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β-adrenergic-stimulated macrophages: Comprehensive localization in the M1–M2 spectrum

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Abstract

β-adrenergic signaling can regulate macrophage involvement in several diseases and often produces anti-inflammatory properties in macrophages, which are similar to M2 properties in a dichotomous M1 vs. M2 macrophage taxonomy. However, it is not clear that β-adrenergic-stimulated macrophages may be classified strictly as M2. In this in vitro study, we utilized recently published criteria and transcriptome-wide bioinformatics methods to map the relative polarity of murine β-adrenergic-stimulated macrophages within a wider M1–M2 spectrum. Results show that β-adrenergic-stimulated macrophages did not fit entirely into any one predefined category of the M1–M2 spectrum but did express genes that are representative of some M2 side categories. Moreover, transcript origin analysis of genome-wide transcriptional profiles located β-adrenergic-stimulated macrophages firmly on the M2 side of the M1–M2 spectrum and found active suppression of M1 side gene transcripts. The signal transduction pathways involved were mapped through blocking experiments and bioinformatics analysis of transcription factor binding motifs. M2-promoting effects were mediated specifically through β₂-adrenergic receptors and were

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associated with CREB, C/EBPβ, and ATF transcription factor pathways but not with established M1–M2 STAT pathways. Thus, β-adrenergic-signaling induces a macrophage transcriptome that locates on the M2 side of the M1–M2 spectrum but likely accomplishes this effect through a signaling pathway that is atypical for M2-spectrum macrophages.

Keywords

macrophage; M1; M2 spectrum; β-adrenergic; transcriptome; bioinformatics; CREB

1. Introduction

Previous studies have found that the sympathetic nervous system (SNS) and β-adrenergic receptor signaling can regulate macrophage involvement in diseases that include cancer,[1,2] heart disease,[3] and AIDS.[4] The earliest investigations came from a line of research that found β-adrenergic signaling can suppress the ability of macrophages to control *Mycobacterium avium* infection, a significant cause of morbidity and mortality in AIDS patients.[4,5] More recently, a mechanism by which macrophage infiltration contributes to increased risk of atherosclerotic cardiovascular disease (i.e., monocyte expression of beta2-integrins for vascular endothelium adhesion) was up-regulated by experimentally-induced anger with higher plasma norepinephrine levels in healthy participants.[3] Accordingly, a mouse model of atherosclerosis showed that SNS-induced β-adrenergic signaling promotes macrophage accumulation in atherosclerotic plaques.[6] In a murine model of breast cancer, we found that chronic stress can promote accumulation of tumor-associated macrophages (TAMs) in the primary tumor of mice through β-adrenergic signaling.[1] Similarly, others have also found that the same chronic stress paradigm in mice will increase TAM prevalence in ovarian tumors through increases in monocyte chemotactic protein 1, a chemokine that is up-regulated in ovarian cancer cells by β-adrenergic signaling.[2]

TAMs often exhibit properties of an M2 macrophage rather than the classical M1 phenotype of microbicidal macrophages.[7] In a non-malignant environment, M2 macrophages facilitate wound healing by tapering immune cell attack, clearing tissue debris, and building new vasculature.[7] However, in malignancy, those M2 properties may forestall immune cell attack, break down normal tissue for malignant expansion, and build new vasculature to support cancer cell metabolism and distant metastasis.[7,8] To this point, we found that β-adrenergic signaling increased gene expression of the prototypical M2 marker, *Arg1*, in primary tumors with elevated accumulation of TAMs and confirmed that norepinephrine increases *Arg1* expression in macrophages outside the tumor microenvironment (i.e., in bone marrow-derived macrophages).[1] Similarly, both norepinephrine and epinephine were found to alter the phenotype of LPS-activated M1-like macrophages by increasing the expression of *Arg1*.[9]

However, it is not clear that β-adrenergic-stimulated macrophages may accurately be called M2 macrophages, because (1) M1 and M2 macrophages are not defined by any single emblematic marker,[10] (2) rather than being just two separate phenotypes, M1 and M2 macrophages are thought to constitute a spectrum of several related phenotypes,[11] and (3)

canonical M1 and M2 macrophage activation (i.e., stimulated by IFNγ and IL-4, respectively) is driven primarily by transcription factors that are different from β-adrenergic-related transcription factors recently found to mediate expression of *Arg1* and other anti-inflammatory genes.[12,13,14] In this latter regard, whereas M1 macrophages exhibit high levels of STAT1 transcription factor and M2 macrophages exhibit high levels of STAT6,[10] the well-defined downstream transcription factor of β-adrenergic signaling, CREB,[15] in conjunction with the transcription factor C/EBPβ, has been found to regulate expression of genes in macrophages that are associated with an anti-inflammatory phenotype, including *Arg1* and *Il10*.[12,13] ATF1, a transcription factor closely-related to CREB, has also been linked to *Il10* transcription.[14]

In order to facilitate accurate description of macrophage polarity phenotypes across diverse experimental scenarios, macrophage biologists have proposed standards for defining multiple distinct categories of M2-like and M1-like macrophages in a linear spectrum (see Figure 1 and Murray et al., 2014),[10] which may be used to approximate the spectrum location of β-adrenergic-stimulated macrophages. These categories are based on the extracellular macrophage *activator* and subsequent transcriptional regulators. For example, canonical M2 macrophages activated by IL-4 are classified as *M(IL-4)* macrophages, but macrophages activated by other established activators are also considered M2-like. These include immune complex-activated macrophages, *M(Ic)*, IL-10-activated macrophages, *M(IL-10)*, glucocorticoid and transforming growth factor β-activated macrophages, *M(GC+TGF-β)*, and glucocorticoid alone-activated macrophages, *M(GC)*. On the M1 side of the spectrum, canonical M1 macrophages activated by IFNγ are classified as *M(IFNγ)*, and other closely-related M1-like macrophages include lipopolysaccharide and IFNγ-activated macrophages, *M(LPS+IFNγ)*, and lipopolysaccharide alone-activated macrophages, *M(LPS)*. Each of the categories are further defined by the activator's consensus set of gene expression markers to facilitate comparison of the categories to gene expression from any novel macrophage stimulation scenario.

Given these newly proposed standards for defining the relative polarity of a specifically-stimulated macrophage, we selected gene transcripts *a priori* from each category in the spectrum, as defined for mouse macrophages, to test for differential induction by β-adrenergic signaling in mouse bone marrow-derived macrophages (BMDMs) using quantitative real-time RT-PCR. Furthermore, because no study has examined the effect of β-adrenergic signaling on the total array of transcriptome dynamics involved in M1–M2 polarity, we followed this targeted *a priori* approach with genome-wide transcriptional profiling in order to more comprehensively assess the spectrum location of β-adrenergic-stimulated macrophages relative to canonical IL-4-stimulated M2 macrophages vs. canonical IFNγ-stimulated M1 macrophages. Finally, given that STAT family transcription factors are established as primary drivers of gene expression in the M1–M2 spectrum,[10] we utilized bioinformatic inferences of activity for these transcription factors, as well as for transcription factors related to β-adrenergic-signaling, to assess their role in the macrophage polarization response to β-adrenergic-stimulation. Given the previous findings noted above, we hypothesized that β-adrenergic-stimulated macrophages exhibit a transcriptome that locates on the M2 side of the M1–M2 spectrum and that this M2-spectrum transcriptome is regulated in part by CREB, C/EBPβ, ATF, and STAT family transcription factors.

2. Methods

2.1. Bone marrow derived macrophages (BMDMs)

Flushed bone marrow from female Balb/c mice (Charles River, 8–10 weeks) in RPMI-1640 with L-glutamine (Cellgro-Corning, Inc., #10-040-CV) was passed through a 30-μm cell strainer (Miltenyi, #130-041-407) and subjected to red blood cell lysis buffer (BD Biosciences, #555899). White blood cells were counted by hemocytometry and seeded at 0.25×10^6 cells/mL in a total of 4 mL/well of RPMI-1640 with L-glutamine supplemented with 10% FBS (Atlanta Biologicals, #S11550H), 100 IU penicillin/mL, 100 μg streptomycin/mL (Cellgro-Corning, #30-002-CI), at 37°C, 5% CO_2, in a 6-well polystyrene low attachment plate (Costar, #3471) with 10 ng/mL of recombinant mouse M-CSF (Gibco, #PMC2044) for 7 days (media replenished after 2, 5, and 7 days). Flow cytometry was used to confirm macrophage phenotype of resultant BMDMs with fluorescence-conjugated antibodies against murine F4/80 (BD Biosciences, #565411) after mouse Fc blocking (BD Bioscience, #553141) using a FACSAria II High-Speed Cell Sorter with FACSDiva software (BD Biosciences) for analysis of total live cells from gating based on forward- versus side-scatter profiles.

2.2. Macrophage stimulation

To examine the effect of β-adrenergic agonism on gene transcripts indicative of macrophages in the M1–M2 spectrum, BMDMs were incubated with the non-selective β-adrenergic agonist isoproterenol (Sigma, #I2760) for 24 hours. Control BMDMs were incubated in media only during the same 24 hour period. To comprehensively compare the global gene expression profile of β-adrenergic-stimulated macrophages to M1 and M2 transcriptomes, separate BMDMs were incubated with either 20 ng/mL of recombinant mouse IFN-γ (Gibco, #PMC4034) or 20 ng/mL of recombinant mouse IL-4 (Gibco, #PMC0045) for 24 hours. In each of three independent experiments, duplicate wells were prepared for each condition (control, isoproterenol, IL-4, IFN-γ).

To determine the specific receptor subtype necessary for isoproterenol to induce up-regulation of select gene transcripts differentially regulated in *a priori* experiments and in the global gene expression profile, selective β$_1$-, β$_2$-, and β$_3$-adrenergic antagonists, atenolol (Sigma, #A7655), ICI 118,551 (Sigma, #I127), and L-748,337 (Santa Cruz, #sc-204044), respectively, were used. Antagonists were added to wells 15 minutes before isoproterenol. In each of three independent experiments, duplicate wells were prepared for each condition (control, isoproterenol, control+atenolol, isoproterenol+atenolol, control+ICI 118,551, isoproterenol+ICI 118,551, control+L-748,337, isoproterenol+L-748,337). To determine whether β$_2$-adrenergic receptor signaling was sufficient to induce up-regulation of select gene transcripts, the selective β$_2$-adrenergic agonist, formoterol (Sigma, #F9552), was used. In each of three independent experiments, duplicate wells were prepared for each condition (control, formoterol).

2.3. qRT-PCR

To quantify gene expression, total RNA from BMDMs was extracted (Qiagen RNeasy Mini Kit, #74104), cleared of contaminating DNA with on-column DNase digestion (Qiagen

RNase-Free DNase Set, #79254), and quantified by spectrophotometry (NanoDrop ND-1000, Thermo Scientific). Gene transcripts indicative of recently suggested key marker systems for activated macrophages in the M1–M2 spectrum (Murray et al., 2014; Figure 1) were selected *a priori* for examination in β-adrenergic-stimulated macrophages by qRT-PCR using one-step assay reagents (Qiagen Quantitect Probe RT-PCR, #204443) and TaqMan Gene Expression Assay primer-probes for mouse *Arg1, Retnla, Il10, Il4ra, Nos2,* and *Ido1* (Life Technologies/Applied Biosystems, Mm00475988_m1, Mm00445109_m1, Mm00440502_m1, Mm00439614_m1, Mm01275139_m1, Mm00492586_m1, respectively). Five gene transcripts with previously reported importance for M1–M2 macrophage biology were selected for independent verification of differential expression by qRT-PCR after exhibiting differential expression in the global gene expression profile. These included *Cxcl4/Pf4, Ccl24, Dusp1, Il1rn,* and *Cd74* (Mm00451315_g1, Mm00444701_m1, Mm00457274_g1, Mm00446186_m1, Mm00658576_m1, respectively). Following reverse transcription of RNA template, resulting product underwent 50 PCR amplification cycles of 15 seconds of strand separation at 94°C and 60 seconds of annealing and extension at 60°C. Triplicate determinations were quantified by threshold cycle analysis of FAM fluorescence intensity using iCycler software (Bio-Rad), normalized to values of beta-actin mRNA amplified in parallel (*Actb*, #Mm00607939_s1). Student's *t* test was used to analyze the effects of β-adrenergic-activation on gene expression. Univariate analysis of variance was used to analyze the effects of selective β1-, β2-, and β3-adrenergic antagonists on isoproterenol-induced gene expression with Tukey's adjustment for multiple comparisons.

2.4. Transcript origin analysis (TOA)

Total RNA (~1 μg) was assayed using Illumina MouseRef-8 v2.0 Expression Beadchips in the University of California, Los Angeles Neuroscience Genomics Core (UNGC). Quantile normalization[16] was applied to values of the 18,138 assayed transcripts, and differentially expressed genes were identified by 25% difference in mean (log2) expression levels in macrophages treated with isoproterenol vs. controls. Genes that were differentially expressed by isoproterenol were identified based on biological effect size (i.e., difference = mean isoproterenol – mean control) rather than statistical effect size (e.g., *t* statistic or *P* value), because previous research has shown that biological effect size-based criteria yield more replicable results than do statistical effect size criteria (e.g., *t* statistics, *P* values, or false discovery rate *q* values).[17,18,19,20] Effect size point estimates for individual gene transcripts serve only as input into higher-order bioinformatics analyses testing gene set hypotheses regarding M1- and M2-diagnostic groups in TOA and transcription factor binding motif analysis with TELiS as described below.[21] Gene expression data are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; accession no. GSE80185).

For TOA, we defined a cell type diagnosticity score, as previously described,[22] for each gene (indexed g = 1 to G, g ∈ 18,134 mouse gene transcripts assayed in the Illumina MouseRef-8 v2.0 Expression Beadchip). In the current experiment, a cell type diagnosticity score indicates the extent to which a given gene transcript is predominately expressed by an M2-polarized macrophage relative to an M1-polarized macrophage in the reference wells of our study (i.e., BMDMs stimulated only by IL-4 or IFNγ, respectively). Positive values

indicate relative over-expression of the gene in M2 macrophages and negative values indicate relative over-expression of the gene in M1 macrophages. The mean diagnosticity score for all genes that were up-regulated by β-adrenergic-stimulation and the mean diagnosticity score for all genes down-regulated by β-adrenergic-activation were then tested for statistically significant deviation from the mean population diagnosticity score for all mouse genes using a single-sample *t* test.[23]

2.5. Transcription factor analysis

We used a two-sample variant of the Transcription Element Listening System (TELiS; www.telis.ucla.edu) as previously described[24] to compare the prevalence of transcription factor-binding motifs (TFBMs) for CREB, C/EBPβ, ATF, and STAT transcription factors in the promoters of genes that were up-regulated and down-regulated in macrophages by isoproterenol stimulation relative to control treatment. Binding motif definitions were retrieved from the TRANSFAC database. Analyses averaged results derived from nine parametric variations of promoter length (−300 bp relative to RefSeq transcription start site, −600 bp, and −1000 bp to +200) and target TFBM match stringency (MatSim = 0.80, 0.90, 0.95). Differential TFBM prevalence ratios of up-regulated to down-regulated genes were then tested for significant deviation from a null population mean ratio of 1 by two-tailed *P* values in a single-sample *t* test.

3. Results

3.1. M1–M2 spectrum gene transcripts in the β-adrenergic-stimulated macrophage

To determine whether β-adrenergic-stimulated macrophages fall squarely into one category of the M1–M2 spectrum, we derived F4/80$^+$ macrophages from murine bone marrow cells (Suppl. Figure 1) and exposed them to 1 μM isoproterenol. We then used qRT-PCR to assay expression for two genes from each category's consensus set of gene markers for mouse macrophages (Figure 1B; see Murray et al., 2014 for full gene sets). We chose two genes because one gene can appear in more than one category's consensus gene set. For categories on the M2 side of the spectrum, *Arg1* and *Retnla* were measured for M(IL-4); *Il10* and *Nos2* for M(Ic); *Il10* and *Il4ra* for M(IL-10) (note: M(GC+TGFβ) and M(GC) categories on the M2 side are currently undefined for mouse macrophages). For M(IL-4), isoproterenol induced a 10.4-fold increase in *Arg1* gene expression (*P* < 0.001) but had no significant effect on *Retnla* expression (*P* = 0.316; Figure 2). For M(Ic), isoproterenol induced a 2.2-fold increase in *Il10* gene expression (*P* = 0.005) but decreased *Nos2* expression by 2.1-fold (*P* = 0.022; Figure 2).

For M(IL-10), isoproterenol had no significant effect on *Il4ra* expression (*P* = 0.411; Figure 2). For categories on the M1 side of the spectrum, *Ido1* and *Nos2* were measured for M(IFNγ), and *Nos2* and *Arg1* were measured for both M(LPS+IFNγ) and M(LPS). For M(IFNγ), isoproterenol had no significant effect on *Ido1* expression (*P* = 0.407) and, as already noted above, had a suppressive effect on *Nos2* expression (Figure 2). M(LPS+IFNγ) and M(LPS) are both also defined in part by increases in *Nos2* expression, which was decreased by isoproterenol treatment, and relatively small increases in *Arg1* expression, which was increased significantly by isoproterenol treatment. Collectively, the results of this

targeted approach of M1–M2 spectrum genes suggest that β-adrenergic-stimulated macrophages do not fit discretely into any one pre-defined category in the proposed spectrum. Given the up-regulation of both *Arg1* and *Il10*, it may be that β-adrenergic-stimulated macrophages express a profile that shares elements of both M(IL-4) and M(IL-10) categories on the M2 side. Also, given that β-adrenergic signaling down-regulates *Nos2* but this gene is up-regulated in all three categories of the M1 side, it may be that β-adrenergic signaling suppresses some elements of M1-spectrum macrophages. To address these questions, we followed this targeted *a priori* approach with genome-wide transcriptional profiling in order to more comprehensively define the M1–M2 spectrum location of β-adrenergic-stimulated macrophages.

3.2. Comprehensive localization of the β-adrenergic-stimulated macrophage transcriptome in the M1–M2 spectrum

To provide a more systematic and unbiased approach to localization of β-adrenergic-stimulated macrophages within the M1–M2 spectrum, we used transcript origin analysis (TOA) to examine global gene expression profiles derived from Illumina bead arrays. Diagnosticity scores were calculated for each gene in the array, i.e., scores that quantify the extent to which each and every gene in the array is expressed predominately by M2 macrophages relative to M1 macrophages. These diagnosticity scores are listed in Dataset S1, where positive values indicate predominant expression of that gene in M2 macrophages and negative values indicate predominant expression of that gene in M1 macrophages.

We found that isoproterenol up-regulated the expression of 128 genes by   25% compared to controls (Dataset S2) and that these genes were significantly representative of M2 macrophages (mean diagnosticity score = 2.44, *P* < 0.0001; Figure 3). Conversely, isoproterenol down-regulated the expression of 113 genes by   25% difference (Dataset S2), and these genes were significantly representative of M1 macrophages, although as can be seen in Figure 3, this M1 suppressive effect was smaller in comparison to the M2 promoting effect (mean diagnosticity score = −0.86, *P* < 0.001). Confirmatory qRT-PCR results verified   25% differential expression for 5 target genes assayed (*P* values < 0.05; Figure 4). Thus, this transcriptome-wide approach confirms results from the targeted *a priori* analyses above in locating β-adrenergic-stimulated macrophages on the M2 side of the M1–M2 spectrum and demonstrates that β-adrenergic signaling accomplishes its M2 effect not only by promoting M(IL-4) gene expression on the M2 side but also by actively suppressing M(IFNγ) gene expression on the M1 side.

3.3. Transcriptional regulation in the β-adrenergic-stimulated macrophage

To assess the role of established M1–M2 spectrum STAT family transcription factors, as well as hypothesized β-adrenergic-related transcription factors, in the effect of β-adrenergic signaling on macrophage polarization, we tested for over-representation of transcription factor binding motifs for CREB, C/EBPβ, ATF, and STAT transcription factors in the promoters of genes that were up- and down-regulated by isoproterenol. We found that CREB binding motifs (TRANSFAC V$CREB_01) were significantly more prevalent among genes up-regulated by isoproterenol vs. down-regulated genes (mean fold difference [MFD] = 1.39, *P* = 0.026; Figure 5). Similarly, C/EBPβ and ATF binding motifs (TRANSFAC V

$CEBPB_02 and ATF_01) were significantly more prevalent among up-regulated genes (MFD = 1.36, P = 0.008 and MFD = 1.34, P = 0.039, respectively; Figure 5). These results are in accord with previous research implicating CREB, C/EBPβ, and ATF1 in the up-regulation of M2-spectrum genes.[12–14]

In contrast, STAT3 binding motifs (TRANSFAC V$STAT3_01) were significantly more prevalent among genes down-regulated by isoproterenol (MFD = 0.41, P = 0.024; Figure 5), which may be considered aberrant for an M2-spectrum macrophage, given the established role of STAT3 in promoting M2-like gene expression in M(IL-10) macrophages. STAT1 binding motifs (TRANSFAC V$STAT1_01) were also more prevalent among down-regulated genes, as might be expected, given its established role in mediating gene expression in M1-spectrum macrophages, but this result was not significant (MFD = 0.84, P = 0.421; Figure 5). Binding motifs that are receptive to the whole family of STAT transcription factors, i.e., that don't discriminate STAT3 from STAT1 (TRANSFAC V$STAT_01), were also more prevalent among down-regulated genes (MFD = 0.58, P = 0.003). Considered together, these results suggest that established M1–M2 STAT transcription factors do not play a role in the effect of β-adrenergic signaling on M2-spectrum gene expression in macrophages.

3.4. Receptor specificity in the β-adrenergic signaling pathway for M2 macrophage gene expression

Given that β-adrenergic ligands may signal through any of three receptor subtypes,[25] we used selective $β_1$-, $β_2$-, and $β_3$-adrenergic antagonists to determine which subtype mediated isoproterenol effects on expression of specific gene transcripts that were previously found to be up-regulated by isoproterenol in the *a priori* qRT-PCR assays and in the genome-wide transcriptional profile. As shown in Figure 6A, $β_2$-adrenergic antagonist, ICI 118,551, abrogated isoproterenol-induced M2-spectrum gene expression (average suppression: *Arg1*, 98 ± 2%, P = 0.0005; *Il10*, 92 ± 8%, P = 0.032; *Pf4*, 95 ± 9%, P = 0.0001; *Ccl24*, 86 ± 9%, P = 0.001). In contrast, neither $β_1$- nor $β_3$-adrenergic antagonism significantly inhibited the effect of isoproterenol on the same gene transcripts (P values > 0.97; Figure 6A). Given the evident necessity of $β_2$-adrenergic signaling in the effect of isoproterenol on select gene transcripts, we further determined the sufficiency of $β_2$-adrenergic signaling by examining the effect of selective $β_2$-adrenergic agonist, formoterol, on the same gene transcripts. As shown in Figure 6B, formoterol significantly increased expression of all transcripts (P values < 0.001).

4. Discussion

β-adrenergic-stimulated macrophages did not fit cleanly into any one pre-defined category of the M1–M2 spectrum as previously defined (Murray et al., 2014; Figure 1), but did express genes that are representative of M(IL-4) and M(IL-10) categories on the M2 side of that spectrum. Comprehensive transcript origin analysis of genome-wide transcriptional profiles confirmed that β-adrenergic-stimulated macrophages primarily express transcripts indicative of M2 macrophages while suppressing transcripts indicative of M1 macrophages. The M2-promoting effects were mediated specifically through $β_2$-adrenergic receptors and

were associated with bioinformatic indications of increased CREB, C/EBPβ, and ATF transcription factor pathways, which have previously been shown to regulate M2-associated genes in other contexts.[12–14] However, these effects did not appear to be associated with the more established M1–M2 spectrum transcription factors of the STAT family. Together, these results firmly locate β-adrenergic-stimulated macrophages on the M2 side of the M1–M2 spectrum and suggest a selective transcriptional pathway for their polarity that is atypical for M2-spectrum macrophages.

These results are consistent with previous studies that found β-adrenergic signaling regulates macrophage activity in cancer and AIDS via mechanisms that could be interpreted as M2-promoting and/or M1-suppressing. For example, in the context of *Mycobacterium avium* infection, which disproportionately affects AIDS patients, it was found that epinephrine decreases macrophage expression of MHC class II proteins,[5] which feature prominently in classical macrophage activation scenarios by presenting microbial peptides to T-cells.[26] Further investigation in this line of research found that β-adrenergic signaling also suppresses nitric oxide (NO) production in M(IFNγ) macrophages, which is used to combat infectious organisms in a classical macrophage response.[4] Thus, β-adrenergic mechanisms in these instances are decidedly M1-suppressing. One possible application of the current findings then would be to investigate whether psychological and/or pharmacological interventions aimed at reducing β-adrenergic signaling in AIDS patients could reverse M1 suppression and reduce *Mycobacterium avium* comorbidity in such patients. Although a review of psychological interventions for persons with HIV has found that salutary effects on neuroendocrine regulation tend to associate with improved immune status, specific examination of macrophage function in these studies was not reported.[27]

In the context of cancer, the M2-promoting mechanisms of β-adrenergic signaling are salient. β-adrenergic-stimulated macrophages have been found to up-regulate TGF-β,[1] a well-established immunosuppressive mechanism that is being investigated for potential therapeutic blockade in cancer patients.[28] Consistent with that immunosuppressive result, the current study found that β-adrenergic signaling increased gene expression of platelet factor 4 (PF4) in macrophages (alias, Chemokine C-X-C ligand 4 [CXCL4]), which facilitates the re-growth of colon cancer after chemotherapy by suppressing anti-tumor immunity.[29] Thus, given the evidence that TAMs are necessary partners for cancer cell invasion and metastasis,[8] these findings underscore the notion that β-adrenergic signaling exerts its facilitative effects on cancer progression, at least in part, by promoting a phenotype in TAMs that is on the immunosuppressive M2 side of the M1–M2 spectrum. Although several observational studies of β-blocker usage in cancer patients exemplify possible application of these findings, i.e., where it was found that β-blocker usage associated with increased survival,[30] it is not known to what extent these links may be mediated by inhibition of M2 spectrum properties in tumor associated macrophages.

In the context of heart disease, the effects of β-adrenergic signaling on macrophage polarity are less clear. Although compelling research shows that SNS-induced β-adrenergic signaling increases both myelopoiesis[31] and subsequent macrophage accumulation in the aortas of atherosclerosis-prone mice,[6] how direct β-adrenergic stimulation of such macrophages affects their polarity and subsequent contribution to progression of atherosclerosis is

uncertain. Review of macrophage polarity in atherosclerosis suggests that both M1-like and M2-like macrophages are present in atherosclerotic lesions but also suggests that phenotype plasticity in such lesions is dependent on predominating local factors in the micro-environment.[32] This latter finding could mean that β-adrenergic stimulation continues to pull such macrophages toward the M2 side of the spectrum. However, that even being the case, possible application of the β-adrenergic/M2 finding in this context is still ambiguous because the evidence is mixed as to which macrophage phenotype may be harmful vs. protective in atherosclerosis.[32]

The present finding that β_2-adrenergic receptors mediate the effect of isoproterenol on select gene transcripts from the M2 spectrum (*Arg1, Il10, Pf4, Ccl24*) is consistent with previous studies that show β_2-adrenergic receptors are the predominant functional β-adrenergic receptors in macrophages and induce several anti-inflammatory molecules.[9,33,34] Additional research has shown that pharmacologic inhibition of mediators downstream of β_2-adrenergic receptor signaling, i.e., cAMP and PKA, can block the M1-suppressing effect of β_2-adrenergic signaling on NO production in M(IFNγ) macrophages.[4] Given that CREB transcription factor is a primary target of the cAMP-PKA signaling pathway,[15] those previous results are consistent with the present study's indication that a β_2-adrenergic-induced M2-spectrum transcriptome associates with CREB activation. Also, given the findings by others that CREB can induce C/EBPβ, which in turn can drive expression of genes associated with the M2 spectrum (i.e., *Arg1, Il10, Il13ra, Msr1,* and *Tgm2*),[12,13] the association found between the C/EBPβ transcription factor and the M2-spectrum transcriptome induced by β-adrenergic signaling in the current study is coherent. However, none of the STAT family transcription factors assessed here showed an association with the up-regulation of M2-spectrum genes by β-adrenergic signaling. In fact, the opposite was true for STAT3, as its binding motif prevalence was over-represented among down-regulated genes. Given the established role of STAT3 in mediating M2-like gene expression in M(IL-10) macrophages (see Figure 1), the failure of β-adrenergic signaling to activate this pathway may explain why selected genes from the consensus gene set for M(IL-10) macrophage (*Il10* and *Il4ra*) were not both increased by isoproterenol in our targeted *a priori* approach (i.e., *Il4ra* was not up-regulated; see Figure 2). Similarly, a binding motif that is receptive to the whole family of STAT transcription factors was significantly over-represented among down-regulated genes. Given that result, it is tempting to speculate that the suppressive effect of β-adrenergic signaling on M1-spectrum gene expression may be mediated through suppression of the prototypical M1 transcription factor, STAT1. However, prevalence of the binding motif specific to STAT1, though slightly greater among down-regulated genes, did not reach statistical significance in this study. Thus, the current data on STAT family transcription factors in this study suggests that these factors likely do not play a role in β-adrenergic-induced up-regulation of M2-spectrum gene expression. As for their role in β-adrenergic-induced down-regulation of M1-spectrum genes, future research will be required to address their potential involvement and identify other transcription factors that may be actively inhibited by β-adrenergic signaling.

It should be noted that extrapolation from in vitro models, like the one in the present study, is necessarily limited. Whereas SNS expression of catecholamines in vivo includes both norepinephrine and epinephrine, which can stimulate both α- and β-adrenergic receptors at

varying doses, we utilized only isoproterenol at a dose strong enough to induce reliable effects across experiments in this study. Given the in vivo evidence that suggests β-adrenergic blockade is sufficient to inhibit catecholamine effects on macrophage activity in animal models of cancer,[1] atherosclerosis,[6] and sepsis,[9] we limited our focus to β-adrenergic signaling in this study. However, we note the possibility that α-adrenergic signaling could play a role in those in vivo effects. Also, we only looked at changes in gene expression at one time point (24 hours) to analyze the first order (direct) effects of β-adrenergic signaling on the basal macrophage transcriptome. However, it is possible that ongoing episodes of stimulation lasting days or weeks, such as in a chronic stress paradigm, could have effects on macrophages that are not accounted for in the current study.

In summary, the present study demonstrates that $β_2$-adrenergic-stimulation induces a macrophage transcriptome that locates on the M2 side of the M1–M2 spectrum and implicates an atypical signaling pathway for this macrophage that involves CREB, ATF, and C/EBPβ activation in the absence of STAT family regulators. These findings may help illuminate the pathways by which SNS-induced β-adrenergic signaling affects macrophage-related disease processes and may suggest pharmacologic strategies for redirecting the actions of an "*M(ADRB2)"* macrophage toward more health-promoting trajectories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Genome-wide transcriptional profiles of β-adrenergic-stimulated macrophages were analyzed

- β-adrenergic-stimulated macrophages located on the M2-side of the M1–M2 macrophage spectrum

- β-adrenergic signaling effects were mediated specifically through the $β_2$-adrenergic receptor

- Effects were associated with CREB, C/EBPβ, and ATF transcription factor pathways

- Established M1–M2 spectrum STAT transcription factors were not associated with these effects
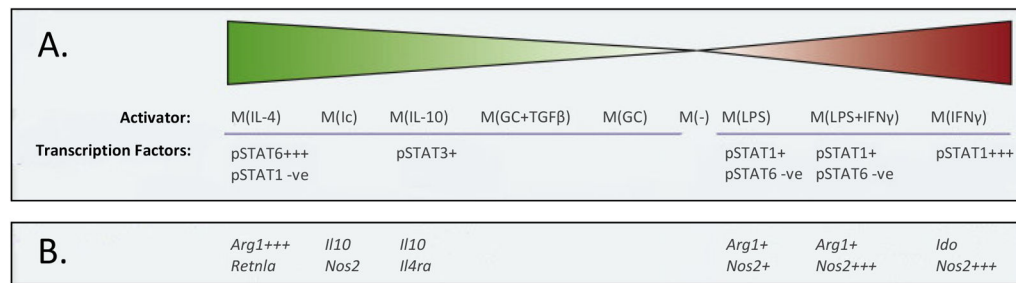
Figure 1.
(A) M1 - M2 spectrum categories of macrophage activation, based on the activator and subsequent known transcription factors, as proposed by Peter J. Murray and colleagues following the International Congress of Immunology in Milan in 2013. At the far end of the M2 side of the spectrum sits IL-4-activated macrophages, M(IL-4), followed by immune complex-activated macrophages, M(Ic), IL-10-activated macrophages, M(IL-10), glucocorticoid and transforming growth factor β-activated macrophages, M(GC+TGF-β), and glucocorticoid alone-activated macrophages, M(GC). At the far end of the M1 side of the spectrum sits IFNγ-activated macrophages, M(IFNγ), followed by lipopolysaccharide and IFNγ-activated macrophages, M(LPS+IFNγ), and lipopolysaccharide alone-activated macrophages, M(LPS). M(-) indicative of non-activated macrophages. (B) Example markers from each category's current consensus gene expression set. + signs indicative of relative expression, -ve sign indicative of no expression. Adapted from *Immunity, Vol 41, Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines, pp. 14–20, Copyright (2014),* with permission from Elsevier.
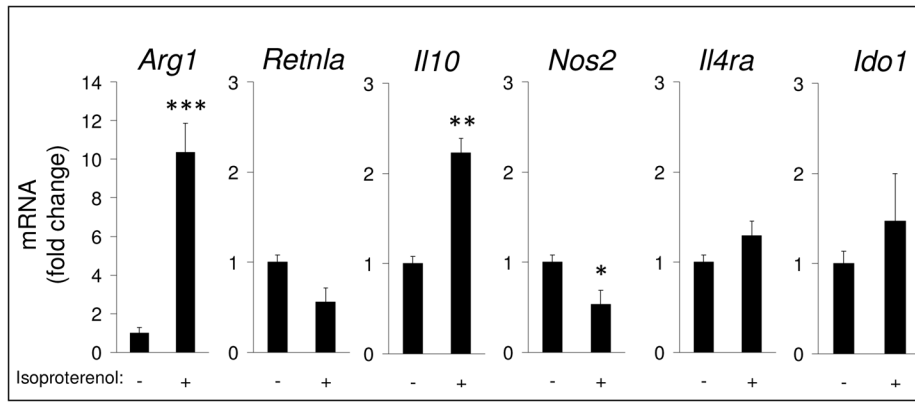
Figure 2.
Effect of β-adrenergic signaling on select gene transcripts that constitute macrophage activation categories along the M1–M2 spectrum in Murray et al., 2014. Isoproterenol at 1 μM. Data represent mean ± SE of three independent experiments. $***P < 0.001$, $**P < 0.01$.
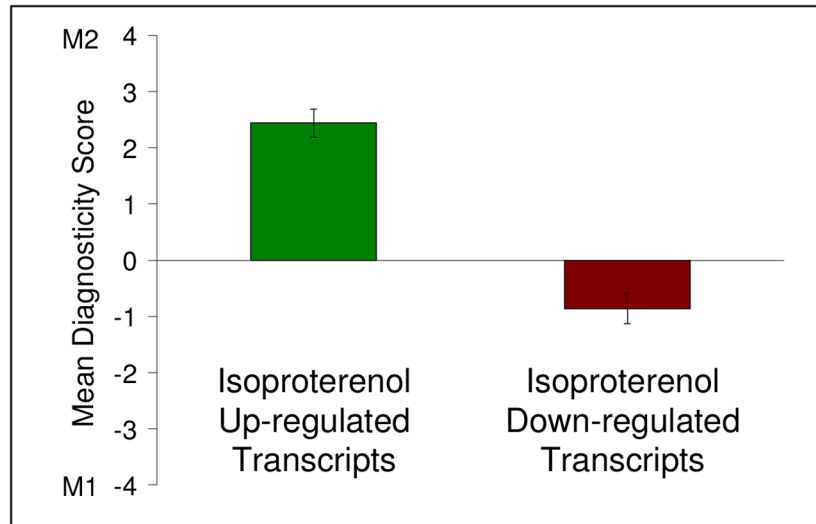
Figure 3.
Mean diagnosticity score for genes that were up-regulated or down-regulated by β-adrenergic signaling. Diagnosticity scores for each gene transcript quantified the extent to which that transcript was predominately expressed by an M2-polarized macrophage relative to an M1-polarized macrophage.
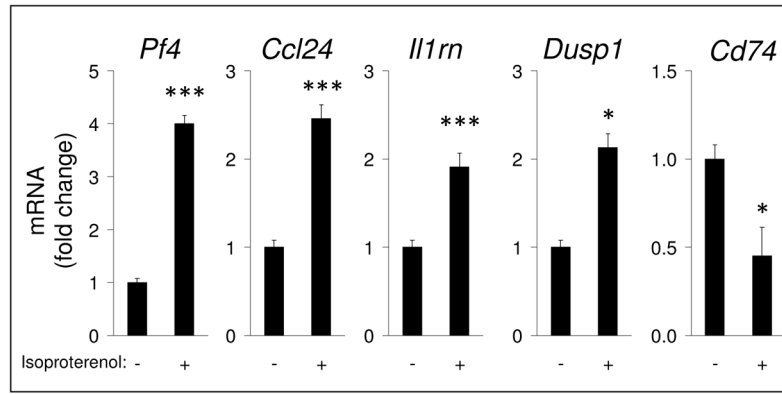
Figure 4.
Independent verification of differential expression by genes in the global gene expression profile with qRT-PCR. Isoproterenol at 1 μM. Data represent mean ± SE of three independent experiments. ***$P < 0.001$, *$P < 0.05$.
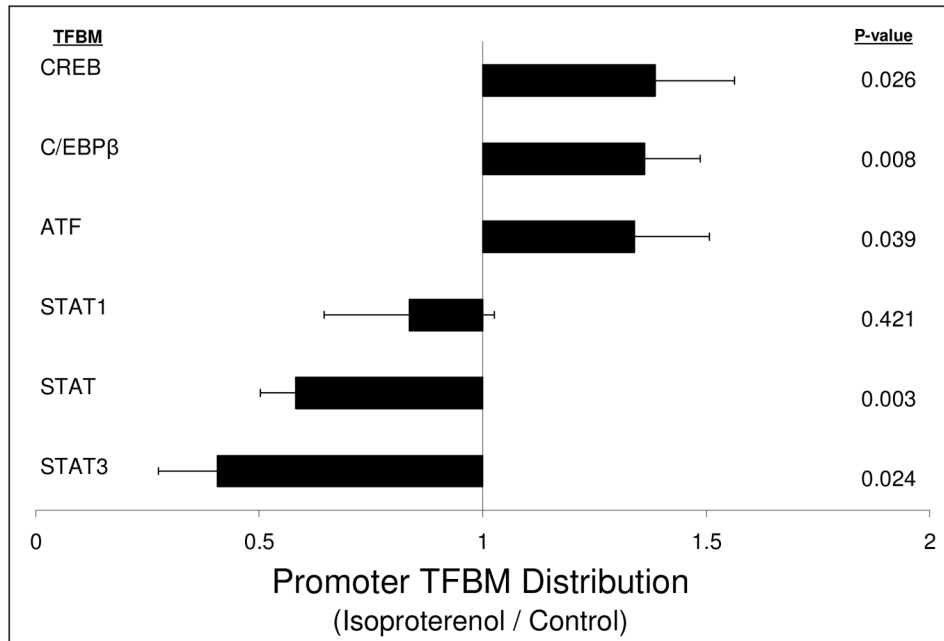
Figure 5.
Promoter-based bioinformatic analysis of transcription factors in genes differentially-regulated in β-adrenergic-stimulated vs. control macrophages. Data represent mean fold difference (mean ratio of isoproterenol/control) ±SE of transcription factor binding motifs, averaged over nine parametric combinations of promoter length and motif detection stringency. *P* values, two-tailed difference from null difference of 1.
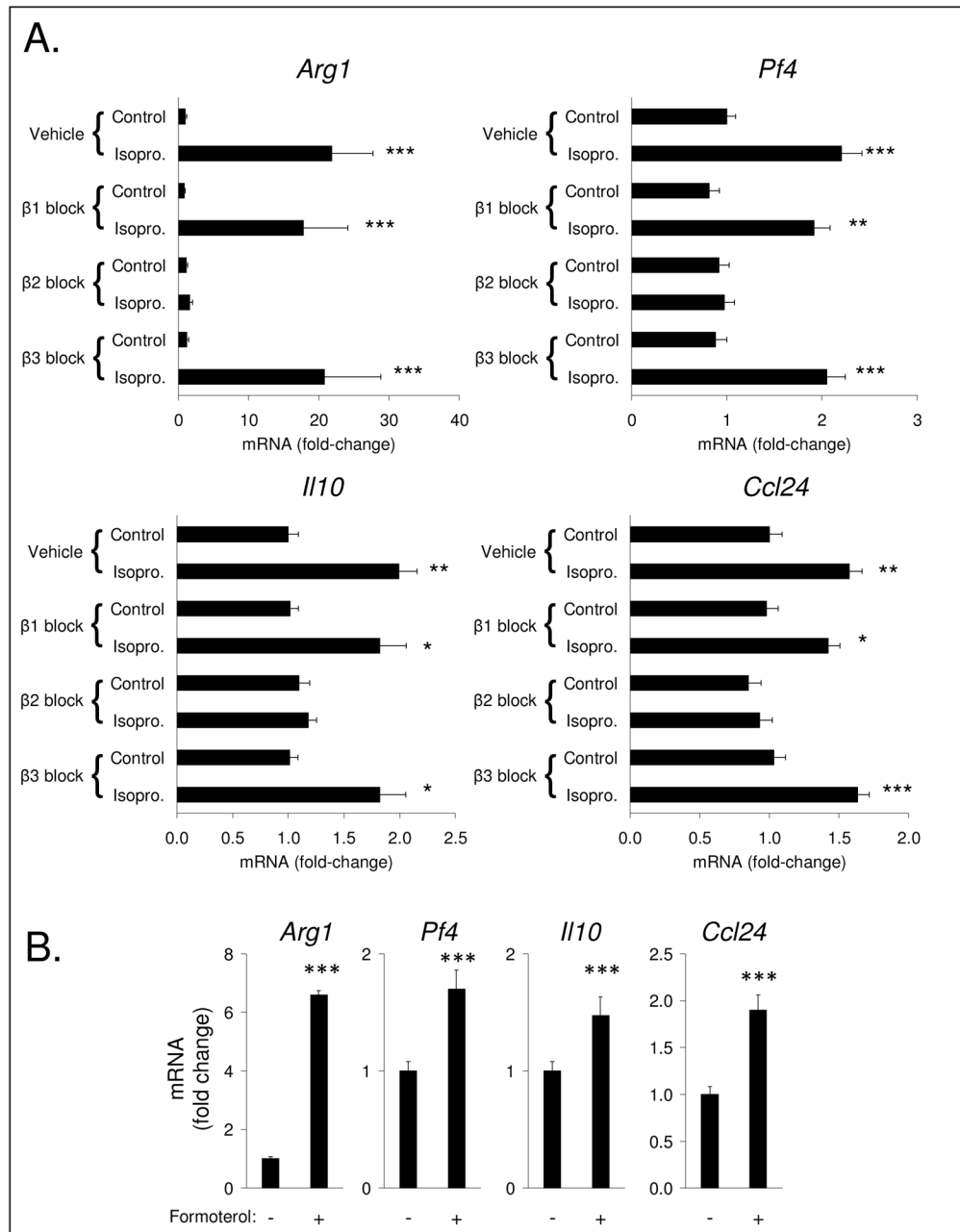
Figure 6.
(A) Effects of selective β-adrenergic antagonists on isoproterenol-induced M2 gene expression. (B) Effects of selective β2-adrenergic agonist, formoterol, on M2 gene expression. All adrenergic reagents at 100 nM. Vehicle DMSO at 0.01%. Data represent mean ± SE of three independent experiments. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. vehicle control.