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Adrenergic receptor expression on RAW264.7 macrophages

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Adrenergic Receptor Expression on RAW264.7 Macrophages

by

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Macrophages are phagocytic cells involved in the immune response. They are derived from circulating monocytes which differentiate into macrophages in the tissues. Despite small phenotypic variations due to the different microenvironments of the tissues, all macrophages act as the first line of defense against invading pathogens.[1]

Macrophages primarily combat infection as a key component of innate immunity. They are involved in both the initiation and maintenance of the inflammatory response, as well as the initiation of the adaptive immune response.[1,15] In innate immunity, macrophages establish the inflammatory response.[1] Specifically, these cells phagocytose bound pathogens to destroy them in phagolysosomes, and produce compounds, such as nitric oxide (NO) and reactive oxygen intermediates (ROS), to aid in destruction of pathogens. Macrophages also produce and secrete pro-inflammatory cytokines after being activated. Release of cytokines such as tumor necrosis factor-α (TNF) or interleukin-6 (IL-6) enhances activation of phagocytes through autocrine and paracrine signaling, and promotes recruitment of additional leukocytes to the site of infection. TNF is also capable of directly killing extracellular microbes.[1,12,17] Through these innate immune response activities the macrophages generally resist further replication of the pathogen and reduce the total number of pathogens in the body.[3]

Before a macrophage can perform these immune functions, it must be activated. Activation may occur via binding of pro-inflammatory cytokines to cell surface receptors or by recognition of pathogen-associated molecular patterns (PAMPs). Lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, is a common macrophage activating PAMP. LPS binds to CD14 and Toll-like Receptor 4 (TLR4) on the macrophage surface, stimulating a cascade that results in the phosphorylation and subsequent degradation of cytosolic IκB. NF-κB is thus released from IκB to enter the cell nucleus, and promote the transcription of pro-inflammatory genes. In response to the LPS signaling pathway, NF-κB promotes the transcription of inducible nitric oxide synthase (iNOS), superoxide-producing enzymes and pro-inflammatory cytokines to combat the infection.[1,3,9,12] Other PAMPs stimulate additional signaling cascades to activate transcription factors to start production of anti-microbial genes.[1,3]

Catecholamines are small monoamines, such as NE which has been shown to be released by the sympathetic nervous system during stress responses.[5,15,19] Although catecholamines are predominantly derived from the neuroendocrine system, recent work has shown that NE and dopamine are also synthesized by activated macrophages.[2,4] Catecholamines mediate their effects by binding to the adrenergic receptors expressed on macrophages. Adrenergic receptors are seven-transmembrane G-protein coupled receptors with cytoplasmic tails that interact with G_s proteins. The activation of G_s proteins initiates signaling cascades that alter the immunological activity level of activated macrophages in response to PAMPs. Primarily, stimulation of ARs induces changes in NO and cytokine production correlating with the identity and concentration of catecholamine to which the macrophages were exposed.[5,15,19]

Adrenergic receptors can be divided into two major subgroups: α-AR and β-AR. Generally, treatment of LPS-activated macrophages with β-ARs agonists has been demonstrated to significantly reduce NO and inflammatory cytokine production.[14,17] This effect can be abrogated by the addition of β-AR antagonists, indicating that the decrease is specific to stimulation of the β-AR signaling cascade by catecholamines.[4] Kizaki et al. indicates that this decrease in immunological activity is due to reduced release of NF-κB from IκB in the treated macrophage's cytoplasm, preventing transcription of pro-inflammatory genes.[11]

However, a small but significant body of primary literature has shown that stimulation of the β-ARs of activated macrophages increases their immunological activity. A study by Szelenyi et al. showed that treatment with isoproterenol, a β-AR agonist, increased TNF production by macrophages activated with phorbol myristyl acetate.[17] It has also been demonstrated that the enhancing effect of NE on macrophage production of NO can be abrogated by the addition of propranolol, a β-AR antagonist.[3]

There is also some disagreement in the primary literature regarding the role of α-ARs in regards to regulating immunological activity of activated macrophages. In general, treatment with α-AR agonists has been demonstrated to enhance the functions of activated macrophages. Stimulation of α-ARs by clonidine during *Mycobacterium avium* or *Toxoplasma gondii* infections significantly increases macrophage resistance to pathogen growth.[6,19] Likewise, UK-14304 (an α-AR agonist) and low concentrations of NE have been shown to enhance TNF production. This effect appears to be mediated at the transcriptional level, as increases in TNF mRNA are seen in activated macrophages treated with NE. Antagonism of α-ARs with yohimbine has been shown to significantly decrease the production of TNF in response to α-AR stimulation with a constant level of agonist, suggesting that the enhancement is mediated exclusively by stimulation of the α-ARs.[9,15] However, another study found that stimulation of α-ARs with NE suppressed phagocytosis by macrophages.[7] Clonidine treatment has also been shown to have no effect on NO production by activated macrophages, leading to the hypothesis that α-ARs do not play a regulatory role in macrophages.[14] The discrepancies in these results clearly point to a need to increase understanding of the role of α-AR signaling in macrophages.

Previous studies have generally demonstrated a distinctive pattern of regulation of primary macrophages by adrenergic receptor stimulation. However, a complete characterization of the regulation of RAW264.7 murine macrophages, an important cell culture model, has yet to be performed. Additionally, a small but significant subset of current primary literature contradicts the generally-accepted model of AR-mediated regulation of macrophages. These functional differences may be due to changes in AR surface expression patterns in response to LPS activation and/or stimulation by catecholamines, but little analyses has been performed in this area. Therefore, further study is necessary to elucidate the roles and expression patters of ARs on macrophages during an immune response.[10,11]

In this study, AR surface expression patterns and regulation via AR-catecholamine interactions were characterized. Macrophage function was shown to be regulated by catecholamines through both α- and β-ARs, as evidenced by corresponding alterations in cytokine production. Treatment of activated macrophages with NE, a general catecholamine, or fomoterol, a β-AR agonist, produced significant decreases in TNF and IL-6 secretion. The effects of clonidine, an α-AR agonist, produced less consistent results, but clonidine-mediated enhancement of cytokine secretion appears to be mediated by α-AR signaling. LPS was shown to noticeably change the surface expression of both types of ARs in RAW264.7 macrophages. However, NE only influenced α-AR signaling. This data adds to the current model of neuroendocrine regulation of macrophage-mediated immunity, specifically presenting functional roles for α- and β-ARs.

Materials and Methods

RAW264.7 Cell Culture

The RAW264.7 murine macrophage cell line was used to model macrophage activity. Cells were stored in liquid nitrogen until needed. After thawing, RAW264.7 macrophages were cultured in RPMI-1640 complete media supplemented with 10% heat-inactivated fetal calf serum, 1.5% sodium bicarbonate, 25 mM HEPES buffer, 1% minimal essential medium vitamins, 1% glutamine, 1% nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in T_{25}-cm^2 or T_{75}-cm2 tissue culture flasks in a humidified incubator at 37°C, 5% CO_2. Fresh media was added and cells were sub-cultured as needed to maintain health.

Sandwich Enzyme-Linked Immunosorbent Assays

RAW264.7 murine macrophage cells were cultured to confluency in T_{75}-cm^2 flasks. For experiments, cells were seeded into the wells of a 12-well tissue culture plate at a density of 1.5 x 10^6 cells per well. Macrophages were treated with 5μM, 2.5μM, 500nM and 50nM clonidine, or 1μM, 500nM, 100nM and 10nM fomoterol (FOM), or 1μM, 500nM, 100nM and 10nM NE. Additional studies were performed by treating cells treated with RS79948 (500 nM), an α-adrenergic receptor antagonist, for 30minutes (37°C, 5% CO_2) prior to the addition of 5μM, 2.5μM, 500nM or 50nM clonidine. Control wells were left untreated and unactivated. All treatment groups were then incubated for 30 minutes (37°C, 5% CO_2) before 30 ng/ml or 10 ng/ml LPS was added to the media to activate the macrophages. The cells were incubated (37°C, 5% CO_2) for the appropriate times (TNF studies: 4 hours; IL-6 studies: 21 hours), then supernatants were harvested from each well and centrifuged at 11,000 rpm for 5 minutes. The pelleted debris was discarded, and clean microfuge tubes of supernatants were frozen at -20°C until analysis.

An OptEIA Mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) was used to measure the quantity of TNF secreted by each treatment group. The ELISA was performed according to the manufacturer's instructions. A 96-well microtiter plate was coated with 1:250 dilution of TNF Capture Antibody, and incubated overnight at 4°C. The wells were then washed three times with 1x Wash Buffer, and blocked with Assay Diluent for 1

hour at room temperature. The wells were washed three times after the incubation. An aliquot of each sample was added to wells in triplicate, and a standard curve of purified TNF (1000 pg/ml to 15.6 pg/ml) was generated. The plate was incubated for 2 hours at room temperature, and then washed five times. A 1:250 dilution of TNF Detection Antibody was added to each well for 1 hour at room temperature. After washing the wells five times, a 1:250 dilution of Streptavidin-HRP secondary antibody was added to the wells. After a 30 minute incubation, the wells were washed seven times with a 30-60 second soak in 1x Wash Buffer between each wash. TMB substrate was then added to each well, and the microtiter plate was incubated in the dark for 30 minutes. Stop solution was added to the wells, and the microtiter plate was read with a plate reader at 450 nm with a correction reading at 570 nm. TNF concentrations in each treatment group were calculated according to the absorbencies of the TNF standard curve.

An OptEIA Mouse IL-6 ELISA kit (BD Biosciences) was used to quantify the amount of IL-6 secreted by each treatment group. This kit followed the same protocol as the TNF-α ELISA Kit with a few notable changes. All antibodies used were specific for the IL-6 cytokine, and the standard curve was prepared using dilutions of purified IL-6 (1000 pg/ml to 15.6 pg/ml). Additionally, the Detection Antibody and Strepavidin-HRP were added in the same step.

Statistical Analysis

Concentrations of TNF and IL-6 were calculated from their respective standard curves. An ANOVA with a Tukey's Analysis using a $p<0.05$ was used to calculate significant changes between sample treatments. All statistical analyses were performed with Graph Pad Prism software.

Immunohistochemistry

RAW264.7 macrophages were cultured to confluency as previously described. Equal quantities of cells were plated onto sterile cover slips placed in the wells of a 12-well tissue culture plate. The cells were incubated (37°C, 5% CO_2) for 2-4 hours, then non-adherent cells were removed by washing with 1x PBS. Some experimental samples were treated with 1μM NE, and incubated for 30 minutes at 37°C, 5% CO_2. All experimental samples were activated with 100 ng/ml LPS. Control samples were not treated or activated. After a 4 hour incubation (37°C, 5% CO_2) cells were fixed with glyoxal (37°C, 15 minutes). Media was removed with three washes using 1x PBS, and cover slips were moved into a clean 12-well plate. The cells were then blocked with a solution of 1% powdered milk in 1x PBS for 30 minutes at room temperature with gentle swirling. The cells were then washed three times, and incubated with either 1:25 mouse anti-α_{2B} adrenergic receptor antibody (Santa Cruz Biotechnology, Inc., sc-1479 or 1:50 mouse anti-β_2 adrenergic receptor antibody (Santa Cruz Biotechnology, Inc., sc-570) in blocking solution for 30 minutes at room temperature. After three more washes, a 1:100 dilution of anti-mouse AlexaFluor 594 or 647 secondary antibody (Invitrogen) in blocking solution was added to the corresponding wells. After a 30 minute incubation in the dark, the cover slips were washed

three times. The cover slips were mounted on slides using Prolong Gold Anti-Fade Mounting Medium, and stored overnight at 4°C. Slides were sealed with nail polish and stored in the dark at 4°C until analyzed. Samples with no antibodies, only primary antibody, only secondary antibody, or serum with secondary antibody were prepared as controls to demonstrate the specificity of the antibodies used.

Confocal Microscopy

Slides were viewed using a Leica SP2 Laser Scanning Confocal Microscope, at excitation wavelengths of 561nm and 633nm for AlexaFluor 594 and 647 samples, respectively. Emission was collected from 600 to 670nm for samples with AlexaFluor 594, and 650 to 750nm for AlexaFluor 647. Gain and offset were set to the same levels for all collected images. Transmitted light images were also collected from the confocal microscope. Leica Confocal Software was used to collect images, and figures were prepared with Adobe Photoshop CS3.

Results

Treatment with high concentrations of NE reduces TNF secretion by RAW264.7 macrophages

Macrophages produce pro-inflammatory cytokines to protect the host from bacterial infections. The secretion of these inflammatory mediators is tightly regulated, such that cytokines are only secreted in response to recognition of an on-going infection. This secretion can be used in studies as a measure correlating to overall immunological activity level of activated macrophages.[1] The secretion of cytokines has also been shown to be regulated by signaling via ARs in activated macrophages, and can thus be used as a measure of the effects of catecholamine treatment.[3,14,17]

To study the ability of catecholamines to regulate macrophage function, we examined the effect of treatment with the catecholamine NE on TNF production by activated RAW264.7 macrophages. A sandwich ELISA was used to measure the amount of TNF produced by the LPS-activated macrophages pre-treated with NE. Analysis demonstrated that activation with LPS elicits dose-dependent secretion of TNF from RAW264.7 macrophages. At both concentrations of LPS, pre-treatment with 1μM or 500 nM NE results in a significant decrease in the amount of TNF secreted. Macrophages activated with 30 ng/ml LPS still show a significant decrease in TNF secretion when treated with 100 nM NE, but the same NE concentration in the presence of 10 ng/ml LPS does not alter TNF secretion from baseline levels. At either LPS concentration, addition of 10 nM NE has no effect on the production of TNF by activated macrophages (Figure 1). As NE is known to bind both α- and β-ARs, it was expected that treatment would alter cytokine secretion.[16] The reduction of TNF levels in response to treatment with high concentrations of NE suggests that the β-AR signaling cascade was strongly activated. However, low concentrations of NE did not alter TNF production so these studies do not provide evidence for α-AR expression or function on RAW264.7 macrophages. Given these results, further

research was needed to study the expression pattern of both types of ARs on RAW264.7 macrophages.

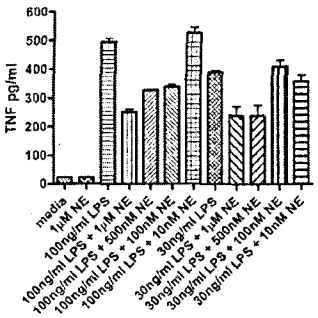

Figure 1. Concentrations of TNF produced in response to NE treatment of activated RAW264.7 macrophages. RAW264.7 macrophages were plated into 12-well tissue culture plates at a density of 1.5×10^6 cells/well. Cells were then treated with 1 μM, 500 nM, 100 nM or 10 nM NE for 30 minutes and then activated with 30 ng/ml or 10 ng/ml LPS. Cells were incubated for 4 hours (37°C, 5%CO_2) before supernatants were harvested. The concentration of TNF in each sample was quantified using a TNF sandwich ELISA. Statistical analysis was performed using Graph Pad Prism software.

Treatment with the β-adrenergic receptor agonist fomoterol reduces cytokine secretion by RAW264.7 macrophages

The inhibitory effect of NE on TNF production is known to be mediated by stimulation of β-ARs on RAW264.7 macrophages.[3] To better understand the role of β-ARs in macrophage activity, the macrophages were exposed to the specific β-AR agonist fomoterol (FOM) prior to macrophage activation. After a 4 or 21 hour incubation period, TNF and IL-6 secretion, respectively, was measured using an ELISA specific to each cytokine. ELISA analysis showed that treatment with FOM significantly reduces the amount of both IL-6 and TNF secreted (Figure 2). A trend toward dose-dependent inhibition of IL-6 secretion is apparent from these studies (Figure 2A and 2B). However, secreted IL-6 levels do not return to baseline levels at 10 pM FOM, indicating that even extremely low concentrations of the β-AR agonist have a strong negative effect (Figure 2B). These dose-dependent changes are only seen when RAW264.7 macrophages are activated with 30ng/ml LPS. When the cells are activated with 10ng/ml LPS, all the tested concentrations of FOM resulted in significantly decreased levels of secreted IL-6 (Figure 2A and B). Figure 2C demonstrates that FOM also effectively reduces TNF levels produced by activated macrophages. Since FOM is known to selectively activate β-ARs, it can be concluded that the cytokine decreases are due to catecholamine-mediated signaling through the β-AR pathway.

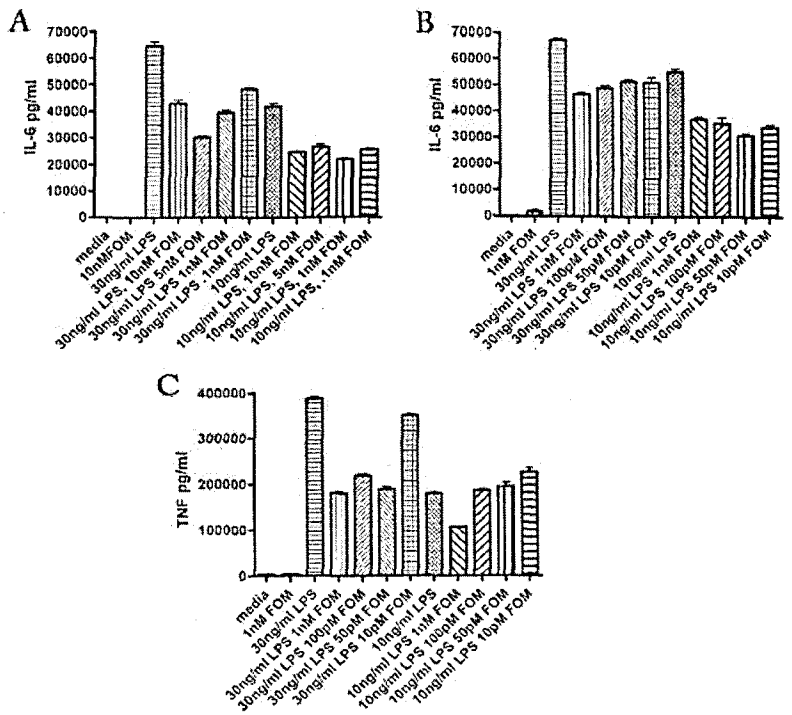

Figure 2. Concentrations of IL-6 and TNF produced in response to FOM treatment of activated RAW264.7 macrophages. RAW264.7 macrophages were plated into 12-well tissue culture plates at a density of 1.5×10^6 cells/well. Cells were then treated with FOM (10 nM FOM – 10 pM) for 30 minutes and then activated with 30 ng/ml or 10 ng/ml LPS. Cells were incubated (37°C, 5%CO_2) for 21 hours prior to IL-6 ELISA analysis (A and B); or 4 hours prior to TNF ELISA analysis (C). Statistical analysis was performed using Graph Pad Prism software.

Treatment with the α-adrenergic receptor agonist clonidine tends to enhance cytokine secretion by RAW264.7 macrophages

Primary macrophages also express α-ARs, which are generally believed to enhance the activity of activated macrophages.[6,9,15,19] However, a small portion of primary literature has also suggested that stimulation of the α-AR may have no effect on or decrease macrophage activity.[7,14] To attempt to address this discrepancy in the literature, and to characterize the role of α-ARs in regulating the function of RAW264.7 macrophages during an immune response, we analyzed the production of TNF and IL-6 by LPS-activated macrophages treated with clonidine, an α-AR agonist.

Macrophages were exposed to the specific α-AR agonist clonidine prior to macrophage activation. After a 4 or 21 hour incubation period, TNF and IL-6 secretion, respectively, were measured using an ELISA specific to each cytokine. Analysis of the ELISA data revealed that clonidine stimulates increases in IL-6 production at specific concentrations. A dramatic enhancement of IL-6 secretion was seen when RAW264.7 macrophages were treated with 500 nM clonidine. This effect was found when the macrophages were activated with either concentration of LPS (30 ng/ml or 10 ng/ml). Additionally, macrophages activated with 10 ng/ml

LPS also showed a small but significant increase in IL-6 secretion in response to treatment with 50 nM clonidine (Figure 3A).

The effects of clonidine treatment on TNF secretion were less clear. Analysis of the ELISA data indicated that TNF secretion is sometimes enhanced, while in other treatments it is reduced or unaffected. Similar results were found with several repeated studies (Figure 3B). Although signaling via the α-AR pathway typically induces a stimulatory effect, a few studies have found that α-agonists can also negatively influence macrophage activity.[7,14] Further research is necessary to determine what factors may influence the ambivalent behavior of α-AR signaling.

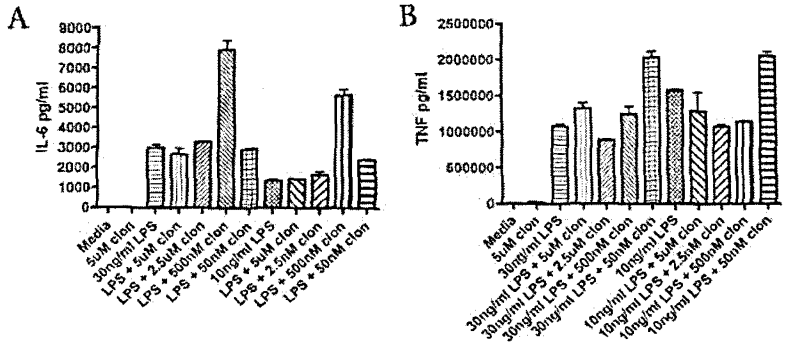

Figure 3. Concentrations of IL-6 and TNF secreted in response to clonidine treatment of activated RAW264.7 macrophages. RAW264.7 macrophages were plated into 12-well tissue culture plates at a density of 1.5×10^6 cells/well. Cells were treated with 5 μM, 2.5 μM, 500 nM or 50 nM clonidine for 30 minutes and then activated with 30 ng/ml or 10 ng/ml LPS . Cells were incubated (37°C, 5%CO_2) for 21 hours prior to IL-6 ELISA analysis (A); or 4 hours prior to TNF ELISA analysis (B). Statistical analysis was performed using Graph Pad Prism software.

Treatment with the α-adrenergic receptor antagonist RS79948 inhibits the enhancement of cytokine production seen in clonidine-treated, LPS-activated RAW264.7 macrophages

In an effort to further characterize the role of α-ARs in the regulation of macrophage immunological activity, RAW264.7 macrophages were treated with RS79948, an α-AR antagonist, prior to treatment with clonidine. The addition of RS79948 blocks any signaling through α-ARs, and thus abrogates any effects which are mediated by α-AR signaling.

ELISA analysis demonstrated that treatment of activated macrophages with 500 nM or 50 nM clonidine increases the concentration of cytokine secreted by the activated RAW264.7 cells (Figures 3 and 4). The addition of RS79948 prior to clonidine treatment results in a significant decrease in IL-6 production, returning the amount of secreted cytokine to levels equivalent to or below those measured in untreated, activated macrophages (Figure 4A). Likewise, TNF secretion is significantly impacted by blocking the α-AR pathway. RS79948 antagonism of the α-AR pathway significantly decreases the amount of TNF secreted from the activated macrophages to below positive control levels (Figure 4B). These results suggest that the positive effect of

clonidine is mediated by signaling through the α-AR pathway, as blocking the stimulation of α-ARs abrogates this effect. Furthermore, the observation that cytokine levels decrease below that of positive controls suggests that α-AR signaling may normally be involved in enhancing macrophage activity in response to LPS activation.

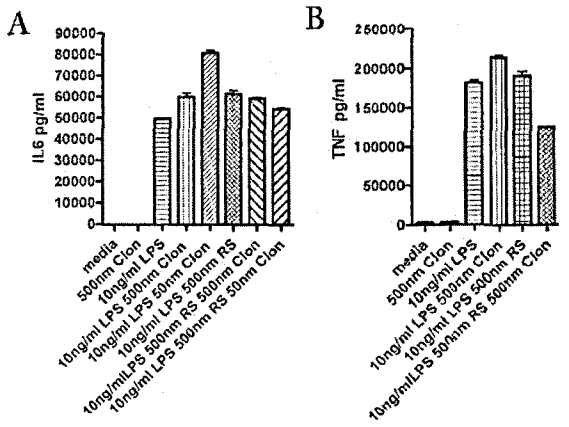

Figure 4. Concentrations of IL-6 and TNF secreted in response to treatment of activated macrophages with clonidine and the α-AR antagonist RS79948. RAW264.7 macrophages were plated into 12-well tissue culture plates at a density of 1.5 x 10^6 cells/well. Cells were then treated with 500 nM or 50 nM clonidine after a 30 minute incubation with RS79948 and then activated with 10 ng/ml LPS. Cells were incubated (37°C, 5%CO_2) for 21 hours prior to IL-6 ELISA analysis (A); or 4 hours prior to TNF ELISA analysis (B). Statistical analysis was performed using Graph Pad Prism software.

Regulation of surface expression of β-adrenergic receptors by LPS activation and NE exposure

In addition to studying the functional role of ARs, studies were also performed to characterize the expression pattern of ARs on RAW264.7 macrophages. These immunofluorescence studies were performed with mouse anti-β-AR primary antibody and anti-mouse IgG-AlexaFluor 594 conjugated secondary antibody. All labeled macrophages were examined with a confocal microscope. Appropriate controls were also included to demonstrate that each antibody was specific to its indicated protein, and to control for autofluorescence of the RAW264.7 macrophages (data not shown).

Immunofluorescence reveals a distinctive pattern of β-AR surface expression in RAW264.7 macrophages. Resting macrophages exhibit high levels of surface β-ARs (Figure 5 A1-A2'). However, the expression noticeably decreases in activated macrophages, as these images are characterized by reduced staining intensity and less overall staining (Figure 5 B1-B2'). NE treatment of activated macrophages does not visibly alter β-AR expression from the reduced levels seen in activated macrophages (Figure C1-C2'). These results suggest a mechanism of cross-talk between pathways which regulates β-AR expression in response to recognition of extracellular LPS.[11]

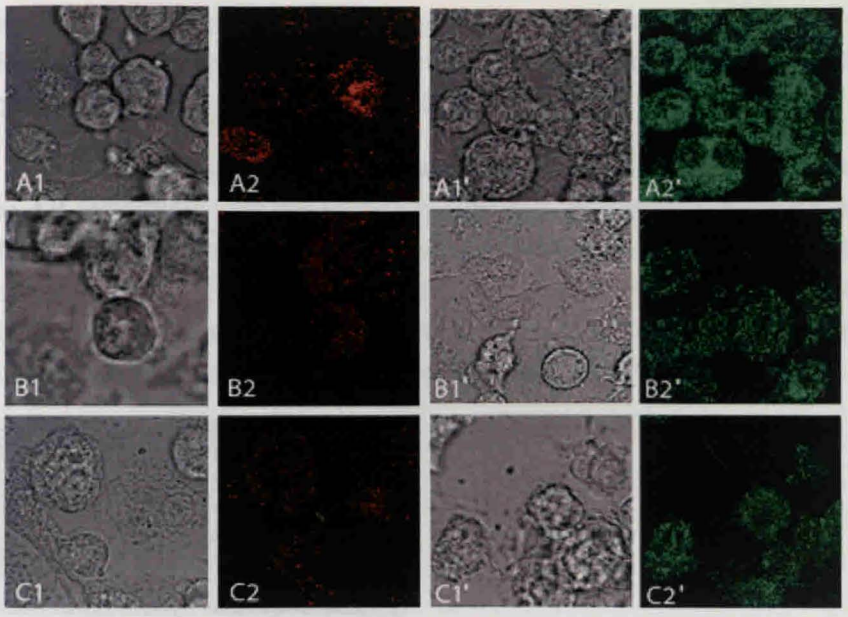

Figure 5. Surface expression of β-ARs by RAW264.7 macrophages in response to LPS activation and NE treatment. RAW264.7 macrophages were plated in wells containing sterile glass coverslips. Cells were treated with 1μM NE, then activated with 100 ng/ml LPS and incubated for 4 hours. Mouse anti-β-AR antibody was used as primary antibody, and anti-mouse IgG-AlexaFluor conjugated antibody was used as the secondary antibody. Immunofluorescence images were collected using Leica Confocal Software with a Leica SP2 Laser Scanning Confocal Microscope, and the contrast in transmitted light images was enhanced using Adobe Photoshop CS3 Extended software. Red images (A1-C2) were obtained using anti-mouse IgG secondary antibody conjugated with AlexaFluor 647; Green images (A1'-C2') were obtained using anti-mouse IgG secondary antibody conjugated with AlexaFluor 594. A1, A2, A1', A2' represent resting RAW264.7 macrophages; B1, B2, B1', B2' represent RAW264.7 macrophages activated with 100ng/ml LPS; C1, C2, C1', C2' represent RAW264.7 macrophages activated with 100ng/ml LPS and treated with 1μM NE

Regulation of surface expression of α-adrenergic receptors by LPS activation and NE exposure

Studies were also performed to characterize the surface expression of α-ARs in response to LPS activation and NE exposure. Immunofluorescence was performed using mouse anti-α-AR primary antibody and anti-mouse IgG-AlexaFluor594 conjugated secondary antibody. All images were collected using confocal microscopy. Controls were again performed to demonstrate that the antibodies used were specific to their advertised epitopes and to control for autofluorescence of the RAW264.7 cells (data not shown).

Immunofluorescence reveals that quiescent RAW264.7 macrophages express extremely low levels of α-ARs on their surface. This pattern is characterized by very low intensity staining, and widely dispersed points of fluorescence (Figure 6 A1-2). Activation with LPS noticeably increases the intensity of surface staining, as well as the number of points emitting fluorescence. This indicates that activated macrophages increase the expression of α-ARs on their surfaces (Figure 6 B1- B2). Treatment with NE appears to result in a slight decrease in the amount and

intensity of fluorescence, indicating a slight decrease in α-AR expression (Figure 6 C1-2). However, more quantitative immunofluorescence studies are needed to determine if this apparent change represents a significant decline.

Most images are collected at the surface of the cells to best demonstrate visible changes in AR expression. Optical sectioning through the center of a cell reveals a distinctive ring of fluorescence around the perimeter of a cell, and that staining is generally specific to the plasma membrane of RAW264.7 macrophages. This indicates that changes seen in the immunofluorescence studies reflect changes in surface protein expression, rather than global changes in protein expression throughout the cell (Figure 6 D1-2).

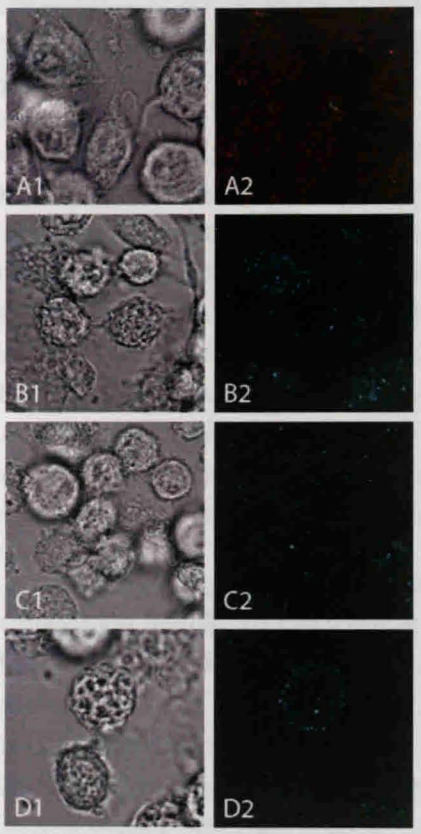

Figure 6. Surface expression of α-ARs by RAW264.7 macrophages in response to LPS activation and NE treatment. RAW264.7 macrophages were plated in wells containing sterile glass coverslips. Cells were treated with 1μM NE, then activated with 100 ng/ml LPS and incubated for 4 hours. Mouse anti-α-AR antibody was used as primary antibody, and anti-mouse IgG-AlexaFluor 594 conjugated antibody was used as the secondary antibody. Immunofluorescence images were collected using Leica Confocal Software with a Leica SP2 Laser Scanning Confocal Microscope, and the contrast in transmitted light images was enhanced using Adobe Photoshop CS3 Extended software. A1, A2 represent resting macrophages; B1, B2 represent RAW264.7 macrophages activated with 100 ng/ml LPS; C1, C2, D1, D2, represent RAW264.7 macrophages activated with 100 ng/ml LPS and treated with 1μM NE.

Discussion

This study was designed to characterize the expression patterns of α- and β-ARs, and the role they play in regulating RAW264.7 macrophage activity. This cell line is commonly used as a model of macrophage behavior, but much of the research regarding catecholamine-mediated regulation of macrophages has been performed with primary macrophages. Given the relevance of RAW264.7 macrophages as a model, it is important to understand their regulation. This data indicates that signaling through the β-AR decreases macrophage function, and it is thus in agreement with most previous studies.[3,11,14,17,18] Cytokine secretion, a common measure used to represent the immunological activity level of macrophages, was significantly decreased after treatment with NE or FOM, a β-AR agonist. B-ARs are also strongly expressed in quiescent macrophages, and their surface expression decreases in response to LPS activation of the macrophages.

Conversely, α-AR surface expression is extremely low in quiescent macrophages, but noticeably increases after LPS activation. NE treatment also appears to slightly decrease α-AR surface expression on activated macrophages. While the changes in surface expression of α-ARs appear relatively straightforward, the role of α-ARs in regulating macrophage immunological activity has yet to be fully characterized. This research suggests that enhancement of cytokine secretion by catecholamines is mediated through α-ARs, as antagonizing the receptors abrogated this effect. However, TNF studies exhibited both an enhancement and a decline in cytokine secretion after treatment with the α-AR agonist. These results suggest that changes in α-AR surface expression may alter the regulation of cytokine secretion. Additionally, some changes in expression may be due to conditions that have yet to be clearly defined.

Macrophages are cellular mediators of innate immunity, and play an essential role in maintaining health. To carry out this function, macrophages must be capable of responding to PAMPs displayed in MHCs and free in the extracellular matrix. Additionally, the body must carefully regulate these cells to avoid over-activity or excessive suppression, both of which can result in disease states.[1] Recent research has demonstrated that macrophages are regulated by direct immunological signals as well as neuroendocrine signaling via catecholamines. Catecholamines regulate inflammatory response behavior in macrophages by binding to surface ARs, initiating signaling cascades that are hypothesized to alter macrophage function. Current research indicates that stimulation of these two ARs tends to have different, if not opposite, effects on macrophages.[15] In this study, strong evidence was found to support a negative effect on immunological activity by the β-AR stimulation. However, the role of the α-AR remains more elusive.

In the body, NE can bind to both α- and β-ARs to alter immunological function of macrophages. The preference for one type of AR over the other has been shown to be concentration dependent, with high concentrations of NE inducing a predominantly β-AR effect. The results shown here support this model, as decreases in TNF secretion were seen as a result of

NE treatment (Figure 1). However, at very low concentrations, NE binds predominantly to the higher affinity α-AR and can enhance immunological function.[3,15,16,18] Epinephrine treatment similarly produces a predominantly β-AR response, enhancing the production of anti-inflammatory cytokines such as IL-10 as well as down-regulating pro-inflammatory cytokine production in LPS-activated primary macrophages.[13,18]

Kizaki et al. recently proposed a model detailing the cross-talk between the TLR4/LPS pathway and the β-AR pathway. Their study demonstrated that stimulation of β_2-ARs decreases the degradation of IκBα in the cytoplasm of macrophages. This effect is mediated by a direct interaction between IκBα and β-arrestin 2, a protein that is activated by the β_2-AR signaling cascade. This interaction stabilizes IκBα, and thus prevents the release of NF-κB from the complex. Lower NF-κB release ultimately results in lower levels of this transcription factor in the nucleus, and less transcription of pro-inflammatory genes in response to the TLR4 activation signal.[11] The results of these ELISA studies with the β-AR agonist FOM strongly support the model that β-AR stimulation decreases cytokine release. According to the model proposed by Kazaki et al. it would appear that this decrease is a result of decreased transcription of cytokine genes.[11] However, further study is necessary to conclusively show that decreases in secreted cytokine concentrations are a result of transcriptional regulation.

The immunofluorescence data which details surface expression of β-ARs offers a possible explanation for how the body may minimize the influence of β-ARs on macrophage function during an infection. High levels of β-ARs are expressed on the surface of resting RAW264.7 macrophages, and that surface expression decreases in response to LPS activation (Figure 5). Down-regulation of β-AR expression reduces the quantity of negative signals that can be sent to the macrophage. With fewer β-ARs expressed, less signal will be sent and thus less β-arrestin 2 will be activated. This will allow for the degradation of IκBα, releasing more NF-κB to enter the nucleus of activated RAW264.7 macrophages. Higher levels of pro-inflammatory genes will then to be produced, leading to a more efficient response to infection.[11] Down regulation of β-ARs would also thus prevent attenuation of the immune response before the pathogen is completely cleared from the organism. Additionally, signaling through β-ARs might be a mechanism to reduce the level of TNF secretion that causes serious injury during acute sepsis.[18] These studies help to explain how β-ARs influence macrophage regulation, as well as a potential reason for this pattern of regulation, but further research is needed to understand the mechanism regulating expression of β-ARs.

Immunofluorescence images studying the α-AR also suggest a potential functional explanation for the documented expression pattern of α-ARs when combined with the current model of catecholamine effects on macrophage function.[6,19] Resting macrophages express very low levels of α-ARs on their surfaces, which may be a mechanism for preventing improper activation of the macrophages. Previous work with macrophages is in agreement with these results. RT-PCR studies demonstrate that resting macrophages express α_1-AR transcripts at

undetectable levels, but that transcripts then become detectable after activation with LPS.[10] These low levels, coupled with the high level of β-AR expression, might be maintained to ensure that any catecholamines present will signal predominantly through β-ARs when no pathogen is present and the macrophages have not been activated, ensuring that the macrophages remain inactive. When macrophages become activated, there is a noticeable increase in α-AR expression. This increase alters the balance between α- and β-ARs, increasing the likelihood of catecholamines binding to α-ARs. This change in expression may function as a mechanism to establish a positive feedback loop. With higher expression of α-ARs, more stimulatory signals will be sent to the macrophage. This may result in a subsequent enhancement of macrophage function, and increased efficiency of pathogen clearance.[19]

Treatment with NE appears to induce a slight decrease in surface expression of α-ARs. As NE predominantly signals through β-ARs, this suggests that there may be some cross-talk occurring between the two AR pathways that regulates the expression of the α-AR.[15] However, previous studies with THP-1 monocytes have shown that treatment with a β_2-AR agonist enhances the amount of α_1-AR transcript in the monocytes.[10] Further study is therefore necessary to identify the pathway involved in this cross-talk, and to determine its outcome. Current work is also continuing with characterizing α-AR expression through flow cytometry and western immunoblotting analysis. As with the β-AR, additional studies are needed to fully understand how α-AR surface expression changes in response to exposure to pathogens (LPS activation) and exposure to catecholamines.

The majority of studies have found that stimulation of α-ARs enhances immunological activity of macrophages.[6,15,19] Spengler et al. showed that treatment of LPS-activated macrophages with UK-14304, a different α-AR agonist, resulted in an increase in TNF mRNA levels and secreted TNF levels.[15] Treatment with the α-AR antagonist yohimbine suppresses the normal increase in iNOS expression, and thus NO production, in macrophages activated with LPS. This indicates that the enhancing effects are a direct result of stimulation of α-ARs.[8] The results of our studies demonstrate an increase in IL-6 production after α-AR stimulation, in agreement with these previous studies showing that α-AR signaling enhances macrophage function. However, not all of our results support the conclusion that stimulation of the α-AR enhances cytokine secretion. Specifically, the production of TNF was reduced by activated macrophages after exposure to the α-AR agonist clonidine. Likewise, some previous studies have found that stimulation of α-ARs is not involved in enhancing cytokine production or, in some cases, actually decreases macrophage function.[7,14]

These variable results may be explained by differences in the expression patterns of α-AR sub-types on the surface of macrophages during an immune response (LPS activation). Previous work has shown that macrophages express α_1- and α_2-ARs, and that only stimulation of α_2-ARs enhances macrophage immunological function.[6,19] However, these sub-types have some overlapping agonist affinities, and stimulation of both sub-types may contribute to the variable

results since clonidine is a general α-AR agonist.[10] Additionally, macrophage α_2-ARs have also been shown to have both high and low affinity catecholamine binding sites. Similar sites on neutrophils have been shown to initiate different signaling cascades when bound. At high concentrations the agonist binds to the low affinity site, while at lower concentrations (approximately 1 nM) the agonist binds to the high affinity site. The intermediate concentrations of clonidine used for these experiments may be stimulating both sites, and producing inconsistent cytokine secretion. Alternatively, high concentrations of clonidine have been shown to lead to non-specific binding to other surface receptors. Non-specific binding by clonidine to the β-AR may also explain the measured decreases in TNF secretion.[15] Further study elucidating the signaling pathways initiated by α-AR stimulation might provide a better understanding of the α-AR signaling pathway in macrophages.

Despite these inconsistencies, studies with the α-AR antagonist RS79948 indicate that the stimulatory effects of clonidine are a result of signaling through the α-AR pathway, as addition of the antagonist abrogated this effect. Measurements of cytokine secretion show that addition of the antagonist reduced both TNF and IL-6 levels to below that of RAW264.7 macrophages treated only with LPS (Figure 4). A similar decrease in IL-1β secretion to below baseline levels was seen when LPS-activated macrophages were treated with the α-AR antagonist yohimbine in previous studies.[4] Previous work has shown that macrophages produce the catecholamines NE and epinephrine in response to LPS treatment. This suggests that these macrophage-derived catecholamines may act in an autocrine manner to further enhance inflammatory functions through α-AR signaling in the macrophage. Loss of this autocrine signaling by antagonism of α-ARs thus results in lower levels of cytokine production than those measured for RAW264.7 macrophages treated with just LPS due to the loss of the positive feedback loop. Alternatively, autocrine signaling with the macrophage-produced cytokines may also decrease the inflammatory response via signaling through exclusively β-ARs, as the α-ARs are blocked by the antagonist.[2,15]

The results of this study support the current model that neuroendocrine signaling plays an essential role in regulating immune function. This regulation has been shown to be the result of interactions between catecholamines and the ARs expressed on the surface of macrophages. It appears that the sympathetic nervous system can enhance immune function through α-ARs.[19] However, as NE is shown to have a predominately negative effect on cytokine secretion, it appears more likely that neuroendocrine signaling acts as an additional signal to reduce the strength of the immune response to protect the host from excessive damage.[18] With more research, the regulatory power of macrophage-expressed ARs may be used therapeutically to artificially enhance or shut down the immune system.

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