

# A pertussis toxin sensitive G-protein-independent pathway is involved in serum amyloid A-induced formyl peptide receptor 2-mediated CCL2 production

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Abbreviations: FPR2, formyl peptide receptor 2; HUAECs, human umbilical artery endothelial cells; HUVECs, human umbilical vein endothelial cells; PTX, pertussis toxin; SAA, serum amyloid A

## Abstract

**Serum amyloid A (SAA) induced CCL2 production via a pertussis toxin (PTX)-insensitive pathway in human umbilical vein endothelial cells (HUVECs). SAA induced the activation of three MAPKs (ERK, p38 MAPK, and JNK), which were completely inhibited by knock-down of formyl peptide receptor 2 (FPR2). Inhibition of p38 MAPK and JNK by their specific inhibitors (SB203580 and SP600125), or inhibition by a dominant negative mutant of p38 MAPK dramatically decreased SAA-induced CCL2 production. Inactivation of G<sub>i</sub> protein(s) by PTX inhibited the activation of SAA-induced ERK, but not p38 MAPK or JNK. The results indicate that SAA stimulates FPR2-mediated activation of p38 MAPK and JNK, which are independent of a PTX-sensitive G-protein and are essential for**

**SAA-induced CCL2 production.**

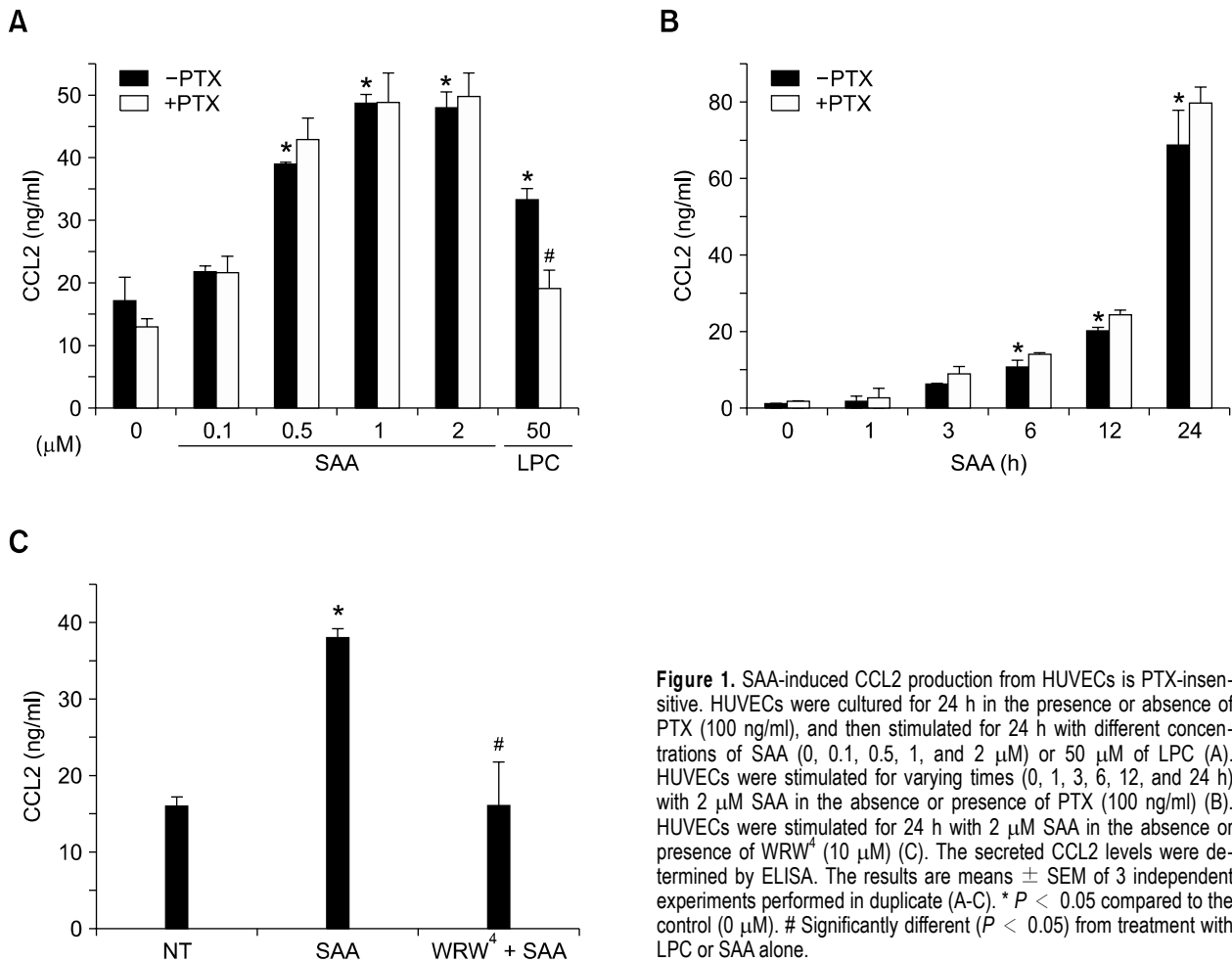
**Keywords:** atherosclerosis; chemokine CCL2; endothelial cells; FPR2 protein, human; pertussis toxin; serum amyloid A protein

## Introduction

Serum amyloid A (SAA), a major acute-phase protein, is an important component of responses to infection or injury (Uhlir and Whitehead, 1999). Previous studies have shown that the local concentration of SAA is increased in a pro-inflammatory environment by the actions of several cytokines, such as interleukin-1 $\beta$ , resulting in an elevation of about 1000-fold during acute-phase reactions compared to the normal state (Malle *et al.*, 1993; Uhlir and Whitehead 1999; Urieli-Shoval *et al.*, 2000). Recently, it has been shown that SAA has cytokine-like properties by modulating several cellular responses (Badolato *et al.*, 1994; Jijon *et al.*, 2005; Lee *et al.*, 2006a; Koga *et al.*, 2008). SAA regulates chemotactic migration of some leukocytes, including monocytes and neutrophils (Badolato *et al.*, 1994). SAA also stimulates production of several cytokines from different cell types, including monocytes, epithelial cells, and synovial cells (Jijon *et al.*, 2005; Lee *et al.*, 2006a; Koga *et al.*, 2008). We have recently reported that SAA stimulates CCL2 production by human umbilical vein endothelial cells (HUVECs; Lee *et al.*, 2009).

SAA stimulates formyl peptide receptor 2 (FPR2), which results in Ca<sup>2+</sup> mobilization and cell migration (Su *et al.*, 1999; Ye *et al.*, 2009). Several studies have shown that pertussis toxin (PTX)-sensitive G-proteins are involved in FPR2-mediated cellular responses by SAA, including chemotactic migration (Badolato *et al.*, 1995; Su *et al.*, 1999; He *et al.*, 2003).

Despite the role of SAA in atherosclerosis and the role of FPR2 in SAA-induced CCL2 production by endothelial cells, the signaling pathways involved in this process have not been clearly elucidated. In this study we investigated the role of PTX-sensitive G-proteins and the downstream signaling of FPR2 on SAA-induced CCL2 production by HUVECs.



**Figure 1.** SAA-induced CCL2 production from HUVECs is PTX-insensitive. HUVECs were cultured for 24 h in the presence or absence of PTX (100 ng/ml), and then stimulated for 24 h with different concentrations of SAA (0, 0.1, 0.5, 1, and 2  $\mu$ M) or 50  $\mu$ M of LPC (A). HUVECs were stimulated for varying times (0, 1, 3, 6, 12, and 24 h) with 2  $\mu$ M SAA in the absence or presence of PTX (100 ng/ml) (B). HUVECs were stimulated for 24 h with 2  $\mu$ M SAA in the absence or presence of WRW<sup>4</sup> (10  $\mu$ M) (C). The secreted CCL2 levels were determined by ELISA. The results are means  $\pm$  SEM of 3 independent experiments performed in duplicate (A-C). \*  $P < 0.05$  compared to the control (0  $\mu$ M). # Significantly different ( $P < 0.05$ ) from treatment with LPC or SAA alone.

## Results

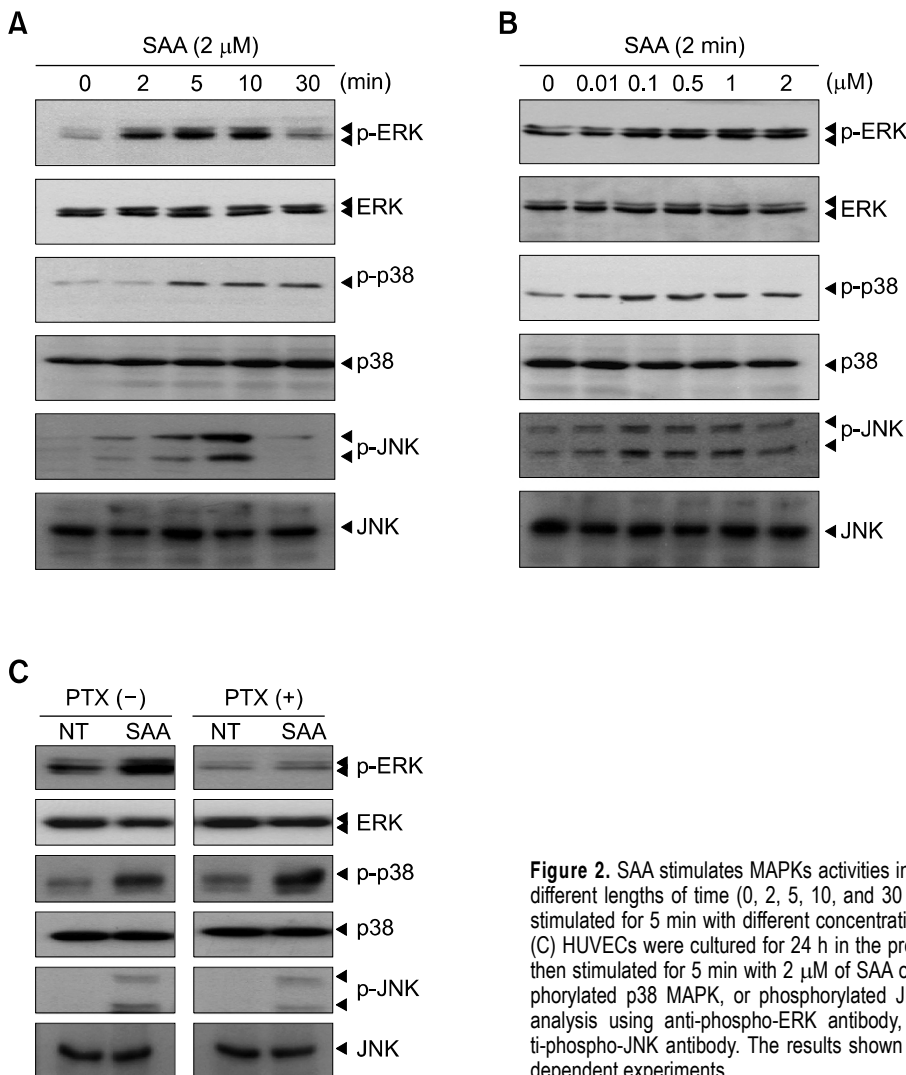
### SAA stimulates CCL2 production in HUVECs by a PTX-independent pathway

PTX-sensitive G-proteins have been reported to play a role in SAA receptor-mediated  $\text{Ca}^{2+}$  signaling (Badolato *et al.*, 1995). We previously demonstrated that stimulation of HUVECs with several concentrations of SAA (0, 0.1, 1, and 2  $\mu$ M) for 24 h induced CCL2 production in a concentration-dependent manner (Lee *et al.*, 2009). To examine the role of PTX-sensitive G-proteins on the SAA-induced CCL2 production by HUVECs, HUVECs were first incubated in the presence or absence of 100 ng/ml of PTX for 24 h. HUVECs were then stimulated with several different concentrations (0, 0.1, 0.5, 1, and 2  $\mu$ M) of SAA for an additional 24 h. We found that SAA-induced CCL2 production was not inhibited by pre-incubating the cells with 100 ng/ml of PTX (Figure 1A). However, lysophosphatidylcholine-induced CCL2 production

was almost completely inhibited by PTX (Figure 1A). Thus, SAA-induced CCL2 production does not appear to be mediated by a PTX-sensitive G-protein. HUVECs were also stimulated with 2  $\mu$ M of SAA for several different times (0, 1, 3, 6, 12, and 24 h) in the absence or presence of PTX. SAA-stimulated CCL2 production from HUVECs was not inhibited by PTX (Figure 1B). Since SAA has been reported to act on FPR2, we tested the effect of WRW<sup>4</sup>, an FPR2 antagonist, on SAA-induced CCL2 production in HUVECs. Pre-incubation of HUVECs with WRW<sup>4</sup> prior to SAA addition caused dramatic inhibition of CCL2 production induced by SAA (Figure 1C), indicating that SAA acts on FPR2, resulting in CCL2 production in HUVECs.

### SAA induces MAPK activity via PTX-sensitive and -insensitive pathways in HUVECs

When HUVECs were stimulated for 2-10 min with 2



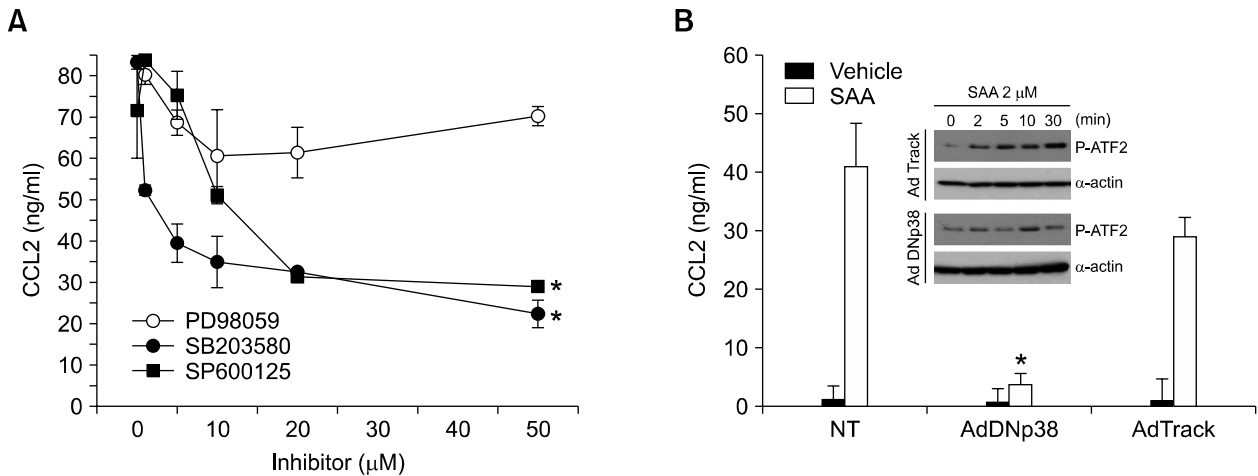
**Figure 2.** SAA stimulates MAPKs activities in HUVECs. (A) HUVECs were stimulated for different lengths of time (0, 2, 5, 10, and 30 min) with 2 μM of SAA. (B) HUVECs were stimulated for 5 min with different concentrations of SAA (0, 0.01, 0.1, 0.5, 1, and 2 μM). (C) HUVECs were cultured for 24 h in the presence or absence of PTX (100 ng/ml), and then stimulated for 5 min with 2 μM of SAA or vehicle. (A-C) Phosphorylated ERK, phosphorylated p38 MAPK, or phosphorylated JNK levels were determined by immunoblot analysis using anti-phospho-ERK antibody, anti-phospho-p38 MAPK antibody, or anti-phospho-JNK antibody. The results shown in (A-C) are representative of at least 3 independent experiments.

μM of SAA, the phosphorylation levels of three MAPKs (ERK, p38 MAPK, and JNK) were dramatically increased (Figure 2A). Moreover, when HUVECs were stimulated with different concentrations of SAA, these 3 MAPKs were activated at 100 nM - 2 μM; (Figure 2B). To examine a role for PTX-sensitive G-proteins on SAA-induced MAPK activation, we pre-treated HUVECs with 100 ng/ml of PTX prior to SAA stimulation. Figure 2C shows that PTX completely blocked SAA-induced ERK activity. However, SAA-induced p38 MAPK and JNK activities were not affected by PTX (Figure 2C).

**P38 MAPK and JNK regulate SAA-induced CCL2 production**

To determine the roles of individual MAPKs on

SAA-induced CCL2 production, we pre-incubated HUVECs with either PD98059 (a selective MEK inhibitor), SB203580 (a selective p38 MAPK inhibitor), or SP600125 (a selective JNK inhibitor) prior to SAA treatment. We confirmed that all the tested inhibitors specifically inhibited each enzyme (data not shown). SAA-induced CCL2 production was dramatically inhibited by SB203580 and SP600125, but not by PD98059 (Figure 3A), indicating that p38 MAPK and JNK are essential for SAA-induced CCL2-production. However, MEK-dependent ERK was not involved with SAA-induced CCL2 production. To support the hypothesis that p38 MAPK is essential for the production of CCL2 by HUVECs, we used a recombinant adenovirus expressing the dominant negative mutant of p38 MAPK (AdDNp38). We first confirmed that transfection of AdDNp38 resulted in



**Figure 3.** p38 MAPK and JNK are essential for SAA-induced CCL2 production by HUVECs. (A) HUVECs were pre-incubated with several concentrations (0, 1, 5, 10, 20, or 50  $\mu$ M) of PD98059 (60 min), SB203580 (15 min), or SP600125 (15 min) prior to stimulation with 2  $\mu$ M of SAA for 24 h. (B) The genes for GFP (AdTrack) or dominant negative p38 (AdDNp38) (MOI; 50) were transferred into HUVECs using recombinant adenoviruses prior to SAA (2  $\mu$ M) stimulation for 24 h (B) or for 2, 5, 10, or 30 min (B insert). The amounts of secreted CCL2 were measured by ELISA. Phosphorylated ATF-2 levels were measured by Western blot analysis (B insert). Results are means  $\pm$  SEM of 3 independent experiments performed in duplicate (A, B). \* $P < 0.05$  compared to the control (SAA only treated).

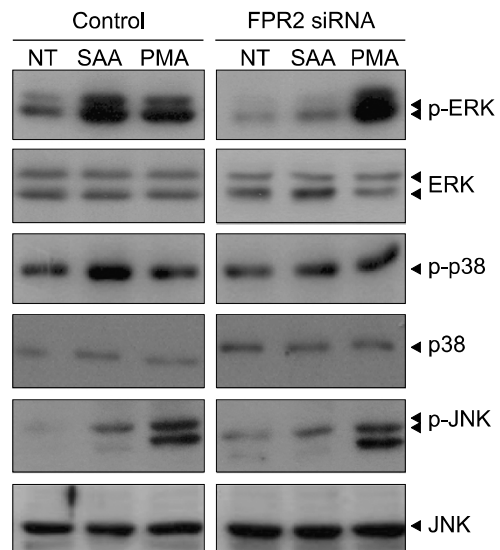
near-complete inhibition of the phosphorylation of ATF2, which is a well-known p38 MAPK substrate (Figure 3B insert). Further, transfection of AdDNp38 into HUVECs completely inhibited SAA-induced CCL2 production. As a negative control, the transfer of a recombinant adenovirus expressing GFP (AdTrack) did not affect CCL2 production by SAA (Figure 3B). These results strongly suggest that p38 MAPK and JNK are essential for SAA-induced production of CCL2 by HUVECs.

#### SAA stimulates MAPK activity via FPR2

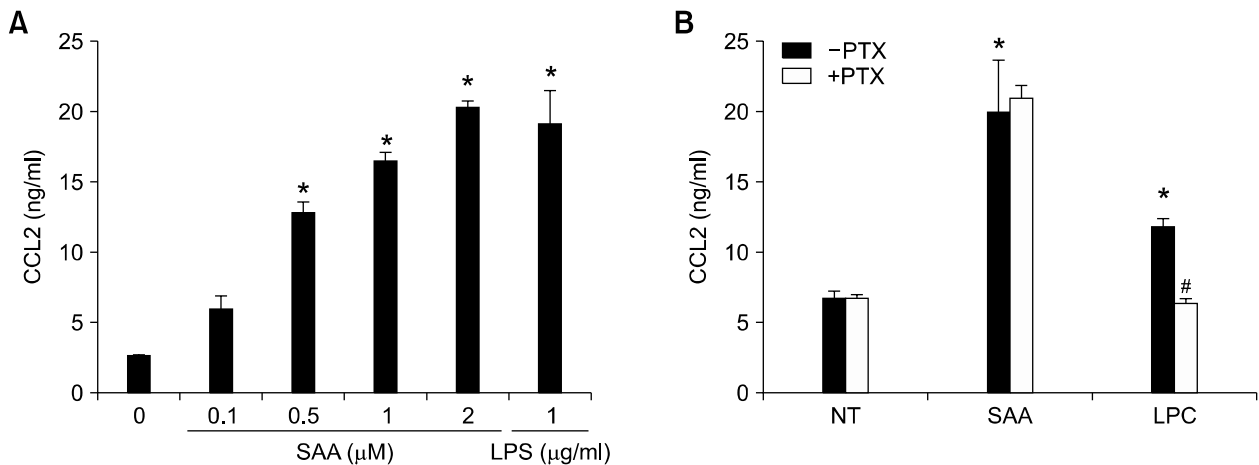
The finding that SAA, an FPR2 agonist, induced CCL2 production by a PTX-insensitive pathway in HUVECs led us to examine if FPR2 was involved in SAA-induced MAPK activation in these cells. To verify that SAA-induced MAPK activation was mediated by FPR2 in HUVECs, we used siRNA for FPR2. The down-regulation of FPR2 by siRNA transfection resulted in a near-complete inhibition of SAA-induced ERK phosphorylation (Figure 4). Knock-down of FPR2 using FPR2 siRNA caused inhibition of SAA-induced p38 MAPK and JNK phosphorylation (Figure 4), whereas siRNA for luciferase did not affect SAA-induced MAPK phosphorylation (Figure 4). These results strongly indicate that SAA stimulates MAPK activity via FPR2 in HUVECs.

#### SAA stimulates CCL2 production in HUAECs via a PTX-independent manner

Since we observed that SAA stimulates CCL2 production via a PTX-insensitive pathway down-



**Figure 4.** SAA-induced MAPK activity is mediated by FPR2. HUVECs were transfected with 20 nM of FPR2 siRNA or control siRNA for 48 h. The transfected cells were stimulated with vehicle, 2  $\mu$ M of SAA, or 100 nM of PMA for 5 min. Phosphorylated ERK, phosphorylated p38 MAPK, or phosphorylated JNK levels were determined by immunoblot analysis using anti-phospho-ERK antibody, anti-phospho-p38 MAPK antibody or anti-phospho-JNK antibody. The results shown are representative of 3 independent experiments.



**Figure 5.** SAA-induced CCL2 production from HUAECs is PTX-insensitive. HUAECs were stimulated for 24 h with different concentrations of SAA (0, 0.1, 0.5, 1, and 2  $\mu$ M) or 1  $\mu$ g/ml of LPS (A). HUAECs were cultured for 24 h in the presence or absence of PTX (100 ng/ml), and then stimulated for 24 h with 2  $\mu$ M SAA or 50  $\mu$ M LPC (B). The secreted CCL2 levels were determined by ELISA. Results are the means  $\pm$  SEM of 3 independent experiments performed in duplicate (A, B). \* $P$  < 0.05 compared to the control (0  $\mu$ M). # Significantly different ( $P$  < 0.05) from treatment with LPC alone.

stream of FPR2 (Figure 1), we investigated whether this event is common in other endothelial cells. We first tested the effect of SAA on the production of CCL2 in HUAECs. As expected, SAA stimulated CCL2 production in a concentration-dependent manner (Figure 5A). The potency of SAA-induced CCL2 production was comparable with CCL2 production by LPS in HUAECs. We then investigated the effect of PTX on SAA-induced CCL2 production in the cells. Pre-incubation of HUAECs with 100 ng/ml of PTX prior to the addition of SAA did not inhibit SAA-induced CCL2 production. In contrast, lysophosphatidylcholine (LPC)-stimulated CCL2 production resulted in near-complete inhibition by PTX in HUAECs (Figure 5B). The results indicate that SAA stimulates CCL2 production from HUAEC in a PTX-insensitive manner.

## Discussion

Previous studies have shown that many SAA-induced signals are inhibited by PTX, suggesting that PTX-sensitive  $G_i$ -proteins are involved with SAA-induced signaling (Badolato *et al.*, 1995; Lee *et al.*, 2005; Zhao *et al.*, 2007; Björkman *et al.*, 2008). SAA has been reported to stimulate chemotactic migration of some leukocytic cells in a PTX-sensitive manner (Lee *et al.*, 2005; Zhao *et al.*, 2007; Björkman *et al.*, 2008). The increase in intracellular calcium induced by SAA is also mediated by PTX (Badolato *et al.*, 1995). Recently, we also demonstrated that SAA-induced CCL2

expression resulted in near-complete inhibition by PTX in human monocytes (Lee *et al.*, 2008). However, in the present study, we found that SAA-induced CCL2 expression was not affected by PTX (Figure 1). Because many FPR2-mediated signals are known to be mediated by PTX-sensitive G-proteins, these results are very interesting for FPR2-mediated signaling. At this point, it is not clear what causes different signaling in human monocytes and HUVECs. One possible explanation can be derived from the differential cellular context from the two different cell types.

We also examined the intracellular signaling mechanism involved in SAA-induced cytokine production. In the present study, we found that SAA-induced CCL2 production was significantly inhibited by either SB203580, a dominant negative mutant of p38 MAPK or SP600125 (Figures 3A and 3B). These results indicate that p38 MAPK and JNK, but not ERK, are essential activation components for SAA-induced CCL2 production by human endothelial cells. Pre-incubation of HUVECs with PTX prior to SAA treatment completely inhibits SAA-induced activation of ERK, but not p38 MAPK or JNK (Figure 2C). These results indicate that activation of p38 MAPK and JNK, which are themselves activated by FPR2 in a pathway that is insensitive to PTX, is a prerequisite for the induction of CCL2 by SAA. It also indicates that the activation of ERK activity by SAA, which is mediated by PTX-insensitive signaling, is not necessary for SAA-induced CCL2 production in HUVECs. This is the first report to demonstrate that SAA regulates a cellular response via FPR2 in a

PTX-independent pathway, and suggests a new mechanism of action for FPR2 in endothelial cells. Since SAA induced CCL2 production, and p38 MAPK and JNK activation are not inhibited by PTX, we can rule out the possible involvement of G<sub>i</sub> protein in the processes. However, SAA-induced ERK activation was inhibited by PTX. From these results, it may be possible that SAA stimulates p38 MAPK and JNK activation and CCL2 production via other G-protein(s), except G<sub>i</sub>.

In addition to FPR2, several other targets have been reported as specific receptors for SAA, including receptor for advanced glycation end products (Yan *et al.*, 2000), the HDL receptor, scavenger receptor class B type I (Cai *et al.*, 2005), CLA-1 (CD36 and LIMPII analogous-1), human orthologue of the Scavenger Receptor Class B Type I (Baranova *et al.*, 2005), P2X7 (Christenson *et al.*, 2008), TLR4 (Sandri *et al.*, 2008), and TLR2 (He *et al.*, 2009). Because we found that SAA stimulated CCL2 production via a PTX-insensitive pathway, we confirmed a role for FPR2 in SAA-induced cellular signaling in HUVECs. Transfection of FPR2 siRNA into HUVECs completely inhibited SAA-induced MAPK activities, which are important mediators for the induction of CCL2 by SAA in HUVECs (Figure 4). In a previous report, we also demonstrated that knock-down of FPR2 by siRNA completely blocked SAA-induced CCL2 production by HUVECs (Lee *et al.*, 2009). Taken together, our combined results show that SAA induces CCL2 production via a PTX-insensitive pathway downstream of FPR2 in HUVECs.

In conclusion, we showed that SAA induced CCL2 production via FPR2 in a PTX-insensitive pathway in endothelial cells. This stimulatory effect of SAA was mediated by p38 MAPK and JNK-dependent pathways. We also showed a molecular mechanism for FPR2-mediated signaling that was PTX-independent.

## Methods

### Materials

Recombinant human SAA (endotoxin level < 0.1 ng/μg) was purchased from Peprotech (Rocky Hill, NJ). PTX, PD98059, SB203580, and SP600125 were from Calbiochem (San Diego, CA). All antibodies for phospho-MAPKs were from Cell Signaling Technology, Inc. (Beverly, MA).

### Cell culture

HUVECs (Cambrex Corporation) and human umbilical artery endothelial cells (HUAECs) were cultured on 0.02% gelatin-coated (Sigma, St. Louis, MO) 10 cm-dishes in endothelial basal medium supplemented with 10% FBS

and supplements (Cambrex Corporation). Cells between passages 3 and 10 were used for experiments.

### Cytokine assay

Cytokine measurements were performed as previously described (Jo *et al.*, 2004). HUVECs ( $3 \times 10^5$  cells/0.3 ml) in EGM-2 medium containing 1% FBS were plated in 24-well plates and kept in a 5% CO<sub>2</sub> incubator at 37°C. After stimulation, the cell-free supernatants were collected, centrifuged, and assayed for CCL2 by an ELISA (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions. To test the role of MAPKs on the SAA-induced CCL2 production, we pre-incubated HUVECs with each MAPK inhibitor (PD98059, SB203580, or SP600125) prior to SAA treatment. For the entire period, the inhibitors were present.

### Western blot analysis

After HUVEC stimulation with SAA, the cells were lysed in lysis buffer (20 mM HEPES [pH 7.2], 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Detergent insoluble materials were pelleted by centrifugation (12,000 × *g* for 15 min at 4°C), and the soluble supernatant fraction was removed and stored at either -80°C or used immediately. Proteins were separated in 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. Subsequently, the membranes were incubated with specific 1:5000 diluted goat anti-rabbit IgG antibodies, combined with horseradish peroxidase and antigen-antibody complexes were visualized by enhanced chemiluminescence (Cho *et al.*, 2008).

### Construction of recombinant adenovirus expressing dominant negative mutant p38 MAPK

Recombinant adenoviruses were prepared via homologous recombination in *E. coli* using the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA). The dominant negative p38 mutant from pcDNA3 p38 (TY > AF; kindly provided by Dr. J. Han, The Scripps Research Institute, La Jolla, CA) was cloned into the Shuttle vector, linearized using *Pme* I and transfected with the pAdEasy-1 vector into *E. coli* BJ5183. Recombinant adenoviral DNA was then transfected into 293 cells, followed by the generation of replication-deficient recombinant adenoviruses. The adenoviruses harboring genes of interest were isolated using a commercial adenovirus purification kit (Cell Biolabs Inc., San Diego, CA) according to the manufacturer's instructions. The viral titer was determined using a QuickTiter™ Adenoviral Quantitation Kit (Cell Biolabs Inc.).

### Transfection of siRNA for FPR2 transcripts

The down-regulation of FPR2 by siRNA was performed, as described previously (Lee *et al.*, 2006b). The HUVECs were transfected with a final concentration of 20 nM FPR2 siRNA, or luciferase siRNA as a control, using electroporation with a Microporator (Digitalbiotechnology, South Korea). The cells were washed with serum-free medium,

followed by incubation with a transfection mixture for 4 h, and the addition of complete endothelial basal medium.

### Data analysis

The results are expressed as the mean  $\pm$  SEM for the number of indicated determinations. Statistical comparisons used Student's *t*-test or ANOVA and statistical significance was considered at a  $P < 0.05$ .

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