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Characterization of Catecholamine Receptors and Transporters in Murine Macrophages

by

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1. Introduction

Macrophages are a critical part of the immune response. When circulating monocytes move into tissues they differentiate into macrophages to mount the first line of defense against pathogens. Macrophages are primarily phagocytic cells, physically engulfing pathogens and cell debris at the site of an infection (Abbas et al. 2009). They play a key role in the innate immune system, initiating and maintaining the inflammatory response and acting as antigen presenting cells to active adaptive immunity (Abbas et al. 2009). Macrophages are activated in two different ways: the binding of a pro-inflammatory cytokine or by the recognition of a pathogen-associated molecular pattern (PAMP) by a receptor on the macrophage's surface (Abbas et al. 2009, Chi et al. 2003). Lipopolysaccharide (LPS) is a component of gram-negative bacterial cell walls and is a common activating PAMP that triggers NF-κB mediated pro-inflammatory effects (Abbas et al. 2009). When an immune response is mounted, macrophages act by phagocytosing pathogens and by releasing nitric oxide (NO) and other reactive intermediates as well as by secreting pro-inflammatory cytokines (Abbas et al. 2009). Cytokine release enhances the activation of phagocytes and promotes the recruitment of other immune cells to the site of infection (Abbas et al. 2009). Macrophages are able to control the spread of the pathogen through these non-specific destructive effects as well as by recruiting other immune cells to initiate a targeted immune response.

Catecholamines are small monoamines that act as neurotransmitters in the central and peripheral nervous systems. Norepinephrine (NE) is a specific catecholamine that plays a critical role in the stress response as it is mainly responsible for initiating the “fight or flight” response (Flierl et al. 2008). While catecholamines were previously thought to only initiate changes in the nervous system, recent work has shown that activated macrophages are able to synthesize and release catecholamines (Brown et al. 2003, Engler et al. 2004). In the nervous system, catecholamines initiate changes in physiology by binding to adrenergic receptors (ARs) found on post-synaptic cells. ARs are seven-transmembrane G-protein coupled receptors that interact with G proteins (Flierl et al. 2008). There are two main isoforms of ARs, α and β which can then be divided further into subtypes. Treatment of immune cells with AR agonists, including NE, has been shown to have an effect on NO and cytokine production which differs depending on the receptors being activated (Spengler et al. 1994, van der Poll et al. 1994). Treatment with β-AR agonists has been shown to decrease NO and pro-inflammatory cytokine production which is thought to be mediated through reduced activation of the NF-κB pathway (Sigola et al. 2001). In peritoneal macrophages, treatment with β-AR antagonists consistently causes an increase in TNF production clearly indicating the anti-inflammatory role of β-AR signaling (Spengler et al. 1994). Treatment of macrophages with an α-AR agonist clonidine has been shown to significantly increase macrophage resistance to pathogen growth in Mycobacterium avium infection (Weatherby et al. 2003). In general, stimulation of β-ARs is thought to have an anti-inflammatory effect on activated macrophages and stimulation of α-ARs is thought to have a pro-inflammatory effect.
However, these patterns are not always consistent with the opposite being observed under certain conditions (Szelenyi et al. 2006, Hamano et al. 2007). In mice treated with NE there was an observed decrease in survival of *Staphylococcus aureus* infection even though there was an observed increase in macrophage maturation (Grebe et al. 2010). Additionally, the concentration of NE or other AR agonists present has been shown to cause fluctuations in observed data with low concentrations having more pronounced anti-inflammatory effects and higher concentrations mediating pro-inflammatory changes (Baccan et al. 2010). Additionally, stimulation of different receptor subtypes can have widely varying effects depending on complex environmental factors. It is possible that the activation state of the macrophage as well as the mode of activation may influence the expression of the adrenergic receptors and play an important role in determining how the macrophage responds to AR stimulation. Therefore, characterization of the receptors expressed by macrophages is important for understanding immune regulation by catecholamines.

In addition to the adrenergic receptors, vesicular transporters are critical for catecholamine signaling. When catecholamines are released into the synaptic cleft to propagate a nerve impulse, they must be quickly recycled to ensure proper signaling. In the nervous system, a plasma membrane transporter pumps catecholamines back into the cytoplasm where they are then packaged into vesicles by a vesicular monoamine transporter (VMAT). There are two isoforms of the VMAT protein. VMAT2 is a high affinity transporter found mainly in the central nervous system where the time course for signaling is usually very rapid. VMAT1 is the lower affinity transporter and is found mainly in the neuroendocrine system where signals usually occur on a longer time scale. It is known that macrophages can synthesize and release catecholamines under infection conditions but it is unclear whether they can store these molecules for release at a later time (Spengler et al. 1994). It is possible that VMATs could be involved in the storage and release of catecholamines from immune cells.

### 2. Materials and Methods

#### 2.1. Cell culture

The RAW264.7 murine macrophage cell line was used as a model for macrophage activity. Cells were cultured in RPMI-1640 complete media supplemented with 10% heat-inactivated fetal calf serum, 1.5% sodium bicarbonate, 25 mM HEPES buffer, 1% minimum essential medium vitamins, 1% nonessential amino acids, 1% glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained in tissue culture flasks in a humidified incubator at 37°C in 5% CO2. Fresh media was added and cells were sub-cultured as needed to maintain health.

#### 2.2. Cytokine measurement by sandwich ELISA

RAW264.7 murine macrophage cells were seeded into the wells of a 12-well tissue culture plate at a density of 1.5×10^6 cells per well. The macrophages were treated with 5 µM, 2.5
µM, 500 nM, or 50 nM of the α-adrenergic receptor agonist, clonidine (CLN), or 1 µM, 500 nM, 100 nM, or 10 nM of the β-adrenergic receptor agonist, formoterol (FOM). Additional studies were performed using RS79948, an α2-adrenergic receptor antagonist, at a concentration of 500 nM for 30 minutes at 37°C in 5% CO2 prior to the addition of clonidine. Control wells were left untreated and unactivated. All treatment groups were then incubated for 30 minutes at 37°C in 5% CO2 before 10 ng/mL or 30 ng/mL of LPS was added to activate the cells. Cells were then incubated at 37°C in 5% CO2 for the appropriate times (TNF: 4 hours, IL-6: 21 hours). Supernatants were harvested from each well and centrifuged at 11,000 rpm for 5 minutes. The pellet was then discarded and the supernatant was held at -20°C until use. To quantify levels of TNF and IL-6 in supernatants, OptEIA Mouse TNFα enzyme-linked immunosorbent assay (ELISA) kits and OptEIA Mouse IL-6 ELISA kits were obtained from BD Biosciences. The kits were executed according to manufacturer’s instructions using collected supernatants. Concentrations of TNFα 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 determine significant changes between sample treatments. Statistical analysis was performed with GraphPad Prism software.

2.3. RT-qPCR of adrenergic receptor genes

RAW264.7 murine macrophage cells were seeded into the wells of a 6-well tissue culture plate at a density of 1.0×10⁶ cells/well. Cells were allowed to adhere overnight at 37°C in 5% CO2. Cells were then activated with 500 ng/mL LPS for 24 hours. Control wells were not activated with LPS. RNA was isolated from cells using the Qiagen RNeasy Mini kit according to the manufacturer’s instructions with an additional 15 minute DNase step (Promega, M610A). RNA concentrations were measured using a NanoDrop Lite instrument (Thermo Scientific). For each sample, 500 ng of RNA was reverse transcribed using a qScript cDNA synthesis kit from Quanta Biosciences according to manufacturer’s instructions. To detect AR gene expression levels, qPCR primers (β2, MP200467; β1, MP200466; α1A, MP200460; α1B, MP200461; α2A, MP200463) were purchased from Origene. Actin primers (MP200232, Origene) were also obtained to use as the housekeeping gene for normalization. Reactions containing primers and SYBR Green Master Mix (Quanta Biosciences, 170-8880BR) were assembled using manufacturer’s instructions. Reactions were run on a Bio-Rad CFX Connect Real-Time System using the following protocol: 95°C 3 minutes, repeat 35 times: 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds. Cycling was followed by a melting curve analysis to ensure specific amplification of the desired product. RT-qPCR data was analyzed using -2ΔΔCt with actin as the control gene. To analyze significance of differences between native and activated expression levels, a paired Student’s t-test was performed using GraphPad Prism (GraphPad Software).

2.4. Immunofluorescence

Glass cover slips were coated with a solution of 0.01% poly-lysine in dH2O. RAW264.7 cells were plated onto sterile coverslips in a 6-well tissue culture plate at a density of 1.5×10⁵
cells/well. The cells were incubated overnight at 37°C in 5% CO2 to allow for cell adherence. Cells were then activated with 500 ng/mL LPS and incubated at 37°C in 5% CO2 for 24 hours. Control samples were not activated. After incubation, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. After fixation, cover slips were blocked with a solution of 5% donkey serum and 0.001% Triton-X in PBS. After blocking, cover slips were washed with PBS and incubated with primary antibody (α: sc-1479, Santa Cruz Biotechnology; β: sc-570, Santa Cruz Biotechnology) diluted 1:100 in blocking solution. After primary antibody incubation, cover slips were washed again with PBS and incubated with secondary antibody diluted 1:5000 (Alexa647 donkey anti-rabbit IgG, Alexa488 donkey anti-goat IgG), DAPI diluted 1:4000, and Alexa594-Phalloidin diluted 1:1000 in blocking solution in the dark. After secondary incubation, coverslips were washed with PBS and mounted on slides using 80% glycerol, 0.5% n-propyl gallate in PBS and sealed with nail polish. Slides with secondary antibody alone were prepared as controls to ensure specificity of primary antibodies.

Slides were imaged using an Olympus Fluoview 1200 Laser Scanning Confocal Microscope with excitation wavelengths of 358 nm, 488 nm, 581 nm, and 633 nm. Images were collected using Olympus software and were manipulated using ImageJ to produce figure images.

2.5. PCR of VMAT genes

RAW264.7 murine macrophage cells were seeded into the wells of a 6-well tissue culture plate at a density of 1.0×10^6 cells/well. Cells were allowed to adhere overnight at 37°C in 5% CO2. For NE treatments, cells were treated with NE at a concentration of 1 µM for varying amounts of time (1, 4, 8 hours). For LPS activation, cells were activated with 500 ng/mL LPS for varying amounts of time (1, 4, 8, 24, 48 hours). Control wells were not treated with LPS or NE. RNA from cell samples was isolated according to the procedure described in 2.3. For each sample, 500 ng of RNA was reverse transcribed using a qScript cDNA synthesis kit according to manufacturer’s instructions (Quanta Biosciences). cDNA was amplified using primers for VMAT1 (Origene, MP215693), VMAT2 (Origene, MP215694), actin (Origene, MP200232), or GAPDH (Origene, MP205604) depending on treatment. PCR samples were assembled using GoTaq Green (Quanta Biosciences) according to manufacturer’s instructions and were cycled using the same protocol as outlined in 2.3. PCR products were run on a 3% agarose gel stained with EtBr with a ladder (Quik-Load 50 bp ladder, New England Biolabs) to ensure correct product size. Expected size of VMAT1 and VMAT2 products were 132 bp and 138 bp respectively. Products were visualized with UV to generate figures.

3. Results

3.1. Treatment with βAR agonist formoterol decreases pro-inflammatory cytokine production in RAW264.7
The inhibitory effect of NE on pro-inflammatory cytokine production is known to be mediated by stimulation of β-ARs on primary macrophages (Brown et al. 2003). To better characterize the role of β-ARs in RAW264.7 cells, formoterol, a specific β2 agonist, was added to cells prior to activation with LPS. Both IL-6 and TNF secretion levels were significantly decreased after treatment with formoterol (Fig. 1A-B). Formoterol selectively activates β2-ARs, so it is likely that the observed effects are due to catecholamine-mediated signaling through the β2-AR pathway.

3.2. Treatment with α-AR agonist clonidine enhances pro-inflammatory cytokine production in RAW264.7 cells

Primary macrophages are known to express α-ARs, but the effect of α-AR stimulation on macrophage activity is not well characterized. Across most primary literature, α-ARs are thought to enhance the pro-inflammatory activity of activated macrophages. However, under certain conditions, it has been show that α-AR stimulation may have no effect on macrophage activity. To characterize the role of α-ARs in RAW264.7 model macrophages, cells were treated with clonidine, a non-specific α-AR agonist, and levels of TNF-α and IL-6 released were measured by ELISAs. For TNF, data showed that α-AR stimulation led to a significant increase at all doses tested (Fig. 2A) while IL-6 secretion increased only at the 500 nM dose of clonidine (Fig. 2B). This range specific functionality has been observed in other immune cells, but the exact mechanism or factors involved in this ambivalent α-AR signaling are not well understood.
Figure 2—Treatment with α-AR agonist clonidine increases TNF and IL-6 production in RAW264.7 cells
RAW264.7 macrophages were plated into 12-well tissue culture plates at a density of 1.5×10⁶ cells/well. Cells were treated with different concentrations of clonidine for 30 minutes and then activated with LPS. Cells were incubated (37°C, 5% CO₂) for 4 hours prior to TNF ELISA analysis (A); or 21 hours prior to IL-6 ELISA analysis (B). Paired Student's t-test analysis was performed using GraphPad Prism software (* = p < 0.001 compared to LPS alone).

3.3. Treatment with α-AR antagonist R279948 in conjunction with clonidine inhibits enhancement of pro-inflammatory cytokine production in RAW264.7 cells

To better characterize the role of the α-ARs in modulating macrophage function, RAW264.7 cells were treated with RS79948, an α₂-AR antagonist, prior to treatment with clonidine. The addition of RS79948 blocks signaling through α₂-ARs. The addition of RS79948 prior to clonidine treatment resulted in a significant decrease in TNF production, bringing levels back down to or below baseline activation levels (Fig. 3A). IL-6 secretion levels are similarly affected with secretion levels of cells treated with RS79948 and clonidine returning to baseline activation levels (Fig. 3B). These results suggest that the clonidine agonist effect is mediated through the α₂-ARs specifically. They also may suggest that α₂-AR signaling is normally involved in the enhancement of macrophage activity in response to LPS activation due to the decrease below baseline levels for TNF secretion seen in populations treated with RS79948.
Figure 3—Pre-treatment with α-AR antagonist RS79948 inhibits TNF and IL-6 production in RAW264.7 cells

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 RS79948 for 30 minutes, then subsequently treated with different concentrations of clonidine for 30 minutes and then activated with LPS. Cells were incubated (37°C, 5% CO₂) for 4 hours prior to TNF ELISA analysis (A); or 21 hours prior to IL-6 ELISA analysis (B). Paired Student's t-test analysis was performed using GraphPad Prism software (* = p < 0.05, ** = p < 0.01 compared to LPS alone).

3.4. RAW264.7 cells express genes for multiple subtypes of ARs

There are many different subtypes of both the α and β-ARs that exist in central and peripheral nervous tissue. The expression pattern of these receptors is not well characterized in macrophages. RAW264.7 cells were used as a model to determine both native and activated expression levels for multiple subtypes of both α and β-ARs. RAW264.7 cells were found to natively express both β₁ and β₂-ARs (Fig. 4A-B). The level of β₂-AR gene expression was shown to significantly decrease in cells activated with LPS which is consistent with β₂-ARs' proposed anti-inflammatory effects (Fig. 4B). Interestingly, the level of β₁-AR expression was shown to increase in cells activated with LPS (Fig. 4A). The effect of β₁-AR stimulation has not yet been characterized so it is unclear what role these receptors may play in regulating macrophage function.

RAW264.7 cells were also found to express the genes for multiple subtypes of the α-AR isoform. RAW264.7 cells natively express both α₁A and α₂B-AR subtypes (Fig. 4C-D). These cells were found not to express α₂A-AR subtypes under native or activated conditions (results not shown). When cells were activated with LPS, the levels of both α₁A and α₂B-AR gene expression significantly increased (Fig. 4C-D). The level of α₁A-AR expression was found to be more than 10-fold higher in activated cells than in the native (Fig. 4C) suggesting a possible important role for these receptors in regulating macrophage function. Additionally, the gene expression levels of all α-AR subtypes tested were shown to increase in activated cells mirroring previous results pinpointing the α-ARs as mediating pro-inflammatory processes.
Figure 4—RAW264.7 cells express multiple AR subtypes and gene expression changes under infection conditions. RAW264.7 macrophages were plated into tissue culture plates at a density of $1.0 \times 10^6$ cells/mL. Cells were activated with 500 ng/mL of LPS for 24 hours prior to RNA isolation. Expression patterns of $\beta_1$ (A), $\beta_2$ (B), $\alpha_1$ (C), and $\alpha_2$ (D) ARs were detected. qPCR data were analyzed according to $\Delta\Delta C_t$ method with $\beta$-actin as the control gene. Significance was determined by a paired Student's t-test performed with Graph Pad Prism software (* p < 0.05, ** p < 0.01, *** p < 0.001).

3.5. RAW264.7 cells display multiple AR proteins

In addition to studying gene expression patterns of ARs in RAW264.7 cells, protein levels for different AR isoforms were also analyzed using immunofluorescence techniques. Non-active macrophages were shown to express both $\beta_2$ and $\alpha_2$-ARs (Fig. 5A-B). The levels of $\beta_2$-AR expression appear to be higher than that of the $\alpha_2$-AR qualitatively, but the difference in fluorescence levels was not quantified.
Figure 5--RAW264.7 cells express both AR isoform proteins
Blue (DAPI) shows nucleus, yellow (phalloidin) F-actin, red \( \beta_2 \)-AR (A), and green \( \alpha_2 \)-AR (B). Slides were imaged using a Leica SP2 Laser Scanning Confocal Microscope.

3.6. RAW264.7 cells express a vesicular catecholamine transporter

Macrophages are known to synthesize and release catecholamines after LPS activation (Brown et al, 2003). The release mechanism of catecholamines by macrophages may be similar to that found in neurons, with catecholamines being pumped into vesicles by VMATs to be released later by exocytosis but the expression of VMATs in macrophages has not been characterized. Under native conditions, these cells were found to only express VMAT1 (not VMAT2) which is the lower affinity and less efficient transporter (Fig 6A-B). Additional studies examined VMAT1 expression after LPS activation or NE exposure (Fig. 6A-B). Since these experiments were not done quantitatively it is difficult to determine whether activation or NE exposure alters VMAT1 expression so further analysis with qPCR is warranted.

Figure 6--RAW264.7 cells express the VMAT1 gene
Cells were activated with LPS (A) or treated with norepinephrine (B) for the times in hours indicated. cDNA from cells was amplified with PCR using primers for VMAT1 with actin/GAPDH as controls. PCR products were separated on a 3% agarose gel stained with EtBr and imaged with UV.
4. Discussion

This study was designed to better characterize the expression of proteins involved in catecholamine signaling in the RAW264.7 macrophage model cell line. This cell line is a commonly used model for macrophages since these cells are known to possess macrophage markers and retain functionality of primary macrophages. Therefore, characterization of the catecholamine transporters and receptors in these cells is important to better understand how catecholamines may regulate macrophage function. The data reported here provides a detailed AR expression profile for these cells in quiescent and activated states and also provides evidence for the expression of VMAT proteins possibly involved in the storage of catecholamines in the cytoplasm of the macrophages.

Earlier studies (by a Lindsay Ward) demonstrated that stimulation of β-ARs leads to a decrease in the release of pro-inflammatory cytokines providing evidence for the dominant anti-inflammatory effect of β-AR stimulation. In conjunction with these studies, preliminary studies suggested that the β2-AR protein is highly expressed in native macrophages but decreases after LPS activation which may help to explain the anti-inflammatory effects of β2-AR stimulation. Ward's work also demonstrated that stimulation of α-ARs appears to have the opposite effect, with a significant increase in pro-inflammatory cytokine production when cells are treated with an α-AR agonist. The preliminary work also suggested that RAW264.7 cells express low levels of the α2B-AR when quiescent and the level of α-AR protein expression was observed to increase upon activation with LPS. To better understand the AR expression pattern in these cells, we performed PCR studies. RAW264.7 cells were found to express multiple subtypes of both α and β-ARs and the expression pattern of the receptors was shown to change when cells were activated with LPS. Additionally, PCR analysis demonstrated that these cells also express the vesicular catecholamine transporter VMAT1 which could be involved in catecholamine storage suggesting that macrophages have the ability to carry out regulated release of catecholamines under certain conditions. Characterization of this transporter's protein expression in native cells and how gene and protein expression changes after activation or after treatment with AR agonists could lead to a better understanding of the role of catecholamine storage and release in immune functionality. These results together suggest an important role of catecholamines in regulating macrophage function and carefully controlling the immune response.

In the body, cross-talk between signaling pathways and simultaneous expression of different cell-surface receptors leads to the complex behavior of cells that can be fine-tuned based on tiny fluctuations in environmental conditions. When NE is released in the vicinity of macrophages it binds to both α and β-ARs simultaneously since both are expressed in the inactive state. The concentration of NE released is thought to influence which receptor dominates the response of the macrophages. If high concentrations of NE are present, the low affinity β-ARs are more active and initiate an anti-inflammatory effect (Hetier et al. 1991). If lower concentrations of NE are present, the higher affinity α-ARs are more likely to be activated and initiate changes in macrophage function (Elenkov et al. 1996). Additionally, α-ARs have been
suggested to contain multiple catecholamine binding sites of different affinities which activate different signaling pathways upon binding NE (Spengler et al. 1990). This concentration specific activity can help to carefully regulate the immune response when catecholamines are present. It also may point to a link between chronic stress, in which higher levels of NE are released by the nervous system, and decreased immune functionality as these high NE levels may have a pronounced anti-inflammatory effect (Baccan et al. 2010).

In addition to simultaneous expression of multiple AR subtypes, cross talk between AR signaling pathways and Toll-like receptor (TLR) pathways responsible for recognizing pathogens has been observed in macrophages and could possibly be a mechanism for catecholamine regulation of immune function. In a classic innate immune response, LPS binds to TLR4 on the macrophage surface which initiates a signaling cascade that eventually leads to the phosphorylation of an inhibitory protein I kB leading to its degradation and release of NF-κB into the nucleus to initiate transcriptional changes (Abbas et al. 2009). A recent study on the role of β2-ARs in macrophages showed that stimulation of β2-ARs led to a decrease in degradation of I kB (Kizaki et al. 2008). These results were mediated through a direct interaction between I kB and β-arrestin 2, a protein that is activated in the β2-AR signaling cascade. By stabilizing I kB, lower amounts of NF-κB are released leading to a decrease in transcription of pro-inflammatory genes. This study points to an important interaction between pathways that could be confirmed by future studies examining the regulation of cytokine genes in activated macrophages treated with AR agonists. Similar crosstalk could also lead to changes in VMAT expression levels which could change macrophage catecholamine release patterns in stress or infection environments.

The results of this study highlight a clear link between the neuroendocrine and immune systems. Catecholamines appear to play an important role in regulating immune function through AR binding. Additionally, catecholamines released from macrophages could have an autocrine effect to further regulate macrophage functionality. This link between the nervous and immune systems provides a new perspective on the role of stress and hormone imbalances leading to changes in immune functionality and could provide a new avenue for catecholamine-based treatment of immune disorders.

Works Cited


