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Somatostatin Regulates Raw 264.7 Cell Cytokine Release

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SOMATOSTATIN REGULATES RAW 264.7 CELL CYTOKINE RELEASE

BY

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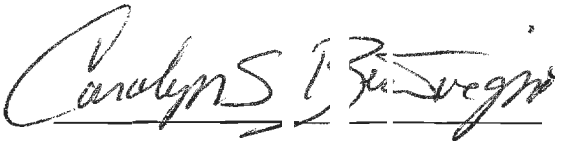

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ABSTRACT

Immigrant monocytes and resident macrophages propagate the vertebrate innate immune response through cell migration and cytokine production. Monocytes responding to inflammatory challenge migrate into tissues, and as resident macrophages, release a major pro-inflammatory cytokine, tumor necrosis factor α (TNF-α). Macrophge cytokine synthesis is known to be under both negative and positive regulatory control. Recent studies have shown that somatostatin (SRIF) regulates monocyte and peritoneal macrophages. However, the effects of SRIF on RAW 264.7 cells, a transformed monocyte/macrophage cell line, have not been investigated. In the present study, SRIF effect on cytokine release in LPS stimulated RAW 264.7 cells was examined. Somatostatin regulated monocyte response to LPS stimulation as reflected by decrease in TNF-α release. In particular, LPS showed temporal TNF-α release peaking at 2h. SRIF, at physiological concentrations (10^{-7} M to 10^{-10} M) showed dose independent reduction on TNF-α release. We found SRIF 10^{-10} M concentration inhibited TNF-α release to the maximum at 2 h. Finally, we show SRIF 10^{-10} Mover a time course inhibited maximum TNF-α release at 2 h in LPS stimulated RAW 264.7 cells. Taken together, our results show that SRIF modulates TNF-α release in LPS stimulated RAW 264.7 cells.

INTRODUCTION

Inflammation is a highly regulated process initiated as a response of the immune system to infection. While the regulated response, acute inflammation, protects the host tissue from damage and injury, an unregulated response, chronic inflammation, is implicated in a spectrum of major diseases (Fujiwara and Kobayashi, 2005). Chronic inflammation is associated with the presence of immigrant monocytes becoming activated resident macrophages in inflamed tissues (Shacter, 1993; Kasahara and Matsushima, 2001). Cytokines such as interferon γ, TNF-α and granulocyte-macrophage colony stimulating factor activate the resident macrophages, which in turn respond by producing inducible nitric oxide synthase, TNF-α, IL-6 and IL-1β thus playing a key role in the propagation of the inflammation response (Denis, 1991; Fujiwara and Kobayashi, 2005).

Activated macrophages also participate in the resolution of inflammation through increased endocytotic activity, although this cellular activation is dependent upon other stimuli, such as IL-4 and IL-13 (Martinez *et al.*, 2008). Apoptosis, as well as the formation and release of anti-inflammatory cytokines, antioxidants and suppressors of inflammation are also critical components of anti-inflammatory mechanism (Fujiwara and Kobayashi, 2005). Indeed, the balance between pro-inflammatory and anti-inflammatory macrophage responses are poorly understood, although Toll-like receptors (TLRs) are clearly involved in initiating the innate immune responses.

Macrophages also respond to endogenous circulating peptides that were originally characterized as neuroendocrine mediators. Notable among these peptide hormones is somatostatin. Somatostatin (somatotropin releasing-inhibitory factor, SRIF) has been identified locally at sites of inflammation (Pinter *et al*, 2006). Indeed, SRIF and SRIF peptide analogs have shown to exert anti-inflammatory effects in vivo (Karalis *et al*, 1994). Recent studies have also identified SRIF receptors on monocytes and macrophages from human and mouse (Armani *et al*., 2007; Perez *et al*., 2003). SRIF regulates monocyte pro-inflammatory responses, yet the exact mechanism by which this reduction in inflammatory activities occurs remains unknown.

SRIF is a cyclic peptide hormone widely distributed in the central and peripheral nervous systems as well as in the pancreas and gastrointestinal system (Reichlin, 1983). A 116 amino acid preprosomatostatin formed from a single-gene product is cleaved into the biologically active peptides SRIF-14 and SRIF-28. SRIF-28 contains 14 additional residues at the amino acid terminus. Both SRIF-14 and SRIF-28 are found in mammals and produced in a cell-specific manner, with SRIF-14 found predominately in the CNS and immune system and SRIF-28 localized in the gut (Reichlin, 1983). In addition, a SRIF-like peptide, cortistatin, is also widely produced and appears to act primarily through SRIF membrane receptors (de Lecea, 2006).

SRIF has a wide range of biological actions; all mediated through a highly conserved family of receptors which couple to heterotrimeric guanine nucleotide regulatory proteins (GPCRs). As members of the GPCR super family, SRIF receptors possess seven transmembrane receptor domains. Molecular pharmacology studies have shown that SRIF receptors bind SRIF, cortistatin and SRIF derived analogs with high affinity (Reisine and Bell,1995). SRIF mediates its biological effects via five receptor subtypes (sst1-5) which are encoded by five genes located on different chromosomes. SRIF receptor genes sst1, 3, 4 and 5 are intronless. Sst2 is alternatively spliced to generate sst2A and sst2B (Lahlou and Guillermet, 2004). SRIF binding triggers a variety of G-protein dependent pathways resulting in physiological responses which are both tissue and receptor specific (Lahlou and Guillermet, 2004). In general, SRIF receptor activation results in a reduction of cellular secretion and proliferation via reductions in intracellular cyclic nucleotide metabolism, protein phosphorylation and ion concentrations (Blake et al, 2004). Species differences exist in SRIF expression patterns with significant receptor subtype expression patterns apparent in endocrine and exocrine tissues (Strowski and Blake, 2008).

Since macrophages act as the predominant source of proinflammtory cytokines in local and systemic inflammation, the control of these responses is important in organismal homeostasis. Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 can in turn induce a defensive inflammatory response (Chowers *et al.*, 2000; Agelaki *et al.*, 2002). TNF-α is a primary inflammatory mediator which initiates the production and release of other cytokine mediators. However TNF-α overproduction leads to variety of

human diseases such as arthritis and cancer (Locksley *et al.*, 2001). SRIF has been reported to exhibit immunomodulatory and anti-inflammatory properties, such as the ablity to suppress the production of interferon-gamma in human monocytes (Niedermuhlbichler and Weidermann, 1992), TNF-α release in LPS-treated human macrophages (Bermudez *et al.*,1990), and proliferation of T- lymphocytes (Payan *et al.*, 1984). Therefore, understanding the mechanism of SRIF action is of considerable importance. SRIF and its analogs have been used as therapeutic agents in human immune mediated inflammatory disease such as rheumatoid arthritis (Badway *et al.*, 2004).

In order to better understand SRIF control of monocyte/macrophage functions, we have chosen to study SRIF actions on a transformed, clonal mouse monocytic cell line. RAW 246.7 cells are murine monocytes that have been extensively used as a renewable surrogate for mouse primary monocytes. RAW 264.7 cells been used to study cellular signaling interactions, calcium mobilization, 3',5' cyclic adenosine monophosphate (cAMP) synthesis and cytokine production. Using a broad range of endogenous ligands, ongoing research on the RAW 264.7 cell seeks to decipher the monocyte/macrophage's complex intracellular signaling pathways (Natarajan *et al.*, 2006). To date, the molecular target of SRIF action, the receptor subtype(s) involved and the intracellular signaling pathways employed have yet to be elucidated in RAW 264.7 cells. RAW 264.7 cells respond to SRIF stimulation (Bellocq *et al.*, 1999) and, recently ,the SRIF receptor subtype present in these cells was identified (Sung, 2007). In the current study, we examine the effect of SRIF-14 on cytokine release in RAW 264.7 cells. Using the potent cytokine secretagogue, bacterial lipopolysaccharide (LPS) to stimulate RAW 264.7 cells,

we then examine the effect of SRIF on the LPS-induced release of TNF-α. Our results show that SRIF, acting through an endogenous sst2b receptor, diminishes TNF-α release from RAW 264.7 cells. This study provides the first direct evidence for SRIF regulation of cytokine release in RAW 264.7 cells.

MATERIALS AND METHODS

Materials:

Cell culture plasticware, including pipettes, multiwell dishes and Petri dishes were obtained from MidSci (St Louis, MI). Cell culture growth medium, salt solutions, fetal calf serum and antibiotics were purchased from Invitrogen (Carlsbad, CA). Enzyme linked immunoassay kits and reagents were obtained from BioLegend (San Diego, CA). Lipopolysaccharide (LPS; serotype 055:B5, cat #L2880) was obtained from Sigma Aldrich (St. Louis, MO). All reagents were cell culture or analytical grade, unless otherwise noted.

Cell Culture:

RAW 246.7 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10 mM l-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin , at 5% CO_2 and 37 ^{o}C. The cell cultures were passaged at 75% confluence.

Cell treatment:

Cells were plated in 24-well plates at a concentration of 3 x 10^5 /ml (1ml/well final volume per well) and cultured for 48 hours at 5% CO_2 and 37 ^{o}C incubator. One plate was prepared for each time point of the treatment protocol. After 48 hours, the

media was aspirated and the cells were washed with 300 µl/well of PBS (plus calcium and magnesium) after which 1 ml of OptiMEM media was added to each well in order starve the cells. After an additional 24 hours the cells were stimulated with 10 µg/ml E. Coli-derived LPS. In order to study the effect of somatostatin (SRIF) on TNF-alpha production, SRIF was serially diluted in the OptiMEM culture medium to achieve concentrations of 10^{-7} M to 10^{-10}M with and without LPS. The supernatant from each well was collected individually in a1.5 ml Eppendorf tube. The supernatants were collected from each plate at 1 hr, 1.5 hr, 2 hr, 4 hr and 8 hr time points to measure released TNF-α. OptiMEM medium alone used to treat the cells served as the negative control (No treatment =NT).

24-well plate layout for treatment of RAW 246.7 cells:

NT	LPS	Sm 10^{-7}	Sm 10^{-9}	LPS+Sm 10^{-7}	LPS+ Sm 10^{-9}
NT	LPS	Sm 10^{-7}	Sm 10^{-9}	LPS+Sm 10^{-7}	LPS+Sm 10^{-9}
NT	LPS	Sm 10^{-8}	Sm 10^{-10}	LPS+ Sm 10^{-8}	LPS+Sm 10^{-10}
NT	LPS	Sm 10^{-8}	Sm 10^{-10}	LPS+Sm 10^{-8}	LPS+Sm 10^{-10}

NT: No treatment

LPS: LPS (10µg/ml)

SRIF: 10^{-7} to 10^{-10} M concentration

TNF-α ELISA assay:

TNF-α secretion was measured in the supernatant collected from the LPS-stimulated RAW 346.7 cells using ELISA Max mouse TNF-alpha kit. The ELISA assay

was carried out according to the manufacturer's instructions. In brief, 96 well plate(s) were coated with the capture antibody (anti TNF-α) before performing the assay. The assay procedure was divided into two parts 1) Reagent preparation, 2) Assay execution.

1. Reagent preparation:
    a. **1x Assay Diluent**: For one 96 well plate assay, a total volume of 50 ml of 1x assay diluent was prepared from the concentrated stock of 5x Assay diluent provided in the kit by adding 10 ml of the 5x assay diluent to 40 ml of deionized (DI) water.
    b. **1x Coating Buffer**: In order to coat the provided 96 well plate with the capture antibody, 15 ml of 1x coating buffer was prepared from 5x coating buffer by adding 3 ml of 5x coating buffer to 12 ml deionized water (DI) water
    c. **Capture antibody solution:** 12 µl of capture antibody was added to 11.94 ml of 1x coating buffer
    d. **Diluted Av-Horse Radish Peroxidase (Av-HRP) solution**: 12 µl of Avidin-HRP was added to 11.99 ml 1x assay diluent.
    e. **Diluted Detection antibody solution**: 60 µl of detection antibody was added to 11.94 µl of 1x assay diluent.
    f. **Freshly prepared** 3,3',5,5'-tetramethylbenzidine **(TMB) substrate**: 6 ml of each of substrate A and substrate B (provided in the kit) was mixed together in a 15 ml conical tube wrapped with aluminum foil.

g. **Wash buffer:** 0.05% Tween 20 (Fisher Scientific, cat# BP337-500) in DPBS (calcium and magnesium) (Gibco, cat# 14040).

h. **Stop solution:** 50 ml of 2N H_2SO_4 was prepared by adding 47.3 ml of DI water to 2.704 ml of stock solution of 36.98 N H_2SO_4 (Pharmaco-aaper, cat # 290000ACS).

2. Procedure:

The coating buffer, assay diluent, TMB substrates and Av-HRP solution were warmed to room temperature before starting the assay. The coating antibody and the detection antibody solution were kept at 4°C until used. The capture antibody solution (100 µl) was added to the wells of the 96 well plate. The plate was sealed and incubated at 37° C for 2 hours. The plate was washed four times with wash buffer (200 µl/well) and blotted dry on a paper towel. Thereafter, 200 µl of 1x assay diluent was added per well of the plate. The plate was sealed and incubated at room temperature for an hour with shaking. About 15 minutes before the completion of the incubation, standard dilutions were prepared. The TNF alpha standard provided in the kit was a stock of 50 µl of 5 ng protein, which was aliquoted into five 1.5 ml Eppendorf tubes upon receipt of the kit. A fresh standard was generated with each assay. In order to prepare the dilutions from one aliquot, a dilution of 500 pg/ml (1000 µl) was prepared by adding 5 µl from the stock aliquot to a 1.5 Eppendorf tube with 995 µl of deionized (DI) water. Two hundred and fifty µl of this dilution (500 pg/ml) was added to 250 µl of DI water to prepare the next dilution (250 pg/ml). Similarly, TNF-alpha concentrates of 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml and 7.8 pg/ml were generated. Assay diluent (1x) served as the

blank/zero standards. The standards and the samples were run in duplicate and the plate was sealed and incubated at the room temperature for 2 hours with shaking. Ten minutes before the completion of the incubation, biotinylated detection antibody diluted solution was prepared. After the incubation the plate was washed four times with wash buffer and 100 µl of the diluted detection antibody solution was added to each well. The plate was sealed and incubated at the room temperature for an hour with shaking. Ten minutes before the completion of the incubation, diluted solution of Av-HRP was prepared. The plate was sealed and incubated at room temperature for 30 minutes with shaking. Thereafter, the plate was washed with washing buffer five times and kept inverted on the paper towel. Meanwhile a fresh solution of TMB substrate was prepared. 100 µl of this freshly prepared TMB substrate solution was added to each well of the plate and the plate was incubated in dark for 15 minutes (by keeping the plate in the cabinet). The reaction was stopped after 15 minutes by adding 2N H_2SO_4 to each well. The plate was read at 450 nm absorbance with a SpectraMax Absorbance plate reader. Data were recorded as OD readings and retained for determining TNF-α concentrations based upon the standard curve generated for each experiment.

Statistical analysis:

Data analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego CA). One way ANOVA was performed and values at $p<0.05$ was considered to be significantly different.

RESULTS

LPS stimulated Raw 246.7 cells release maximum TNF-α at 2 h:

RAW 264.7 cells respond to LPS challenge with a range of pro-inflammatory signaling events, including the up-regulation of inducible nitric oxide and pro-inflammatory cytokine synthesis and release (Chao *et al.*, 1995). Given the importance of TNF-α in promoting inflammation and its pivotal role in sustaining inflammation, we first assessed the time course of TNF-α release from LPS stimulated RAW 246.7 cells. The levels of TNF-alpha in response to stimulation of RAW 264.7 cells with LPS (10 μg/ml) were examined over 8-h time period. As shown in figure 1, LPS treatment induced a rapid and marked increase in TNF-α from the basal level of 315 pg/ml at 1.5 h to a maximal stimulation of 2678 pg/ml at 2h with no further increase in release up to and including 8h. Statistically significant increase in TNF-α were observed with LPS treatment incubations of 2h, 4h, 6h and 8h ($p < 0.005$) (Figure 1). In contrast, parallel control cells showed a modest increase in basal TNF-α release in the absence of LPS (Figure 1).

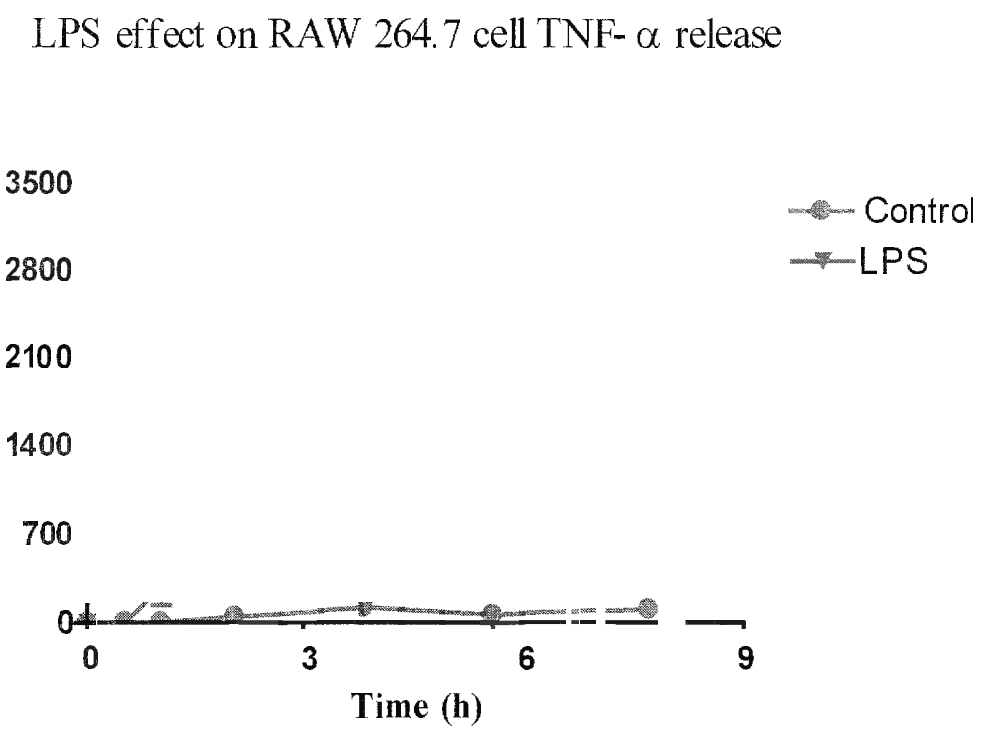

Figure1. *LPS stimulated RAW 264.7 cells release maximum TNF- α at 2 h.* 0.3 x 10^6 RAW 264.7 cells (ATCC, Rockville, MD) were seeded in 24 well plate(s) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37° C, 5% CO_2, 95% air for 48 h and then treated with OptiMEM media for 24h. The cells were treated with 10 µg/ml LPS at 1h, 1.5h, 2h, 4h and 8h and TNF- α was measured in the culture supernatants at each time point using ELISA. Control cells were treated with OptiMEM medium without LPS. Data shown are the replicate mean+/- SEM from 3 separate 24 well plates, with each well assayed in duplicate.

SRIF-14 reduces the release of TNF-α in LPS stimulated RAW 246.7 cells:

SRIF is known for its immunomodulatory effects in activated primary monocytes (Chao *et al*., 1995; Peluso *et al*. 1996)). Thus, it was of interest to evaluate first, if SRIF could modulate TNF-alpha response of LPS stimulated RAW 264.7 cells and second, if the regulation is SRIF dose dependent as per previous work (Chao *et al*, 1995; Peluso *et al*, 1996;). To confirm whether the TNF-alpha release was responsive to SRIF and its graded dose, studies were conducted in which RAW 264.7 cells were stimulated with LPS (10 μg/ml) in the presence and absence of SRIF concentrations ranging between 10^{-10} M and 10^{-7} M. Maximum TNF-α response time point, 2h, (Figure 1) was selected for this study. As expected, the release of TNF-α from LPS activated monocytes was decreased by cell treatment with SRIF and the effect was observed to be dose independent (Figure 2). Cells treated with 10^{-10} M ($p<0.01$) and 10^{-8} M ($p<0.05$) of SRIF showed significantly less TNF-α release compared to LPS treated cells. Further, SRIF 10^{-10} M concentration had more regulatory effect compared to SRIF 10^{-8} M effect on TNF-α release. However, TNF-α release in cells treated with 10^{-7} M and 10^{-9} M of SRIF was not significantly different from LPS treated cells. Cells treated with LPS alone released significantly ($p<0.05$) greater amounts of TNF-α than did the untreated control cells (2299+_ 679 pg/ml vs 53.92+_12.86 pg/ml) and SRIF treated control cells. Control cells used to asses the effect of SRIF showed that SRIF, at all the four concentrations, had no effect on TNF-α release (Figure 2: SRIF-7, SRIF-8, SRIF-9 and SRIF-10). Taken

together, SRIF at 10^{-10} M concentration showed potent TNF-α regulatory effect in LPS activated RAW 264.7 cells (Figure 2).

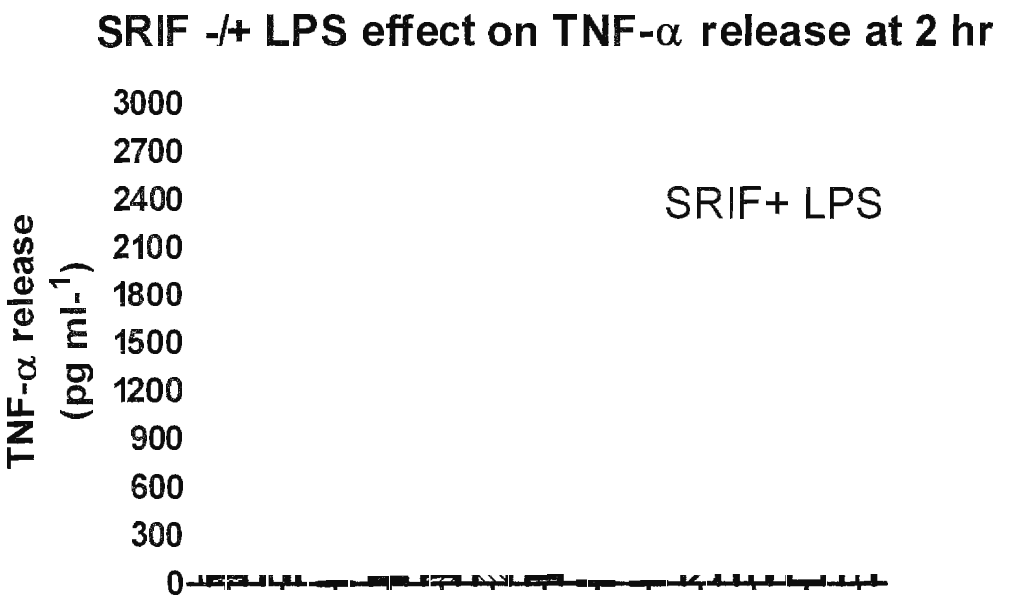

Figure 2: *SRIF-14 reduces the release ofTNF- α in LPS stimulated RAW 264.6 cells at 2h.* 24 well plate(s) were seeded with RAW 264.7 cells similar to the protocol outlined in Figure 1. The cells were treated with 10 μg/ml LPS alone, 10 μg/ml LPS in the presence and absence of SRIF-14 at physiological concentrations of 10^{-7} M, 10^{-8}M, 10^{-9} Mand 10^{-10} Mand released TNF-α was measured in the cell culture supernatants at 2h. (*P< 0.05, # P<0.01 denote statistically significant differences compared to LPS-only treated cells). Control cells were treated with OptiMEM medium in the absence of LPS. The results shown are the mean +/- SEM for 3 individual plates; with 4 replicate wells assayed (in duplicate) from each plate.

10^{-10} M SRIF 14 reduces TNF-α to maximum at 2 h

The results presented in Figure 1 and Figure 2 show different kinetic pattern of TNF-α release and dose-independent SRIF inhibition of TNF-α release in LPS stimulated monocytes compared to previous studies (Peluso *et al*., 1996; Chao *et al*, 1995). Considering that this might be typical response of RAW 246.7 cells to LPS and SRIF, we next wanted to study the time course of SRIF 10^{-10} M on LPS activated RAW 246.7 cells. The Raw 264.7 cells were treated with LPS with or without 10^{-10} M SRIF-14 at 1h, 1.5 h, 2h and 4h. Maximum inhibition of TNF-α release was observed at 2h (by 17.53% or 403.03 pg/ml) compared to other time points [by 11.31 % or 8.62 pg/ml at 1h, 3.7 % or 17 pg/ml at 1.5 h, and 6.64 % or 88.51 pg/ml at 4 h] (Figure 3). Statistically significant decrease in TNF-α release was observed at LPS incubations of 1.5 h, 2h and 4h time points ($p< 0.05$). Again, LPS alone treated cells showed maximum TNF-α release at 2 h followed by decline at 4h. There was minimal response of SRIF alone treated cells and control cells to TNF-α release at all the time points (Figure 3). Taken together, the results from these experiments demonstrate an SRIF inhibition of TNF-α release from RAW 264.7 cells, adding a new direction for studying the mechanism of pro-inflammatory cytokine secretion.

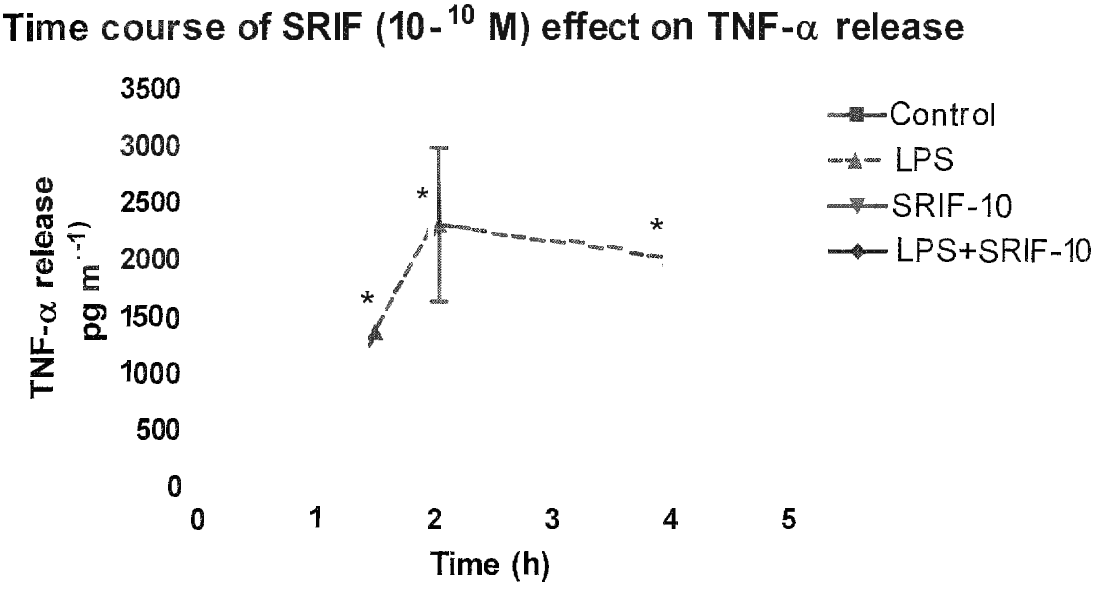

Figure 3.*SRIF-14 reduces TNF- α release in RAW 264.7 cells to maximum at 10^{-10} M concentration at 2h.* 24 well plate(s) were seeded with RAW 264.7 cells similar to the protocol outlined in Figure 1. The cells were treated with 10 μg/ml LPS alone, 10 μg/ml LPS in the presence and absence of SRIF-14 at physiological concentrations of 10^{-10} M and released TNF-α was measured in the cell culture supernatants at the indicated times. Control cells were treated with OptiMEM medium in the absence of LPS. A maximum release of LPS-induced TNF-α release was observed at 2 h. SRIF-14 showed maximum reduction in TNF-α release at 10^{-10} M concentration out of four physiological concentrations (10^{-7} M, 10^{-8}M, 10^{-9} M and 10^{-10} M). The results shown are the mean+/- +/- SEM for 3 separate 24 well plates; with 4 replicate wells from each plate assayed in duplicate.(*) indicate statistically significant difference between TNF- α released in LPS treated cells and LPS± SRIF treated cells ($p<0.05$).

Figure 4: A proposed schematic model of TNF-α inhibition by SRIF in RAW 264.7 cells: LPS binds to toll like receptors (TLR 4) to increase intracellular calcium level, activating NF-κB signal pathway resulting in increase in TNF-α release (pathway indicated by black arrows). SRIF, immunoregulatory in function, might alter LPS pathway via decreasing cellular calcium level by regulating membrane/intracellular calcium ion channels. Consequently, decreasing TNF-α release from RAW 264.7 cells (pathway indicated by red arrows). (Reference: model is adopted from Zhou *et al.*, 2006 and Weckbecker *et al.*, 2003).

DISCUSSION

SRIF agonists control a host of cellular responses, including cell proliferation and secretion. In addition, SRIF peptides and peptidomimetic possess anti-inflammatory activity which may originate from either one or both of the anti-proliferative and anti-secretory effects (Karalis *et al.*, 1994; Badway *et al.*, 2004; Blake *et al.*, 2007). In innate immune responses, resident macrophages are a primary source of pro-inflammatory cytokines, which govern the intricate intercellular communication responsible for the inflammatory response (Fujiwara and Kobayashi , 2005). However, macrophages are also involved in resolving cellular inflammation, typically through their phagocytic activities, but also through their response to circulating anti-inflammatory signals which arise from both neural and immune sources. SRIF is one such immunomodulatory signaling molecule. SRIF's immunomodulatory effects have been studied *in vitro* using different cell lines such as human blood monocytes (Peluso *et al.*,1996), mouse macrophages (Chao *et al.*, 1995) and epithelial cells (Chowers *et al.*, 2000). SRIF actions are thought to be multifactorial, possibly involving control of cytokine synthesis, processing and release. Limitations in the availability of primary immune cells, as well as technical difficulties in isolating resident tissue macrophages, have led us to examine SRIF effects in a murine transformed monocyte/macrophage cell line. The RAW 264.7 cell provides a valuable model cell line for studying macrophage functions and intracellular signaling

pathways (Natarajan *et al*., 2006). In a previous study we demonstrated that the sst2b receptor was expressed in the RAW 264.7 cells (Sung, 2007). The aim of the present study was to study the effect of SRIF on TNF-α release in LPS stimulated RAW 264.7 cell. LPS is a major component of the outer membrane of Gram-negative bacteria and acts as an endotoxin and a potent activator of the immune cells (Morrison and Ryan, 1979) inducing secretion of inflammatory mediators such as IL-1, TNF-α from the macrophages (Adams and Hamilton, 1984). TNF-α acts as primary mediator of inflammatory response initiating the release of other mediators (Urban *et al*., 1986; Le and Vilcek, 1987). We have found that LPS treated RAW 246.7 cells showed temporal TNF-α release peaking at 2h. Further, SRIF-14 showed marked inhibitory effect on TNF-α release in LPS-activated RAW 264.7 cells. This effect as seen was neuropeptide concentration independent with 10^{-8} M and 10^{-10} M concentrations showing significant reduction on TNF-α release but 10^{-7} M and 10^{-9} M concentrations while reduced, were not significantly reduced TNF-α release. In specific, SRIF-14 10^{-10} M concentration inhibited TNF-α release to the maximum compared to other concentrations in RAW 264.7 cells. Our results are consistent with the previous work where RAW 264.7 cells responded in a similar manner to neuropeptide, corticotrophin-releasing factor, graded doses (Tsatsanis *et al*, 2007). We also studied the time course of SRIF 10^{-10} M on LPS stimulated RAW 264.7 cells and found that SRIF-14 10^{-10} M concentration showed maximum reduction in TNF-α release at 2 hr.

Although in general, SRIF is known to suppress monocyte and macrophage functions, there exists a contradictory data on modulation of cytokine release by SRIF

from human and mouse monocytes. While Komorowski and Stepien have reported that SRIF stimulates IL-6 and IL-1 α secretion from LPS stimulated human monocytes (Komorowski and Stepien, 1995), Peluso *et al* have shown that SRIF decreases cytokine secretion in LPS stimulated human monocytes (Peluso *et al*., 1996). Further, SRIF and its analogs have shown to diminish the levels of inflammatory mediators such as TNF-α in vivo (Karalis *et al*., 1994) and in rat peritoneal macrophages (Chao *et al*.,1995). Our results are in consistent with the studies showing SRIF regulation of cytokine release in monocytes/macrophages or in vivo.

Taken together, in this study we have shown the marked effect of SRIF (10^{-10} M) upon LPS activated RAW 264.7 cells, which is neuropeptide concentration-independent. We speculate that SRIF might mediate its regulatory effect via regulation of calcium channels present in the cells (Weckbecker *et al*., 2003). Increase in TNF-α in LPS stimulated macrophage cell line has been related to transient increase in calcium (Watanabe *et al*., 1996) and decrease in TNF-α in vivo has been related to blocking of calcium ion channel (low calcium levels) (Hotchkiss *et al*.,1995). Recently, Zhou *et al* have showed LPS activating PKC-dependent pathway resulting in increased intracellular calcium and TNF-α production in LPS-stimulated rat peritoneal macrophages (Zhou *et al*., 2006). In parallel, it is likely that SRIF regulates LPS activated pathways via regulating calcium channels in RAW 264.7 cells, with a final consequence of inhibition of TNF-α release (Figure 4).

This study gives way to future work to study effect of SRIF-14 on release/secretion of other proinflammatory and anti-inflammatory cytokines in this cell

line; mechanism of cytokine regulation; and SRIF intracellular signaling pathway cross talk with LPS signaling pathway. This cell line has been used as a model cell line to delineate intricate intracellular signaling pathways and SRIF signaling pathway is one of the pathways to be unraveled. Our present work might work as an initiating point for such exhaustive study.

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