Research Article

Altered Sympathetic-to-Immune Cell Signaling via $\beta_2$-Adrenergic Receptors in Adjuvant Arthritis

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Adjuvant-induced arthritic (AA) differentially affects norepinephrine concentrations in immune organs, and in vivo $\beta$-adrenergic receptor ($\beta$-AR) agonist treatment distinctly regulates ex vivo cytokine profiles in different immune organs. We examined the contribution of altered $\beta$-AR functioning in AA to understand these disparate findings. Twenty-one or 28 days after disease induction, we examined $\beta_2$-AR expression in spleen and draining lymph nodes (DLNs) for the arthritic limbs using radioligand binding and western blots and splenocyte $\beta$-AR-stimulated cAMP production using enzyme-linked immunoassay (EIA). During severe disease, $\beta$-AR agonists failed to induce splenocyte cAMP production, and $\beta$-AR affinity and density declined, indicating receptor desensitization and downregulation. Splenocyte $\beta_2$-AR phosphorylation ($p$$\beta_2$-AR) by protein kinase A ($p$$\beta_2$-AR PKA) decreased in severe disease, and $p$$\beta_2$-AR by G protein-coupled receptor kinases ($p$$\beta_2$-AR GRK) increased in chronic disease. Conversely, in DLN cells, $p$$\beta_2$-AR rose during severe disease, but fell during chronic disease, and $p$$\beta_2$-AR GRK increased during both disease stages. A similar $p$$\beta_2$-AR pattern in DLN cells with the mycobacterial cell wall component of complete Freund’s adjuvant suggests that pattern recognition receptors (i.e., toll-like receptors) are important for DLN $p$$\beta_2$-AR patterns. Collectively, our findings indicate lymphoid organ- and disease stage-specific sympathetic dysregulation, possibly explaining immune compartment-specific differences in $\beta_2$-AR-mediated regulation of cytokine production in AA and rheumatoid arthritis.

1. Introduction

The sympathetic nervous system (SNS) via its modulation of the immune system is an important player in determining the disease onset, progression, and severity in rheumatoid arthritis (RA) and animal models of RA [1, 2]. Autonomic dysregulation is reported in RA, juvenile chronic arthritis, and arthritic animals (reviewed in [3]) that support increased sympathetic activity. Treatment of rodent RA models with adrenergic drugs suggests both a permissive and suppressive role for the SNS in the pathogenesis of RA [4–6]. Blocking sympathetic signaling at disease initiation with an arthritogenic challenge or disease onset exacerbates or attenuates disease severity [5, 7], respectively, suggesting a complex role for the SNS in RA.

SNS dysregulation in RA patients is supported by reports of reduced numbers of $\beta_2$-AR receptors and functional responses of peripheral blood mononuclear cells (PBMCs) to sympathomimetic agents compared with nonarthritic, age-matched controls [8–12]. The anti-CD3 antibody, OKT3-induced PBMC proliferative responses in the presence of catecholamines are attenuated in RA compared with healthy subjects [8]. Diminished expression and activity of G protein-coupled receptor kinases (GRKs) in PBMCs occur in RA patients [13] and in animal models of RA [14]. In RA patients, PBMC
GRK-2 and GRK-6 levels are reduced, whereas, GRK-5 levels are not altered. Given that receptor ligand concentration and GRKs regulate β-AR functions, these findings indicate that sympathetic signaling in targeted immune cells is dysregulated in RA and animal models of RA.

Sympathetic nerves signal immune cells in lymphoid organs primarily through the release of norepinephrine (NE). The primary adrenergic receptors (ARs) expressed on lymphocytes are β2-ARs. β-ARs are G coupled-protein receptors that classically use the cAMP/protein kinase A (PKA) signaling pathway. The expression of β2-AR on target cells is dynamically regulated, in part by ligand availability, but also intracellularly by PKA, β-arrestins, and different GRKs [15].

β2-AR function is regulated by PKA and GRK through β2-AR phosphorylation (pβ2-AR) at different serines [16–18]. Desensitization is induced by PKA, and at higher agonist concentrations, by PKA and GRK [19–21]. PKA-induced pβ2-AR (pβ2-ARPKA) alters receptor conformation, impairing Gs and enhancing Gi protein coupling to induce transient activation of extracellular signal-regulated kinases (ERK1/2) [21]. pβ2-AR by GRK (pβ2-ARGRK), specifically GRK2, leads to β-arrestin binding to the β2-AR [21–23], altering the conformation of the β2-AR, further impeding Gs protein coupling [21] and inducing receptor internalization. Internalized β2-AR are dephosphorylated and recycled to the plasma membrane or degraded [24, 25].

Additionally, β2-AR signaling can induce sustained ERK 1/2 signaling in a β-arrestin- [15, 20, 26] and GRK-dependent manner [27, 28]. pβ2-ARGRK occurs in an agonist concentration-dependent manner by GRK 2, 5, and 6 to induce different receptor functions [18, 28, 29]. High agonist concentrations induce PKA- and GRK5/6-mediated pβ2-AR and subsequent binding to β-arrestin [30], a scaffold/adaptor protein for mitogen-activated protein kinase (MAPK) activation [31–33]. In this manner, high agonist concentrations can induce sustained ERK activation independent of the G protein pathway [27, 34, 35]. Therefore, chronically elevated sympathetic tone in patients with RA [36–39] may provide conditions for differential pβ2-AR that result in β2-AR-induced ERK 1/2 signaling.

Previously, our laboratory has reported reduced and elevated NE concentration in the spleen and draining lymph nodes (DLN) of adjuvant-induced arthritis (AA) rats, respectively, compared with non-AA controls [40]. These findings suggest differential activational states of splenic and DLN sympathetic nerves in AA, which may induce differential β2-AR signaling in immune cells from these two lymphoid organs. Consistent with this hypothesis, in rats challenged to induce AA, a model of RA, we find that TH1 versus TH2 cytokine production differs depending on the source of immune cells cultured ex vivo [41]. Despite findings that support the downregulation and desensitization of β2-ARs in peripheral blood leukocytes from RA patients, no studies have examined the extent to which leukocyte β2-ARs in animal models of RA mimic the receptor changes observed in RA. It is also not clear if leukocytes from relevant lymphoid organs, where autoreactive T cells differentiate, also demonstrate changes in β2-AR indicative of altered signaling and how such changes impact immune functions in different lymphoid organs in RA or animal models of this disease. In the present study, we tested the hypothesis that disparate TH cell cytokine profiles in different lymphoid organs reflect disease-induced and organ-specific changes of β2-AR functions. We examined β2-AR numbers, affinity, signaling, and phosphorylation patterns in immune cells from spleens and DLN of AA rats challenged with complete Freund’s adjuvant (CFA) and non-AA rats challenged with components of the CFA (mineral oil or bacterial cell wall) or saline vehicle. We report here immune organ-dependent changes in β2-AR expression, intracellular signaling, and receptor phosphorylation in AA rats and nonarthritic rats treated with one of the components of CFA, mineral or the heat-killed bacterial cell wall in saline.

2. Materials and Methods

2.1. Materials. Dried and heat-killed Mycobacterium butyricum (M. butyricum) and mineral oil were obtained from Difco (Detroit, MI) and Sigma-Aldrich Company (St. Louis, MO), respectively. CFA and M. butyricum in saline were prepared as previously described [5]. All tissue culture media and supplements were obtained from Gibco BRL (Rockville, MD), unless otherwise stated. EIA kits for cAMP determination and (125I)CYP (2200 Ci/mMole) were purchased from R&D Systems and Amersham International (Amersham Bucks, UK), respectively. OptEIA kits for the detection of interferon-γ (IFN-γ) were obtained from BD Pharmingen (Los Angeles, CA). Antibodies recognizing the β2-AR (no. sc-569) and pβ2-AR at serines 345 or 346 (PKA site; no. 16718) or 355 or 356 (GRK site; no. 16719) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CGP-12177, propranolol, and all chemicals, reagents, and buffers used for the western blotting were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

2.2. Animals. Male Lewis rats (200–250 g) were obtained from Charles River Laboratory (Wilmington, MA). Rats were housed two per cage in plastic bottom cages with soft CareFRESH bedding (Absorption, Bellingham, WA), allowed access to water and food pellets ad libitum, and placed on a 12-h-on 12-h-off light schedule. Rats were allowed to adjust to conditions in the Banner Sun Health Research Institute’s AAALAC accredited vivarium for one week prior to starting the experiments. For AA rats, food (Purina Lab Diet 5001) was placed in the bottom of the cage, and water was supplied using long-stemmed sipper tubes to ensure easy access to food and water. All rats were observed to eat and drink throughout the study. Animals were weighed weekly and observed daily to verify adequate weight gain and overall good health. Other than the development of arthritis, the general health of the animals was maintained during the course of the experiments. Protocols for the use and care of animals in this study were approved prior to beginning the experiments by the Banner Health Research Institute’s Institutional Animal Care and Use Committee and complied
with NIH guidelines for the humane use and care of research animals.

2.3. Induction of AA and Experimental Design. Rats were randomly assigned to 1 of 4 groups (n = 8 per group per sacrifice day) based on the treatment they received. The experiment was repeated twice per time point. No differences were seen between the experiments so the data for the two experiments was collapsed giving an n = 8 per group, per time point. Rats were given 0.1 mL (1) complete Freund's adjuvant (CFA); (2) mineral oil (MO); (3) M. butyricum (SMB; 30 mg/10 mL sterile endotoxin-free saline); or (4) sterile endotoxin-free saline by intradermal injection into the base of the tail. Only rats that received CFA developed AA. Control rats treated with MO or SMB served to control for the CFA vehicle or the effects of mycobacterium challenge in the absence of inflammatory arthritis, respectively. Saline-treated rats controlled for the stress of the injection and handling. A single preparation of CFA and SMB was used for this study. All CFA-immunized animals developed arthritis with similar timing of disease onset and severity. After overdose-induced anesthesia with 8% chloral hydrate, rats were sacrificed on day (D) 21 or 28 after CFA challenge or control treatments. The DLN (popliteal and inguinal lymph nodes) and spleens were harvested, and single cell suspensions were prepared. D21 and D28 represent acute and severe disease, respectively.

A separate experiment was performed to evaluate whether altered β2-AR phosphorylation in immune organs from AA rats affects the production of a cytokine that is regulated by β2-AR under normal conditions and in AA. The effects of in vivo treatment of arthritic rats with a β2-AR agonist, terbutaline, or vehicle on ex vivo production of IFN-γ by spleen and DLN cells were examined. Terbutaline (1200 µg/day) was administered by intraperitoneal (i.p.) injections twice a day in a total volume of 250 µL per injection (n = 8). Control arthritic rats were given i.p. injections of the same volume of vehicle (0.01 mM ascorbic acid in 0.9% sterile, endotoxin-free saline; n = 8). Terbutaline or vehicle treatments were started on day 12 after immunization, the time of disease onset, and continued until sacrifice. The animals were sacrificed using an overdose of 8% chloral hydrate 10.0 mL/kg body weight. On day 28, DLN and spleen were dissected and collected in preparation for culturing immune cells.

2.4. Disease Assessment. The onset and progression of arthritis was apparent based on the development of inflammation and swelling of the hind and fore limbs. The inflammatory response in arthritic rats was assessed by routine methods, as previously described [42]. Dorsoplantar widths of the hind feet were measured using a dial thickness gauge (Mitutoyo Corporation, Chicago, IL), beginning before CFA immunization and continuing approximately every other day until sacrifice. After sacrifice, the hind limbs were removed, and radiographs were taken to assess disease severity using the following settings: 400 nN, 50 kVp, and 0.4 s exposure time at 40 cm. The digitized images are printed using a Fujifilm model FM-DPL printer for analysis.

X-rays were evaluated using a grading scale, as previously reported [5]. In brief, the radiographs were coded to obscure the treatment groups, and then two independent observers subjectively rated each of the radiographs using the scale: 0 (normal), 1 (slight), 2 (mild), 3 (moderate), and 4 (severe) abnormalities in the tissue. The radiographs were scored using the 4 point scale for each of the following characteristics: (i) swelling as indicated by the width of soft tissue shadows and changes in the normal configuration of the soft tissue planes; (ii) osteoporosis as measured by bone density (recognized by increases in radiolucency relative to uninvolved adjacent bone); (iii) cartilage loss shown by narrowing of the joint spaces; (iv) heterotopic ossification defined as proliferation of new bone tissue (fine ossified line paralleling normal bone but not contiguous with calcified area of the bone itself); and (v) bone erosions. The radiographic scores for each category were added for both hind limbs giving a maximum score of 40.

2.5. Harvesting Spleen and Lymph Node Cells. After anesthetic overdose, the spleens and DLN were aseptically removed. Spleens were placed into stomacher bags containing 10 mL Hank's balanced salt solution (HBSS) and homogenized for 2 × 30 sec. Spleen cells were triturated with a 10 mL pipette, then washed with an additional 10 mL of HBSS, and passed through a 70 µm nylon mesh (Marsh Industries, Plano, TX) to remove the extraneous connective tissue. The collected cells were centrifuged at 3,000 g for 7 min and then resuspended in 5 mL of a NH₄Cl hypotonic buffer for 3 min to lyse the red blood cells. The immune cells were washed 2X with 10 mL HBSS, centrifuged, and resuspended into complete media (RPMI 1640 supplemented with 5% fetal calf serum and 1% antibiotic/antimycotic). Splenocytes were counted using a hemocytometer and then brought to a final concentration of 2 × 10⁶ cells/mL in complete media. Single cell lymph node suspensions were prepared by teasing apart the harvested lymph nodes in 5 mL HBSS using fine forceps. These cells were then triturated with a 5 mL pipette to disperse immune cells and washed with an additional 5 mL HBSS. Cells and tissue debris were separated by filtering the cell suspension through a 70 µm nylon mesh. The cells were pelleted and resuspended in 5 mL HBSS. The cells were pelleted and resuspended into complete media, counted using a hemocytometer, and brought to a final concentration of 2 × 10⁶ cells/mL.

2.6. Measurement of cAMP by Enzyme Immunoassay (EIA). To measure cAMP production, spleen cells were incubated in the presence or absence of 10⁻⁵ M isoproterenol (ISO) or forskolin, a direct activator of adenyl cyclase that bypasses the G-coupled protein receptors, in 1.0 mL of HBSS containing 1.0 mM IBMX (a cAMP phosphodiesterase inhibitor) at 37°C for 10 min. The reaction was then stopped by addition of 3 mL ice cold RPMI 1640 to the tubes. Samples then were centrifuged at 4°C for 8 min at 1,000 rpm, and the supernatants decanted. The cell pellets were resuspended in 0.5 mL 50 mM sodium acetate buffer. Splenocytes were lysed by two cycles of freezing (dry ice) and boiling (100°C water
bath) for 5 min each. The samples were centrifuged for 10 min at 3,000 rpm to remove cellular debris, and the supernatants were collected and stored at −70°C until cAMP analysis by EIA according to the manufacturer’s instructions.

2.7. Radioligand Binding Assays. Radioligand binding studies were performed on whole splenocytes from CFA- and saline-treated rats (n of 8 per group) on D21 after challenge. Binding studies were completed using the ligand (125)I-cyanopindolol, a β-AR antagonist with equal affinity for the β₁- and β₂-AR subclones. Spleen cells used for β-AR binding assays were resuspended to 5 × 10⁶ cells/mL RPMI 1640. (125)I-CYP (2200 Ci/mMole) was diluted in 1% ethanol, 5 mM HCl, and 0.2% bovine serum albumin (BSA). Assays were performed in duplicate in 13 × 100 mm polypropylene tubes containing 1 × 10⁶ spleen cells with 8 concentrations of (125)I-CYP ranging from 15.8 to 333 pM. Nonspecific binding was determined using parallel assays incubated in the presence of 10⁻⁶ M CGP-12177, a hydrophilic β-AR antagonist. Tubes were incubated at 37°C for 60 min in a shaking water bath (100 oscillation/min) to ensure that equilibrium was reached. The reaction was terminated with the addition of 3 mL of ice cold hypotonic buffer (3.8 mM KH₂PO₄, 16.2 mM K₂HPO₄, and 4 mM MnSO₄) for 20 min to lyse the cells. The reaction mixture was filtered using a cell harvester (Brandel Corp., Gaithersburg, MD) and bound radioactivity collected on Whatman fiberglass filters (GF/B) (Brandel Corp., Gaithersburg, MD). Filters were washed with 16 mL (4 × 4 mL) of ice-cold Tris-EGTA buffer to remove the unbound radioligand. Filters were removed, placed in 12 × 75 mm tubes, and counted in a gamma counter (Whatman) at 82% efficiency.

Specific binding was defined as the difference between binding of the radioligand at each concentration in the absence or presence of 1 μM propranolol. Nonspecific binding ranged from 8 to 10% of total binding. Receptor density (Bmax) and affinity (Kd) were determined using an iterative nonlinear regression curve fitting program, Prism, version 4, to a model of a single class of homogenous binding sites. Data were transformed into linear form by Scatchard analysis. Lines of best fit were generated using the Bmax and KD to determine the X and Y axis intercepts. The maximal number of binding sites per cell was calculated based on simple stoichiometric assumptions (1 molecule of ligand binding to 1 receptor site) and expressed as sites/cell.

2.8. β₂-AR and β₂-AR Western Blots. Spleen and DLN cell pellets containing 5 × 10⁶ cells were lysed by addition of 1 mL of ice cold RIPA buffer (50 mM Tris 1% IGEPAL, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM EGTA, and 1 mM NaF; pH 7.4) containing a cocktail of protease inhibitors (Complete EDTA-free, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor (Phosstop, Roche Diagnostics). Cell samples were placed on ice, vortexed every 10 min for 30 min, and then centrifuged at 14,000 rpm for 5 min. Supernatants were collected for western blot analysis and Bradford protein determination. Samples (20 μg protein) were treated with reducing agent, denatured at 95°C for 5 min, and then cooled on ice. The samples were loaded onto a 4–12% acrylamide gel, ran at a constant 70 V through stacking, and then increased to 200 V for resolving of the protein bands. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 400 mA for 1 h. The membranes were blocked for 1 h using Tris-buffered saline and Tween 20 (TBST, 10 mM Tris HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.4)/5% milk and washed in TBST for 5 min (six times). The membranes were then placed in TBST/5% milk containing primary antibody (β₂-AR) (catalog number, SC569; 1:200), pβ₂-AR (Ser 345/346; catalog number, SC16718; 1:300), or pβ₂-AR (Ser 355/356; catalog number, SC16719R; 1:300) and then incubated overnight at 4°C. The blots were washed in TBST for 5 min (six times) at room temperature and then placed into TBST/5% milk containing biotinylated anti-rabbit (1:3,000) secondary antibody for 1 h. The membranes were blocked for 1 h with TBST/5% milk and then incubated with Supersignal Chemi Substrate (Pierce Chemical Company, Rockford, IL) for 5 min at room temperature then apposed to CL-Xposure Film (Pierce Chemical Company). Antibodies were stripped from the blots by applying Restore Stripping Buffer (Pierce Chemical Company) for 15 min at 37°C with gentle agitation. The blots were then washed twice in TBST for 10 min each and blocked for 1 h with TBST/5% milk. The blots were then reprobed for β-actin (1:5,000) as a loading control. Analysis of blots was completed using MCID 7.0 software (Image Research Inc., ON, Canada), and GraphPad PRISM software was used for data analyses.

2.9. Cell Culture for Cytokine Production. Spleen and DLN cells suspended at a final concentration of 2 × 10⁶ cells/mL were plated into 24-well plates (Falcon, Oxnard, CA) and then placed into an incubator set at 7% CO₂ and 37°C for 24 h. After 24 h, the supernatants were harvested and stored in the freezer at −80°C. ELISAs for IFN-γ were completed with standards run on each plate and according to the manufacturer’s directions. Plates were read at 450 nm within 30 min with λ correction 570 nm using an ELISA plate reader (Ceres 900 HDI, Bio Tek Instruments Incorporated, Winooski, VT) and cytokine levels determined by comparing optical densities from a standard curve created using known concentrations of IFN-γ present on each plate.

2.10. Statistical Analysis

2.10.1. Disease Outcome Measures. The average of the right and left foot pad measurements was obtained for each animal, and then these individual means were averaged within each group, expressed as a mean ± standard error of the mean (SEM), and analyzed using a two-way analysis of variance (ANOVA) with repeated measures. Significant ANOVA (P < 0.05) values were subjected to Bonferroni posthoc testing. The sum of the radiographic scores for the hind limbs was averaged within the treatment groups and expressed as a group mean ± SEM and subjected to Kruskal-Wallis statistical analysis (nonparametric statistic equivalent to an ANOVA; P < 0.05) followed by Dunn posthoc testing.
2.10.2. cAMP Production, β2-AR Binding Assays, Cytokine Production, and β2-AR Protein Levels. Replicated individual optical densities (OD) from β2-AR western blots normalized to β-actin, β2-AR $K_D$ and $B_{max}$, or fmol cAMP/2 × 10^6 cells were averaged for each animal and then for each treatment group. Data were expressed as a mean ± SEM and analyzed using one-way ANOVA with Bonferroni post hoc testing. OD obtained for pβ2-AR$_{PKA}$ and pβ2-AR$_{GRK}$ were also expressed as a ratio of the β2-AR$_T$ OD. IFN-γ concentrations from duplicate wells were averaged, group means were calculated, and the data are expressed as a mean in pg/mL. Significant differences between treatment groups for mean β2-AR, pβ2-AR$_{PKA}$ and pβ2-AR$_{GRK}$ OD and the ratios for pβ2-AR$_{PKA}$ and pβ2-AR$_{GRK}$ to β2-AR$_T$ were determined using one-way ANOVA ($P < 0.05$) followed by Bonferroni post hoc analysis. Differences between treatment groups for production of IFN-γ were determined using a Student's t-test ($P < 0.05$). Only data where significant differences ($P < 0.1$) between treatment groups were observed will be described.

3. Results

3.1. Disease Severity. Animals challenged with saline, SMB, or MO developed no clinical signs of arthritis throughout the duration of the experiment (Figure 1(a)). Erythema was apparent in CFA-challenged rats by D8 after CFA challenge. Dorsoplantar foot pad width increased in the CFA-challenged animals, reaching significance ($P < 0.0001$) between D12–14 compared with Saline-, SMB-, or MO-treated rats (Figure 1(a)) (CFA versus Saline, CFA versus SMB, and CFA versus MO: $P < 0.0001$ at each time point). Dorsoplantar foot pad widths of CFA-challenged rats continued to increase through D21–24 (severe disease) and then stabilized through D28 (chronic disease). Radiographic analysis of the ankle joints (Figure 1(b)) revealed significant differences between treatment groups ($P < 0.0004$). CFA-challenged rats had increased soft tissue swelling, bone loss, periosteal bone formation, narrowing of their joint spaces, and reduced bone density by D28 after CFA challenge compared with the Saline- ($P < 0.01$), MO- ($P < 0.001$), or SMB- ($P < 0.01$) challenged rats.

3.2. β-AR-Stimulated cAMP Production in Spleen Cells. On D21, unstimulated splenocytes from Saline- and CFA-challenged rats contained similar baseline levels of cAMP. Treatment with forskolin (Figure 2) significantly increased splenocyte cAMP production in nonarthritic and CFA-treated rats from basal levels (40 and 54%, resp., $P < 0.05$). Similarly, isoproterenol-stimulated cAMP production rose 40% ($P < 0.05$) higher than in unstimulated spleen cells from nonarthritic rats. In contrast, adding isoproterenol to the spleen cell suspension from arthritic rats had no effect on cAMP production.
β₂-AR density compared with Saline or MO treatment. There also 
was a significant difference (P < 0.005) in splenocyte β₂-
AR density between treatment groups on D28 (Figure 4(b)). 
Spleen cells from CFA-challenged rats had significantly 
decreased β₂-AR density compared with Saline (35%, P < 
0.001), MO (26%, P < 0.05), or SMB (29%, P < 0.01) 
treatment groups. A representative immunoblot depicting 
β₂-AR expression in splenocytes from each treatment group 
on D28 after CFA challenge is shown in Figure 4(c). No 
difference in β₂-AR expression in DLN cells was observed 
between any of the treatment groups at either time point after 
CFA challenge (Figures 5(a)–5(c)).

3.5. AA Reduces Phosphorylation of Splenocyte β₂-AR at 
Ser 345–346 (PKA Site). To determine if the lack of 
isoproterenol-induced cAMP response observed in spleno-
cytes from rats with established arthritis could be due to 
pβ₂-ARPKA with subsequent uncoupling of the receptor to 
Gs proteins, splenocyte pβ₂-ARPKA expression was assessed 
using western blots. On D21 and D28 after immunization, 
significant main effects of treatment (P < 0.0001 at both time 
points) were observed (Figures 6(a)–6(c)). On D21, expres-
sion of pβ₂-ARPKA in splenocytes from CFA-challenged rats 
was significantly reduced (P < 0.001) by 43, 47, and 
42% compared with Saline-, MO-, and SMB-challenged rats, 
respectively (Figure 6(a)). Similarly, on D28, pβ₂-ARPKA 
in splenocytes from CFA-challenged rats were significantly 
reduced (P < 0.001) by 52, 52, and 49% compared with 
Saline-, MO-, and SMB-challenged rats, respectively (Fig-
ure 6(b)). A representative immunoblot depicting pβ₂-ARPKA 
expression in splenocytes from each treatment group on D28 
after CFA challenge is shown in Figure 6(c).

ANOVA revealed significant differences in the pβ₂-
ARPKA on D21 (P < 0.0001) and D28 (P < 0.0042), 
when expressed as a pβ₂-ARPKA/β₂-AR expression (Figures 6(d) 
and 6(e)). There was a trend (P < 0.1) towards lower pβ₂-
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was significantly reduced (P < 0.001) by 43, 47, and 
42% compared with Saline-, MO-, and SMB-challenged rats, 
respectively (Figure 6(a)). Similarly, on D28, pβ₂-ARPKA 
in splenocytes from CFA-challenged rats were significantly 
reduced (P < 0.001) by 52, 52, and 49% compared with 
Saline-, MO-, and SMB-challenged rats, respectively (Fig-
ure 6(b)). A representative immunoblot depicting pβ₂-ARPKA 
expression in splenocytes from each treatment group on D28 
after CFA challenge is shown in Figure 6(c).

3.6. Spleen Cell pβ₂-AR at Ser 355–356 (GRK Site) Is Increased 
in AA Rats. Analysis of western blots revealed significant 
treatment effects on expression of pβ₂-ARGRK for both D21 
and D28 after immune challenge (P < 0.0154 and P < 0.0134, 
resp.; Figures 7(a)–7(c)). At D21, pβ₂-ARGRK in splenocytes 
from rats challenged with MO, SMB, or CFA were signif-
icantly increased (43%; P < 0.05) compared with Saline-
treated rats (Figure 7(a)). On day 28, pβ₂-ARGRK trended 
towards being reduced (P < 0.1) in SMB- compared with 
Saline-treated rats (Figure 7). Only pβ₂-ARGRK in splenocytes 
from arthritic rats were significantly increased compared 
with MO (28%) and SMB (32%; P < 0.05). A representative
Figure 3: Specific binding and Scatchard plots of (-)-[^125]I-cyanopindolol ([^125]ICYP) in whole spleen cells from rats treated with (a) Saline, (b) MO, (c) SMB, and (d) CFA. Spleen cells were incubated under equilibrium binding conditions at 37 °C with [^125]ICYP (15.8–333 pM) for 60 min, then the reaction was stopped, and the radioactivity was quantified by gamma scintillation counting. Binding assays were run in duplicate. Specific binding (sites/cell) and inset Scatchard plots (bound/free) represent mean values obtained from 8 rats in each treatment group. (e)-(f). The number of β-AR (B_{max}) expressed as sites/cell (e) and K_{D} (f) on spleen cells from Saline-, MO-, SMB-, and CFA-treated rats. (e) The number of β-AR sites per splenocyte is significantly decreased in SMB- and CFA-treated rats compared with both Saline- and MO-treated rats. (f) The mean K_{D} was increased in MO- and CFA-treated rats compared with rats treated with SMB. Mean values were calculated for the B_{max} and K_{D} determined from specific binding curves generated for each rat from each treatment group. Data are expressed as a mean B_{max} or K_{D} ± SEM with an n of 8 rats per treatment group. Statistics: one-way ANOVA with Bonferroni multiple comparison tests (\( * P < 0.05; ** P < 0.01; *** P < 0.001 \)).
Figure 4: Total $\beta_2$-AR expression in splenocytes from AA and non-AA rats at (a) D21 and (b) D28 after immune challenge. (a) Expression of $\beta_2$-AR was increased on D21 in splenocytes from SMB- and CFA-treated rats compared with Saline- and MO-treated rats. (b) In contrast, on D28, $\beta_2$-AR expression was significantly decreased in CFA-treated compared with all other treatment groups. (c) Representative immunoblot depicting $\beta_2$-AR expression in splenocytes from each treatment group on D28 after CFA challenge. Spleen cells were harvested, lysed, and proteins resolved by SDS-PAGE. The data were normalized to $\beta$-actin. Cellular extracts were probed with a $\beta_2$-AR receptor antibody to determine $\beta_2$-AR expression and quantified by densitometry. Each bar represents the mean optical density ± SEM with an $n$ of 8 rats per treatment group. Statistics: one-way ANOVA with Bonferroni multiple comparison tests. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Figure 5: Total $\beta_2$-AR expression in DLN cells from AA and non-AA rats at (a) D21 and (b) D28 after immune challenge ((a)–(c)). In contrast to splenocytes, expression of $\beta_2$-AR was unchanged in DLN cells from CFA-, SMB-, and MO-challenged rats on D21 or D28 compared with Saline-treated controls. DLN cells were harvested, lysed, and proteins resolved by SDS-PAGE. The data were normalized to $\beta$-actin. Cellular extracts were probed with a $\beta_2$-AR receptor antibody, and $\beta_2$-AR expression was quantified by densitometry. An example of the western blots at D28 is shown in (c). Each bar represents the mean optical density ± SEM with an $n$ of 8 rats per treatment group. A one-way ANOVA with Bonferroni multiple comparison tests was used to determine statistically significant differences between treatment groups.
Figure 6: Expression of PKA-phosphorylated-$\beta_2$-AR (p$\beta_2$-ARPKA) in splenocytes from arthritic and nonarthritic control rats. ((a)–(e)). Expression of p$\beta_2$-ARPKA in splenocytes was reduced in CFA- compared with SMB-, Saline-, and MO-challenged rats D21 (a) and D28 (b) after immune challenge. Expression of p$\beta_2$-ARPKA in splenocytes from AA and non-AA rats at D21 (d) and D28 (e) after immune challenge was normalized to total receptor (p$\beta_2$-AR) levels. p$\beta_2$-ARPKA/p$\beta_2$-AR was reduced in CFA-challenged rats compared with SMB-, MO-, and Saline-treated rats at both time points examined, indicating a disease-specific effect. Spleen cells were harvested, lysed, and total protein collected, then resolved by SDS-PAGE. The data were normalized to $\beta$-actin. Cellular extracts were probed with an antibody against phosphorylated Ser345, Ser346 of the $\beta_2$-AR and quantified by densitometry. An example of the western blots at D21 is shown in (c). Each bar represents the mean optical density ± SEM (a)-(b) or mean p$\beta_2$-ARPKA normalized to p$\beta_2$-AR ± SEM (d)-(e) with an n of 8 rats per treatment group. Data were analyzed using one-way ANOVA with Bonferroni posthoc testing (*P < 0.05; **P < 0.01; ***P < 0.001).

Western blot data for p$\beta_2$-ARGRK were also expressed as p$\beta_2$-ARGRK/β2-ARt (Figures 7(d) and 7(e)). ANOVA revealed significant differences in the p$\beta_2$-ARGRK/β2-ARt on D21 (P < 0.0181) and D28 (P < 0.0001) (Figure 7(d)). On D21, p$\beta_2$-ARGRK/β2-ARt were elevated 27% in MO-compared with Saline- or CFA-treated rats (P < 0.05) (Figure 7(d)). In MO-treated rats, this ratio trended toward an increase compared with SMB- treated rats (23%, P < 0.1). p$\beta_2$-ARGRK/β2-ARt in CFA-treated rats significantly increased (P < 0.001) compared with Saline- (44%), MO- (46%) or SMB- (52%) treated rats on D28 after immune challenge (Figure 7(e)).
Figure 7: Expression of GRK phosphorylated-β2-AR (pβ2-ARGRK) in splenocytes from arthritic and nonarthritic control rats. (a) Expression of pβ2-ARGRK in splenocytes was increased in CFA-, SMB-, and MO-challenged rats compared with saline-treated rats on D21. By D28, pβ2-AR GRK expression in spleen cells from SMB- and MO-challenged rats was reduced compared with CFA-challenged rats. No difference in pβ2-ARGRK expression between CFA- and saline-treated rats was observed. (d) Expression of pβ2-ARGRK in splenocytes from AA and non-AA rats normalized to β2-AR levels. MO-treated rats expressed greater pβ2-ARGRK/β2-AR T levels compared with saline- and CFA-treated rats on D21 after immune challenge. (e) On D28 after challenge, pβ2-ARGRK/β2-AR T was significantly increased in CFA-challenged rats compared with all other treatment groups. Splenocytes were harvested, lysed, and proteins resolved by SDS-PAGE. Cellular extracts were probed with an antibody against phosphorylated Ser355/Ser356 of the β2-AR. (c) Western blot shown is representative of the blots within each treatment group. The data were normalized to β-actin. Data are expressed as a mean pβ2-ARGRK ± SEM (a)-(b) or mean pβ2-ARGRK normalized to β2-AR T ± SEM (d)-(e) with an n of 8 rats per treatment group. Data were analyzed using one-way ANOVA with Bonferroni posthoc testing (*P<0.05; **P<0.01; ***P<0.001).

3.8. Phosphorylation of β2-AR at Ser 355-356 (GRK Site) in DLN Cells Is Increased in AA Rats. Analysis of western blots revealed significant treatment effects on expression of pβ2-AR GRK/β2-AR T in DLN cells from CFA- and SMB-challenged rats were decreased (P<0.05) on D28 compared with saline-treated rats (Figure 8(b)). In contrast, pβ2-AR GRK/β2-AR T in DLN cells from CFA- and SMB-challenged rats were decreased (P<0.05) on D28 compared with saline-treated rats (Figure 8(b)). On D28, pβ2-ARGRK/β2-AR T significantly increased (P<0.05 or P<0.001, resp.) in CFA-challenged rats compared with either the saline- or MO-treated rats (Figure 9(b)). pβ2-ARGRK/β2-AR T also significantly (P<0.01) increased in SMB- compared with MO-treated rats. There was a trend (P<0.1) for a higher ratio in SMB- compared with saline-treated rats.

3.9. Terbutaline Treatment Differentially Affected IFN-γ Production in Spleen and DLN Cells. Figure 10 shows the ex vivo secretion of IFN-γ in spleen (a) and DLN (b). IFN-γ production did not differ significantly between spleen cells obtained from arthritic rats treated with terbutaline compared with arthritic rats treated with the vehicle
In the present study, we have determined the effects of CFA, and its components, on β2-AR expression and signaling in immunocytes from lymphoid organs that mediate disease processes for RA. We report that in AA, during severe disease, β-AR agonists fail to induce cAMP production in splenocytes. Loss of β-AR-induced cAMP is accompanied by reduced affinity and expression of β-AR, indicative of receptor desensitization and downregulation. β2-AR in splenocytes and DLN cells from AA rats. In splenocytes, β2-AR in DLN cells from AA rats are desensitized and downregulated in severe AA, supporting a role for the bacterial cell wall component of CFA in this response. Collectively, our data indicate that splenocyte β2-ARs are desensitized and downregulated in severe AA, effects that are mediated via MO and SMB, respectively.

(Figure 10(a)). In contrast, terbutaline treatment in arthritic rats significantly increased DLN cell IFN-γ production compared with vehicle treatment (Figure 10(b); P < 0.05).

4. Discussion

In the present study, we have determined the effects of CFA, and its components, on β2-AR expression and signaling in immunocytes from lymphoid organs that mediate disease processes for RA. We report that in AA, during severe disease, β-AR agonists fail to induce cAMP production in splenocytes. Loss of β-AR-induced cAMP is accompanied by reduced affinity and expression of β-AR, indicative of receptor desensitization and downregulation. β2-AR in splenocytes and DLN cells from AA rats. In splenocytes, β2-AR in DLN cells from AA rats are desensitized and downregulated in severe AA, supporting a role for the bacterial cell wall component of CFA in this response. Collectively, our data indicate that splenocyte β2-ARs are desensitized and downregulated in severe AA, effects that are mediated via MO and SMB, respectively.
Figure 9: Expression of the pβ2-ARGRK/β2-ARγ in DLN cells from rats challenged with CFA, SMB, or MO compared with Saline-treated controls on D21 (a) and D28 (b). No difference in pβ2-ARGRK/β2-ARγ was observed between the MO- and Saline-treatment groups at either time point. Expression of pβ2-ARGRK/β2-ARγ was increased in CFA-, SMB- compared with MO-challenged and Saline-treated rats on D21 and D28. DLN cells were harvested, lysed, and proteins resolved by SDS-PAGE. Cellular extracts were probed with an antibody against GRK phosphorylated Ser355/Ser356 of the β2-AR. A western blot is shown (c) that is representative of the blots seen within each treatment. The data were normalized to β-actin, and pβ2-ARGRK expression was normalized to β2-ARγ. Data are expressed as a mean ± SEM with an n of 8 rats per treatment group. Data were analyzed using one-way ANOVA with Bonferroni posthoc testing (** P < 0.01; *** P < 0.001).

Western blots revealed an increase in β2-ARγ expression (membrane and intracellular) in splenocytes obtained from AA rats on D21, an effect also observed in SMB-treated controls. In contrast, our binding data show reduced cell surface expression of β2-ARs, suggesting increased internalization of β2-ARs. By D28, β2-ARs were decreased in AA rats compared with all controls, indicating a disease-specific internalization and degradation of receptors between D21 and D28. In contrast, β2-ARs levels returned to baseline in SMB-treated rats at D28, supporting recycling of β2-AR to the membrane or β2-AR synthesis keeps pace with degradation.

Others have reported reduced β-AR agonist-induced cAMP and β-AR densities in PBMC and B cells in RA patients that negatively correlate with systemic disease activity [9, 43]. In contrast, β-AR agonist-induced cAMP do not differ in CD4+ and CD8+ T cells and are increased in PBMCs in RA patients compared with healthy controls in other studies [13, 43]. These seemingly inconsistent findings may be explained, in part by, the duration of β2-AR stimulation, use of membranes versus whole cells, and/or reported increase in the incidence of polymorphisms in the β2-AR in RA patients. RA patients have a greater incidence of the Arg16 Gly polymorphism in the β2-AR compared with healthy controls, which in association with HLA-DR alleles imparts a greater risk for developing RA [44]. Further, these polymorphisms modulate the age of disease onset, with additional polymorphisms at Gln27Glu and Thr164Ile imparting an increased risk for developing arterial hypertension in RA patients [44]. Ahles et al. [45] have demonstrated that the Arg16 Gly polymorphism imparts differential signaling of β2-ARs. Similar to Arg16 encoded receptors, the polymorphisms Arg16 →Gly or Gln27 →Glu is recruited to the cell membrane to a similar extent with similar ligand binding affinities. They also have similar activation and deactivation kinetics after a single stimulation with an β-AR agonist. However, receptors encoded by Arg16, Arg16Gly, and Gln27Glu β2-AR genes display different activation kinetics after repeat activation that result in differential efficacies to generate cAMP [45] reviewed in [46]. The Arg16Gly polymorphism result in faster, more persistent, and more effective downstream signaling via cAMP regardless of its association with Gln27 or Gly27. In contrast, the Arg16 Gln27 variant demonstrates slower activation kinetics and reduced cAMP production, an effect independent of receptor internalization, as this did not differ among the three β2-AR variants. Enhanced activation of the Arg16 Gly is coupled with greater phosphorylation of the receptor by GRK-2 and a more rapid recruitment of β2-arrestin to β2-AR, which is required for enhanced activation kinetics. Once β2-AR is stimulated the altered activation kinetics is independent of activation by G proteins. Thus, reported inconsistencies in lymphocyte β2-AR-induced cAMP between RA and
patients. An understanding of the physiological and clinical relevance of $\beta_2$-AR variants could substantially enhance our understanding of mechanisms for differences among experimental subjects and patients in their response to $\beta_2$-AR agonists reviewed in [47]. Collectively, our findings in AA and reported findings in RA suggest that complex site- and cell-specific changes in signal transduction and $\beta_2$-AR polymorphisms impart different catecholamine-induced effects on lymphocyte functions in RA.

Since the SNS dampens inflammation and cell-mediated responses, impaired $\beta$-AR signaling in splenocytes of AA rats is expected to promote disease processes. However, previous studies from our group [5] and others [6] have shown that $\beta$-AR agonists reduce disease severity if administered from disease onset through severe disease. Disruption of $\beta$-AR signaling in splenocytes after disease onset suggests that the attenuating effects of $\beta$-agonists on disease outcome are not due to regulation of splenocyte function via the $\beta$-AR-Gs-cAMP pathway. Alternatively, the attenuating effects of $\beta$-AR agonists could reflect signaling via cAMP in other secondary lymphoid organs and/or at the affected joints.

Loss of $\beta$-agonist-induced cAMP response and $\beta_2$-AR desensitization observed in this study is likely mediated, in part by, altered NE availability. While sympathetic activity is increased in RA (reviewed in [39]), no studies have directly assessed sympathetic nerve activity or NE turnover locally in lymphoid organs from animal models of RA; however, other data provide indirect support for this hypothesis. Loss and reorganization of sympathetic nerves in the spleen [48] are consistent with chronically high NE levels [40, 49] that subsequently auto-destroy NA nerves in target tissues [50]. Elevated plasma NE levels in AA rats at D1–5 or D10–21 after adjuvant challenge have been reported [49] and reflect spillover of NE released from sympathetic nerves in innervated tissues. Splenic and DLN NE concentrations in AA rats are reduced and elevated in chronic disease, respectively, supporting altered sympathetic nerve activity that is site specific [40]. Further, central and peripheral injection of proinflammatory cytokines, particularly, IL-1 and IL-6 (cytokines elevated in RA) increase SNS activity in the spleen [51–53].

Desensitization and downregulation of $\beta$-AR are dependent on p$\beta_2$-AR by PKA and GRK, which occurs via site-specific phosphorylation at different serines/threonines [16–18]. Patterns of p$\beta_2$-AR in splenocytes are disease specific. In AA rats, p$\beta_2$-ARs at the PKA site are reduced on D21 and D28, while p$\beta_2$-ARs at the GRK site are unchanged on D21 and elevated on D28 compared with each control group. p$\beta_2$-AR by GRK2 uncouples $\beta_2$-AR from Gs protein and facilitates the binding of $\beta$-arrestin to the receptor causing receptor downregulation by internalization [21]. Thus, the observed changes in splenocyte p$\beta_2$-AR by PKA and GRK provide a mechanism for $\beta_2$-AR downregulation and alternative second messenger signaling observed in splenocytes from AA rats.

Total $\beta_2$-AR protein and its p$\beta_2$-AR patterns in splenocytes and DLN cells are strikingly different in AA rats. On D21 and D28, the $\beta_2$-AR$_F$ protein levels in DLN cells are similar in all treatment groups, indicating no effect of AA induction on $\beta_2$-AR expression. Unlike the spleen, p$\beta_2$-AR$_{PKA}$ are

healthy controls may result from differences in duration and repetitiveness of receptor stimulation.

It is not known whether different rodent strains also have variants in genes encoding $\beta_2$-AR. If they do, they could explain differences in strain susceptibility to induction of inflammatory arthritis. If so, these strains could provide models for understanding the contribution of $\beta_2$-AR polymorphisms to disease onset and severity in RA.
increased on D21 in DLN cells from CFA- and SMB-treated rats compared with the other control groups (MO and Saline). These findings suggest that the cell wall component of the adjuvant is responsible for increased pβ2-AR PKA. By D28, pβ2-AR PKA site is reduced in CFA-, SMB-, and MO-treated rats compared with the Saline control group. In contrast, on D21 and D28, pβ2-AR GRK is greater in CFA- and SMB-treated rats than in rats treated with MO or Saline. These findings indicate a role for the mycobacterial cell wall component of CFA in driving pβ2-AR GRK and therefore implicate TLRs in regulating pβ2-AR GRK, particularly TLR2 and TLR4 [54, 55]. Different GRKs (i.e., GRK2 versus GRK5/6) may be responsible for the disparate pβ2-AR GRK patterns in spleen and DLN cells [18, 27, 28]. pβ2-AR by GRK5/6 shifts signaling into the ERK 1/2 cascade in HEK-293 cells treated with high physiological concentration of β-agonists [15, 20, 26].

Proinflammatory cytokines elevated in inflammatory arthritis may also contribute to the altered pβ2-AR GRK as IFN-γ and IL-6 or TNF-α, IL-1, and IL-12 induce different expression of GRKs and pβ2-AR in PBMC from healthy controls [56–58]. Given that the SNS is crucial for maintaining homeostasis of the immune system, future research aimed at elucidating the mechanisms responsible for differential pβ2-AR by PKA and GRKs and their consequences for immune function in AA is required to understand disease-specific SNS-immune crosstalk in RA. Of significance for RA, the ERK 1/2 signaling pathway increases IFN-γ production in AA CD4+ TH cells, cells that are prevalent in the arthritic joints and drive disease processes [59–61]. It is unclear whether stimulation of T cell β2-AR can increase IFN-γ by activating ERK 1/2 under conditions of chronically elevated NE release. If so, it would provide a mechanism to explain the increased IFN-γ production after β2-AR stimulation in activated T cells [62], in splenic T cells shortly after onset of collagen-induced arthritis [7, 63] and DLN on day 28 after CFA-challenge in AA rats [41]. Many lymphocyte subsets are well known to express β2-AR, including CD4+TH1, CD4+ T cells, NK cells and B cells. Recently, CD4+FoxP3+ T regulatory (Treg) cells have been demonstrated to express β2-AR, and when stimulated, enhances their suppressive activity in a cAMP-PKA-dependent manner [64]. Further, the activation of β2-ARs on Treg cells enhances the ability of Treg cells to convert from CD4+CD62L+FoxP3+ to cells FoxP3+ inducible Treg (iTreg) cells, an effect mediated by cAMP-PKA pathways. These cells are essential for maintaining tolerance and regulating adaptive immune responses [64, 65]. A lack of Treg cells is linked to the promotion of autoimmune disease development in both mice and humans [66–70]. Consistent with a role for the SNS in regulating Treg cell functions and their importance in autoimmune diseases, chemical sympathectomy with 6-hydroxydopamine increases the number of these cells in the spleen and lymph nodes in experimental autoimmune encephalomyelitis, a model for multiple sclerosis, an effect associated with reduced disease severity [71]. Cytokine profiles in AA rats indicate multiple lymphocyte subsets are likely to contribute to the β2-AR changes observed in this study. Given that multiple T lymphocytes express β2-AR and the importance of interactions between various T cell subtypes, it will be necessary to determine the T cell subtypes responsible for the altered β2-AR expression, signaling and phosphorylation reported here. Future studies to understand the mechanism for altered β2-AR signaling in different lymphoid compartments in specific immune cell subsets in our model and in RA are required. An understanding of β2-AR signaling changes that occur in RA also will be required before the potential to use β2-AR agonists are realized for treatment of RA. Understanding the mechanisms for altered β2-AR signaling may also lead to the improvement of current clinical use of β2-AR agonists.

Conflict of Interests

The authors declare that they have no conflict of interests.

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References

[1] C. G. O. Baerwald, G. R. Burmester, and A. Krause, “Interactions of autonomic nervous, neuroendocrine, and immune systems in rheumatoid arthritis,” Rheumatic Disease Clinics of North America, vol. 26, no. 4, pp. 841–857, 2000.
[2] D. Lorton, C. Lubahn, and D. L. Bellinger, “Potential use of drugs that target neural-immune pathways in the treatment of rheumatoid arthritis and other autoimmune diseases,” Current Drug Targets. Inflammation and Allergy, vol. 2, no. 1, pp. 1–30, 2003.
[3] F. A. Koopman, S. P. Stooft, R. H. Straub, M. A. Van Maanen, M. J. Vervoordeldonk, and P. P. Tak, “Restoring the balance of the autonomic nervous system as an innovative approach to the treatment of rheumatoid arthritis,” Molecular Medicine, vol. 17, no. 9–10, pp. 937–948, 2011.
[4] J. D. Levine, S. J. Dardick, and M. F. Roizen, “Contribution of sensory afferents and sympathetic efferents to joint injury in experimental arthritis,” Journal of Neuroscience, vol. 6, no. 12, pp. 3423–3429, 1986.
[5] C. L. Lubahn, J. A. Schaller, D. L. Bellinger, S. Sweeney, and D. Lorton, “The importance of timing of adrenergic drug delivery in relation to the induction and onset of adjuvant-induced arthritis,” Brain, Behavior, and Immunity, vol. 18, no. 6, pp. 563–571, 2004.
[6] A.-M. Malfait, A. S. Malik, L. Marinova-Mutafchieva, D. M. Butler, R. N. Maini, and M. Feldmann, “The β2-adrenergic agonist salbutamol is a potent suppressor of established collagen-induced arthritis: mechanisms of action,” *Journal of Immunology*, vol. 162, no. 10, pp. 6278–6283, 1999.

[7] P. Härlé, D. Möbius, D. J. Carr, J. Schölmerich, and R. H. Straub, “An opposing time-dependent immune-modulating effect of the sympathetic nervous system conferred by altering the cytokine profile in the local lymph nodes and spleen of mice with type II collagen-induced arthritis,” *Arthritis and Rheumatism*, vol. 52, no. 4, pp. 1305–1313, 2005.

[8] C. G. O. Baerwald, M. Laufenberg, T. Specht, P. Von Wichert, G. R. Burmester, and A. Krause, “Impaired sympathetic influence on lymphocyte subset-specific modulation of β2-adrenergic receptors,” *British Journal of Rheumatology*, vol. 36, no. 12, pp. 1262–1269, 1997.

[9] C. G. O. Baerwald, M. Wahle, T. Ulrichs et al., “Reduced catecholamine response of lymphocytes from patients with rheumatoid arthritis,” *Immunobiology*, vol. 200, no. 1, pp. 77–91, 1999.

[10] M. Wahle, A. Krause, T. Ulrichs et al., “Disease activity related catecholamine response of lymphocytes from patients with rheumatoid arthritis,” *Annals of the New York Academy of Sciences*, vol. 876, pp. 287–297, 1999.

[11] M. Wahle, A. Krause, S. Köker, P. Von Wichert, and C. G. O. Baerwald, “Intracellular cAMP and β2-adrenergic receptors on CD19+ lymphocytes in patients with rheumatoid arthritis,” *Annals of the New York Academy of Sciences*, vol. 876, pp. 309–311, 1999.

[12] M. Wahle, S. Köker, A. Krause, G. R. Burmester, and C. G. O. Baerwald, “Impaired catecholaminergic signalling of B lymphocytes in patients with chronic rheumatic diseases,” *Annals of the Rheumatic Diseases*, vol. 60, no. 5, pp. 505–510, 2001.

[13] M. S. Lombardi, A. Kavelaars, M. Schedlowski et al., “Decreased expression and activity of G-protein-coupled receptor kinases in peripheral blood mononuclear cells of patients with rheumatoid arthritis,” *The FASEB Journal*, vol. 13, no. 6, pp. 715–725, 1999.

[14] M. S. Lombardi, A. Kavelaars, P. M. Cobeles, R. E. Schmidt, M. Schedlowski, and C. J. Heijnen, “Adjuvant arthritis induces down-regulation of G protein-coupled receptor kinases in the immune system,” *Journal of Immunology*, vol. 166, no. 3, pp. 1635–1640, 2001.

[15] R. J. Lefkowitz and S. K. Shenoy, “Transduction of receptor signals by β-arrestins,” *Science*, vol. 308, no. 5721, pp. 512–517, 2005.

[16] R. B. Clark, B. J. Knoll, and R. Barber, “Partial agonists and G protein-coupled receptor desensitization,” *Trends in Pharmacological Sciences*, vol. 20, no. 7, pp. 279–286, 1999.

[17] H. A. Rockman, W. J. Koch, and R. J. Lefkowitz, “Seven-transmembrane-spanning receptors and heart function,” *Nature*, vol. 415, no. 6868, pp. 206–212, 2002.

[18] T. M. Tran, J. Friedman, E. Qunaibi, F. Bameur, R. H. Moore, and R. B. Clark, “Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the beta2-adrenergic receptor using phosphoserine-specific antibodies,” *Molecular Pharmacology*, vol. 65, no. 1, pp. 196–206, 2004.

[19] W. P. Hausdorff, M. Bouvier, B. F. O’Dowd, G. P. Irons, M. G. Caron, and R. J. Lefkowitz, “Phosphorylation sites on two domains of the β2-adrenergic receptor are involved in distinct pathways of receptor desensitization,” *Journal of Biological Chemistry*, vol. 264, no. 21, pp. 12657–12665, 1989.

[20] M. J. Lohse, J. L. Benovic, J. Codina, M. G. Cargon, and R. J. Lefkowitz, “β-Arrestin: a protein that regulates β-adrenergic receptor function,” *Science*, vol. 248, no. 4962, pp. 1547–1550, 1990.

[21] R. J. Lefkowitz, “G protein-coupled receptors: III. New roles for receptor kinases and β-arrestins in receptor signaling and desensitization,” *Journal of Biological Chemistry*, vol. 273, no. 30, pp. 18677–18680, 1998.

[22] N. J. Freedman and R. J. Lefkowitz, “Desensitization of G protein-coupled receptors,” *Recent Progress in Hormone Research*, vol. 51, pp. 319–351, 1996.

[23] J. G. Krupnick and J. L. Benovic, “The role of receptor kinases and arrestins in G protein-coupled receptor regulation,” *Annual Review of Pharmacology and Toxicology*, vol. 38, pp. 289–319, 1998.

[24] J. Goodman O.B. Jr., J. G. Krupnick, F. Santini et al., “β-Arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor,” *Nature*, vol. 383, no. 6599, pp. 447–450, 1996.

[25] S. A. Laporte, R. H. Oakley, J. Zhang et al., “The β2-adrenergic receptor/βarrestin complex recruits the clathrin adaptor AP-2 during endocytosis,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3712–3717, 1999.

[26] S. G. Ferguson, W. E. Downey III, A.-M. Colapietro, L. S. Barak, L. Ménard, and M. G. Caron, “Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization,” *Science*, vol. 271, no. 5247, pp. 363–366, 1996.

[27] S. K. Shenoy, M. T. Drake, C. D. Nelson et al., “β-arrestin-dependent, G protein-independent ERK1/2 activation by the β2 adrenergic receptor,” *Journal of Biological Chemistry*, vol. 281, no. 2, pp. 1261–1273, 2006.

[28] M. Trester-Zedlitz, A. Burlingame, B. Kobilka, and M. Von Zastrow, “Mass spectrometric analysis of agonist effects on posttranslational modifications of the β2 adrenergic receptor in mammalian cells,” *Biochemistry*, vol. 44, no. 16, pp. 6133–6143, 2005.

[29] E. E. Millman, J. L. Rosenfeld, D. J. Vaughan et al., “Endosome sorting of β2-adrenoceptors is GRK5 independent,” *British Journal of Pharmacology*, vol. 141, no. 2, pp. 277–284, 2004.

[30] L. M. Luttrell, F. L. Roudabush, E. W. Choy et al., “Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 5, pp. 2449–2454, 2001.

[31] P. H. McDonald, C.-W. Chow, W. E. Miller et al., “β-Arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3,” *Science*, vol. 290, no. 5496, pp. 1574–1577, 2000.

[32] L. M. Luttrell, S. S. G. Ferguson, Y. Daaka et al., “β-arrestin-dependent formation of β2 adrenergic receptor-src protein kinase complexes,” *Science*, vol. 283, no. 5402, pp. 655–661, 1999.

[33] Y. Sun, Z. Cheng, L. Ma, and G. Pei, “β-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation,” *Journal of Biological Chemistry*, vol. 277, no. 51, pp. 49212–49219, 2002.

[34] K.-S. Kim, D. H. Park, T. C. Wessel, B. Song, J. A. Wagner, and T. H. Joh, “A dual role for the cAMP-dependent protein kinase in tyrosine hydroxylase gene expression,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 8, pp. 3471–3475, 1993.
[35] J. Pitcher, M. J. Lohse, J. Codina, M. G. Caron, and R. J. Lefkowitz, “Desensitization of the isolated β2-adrenergic receptor by β-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms,” *Biochemistry*, vol. 31, no. 12, pp. 3193–3197, 1992.

[36] W. Kuis, C. C. E. Van Steenwijk, G. Sinnema et al., “The autonomic nervous system and the immune system in juvenile rheumatoid arthritis,” *Brain, Behavior, and Immunity*, vol. 10, no. 4, pp. 387–398, 1996.

[37] I. Leden, A. Eriksson, and B. Lilja, “Autonomic nerve function in rheumatoid arthritis of varying severity,” *Scandinavian Journal of Rheumatology*, vol. 12, no. 2, pp. 166–170, 1983.

[38] F. Perry, P. H. Heller, J. Kamiya, and J. D. Levine, “Altered autonomic function in patients with arthritis or with chronic myofascial pain,” *Pain*, vol. 39, no. 1, pp. 77–84, 1989.

[39] L. Stojanovich, “Autonomic dysfunction in autoimmune rheumatic disease,” *Autoimmunity Reviews*, vol. 8, no. 7, pp. 569–572, 2009.

[40] D. Lorton, C. Lubahn, S. Y. Felten, and D. L. Bellinger, “Norepinephrine content in primary and secondary lymphoid organs is altered in rats with adjuvant-induced arthritis,” *Mechanisms of Ageing and Development*, vol. 94, pp. 145–163, 1997. Erratum in, *Mechanisms of Ageing and Development*, vol. 98, pp. 181–183, 1997.

[41] D. Lorton, D. L. Bellinger, T. Osredkar, J. Schaller, and C. Lubahn, “Sympathetic signaling in spleens is altered in adjuvant-induced arthritis (AA),” *Brain Behavior and Immunology*, vol. 25, p. S209, 2011.

[42] D. Lorton, C. Lubahn, N. Klein, J. Schaller, and D. L. Bellinger, “Dual role for noradrenergic innervation of lymphoid tissue and articular joints in adjuvant-induced arthritis,” *Brain, Behavior, and Immunity*, vol. 13, no. 4, pp. 315–334, 1999.

[43] M. Wahle, G. Hanefeld, S. Brunn et al., “Failure of catecholamines to shift T-cell cytokine responses toward a Th2 profile in patients with rheumatoid arthritis,” *Arthritis Research and Therapy*, vol. 8, no. 5, article R138, 2006.

[44] O. Malysheva, M. Pierer, U. Wagner, M. Wahle, U. Wagner, and C. G. Baerwald, “Association between β2 adrenergic receptor polymorphisms and rheumatoid arthritis in conjunction with human leukocyte antigen (HLA)-DRB1 shared epitope,” *Annals of the Rheumatic Diseases*, vol. 67, no. 12, pp. 1759–1764, 2008.

[45] A. Ahles, F. Rochais, T. Frambach, M. B¨unemann, and S. Lefkowitz, “A polymorphism-specific “memory” mechanism in the β2-adrenergic receptor,” *Science Signaling*, vol. 4, no. 185, article ra53, 2011.

[46] K. M. Small, D. W. McGraw, and S. B. Liggert, “Pharmacology and physiology of human adrenergic receptor polymorphisms,” *Annual Review of Pharmacology and Toxicology*, vol. 43, pp. 381–411, 2003.

[47] P. A. Insel, “β2-adrenergic receptor polymorphisms and signaling: Do variants influence the “memory” of receptor activation?” *Science Signaling*, vol. 4, no. 185, article pe37, 2011.

[48] D. Lorton, C. Lubahn, C. A. Lindquist, J. Schaller, C. Washington, and D. L. Bellinger, “Changes in the density and distribution of sympathetic nerves in spleens from Lewis rats with adjuvant-induced arthritis suggest that an injury and sprouting response occurs,” *Journal of Comparative Neurology*, vol. 489, no. 2, pp. 260–273, 2005.

[49] H. Tanaka, Y. Ueta, U. Yamashita, H. Kannan, and H. Yamashita, “Biphasic changes in behavioral, endocrine, and sympathetic systems in adjuvant arthritis in Lewis rats,” *Brain Research Bulletin*, vol. 39, no. 1, pp. 33–37, 1996.

[50] A. A. Teixeira, I. Azevedo, D. Branco, E. Rodrigues-Pereira, and W. Osswald, “Sympathetic denervation caused by long-term noradrenaline infusions; prevention by desipramine and superoxide dismutase,” *British Journal of Pharmacology*, vol. 97, no. 1, pp. 95–102, 1989.

[51] A. Terao, M. Oikawa, and M. Saito, “Tissue-specific increase in norepinephrine turnover by central interleukin-1, but not by interleukin-6, in rats,” *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 266, no. 2, pp. R400–R404, 1994.

[52] J. C. Meltzer, B. J. MacNeil, V. Sanders et al., “Stress-induced suppression of in vivo splenic cytokine production in the rat by neural and hormonal mechanisms,” *Brain, Behavior, and Immunity*, vol. 18, no. 3, pp. 262–273, 2004.

[53] B. G. Helwig, R. A. Craig, R. J. Fels, F. Blecha, and M. J. Kenney, “Central nervous system administration of interleukin-6 produces splenic sympathoexcitiation,” *Autoimmune Neuroscience*, vol. 141, no. 1-2, pp. 104–111, 2008.

[54] J. Fan and A. B. Malik, “Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors,” *Nature Medicine*, vol. 9, pp. 315–321, 2003.

[55] K. Loniewski, Y. Shi, J. Pestka, and N. Parmeswaran, “Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages,” *Molecular Immunology*, vol. 45, no. 8, pp. 2312–2322, 2008.

[56] A. Krause, A. Steitz, P. Von Wichert, and C. Baerwald, “Influence of cytokines on the density of β2-adrenergic receptors on peripheral blood mononuclear cells in vitro,” *Cytokine*, vol. 7, no. 3, pp. 273–276, 1995.

[57] I. L. Korichnev and V. A. Tkachuk, “Alterations in beta-adrenoreceptor density on T-lymphocytes upon activation with interleukin-2 and phytohaemagglutinin,” *Biomedical Science*, vol. 1, no. 1, pp. 84–88, 1990.

[58] T. Nakane, T. Szentendrei, L. Stern, M. Virmani, J. Seely, and G. Kunos, “Effects of IL-1 and cortisol on β-adrenergic receptors, cell proliferation, and differentiation in cultured human AS49 lung tumor cells,” *Journal of Immunology*, vol. 143, no. 1, pp. 260–266, 1990.

[59] P. Larsson, R. Holmdahl, D. Dencker, and L. Klarekskog, “In vivo treatment with W3/13 (anti-pan T) but not with OX8 (anti-suppressor/cytotoxic T) monoclonal antibodies impedes the development of adjuvant arthritis in rats,” *Immunity*, vol. 56, no. 3, pp. 383–391, 1985.

[60] C. Pelegri, M. P. Morante, C. Castellote, M. Castell, and A. Franch, “Administration of a nondepleting anti-CD4 monoclonal antibody (W3/25) prevents adjuvant arthritis, even upon rechallenge: parallel administration of a depleting anti-CD8 monoclonal antibody (OX8) does not modify the effect of W3/25,” *Cellular Immunology*, vol. 165, no. 2, pp. 177–182, 1995.

[61] C. Pelegri, M. P. Morante, C. Castellote, A. Franch, and M. Castell, “Treatment with an anti-CD4 monoclonal antibody strongly ameliorates established rat adjuvant arthritis,” *Clinical and Experimental Immunology*, vol. 103, no. 2, pp. 273–278, 1996.

[62] M. A. Swanson, W. T. Lee, and V. M. Sanders, “IFN-γ production by Th1 cells generated from naive CD4+ T cells exposed to norepinephrine,” *Journal of Immunology*, vol. 166, no. 1, pp. 232–240, 2001.

[63] R. H. Straub, L. Rauch, A. Fassold, T. Lowin, and G. Pongratz, “Neuronally released sympathetic neurotransmitters stimulate splenic interferon-γ secretion from T cells in early type II
collagen-induced arthritis,” *Arthritis and Rheumatism*, vol. 58, no. 11, pp. 3450–3460, 2008.

[64] M. G. Guereschi, L. P. Araujo, J. T. Maricato et al., “Beta2-adrenergic receptor signaling in CD4+ Foxp3+ regulatory T cells enhances their suppressive function in a PKA-dependent manner,” *European Journal of Immunology*, vol. 43, no. 4, pp. 1001–1012, 2013.

[65] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, “Foxp3 programs the development and function of CD4+CD25+ regulatory T cells,” *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[66] S. Hori, T. Nomura, and S. Sakaguchi, “Control of regulatory T cell development by the transcription factor Foxp3,” *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[67] M. E. Brunkow, E. W. Jeffery, K. A. Hjerrild et al., “Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse,” *Nature Genetics*, vol. 27, no. 1, pp. 68–73, 2001.

[68] J. Lafaille, K. Nagashima, M. Katsuki, and S. Tonegawa, “High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice,” *Cell*, vol. 78, no. 3, pp. 399–408, 1994.

[69] F. Van De Keer and S. Tonegawa, “CD4+ T cells prevent spontaneous experimental autoimmune encephalomyelitis in anti-myelin basic protein T cell receptor transgenic mice,” *Journal of Experimental Medicine*, vol. 188, no. 10, pp. 1875–1882, 1998.

[70] T. A. Chatila, F. Blaeser, N. Ho et al., “JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome,” *Journal of Clinical Investigation*, vol. 106, no. 12, pp. R75–R81, 2000.

[71] S. Bhowmick, A. Singh, R. A. Flavell, R. B. Clark, J. O’Rourke, and R. E. Cone, “The sympathetic nervous system modulates CD4+ FoxP3+ regulatory T cells via a TGF-β-dependent mechanism,” *Journal of Leukocyte Biology*, vol. 86, no. 6, pp. 1275–1283, 2009.