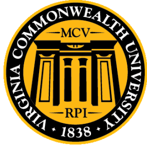

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Autocrine Effects of Catecholamines on Macrophage Release of Interleukin-6 (IL-6)

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science at Virginia Commonwealth University

By

Shaunta D. Poe

B.A in Biology

Mary Baldwin College, Staunton, VA

1999

Director: Jennifer K. Stewart, Ph.D.

Associate Professor, Department of Biology

Virginia Commonwealth University

Richmond, Virginia

May 2006

Acknowledgment

I would like to take the opportunity to thank the individuals who have supported me through this journey. First, I would like thank God for answering my prayers and allowing me to keep my sanity. Thanks to my Daddy, Alonza Poe for believing in me and teaching me to build mental strength and to my fiancé Kevin Williams for his encouragement. I thank my thesis committee members: Dr. Stenger, Dr. Conway, Dr. Ryan and Dr. McCoy. Thank you also to my lab partners: George Georges and Sienna Malubay for their tremendous assistance and laboratory humor. Finally, I would like to thank, my mentor, Dr. Stewart, for her patience, guidance, and confidence in me and most importantly for the "higgly piggly" stuff.

Table of Contents

Acknowledgment....ii

List of Figures....v

List of Abbreviations....vi

Abstract....vii

General Introduction....1

Interleukin 6....2

Catecholamines in Macrophages....3

Actions of Epinephrine and Norepinephrine on Macrophages....3

Methods and Materials....6

Primary Macrophage Recruitment....6

RAW 264.7 Macrophage-Like Cell Line....7

Effects of LPS and Adrenergic Antagonists or Agonists on IL-6....7

Measurement of IL-6....8

MTT Viability Assay....8

Data Analyses....9

Results....10

β_2-adrenergic modulation of IL-6....10

α_2-adrenergic modulation of IL-6....11

Discussion....................12

List of Reference....................16

Figures....................21

Vita....................29

List of Figures

Figure **Page**

1. Interactions of macrophage cytokines..2

2. Effects of LPS on extracellular IL-6 released by murine peritoneal macrophages.....21

3. Effects of LPS and the β_2 adrenergic antagonist ICI 118,551 on extracellular IL-6 released by murine peritoneal macrophages..22

4. Effects of LPS and the β_2 adrenergic antagonist ICI 118,551 on the extracellular IL-6 released by murine peritoneal macrophages..23

5. Effects of the β_2 adrenergic antagonist ICI 118,551 (ICI) on the LPS-stimulated IL-6 released by murine peritoneal macrophages..24

6. Effects of LPS on extracellular IL-6 produced by RAW 264.7 cell line..................25

7. Effects of norepinephrine (NE) and the β_2 adrenergic antagonist ICI 118,551 (ICI) on the LPS-stimulated IL-6 released by RAW 264.7 cell line..............................26

8. Effects of the β adrenergic agonist isoproteranol on the LPS stimulated IL-6 released by RAW 264.7 cell line..25

9. Effects of LPS and the α_2 adrenergic antagonists yohimbine (YOH) and RS79948 (RS) on LPS-stimulated IL-6 released by murine peritoneal macrophages..................27

List of Abbreviations

cAMP........cyclic adenosine monophophate

CFA........complete Freund's Adjuvant

EDTA........ethylenediaminetetraacetic acid

ELISA........enzyme-linked immunosorbent assay

IACUC........Institutional Animal Care and Use Committee

ICI........ICI 118,551

IL-1β........interleukin-1 beta

IL-6........interleukin-6

IL-8........interleukin-8

IL-10........interleukin-10

IL-12........interleukin-12

Iso........isoproteranol

LPS........lipopolysaccharide

NE........norepinephrine

PBS........phosphate buffered saline

TNF-α........tumor necrosis factor alpha

YOH........yohimbine

Abstract

AUTOCRINE EFFECTS OF CATECHOLAMINES ON MACROPHAGE RELEASE OF INTERLEUKIN-6

Shaunta D. Poe, B.A in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: Jennifer K. Stewart, Ph.D., Associate Professor, Department of Biology

Effects of norepinephrine (NE) on macrophage cytokine release are complex because the cells have both α_2 and β_2 adrenergic receptors, which mediate opposing actions. Furthermore, β_2 -adrenergic agonists are reported to have both stimulatory and inhibitory effects on interleukin-6 (IL-6). This study was designed to clarify the autocrine role of macrophage-derived NE on IL-6 production in activated peritoneal macrophages. Effects of NE on IL-6 production in the RAW264.7 macrophage cell-line also were investigated. Treatment of activated peritoneal macrophages with endotoxin, the α_2-adrenergic

antagonists yohimbine or RS79948 revealed that the α_2-adrenergic receptor mediates a stimulatory autocrine action of catecholamines on IL-6 production. When peritoneal macrophages were treated with the β_2 antagonist ICI-118,551 (ICI), there was both inhibition and stimulation of IL-6. Treatment of RAW264.7 macrophages with high and low concentrations of NE and various concentrations of ICI provided evidence that the concentration of NE determines whether the β_2-adrenergic receptor mediates stimulation or inhibition of IL-6 production.

Introduction

Macrophages are antigen-presenting cells and specialized phagocytes that are dispersed throughout the tissues of the body. They play an important role in host defense against infection (11). When an immune response is elicited, macrophages become activated by interferon gamma or bacterial lipopolysaccharide (LPS); this stimulates macrophage release of cytokines, which are signaling proteins. Macrophage cytokines include interleukins and tumor necrosis factor alpha (IL-1, IL-6, IL-8, IL-10, IL-12 and TNFα) (30). These cytokines mediate and regulate many immune responses. Actions of TNFα, IL-1β, and IL-12 are pro-inflammatory, whereas IL-10 has anti-inflammatory actions, and IL-6 functions are mixed. Furthermore, one cytokine can affect production and release of another cytokine. For example: the anti-inflammatory cytokine IL- 10 inhibits the release of IL-1β, and IL-12. IL-1β and TNFα, each facilitate production of the other and stimulate production of IL-6, which then has suppressive effects on TNFα expression(16; 28). Studies with IL-6 knockout mice indicate that IL-6 also inhibits macrophage production of IL-10 and promotes IL-12 production (2; 21). These cytokine interactions are illustrated in Figure 1.

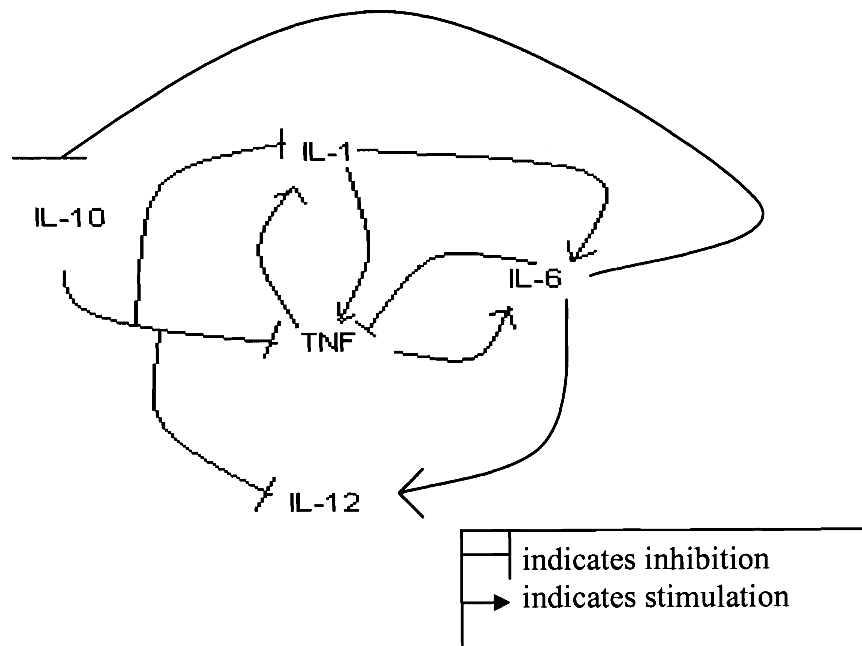


Figure 1: Interactions of macrophage cytokines.

Interleukin 6 (IL-6)

IL-6 is a 26-kD pleiotropic cytokine secreted by lymphoid and nonlymphoid cells. IL-6 functions in haematopoiesis; specifically in the regulation of T cell and B cell growth and differentiation (10; 17). It is released as a pro-inflammatory cytokine in response to infection, trauma, neoplasia and other cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF- α) (28). IL-6 also is known to have a key function as a mediator in inflammation during the acute-phase response (10; 15; 20; 27).

Catecholamines in Macrophages

More than twenty years ago Balter and Schwartz showed that macrophages take up the catecholamine norepinephrine (NE) (3). More recently, Scott Brown in our laboratory demonstrated that the RAW 264.7 macrophage cell line synthesizes norepinephrine and dopamine (4). Several studies now suggest that both mouse and human macrophages release NE (4; 18; 24). Furthermore, our laboratory and others have demonstrated that macrophage-derived norepinephrine has autocrine effects on macrophage release of cytokines (6; 9; 23; 24).

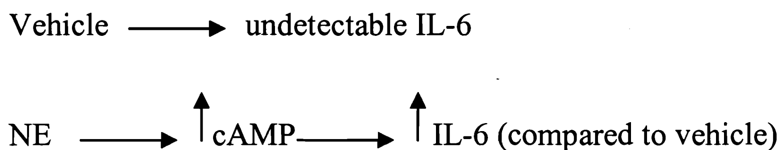
Actions of Epinephrine and Norepinephrine on Macrophages

Specific effects of the catecholamines epinephrine and norepinephrine are determined by adrenergic receptor activation. Macrophages express two adrenergic receptors, α_2 and β_2, which mediate different effects on synthesis and release of cytokines (1; 7; 8; 13). Previous studies indicate that the binding of NE to the β_2 adrenergic receptor inhibits the production of TNFα, IL-12, IL-1β and stimulates IL-10 production, whereas binding to the α_2 receptor has opposite effects on the production of these cytokines (6; 8; 13; 13).

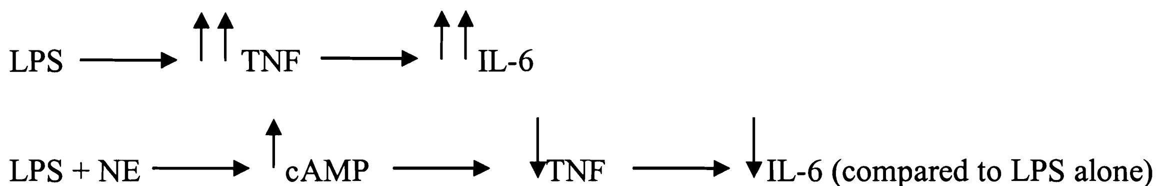
Adrenergic effects on IL-6 are unclear. β_2-adrenergic agonists are reported to have both stimulatory and inhibitory effects on IL-6 (20; 26; 31), and these effects are hypothesized to be dependent on the concentration of agonist and the concentration of TNFα that is present (20; 28). For example, in the absence of TNFα, norepinephrine

binding to the β_2 adrenergic receptor stimulates increased production of cyclic AMP (cAMP), which directly stimulates IL-6 production in several types of cells (27). Nevertheless, this stimulation of IL-6 is small compared to stimulation by TNFα, a pro-inflammatory cytokine that stimulates a large increase in IL-6 production. β_2-adrenergic agonists inhibit TNFα production and, therefore, inhibit TNF-stimulated IL-6. The chart below clarifies the current hypothesis of IL-6 regulation in the absence of signals that trigger pro-inflammatory cytokines, such as TNFα, and in the presence of pro-inflammatory stimuli, such as the bacterial endotoxin, lipopolysaccharide (LPS).

No Inflammatory Signals:



Pro-Inflammatory Signals:



As mentioned above, macrophages have both α_2 and β_2 adrenergic receptors, which mediate opposite effects on cAMP and opposite effects on TNFα (23). Based on the chart above, one would expect opposite effects of these adrenergic receptors on IL-6. If NE exerted equal but opposite effects on α_2 and β_2 receptors, any overall effect would be canceled out; however, low concentrations of NE (< 100 nM) usually stimulate only α_2-adrenergic receptors (13; 23), and at higher NE concentrations, effects on

β_2-adrenergic receptors are usually dominant. These concentration-dependent effects may be particularly complex in recruited peritoneal macrophages that release NE and are exposed *in vivo* to NE from other sources. Exposure of these cells to high concentrations of NE or epinenephrine in the animal prior to harvest of macrophages would be expected to down-regulate β_2-adrenergic receptors(14). The goal of this study was to investigate effects of adrenergic stimulation on IL-6 production in activated peritoneal macrophages and the RAW264.7 macrophage cell-line.

Methods and Materials

Primary Macrophage Recruitment

Female CBA/J mice, age 6-8 weeks, were obtained from Harlan (Indianapolis, IN) and housed in the animal facility of Virginia Commonwealth University under the guidelines of the University Institutional Animal Care and Use Committee (IACUC). All Animal protocols were approved by the IACUC.

A single intra-peritoneal injection (0.5 ml) of Complete Freund's Adjuvant (CFA), diluted 1:1 with Dulbecco's Phosphate-Buffered Saline (Sigma, St. Louis, MO), was used for recruitment of macrophages. Fourteen days following injection, the mice were euthanized with CO_2, and peritoneal exudates were harvested and washed twice with Hanks' balanced salt solution (Invitrogen, Carlsbad, CA). The cells were resuspended in complete RPMI (cRPMI), consisting of RPMI 1640 (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, 1% minimal essential medium vitamins, 100 units/ml penicillin and 100 μg/ml streptomycin, and allowed to adhere for 4 hours. Non-adherent cells were discarded, and adherent cells were washed with cRPMI and incubated at 37° C in 5% CO_2.

RAW 264.7 Macrophage Cell Line

Murine macrophages from the RAW264.7 cell line (ATTC, Rockville, MD) were cultured in cRPMI. Cell cultures were maintained at 37° C in 5% CO_2 in 75 cm^2 tissue culture flasks (Costar Brand, Fisher Scientific, Suwanee, GA). The cultures were sub-cultured twice weekly.

Effects of LPS and Adrenergic Antagonists or Agonists on IL-6

Primary exudate cells (3.0 x 10^6) or RAW264.7 macrophages (0.75 x 10^6) were added to each well of a 24-well tissue culture plate and incubated for 24 hrs at 37°C in 5% CO_2 to allow for macrophage adherence. Approximately 50% of the exudate cells were adherent, and the cells did not proliferate in culture. The RAWS264.7 cells doubled within 24 hours; therefore, the final concentration of all macrophages was (1.5 x 10^6) cells per well. The cells were washed twice with warm (37°C) phosphate buffered saline (PBS), pH 7.4, then incubated for 24 hrs in 500 µl of serum-free RPMI 1640 medium with and without LPS (*E. coli* – serotype 055:B5, Sigma, St. Louis, MO) and adrenergic agonists or antagonists, including ICI 118,551, RS79948 and isoproterenol hydrochloride from Tocris, (Ellisville, MO) and yohimbine from Sigma-RBI, (St. Louis, MO) at concentrations indicated in the figure legends. Extracellular fluid was removed and centrifuged at 500 xg for 10 minutes at 5°C to remove cellular debris, and the supernatant was frozen at -20°C until assayed for IL-6. The remaining macrophage monolayer within each well was washed once with RPMI 1640 and twice with PBS. All PBS was removed, and the monolayer was lysed for subsequent measurement of total cellular protein. The

cell monolayer was lysed with 150 μl of ice cold buffer containing 0.05 M Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, 1.0% Triton-X 100, 2 μg/ml leupeptin, 1 μg/ml aprotinin and 0.2 mM phenylmethylsulfonylfluoride. Protein concentrations of the lysates were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and read at 590 nm in the μQuant Universal Microplate reader purchased from Bio-Tek Instruments (Winooski, VT).

Measurement of IL-6

Extracellular IL-6 concentrations were assayed with the OptEIA Multi Component ELISA Set for Mouse IL-6 from BD Biosciences, (San Diego, CA). The assays were performed in accordance with the manufacturer's instructions. The plates were read at 450 and 570 nm with a μQuant Universal Microplate Spectrophotometer. IL-6 concentrations were calculated with the manufacturer's KC4 software.

MTT Viability Assay

This MTT assay protocol is an adaptation of Mosmann (19), Carmichael et al. (5), and Pozzolini et al. (22). RAW264.7 macrophages were plated in 96-well tissue culture treated plates (Costar) at a density of 2.0×10^5 cells/well. The cells were treated with vehicle (media alone), LPS (30 ng/ml), ICI 118,551(10 μM), or 10% DMSO (a concentration that kills cells) and incubated at 37°C in 5% CO_2 for 24 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, (St. Louis, MO) was added to each well at a final concentration of 0.5 mg/ml and incubated for an additional 4 h. After 4 h, all media was aspirated and the cells were lysed with a buffer containing sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, MO) dissolved in

50% N, N-dimethyl-formamide (DMF; Sigma-Aldrich, St. Louis, MO) and incubated overnight at 37°C. The plate was read at 570 nm with the μQuant Universal Microplate Spectrophotometer. Absorbance was proportional to the number of viable cells.

Data Analyses

Analysis of variance (ANOVA) and the Neuman-Keuls test were used to compare effects of LPS and LPS plus ICI adrenergic antagonists on macrophage production of IL-6. The analyses were performed with Prism software (Graphpad, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

Results

β_2-adrenergic modulation of IL-6

Recruited peritoneal macrophages stimulated with 10 ng/ml and 30 ng/ml of LPS consistently released elevated levels of IL-6 over that observed with vehicle (figure 2). We have previously shown that norepinephrine (NE) released by these cells has autocrine effects on the production of IL-1β (9). To test whether LPS-stimulated IL-6 is modulated by autocrine actions of NE on the β_2-adrenergic receptor, the following experiment was carried out with the β_2 antagonist ICI 118,551(ICI). Macrophages were stimulated with 30 ng/ml of LPS, and the β_2 receptors were blocked with ICI. There was an increase in IL-6 production in cells treated with ICI plus LPS over that observed with LPS alone (Figure 3). In contrast, there was a significant decrease in IL-6 compared to LPS alone when cells were stimulated with 10 ng/ml of LPS plus ICI (Figure 4A). Although these findings initially suggested opposite effects of the β_2 antagonists at low and high concentrations of LPS, repeated studies showed inconsistent modulation of IL-6 release that was not dependent on the concentration of LPS (Figure 4B). Subsequently peritoneal macrophages were treated with LPS and varying concentrations of ICI. There was a significant inhibition of IL-6 production with 0.5 μM and 1.0 μM concentrations of ICI

(Figure 5). Although these findings seem to suggest that low and high concentrations of ICI exert opposite effects on IL-6 production, repeated studies again revealed inconsistent modulation of IL-6 by both low and high concentrations of ICI (Figure 5B). An MTT assay of cell viability revealed no effects of ICI on cell numbers that could account for these results (data not shown).

Peritoneal macrophages take up NE from extracellular fluids (3) and exposure to NE from various sources *in vivo* may influence the amount of NE released by these cells. The extracellular NE concentration is expected to affect the actions of the β_2 antagonist ICI, but it was not possible to measure extracellular NE released by only 3 x 10^6 macrophages in a 24 well culture plate. Because RAW264.7 macrophages do not release significant quantities of NE until 48 h after LPS (4), these cells were used to determine the effects of exogenous NE and ICI on the release of IL-6 that peaks 24 h after LPS stimulation. Figure 6 shows the effects of various concentrations of LPS on the RAW264.7 macrophage release of IL-6. Similar to the peritoneal macrophages, there was a consistent elevation in the production of IL-6 with increasing amounts of LPS. RAW264.7 cells were then treated with exogenous NE to determine the effects of NE on the LPS-stimulated release of IL-6, and in selected experiments, β_2-adrenergic receptors were blocked with ICI. When cells were treated with 1 μM NE and LPS, there was a significant decrease in IL-6 production compared to LPS alone (Figure 7A) and blocking the β_2 receptor with ICI increased IL-6 production. This finding suggests that the β_2-adrenergic receptor mediates inhibitory effects of 1 μM NE on IL-6. When cells were treated with 0.01 μM NE and LPS, there was an increase in IL-6 production compared to

LPS alone (Figure 7B). This finding is consistent with observations that low concentrations of NE bind to α_2 receptors and stimulate production of TNFα (23). However, blocking the β_2 receptor in RAW264.7 cells with ICI appeared to reverse this effect suggesting that the β_2 receptor also mediates stimulatory effects of low concentrations of NE (Figure 7B).

RAW264.7 cells were then treated with LPS and the non-selective β agonist isoproterenol. When the cells were given 1 μM Isoproterenol or 10 μM Isoproterenol there was a significant decrease in the release of IL-6 (Figure 8). This finding is consistent with the results shown in figure 7A, suggesting that β agonists at 1 μM inhibit IL-6 production.

α_2- adrenergic modulation of IL-6

To test the autocrine effects of NE on IL-6 that are mediated by the α_2-adrenergic receptor, peritoneal macrophages were treated with LPS and the α_2-adrenergic antagonist yohimbine or RS79948. Both yohimbine and RS79948 cells decreased IL-6 production, (Figure 9), which suggest the α_2-adrenergic receptor mediates increased production of IL-6.

Discussion

Effects of NE and epinephrine on macrophage cytokine release are complex because the cells have both α_2 and β_2 adrenergic receptors, and these receptors usually mediate opposing actions. The actions of adrenergic receptor modulation of IL-6 are controversial and difficult to interpret because β_2 -adrenergic agonists are reported to have either stimulatory or inhibitory effects on IL-6, depending on the duration and concentration of the agonist, the effects on TNFα, which modulates IL-6 transcription, and interactions with the cAMP signal transduction pathway (15; 20). Previous studies also indicate that TNFα mediates inhibitory effects of the β-adrenergic receptor on IL-6 production (27). A few studies have also suggested that α-adrenergic agonists may influence macrophage IL-6 production (25; 27; 28), but no clear pattern of modulation has emerged. This study was designed to help clarify the autocrine role of macrophage derived NE on IL-6 production.

In this study, treatment of peritoneal macrophages with LPS plus α_2-adrenergic antagonists yohimbine or RS79948 over 24 hour revealed that the α_2-adrenergic receptor mediates a stimulatory autocrine action of catecholamines on IL-6 production (9). These findings are consistent with previous evidence that this receptor mediates stimulatory autocrine effects on TNFα and IL-1β (23; 24). However, when peritoneal macrophages were treated with the β_2 antagonist ICI, there was both inhibition and stimulation of IL-6.

Treatment of RAW264.7 macrophages with high (1 μM) and low concentrations of NE and various concentrations of ICI suggested that effects of ICI depend on the concentration of NE. Our observations that ICI blocked the inhibitory effects of the higher concentration of NE are consistent with β_2-adrenergic inhibition of TNFα production (25) which should inhibit production of IL-6. It is surprising, however, to find that the β_2 antagonist ICI blocked stimulatory effects of a low concentration of NE. This concentration of NE was expected to bind only to α_2 -adrenergic receptors (12). It is conceivable (although unlikely) that binding of 0.01 μM NE to the β_2-adrenergic receptor increased cAMP sufficiently to directly stimulate IL-6 production (28), but not enough to decrease TNFα, which would decrease IL-6 production. Alternatively, the macrophage β_2-adrenergic receptor may mediate effects on other unidentified pathways that regulate IL-6 production.

It is important to recall that release of IL-6 may be directly mediated by TNFα or directly by LPS stimulation (27). It is known that TNFα is released early during an inflammatory response and reduced in the presence of NE. An increase in intracellular cAMP occurs after β-adrenergic receptor stimulation, which decreases the levels of TNFα, which then decreases the levels of IL-6. In the absence of TNFα, however, binding of NE to β_2-adrenergic receptors results in an increase in cAMP and an increase in IL-6 (8; 28).

Other laboratories also have reported opposite effects of different concentrations of β_2 agonists on IL-6. High concentrations of the β_2 agonist terbutaline (10^{-6} M) significantly increase IL-6 production in renal macrophages; whereas lower concentrations (10^{-8} M)

decrease IL-6 production (20; 28). However, these concentration effects are opposite those we observed with NE. Treatment of the human macrophage cell line, U-937, with the β agonist clenbuterol decreases LPS-stimulated IL-6 after 6 hours (15), which is consistent with our observation that isoproterenol decreases IL-6 production in RAW264.7 macrophages. In other cells, such as rat thymic epithelial cells, in the absence of LPS or inflammatory cytokines the β agonist isoproterenol increases IL-6 production in a dose dependent manner (31). Also, IL-6 production *in-vivo* is increased by isoproterenol (29).

In summary, the results of this study suggest that catecholamines released by peritoneal macrophages have stimulatory autocrine effects on IL-6 production that is mediated by α_2-adrenergic receptors and both stimulatory and inhibitory autocrine effects mediated by β_2-adrenergic receptors. The inhibitory effects are postulated to require relatively high concentrations of NE (~ 1 μM), based on concentration-response studies with RAW264.7 macrophages.

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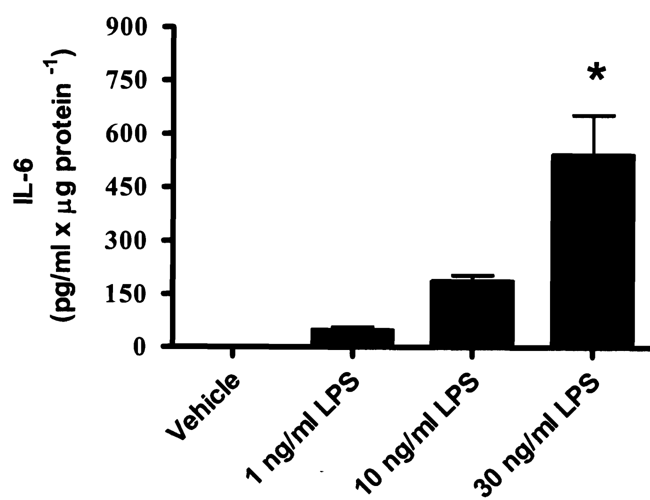

Figure 2. Effects of LPS on extracellular IL-6 released by murine peritoneal macrophages. Cells were plated and treated with vehicle (medium) or the concentrations of LPS shown below the bars. Macrophages were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. * $P \leq 0.001$ compared to vehicle by ANOVA and the Newman-Keuls test. Bars represent mean $\pm$ SE of 3-4 replicates.

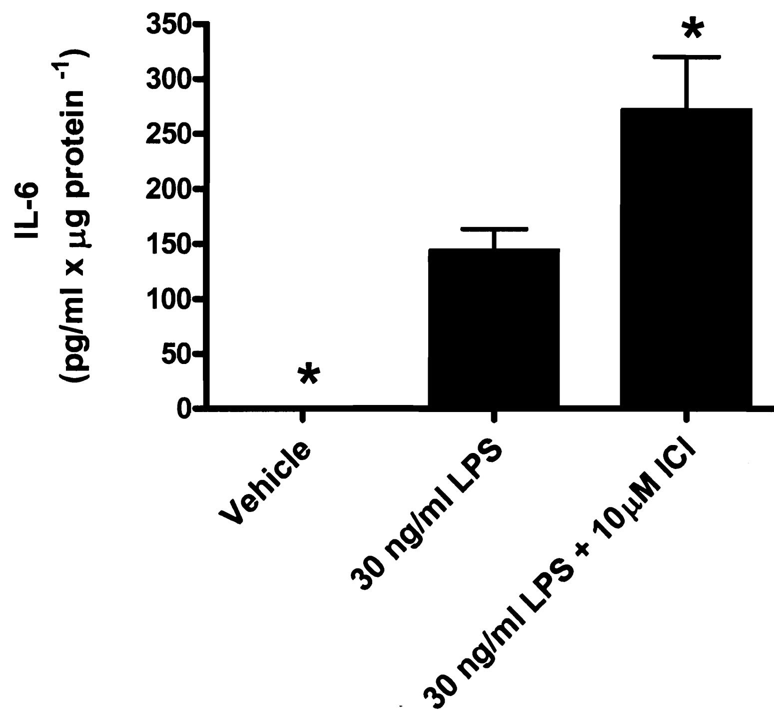

Figure 3. Effects of LPS and the β_2 adrenergic antagonist ICI 118,551 on extracellular IL-6 released by murine peritoneal macrophages. Cells were plated and treated with vehicle (medium), the concentration of LPS shown below the bar, or LPS + 10 µM concentration of antagonist ICI 118,551. Macrophages were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. *P ≤ 0.05 compared to the same concentration of LPS alone by ANOVA the Newman-Keuls test. Bars represent mean ± SE of 3-5 replicates.

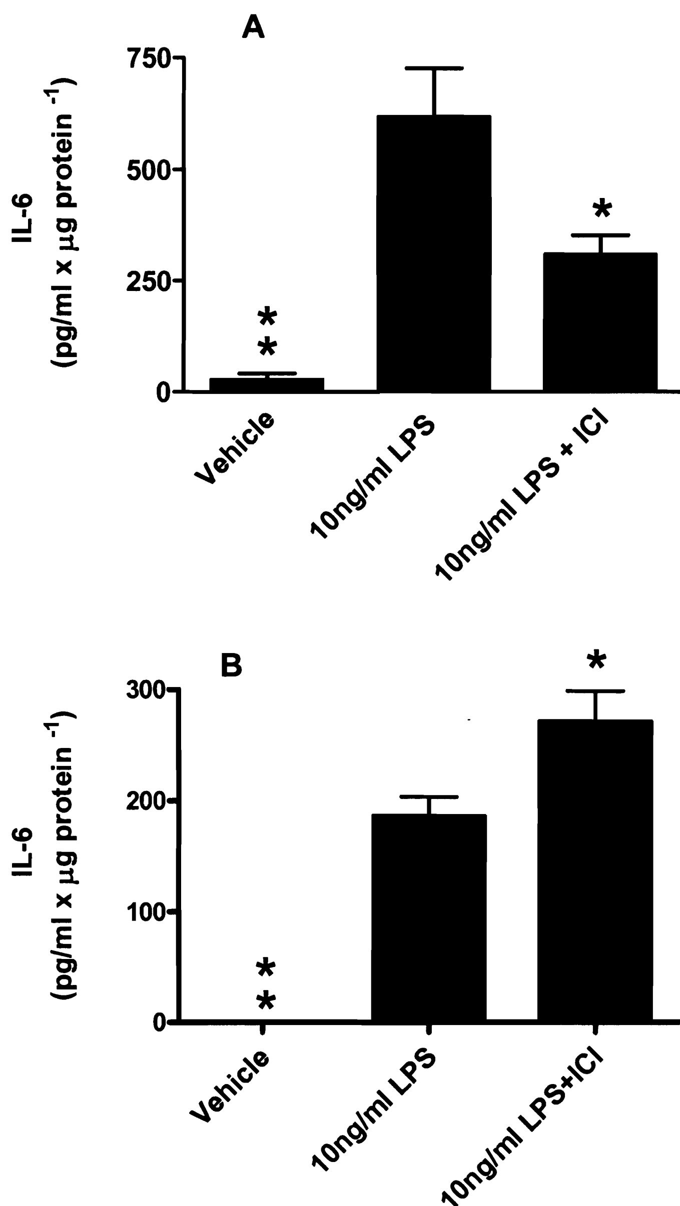

Figure 4. Effects of the β_2 adrenergic antagonist ICI 118,551 (ICI) on LPS-stimulated IL-6 released by murine peritoneal macrophages. A and B represent duplicate experiments. Cells were plated and treated with vehicle (medium), the concentrations of LPS shown below the bar, or LPS + 10 µM ICI 118,551. Macrophages were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. $^{*}P \leq 0.01$ and $^{**}P \leq 0.001$ compared to the same concentration of LPS alone by ANOVA and the Newman-Keuls test. Bars represent mean ± SE of 3-4 replicates.

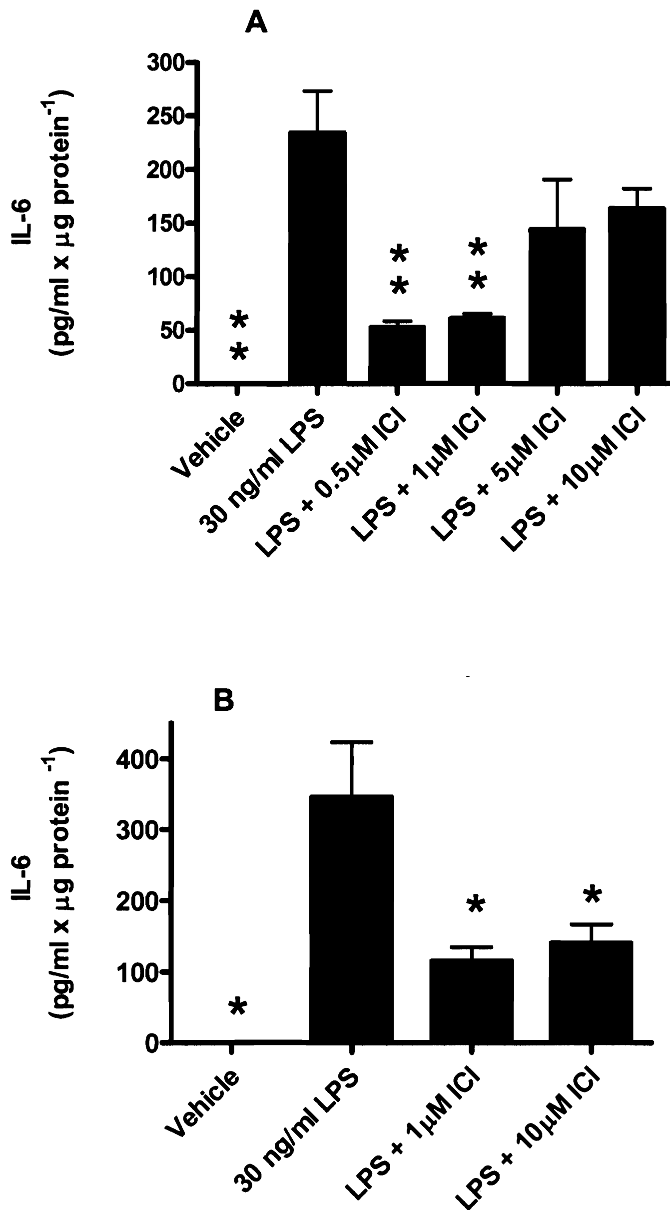

Figure 5. Effects of the β_2 adrenergic antagonist ICI 118,551 (ICI) on the LPS-stimulated IL-6 released by murine peritoneal macrophages. A. Cells were plated and treated with vehicle (medium), concentrations of LPS shown below the bars or LPS + the concentration of the antagonist shown below the bars. Macrophages were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. B. Repeat of experiment A with two concentrations of ICI. *$P \leq 0.01$ and **$P \leq 0.001$ compared to LPS alone by ANOVA and the Newman-Keuls test. Bars represent mean ± SE of 4-5 replicates.

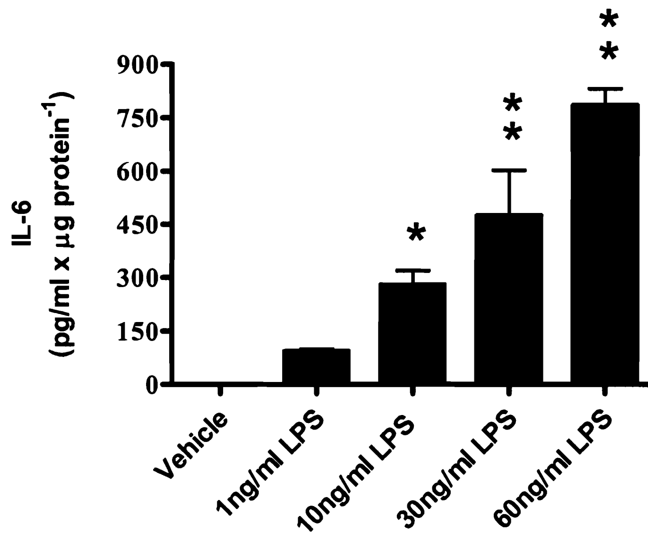

Figure 6. Effects of LPS on extracellular IL-6 produced by RAW 264.7 cell line. Cells were plated and treated with vehicle (medium) or the concentrations of LPS shown below the bars. RAW 264.7 cells were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. $*P \leq 0.05$ and $**P \leq 0.001$ compared to vehicle by ANOVA and the Newman-Keuls test. Bars represent mean ± SE of 3-4 replicates.

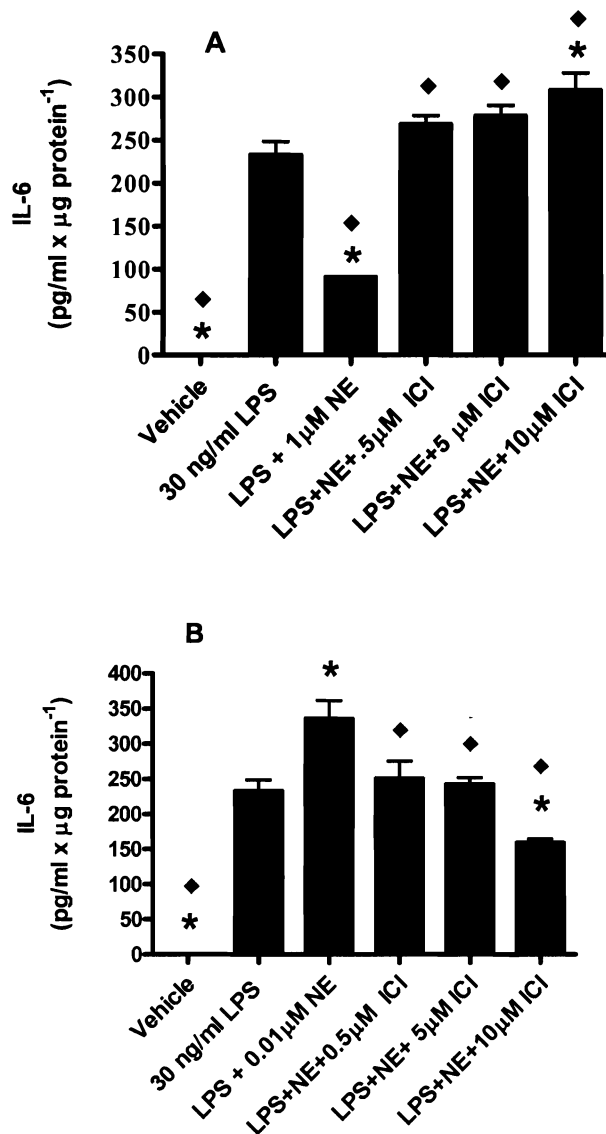

Figure 7. Effects of Norepinephrine (NE) and the β_2 adrenergic antagonist ICI 118,551 (ICI) on the LPS-stimulated IL-6 released by RAW 264.7 cell line.
A. NE = 1 ⊠M; B. NE = 0.01 ⊠M. Cells were plated and treated with vehicle (medium), 1uM NE, .01 uM NE or LPS + NE + the concentrations of ICI 118,551 shown below the bars. RAW 264.7 cells were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. *$P \leq 0.01$ compared to LPS alone and ♦$P \leq 0.01$ compared to LPS + NE by ANOVA and the Newman-Keuls test. Bars represent mean ± SE of 4-5 replicates.

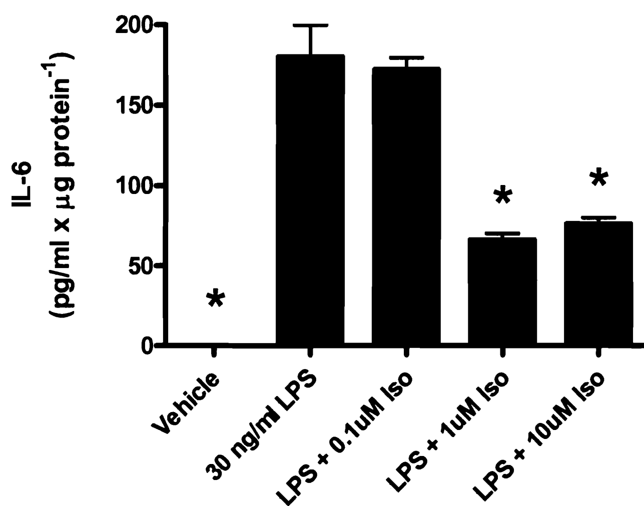

Figure 8. Effects of the β adrenergic agonist Isoproteranol on the LPS stimulated IL-6 released by RAW 264.7 cell line. Cells were plated and treated with vehicle (medium), LPS or LPS + the concentrations of Isoproteranol shown below the bars. RAW 264.7 cells were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. *P ≤ 0.001 compared to LPS alone by ANOVA and the Newman-Keuls test. Bars represent mean ± SE of 5 replicates.

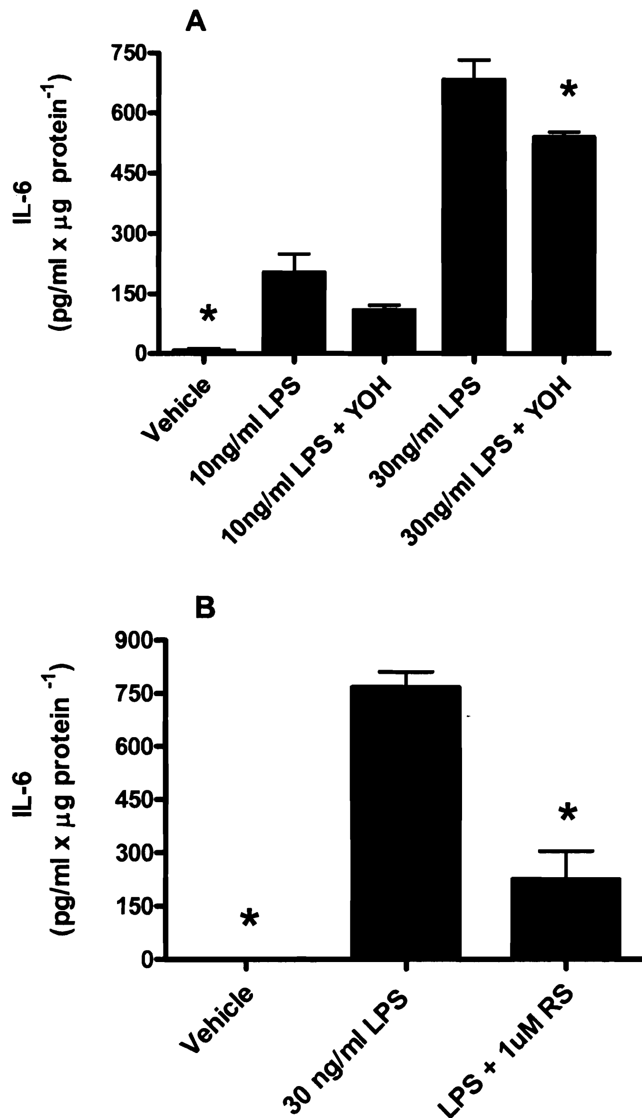

Figure 9. Effects of LPS and the α_2 adrenergic antagonist yohimbine (YOH) and RS79948 (RS) on LPS-stimulated IL-6 released by murine peritoneal macrophages. A. Experiments with Yoh; B. Experiments with RS. Cells were plated and treated with vehicle (medium), the concentrations of LPS shown below the bar, LPS + 10 µM yohimbine or LPS + 1 µM RS79948. Macrophages were incubated for 24 h after treatment, and extracellular IL-6 was measured with an ELISA. *$P \leq 0.01$ compared to the corresponding concentration of LPS alone by ANOVA and the Newman-Keuls test. Bars represent mean ± SE of 3-5 replicates.

Vita

Shaunta Danielle Poe was born on March 11, 1977 in Lynchburg, Virginia. She graduated from E.C Glass High School in 1995 before receiving her Bachelor of Arts degree in Biological Sciences from Mary Baldwin College in 1999. In 2004, she entered the Master of Science program in Biology at Virginia Commonwealth University, Richmond, Virginia. After graduation, she plans to pursue a career in medicine.