type 1/type 2 inflammatory responses by either inhibiting type 1 or enhancing type 2 cytokine production. In *P. aeruginosa* keratitis, this may be an important determinant of whether a type 1 or type 2 inflammatory response prevails and whether or not the cornea perforates or heals.

In vitro studies with RAW cells also were used to further define how SIGIRR provides anti-inflammatory signals to down-regulate type 1 responses. Low SIGIRR mRNA expression was detected in cultured RAW cells, and these data agreed with a previous study showing that SIGIRR expression is low in monocytes and macrophages, while it is highly expressed in epithelial cells and immature dendritic cells (27). After LPS or HK-PA challenge, mRNA levels of SIGIRR were down-regulated at 6 and 18 h. These in vitro data further confirmed our in vivo Ab neutralization studies and were consistent with a previous study demonstrating that LPS stimulation leads to down-regulation of SIGIRR expression in various types of murine cells (16, 40). In addition, overexpression of SIGIRR by transient transfection of SIGIRR constructs into RAW cells markedly reduced mRNA expression of IL-1R1 and TLR4, but not TLR3. SIGIRR overexpression also significantly reduced gene expression of type 1 immune response-associated cytokines such as IL-12, IL-18, and IFN-γ, but did not affect levels of type 2 cytokines IL-4, IL-5, and IL-10. This also confirmed our in vivo Ab neutralization data and provided further evidence that SIGIRR negatively regulates type 1, but not type 2 immune responses. How SIGIRR functions to do this mechanistically is still not fully elucidated. However, data from SIGIRR-transfected RAW cells suggest that SIGIRR signaling can be blocked by Ab binding to the receptor or that one can interfere with signaling by consuming the ligand with recombinant protein. Consistent with this, dendritic cells from SIGIRR$^{-/-}$ mice markedly increased type 1 immune response-associated cytokines such as IL-6 and IL-12 in response to LPS (TLR4 ligand) or CpG oligodeoxynucleotides (TLR9 ligand) (27). Thus, these findings strongly indicate that SIGIRR may contribute to host resistance by dampening type 1, but not affecting a type 2, response.

The ligand for SIGIRR remains unknown, but it has been shown that SIGIRR cannot bind to any known ligand of the Toll/IL-1R (TIR) superfamily such as LPS or IL-1, subsequently activating NF-κB signaling (28). Although other studies have shown that SIGIRR was unable to directly induce NF-κB or AP-1 activation (28, 41), data in the current study provide evidence that SIGIRR overexpression in NF-κB stable transfected cell lines inhibits IL-1R- and TLR4-, but not TLR3-mediated NF-κB activation. Similarly, Brint et al. (42) reported that ST2 also exerts its negative regulatory function on IL-1R1- and TLR4-mediated NF-κB activation. They also showed that this occurred through sequestration of the TLR signaling components MyD88 and Mal, neither of which was tested in our study. However, Qin et al. (43) also demonstrated that SIGIRR may be able to inhibit NF-κB activation by sequestering recruitment of these two downstream adaptor molecules by interacting with their extracellular Ig domain and/or intracellular TIR domain of the TIR superfamily. Thus, it appears that SIGIRR acts as a decoy receptor for the formation of a signaling complex, which subsequently inhibits IL-1 and TLR4 (LPS) signaling through NF-κB activation, and thereby fine-tunes type 1/type 2 inflammatory responses.

In summary, our data provide direct evidence that SIGIRR is disparately expressed in the cornea of BALB/c vs B6 mice after *P. aeruginosa* infection. In addition, we present evidence that SIGIRR is required for host resistance against bacterial infection and functions to negatively regulate type 1, but not type 2 cytokine/chemokine production, reducing IL-1R and TLR4 expression and inhibiting IL-1 and TLR4 (LPS) signaling. Potentially, SIGIRR may provide a novel target for treatment of *P. aeruginosa* keratitis that will allow better control of excessive inflammation and corneal destruction.

Disclosures

The authors have no financial conflict of interest.

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