ST2 Is Essential for Th2 Responsiveness and Resistance to *Pseudomonas aeruginosa* Keratitis

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

Purpose. To elucidate the role of ST2, a member of the TLR/IL-1R (TIR) superfamily, in protecting against *Pseudomonas aeruginosa* keratitis in BALB/c mice.

Methods. ST2 mRNA and protein expression levels were tested by real-time PCR and Western-blot in C57BL/6 (B6; susceptible) versus BALB/c (resistant) mice before and after *P. aeruginosa* (strain 19660; American Type Culture Collection, Philadelphia, PA) challenge. Infected BALB/c mice also were tested after subconjunctival injection with recombinant murine (rm)ST2 or PBS. Disease was monitored by clinical score, slit lamp, bacterial plate count, a myeloperoxidase (MPO) assay to measure polymorphonuclear neutrophil (PMN) infiltrate, real-time RT-PCR, and ELISA.

Results. ST2 mRNA and protein were constitutively expressed in the uninfected normal corneas of both mouse groups. ST2 levels in the cornea of BALB/c compared with B6 mice were elevated significantly at 1 to 3 days post infection (PI), peaked at 3 and decreased at 5 days PI. BALB/c mice treated with rmST2 showed increased corneal opacity and perforation (at 5 days PI) when compared with PBS controls. rmST2- versus PBS-injected mice exhibited increased bacterial load, PMN infiltrate, and higher corneal mRNA levels for IL-1β, MIP-2, IL-6, IL-1R1, and Th1-type cytokine such as IFN-γ. Protein levels for IL-1β, MIP-2, and IL-6 also were significantly upregulated, whereas the Th2 cytokines IL-4 (mRNA), IL-5 (mRNA), and IL-10 (mRNA and protein) were significantly reduced.

Conclusions. ST2 is critical in resistance to *P. aeruginosa* keratitis, functioning to reduce corneal infection (bacterial load) and inflammation by negatively regulating proinflammatory cytokines and inhibiting type-1 immunity, but upregulating type-2 cytokine production, particularly IL-10. (*Invest Ophthalmol Vis Sci.* 2007;48:4626–4633) DOI:10.1167/iovs.07-0316

Keratitis induced by *Pseudomonas aeruginosa* is one of the most common and destructive of bacterial diseases, especially in extended-wear contact lens users.[1,2] Compelling evidence suggests that innate and adaptive immune responses play a critical role in bacterial keratitis,[3,4] and both bacterial (e.g., LPS) and host factors released from infiltrating cells during infection contribute to a rapidly progressing liquefactive stromal necrosis.[5,6] Studies also have shown that dominant T-helper type-1 (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.[3,7] Host innate responses to bacterial infection are primarily mediated by polymorphonuclear neutrophils (PMNs) and monocytes/macrophages.[8,9] The initial phase of host defense against many invading microbes such as *P. aeruginosa* also involves a family of proteins called Toll-like receptors (TLRs), which sense microbial products and trigger an innate immune response[10,11] that leads to the expression of various proinflammatory cytokines and chemokines,[12,13] including, but not limited to, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, macrophage inflammatory protein (MIP)-2, IL-4, IL-5, IL-6, IL-10, IL-12, interferon IFN-γ, and nitric oxide (NO). These inflammatory mediators may promote elimination of bacteria and induce tissue repair, but also, if unbalanced or uncontrolled, may amplify the inflammatory response leading to tissue damage and corneal perforation.[1]

In this regard, TLR-activation is a double-edged sword, and negative regulation for TLR signaling may be necessary to avoid detrimental and inappropriate inflammatory responses,[14] as the immune system must strike a constant balance between activation by TLR signaling and inhibition by negative regulators. Several negative regulators of TLR signaling, including ST2, have been identified over the past several years. The negative regulatory response is achieved at multiple levels.[15] The first level is the production of soluble TLRs, such as sTLR2 and sTLR4, acting as soluble decoy receptors by binding its ligand and competitively blocking TLR2 and TLR4 signaling, respectively. The second level involves transmembrane proteins, such as ST2 and single Ig IL-1R-related molecule (SIGIRR), which sequester recruitment of adaptor molecules such as MyD88 and IRAK.[16] Once TLR and ligand interaction occurs, the third level of negative regulation that controls TLR signaling is mediated by intracellular regulators such as MyD88s, SOCS1, and IRAK-M, whereas the fourth level of negative regulation reduces TLR expression or increases degradation of TLRs. The fifth level of negative regulation may activate TLR-induced apoptosis to control pathogenesis and sepsis caused by bacterial infection.

In several studies, investigators have detected the expression of TLRs such as TLR2,[17] TLR3,[18] TLR4,[19,20] TLR5,[21] TLR9,[5,17] and SIGIRR[22] in mouse cornea and cultured human corneal epithelial cells, but the corneal localization and functional role of ST2 in *P. aeruginosa* keratitis remains unexplored. In the present study, we investigated the expression of ST2 in the cornea of susceptible (B6) and resistant (BALB/c) mice before and after *P. aeruginosa* infection. Our hypothesis is that ST2 is an important negative regulator of TLR signaling in *P. aeruginosa* keratitis. Our data provide direct evidence that ST2 is detected in the normal cornea of both mouse groups, but is disparately expressed after infection. Furthermore, our data indicate that ST2 is critical in resistance to *P. aeruginosa* keratitis, functioning to reduce corneal infection and inflammation by negatively regulating proinflammatory cytokines, inhibiting type-1 immunity, and upregulating type-2 cytokine production.

From the Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan.

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Corresponding author: Linda D. Hazlett, Department of Anatomy and Cell Biology, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, MI 48201; lhazlett@med.wayne.edu.

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Materials and Methods

Corneal Infection

Eight-week-old female B6 and BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized with ether and placed beneath a stereoscopic microscope at 40× magnification. The cornea of the left eye was wounded with three 1-mm incisions with a sterile 25-gauge needle. A bacterial suspension (5 μL) containing 1×10^6 colony forming units (CFU)/μL of *P. aeruginosa* (strain 19660; ATCC, Manassas, VA), prepared as described before,[7,23] was topically applied to the scarified cornea. The eyes were examined macroscopically at 1 day post infection (PI) and/or at times described later, to ensure that mice were similarly infected and to monitor disease. Animals were treated humanely and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ocular Response to Infection

After bacterial infection, corneal disease (clinical score) was graded as described before[24]: 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. Five mice from each group, together with a similar number of control animals were examined at 1 to 5 days PI and slit lamp photography was used to illustrate the disease response.

Real-Time PCR

Corneas were removed from normal and infected (1, 3, and 5 days PI) B6 and BALB/c mice and from BALB/c mice treated with rmST2 protein, PBS, or an irrelevant fusion protein (described later). Corneas were immediately frozen in liquid nitrogen and homogenized (RNA Stat-60; Tel-Test, Friendsville, TX), and total RNA was isolated per the manufacturer's instructions. Then, 1 μg of total RNA was reverse transcribed to produce a cDNA template for PCR reaction. For real-time PCR amplification, 1 μL of each cDNA sample was used per 25 μL of PCR reaction. Sequences of primer sets for real-time PCR are shown in Table 1. PCR measurements were analyzed in duplicate in three independent runs of a real-time detection system (Bio-Rad Laboratories, Hercules, CA). Relative mRNA levels of ST2, IL-1β, MIP-2, IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-1R1, TLR3, TLR4, and TLR9 were calculated after normalizing to β-actin, as described before.[4,5]

Western Blot Analysis

Corneas were collected from normal, uninfected B6 and BALB/c mice, and at 1, 3, and 5 days PI, pooled corneas (n = 5/group/time) were lysed and homogenized by means of a glass 1-mL tissue homogenizer in ice-cold tissue protein extraction reagent (Pierce, Rockford, IL) for 30 minutes. Protein concentration of the supernatant was determined by bicinchoninic assay (BCA; Pierce) protein assay. Supernatants were separated on 10% SDS-PAGE and 50 μg of corneal protein sample was added to each lane. The electrophoretically separated material was transferred to a supported nitrocellulose membrane (Millipore, Bedford, MA) and blocked in a 5% solution of nonfat dry milk prepared in 1× Tris-buffered saline (TBS) and 0.05% Tween 20. Blots were incubated with primary goat anti-mouse ST2 primary Ab (R&D Systems, Minneapolis, MN) diluted in PBS overnight at 4°C, washed three times for 10 minutes each with PBS, detected with horseradish peroxidase–conjugated secondary Ab (R&D Systems) diluted 1:2000 in PBS+5% nonfat milk, and developed by enhanced chemiluminescence (ECL Plus; GE Healthcare), according to the manufacturer's protocol and as described before.[22] Band intensity on the gels was quantitated and normalized to the β-actin control.

Table 1. Primer Sequences for Real-Time PCR

Gene	Primer Sequences
ST2	Forward TGA CGG CCA CCA GAT CAT TCA CAG
	Reverse GCC AAA GCA AGC TGA ACA GGC AAT AC
IL-1β	Forward CGC AGC AGC ACA TCA ACA AGA GC
	Reverse TGT CCT CAT CCT GGA AGG TCC ACG
MIP-2	Forward TGT CAA TGC CTG AAG ACC CTG CC
	Reverse AAC TTT TTG ACC GCC CTT GAG AGT GG
IFN-γ	Forward GTT ACT GCC ACG GCA CAG TCA TTG
	Reverse ACC ATC CTT TTG CCA GTT CCT CCA G
IL-4	Forward GAA GAA CAC CAC AGA GAG TGA GC
	Reverse CTT TCA GTG ATG TGG ACT TGG AC
IL-5	Forward AAA GAG AAG TGT GGC GAG GAG AGA C
	Reverse CCT TCC ATT GCC CAC TCT GTA CTC ATC
IL-6	Forward CAC AAG TCC GGA GAG GAG AC
	Reverse CAG AAT TGC CAT TGC ACA AC
IL-10	Forward TGC TAA CCG ACT CCT TAA TGC AGG AC
	Reverse CCT TGA TTT CTG GGC CAT GCT TCT C
IL-1R1	Forward CTC TGC TTC TTG ACA ACG TGA GCT TC
	Reverse TAT AGT CCC CTC TGT GCT CTT CAG CC
TLR3	Forward AGT GAG CAA GGG AGA ATG AGC AAG
	Reverse TCA CGG GAT TGG TGA GTC TGA AG
TLR4	Forward CGC TTT CAC CTC TGC CTT CAC TAC AG
	Reverse ACA CTA CCA CAA TAA CCT TCC GGC TC
TLR-9	Forward AGC TCA ACC TGT CCT TCA ATT ACC GC
	Reverse ATG CCG TTC ATG TTC AGC TCC TGC
β-Actin	Forward GAT TAC TGC TCT GGC TCC TAG C
	Reverse GAC TCA TCG TAC TCC TGC TTG C

Fusion Protein Treatment

BALB/c mice (n = 5/group/treatment) were injected subconjunctivally with 1 μg rmST2/Fc fusion protein (R&D Systems) or PBS 1 day before infection. At 1, 3, and 5 days PI, each mouse was injected intraperitoneally with a similar amount of rmST2 diluted in PBS. Control mice similarly received an equal volume and amount of PBS. An irrelevant fusion protein control (1 μg, hIgG/Fc; R&D Systems) was injected similarly (n = 5 mice) for selective experimental evaluation of mRNA levels to validate the use of PBS as a control in this model.

Quantitation of PMN

Samples were assayed for myeloperoxidase (MPO) activity, as described before.[25] Corneas from BALB/c mice were collected at 3 and 5 days PI and homogenized in 1.0 mL of 50 mM phosphate buffer [pH 6.0], containing 0.5% HTAB (hexadecyltrimethylammonium bromide). Samples were freeze thawed three times and after centrifugation, 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 minutes (Helios-α; Thermo Spectronics, Rochester, NY) and the results expressed as units of MPO/cornea. One unit of MPO activity = $\sim 2.0 \times 10^5$ PMNs.[25]

Quantitation of Viable Bacteria in the Cornea

Bacteria were quantitated in the infected cornea of BALB/c mice treated with rmST2 or PBS, at 3 and 5 days PI (n = 5/group/time). Corneas were collected from both groups and individually homogenized in sterile 0.9% saline containing 0.25% BSA. 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, Detroit, MI) in triplicate and the plates 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 as described before.[3]

ELISA Analysis of Cytokines

Protein levels for proinflammatory cytokines and chemokines were quantitated using enzyme-linked immunosorbent assay (ELISA) kits

(R&D Systems), as described before.[23] Infected corneas of BALB/c mice (n = 5/group/time) treated with rmST2 or PBS were collected at 3 and 5 days PI and tested for IL-1β, MIP-2, IL-6, and IL-10 protein levels. Individual samples were homogenized with a glass pestle (Fisher, Itasca, IL) in 1.0 mL PBS with 0.1% Tween 20 and centrifuged. A 50-μL aliquot of the supernatant was assayed per the manufacturer's instruction. The reported sensitivity of these assays is <3 pg/mL for IL-1β, 1.5 pg/mL for MIP-2, 1.6 pg/mL for IL-6, and 4 pg/mL for IL-10.

Statistical Analysis

The difference in clinical score between two groups at each time point was tested by the Mann-Whitney test. An unpaired, two-tailed Student's *t*-test was used to determine statistical significance for real-time PCR, ELISA, MPO, bacterial plate counts, and Western blot analyses. Data were considered significant at $P < 0.05$. All experiments were performed at least twice to ensure reproducibility, and the data from a single typical experiment are shown.

Results

ST2 Expression in *P. aeruginosa*–Infected Cornea

Although ST2 was constitutively similarly expressed ($P = 0.26$ for mRNA, $P = 0.35$ for protein) in the uninfected normal cornea of both mouse groups, its expression pattern was disparate after bacterial infection (Fig. 1). In the infected cornea, ST2 mRNA expression (Fig. 1A) in BALB/c compared with B6 mice was significantly upregulated at 1 ($P < 0.05$), peaked at 3 ($P < 0.01$), and maintained at a higher level at 5 ($P < 0.01$) days PI. ST2 protein (Figs. 1B, 1C, for Western blot and band intensity, respectively) was constitutively expressed in the uninfected cornea of both groups, and significantly increased at 3 ($P < 0.001$) and 5 ($P < 0.01$) days PI in the cornea of BALB/c compared with B6 mice.

A Protective Role of ST2 in Host Defense against Corneal Infection

Since ST2 mRNA and protein levels were differentially expressed in infected cornea of BALB/c and B6 mice, the next series of in vivo studies tested whether ST2 was protective in bacterial keratitis. For this, BALB/c mice were injected subconjunctivally with rmST2 (as a soluble decoy receptor) to determine whether this would impair host resistance. Clinical score (Fig. 2A) showed that BALB/c mice injected with rmST2 exhibited significantly increased corneal disease ($P < 0.05$ and <0.001 at 3 and 5 days PI, respectively) when compared with PBS treatment. A representative slit lamp photograph at 5 days PI revealed more corneal opacity and disease in the rmST2-treated (Fig. 2B) compared with the PBS-treated (Fig. 2C) group. To determine whether rmST2 treatment impairs bacterial killing, viable bacterial plate counts were performed. Bacterial load (Fig. 2D) was significantly increased ($P < 0.01$ at both 3 and 5 days PI) in the corneas of rmST2- compared with PBS-treated mice. We also tested the effect of rmST2 treatment on PMN infiltration of the cornea by quantitation of MPO activity (Fig. 2E). There was a significant increased number of PMN in the rmST2- compared with PBS-treated mouse corneas ($P < 0.01$ and <0.001 at 3 and 5 days PI, respectively).

ST2 Regulation of Proinflammatory Cytokine Production and Type-1/Type-2 Immune Response

Since blocking ST2-signaling by rmST2 conferred susceptibility to corneal infection, we next investigated the mechanisms involved. Treatment with rmST2 led to a significant increase in mRNA levels of IL-1β (Fig. 3A; $P < 0.05$, <0.05, and <0.01 at 1, 3, and 5 days PI, respectively), MIP-2 (Fig. 3B; $P < 0.05$ at 1, 3, and 5 days PI), and IL-6 (Fig. 4B; $P < 0.01$, <0.05, and

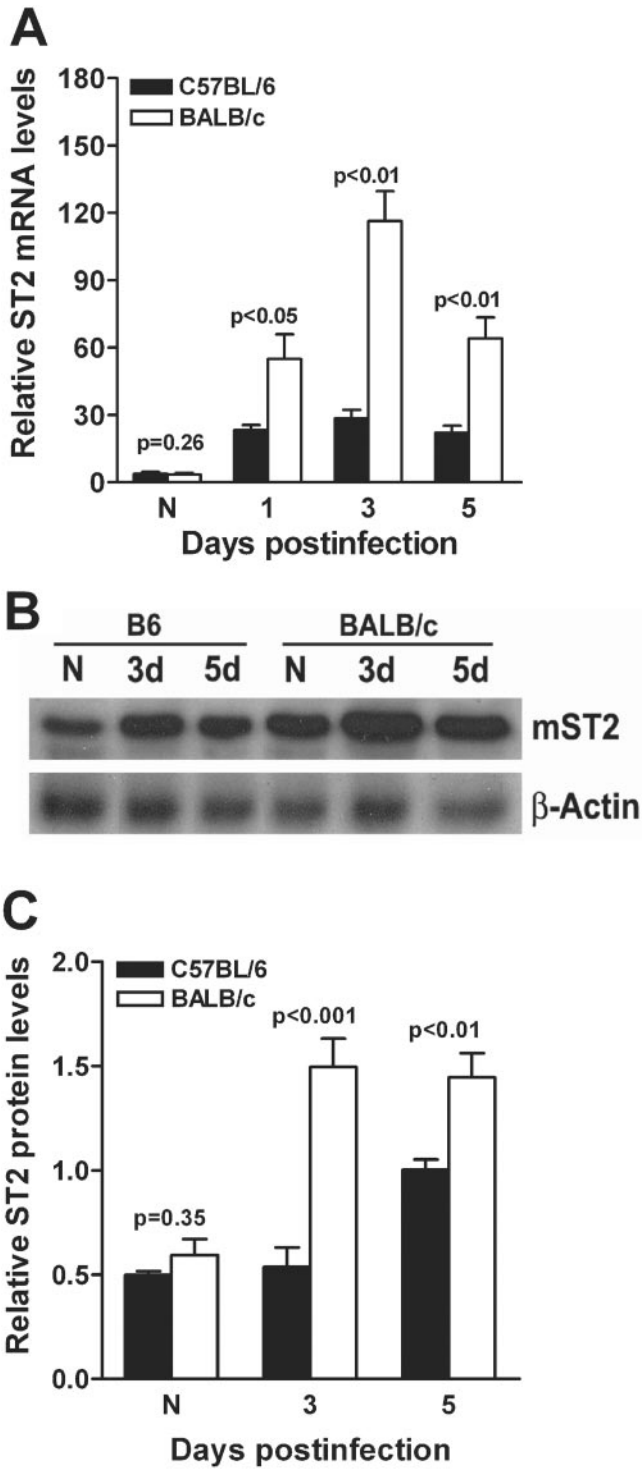

Figure 1. ST2 expression in the cornea of B6 and BALB/c mice. (**A**) ST2 mRNA levels in B6 and BALB/c normal (N) and infected corneas at 1, 3, and 5 days PI. (**B**) Western blot of ST2 protein levels in the corneas of B6 and BALB/c normal (N) and infected mice at 3 and 5 days PI. Equivalent protein loaded (50 μg total protein per lane). (**C**) The intensity of bands was quantitated and normalized to the β-actin control. Data, expressed as the mean ± SEM integrated density values at each time point, are significant at 3 and 5 days PI.

<0.001 at 1, 3, and 5 days PI, respectively) when compared with PBS treatment. To confirm the mRNA data, ELISA analysis was performed and showed that protein levels of IL-1β (Fig.

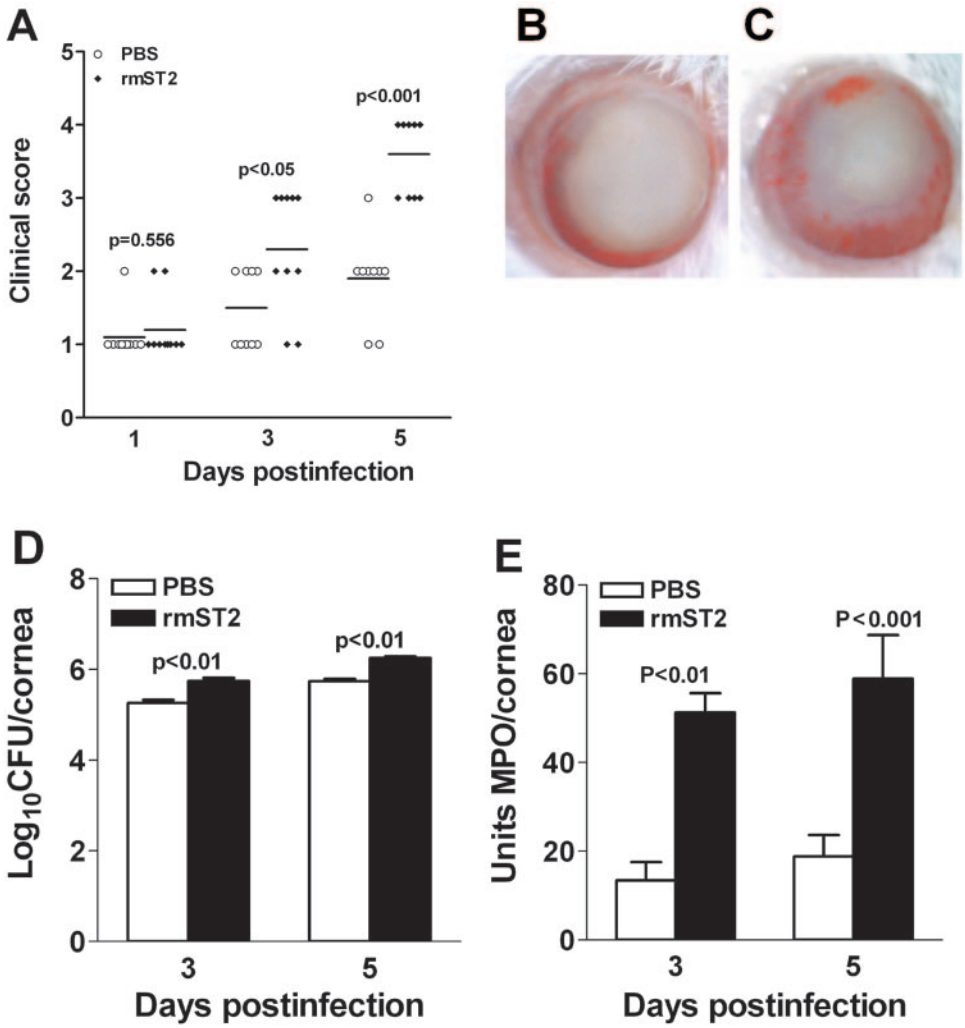

FIGURE 2. In vivo studies of ST2 in host resistance. Clinical score (**A**) shows that more corneas perforated in the rmST2 compared with the PBS-treated group. Slit lamp at 5 days PI (**B**, **C**) shows more opacity and disease in the (**B**) rmST2-treated mouse cornea than in the control (**C**). Significantly increased bacterial counts (**D**) and recruitment of PMNs (**E**, MPO activity) were observed in the rmST2- versus PBS-treated mice.

3C; $P < 0.05$ and <0.01 at 3 and 5 days PI, respectively), MIP-2 (Fig. 3D; $P < 0.01$ at both 3 and 5 days PI), and IL-6 (Fig. 4C; $P < 0.01$ at 5 days PI) also were significantly upregulated in the cornea of rmST2- versus PBS-treated BALB/c mice. Significantly elevated mRNA expression levels for the type I cytokine IFN-γ (Fig. 4A; $P < 0.05$, <0.05 and <0.01 at1, 3, and 5 days PI, respectively) also were detected in the cornea of rmST2 compared with PBS-treated mice. We also tested whether rmST2 attenuated type-2-associated cytokine production in vivo. The mRNA expression of type-2 immune response–associated cytokines, including IL-4 (Fig. 4D; $P < 0.05$, <0.05, and <0.01 at 1, 3, and 5 days PI, respectively), IL-5 (Fig. 4E; $P < 0.05$ at 1, 3, and 5 days PI) and IL-10 (Fig. 4F; $P < 0.05$ at 3 and 5 days PI) were significantly downregulated in rmST2 compared with PBS-treated BALB/c mice. ELISA was also used to detect IL-10 protein (Fig. 4G) at 3 ($P < 0.01$) and 5 ($P < 0.05$) days PI, and confirmed the mRNA data. We also selectively tested an irrelevant fusion protein, hIgG/Fc (to compare with PBS injection). No differences were detected between the two controls in clinical score (data not shown) or mRNA expression levels of IL-10, IL-1β, and IFN-γ at 5 days PI (compare Fig. 5 with Figs. 3A, 4A, 4F).

Effect of ST2 on TLR Expression

Because rmST2 treatment in BALB/c mice enhanced proinflammatory cytokine production in the cornea and worsened disease, the next series of studies was initiated to determine further whether TLR expression changes after rmST2 treatment. Expression levels of IL-1R1, TLR3, TLR4, and TLR9 in the cornea of rmST2- versus PBS-treated BALB/c mice were tested. Data showed that rmST2 treatment led to a significant increase in mRNA for IL-1R1 (Fig. 6A; $P < 0.01$, <0.05, and <0.05 at 1, 3, and 5 days PI, respectively). In contrast, no significant difference in mRNA expression levels was detected for TLR3 (Fig. 6B; $P = 0.1$, 0.67, and 0.95 at 1, 3, and 5 days PI, respectively), TLR4 (Fig. 6C; $P = 0.95$, 0.1, and 0.4 at 1, 3, and 5 days PI, respectively), and TLR9 (Fig. 6D, $P = 0.49$, 0.9, and 0.63 at 1, 3, and 5 days PI, respectively) after infection.

DISCUSSION

TLRs are a family of proteins that are involved in the initial phase of host defense against invading pathogens.[26] The best characterized TLRs are TLR2, 3, 4, 5, 7/8, and 9, which sense lipoprotein,[27] dsRNA,[28] LPS,[12] flagellin,[29] ssRNA,[30] and unmethylated CpG-DNA,[31] respectively. TLRs act as primary sensors of microbial products and initiate innate immunity through activation of transcription factors leading to induction of strong immune and proinflammatory responses.[32] In contrast, ST2 is a novel member of the TLR superfamily with unique anti-inflammatory properties.[33] Although ST2 is ubiquitously expressed in various human and murine tissues,[34] with higher expression levels on the surface of fibroblasts,[35] mast

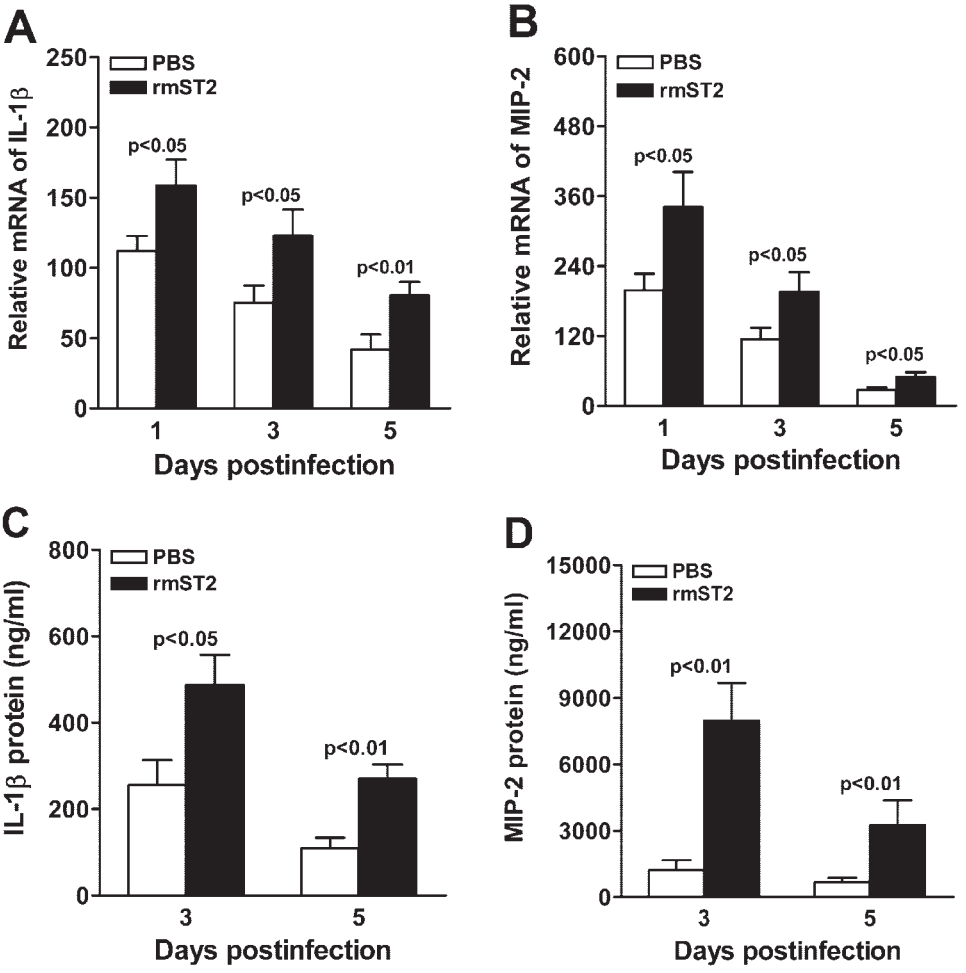

FIGURE 3. Production of proinflammatory cytokines after rmST2 treatment. mRNA levels for IL-1β (**A**) and MIP-2 (**B**) as well as protein levels for each (**C**, **D**) were significantly upregulated in the cornea of rmST2- versus PBS-treated mice.

cells,[36] and Th2 cells,[37,38] this is the first study to show expression of ST2 in the normal cornea and to test its function after bacterial infection. The results presented herein revealed that murine ST2 (mRNA and protein) is constitutively expressed in the uninfected normal cornea of B6 and BALB/c mice and that, after *P. aeruginosa* infection, its expression levels in the cornea of BALB/c compared with B6 mice were significantly upregulated. These data, suggesting that ST2 was involved in bacterial keratitis, are consistent with results in a previous study showing that gene and protein expression of ST2 was increased in a murine alveolar macrophage (AM) cell line reacting to inflammatory stimuli in vitro and in the lung tissue of an acute lung injury model in vivo.[39] In that study, both lipopolysaccharide (LPS) and proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, induced ST2 mRNA expression in MH-S cells. In an acute lung injury model, protein and mRNA expression levels of ST2 significantly increased after LPS challenge. Our findings also are consistent with those in a previous study in a murine model of asthma, showing that sST2 protein levels in murine sera and the mRNA expression levels of ST2 in lung tissue were increased after allergen challenge.[40] In line with data from animal studies, ST2 protein expression levels also were elevated in the sera of patients with asthma, and correlated well with the severity of asthma exacerbation.[41,42]

Although mRNA and protein expression levels of ST2 were significantly increased in the cornea of resistant BALB/c mice after infection, there is no published information as to whether ST2 is protective in the cornea after *P. aeruginosa* infection. To test this notion, we injected rmST2 protein, which acts as a soluble decoy receptor by binding its ligand (IL-33),[43] thereby competitively blocking ST2 signaling. A dose–response (100 ng, 1 μg, and 5 μg) study indicated that 1 μg gave an optimum decoy effect in the cornea by increasing the disease response (data not shown). Data from clinical score and slit lamp showed that BALB/c mice treated with rmST2 (compared with PBS) exhibited significantly increased corneal disease with more opacity and more perforated corneas after infection. A similar effect was achieved with injection of an irrelevant fusion protein (data not shown). These findings are consistent with a study showing that blocking ST2 signaling through administration of anti-ST2 monoclonal antibody exacerbated the toxic effects of LPS.[16] In addition, we found that bacterial load (more than sixfold higher) and PMN recruitment, indicated by detecting MPO activity in cornea, also were significantly increased in the cornea of rmST2- compared with control-treated mice. This result was not surprising to us, because data presented in this study also showed that expression levels of proinflammatory cytokines and chemokines such as IL-1β and MIP-2, chemoattractants for PMNs,[44–46] were significantly upregulated in the cornea of rmST2- compared with PBS (or irrelevant fusion protein, IL-1β tested)-treated BALB/c mice. These data are consistent with data obtained when testing SIGIRR, another negative regulator of the TLR superfamily. In that study, we found that SIGIRR was upregulated in the cornea of BALB/c mice after bacterial challenge and is necessary for host resistance against bacterial infection.[22]

Overall, the results presented in this study provide direct evidence that blocking ST2 signaling by rmST2 exacerbated corneal infection, but the molecular mechanisms remained obscure. In this regard, pathogenesis studies have shown that

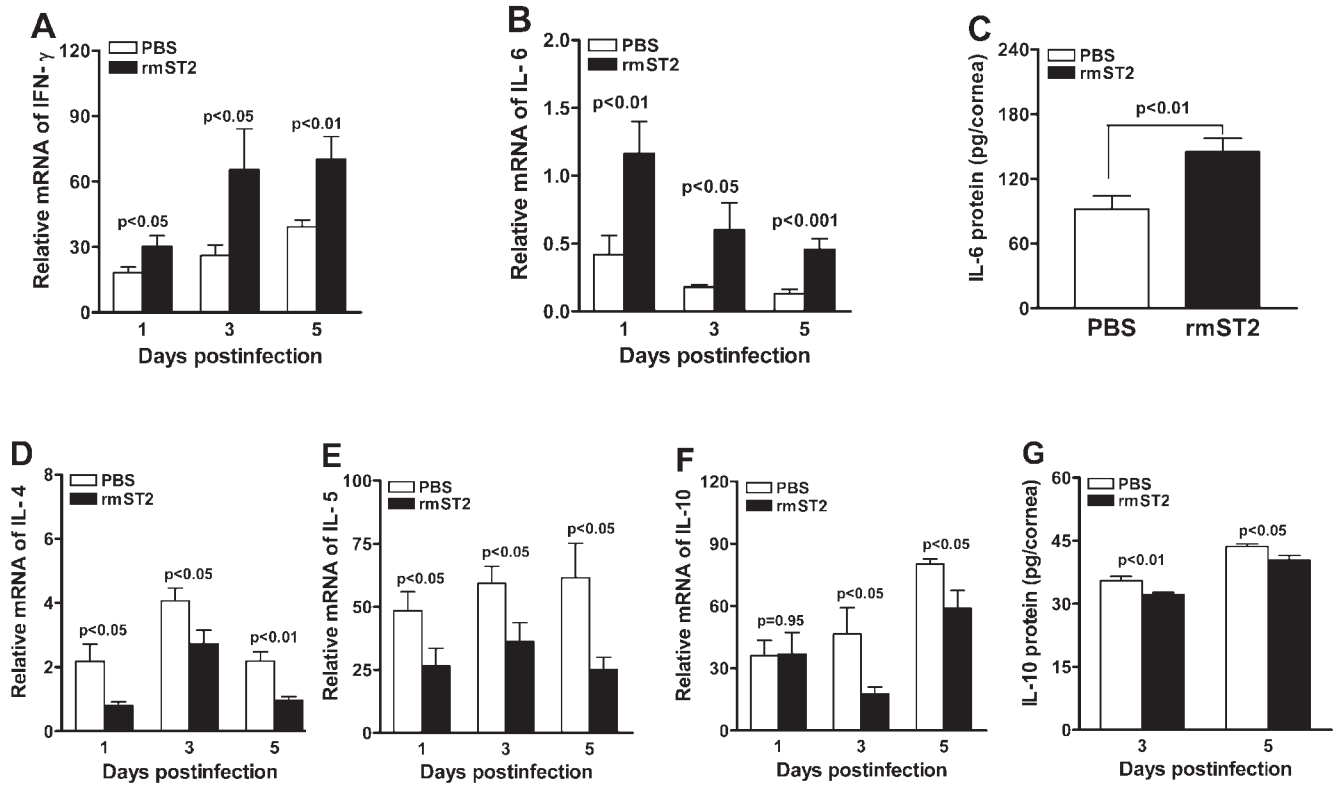

FIGURE 4. Type-1/type-2 cytokine production after rmST2 treatment. In the cornea of rmST2 compared with PBS-treated mice, mRNA expression levels of IFN-γ (**A**) and IL-6 (**B**) increased significantly at 1, 3, and 5 days PI, whereas IL-4 (**D**), IL-5 (**E**), and IL-10 (**F**) mRNA levels decreased significantly at 1 (except IL-10), 3 and 5 days PI. IL-6 (**C**) and IL-10 (**G**) protein levels were also tested by ELISA to confirm the mRNA data. IL-10 decreased significantly at 3 and 5 days PI, whereas IL-6 increased significantly in rmST2- compared with PBS-injected mice when tested at 5 days PI.

type-1/type-2 immune responses control resistance and susceptibility to many organisms including *P. aeruginosa*.[47,48] Th1 T cells promote type-1 immune responses by secreting cytokines such as IFN-γ and IL-2, and Th2 T cells mediate type-2 immunity by secreting the cytokines IL-4, IL-5, IL-10, and IL-13. Microarray studies from this laboratory have confirmed that susceptible B6 mice are dominant type-1, and resistant BALB/c mice are dominant type-2 responders to *P. aeruginosa* infection.[4] Since the TLR superfamily are involved, not only in innate but also in Th1/Th2 adaptive immune responses after induction of inflammation,[10,49] we tested whether ST2 modulates Th1/Th2 cytokine expression. Data provided evidence that type-1 associated cytokines such as IFN-γ were significantly upregulated in the cornea of rmST2- versus control-treated mice. These data are consistent with studies by Kropf et al.,[50] showing that interfering with ST2 signaling by injection of anti-T1/ST2 mAb or rmST2/Fc fusion protein during the course of *Leishmania major* infection resulted in increased IFN-γ production. Meanwhile, type-2 cytokines such as IL-4, IL-5, and IL-10 were markedly downregulated after similar treatment. These data also are consistent with results in a previous study in which pretreatment with rmST2 protein significantly inhibited the production of IL-4 and IL-5 from OVA-stimulated splenocytes.[40] Using ST2-deficient mice, Townsend et al.[38] also demonstrated that ST2 expression plays a critical role in the development of Th2-like cytokine responses. These data are consistent with our findings in BALB/c mouse cornea and suggest that rmST2 treatment prevented ST2 signaling, increased the production of Th1-type cytokines, but attenuated Th2-type cytokine production. Our data also suggest that ST2 may participate in the regulation and balance of Th1 and Th2 cytokines in the infected BALB/c cornea and that, when this balance is disrupted, pathologic events increase. In contrast, in studies of B6 IL-10-knockout mice, Cole et al.[51] demonstrated that the absence of IL-10 led to increased bacterial load and reduced PMN in cornea. This group also has reported that at 24 hours after infection, expression of IL-6 and

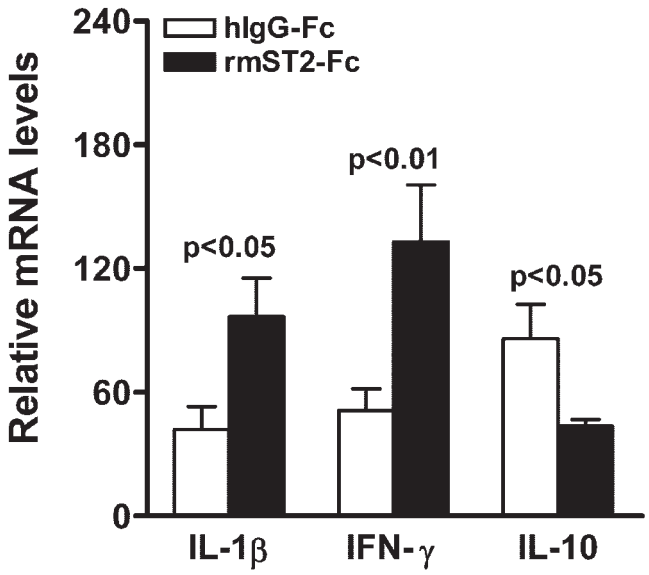

FIGURE 5. mRNA expression levels for IL-1β, IFN-γ, and IL-10 in the cornea of rmST2/Fc- versus hIgG/Fc-treated mice at 5 days PI.

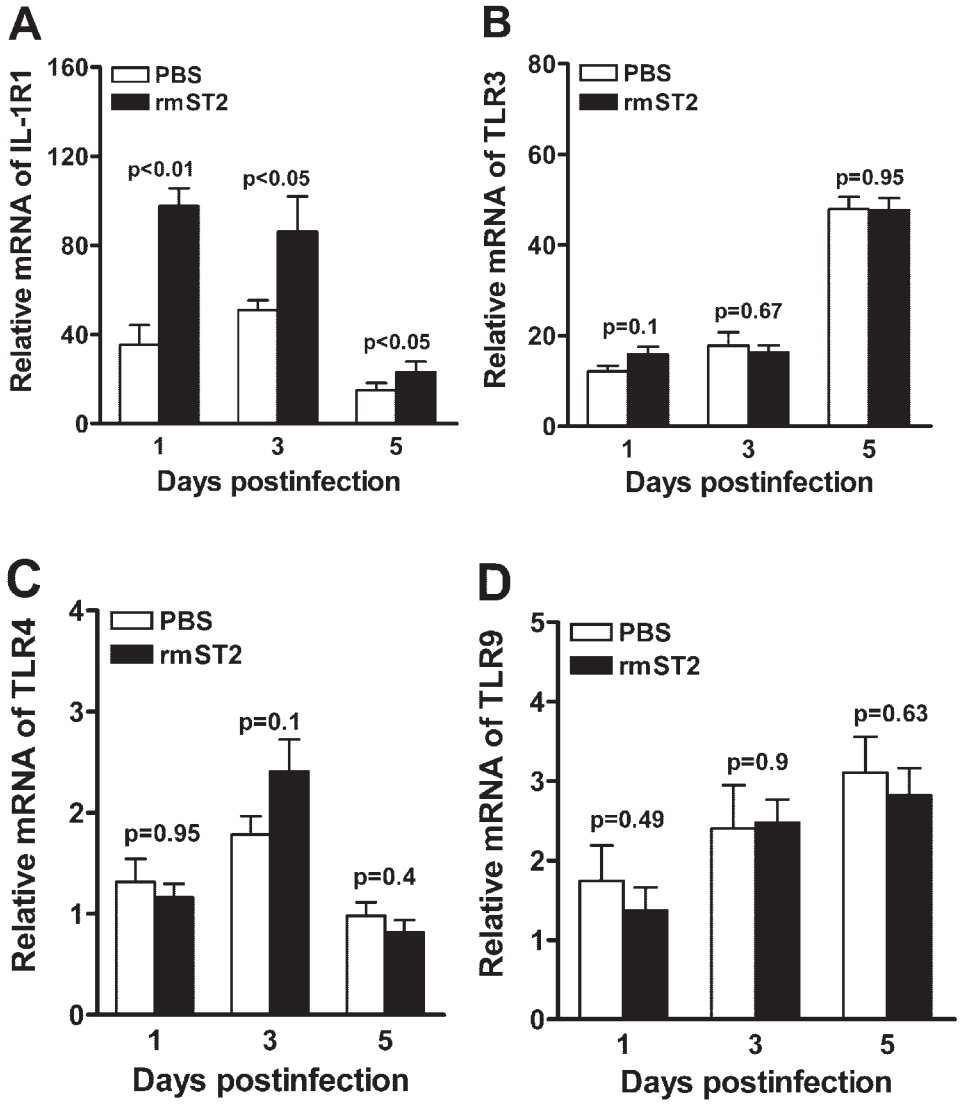

FIGURE 6. IL-1R1, TLR3, TLR4, and TLR9 expression after rmST2 treatment. IL-1R1 mRNA levels (**A**) were significantly elevated at 1, 3, and 5 days PI in the corneas of rmST2 compared with PBS-treated BALB/c mice. mRNA expression levels for TLR3 (**B**), TLR4 (**C**), and TLR9 (**D**) were similar at 1, 3, and 5 days PI in both groups.

-10 was significantly greater in whole eyes than in the cornea of infected eyes.[52] Perhaps the use of B6 versus BALB/c mice, and testing of whole eyes versus only the cornea, as in our studies, account for some of these divergent results.

It also is intriguing that SIGIRR, another inhibitory molecule similar to ST2, significantly downregulated the expression of type-1-associated cytokines, such as IL-18 and IFN-γ, but had no effects on type-2 immune response–associated cytokines, such as IL-4, -5, and -10. These data suggest that ST2 and SIGIRR differentially regulate the immunologic homeostasis of type-1/type-2 inflammatory responses. In *P. aeruginosa* keratitis, this may be an important determinant of whether a type-1 and/or type-2 inflammatory response prevails and whether the cornea perforates or heals.

In summary, the data presented herein indicate that ST2 is disparately expressed in the cornea of B6 versus BALB/c mice after *P. aeruginosa* infection. In addition, we provide substantial evidence that ST2 is critical for host resistance and functions to promote type-2 (e.g., IL-10 production), while negatively regulating type-1 cytokine production, reducing IL-1R1 expression, decreasing PMN infiltration, and impairing bacterial killing. These data suggest that ST2 plays a protective role in corneal defense against bacterial infection and that manipulation of ST2 levels may provide a novel approach for treatment of *P. aeruginosa* keratitis.

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