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A Novel Pathway Regulating Lipopolysaccharide-Induced Shock by ST2/T1 Via Inhibition of Toll-Like Receptor 4 Expression

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ST2/ST2L, a member of the IL-1R gene family, is expressed by fibroblasts, mast cells, and TH2, but not TH1, cells. It exists in both membrane-bound (ST2L) and soluble forms (ST2). Although ST2L has immunoregulatory properties, its ligand, cellular targets, and mode of action remain unclear. Using a soluble ST2-human IgG fusion protein, we demonstrated that ST2 bound to primary bone marrow-derived macrophages (BMM) and that this binding was enhanced by treatment with LPS. The sST2 treatment of BMMs inhibited production of the LPS-induced proinflammatory cytokines IL-6, IL-12, and TNF-α but did not alter IL-10 or NO production. Treatment of BMMs with sST2 down-regulated expression of Toll-like receptors 4 and 1 but induced nuclear translocation of NF-κB. Administration of sST2 in vivo after LPS challenge significantly reduced LPS-mediated mortality and serum levels of IL-6, IL-12, and TNF-α. Conversely, blockade of endogenous ST2 through administration of anti-ST2 Ab exacerbated the toxic effects of LPS. Thus, ST2 has anti-inflammatory properties that act directly on macrophages. We demonstrate here a novel regulatory pathway for LPS-induced shock via the ST2-Toll-like receptor 4 route. This may be of considerable therapeutic potential for reducing the severity and pathology of inflammatory diseases. The Journal of Immunology, 2001, 166: 6633–6639.

Previously, ST2/T1 was independently identified as an oncogene and serum-responsive gene expressed in fibroblasts (1–3). It was subsequently found to be expressed by mast cells (4) and by TH2, but not TH1, cells (5–7). Although ST2/T1 is a member of the IL-1R family, it does not bind IL-1α, IL-1β, or IL-1R antagonist (8, 9). Functional ligands for ST2 have not been demonstrated, although ST2 binding proteins have been reported (8, 9). In the mouse, differential mRNA processing within the ST2 gene allows for the production of both a membrane-bound form of ST2, which is expressed primarily by hemopoietic cells (ST2L), and a soluble (s) form of ST2, which is predominantly expressed by fibroblasts (10). Hence, ST2 is identical with the extracellular region of ST2L except for an additional nine amino acids, which are present at the C terminus of ST2. More recently, another alternatively spliced ST2 transcript has been described in humans, the functional consequences of which are unclear (11).

Transcription of ST2/ST2L is controlled by two distinct promoters: an upstream promoter directs transcription in hemopoietic cells such as mast cells, while a promoter 10.5 kb downstream directs fibroblast-specific expression (10). Regulation of ST2/ST2L expression between mice and humans appears to be conserved, because two promoters also control ST2/ST2L expression in human cells (12). Studies using either anti-ST2 Abs or a recombinant s form of ST2 have demonstrated important roles for this molecule in regulating Th1/Th2-associated immune responses in vitro and in experimental disease models in vivo (6, 13). Such data are supported by studies using ST2-deficient mice (14).

Macrophages respond to bacterial products such as LPS, bacterial or CpG-containing DNA, peptidoglycan, and muramyl dipeptide by producing a cascade of proinflammatory cytokines including IL-1αβ, IL-6, IL-12, IL-18, and TNF-α, adhesion molecules, and inflammatory mediators that mediate innate immunity and prime the acquired immune response. Systemic bacterial infections trigger dysregulated production of these molecules, ultimately leading to disseminated intravascular coagulation, multiple organ failure, and mortality. The toxic effects of LPS in vivo are primarily conferred by macrophages (15). LPS-mediated activation of macrophages involves presentation of LPS by CD14 to members of the Toll-like receptor (TLR) family, particularly TLR4 (16–18). Signaling through TLR members involves recruitment of the IL-1R-associated kinase signaling complex, leading to activation of NF-κB and transcription of responsive genes (19). It is clear that a multitude of other signaling pathways including mitogen-activated protein kinase, erk-1/2, p38, c-Jun N-terminal kinase, and ceramide-activated protein kinase are also activated by LPS in macrophages, and these pathways are critical in instigating and regulating the LPS response (20).

Apart from regulation of disease outcome through modulation of TH1/TH2 bias, there is indirect evidence to suggest that ST2 may also be involved in inflammatory responses. ST2 expression was detected in human monocytes after LPS stimulation in vitro and in...
the muscle and spleen of mice after LPS injection (21). Proinflammatory stimuli including TNF-α, IL-1α, and IL-1β induced ST2 expression in 3T3 cells and, exposure to UV light triggered ST2 expression in vivo (22). We have investigated the role of ST2 in regulating the LPS response using a sST2-human (h)IgG-Fc fusion protein (sST2). We report here that macrophages expressed a sST2-binding activity that was up-regulated by LPS. Treatment of macrophages with sST2 down-regulated expression of TLR4 and TLR1 but induced nuclear translocation of NF-κB. Furthermore, sST2 suppressed inflammatory responses induced by LPS both in vitro and in vivo. Thus, sST2 represents a novel pathway for regulating endotoxic shock and may have considerable therapeutic potential as an anti-inflammatory agent through its ability to deactivate macrophages.

Materials and Methods

Cell lines and reagents

RPMI 1640 medium (Life Technologies, Paisley, U.K.), containing 10% FCS (Hyclone/serum, and glutation (complete medium) was used for culture of primary cells and cell lines. Marine bone marrow-derived macrophages (BMMs) were derived from cells of the femurs of adult BALB/c mice (Harlan Olac, Bicester, U.K.). Briefly, femurs were flushed with complete medium, and cells were plated in complete medium containing 105 U/ml rhCSF-1 (a gift from Chiron, Emeryville, CA, provided by D. Hume, Brisbane, Australia) on 10-cm bacteriological plastic plates (Bibby Sterillium, Staffordshire, U.K.) for 7 days in a 37°C incubator containing 5% CO2. In some experiments, 10% L929 cell-conditioned medium was used as a source of CSF-1 instead of rhCSF-1. The marine macrophage-like cell line RAW264 was obtained from American Type Culture Collection (Manassas, VA) and was cultured in complete medium on 10-cm bacteriological plastic plates. For in vitro experiments, LPS from S. enteritidis (Sigma, Poole, U.K.) was cultured in complete medium on 10-cm bacteriological plastic plates. For in vitro experiments, LPS from Salmonella minnesota (Sigma, Poole, U.K.) was used at a concentration of 100 ng/ml in all in vitro experiments. For in vivo experiments, LPS from S. enteritidis (Sigma) was used at a dose of 18 mg/kg body weight. The sST2-IgG fusion protein was prepared as previously described (13). Briefly, a mammalian expression plasmid containing the sST2 cDNA linked to the hlgG1 constant region and containing the CS signal sequence was stably transfected into Chinese hamster ovary cells. The sST2-IgG was purified from culture medium (low IgG) via protein A affinity chromatography, and purity was confirmed by SDS-PAGE. LPS was not detected in the sST2-IgG preparation (<0.01 ng/µg protein) by an amoebocyte Limulus test; E-toxate, Sigma). The hlgG control protein (Sigma) was resuspended in PBS and stored at −20°C. Polyclonal rabbit anti-ST2 Ab and rabbit IgG control have been described previously (6). Monoclonal anti-ST2 Ab for flow cytometry was purchased from Morwell Diagnostics GmbH (Post- fach, Switzerland). MTT assay reagent was from Sigma.

Flow cytometric analysis

BMMs were plated at 15 × 105 cells/15 ml complete medium in 10-cm bacteriological plastic plates and were treated with LPS or left untreated for 20 h. Adherent cells were harvested by scraping in ice-cold PBS, and 5 × 105 cells were resuspended in ice-cold complete medium and incubated at 4°C in the presence of 5 µg/ml of Fc block (BD PharMingen, San Diego, CA) for 20 min. The sST2-Ig fusion protein (35 µg/ml) or hlgG (25 µg/ml, purified as for ST2-Ig using a protein A column) was then added, and cells were incubated for 30 min at 4°C. Cells were then washed in FACS buffer (1X PBS supplemented with 2% FCS, 0.1% sodium azide) and stained with anti-hlgG1 mAb (26 µg/ml; Sigma). Cells were fixed and analyzed on a FACSciCalibur flow cytometer (BD Biosciences, Mountain View, CA).

In vitro treatment of cells, ELISA, NO, and MTT assays

For all in vitro experiments, cells were plated in 24-well plates at 5 × 105 cells/well in 1 ml complete medium plus CSF-1 overnight. Cells were then treated with sST2, hlgG, or medium for 1 h (unless otherwise stated) followed by stimulation with LPS or medium alone. After 8 h (unless otherwise stated), supernatants were collected and stored at −20°C. ELISA was performed using paired Abs (BD PharMingen), and nitrite levels in culture medium were analyzed by Griess reagent (23). Cell viability was assessed by MTT assay (24).

Nuclear extract preparation and gel shift assays

Nuclear extract preparation and gel shift assays were performed as described previously (25). Double-stranded oligonucleotides (Sigma-Geno-
with anti-ST2 Ab. Cells incubated with hlgG showed no difference in binding between anti-ST2 Ab and the isotype control, thus, indicating that macrophages do not express detectable levels of cell surface ST2L (data not shown).

Treatment with sST2 inhibits LPS-induced proinflammatory cytokine production from macrophages

Because binding of sST2 to BMMs was up-regulated by LPS treatment, we investigated the reciprocal involvement of sST2 in regulating the LPS response. BMMs were pretreated for 1 h with sST2 or hlgG (both at 50 μg/ml) or medium alone and then stimulated with LPS (100 ng/ml) for up to 48 h. Culture supernatants were harvested at regular intervals, and concentrations of cytokines were analyzed by ELISA. Treatment with sST2 markedly inhibited the production of IL-6, IL-12, and TNF-α (Fig. 2). This was apparent at all time points examined, indicating that inhibition by sST2 was not overcome by persistent exposure to LPS. In contrast, NO and IL-10 (data not shown) production in response to LPS was not inhibited by treatment with sST2. Production of IL-6 and TNF-α, but not IL-12, in response to LPS in the murine macrophage-like cell line RAW264 was also suppressed by sST2 treatment (data not shown). Hence, the inhibition mediated by ST2 appears to be selective.

We then conducted dose-response studies. BMMs were preincubated with increasing concentrations (0.1–50 μg/ml) of sST2 or hlgG for 1 h and then cultured with LPS for 8 h before supernatants were assayed for IL-6 by ELISA. Treatment with sST2 caused a dose-dependent decrease in LPS-induced IL-6 production compared with the hlgG control, with significant effect from 0.1 μg/ml and a 6-fold reduction at 50 μg/ml (data not shown). This effect was not due to toxicity induced by sST2 because cell viability (assessed by MTT assay) was not altered by treatment with sST2 (data not shown). The dose of sST2 used for additional experiments (50 μg/ml) was similar to or lower than the concentration of sST2 used by others in an earlier report on Th1/Th2 cell differentiation (13).

Although we have sometimes detected very low levels of expression of ST2L mRNA in BMMs by RT-PCR, we have been unable to detect membrane-bound ST2L by flow cytometry (data not shown). To confirm that the effect of sST2 on macrophages did not involve competition between sST2 and presumptive low levels of ST2L for an LPS-inducible protein, we pretreated BMMs for 2 h with sST2, washed the cells thoroughly to remove sST2 in culture medium, and then stimulated cells with LPS for 8 h. We found that sST2 inhibited LPS-induced IL-6 production irrespective of whether sST2 had been removed before stimulation with LPS (data not shown). Hence, sST2 appears to trigger anti-inflammatory signals in macrophages directly.

NF-κB is activated in BMMs by sST2

One mechanism by which sST2 could suppress LPS-induced proinflammatory cytokine production is by blocking LPS-initiated nuclear translocation of NF-κB or specifically mobilizing NF-κB p50 homodimers, which can have inhibitory effects on gene expression (27). To test this possibility, we assessed levels of NF-κB in BMMs pretreated with 50 μg/ml of hlgG or sST2 for 30 min before stimulation with LPS for 45 min. Unexpectedly, sST2 was itself able to activate NF-κB in BMMs to the same extent as LPS, and no additional activation or repression by LPS was observed (Fig. 3A). Cold competition analysis indicated that the induced band was indeed NF-κB, because unlabeled self oligonucleotide, but not an unlabeled oligonucleotide containing a GATA-1 site, could compete for binding (Fig. 3A). NF-κB induced by sST2 was primarily composed of p65, c-rel, and p50 as assessed by supershift analysis (Fig. 3B). The inducible complex did not contain the rel family member p52, because treatment with an anti-p52 Ab did not alter binding. On the basis of supershift analysis, LPS-induced NF-κB could not be distinguished from sST2-induced NF-κB (data not shown). Hence, LPS and sST2, which clearly have opposing effects on macrophage cytokine production, both induced p65/c-rel/p50-containing NF-κB.

TLR4 and TLR1 levels in BMMs are suppressed by sST2

Because members of the TLR family, particularly TLR4, are essential for macrophage responses to LPS, we examined the effect
of sST2 on TLR mRNA levels in BMMs using quantitative real-time PCR. Fig. 4 shows that treatment of BMMs with either sST2 or LPS down-regulated expression of TLR4 mRNA 3-fold, and the combination of these stimuli suppressed TLR4 mRNA levels further. Interestingly, TLR1, which may also be involved in the response to LPS (28), was differentially regulated by LPS and sST2; sST2 repressed TLR1 mRNA levels 4-fold, whereas LPS induced expression 2-fold. To confirm that sST2 inhibited LPS-induced proinflammatory gene expression in the same RNA samples, mRNA levels of IL-12 p40 and IL-12 p35 were also assessed. Both IL-12 p40 and p35 mRNA levels were almost undetectable in unstimulated cells or cells treated with sST2. LPS induced expression of both IL-12 p40 and p35, and sST2 repressed this LPS-induced expression (p40, 2.6-fold and p35, 5.5-fold).

**LPS-induced mortality in vivo is blocked by sST2**

The observation that sST2 could inhibit LPS-induced proinflammatory cytokine production in vitro suggested that this molecule might have therapeutic potential as an LPS antagonist. To test this hypothesis, a murine LPS shock model was used. Mice were injected i.p. with LPS and then treated 1 h later with either sST2 or hlgG. Although only 4 of 12 mice in the control hlgG-treated group survived over a 4-day period, mice treated with sST2 had a significantly enhanced survival rate (10 survived of 12; \( p < 0.01 \)) (Fig. 5A). Treatment with sST2 clearly down-regulated the levels of serum IL-6, IL-12, TNF-\( \alpha \), and IFN-\( \gamma \) in LPS-challenged mice (Fig. 5B). No significant differences in IL-10 levels were seen between sST2- and hlgG-treated mice. Because there is evidence that IL-18 may be involved in controlling the onset of LPS shock (29), we also measured serum IL-18 in the two groups. IL-18 levels were not altered by treatment with sST2 (Fig. 5B).

FIGURE 5. LPS-induced mortality and proinflammatory cytokine production in vivo are reduced by sST2. Age- and sex-matched BALB/c mice were injected i.p. with 18 mg/kg LPS, and then, 1 h later, 100 \( \mu \)g/mouse sST2 or hlgG was administered i.p. After a further 2 h, serum was collected from six mice from each group and pooled. Levels of IL-6, IL-10, IL-12, IL-18, IFN-\( \gamma \), and TNF-\( \alpha \) were quantitated by ELISA (presented as the mean of triplicates ± SD), and survival in each group was monitored (\( n = 12 \)).

**FIGURE 4.** TLR4 and TLR1 mRNA in BMMs is down-regulated by sST2. BMMs were pretreated with either 50 \( \mu \)g/ml hlgG or 50 \( \mu \)g/ml sST2 for 1 h and then treated with LPS (100 ng/ml) or medium for a further 3 h. RNA and cDNAs were prepared, and the levels of TLR4, TLR1, IL-12 p40, IL-12 p35, and HPRT mRNAs were determined by quantitative real-time PCR. Results (displayed as the mean of triplicates ± SD relative to HPRT) are representative of two separate experiments, and, for each experiment, two separate real-time PCR experiments were performed.

**FIGURE 3.** Soluble ST2 activates NF-\( \kappa \)B but does not inhibit LPS-induced NF-\( \kappa \)B in BMMs. BMMs were treated for 30 min with 50 \( \mu \)g/ml of either hlgG or sST2 and then activated with LPS for 45 min or left untreated. Nuclear extracts were prepared and levels of NF-\( \kappa \)B determined by EMSA. A, Cold competition analysis. EMSA was performed using an NF-\( \kappa \)B binding site from the murine TNF-\( \alpha \) promoter, and specificity of binding was demonstrated by cold competition with a 10-fold excess of either self oligonucleotide or an unrelated oligonucleotide (GATA-1 binding site). B, Characterization of rel family members induced by sST2. EMSA was performed using nuclear extracts from sST2-treated BMM. Before electrophoresis, binding reactions were incubated with Abs against the rel members p50, p65, p52, or c-rel.
**Anti-ST2 Ab exacerbates LPS shock**

The above-described data demonstrate that sST2 has potent anti-inflammatory activity and blocks LPS shock in vivo. Therefore, it might be expected that inhibition of endogenous sST2 action would exacerbate mortality. We tested this hypothesis by examining the effects of an anti-ST2 Ab that has been used previously to block Th2 responses in vivo (6). Mice were injected i.p. with either anti-ST2 Ab or an isotype control on day –1, 0, and +1 of LPS challenge. Although 9 of 25 from the control group succumbed to LPS-induced shock, the level of mortality in the anti-ST2 Ab-treated group was significantly higher (20 of 25; p < 0.01) than in the control group (Fig. 6A). Levels of the proinflammatory cytokines IL-6, TNF-α, and IFN-γ were also elevated in sera from mice treated with anti-ST2 Ab compared with the control group (Fig. 6B). The level of IL-10 was similar in both groups (3.5 vs 3.8 ng/ml).

**Discussion**

Data presented here demonstrate for the first time that sST2, the product of a gene previously found to be selectively expressed in BALB/c mice, is able to down-regulate TLR4 expression in virus- and endotoxin challenge of cultured macrophages. Our data show that sST2 binds to TLR4 and, in turn, decreases its expression on the cell surface. Furthermore, we have demonstrated that IL-6, IFN-γ, and TNF-α, which are known to induce TLR4 expression, are down-regulated by sST2 treatment (21). This down-regulation of TLR4 was confirmed by flow cytometry analysis. The mechanism of sST2-induced down-regulation of TLR4 is currently being addressed.

**FIGURE 6.** Anti-ST2 Ab exacerbates LPS-induced mortality and augments proinflammatory cytokine production in vivo. Age- and sex-matched BALB/c mice were given three i.p. doses of an anti-ST2 polyclonal Ab (250 μg/mouse) or an isotype control and challenged i.p. with LPS (18 mg/kg). Two hours after LPS treatment, serum was collected from six mice in each group and pooled. Levels of IL-6, TNF-α, and IFN-γ were quantitated by ELISA (presented as the mean of triplicates ± SD), and survival in each group was monitored (n = 25). *, p < 0.05; **, p < 0.01, anti-ST2 vs isotype control.
factor, because LPS-induced NO and IL-10 levels were not altered by sST2 treatment, and we were unable to detect significant levels of TGF-β1 in sST2-treated BMMs (data not shown). Furthermore, kinetic studies showed that sST2-mediated suppression was apparent 4 h after LPS treatment at the protein level. This is consistent with the effect of sST2 in vivo, where IL-6, IL-12, IFN-γ, and TNF-α levels were suppressed 2 h after administration of sST2. Similarly, IL-6, TNF-α, and IFN-γ levels in the serum were markedly elevated in mice treated with anti-ST2 Ab 2 h after LPS challenge.

In conclusion, apart from acting as a marker for Th2 cells and directly modulating Th1/Th2 balance by influencing Th2 cell functions (6, 7), the gene encoding T1/ST2 may also have an important regulatory role in the inflammatory response by acting directly on macrophages. Fig. 7 summarizes the regulatory pathway of sST2 in LPS-induced inflammatory responses. LPS activates macrophages via TLR4, inducing production of proinflammatory cytokines including IL-1β and TNF-α, which activate fibroblasts and other cell types to produce sST2. Then sST2 binds to macrophages via a yet undefined receptor and directly and selectively represses the expression of proinflammatory cytokines, including IL-6, IL-12, and TNF-α, possibly through the down-regulation of TLR4. Thus, sST2 may function as an important mediator in this negative feedback loop for preventing uncontrolled inflammatory reactions. Consistent with this is our demonstration that ST2-IgG, an effective surrogate of ST2, markedly blocked LPS-induced shock and down-regulated proinflammatory cytokine production in vivo. Conversely, anti-ST2 Ab, which neutralized ST2, exacerbated LPS-mediated mortality and enhanced production of proinflammatory cytokines in vivo. However, it should be noted that the ST2-Fc construct used here may exist as a dimeric molecule, which may or may not be identical with native sST2. Nevertheless, it would be of considerable interest to define the ST2 receptor and the mechanism by which it transmits signal leading to the inhibition of TLR4 expression. These data also strongly suggest that ST2-IgG could be a novel therapeutic agent against septic shock and other inflammatory diseases.

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