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Serum amyloid A induces contrary immune responses via formyl peptide receptor like-1 in human monocytes

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Abbreviations used in this paper: TNF-α, tumor necrosis factor-α; FPRL1, formyl peptide

receptor like 1; BAY 11-7082, (E)-3-[4(4-methylphenylsulfonyl)-2-propenenitrile; SC-514, 5-

(Thien-3-yl)-3-aminothiophene-2-carboxamide; PD98059, 2´-Amino-3'-methoxyflavone;

SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole;

MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase;

PI3K, phosphoinositide-3-kinase.

Abstract

Although the level of serum amyloid A has been reported to be up-regulated during inflammatory response, the role of serum amyloid A on the regulation of inflammation and immune response has not been elucidated. We found that serum amyloid A stimulated the production of tumor necrosis factor-α (TNF-α) and IL-10, which are, proinflammatory and anti-inflammatory cytokines, respectively in human monocytes. Low concentrations of serum amyloid A stimulated TNF-α production with maximal activity at 6 hr after stimulation, whereas high concentrations of serum amyloid A stimulated IL-10 production with maximal activity at 12 hr. The activations of the two cytokines by serum amyloid A occurred at both the transcription and translational levels. Signaling events induced by serum amyloid A included the activation of two mitogen activated protein kinase (extracellular signal-regulated kinase and p38 kinase), which were found to be required for TNF-α and IL-10 production, respectively. The stimulation of formyl peptide receptor like 1-expressing RBL-2H3 cells, but not of vector-expressing RBL-2H3 cells with serum amyloid A, induced mitogen activated protein kinases activation and the accumulation of the RNAs of these two cytokines. Taken together, our findings suggest that serum amyloid A modulates contrary immune responses via formyl peptide receptor like 1, by inducing TNF-α or IL-10, and demonstrate that extracellular signal-regulated kinase and p38 kinase play counteracting roles in this process.

Introduction

Immune responses are modulated by the regulation of cytokine balances. For example, when challenged by pathogens, host cells respond by releasing several kinds of cytokines that enhance the defense mechanism. These cytokines include pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and the Th1 cytokines (IL-12, and interferon-γ) (Titus et al., 1991; Netea et al., 2003; Jankovic et al., 2001; Strieter et al., 2003). However, the immune system is maintained in a state of homeostasis by other soluble cytokines that counteract the effects of these pro-inflammatory species. These anti-inflammatory cytokines include IL-10, which is known to inhibit Th1 cells and exacerbate pathogen infection (Groux et al., 1999; Mosmann and Moore, 1991). Previous reports have demonstrated that different cell types and surface molecules maintain immune system homeostasis by reciprocal regulation (Fallarino et al., 2002; Harris et al., 2000). Moreover, it is well known that proinflammatory Th1 cytokines, such as interferon-γ, inhibit Th2, and that the Th2 cytokines (IL-4 and IL-10) inhibit Th1 production (Liu et al., 2001). Some G-protein coupled receptors also play important roles in the modulation of immune response against invading pathogens; however, their roles on cytokine homeostasis have not been intensively studied.

Serum amyloid A was initially described as the main constituent of amyloid fibrils in secondary amyloidosis (Uhlar and Whitehead, 1999), and several studies have shown that serum amyloid A production is triggered when liver cells are exposed to a pro-inflammatory environment by several cytokines, like TNF-α (Rienhoff et al., 1990). Moreover, the concentrations of serum amyloid A were reported to increase during acute-phase inflammatory reactions to 1,000-fold their levels under normal conditions (Schultz and Arnold, 1990). Serum amyloid A has been reported to have a number of cytokine-like immunomodulatory roles, and recently Su et al. demonstrated that serum amyloid A is a

potent chemoattractant for human leukocytes, such as monocytes, neutrophils, and T lymphocytes (Su et al., 1999). In terms of serum amyloid A cell surface receptors, Su et al. demonstrated that serum amyloid A selectively stimulates formyl peptide receptor like 1 (FPRL1), and that this results in Ca^{2+} mobilization and cell migration (Su et al., 1999). These workers also found that radiolabeled serum amyloid A bound specifically to human phagocytes and FPRL1-transfected 293 cells, and thus demonstrated that FPRL1 as a specific receptor for serum amyloid A (Su et al., 1999). However, the role of serum amyloid A on cytokine production, and the role of FPRL1 in this process have not been elucidated.

In this study, we investigated whether serum amyloid A induces pro-inflammatory or anti-inflammatory cytokines production in human monocytes, and interestingly, we found that serum amyloid A stimulates both TNF-α and IL-10 production. We then further investigated the involvement of FPRL1 in TNF-α and IL-10 production induced by serum amyloid A stimulation, and the signaling pathways involved in these processes.

Materials and methods

Isolation of human peripheral blood monocytes

Peripheral blood was collected from healthy adult donors. The donors were confirmed not to have taken anti-inflammatory drugs for at least 4 weeks before sampling. Venous blood was collected and peripheral blood mononuclear cells were separated on a Histopaque-1077 gradient. After two washings with Hank's buffered saline solution, without Ca^{2+} and Mg^{2+}, the peripheral blood mononuclear cells were suspended in RPMI 1640 medium containing 10% FBS and incubated for 60 min at 37°C to allow the monocytes to attach to the culture dish. The attached monocytes were then collected as described previously (Bae et al., 2001). The isolated cells were used immediately.

Cell culture

FPRL1-expressing RBL-2H3 cells and vector-transfected RBL-2H3 cells were maintained as previously described (Bae et al., 2003). Cells were maintained at about 1×10^6 cells/ml under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, 37^oC).

Cytokine assay

Cytokine measurement was performed as previously described (Jo et al., 2004). Monocytes (3 $\times 10^6$ cells/0.3 ml) were placed in RPMI 1640 medium containing 10% FBS in 24-well plates and kept in a 5% CO_2 incubator at 37^oC. After stimulation of the cells with serum amyloid A (Peprotech, Rocky Hill, NJ), cell-free supernatants were collected, centrifuged, and measured for TNF-α or IL-10 by enzyme-linked immunosorbent assay (BD Biosciences Pharmingen, San Diego, CA) according to the instruction of the vender.

Real-time PCR

Monocytes (1×10^6 cells) were stimulated with 2 μM serum amyloid A for the indicated times. mRNA was isolated by using a QIAshredder and an RNeasy kit (Qiagen, Hilden, Germany). mRNA, moloney murine leukemia virus reverse transcriptase, and pd(N)6 primers (GIBCO BRL, Gaithersburg, MD) were used to obtain cDNA. 1 μg of the cDNA and TaqMan real time primers and probes was used for amplification. A Set of primers and a probe for each gene tested was obtained from Applied Biosystems. Assay ID for genes are as follows: IL-10, Hs0017086_m1; TNF-α, Hs00174128_m1; GAPDH, Hs99999905_m1. All PCR reactions were carried out in TaqMan Universal PCR master Mix (Applied Biosystems) with 900 nM of each primer and 250 nM of probe. Sequence-specific amplification was

detected with an increased fluorescent signal of FAM (reporter dye) during the amplification cycles by ABI Prism 7000 real-time PCR system (Applied Biosystems Inc. California). PCR was performed for 15 s at 95°C and 1 min at 60°C for 50 cycles followed by the thermal denaturation protocol. Amplification of human GAPDH was used in the same reaction of all samples as an internal control. Gene-specific mRNA was subsequently normalized to GAPDH RNA. The expression of each mRNA was determined using the $2^{-\Delta\Delta CT}$ Method (Livak and Schmittgen, 2001).

Preparation of nuclear extracts

Nuclear extract of human monocyte were prepared as described previously (Kettritz et al., 2004). Briefly, human monocytes (1 X 10^6 cells) were incubated with 2 μM serum amyloid A for several times as indicated. Cells were harvested in PBS containing 2% FBS, washed twice with PBS, and resuspended in 400 μl of buffer (10 mM HEPES, pH 7.9, 5 mM MgCl$_2$, 10 mM KCl, 1 mM ZnCl$_2$, 0.2 mM EGTA, 1 mM Na$_3$VO$_4$, 10 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). After the cells were incubated on ice for 10 min and then lysed by the addition of 50 μl of 10% Nonidet P-40 (1.1% final concentration), the nuclei were harvested by centrifugation. The nuclear pellets were resuspended in 60 μl of extraction buffer (10 mM HEPES, pH 7.9, 5 mM MgCl$_2$, 300 mM NaCl, 1 mM ZnCl$_2$, 0.2 mM EGTA, 25% glycerol, 1 mM Na$_3$VO$_4$, 10 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A) and incubated for 15 min on ice. Nuclear debris was removed by centrifugation (13,000 rpm for 10 min), and the nuclear protein extract was used for gel-shift analysis.

Electrophoretic mobility shift assay

Gel-shift analysis of nuclear extracts was performed using oligonucleotides containing the

consensus sequence for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG-3'; Santa Cruz

Biotechnology) end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega), as

described previously (Cowland et al., 2003).

Stimulation of cells with serum amyloid A for Western blot analysis

Human monocytes or FPRL1-expressing RBL-2H3 cells (2 X 10^6) were stimulated with the

indicated concentrations of serum amyloid A for predetermined times. After stimulation, the cells

were washed with serum free RPMI 1640 medium and 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$_3$VO$_4$, 10 μg/ml leupeptin,

10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Detergent insoluble materials were

pelleted by centrifugation (12,000 X g, 15 min, at 4°C), and the soluble supernatant fraction was

removed and stored at either -80°C or used immediately.

Electrophoresis and Western blot analysis

Proteins were separated in 10% SDS-polyacrylamide gel and, the proteins were blotted onto a

nitrocellulose membrane, which was then blocked by incubating with TBST (Tris-buffered

saline, 0.05% Tween-20) containing 5% non-fat dry milk. Subsequently, membranes were

incubated with specific antibodies and washed with TBST. Antigen-antibody complexes were

visualized after incubating the membrane with 1:5,000 diluted goat anti-rabbit IgG antibody

coupled to horseradish peroxidase and detected by enhanced chemiluminescence.

RT-PCR analysis

FPRL1- or vector-expressing RBL-2H3 cells (1 X 10^6 cells) were stimulated with 2 μM SAA

for the indicated times. For RT-PCR, we ran 35 PCR cycles at 94°C (denaturation, 1 min),

55°C (annealing, 1 min), and 70°C (extension, 1 min). The sequences of the primer used

were as follows; rat TNF-α (396 bp product): forward, 5'-CATCTGCTGGTACCACCAGTT-

3', reverse, 5'-TGAGCACGAAAAGCATGATC-3'. Rat IL-10 (346 bp product): forward, 5'-

AAACTCATTCATGGCCTTGTA-3', reverse, 5'-TGCCTTCAGTCAAGTGAAGACT-3'.

Rat actin (250 bp product): forward, 5'-ATGGATGATGATATCGCCGCG-3', reverse, 5'-

TCTCCATGTCGTCCCAGTTG-3'. PCR products were electrophoresed on a 2% agarose gel

and visualized by ethidium bromide staining.

Results

Serum amyloid A stimulates TNF-α and IL-10 production in human monocytes.

To investigate the effect of serum amyloid A on the production of several cytokines in human

monocytes, freshly isolated human monocytes were stimulated with 2 µM of serum amyloid

A for various lengths of time. serum amyloid A dramatically stimulated TNF-α and IL-10

production in human monocytes (Fig. 1A and 1B). Serum amyloid A also slightly stimulated

IL-1β, IL-8, and IL-12 production in human monocytes (data not shown). In this study we

focused on TNF-α and IL-10, because they were dramatically produced by serum amyloid A.

As shown in Fig. 1A, serum amyloid A induced TNF-α production in a time-dependent

manner and showed maximal activity 6 hr after stimulation, whereas IL-10 production by

serum amyloid A peaked at 12 hr after stimulation (Fig. 1B). We also tested the

concentration-dependency of TNF-α or IL-10 production by serum amyloid A. Serum

amyloid A induced TNF-α production in a concentration-dependent manner, showing

maximal activity at 100 nM (Fig. 1C), and also induced IL-10 production in a concentration-

dependent manner and showed maximal activity at 2 µM (Fig. 1D). These results suggest that

serum amyloid A stimulates both cytokines, at different concentrations and with different kinetics. We also tested the effect of serum amyloid A on the expression of c-jun and c-fos. Stimulation of human monocytes with 2 μM of serum amyloid A did not affect on the expression of c-jun and c-fos (data not shown).

TNF-α and IL-10 productions by serum amyloid A are transcriptionally and translationally mediated.

To investigate the mechanism involved in serum amyloid A-induced TNF-α and IL-10 production, we pretreated human monocytes with the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide. When human monocytes were pretreated with actinomycin D or cycloheximide prior to serum amyloid A, serum amyloid A-induced TNF-α and IL-10 productions were almost completely inhibited (Fig. 2A and 2B). We also examined the effect of serum amyloid A on the accumulations of TNF-α and IL-10 mRNA by quantitative real-time PCR. As shown in Fig. 2C, stimulation with 2 μM serum amyloid A caused TNF-α and IL-10 mRNA transcript accumulation in a time-dependent manner. Moreover, TNF-α mRNA levels were significantly increased after stimulating cells for 1-3 hr with serum amyloid A, and returned to the basal level after 6 hr (Fig. 2C), whereas IL-10 mRNA levels were significantly increased after stimulation for 3-6 hr and were maintained for 12 hr (Fig. 2D). These results indicate that TNF-α and IL-10 inductions by serum amyloid A require transcriptional activation and *de novo* protein synthesis. These results correlate well with Fig. 1 in terms of the different expressional kinetics of these two cytokines when induced by serum amyloid A.

Serum amyloid A stimulates NF-κB activity and this leads to TNF-α and IL-10

production in human monocytes.

The expressions of TNF-α and IL-10 have been reported to require NF-κB activation (Kawai et al., 2001; Lin et al., 2005; Badou et al., 2000). Thus, we examined the effect of serum amyloid A on NF-κB activity in human monocytes. To investigate the involvement of NF-κB in the inductions of TNF-α and IL-10 by serum amyloid A, we adopted an electrophoretic mobility shift assay approach. Accordingly, human monocytes were stimulated for several lengths of time with serum amyloid A. As shown by Fig. 3A, the intensities of DNA-protein complexes increased after exposing monocytes to serum amyloid A, and showed dramatic activity 15-60 min after stimulation (Fig. 3A). In addition, this serum amyloid A-induced NF-κB-specific DNA-protein complex formation was inhibited by a 100-fold excess of unlabelled NF-κB probe (Fig. 3A). NF-κB activation has been reported to be associated with I-κB phosphorylation and subsequent degradation (Bonizzi and Karin, 2004; Rothwarf and Karin, 1999), thus the effect of serum amyloid A on I-κB degradation was also examined. Stimulation of monocytes with 2 μM of serum amyloid A for 30 min elicited a dramatic degradation of I-κB, thus supporting our notion of NF-κB activation by serum amyloid A (Fig. 3B).

To investigate the role of NF-κB on TNF-α and IL-10 production by serum amyloid A, we pretreated human monocytes with several types of NF-κB inhibitor, i.e., (E)-3-[4(4-methylphenylsulfonyl)-2-propenenitrile (BAY 11-7082), 5-(Thien-3-yl)-3-aminothiophene-2-carboxamide (SC-514), or cell-permeable NF-κB inhibitory peptide (SN50) (Biomol, Plymouth Metting, PA) prior to adding serum amyloid A. As shown in Fig. 3C, BAY 11-7082 (20 μM), SC-514 (20 μM), or SN50 (50 μg/ml) blocked serum amyloid A-induced TNF-α production by 72, 87, and 64%, respectively. BAY 11-7082, SC-514, or SN50 also blocked serum amyloid A-induced IL-10 production by 78, 69, and 34%, respectively. These results

indicate that NF-κB activation is essentially required for TNF-α and IL-10 production by serum amyloid A in human monocytes.

Serum amyloid A stimulates ERK or p38 kinase at different concentrations.

Mitogen-activated protein kinase (MAPK) has been reported to mediate extracellular signals to the nucleus in several cell types (Johnson and Lapadat, 2002). In this study, we used Western blotting to determine whether serum amyloid A stimulates MAPKs using anti-phospho-specific antibodies against each enzyme. When human monocytes were stimulated with 2 μM of serum amyloid A for different times, extracellular signal regulated kinase (ERK) phosphorylation levels transiently increased, i.e., they peaked at 10 min (Fig. 4A) and returned to baseline at 30 min (Fig. 4A). Moreover, when these cells were stimulated with different concentrations of serum amyloid A, ERK was found to be activated in a concentration-dependent manner (Fig. 4A), i.e., significantly at 10 nM and maximally at 2 μM (Fig. 4A). Moreover, another important MAPK, p38 kinase was also activated with time-kinetics similar to those of ERK (Fig. 4A). We also examined the concentration-dependency of p38 kinase phosphorylation by serum amyloid A. When human monocytes were stimulated with various concentrations of serum amyloid A, p38 kinase was found to be activated in a concentration-dependent manner, i.e., significantly at 500 nM and maximally at 2 μM (Fig. 4A).

Distinct MAPK regulates the productions of TNF-α or IL-10 by serum amyloid A.

To determine the roles of MAPKs on serum amyloid A-induced TNF-α or IL-10 production, we preincubated human monocytes with 2´-Amino-3'-methoxyflavone (PD98059, Calbiochem San Diego, CA), a selective MEK inhibitor, or with 4-(4-Fluorophenyl)-2-(4-

methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580, Calbiochem San Diego, CA), a selective p38 kinase inhibitor, prior to serum amyloid A treatment. At first, we examined whether PD98059 and SB203580 effectively inhibited MEK1/2 and p38 kinase in our system. Preincubation of human monocytes with PD98059 (50 μM) completely inhibited ERK phosphorylation (data not shown), indicating that PD98059 effectively inhibited MEK1/2 in the cells. We also found that SB203580 (20 μM) completely inhibited ATF-2 (a p38 kinase substrate) phosphorylation (data not shown), indicating that SB203580 inhibited p38 kinase in the cells. We then, investigated the roles of ERK and p38 kinase on serum amyloid A-induced TNF-α production. As shown in Fig. 4B, serum amyloid A-induced TNF-α production was dramatically inhibited by PD98059, but SB203580 had no effect on serum amyloid A-induced TNF-α production. These results demonstrate that MEK-dependent ERK, but not p38 kinase, is essentially required for serum amyloid A-induced TNF-α production. We also investigated the roles of ERK and p38 kinase on serum amyloid A-induced IL-10 production. In contrast to TNF-α, IL-10 production by serum amyloid A was almost completely inhibited by SB203580, but unaffected by PD98059 (Fig. 4C). These results demonstrate that p38 kinase, but not ERK, is essentially required for serum amyloid A-induced IL-10 production.

Both of TNF-α and IL-10 production by serum amyloid A are PI3K-mediated.

Phosphoinositide-3-kinase (PI3K)-mediated activity has been reported to mediate various cellular responses, including cytokines production in monocytes and macrophages (Martin et al., 2003; Foey et al., 2002; Miller et al., 2003). In this study, we examined whether serum amyloid A stimulate Akt activity by Western blotting using anti-phospho-Akt specific antibodies. When human monocytes were stimulated with 2 μM of serum amyloid A for

different times, Akt phosphorylation levels transiently increased, i.e., they peaked at 5-10

min (Fig. 5A) and returned to baseline at 30 min (Fig. 5A). In addition, we examined the

concentration-dependency of serum amyloid A-induced Akt activation. When human

monocytes were stimulated with various concentrations of serum amyloid A, Akt was found

to be activated in a concentration-dependent manner (Fig. 5A), i.e., significantly at 100 nM

and maximally at 2 μM (Fig. 5A). To determine the role of the PI3K pathway on serum

amyloid A-induced TNF-α or IL-10 production, we preincubated human monocytes with two

PI3K-selective inhibitors (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002,

Calbiochem, San Diego, CA) or wortmannin) prior to serum amyloid A treatment. Both

LY294002 and wortmannin dramatically inhibited TNF-α and IL-10 production by serum

amyloid A (Fig. 5B and 5C), thus demonstrating that PI3K activity is essentially required for

serum amyloid A-induced TNF-α and IL-10 production.

Serum amyloid A stimulates TNF-α and IL-10 production via FPRL1.

To support our notion that serum amyloid A stimulates TNF-α and IL-10 production via

FPRL1 in human monocytes, we investigated the effects of serum amyloid A on TNF-α and

on IL-10 production in FPRL1-expressing RBL-2H3 cells. As shown in Fig. 6A, stimulation

of these cells with 2 μM serum amyloid A caused TNF-α and IL-10 mRNA accumulation.

Since no significant mRNA accumulation was observed in response to serum amyloid A in

vector-expressing RBL-2H3 cells (Fig. 6A), we conclude that serum amyloid A stimulates

FPRL1 and that this leads to TNF-α and IL-10 production.

We also tested the effects of serum amyloid A on ERK and p38 kinase activation in

FPRL1-expressing RBL-2H3 cells. Stimulation of these cells with various concentrations of

serum amyloid A caused the transient concentration-dependent phosphorylations of ERK and

p38 kinase (Fig. 6B). However, when vector-expressing RBL-2H3 cells were stimulated with different concentrations of serum amyloid A, no significant enhancements of ERK or p38 kinase phosphorylation were observed (Fig. 6B and data not shown). These results indicate that serum amyloid A stimulates FPRL1 and that this results in ERK and p38 kinase activation, which are essentially required for TNF-α and IL-10 production by serum amyloid A, respectively. Stimulation of FPRL1- but not vector-expressing RBL-2H3 cells with serum amyloid A also induced Akt phosphorylation, indicating that serum amyloid A stimulates PI3K-mediated Akt activation via FPRL1 (Fig. 6B).

Discussion

In this study, we investigated the effects of serum amyloid A on the regulations of the production of TNF-α and IL-10 in human monocytes. We found that serum amyloid A induced the concentration-dependent production of TNF-α and IL-10 in these cells. Moreover, these inductions of TNF-α and IL-10 by serum amyloid A were found to be associated with the accumulations of the mRNAs of TNF-α and IL-10, which suggests that serum amyloid A induces the production of both of these cytokines at the transcriptional level. We also found that serum amyloid A stimulates TNF-α and IL-10 production via NF-κB activation downstream of FPRL1 activation. Thus, this study provides first evidence that serum amyloid A activates the production of the pro-inflammatory and anti-inflammatory cytokines, TNF-α and IL-10 via FPRL1 activation.

Serum amyloid A is regarded as a proinflammatory mediator, as it is highly elevated in the circulation and locally in tissues during various pathologic conditions, like sepsis. IL-1β and TNFR-II but not TNF-α or IL-6 were found to be induced by serum amyloid A in THP-1

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human monocytic cells (Patel et al., 1998). Moreover, serum amyloid A stimulated TNF-α secretion by forming a serum amyloid A-extracellular matrix complex in human T lymphocytes (Preciado-Patt et al., 1998). Serum amyloid A was also reported to induce IL-8 in human neutrophils (Furlaneto et al., 2000), and a more recent report demonstrated that serum amyloid A-induced IL-8 production is mediated by the activation of its membrane-bound specific receptor, FPRL1 (He et al., 2003). Taken together, previous reports suggest that proinflammatory effect of serum amyloid A, and the role of FPRL1 in the process. In this study, we suggest that serum amyloid A, an endogenous ligand of FPRL1, stimulates the production of IL-10, an anti-inflammatory cytokine in human monocytes.

Several previous reports have documented that two different receptors reciprocally maintain immune homeostasis (Krummel and Allison, 1995; Tachimoto et al., 2000). Moreover, T-cell receptor-induced IL-2 secretion is stimulated by CD28, but suppressed by CTLA-4 (Krummel and Allison, 1995). And, FcϵRI-mediated cytokine and chemokine secretions from mast cells were also reciprocally regulated by IL-4 and interferon-γ (Tachimoto et al., 2000). However, recently, CD40 signaling has been demonstrated to reciprocally regulate both inflammatory (IL-12) and anti-inflammatory (IL-10) cytokine production in macrophages. In the present study, we found that serum amyloid A reciprocally regulates both inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokine production in human monocytes (Fig. 1). The key factor that determines the type of cytokine produced is the serum amyloid A concentration. A low concentration of serum amyloid A was found to stimulate TNF-α production, whereas a high concentration stimulated both TNF-α and IL-10 production (Fig. 1). Moreover, the productions of these cytokine types by serum amyloid A showed differential kinetics (Fig. 1). Serum amyloid A rapidly induced TNF-α which peaked 6 hr after stimulation, whereas IL-10 expressions peaked after 12-24 hr (Fig. 1). The

differential inductions of these two cytokines by serum amyloid A suggests that on infection, synthesized serum amyloid A rapidly stimulates an inflammatory response by inducing TNF-α, and that the subsequent accumulation of serum amyloid A inhibits inflammatory response by inducing IL-10 and promoting immune system homeostasis.

In order to confirm that serum amyloid A stimulates both inflammatory and anti-inflammatory cytokine production via the same target receptor in human monocytes, it is crucial to demonstrate that serum amyloid A stimulates TNF-α and IL-10 production via its specific receptor, FPRL1. Given the data obtained from human monocytes, it was still unclear as to whether another receptor was possibly involved in serum amyloid A-stimulated IL-10 production. However, we subsequently found that serum amyloid A stimulated the inductions of TNF-α and IL-10 in FPRL1-expressing RBL-2H3 cells, but not in vector transfected RBL-2H3 cells (Fig. 6A). These results strongly suggest that serum amyloid A reciprocally stimulates the productions of TNF-α and IL-10 via FPRL1. The serum amyloid A we used is recombinant protein serum amyloid A which is produced in *E. Coli*. Even though the endotoxin content of serum amyloid A preparation is negligible (0.1 ng/μg), we further examined the possible contribution of lipopolysaccharide on the serum amyloid A-induced cytokines production using a potent inhibitor of lipopolysaccharide (polymyxin B). Preincubation human monocytes with polymyxin B (10 μg/ml) prior to addition of lipopolysaccharide completely inhibited cytokines production by lipopolysaccharide, but serum amyloid A-induced cytokines production was unaffected (data not shown). The results indicate that serum amyloid A were not mediated by containing lipopolysaccharide on the cytokines production. According to our results TNF-α production was preceded by IL-10 production by serum amyloid A (Fig. 1). To examine a possible production of IL-10 by the action of secreted TNF-α by serum amyloid A in the cells, we checked the effect of TNF-α

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on the production of IL-10. Stimulation of human monocytes with effective concentrations of

TNF-α (100 nM and 1 μM) for several lengths of time (6, 12, and 24 hr) did not affect on the

IL-10 production (data not shown). From the results we can rule out the possibility of

indirect induction of IL-10 mediating TNF-α production by serum amyloid A, supporting our

notion that serum amyloid A directly stimulates the two cytokines (TNF-α and IL-10)

production via FPRL1.

In terms of the intracellular signaling mechanism of serum amyloid A-induced cytokine

production, we found that the PI3K/ERK and PI3K/p38 kinase pathways are essentially

required for TNF-α or IL-10 expression, respectively, via the activation of FPRL1 by serum

amyloid A in human monocytes (Figs. 4 and 5). Some previous reports have also

demonstrated that ERK activity is essential for the expression of several pro-inflammatory

cytokines, such as TNF-α, IL-1β, and IL-8, induced by extracellular stimuli, such as, by

placenta growth factor (Selvaraj et al., 2003). In this study, we found that FPRL1 activation

by serum amyloid A induces ERK and p38 kinase activations in human monocytes, and that

these activities are essential for the inductions of TNF-α and IL-10, respectively (Fig. 4). On

the activation of ERK and p38 kinase by serum amyloid A, the rapid onsets of these two

kinases phosphorylation suggest that the activation of ERK and p38 kinase by serum amyloid

A is primary events mediated by the activation of FPRL1, rather than events that are

secondary to TNF-α production in the cells. We also found that the activations of ERK and

p38 kinase by serum amyloid A were not affected by preincubation of human monocytes

with cycloheximide which inhibits synthesis of proteins, including TNF-α (data not shown).

It strongly indicates that ERK and p38 kinase activations are primary and directly coupled to

activation of FPRL1. Moreover, it was interesting to find that serum amyloid A modulates

pro- and anti-inflammatory cytokines via distinct intracellular signaling pathways, i.e., ERK

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and p38 kinase. This finding suggests that the regulations of ERK and p38 kinase are important aspects of the modulation of immune responses downstream of FPRL1/ serum amyloid A signaling.

Our results suggest that FPRL1 mediates serum amyloid A-induced cellular signals and TNF-α and IL-10 production. Our results and previous findings indicate that further investigation of FPRL1 is warranted, to determine the potential therapeutic benefits of targeting this receptor as a potential immunomodulator of TNF-α and IL-10, and to elucidate the natures of related pathophysiological responses.

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Footnotes

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Figure legends

Figure 1. Serum amyloid A stimulates the concentration-dependent productions of TNF-α and IL-10 but at different times post-stimulation in human monocytes. Freshly isolated human peripheral blood monocytes were stimulated with serum amyloid A (at 2 μM) for various times (A, B) and at different concentrations for 6 hr (C) or 24 hr (D). Levels of secreted TNF-α and IL-10 were measured by ELISA. Data represent the means $\pm$ SE of three independent experiments performed in duplicate (A-D).

Figure 2. The serum amyloid A-induced productions of TNF-α and IL-10 require transcription and *de novo* protein synthesis. Freshly isolated human peripheral blood monocytes were preincubated for 1 hr with or without actinomycin D (10 μg/ml) or cycloheximide (100 μM), and then stimulated with 2 μM serum amyloid A for 6 hr (A) or 24 hr (B). Secreted levels of TNF-α and IL-10 were measured by ELISA. Data represent the means $\pm$ SE of three independent experiments performed in duplicate (A, B). Human peripheral blood monocytes were stimulated with 2 μM serum amyloid A for 0, 1, 3, 6, 12, or 24 hr. At the end of treatment, cells were harvested and total RNA was extracted, and TaqMan analysis was conducted for TNF-α (C) or IL-10 (D) mRNA. Data are the means $\pm$ SE of 3 determinations (C, D).

Figure 3. The serum amyloid A-stimulated productions of TNF-α and IL-10 are NF-κB-dependent. Freshly isolated human peripheral blood monocytes were stimulated with 2 μM serum amyloid A for different times. Nuclear proteins were extracted and electrophoretic mobility shift assays were performed. The NF-κB/DNA complex was detected by using a ^{32}P-

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labeled NF-κB oligonucleotide probe. An unlabeled NF-κB probe (100 X excess) was used as a competitor to determine the specificity of DNA binding (lane 5). The data shown were obtained from six independent experiments (A). Monocytes were stimulated with 2 μM serum amyloid A for different times. Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and I-κB levels were determined by immunoblot analysis using anti-I-κB antibody (B). Western blot analysis with anti-actin antibody was used to confirm equal protein lodaings. The results shown are representative of three independent experiments (B). Human monocytes were preincubated with vehicle, 20 μM BAY 11-7082, 20 μM SC-514, or 50 μg/ml SN50 at 37°C for 30 min and then stimulated with 2 μM serum amyloid A for 6 hr (for TNF-α) or 24 hr (for IL-10). Secreted levels of TNF-α and IL-10 were determined by ELISA. The data shown represent means ± SE of three independent experiments performed in duplicate (C).

Figure 4. Different MAPKs are required for serum amyloid A-induced TNF-α or IL-10 production in human monocytes. Human monocytes were stimulated with serum amyloid A (2 μM) for different times (left panel) and with different concentrations for 5 min (right panel). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated ERK and p38 kinase levels were determined by immunoblotting using anti-phospho-ERK antibody or anti-phospho-p38 kinase antibody (A). The results shown are representative of at least three independent experiments (A). Cells were preincubated with vehicle, 50 μM PD98059 (60 min), or 20 μM SB203580 (15 min) prior to being treated with 2 μM of serum amyloid A for 6 hr (B) or 24 hr (C). The amounts of TNF-α (B) and IL-10 (C) secreted were measured by ELISA. Data represent the means ± SE of three independent experiments performed in duplicate (B, C).

Figure 5. PI3K activity is essential for the serum amyloid A-induced production of TNF-α or IL-10 in human monocytes. Human monocytes were stimulated with serum amyloid A (2 μM) for different times (left panel), and with various concentrations for 5 min (right panel). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated Akt levels were determined by immunoblotting using anti-phospho-Akt antibody (A). The results shown are representative of at least three independent experiments (A). Cells were preincubated with vehicle, 50 μM LY294002 (15 min), or 1 μM wortmannin (60 min) prior to being treated with 2 μM of serum amyloid A for 6 hr (B) or 24 hr (C). The amounts of TNF-α (B) and IL-10 (C) secreted were measured by ELISA. Data represent the means ± SE of three independent experiments performed in duplicate (B, C).

Figure 6. The serum amyloid A-induced productions of TNF-α and IL-10 are FPRL1-mediated. Vector- or FPRL1-expressing RBL-2H3 cells were stimulated with 2 μM serum amyloid A for 0, 2, 4, or 6 hr. Cells were harvested for RNA preparation. RT-PCR was performed using specific primers for rat TNF-α, IL-10, and actin. PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide (A). The data obtained from one representative experiment performed in quadruplicate is shown (A). Vector- or FPRL1-expressing RBL-2H3 cells were stimulated with various concentrations of serum amyloid A for 5 min. Each sample (30 μg of protein) was subjected to 10% SDS-PAGE and phosphorylated ERK and p38 kinase levels were determined by immunoblotting using anti-phospho-ERK and anti-phospho-p38 kinase antibodies (B). The results shown are representative of three independent experiments (B).
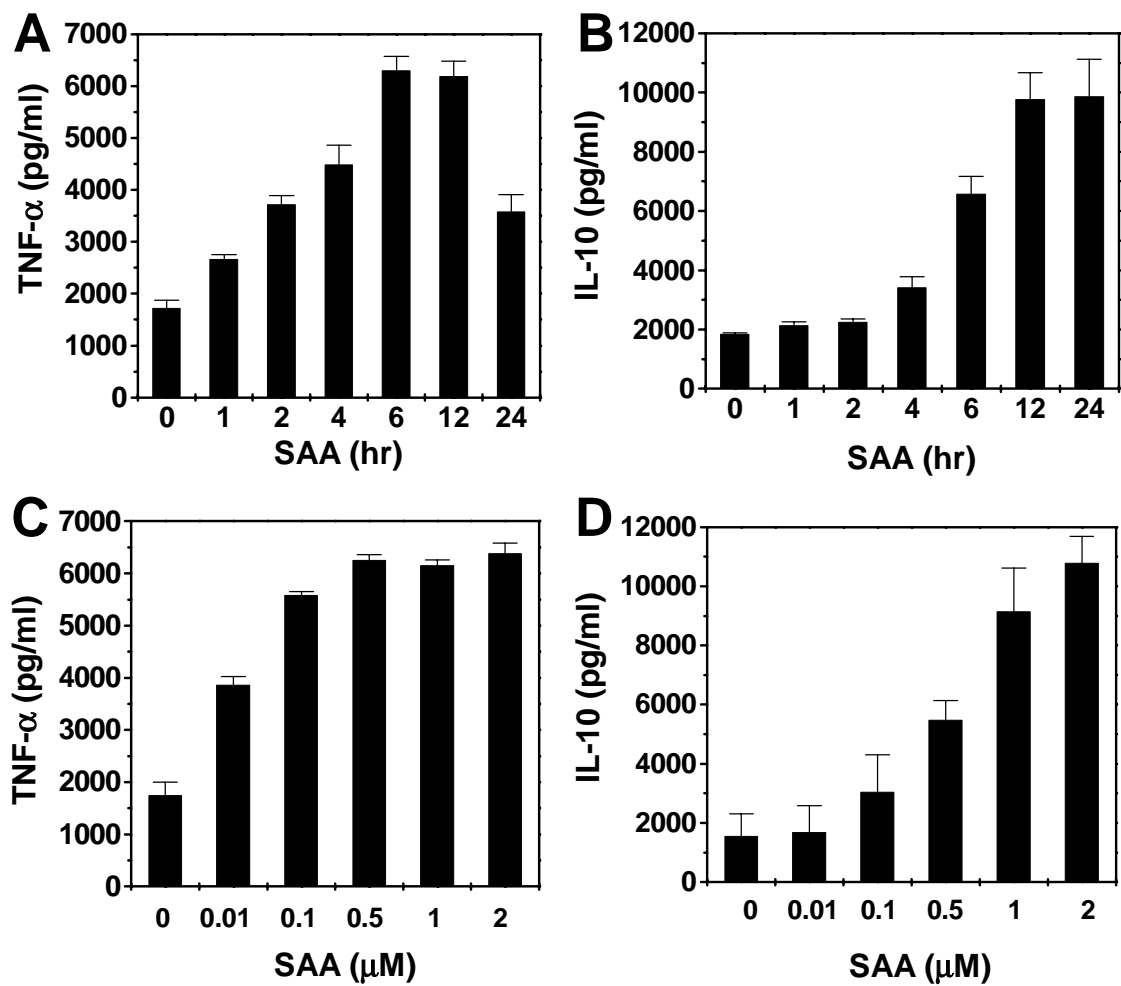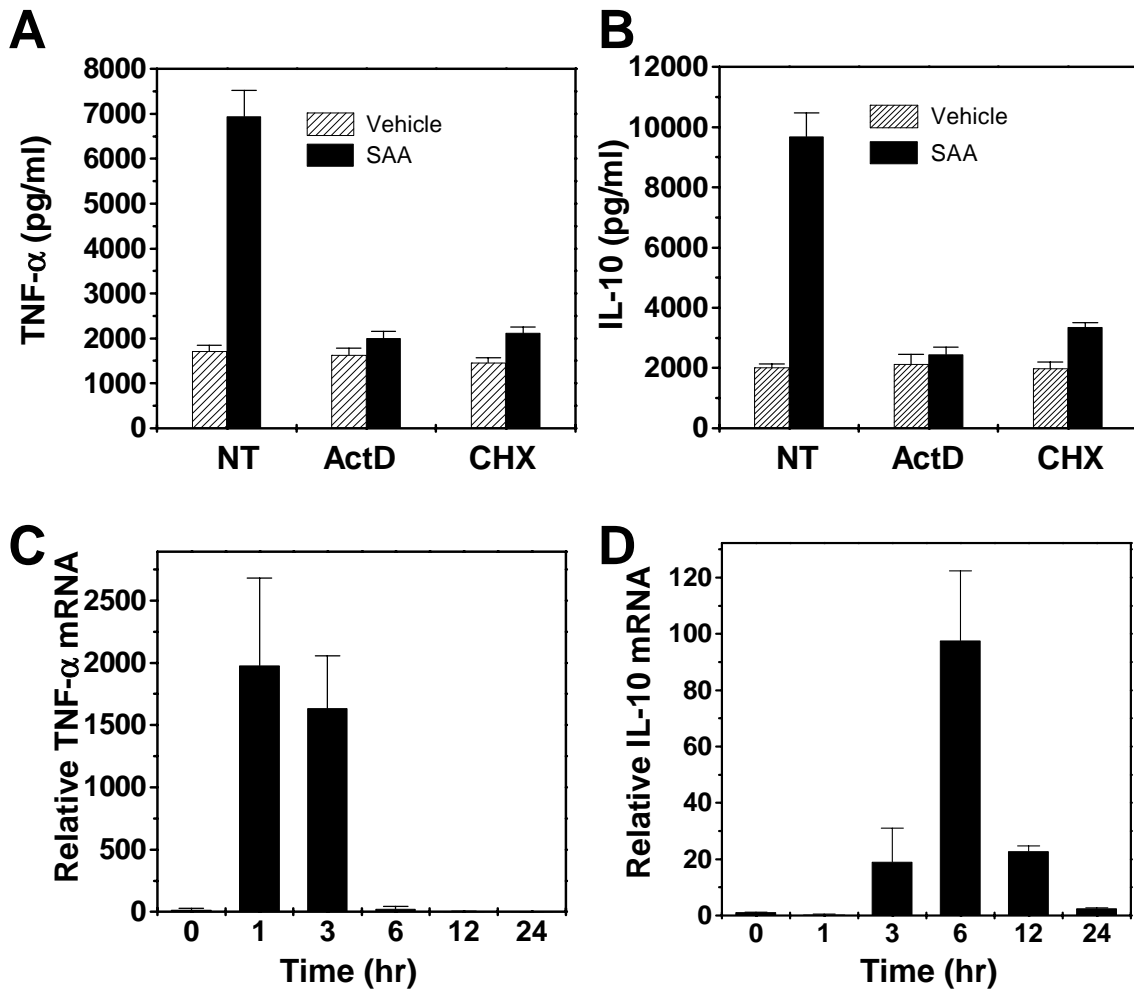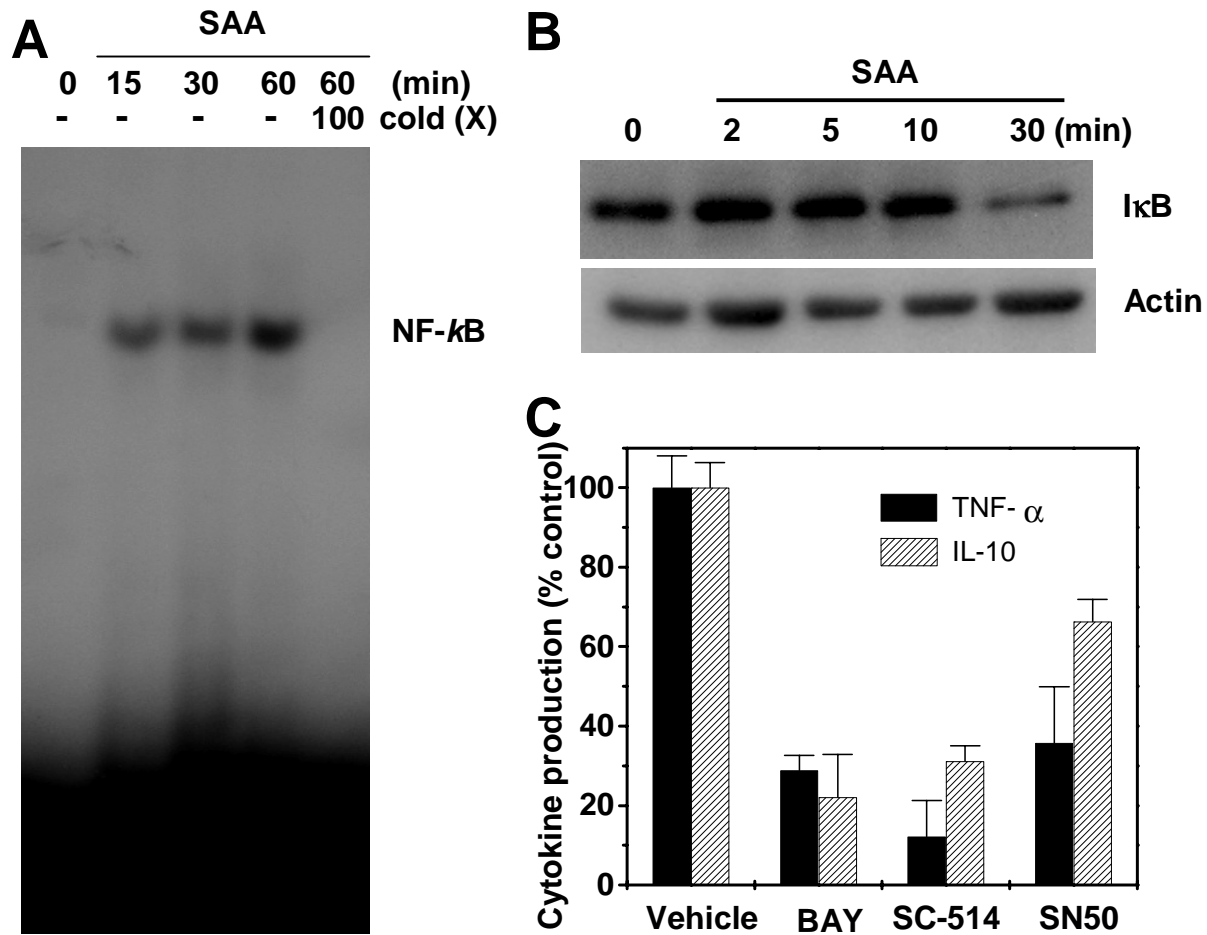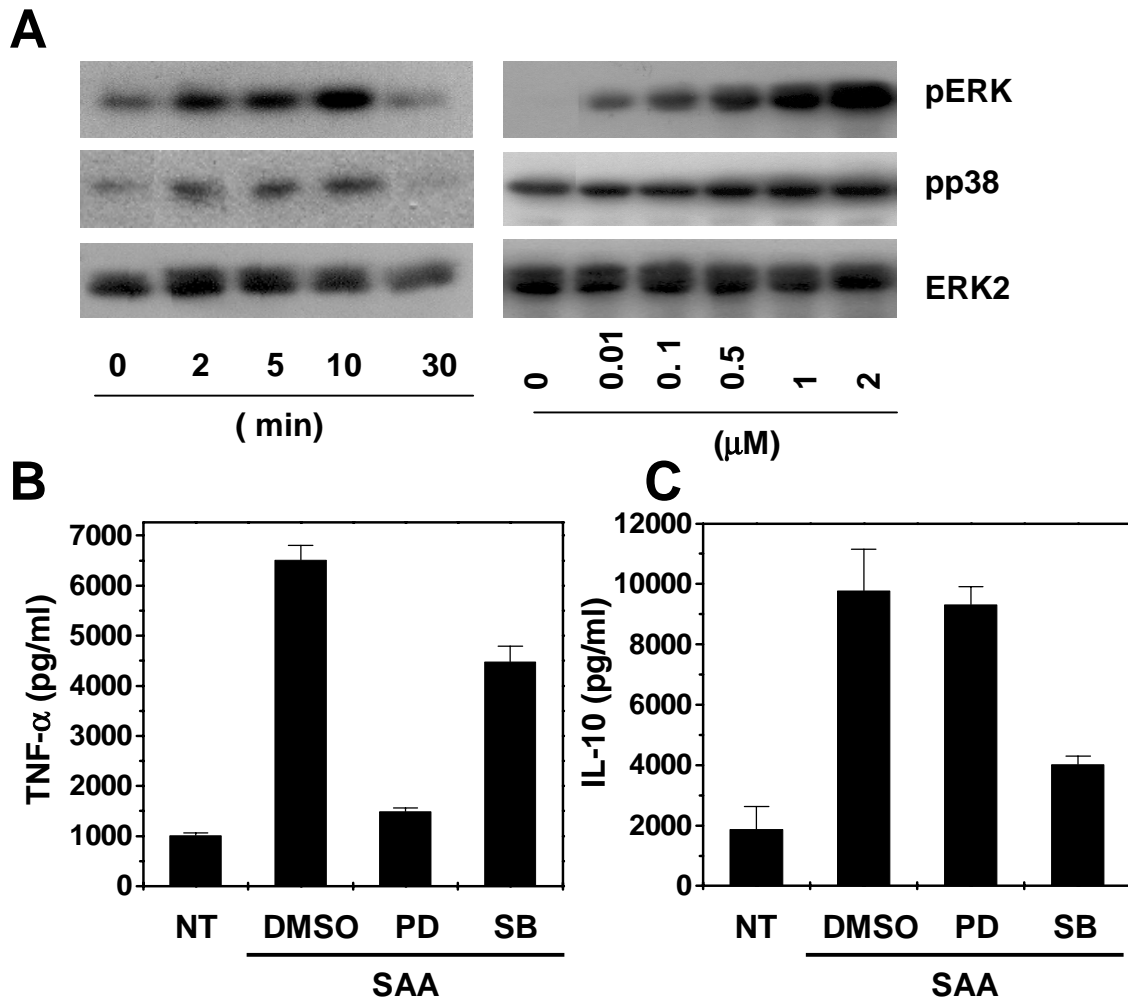
Fig. 1

Fig. 2

Fig. 3

A

pERK

pp38

ERK2

0   2   5   10   30

(min)

0   0.01   0.1   0.5   1   2

(μM)

B

TNF-α (pg/ml)

NT   DMSO   PD   SB

SAA

C

IL-10 (pg/ml)

NT   DMSO   PD   SB

SAA

Fig. 4

Fig. 5

Fig. 6