

RESEARCH ARTICLE

Fusion of the β_2 -adrenergic receptor with either $G_{\alpha s}$ or β arrestin-2 produces constitutive signaling by each pathway and induces gain-of-function in BEAS-2B cells

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Abstract

The β_2 AR is a prototypical G protein-coupled receptor (GPCR) known to orchestrate different cellular responses by the stimulation of specific signaling pathways. The best-established signaling pathways for the β_2 AR are the canonical Gs pathway and the alternative β arrestin 2 (β arr2) pathway. Exploring each pathway separately remains a challenging task due to the dynamic nature of the receptor. Here, we fused the β_2 AR with its cognate transducers, $G_{\alpha s}$ and β arr2, using short linkers as a novel approach for restricting the conformation of the receptor and preferentially activating one of its two signaling pathways. We characterized the behavior of our fusion proteins β_2 AR- $G_{\alpha s}$ and β_2 AR- β arr2 in HEK293 cells by measuring their constitutive activity, transducer recruitment, and pharmacological modulation. Our fusion proteins show (a) steric hindrance from the reciprocal endogenous transducers, (b) constitutive activity of the β_2 AR for the signaling pathway activated by the tethered transducer, and (c) pharmacologic modulation by β_2 AR ligands. Based on these characteristics, we further explored the possibility of a gain-of-function mechanism in the human lung non-tumorigenic epithelial cell line, BEAS-2B cells. This immortalized human bronchial epithelial cell line has immunomodulatory properties through cytokine release mediated by β_2 AR stimulation. Our findings suggest that each signaling pathway of the β_2 AR is biased toward either the Th1 or Th2 inflammatory response suggesting a role in regulating the immune phenotype of respiratory diseases. Our data imply that

Abbreviations: GPCR, G protein-coupled receptor; 7TMR, seven transmembrane domain receptor; β_2 AR, β_2 adrenergic receptor; β arr, β arrestin; $G_{\alpha s}$, G alpha (s) subunit; PKA, protein kinase A; cAMP, cyclic adenosine 3',5'-monophosphate; MAPK, mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated kinase 1/2; ISO, isoproterenol; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate buffer saline; BEGM™, bronchial epithelial growth medium bulletkit™; BEBM, bronchial epithelial cell growth basal medium; [³H] DHA, dihydroalprenolol hydrochloride, levo-[ring, propyl-³H(N)]-; Nluc, nanoluciferase; Kd, dissociation constant; HTRF, homogeneous time-resolved fluorescence; IBMX, 3-isobutyl-1-methylxanthine; pA₂, apparent affinity; Emax, maximal response; SmBiT, small fragment of nanoluciferase; LgBiT, large fragment of Nanoluciferase.

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our fusion proteins can be used as tools to isolate the function elicited by a single signaling pathway in physiologically relevant cell types.

KEYWORDS

biased signaling, cytokine profile, G α s protein, β arrestin 2, β_2 adrenergic receptor

1 | INTRODUCTION

Seven transmembrane domain receptors (7TMR), commonly known as G protein-coupled receptors (GPCR), are biological microprocessors capable of transducing an extracellular signal into particular cellular responses. Over the past 30 years, data has shown that 7TMR can couple to multiple signaling proteins that in turn activate distinct signaling pathways, thereby allowing for a myriad of cellular responses.¹ For example, the β_2 -adrenergic receptor (β_2 AR), one of the most studied 7TMRs, can couple to the G alpha (s) subunit (G α s) protein to increase cyclic adenosine 3',5'-monophosphate (cAMP) production by activating adenylate cyclase. The increased level of cAMP production activates a cAMP-dependent protein kinase (PKA), which phosphorylates multiple effector proteins to produce a cellular response.² Alternatively, the β_2 AR can also couple to β arrestin 2 (β arr2), a scaffolding protein required for desensitization and internalization of G protein signaling, which recruits mitogen-activated protein kinases (MAPK) and increases the extracellular signal-regulated kinase 1/2 (ERK) phosphorylation. The phosphorylated ERK1/2 can activate a different set of effector molecules that ultimately produce an alternative cellular response distinct from G α s.^{3,4} Therefore, isolating each signaling pathway is useful in understanding the role the β_2 AR has in regulating the (patho)physiological processes resulting from the activation of each pathway.

The use of synthetic small molecules targeting the β_2 AR has shown that a given signaling pathway could be preferentially stimulated over other pathways relative to the endogenous hormone or reference ligand, a phenomenon known as biased signaling.⁵ For example, while epinephrine can activate both pathways, carvedilol, a beta-blocker used to treat heart failure, can induce ERK1/2 phosphorylation via the coupling of β arr2 to the β_2 AR, while not activating the cAMP-PKA pathway.⁶ However, ligands that can selectively stimulate the G α s pathway without stimulating the β arr2 pathway are not currently available. Moreover, many ligands targeting the β_2 AR, like the endogenous β_2 AR ligand epinephrine, can stimulate both pathways.^{7,8} As an effort to confine the response to one signaling pathway while minimizing the stimulation of the alternative pathway we developed fusion proteins of the β_2 AR tethered with either G α s or β arr2. Previous reports using different fusion proteins using a similar approach have shown that a 7TMR-transducer

fusion increases the constitutive (basal) activity of the pathway related to the transducer.^{9,10} However, unlike previous fusion proteins, our novel fusion proteins were designed using short linkers between the β_2 AR and the transducers G α s, or β arr2. This approach increases the proximity between the β_2 AR and the fused transducer to favor their interaction while hindering the recruitment of other endogenous transducers with the fused receptor.^{9,10}

We tested the hypothesis that our fusion proteins would restrict the signaling of the β_2 AR to the tethered transducer thus allowing us to explore both signaling pathways separately. We further characterized the functionality of the fusion proteins in HEK293 cells showing that both fusion proteins are still responsive to the pharmacological modulation by the β AR agonist Isoproterenol (ISO) and the β_2 AR inverse agonist ICI 118,551. We also tested if the fusion proteins exhibited constitutive activity, and if so, could they induce a gain-of-function in an immortalized human bronchial epithelial cell line (BEAS-2B). Our data show that each signaling pathway is constitutively activated by the cognate transducer tethered to the β_2 AR in both HEK293 and BEAS-2B cells. That is, cells transfected with the β_2 AR fused to G α s show high basal cAMP levels whereas ERK1/2 phosphorylation is constitutively elevated in cells transfected with β_2 AR fused to β arr2. Finally, given the fact that β_2 AR signaling is fundamental for the development of asthma^{11,12} and regulates cytokine/chemokine release in airway epithelial cells,¹³⁻¹⁵ we tested the proposed gain-of-function mechanism of the fusion proteins on the release of a set of inflammatory cytokines/chemokines by BEAS-2B cells. Our data suggest that the β_2 AR-G α s signaling pathway activates a type 1 immune response whereas the β_2 AR- β arr2 induces a type 2 immune response in BEAS-2B cells. In summary, our fusion proteins appear to be useful tools to constrict the receptor into constitutively activating one signaling pathway and induce a gain-of-function mechanism in a physiologically relevant cell system.

2 | MATERIALS AND METHODS

2.1 | Materials

All β_2 AR ligands were purchased from Sigma-Aldrich. Dulbecco's Modified Eagles Medium (DMEM),

Trypsin/EDTA 0.25%, Dulbecco's phosphate buffer saline (DPBS), Penicillin, G-streptomycin-amphotericin B, and fetal bovine serum (FBS) were purchased from GenDepot. Dihydroalprenolol hydrochloride, levo-[ring, propyl-³H(N)]-([³H] DHA) and FlashPlate® were purchased from PerkinElmer. 384-well small volume white plates were purchased from Greiner Bio-One.

2.2 | Constructs

The β_2 AR, G α s, and β arr2 constructs were obtained from human cDNA. The β_2 AR construct was ligated in-frame to the G α s coding region by substitution of the stop codon at the β_2 AR 3' end using an XhoI site followed by the 5' starting codon methionine of the G α s coding region. For the β_2 AR- β arr2 fusion construct, the stop codon for the β_2 AR was replaced by the sequence: 5' CTCGAGGGGGGCCCGGTACC GAGCTCGGATCCACC 3' (The underlined nucleotides represent a BamHI site for in-frame ligation to the β arr2 construct) and immediately followed by the 5' starting codon encoding for methionine of the β arr2 coding region. The β_2 AR, β_2 AR-G α s, and β_2 AR- β arr2 constructs were subcloned into the expression vector pCDNA3.1(+) (Invitrogen) for cell transfection. All procedures were done by Norclone Biotech Labs, Inc.

2.3 | Cell culture and transfection

Human embryonic kidney 293 (HEK 293) cells, a gift from Dr. Kehe Ruan, were plated in 60-mm cell culture plates and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin B. BEAS-2B cells were purchased from ATCC (ATCC® CRL-9601) plated in 100-mm cell culture plates and maintained with BEGM™ bronchial epithelial growth medium bulletkit™ (Lonza Inc). Both cell lines were kept at 37°C with 5% CO₂. Cells were stably transfected using a pCDNA3.1 vector encoding for the β_2 AR alone or fused to the protein G α s or β arr2. Lipofectamine 3000 transfection reagent (ThermoFisher Scientific) was used for transfection following the manufacturer's instructions. Forty-eight hours after transfection (*where* t = transfected), cells stably expressing the receptor (t β_2 AR), or the fusion proteins (t β_2 AR-G α s or t β_2 AR- β arr2) were selected using Geneticin (G-418) 1 mg/ml for 12–14 days, and the resultant cell colony was later maintained using 600 μ g/ml.

2.4 | RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNA was extracted from all groups using the PureLink™ RNA mini kit (Invitrogen) following the manufacturer's instructions. In brief, cells were detached with Trypsin/EDTA 0.25% and centrifuged at 300g for 5 mins at 4°C. The supernatant was removed, and the pellet was resuspended in an ice-cold lysis buffer containing 1% 2-mercaptoethanol. Mechanical lysis was performed, and the homogenized solution was centrifuged at 10,000g for 3 mins. The supernatant was mixed thoroughly with a similar volume of 70% ethanol. The mix was transferred to a column and a collection tube and centrifuged at 16,000g for 1 min at 4°C. After discarding the flow-through, the column was washed twice with washing buffers and dried by centrifugation. Finally, RNAase-free water was added to the column and centrifuged at max speed for 1 min to detach the RNA and collect the elution. The total RNA was quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific). One-step RT-PCR was then performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Life Technologies) and 200–400 ng RNA template according to the manufacturer's instructions. PCR products were analyzed via agarose gel electrophoresis. PCR samples were resolved on a 1% agarose gel containing ethidium bromide and visualized under UV light using the ChemiDoc MP Imaging System (Bio-Rad, USA). Primer sequences are listed in [Figure 1C](#).

2.5 | Detection of β arr2 recruitment

Using a structural complementation reporter system (NanoBiT®; Promega) to monitor protein-protein interactions across time, β arr2 recruitment to the β_2 AR-LgBiT or fused to the protein G α s-LgBiT or β arr2-LgBiT was measured according to manufacturer instructions. In brief, HEK293 cells were seeded in white 96-well plates at a density of 2.5×10^4 cells per well. The next day, the cells were transfected using a mixture containing 50 ng of SmBiT- β arr2 and 50 ng of the β_2 AR-LgBiT or fused with β arr2-LgBiT or G α s-LgBiT. We prepared and added 0.3 μ l of ViaFect™ Transfection Reagent (Promega Cat. No. E4982) to each well. First, we tested four receptor/ β arr2 plasmid combinations to the plasmid combination with the highest fold increase in luminescence after ligand stimulation. The plasmid combination that showed the highest luminescent signal (Receptor-LgBiT:SmBiT- β arr2, using the same expression promoter, Herpes Simplex Virus) was chosen for further experiments. The LgBiT was attached to

the C-terminal of our fusion proteins whereas the SmBiT was attached to the N-terminal of β arr2. The medium was aspirated 24 h after transfection and replaced with 100 μ l Opti-MEM at room temperature. After a 10 mins incubation, 25 μ l/well of diluted substrate (furimazine) was added and the luminescence was monitored for 10 min in the absence of ligand to obtain the baseline values. After ligand addition, luminescence was immediately recorded. β arr2 recruitment the signal was monitored every 30 s for 1 h. The luminescence values were recorded using a Synergy 2 Multi-Mode Microplate Reader (BioTek).

2.6 | Detection of G α s activity

A GloSensor cAMP biosensor (Promega) which contains a modified form of firefly luciferase was used to indirectly measure G protein activation. In brief, enzyme complementation as a result of cAMP binding to the GloSensor biosensor results in luminescence following incubation with a luciferase substrate. HEK293 cells were plated at 35,000 cells per well in white bottom 96-well plates, and 24 h thereafter, each well was transiently transfected with 50 ng of GloSensor biosensor and 50 ng of pcDNA3.1+ β ₂AR, β ₂AR- β arr2, or β ₂AR-G α s using ViaFect™ Transfection Reagent (Promega Cat. No. E4982). Cells were incubated with GloSensor reagent for 120 min at 37.5°C 48 h post-transfection. Then, cells were treated with a dose of ISO in the absence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX). After ligand addition, luminescence was recorded immediately and the luminescent signal was measured every 45 s for 55 mins. Bioluminescence values were recorded using a Synergy 2 Multi-Mode Microplate Reader (BioTek).

2.7 | Fusion proteins gene expression in the structural complementation system

To determine protein expression levels in our structural complementation constructs, we used reverse transcriptase quantitative PCR using the kit Luna® Universal One-Step RT-qPCR Kit (New England Biolabs Cat. No. E3005S) according to the manufacturer instructions. Briefly, we purified total RNA (RNeasy Micro Kit [50] Cat. No. 74004) and used 0.8 μ g of total RNA for the reverse transcription reaction. The primers used for qPCR reactions were designed by targeting the NLuc expressed at the c-termini of β ₂AR and the fusion proteins. The sequence of the primers is as follows: Forward 5'-CGGAGCGGTGAAAATGCCCTG-3' and Reverse 5'-CGTCCGAAATAGTTCAGCATG-3'.

2.8 | Membrane preparation and saturation binding assays

Non-transfected and transfected cells were grown in a 100-mm culture plate until total confluency before harvesting cells. After adding 3 ml of cold DPBS, cells were detached and centrifuged at 300g for 5 mins at 4°C. The supernatant was discarded, and the pellet was resuspended on ice-cold lysis buffer (Tris-HCl 50 mM; pH 7.4). The homogenate was centrifuged at 22,000g for 30 mins at 4°C to obtain a pellet of the membrane fraction. The pellet was resuspended in lysis buffer, homogenized, and stored at -80°C until needed. Before the saturating binding experiments, protein quantification from the thawed homogenates was performed using the bicinchoninic acid (BCA) protein assay following the manufacturer's instructions. Cell membranes were further diluted in binding buffer (Tris-HCl 50 mM, EDTA 2 mM, MgCl 12.5 mM; pH 7.4) and homogenized using a polytron homogenizer for 2 periods of 30 s at maximum speed. The resultant homogenate was added to 96 well FlashPlate® in a volume of 200 μ l per well and centrifuged at 1000g for 10 mins at 4°C. After centrifugation, the supernatant was discarded and 100 μ l of binding buffer with or without propranolol (30 μ M), to determine non-specific binding (NSB), was added to each well. Increasing concentrations of Dihydroalprenolol Hydrochloride, Levo-[Ring, Propyl-3H(N)]-([³H] DHA), from 0.125 to 8 nM, were added to separate wells of the FlashPlate® in a volume of 100 μ l to detect total binding (TB) at a constant volume of 200 μ l/well and incubated for 2 h at room temperature to reach equilibrium. The radioactivity was measured using a MicroBeta²® microplate counter (PerkinElmer Life and analytical sciences). The final curves are reported as specific binding (SB) where SB = TB - NSB. The dissociation constant (Kd) and the receptor density (Bmax) were determined using one site-specific binding regression curve (Graphpad Prism 9). The values of Kd and Bmax were converted from counts per minute to fmoles/mg of protein.

2.9 | Cyclic adenosine 3',5'-monophosphate (cAMP) measurements

To measure the cAMP accumulation as a direct response of the G α s pathway of the β ₂AR, the cAMP-Gs dynamic kit (Cisbio) was used according to the manufacturer's instructions with few modifications. In brief, cells were detached using trypsin/EDTA 0.25%, counted by the automatic Countess II Automated Cell Counter (Invitrogen), centrifuged at 300g for 5 mins, and resuspended in DMEM or BEBM™ Bronchial Epithelial Cell Growth Basal Medium (Lonza Inc.). For constitutive activity detection, 5×10^4

cells per well were used whereas 3×10^4 cells per well were used for the concentration–response curves. The adjustment of cell number was necessary to detect the sigmoidal curves at the lowest concentration range of the β_2 AR full agonist isoproterenol (ISO). A 45-min preincubation period with 100 nM of the selective β_1 AR antagonist, CGP 20712A, in a 37°C chamber with 5% CO₂ was done in all experiments to isolate the β_2 AR response. Before cells were dispensed in a volume of 5 μ l/well to 384-well small volume white plates, the phosphodiesterase inhibitor, IBMX, was added to the cell suspension for a final concentration of 10 μ M/well. The indicated concentrations of ISO or ICI 118,551 were added to individual wells and incubated for 10 or 20 mins, respectively, at room temperature. Immediately after incubation, 5 μ l of the cAMP-tagged d2 fluorophore followed by 5 μ l of the Anti-cAMP-Cryptate antibodies were added to each well. The plate was incubated for 1 h at room temperature and the fluorescent signal was read using Synergy H1 (BioTek). Since the response measured was inversely proportional to the Homogeneous Time-Resolved Fluorescence (HTRF) signal ratio, all generated data were transformed so the lowest values of the HTRF ratio for each group, equivalent to the highest levels of cAMP in that system, become 100% of the response, and the lowest HTRF ratio for each group, equivalent to the lowest levels of cAMP in the system, become the basal line (0%).

2.10 | Total ERK1/2 and PhosphoERK1/2 measurements

To measure ERK1/2 as a direct response of the β arr2 pathway of the β_2 AR, the alternative pathway to G α s of the β_2 AR, the total-ERK1/2 and the advanced phospho-ERK1/2 (THR202/TYR204) kits (Cisbio) was used to measure total (tERK) and phosphorylated ERK1/2 (pERK), respectively, following the manufacturer's instructions with some modifications. In brief, cells were seeded in a 96-well cell culture plate and incubated with supplemented DMEM or BGEM at 37°C and 5% CO₂ for 22–24 h. A total of 1.5×10^5 cells per well was used for the detection of constitutive activity, whereas 2.4×10^5 cells per well was necessary for robust concentration–response curves of ISO. Then, supplemented media was removed, and cells were starved in DMEM supplemented with 1% FBS or non-supplemented BEBM (starvation media) for 20–22 h. The media was aspirated and 100 nM of CGP 20712A was added to 40 μ l of starvation media for a 45 min preincubation period in a 37°C chamber with 5% CO₂. β_2 AR ligands were freshly prepared in the respective media and added into separate wells for a final volume of 80 μ l/well. For ISO and ICI 118,551, pERK was detected at a 5- and 90-min incubation, respectively, to achieve equilibrium. After

incubation, the media was carefully removed and 60 μ l of the supplemented lysis buffer was added to each well. The 96-well plates were thoroughly shaken at 1000 rpm for 35 mins. Then, 16 μ l of the lysed solution was added to 384-well small volume white plates followed by 4 μ l of the premixed antibody solution (Phospho-ERK1/2-d2 and -cryptate antibodies). The plate was sealed and incubated for 4 h at room temperature before the HTRF signal was detected with Synergy H1 (BioTek). The tERK was measured by adding a 4 μ l premixed solution of antibodies (Total-ERK1/2-d2 and -cryptate antibodies) to the 16 μ l of lysed solution. The data were expressed as relative ERK1/2 = pERK/tERK.

2.11 | Cytokine profile measurements

Non-transfected and transfected BEAS-2B cells were cultured in 12-well plates and grown to ~80% confluency. Then, cells were starved using BEBM™ Bronchial Epithelial Cell Growth Basal Medium (Lonza Inc.) and the medium was collected 24 h later. Samples were centrifuged at 800 g for 5 mins at 4°C and stored at –80°C until needed. The concentration of human TNF α , IL-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, CCL2, GM-CSF, and IFN γ were measured using a commercially available human multiplex cytokine assay (Eve Technologies).

2.12 | Immunofluorescence

Cells were cultured in 35-mm ibidi dishes. Once 70% confluency was reached, the media was extracted and 200 μ l of ice-cold 4% paraformaldehyde (PFA) was incubated for 10 mins. Cells were washed three times with cold PBST and blocked using 1% BSA for 30 mins. Primary antibodies directed against β_2 AR (Abcam; ab182136), G α s (Novus; NB1-49874), or β arr2 (Mybiosource; MB52522670) at a dilution of 1:300 for 1 h at room temperature. Cells were washed three times with PBST and incubated with the secondary antibodies anti-rabbit Alexa 488 (Abcam; ab150077), anti-goat Alexa 568 (Abcam; ab175474) at a concentration of 1:1000, or with anti-rabbit Alexa 594 (Jackson ImmunoResearch; 111-585-003) at a 1:200 dilution. Because two secondary antibodies had reactivity to the same species, the primary and secondary antibody incubation for each targeted protein was done separately with an extra blocking step in between to avoid cross-reactivity of the secondary antibodies. The secondary antibodies were incubated for 1 h at room temperature in a humidified chamber and washed three times with PBST. Finally, cells were mounted using fluoro-gel II with DAPI (electron microscopy services) and imaged using a DMi8 confocal laser scanning microscope (Leica).

2.13 | Statistical analysis

Except for ERK experiments, which were done in duplicate, all experiments were performed in triplicate on at least three separate occasions ($n \geq 3$). Measurements were analyzed using one- or two-way ANOVA according to the nature of the experiment. Tukey's test was used as the post hoc analysis for multiple comparisons. For cytokine experiments, the concentrations were transformed to log2 values and analyzed as mentioned previously. p values lower than 0.05 ($*p \leq 0.05$) were taken as statistically significant.

3 | RESULTS

3.1 | Fusion protein design, expression, and quantification

To independently explore the two of the most studied signaling pathways of the β_2 AR, separate DNA constructs containing either the β_2 AR fused with $G\alpha_s$ protein (β_2 AR- $G\alpha_s$) or β_2 AR fused with β arr2 (β_2 AR- β arr2),

were manufactured by Norclone laboratories. Both constructs were designed to ideally allow structural flexibility between the receptor and the fused protein while also restricting other signaling proteins to be recruited to the receptor (Figure 1A). For the β_2 AR- $G\alpha_s$ fusion protein, a 2 amino acid linker (Leu-Glu) between the C-terminal tail of the β_2 AR and the N-terminal of the $G\alpha_s$ protein was added. A 12 amino acid linker (Leu-Glu-Gly-Gly-Pro-Gly-Thr-Glu-Leu-Gly-Ser-Thr) was added between the C-terminal tail of the β_2 AR and the N-terminal of the β arr2 (Figure 1B). The length of both linkers was chosen by trial and error based on the fewest amino acids needed to avoid cytotoxicity after transfection. After stable transfection of either: the β_2 AR alone (t β_2 AR), the β_2 AR- $G\alpha_s$, or the β_2 AR- β arr2 fusion proteins in HEK 293 cells, RT-PCR was employed to detect the mRNA expression of our constructs. A forward primer targeting the β_2 AR and a reverse primer targeting either the β_2 AR, $G\alpha_s$, or β arr2 was used to detect the receptor alone and the fusion proteins (Figure 1C). As expected, the nucleotide base pairs of the non-transfected (HEK 293) group and the group transfected with the t β_2 AR alone were 1.23 kb, whereas the molecular weights were 2.5 and 2.4 kb for the groups

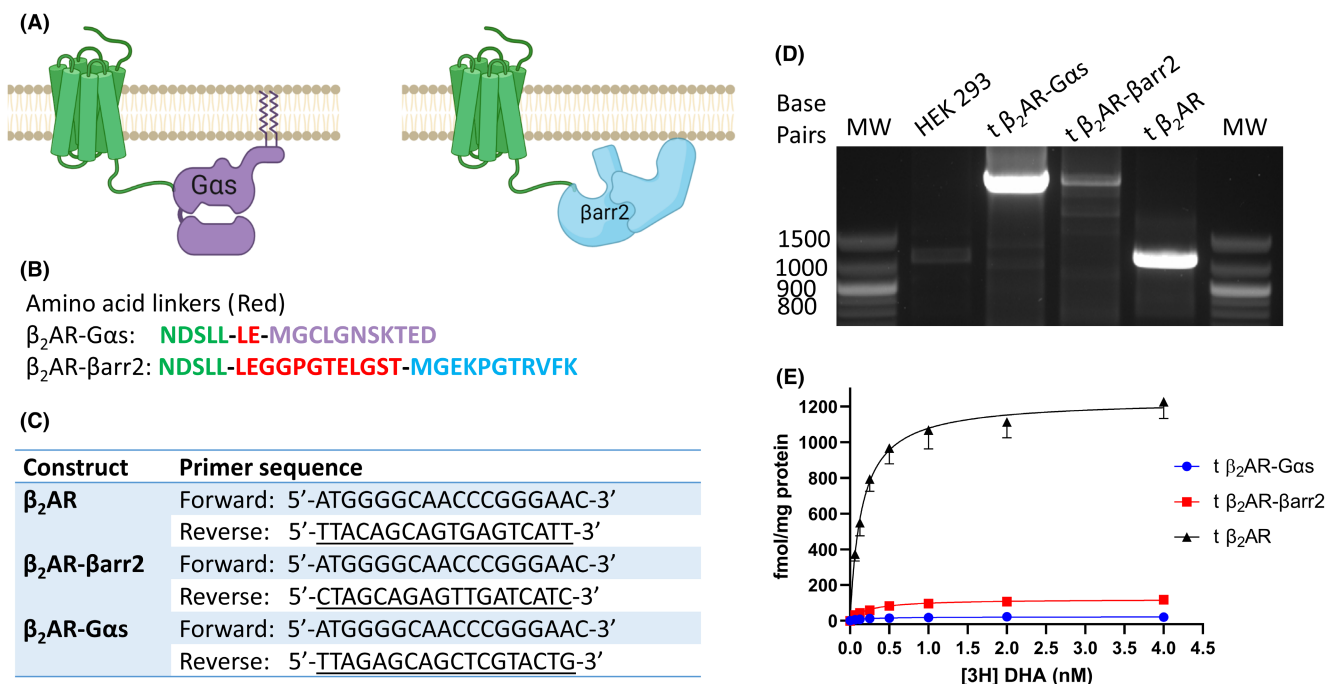


FIGURE 1 Design and expression of the fusion proteins β_2 AR- $G\alpha_s$ and β_2 AR- β arr2 in HEK 293 cells. (A) Schematic representation of the β_2 AR (green) at the membrane tethered by its C-terminal to the N-terminal of the signaling proteins $G\alpha_s$ (purple) or β arr2 (blue). (B) Amino acids (red) are used for protein linkage between the C-terminal of the β_2 AR and the N-terminal of the transducers $G\alpha_s$ and β arr2. (C) Forward and reverse primers were used to detect the genetic expression of β_2 AR alone or when fused to either $G\alpha_s$ or β arr2. (D) Reverse transcriptase-PCR shows the gene expression of either β_2 AR alone (t β_2 AR) and when fused to $G\alpha_s$ (t β_2 AR- $G\alpha_s$) or β arr2 (t β_2 AR- β arr2). The first and last columns show the molecular weight ladder for base pair quantification. (E) Saturation binding assays using increasing concentrations of [3H] dihydroalprenolol (DHA) in all transfected groups. The transfected group with the β_2 AR alone (black) showed a $K_d = 147.6 \pm 28$ nM. The β_2 AR- $G\alpha_s$ (blue) and β_2 AR- β arr2 (red) showed a $K_d = 213 \pm 49$ nM and 232.7 ± 22 nM, respectively. Data shown are the means \pm SEM from three independent experiments. MW, molecular weight

transfected (t) with either the β_2 AR-G α s (t β_2 AR-G α s) or β_2 AR- β arr2 (t β_2 AR- β arr2) fusion proteins, respectively (Figure 1D). To further quantify the protein expression of all transfected groups, we performed saturation binding assays using [3 H] DHA. The expression of the t β_2 AR group was at least 10 times higher (1.238 ± 0.106 pM/mg) than the fusion protein groups (t β_2 AR-G α s = 22.08 ± 1.3 fmol/mg; t β_2 AR- β arr2 = 121.3 ± 6 fmol/mg) (Figure 1E). Although the functional response of the β_2 AR in the wild-type (WT) HEK 293 group was quantifiable (see Suppl. Figures 4 and 5), there was no adequate signal in radioligand binding assays (low signal-to-noise ratio) to allow quantification of the receptor expression.

3.2 | The fusion proteins show constitutive activity for a single pathway and sterically hinder the reciprocal protein from coupling to the receptor

Since the fusion of a signaling protein with the receptor forces both proteins to be in close proximity, we tested the constitutive activity of the fusion proteins in the absence of ligand on the G α s signaling pathway, through cAMP accumulation, and on the β arr2 signaling pathway, through ERK1/2 phosphorylation. For the G α s pathway, all transfected groups showed increased constitutive activity compared to the HEK 293 group (Figure 2A). By adding 10 μ M ICI 118,551 (a selective β_2 AR antagonist/inverse agonist) the constitutive activity of the t β_2 AR- β arr2

was completely inhibited, whereas the t β_2 AR-G α s activity remained highly increased and the t β_2 AR was reduced by half (Figure 2A). For ERK1/2 phosphorylation, the only group with a significant increase in constitutive activity compared to the HEK 293 control group was the t β_2 AR- β arr2 (Figure 2B). To test the possibility that the constitutive increase in ERK1/2 phosphorylation observed in the HEK 293 group transfected with the t β_2 AR- β arr2 fusion was mediated by G α i, we used the G α i/o inhibitor, pertussis toxin. Our results show that pertussis toxin treatment had no effect on the increased constitutive activity of the t β_2 AR- β arr2 fusion protein for ERK1/2 phosphorylation suggesting the enhanced ERK1/2 phosphorylation is not mediated by G α i (Suppl. Figure 2).

Because recent studies showed the formation of a GPCR-G protein- β arrestin super-complex with sustained activation and signaling of G α s proteins,^{16,17} we assessed for the potential protein-protein interactions of the β_2 AR-G α s or β_2 AR- β arr2 fusion proteins with the reciprocal transducers, β arr2 and G α s. The direct recruitment of β arr2 was assessed by a nano luciferase structural complementation reporter system [NanoBiT¹⁸]. For G α s recruitment, this structural complementation assay was difficult to implement due to the heterotrimeric nature of G-proteins and thus an indirect strategy was used by measuring the activation of the G α s with a biosensor for cAMP (GloSensor). The control group (t β_2 AR) showed the well-established transient activation or interaction between the receptor and G α s or β arr2, respectively, after stimulation with 10 μ M isoproterenol (ISO). This response elicited a 20-fold and 10-fold change in the luminescence

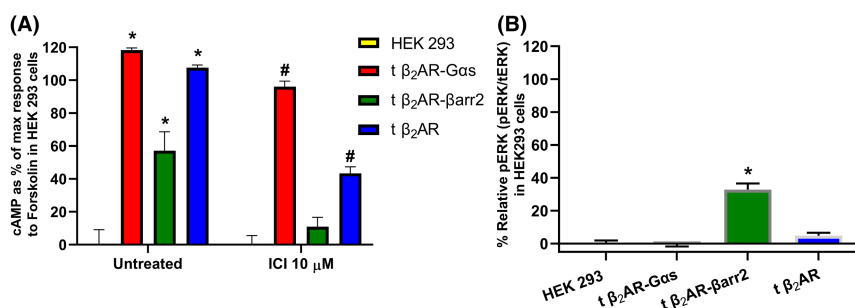


FIGURE 2 Constitutive activity for G α s and β arr2 signaling pathways in HEK 293 cells transfected with the fusion proteins. (A) cAMP measurements represent the G α s pathway using a scale based on the adenylate cyclase activator, forskolin (10 μ M). Forskolin alone was used to define 100% of the response whereas the basal response of the untransfected HEK 293 cells was used to define 0% of the response in all groups. All transfected groups [t β_2 AR-G α s (red), t β_2 AR- β arr2 group (green), and t β_2 AR group (blue)] showed increased constitutive activity when compared to the basal activity of untransfected HEK 293 cells. Under 10 μ M of ICI 118,551, the t β_2 AR-G α s group (red) proved to be the most constitutively active regardless of the low protein expression levels. The highly expressed t β_2 AR group (blue) also shows increased constitutive activity when compared to the basal activity of untransfected HEK 293 cells. Conversely, the t β_2 AR- β arr2 group (green) had a similar basal activity when compared with the untransfected HEK 293 cells. (B) ERK1/2 phosphorylation (pERK) is relative to total ERK1/2 (tERK) representing the β arr2 pathway. Here, only the t β_2 AR- β arr2 group (green) showed a significant increase in constitutive activity compared to the control untransfected HEK 293 cell group. Data are the means \pm SEM from at least three independent experiments. * $p < 0.0001$ versus untreated HEK 293, and # $p < 0.0001$ versus ICI 10 μ M HEK 293 was considered significant by one-way ANOVA and Tukey's test was used as the post hoc test

intensity for $G_{\alpha s}$ activation and β_{arr2} recruitment, respectively (Figure 3A,B). The recruitment of β_{arr2} to the receptor and the activity of $G_{\alpha s}$ after ISO stimulation was blunted by the fusion of the β_2AR to either transducer ($G_{\alpha s}$ or β_{arr2}) suggesting that the fusion proteins exhibit steric hindrance to $G_{\alpha s}$ or β_{arr2} when stimulated by a ligand (Figure 3A,B). Taken together, the data presented in this section indicate that our fusion proteins can be used to restrict signaling to a single pathway and can produce a gain-of-function allowing for the exploration of the physiological role of each pathway in different cell systems.

3.3 | The fusion proteins remain functionally responsive to ligand stimuli

To test if the fusion between the β_2AR with either $G_{\alpha s}$ or β_{arr2} affected the capacity of the receptor to elicit a functional response after ligand binding, we performed concentration–response curves for both signaling pathways using the β_2AR agonist ISO followed by competitive antagonist displacement curves using ICI 118,551. For measurement of cAMP accumulation, we first established the response window for each group by using 10 μM forskolin, an adenylate cyclase activator, to elicit

the maximal response on cAMP (labeled as 100%) independent from the stimulation of any β_2AR ligand (Suppl. Figure 1). To define the basal cAMP levels (labeled as 0%) of each group separately, 300 nM ICI was used to inhibit the constitutive activity of the non- and transfected groups. This set of experiments revealed a unique window for each group for cAMP accumulation that was used to quantify the response of ISO. Alternatively, the β_{arr2} signaling was measured by comparing the amount of phosphorylated ERK1/2 (pERK) relative to the value of total ERK1/2 (tERK) in each group.

For the cAMP measurements representing the $G_{\alpha s}$ pathway, all groups showed classic sigmoidal curves under increasing concentrations of ISO and, as anticipated, increasing concentrations of ICI 118,551 proportionately displaced the ISO concentration–response curve to the right (Suppl. Figure 4). The maximal response (E_{max}) of ISO was decreased $\sim 25\%$ and $\sim 21\%$ in the untransfected HEK 293 and the t β_2AR - β_{arr2} groups, respectively, even at the lowest concentration used of ICI 118,551 (10 nM). Alternatively, the E_{max} of ISO under any ICI 118,551 concentration remained the same for the t β_2AR - $G_{\alpha s}$ and t β_2AR groups. The potency of ISO was increased by more than one logarithmic unit (10-fold increase) in the t β_2AR - $G_{\alpha s}$ and t β_2AR groups compared to the HEK and t β_2AR - β_{arr2} groups (Table 1).

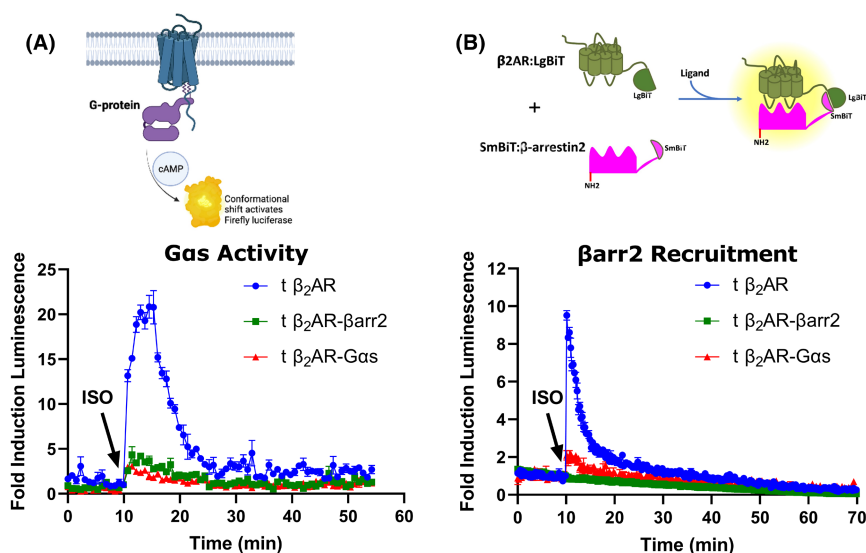


FIGURE 3 $G_{\alpha s}$ activity and β_{arr2} recruitment by the fusion proteins β_2AR - $G_{\alpha s}$ and β_2AR - β_{arr2} in HEK 293 cells. (A) Schematic representation of the $G_{\alpha s}$ activity (upper panel) using GloSensor technology. By increasing the concentrations of cAMP, the genetically engineered firefly luciferase undergoes a conformational change that induces luminescence. Here, the $G_{\alpha s}$ activity is measured by its capacity to produce cAMP after stimulation of the β_2AR with isoproterenol (ISO) (lower panel). The wild-type β_2AR (blue) shows a time-dependent increase in luminescence after stimulation with 10 μM ISO. Such activity is markedly diminished when the β_2AR is fused to either $G_{\alpha s}$ (red) or β_{arr2} (green). (B) Schematic representation of the β_{arr2} recruitment using a structural complementation assay adapted to NanoBiT technology by molecular cloning (upper panel). β_2AR -LgBiT (blue) recruits SmBiT- β_{arr2} after 10 μM isoproterenol (ISO) stimulation (lower panel). This recruitment is drastically blunted when the receptor is fused to either $G_{\alpha s}$ (red) or β_{arr2} (green) (lower panel). All experiments are expressed as the mean \pm SEM from three independent experiments.

Analysis of the rightward shift of the ISO concentration response curves by ICI 118,551 was done by excluding the lowest concentration of ICI and under a linear slope with a value of 1 using the log (concentration ratio - 1).¹⁹ This analysis did not reveal any change in the apparent affinity (pA_2) of ICI 118,551 among groups (Table 2).

Regarding the β arr2 signaling, the same pattern of sigmoidal curves under increasing concentrations of ISO and a rightward shift by ICI 118,551 was observed in all groups when relative ERK1/2 (pERK/tERK) was measured. The right-shift displacement of the ISO curve in the t β_2 AR group was only apparent when 30 nM of ICI 118,551 was used (Suppl. Figure 5). The intrinsic activity of ISO was unaffected in the t β_2 AR- β arr2 and t β_2 AR groups and significantly reduced by ~40% for the HEK 293 and t β_2 AR- $G\alpha_s$ groups by pretreatment with ICI 118,551 (Suppl. Figure 5). ISO displayed similar potency among all groups (Table 1). The pA_2 values of ICI 118,551 were also similar among all groups (Table 2). Together with the cAMP measurements, this data shows that the fusion proteins are functionally responsive to ligand stimuli and each of our chimeras forms a unique pharmacological pattern distinct from the endogenously expressed β_2 AR.

3.4 | BEAS-2B cells also show selective constitutive activity and different patterns of protein expression when transfected with the fusion proteins

First, using a similar approach to that used for HEK 293 cells, BEAS-2B cells were successfully transfected with the receptor alone or with the fusion proteins as observed by RT-PCR (Figure 4A). The protein expression was observed using immunofluorescence. For colocalization of the β_2 AR together with $G\alpha_s$, cells transfected with β_2 AR- $G\alpha_s$ showed protein co-expression at the cell membrane only (Figure 4B). Conversely, the t β_2 AR- β arr2 group showed abundant co-expression at the cell's cytoplasm but no fluorescence was detected at the cellular membrane (Figure 4B). The t β_2 AR group showed a mixed pattern of co-expression at the membrane as well as at the cytoplasm whereas fluorescence was not detected in the control BEAS-2B group (Figure 4B). When colocalization of the β_2 AR with β arr2 was tested, only the t β_2 AR- β arr2 and t β_2 AR groups had a detectable fluorescent signal (Figure 4C). Like the previous experiments, the t β_2 AR- β arr2 group showed co-expression of β_2 AR with β arr2 at

TABLE 1 Competitive antagonism

| Groups | cAMP | | | | | pERK | | |
|------------------------------|---------------|--------------|--------------|--------------|--------------|-------------|-------------|-------------|
| | No ICI | 10 nM | 30 nM | 100 nM | 300 nM | No ICI | 10 nM | 30 nM |
| HEK 293 | 8.43 ± 0.09 | 7.15 ± 0.16 | 6.73 ± 0.16 | 6.26 ± 0.19 | 5.72 ± 0.23 | 6.93 ± 0.21 | 6.61 ± 0.30 | 6.39 ± 0.44 |
| t β_2 AR- $G\alpha_s$ | 10.12 ± 0.30* | 9.02 ± 0.07* | 8.55 ± 0.07* | 8.07 ± 0.05* | 7.57 ± 0.06* | 7.01 ± 0.16 | 6.98 ± 0.17 | 6.66 ± 0.17 |
| t β_2 AR- β arr2 | 8.99 ± 0.12 | 7.58 ± 0.10 | 7.12 ± 0.08 | 6.74 ± 0.09 | 6.21 ± 0.10 | 7.37 ± 0.17 | 6.98 ± 0.22 | 6.42 ± 0.22 |
| t β_2 AR | 10.28 ± 0.65* | 9.84 ± 0.28* | 9.04 ± 0.09* | 8.43 ± 0.07* | 7.91 ± 0.05* | 6.97 ± 0.19 | 6.95 ± 0.29 | 6.79 ± 0.23 |

Notes: The potency of ISO and rightward displacement by the competitive agonists ICI 118,551. Measurements are expressed as $-pEC_{50}$ values obtained from semilogarithmic concentration-response curves of the control HEK 293 and t β_2 AR groups, and the fusion protein groups t β_2 AR- $G\alpha_s$ and t β_2 AR- β arr2. The displacement curves were generated using increasing concentrations of ICI 118,551 (10–300 nM). The two main signaling pathways of the β_2 AR, $G\alpha_s$ and β arr2, are explored by measurements of cAMP accumulation and ERK phosphorylation (pERK), respectively. Data are the mean ± SEM of at least three independent experiments with repeats in triplicate for cAMP and duplicate for ERK. * $p < 0.05$ when compared to both non-transfected and transfected β_2 AR- β arr2 groups. Two-way ANOVA and Tukey's post hoc test.

TABLE 2 Schild regression analysis

| Groups | cAMP | | | pERK | |
|------------------------------|---------------|-------------------|---------------------------|-------------|-------------------|
| | pA_2 | Schild-plot Slope | pA_2 (adapted analysis) | pA_2 | Schild-plot Slope |
| HEK 293 | 9.43 ± 0.16 | 1.191 ± 0.080 | 9.19 | 8.96 ± 0.58 | 0.95 ± 0.44 |
| t β_2 AR- $G\alpha_s$ | 10.16 ± 0.11* | 1.049 ± 0.030 | 9.05 | 8.75 ± 0.30 | 1.11 ± 0.31 |
| t β_2 AR- β arr2 | 10.38 ± 0.16* | 1.031 ± 0.050 | 9.30 | 8.4 ± 0.170 | 1.08 ± 0.23 |
| t β_2 AR | 9.21 ± 0.25 | 2.015 ± 0.060 | 8.80 | 8.04 ± 0.41 | 0.77 ± 0.58 |

Notes: Schild regression analysis for the competitive antagonism of ICI 118,551. The apparent affinity (pA_2) of the competitive antagonist ICI 118,551 was calculated in non-transfected HEK 293 cells and transfected with the wild-type β_2 AR, or either fusion proteins β_2 AR- $G\alpha_s$ or β_2 AR- β arr2 using the Schild analysis. The adapted analysis for pA_2 calculation was necessary as the slopes were different from unity. Data are the mean ± SEM of at least three independent experiments. * $p < 0.05$ when compared to both non- and transfected wild-type β_2 AR control groups. Two-way ANOVA and Tukey's post hoc test.

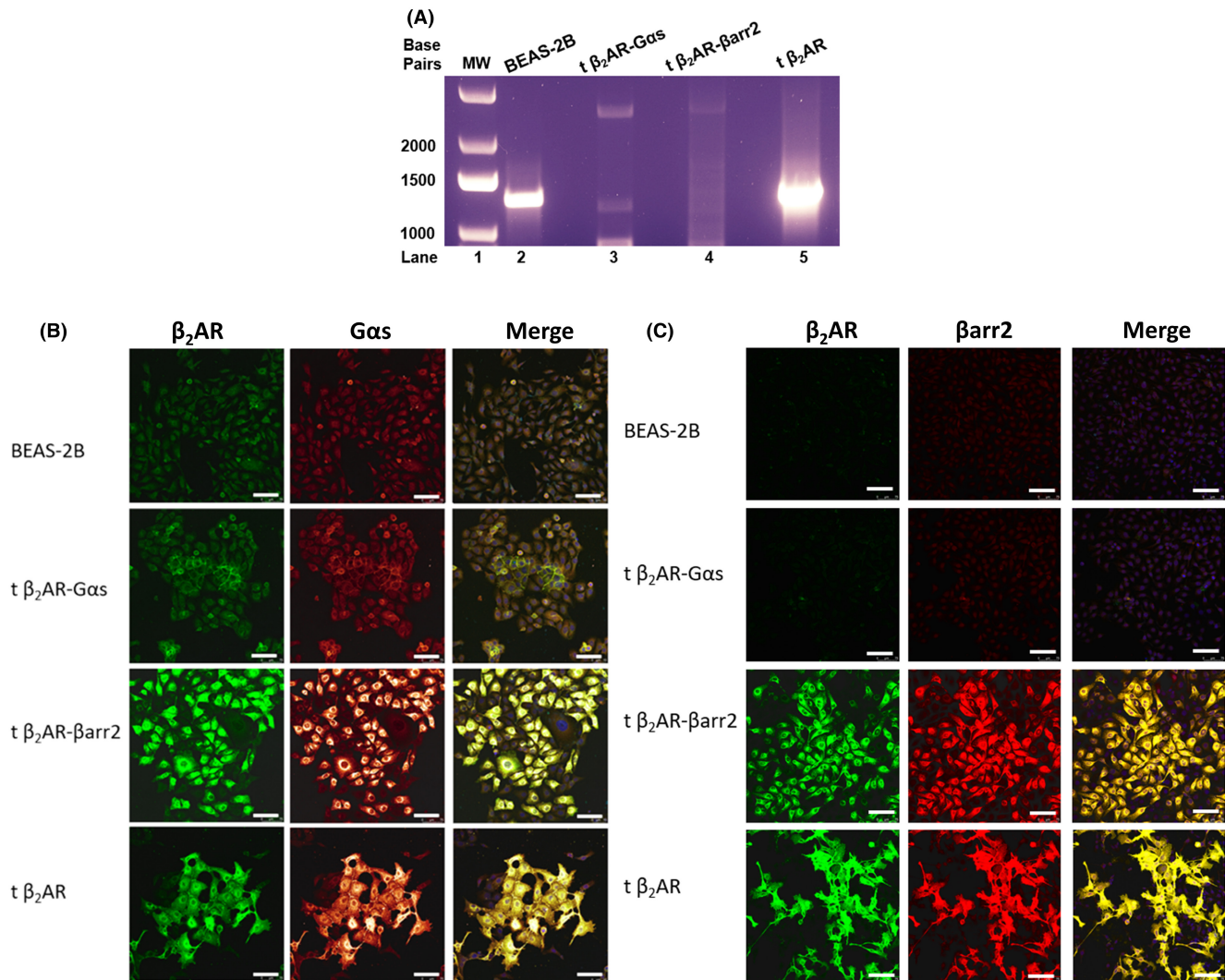


FIGURE 4 Expression of the fusion proteins β_2 AR-G α s and β_2 AR- β arr2 in BEAS-2B cells. (A) Reverse transcriptase-PCR shows the gene expression of the β_2 AR alone (lane-2 and lane-5) and when fused to either G α s or β arr2 (lane-3 and lane-4, respectively). The first lane shows the molecular weight (MW) ladder for base pair quantification. (B) Representative images of protein expression of either β_2 AR (green) or G α s (orange). Both proteins are observed together in the merged image (yellow) showing colocalization for β_2 AR and G α s at the cellular membrane for t β_2 AR-G α s whereas a cytoplasmic colocalization is observed for t β_2 AR- β arr2. The expression of t β_2 AR showed a mixed pattern of colocalization at the membrane, and cytoplasm whereas no pattern was observed for the non-transfected BEAS-2B group (low signal). (C) Representative images of the protein expression of either β_2 AR (green) or β arr2 (red). Both proteins are observed together in the merged image (yellow) showing colocalization for β_2 AR and β arr2 at the cytoplasm of the t β_2 AR- β arr2, a mixed expression pattern for the t β_2 AR, and no expression pattern for either non-transfected BEAS-2B or t β_2 AR-G α s (low IF signal). Proteins were detected by immunofluorescence at a lens magnification of 20 \times using confocal microscopy. Scale bars represent 100 μ m

the cytoplasm only, predominantly at the perinuclear region whereas a mixed pattern of co-expression at the cytoplasm and the cell membrane was observed in the t β_2 AR group (Figure 4C).

Second, the measurements of cAMP levels and ERK1/2 phosphorylation in BEAS-2B cells were conducted using the same methods as with HEK 293 cells. Transfection of BEAS-2B cells with the β_2 AR-G α s fusion protein produced a significant increase in basal cAMP levels when compared to the non-transfected BEAS-2B cells (Figure 5A). The other transfected groups, t

β_2 AR- β arr2 and t β_2 AR, had similar cAMP levels when compared to the control BEAS-2B group. Regarding the β arr2 pathway, non-transfected BEAS-2B cells showed overall increased constitutive activity observed as a high relative ERK1/2 phosphorylation that was comparable to the t β_2 AR- β arr2 group (Figure 5B). Compared to the control BEAS-2B and the β_2 AR- β arr2 groups, the t β_2 AR-G α s and t β_2 AR groups had a significant reduction in the basal ERK1/2 phosphorylation. After treatment with the inverse agonist ICI 118,551, the ERK1/2 phosphorylation of the non-transfected BEAS-2B cells

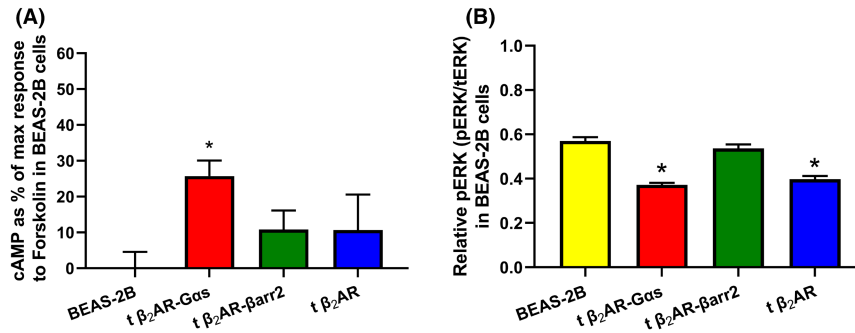


FIGURE 5 Constitutive activity for Gαs and βarr2 signaling pathways in BEAS-2B cells transfected with the fusion proteins. (A) cAMP measurements represent the Gαs pathway using a scale based on the adenylate cyclase activator, forskolin (10 μM). Forskolin was used to define 100% of the response whereas the basal response of the untransfected BEAS-2B cells was used to define 0% of the response in all groups. The constitutive activity of the t β₂AR-Gαs group (red) was significantly increased compared to the untransfected BEAS-2B cells. Conversely, the t β₂AR-βarr2 group (green) and t β₂AR group (blue) had similar basal activity when compared with the untransfected BEAS-2B cells. (B) ERK1/2 phosphorylation (pERK) relative to total ERK1/2 (tERK) as the surrogate measurement of the βarr2 pathway. Constitutively high pERK was observed for the untransfected BEAS-2B (yellow) similar to the basal activity of the t β₂AR-βarr2 group. The t β₂AR-Gαs and t β₂AR had a lower constitutive activity when compared to the control BEAS-2B group and the t β₂AR-βarr2 group. Data are the means ± SEM from at least three independent experiments. **p* < 0.05 was considered significant by one-way ANOVA and Tukey's test was used as the post hoc test

and the t β₂AR-βarr2 group was significantly reduced whereas the t β₂AR-Gαs and t β₂AR groups remained at a similar basal activity (Suppl. Figure 6). These findings suggest that the low constitutive activity observed in the t β₂AR-Gαs and t β₂AR groups is not related to β₂AR signaling.

3.5 | The cytokine profiles and cell size change depending on the fusion protein expressed in BEAS-2B cells

After stable transfection, we noted the cytokine secretion profile changed depending on the fusion protein expressed by the transfected BEAS-2B cells. From the 15 cytokines analyzed (see Section 2), five were not detected in the supernatant of any group (IL-2, IL-4, IL-5, IL-10, and IL-12p70) and, thus, were not included in the present analysis. For the remaining cytokines, a heatmap was used as a visual representation of normalized percentile changes in cytokine secretion by each group (Figure 6A). Specifically, the non-transfected BEAS-2B group showed a high secretion pattern for 7 out of 10 cytokines measured. The secretion of cytokines modulating part of the type 1 (Th1; IL-12p40, IFN-γ) or type 2 (Th2; IL-13, IL-6) immune response was reduced in all transfected groups when compared to the control BEAS-2B group (Figure 6B). For the proinflammatory cytokines, TNFα secretion was increased only in the t β₂AR group, while IL-1β and IL-1Ra were increased only in the t β₂AR-βarr2 group (Figure 6C; upper panel). When the chemoattractants were analyzed, the

secretion of GM-CSF and IL-8 were constitutively decreased in the t β₂AR-Gαs group when compared to the control BEAS-2B group. Conversely, the secretion of the chemoattractant CCL2 was constitutively reduced in the t β₂AR-βarr2 group when compared to the control BEAS-2B group (Figure 6C; lower panel). Finally, the comparison of the cytokine profiles between fusion proteins showed a reciprocal regulation of the chemoattractants CCL2, GM-CSF and IL-8. This is, CCL2 was highly secreted in the t β₂AR-Gαs group when compared to the t β₂AR-βarr2 group, whereas GM-CSF and IL-8 were increased in the t β₂AR-βarr2 group when compared to the t β₂AR-Gαs group (Figure 6C; lower panel). These results indicate that there is differential regulation of cytokine/chemokine secretion by each signaling pathway of the β₂AR.

Finally, we observed changes in the average diameter of detached cells suggesting a differential regulation of the morphology by the fusion proteins. The t β₂AR-βarr2 group showed a larger cell diameter when compared to the untransfected BEAS-2B, the t β₂AR, or the t β₂AR-Gαs groups (Figure 7). Additionally, the attached cells from the t β₂AR group further displayed a change in their morphology compared to the other transfected groups at 100× confocal magnification. Particularly, lamellipodia were observed only in the t β₂AR group where high expression of β₂AR and its cognate transducers, Gαs and βarr2, was also observed (Figure 8). The mechanisms for the change in cell structure and morphology elicited by the βarr2 signaling pathway and β₂AR overexpression, respectively, remain to be investigated.

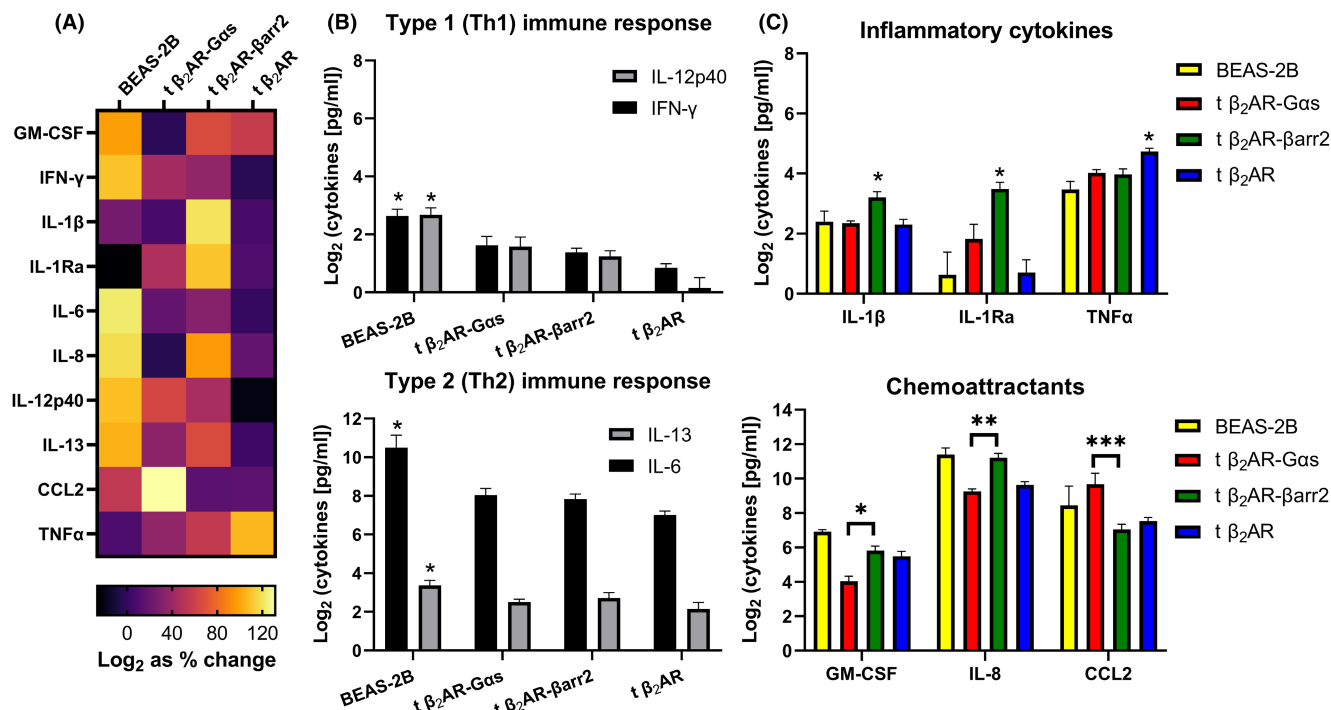


FIGURE 6 Cytokine profile changes for BEAS-2B cells transfected with the wild-type β_2 AR or the fusion proteins. Changes in the secretion of multiple cytokines were measured as [pg/ml] and transformed to \log_2 units for clear visualization. (A) Heatmap of the inflammatory cytokine profile representing the magnitude of secretion relative to each group (percentile change). The cytokine profile of the control BEAS-2B group (high cytokine secretion) was in stark opposition with the profile of the t β_2 AR group (low cytokine secretion). Similarly, the fusion proteins showed distinctive profiles favoring a type-1 (e.g., t β_2 AR-G α s group) or type-2 (e.g., t β_2 AR- β arr2 group) immune response. (B) Cytokines are known to polarize the differentiation of naïve T cells into type-1 (Th1) (upper panel) or type-2 (Th2) (lower panel) helper T cells. Non-transfected BEAS-2B cells show the highest levels for both immune responses. All transfected groups show lower secretion of Th1 (IL-12, IFN- γ) or Th2 (IL-6, IL-13) polarizing cytokines compared to BEAS-2B cells. (C) The proinflammatory cytokines IL-1 β and TNF α are elevated for the t β_2 AR- β arr2 and t β_2 AR group, respectively, when compared to the rest of the groups (upper panel). The lower panel shows a reciprocal activation of chemoattractants by each fusion protein. The t β_2 AR-G α s group has constitutively high CCL2 along with low GM-CSF and IL-8 secretion whereas constitutively low CCL2, and high GM-CSF and IL-8 secretion were observed for the t β_2 AR- β arr2. The control untransfected BEAS-2B cells were not different from the transfected group with the highest secretion. Data are expressed as the mean \pm SEM from three independent experiments ($n = 3$). * $p \leq 0.05$, ** $p \leq 0.0005$, and *** $p \leq 0.0001$ were considered significant by two-way ANOVA and Tukey's test was used as the post hoc test

4 | DISCUSSION

Here, we transfected the β_2 AR-G α s and β_2 AR- β arr2 fusions into two different cell lines: HEK 293 and BEAS-2B cells. The expressed fusion proteins in HEK 293 cells allowed us to independently characterize the constitutive activity of two of the most known signaling pathways of the β_2 AR: G α s and β arr2. We also determined that, after receptor activation, the transducer moiety of the fusion protein dampens the activity and recruitment of the endogenous transducers G α s and β arr2, respectively, suggesting steric hindrance. Finally, the use of the full agonist Isoproterenol and the antagonist/inverse agonist ICI 118,551 on our fusion proteins showed that the receptor remains sensitive to pharmacological manipulation. The experiments with BEAS-2B cells supported the pattern of constitutive signaling by a single pathway that was

observed in HEK 293 cells. This unique constitutive activity was associated with a different profile of cytokine release, as well as an observed change in the average size and shape of cells.

The recruitment of G α s or β arr2 after ISO stimulation is blunted by either fusion protein, β_2 AR-G α s, or β_2 AR- β arr2, respectively, whereas the receptor alone kept the activation or recruitment feature for both signaling proteins (Figure 3). Since the expression levels of both fusion proteins measured by RT-qPCR were almost identical to the control β_2 AR in our structural complementation system (Suppl. Figure 3), our results indicate that each fusion protein excludes the coupling of the alternative signaling pathway, likely by steric hindrance. In contrast with our findings, other studies have shown the formation of a 7TMR-G α s- β arr2 megacomplex.^{16,17} The discrepancy can be reconciled based on the conformational interaction

β arr has with 7TMRs. Based on the strength of the interaction between β -arrestins and 7TMR, receptors have been classified in class A (transient interactions) or class B (stable interactions).^{20,21} Such interactions are dependent on the conformation of β arr whereby the tail conformation (predominantly observed across class B 7TMRs) would allow β arr to remain attached to the C-terminal of the receptor while the intracellular core of the receptor

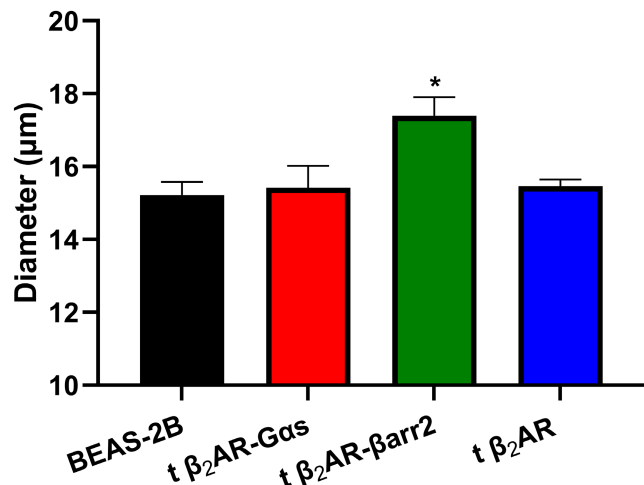


FIGURE 7 The constitutive activity of the β arr2 pathway modifies the cell size of BEAS-2B cells. Cell diameter was measured in live detached cells using trypan blue as a dye to identify living from dead cells. The wild-type BEAS-2B cells (black) or the cells transfected with the β_2 AR alone (blue) or the fusion proteins β_2 AR-G α s (red) show a shorter cell diameter when compared to BEAS-2B cells transfected with the β_2 AR- β arr2 fusion (green). Experiments were performed in duplicate at least three separate times ($n \geq 3$). * $p \leq 0.05$ was considered significant by one-way ANOVA and Tukey's test was used as the post hoc test

is still accessible to the G protein for canonical signaling. Conversely, the core-engaged β arr (observed in class A 7TMRs such as the β_2 AR) would not allow G protein interaction with the receptor.^{17,22} Therefore, the core conformation of β arr is most likely present in the β_2 AR- β arr2 fusion.

The functional data measuring cAMP accumulation as a surrogate of the G α s pathway, and ERK1/2 phosphorylation as a surrogate of the β arr2 pathway revealed that each fusion protein was constitutively active for their respective pathways. We did observe a small increase in cAMP by the β_2 AR- β arr2 fusion that was abolished by low concentrations of ICI 118,551 (300 nM). In contrast, only a small reduction in the cAMP constitutive activity of the β_2 AR-G α s fusion protein is observed even at high ICI 118,551 concentrations (10 μ M) (Figure 2A). Another contributing factor for the increased strength of the β_2 AR-G α s fusion protein on cAMP production could be the length of the linker between the β_2 AR and G α s. Previous reports on the β_2 AR-G α s fusion proteins have shown a variable degree of constitutive activity presumably produced by the change in the linker length.²³⁻²⁵ This variability was particularly addressed by exploring multiple lengths between the tethered proteins, demonstrating that the length of the linker is inversely proportional to the basal activity elicited by the fusion protein.^{9,10} Therefore, the small size of the linker in the β_2 AR-G α s fusion is constraining the receptor to couple to the G α s pathway. Further supporting this hypothesis is the lack of constitutive activity of the β_2 AR-G α s fusion for the β arr2 pathway (Figure 2B) suggesting that the receptor is being tightly held in the conformation coupling to G α s. For the β arr2 pathway, the constitutive activation of ERK1/2 phosphorylation was observed only when the β_2 AR- β arr2 fusion was expressed and our results

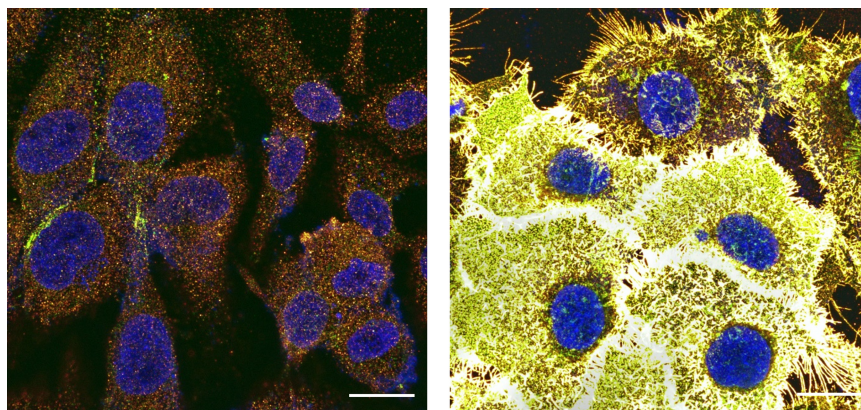


FIGURE 8 The morphology of BEAS-2B cells changes when the β_2 AR is overexpressed. Representative images of the BEAS-2B group (left) with a typical shape characteristic of epithelial cells. Overexpression of the β_2 AR (right) altered the morphology of BEAS-2B cells displaying filamentous extensions resembling cilia. The yellow label represents the merged signal between the expression of the β_2 AR (green) and G α s (orange). Signal was detected by immunofluorescence at a lens magnification of 100 \times using confocal microscopy. Scale bars represent 20 μ m

with pertussis toxin suggest this increased ERK1/2 phosphorylation was independent from the endogenous G α i protein (Suppl. Figure 2).

In addition, ISO generated classical sigmoidal response curves in both fusion proteins for both signaling pathways (Suppl. Figures 4 and 5). Moreover, the potency of ISO was increased in the t β_2 AR-G α s group when compared to the t β_2 AR- β arr2 group suggesting that ISO preferentially binds to the β_2 AR conformation that couples to G α s. Accordingly, the binding affinity of ISO for the β_2 AR increases when the Gs protein is present whereas the induction of other conformations of the receptor by allosteric nanobodies drastically changes the affinity of ISO for the receptor.²⁶ Further experiments showed that the sigmoidal curves induced by ISO in all groups were displaced to the right by increasing concentrations of ICI 118,551. For the Gs pathway, a Schild regression analysis on the displacement of ISO curves by ICI 118,551 initially demonstrated higher pA₂ values for both fusion proteins compared to the controls. However, correction of the Schild analysis due to a slope different from unity decreased the pA₂ values of ICI 118,551 to similar levels in all groups (Table 2). Our results can be interpreted as a preserved Emax of ISO under increasing concentrations of ICI 118,551 because of a high β_2 AR density (overexpression of the β_2 AR; Suppl. Figure 4B) or increased coupling efficiency (when β_2 AR is fused to G α s; Suppl. Figure 4C).^{27,28} Conversely, the reduction of the Emax of ISO by the ICI 118,551-occupied receptors is observed in the untransfected HEK 293 cells with low receptor expression (Suppl. Figure 4A). A similar reduction in the Emax of ISO is observed in the t β_2 AR- β arr2 group by increasing concentrations of ICI 118,551 (Suppl. Figure 4D) suggesting that the endogenously expressed β_2 AR is the predominant subpopulation functionally inhibited in this group. Together with the decoupling of cAMP production via G α s when the β_2 AR is fused to β arr2 these data indicate that the t β_2 AR- β arr2 group is poorly coupled to the β_2 AR-Gs pathway. In contrast, the Emax of ISO collapsed under increasing concentrations of ICI 118,551 in the non-transfected and the t β_2 AR-G α s group when ERK1/2 phosphorylation was measured (Suppl. Figure 5A,C). This is suggestive of a decreased coupling efficiency to β arr2.^{27,28} Accordingly, the increased availability of the receptor subpopulation that couples to β arr2 by the overexpression of the β_2 AR- β arr2 fusion protein show parallel rightward displacements with similar maximal activities of the ISO curves under increasing concentrations of ICI (Suppl. Figure 5D). Thus, the β_2 AR- β arr2 fusion protein shows the highest coupling efficiency for the β arr2 pathway. Taken together, the pharmacological profile of each fusion protein is different from the endogenously expressed receptor. The high degree of responsiveness of our fusion proteins to β_2 AR ligands further allows for more accurate

quantification of the selectivity and preference of β_2 AR ligands for the G α s or the β arr2 pathway.

Regarding the BEAS-2B cells, distinctive cytokine profiles were elicited by each fusion protein, demonstrating a gain-of-function in a physiologically relevant cell line. While activation of the Gs pathway follows the well-described β_2 AR behavior in BEAS-2B cells,²⁹ there is some controversy on the basal activity of BEAS-2B cells elicited by the β_2 AR- β arr2 pathway. A recent study analyzing multiple cell lines of human bronchial epithelial cells, including the BEAS-2B, showed constitutively high ERK1/2 phosphorylation.³⁰ Yet, no clear evidence on the β arr2-dependent ERK1/2 phosphorylation was observed since the β arr2-biased partial agonist, carvedilol, failed to further increase ERK1/2 phosphorylation.^{30,31} In the present study, we observed similar high ERK1/2 phosphorylation between non-transfected BEAS-2B cells and transfected with the β_2 AR- β arr2 fusion protein. Treatment with the selective β_2 AR inverse agonist, ICI 118,551, decreased ERK1/2 phosphorylation in both groups to the basal levels observed in the t β_2 AR-G α s group.

Under pathologic states, the epithelium secretes selective cytokines inducing a local inflammatory response that activates and recruits a myriad of immune cells.³² The inflammatory response can be divided into two types (Th1 and Th2) based on the cytokine-induced polarization of naive T lymphocytes. Here, we show that the human BEAS-2B cell line has a constitutively high secretion of most cytokines measured, except for the proinflammatory cytokines IL-1 and TNF α . This suggests that under basal conditions, there exists an equilibrium at the epithelial microenvironment between the two main immune responses, Th1 and Th2.^{33,34} Additionally, the chemokine profile observed for non-transfected BEAS-2B cells does not suggest a preference for chemoattraction between immune cell types. Overexpression of the β_2 AR altered the cytokine profile of BEAS-2B cells and showed TNF α as the only elevated cytokine. Together with the change to a ciliated-shaped morphology (Figure 8), our findings suggest that BEAS-2B cells overexpressing the β_2 AR became differentiated. Previous observations showing a lack of differentiation of BEAS-2B under similar environmental conditions³⁵ challenge our observations. Further structural and genetic characterization of BEAS-2B cells overexpressing the β_2 AR and comparison with subpopulations of epithelial cells³⁶ are needed.

Regarding the fusion proteins, the cytokine profile of BEAS-2B cells shifted based on the activated downstream signaling of the β_2 AR. When the constitutive activity is high for the G α s signaling, the cytokine profile shifts only to the secretion of the monocyte chemoattractant CCL2 (MCP-1). Conversely, the chemokines GM-CSF and IL-8 (CXCL8), known for eosinophil and neutrophil recruitment,

respectively, are decreased under the β_2 AR- $G_{\alpha s}$ signaling pathway. Accordingly, manipulation of the Gs pathway with the adenylate cyclase activator, forskolin, or a cAMP analog, 8-Br-cAMP, decreases GM-CSF, IL-6, and IL-8 secretion.^{13,15} A reciprocal shift in the cytokine profile of BEAS-2B cells is observed for the t β_2 AR- β arr2 group. In this group, the constitutive activity for the β_2 AR- β arr2 signaling pathway decreases CCL2 secretion while GM-CSF and IL-8 remain at high concentrations. Furthermore, the proinflammatory cytokine IL-1 β , instrumental in the activation and recruitment of eosinophils, mast cells, neutrophils, and dendritic cells,³⁷ is increased only in the t β_2 AR- β arr2 group. This cytokine profile suggests that the β_2 AR- β arr2 signaling axis mediates the Th2 immune response. This profile is consistent with the immune phenotype of asthma reported in human and animal studies.^{14,38,39} Moreover, β_2 AR signaling, predominantly through the β arr2 pathway, is necessary for the development of an asthma phenotype.^{14,40,41} Thus, our results suggest that the β_2 AR- β arr2 signaling pathway in human bronchial epithelial cells favors the development of an asthma-like phenotype by altering the cytokine profile to the Th2 proinflammatory profile.

In summary, here we show that our fusion proteins can elicit a robust and independent activation of each signaling pathway in multiple cellular systems. We transfected our β_2 AR- $G_{\alpha s}$ and β_2 AR- β arr2 fusion constructs separately into HEK 293 cells; cells that are commonly used to characterize the β_2 AR behavior and pharmacology. As predicted, the β_2 AR- $G_{\alpha s}$ and β_2 AR- β arr2 fusion proteins showed steric hindrance from other non-tethered transducers and selectively increased the constitutive activity of the receptor via the transducer fused to the receptor. We also demonstrated that the signaling activity of the receptor for both pathways can still be manipulated by ligands, suggesting that the structure of the receptor fused to any transducer is not locked in its conformational state. Finally, by using the immortalized human bronchial epithelial cell line, BEAS-2B cells, we further demonstrated that the selective constitutive activity of our fusion proteins observed in HEK 293 cells is preserved in a more physiologically relevant cell type. Measurements of β_2 AR-mediated cytokine release in BEAS-2B cells revealed that the constitutive signaling via each pathway is translated into a unique cellular response that differs between the two pathways. Moreover, the β_2 AR- β arr2 signaling pathway induces a strong type 2 immune response not observed for the β_2 AR- $G_{\alpha s}$ signaling pathway. Thus, the fusion proteins can be used to study the pathway-specific pharmacology of β_2 AR ligands and the physiological consequences of inducing a gain-of-function. Additionally, this mechanism can be used as a tool to dissect the most well-known signaling pathways of the β_2 AR and study other physiological systems. The fusion proteins can also facilitate the study and development of biased

ligands that could ultimately increase the therapeutic efficacy and/or decrease adverse effects.

AUTHOR CONTRIBUTIONS

Emilio Y. Lucero-Garcia Rojas, Arfaxad Reyes-Alcaraz, Kehe Ruan, Bradley K. McConnell, and Richard A. Bond designed the research; Emilio Y. Lucero-Garcia Rojas, Arfaxad Reyes-Alcaraz performed the research; Emilio Y. Lucero-Garcia Rojas, Arfaxad Reyes-Alcaraz, Bradley K. McConnell, and Richard A. Bond analyzed and interpreted the research; Emilio Y. Lucero-Garcia Rojas, Bradley K. McConnell, and Richard A. Bond wrote the paper; Kehe Ruan designed the fusion proteins.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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