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Research article

Modulation of IL-17A and IFNγ by β2-adrenergic agonist terbutaline and inverse-agonist nebivolol, influence of ADRB2 polymorphisms

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Abstract: *Background*: Upon activation, helper T (Th) cells produce cytokines such as IL-17A and IFNγ, which may exacerbate inflammatory disease and disorders. Adrenergic drugs are emerging as immunomodulatory agents to treat pro-inflammatory diseases, but their function is not completely understood. Th Cells express the β2-adrenergic receptor (β2AR) that is encoded by *ADRB2*. Agonists of the β2AR decrease IFNγ but can increase IL-17A from Th cells. We compared a β2AR agonist to an inverse-agonist, and assessed the influence of *ADRB2* polymorphisms on IL-17A and IFNγ responses. *Methods*: Peripheral blood mononuclear cells (PBMCs) from venous blood of healthy human participants were cultured with T cell activators anti-CD3 and anti-CD28 antibodies. Terbutaline, a β2AR agonist or nebivolol, a β1AR antagonist and β2AR inverse-agonist, were added *in vitro*. Cytokines IL-17A and IFNγ were measured using enzyme-linked immunosorbent assay. Genomic *ADRB2* and its immediate upstream region were sequenced using Sanger's method. Cytokine response to drug was analyzed based on *ADRB2* polymorphisms. *Results*: Terbutaline consistently inhibited IFNγ from activated PBMC samples. In contrast, it increased IL-17A in PBMC homozygous for Gly16 codon of *ADRB2*. Nebivolol inhibited IL-17A and IFNγ from activated Th cells. When applied to activated-PBMCs, nebivolol inhibited IL-17A but did not significantly inhibit IFNγ although a trend was observed. The ability of nebivolol to inhibit IL-17A was attenuated by a β2AR-specific antagonist. Cellular proliferation and viability was not significantly changed by

nebivolol. Nebivolol suppressed IL-17A in all of the samples regardless of *ADRB2* polymorphisms. *Conclusions*: This data demonstrates that terbutaline inhibited IFNγ, however, it increased IL-17A in samples with the common Gly16 polymorphism of *ADRB2*. Nebivolol inhibited IL-17A regardless of *ADRB2* polymorphisms. Thus, nebivolol is a strong candidate for treating inflammatory diseases or disorders where IL-17A exacerbates symptoms.

Keywords: β2-adrenergic receptor; helper T cells; terbutaline; nebivolol; cytokines

1. Introduction

T helper (Th) cells coordinate adaptive immunity but exacerbate pro-inflammatory autoimmune diseases in susceptible people. Accumulated evidence demonstrates that T helper 1 (Th1) and T helper 17 (Th17) cells are dysregulated in autoimmune diseases. For example Th17 cells are elevated in , rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis where IL-17A and IFNγ play a pro-inflammatory role [1–3]. In this report, we explored the immuno-modulatory capacity of two adrenergic drugs. Adrenergic drugs are indicated for cardiovascular disease or asthma, but their role as immunomodulatory agents is not fully explored. An albuterol add-on study in relapsing remitting multiple sclerosis combined with glatiramer acetate was well tolerated and delayed the time to first relapse [4].

The adrenergic receptor (AR) family is divided into α and β sub-families. The gene encoding β2AR is *ADRB2*, a short, intronless gene located on chromosome 5q31–q32 that is translated into a 413 amino acid protein product forming a classic seven-transmembrane G-protein coupled receptor [5]. Terbutaline is an agonist of β2AR commonly used for treating asthma [6]. Nebivolol is a third generation adrenergic drug used to treat hypertension and congestive heart failure [7]. Nebivolol is a selective β1AR antagonist, and it is an inverse agonist of β2AR [8,9]. The inverse agonist activity of nebivolol, also referred to as biased agonist, involves a GRK/βarrestin pathway that contrasts the classical G protein coupled pathway used by the β2AR in response to agonists [8].

Adrenergic drugs can be anti-inflammatory when used in a clinical setting [10]. These drugs may act directly on pro-inflammatory Th cells because Th1 cells and Th17 cells express β2AR, while anti-inflammatory Th2 cells do not express β2AR [11–14]. Functional studies showed that terbutaline suppressed Th1 cells and IFNγ, leading to an expansion of Th2 cells [15,16]. Our group showed that terbutaline decreased IFNγ from Th1 cells, but increased IL-17A from Th17 cells in the majority of samples. That result suggested that gene polymorphisms in *ADRB2* were causing inter-individual variability to terbutaline drug response *in vitro* [11].

ADRB2 has common polymorphisms within the coding and adjacent regions. There is a region encoding a 19 amino acid β2AR upstream peptide (BUP) within nucleotides -102 to -42 upstream of the open reading frame of the *ADRB2*. The BUP contains one non-synonymous polymorphism at nucleotide position -47 T/C which alters amino acid Cys19Arg [17]. There are nonsynonymous polymorphisms within *ADRB2.* Three such examples are found at nucleotide 46 G/A in the coding region, which alters Gly16Arg, at nucleotide 79 C/G, which alters Gln27Glu, and at nucleotide 491 C/T, which alters Thr164Ile [18–20]. Nucleotide 46 G/A corresponds to amino acid 16 Gly/Arg, and nucleotide 79 C/G corresponds to amino acid 27 Gln/Glu (Table 1). There are additional

polymorphisms in *ADRB2* that define haplotypes of the gene [17]. The common haplotype 4 contains Arg16 Gln27, and haplotype 2 contains Gly16 Glu 27 (Table 2).

Table 1. The numbering system for nucleotide positions in *ADRB2* with the corresponding amino acid position in β2AR protein, and the haplotype designations in which the polymorphisms appear.

Nucleotide	Amino acid	Haplotype
46G	Gly16	2, 5, 6, 7, 10, 11
46A	Arg16	1, 3, 4, 8, 12
79C	Gln27	1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12
79G	Glu27	2

Table 2. SNP localization and haplotype classification of the *ADRB2* of the most common 4 and 2 haplotypes. BUP = β2AR upstream peptide, syn = synonymous, AA = amino acid residue position in β2AR.

SNP	-1023	-709	-654	-468	-406	-367	-47	-20	46	79	252	491	523
Haplotype 4	G	C	A	C	C	T	T	T	A	C	G	C	C
Haplotype 2	A	C	G	G	C	C	C	C	G	G	G	C	C
Location	5'	5'	5'	5'	5'	5'	BUP	5'	AA16	AA27	syn	AA164	syn

Polymorphisms in amino acid position 16 of β2AR are extensively studied in the context of drug responses and exacerbation of disease. Albuterol data from an asthma cohort showed that the FEV1 response was higher in the 2/2 (Gly16/Gly16) compared to 4/4 (Arg16/Arg16) patients. Moreover, transfection studies showed lower expression of the 4 haplotype gene product using luciferase reporters [19]. There was a small decline in albuterol responses in asthma patients with homozygous Arg16 codon found in haplotype 4 [21,22]. Exacerbations were observed in Arg16 homozygous asthma patients taking a short acting beta(2) agonist salbutamol [23]. Thus, the common Arg16 codon in the β2AR may correlate with reduced short-term adrenergic agonist effects on lung function and asthma exacerbations.

ADRB2 polymorphisms have not been extensively studied in the immune system. One study showed that Th1 cells were inhibited by isoproterenol in all of the haplotypes tested [24]. The goal of our study was to determine if gene polymorphisms within or adjacent to the receptor coding regions could account for the variable response to β2-agonist terbutaline. We also determined if an inverse β2-agonist, nebivolol, could suppress both IL-17A and IFNγ. We demonstrated that terbutaline suppressed IFNγ, but increased IL-17A in human peripheral blood samples heterozygous or homozygous for Gly16 in *ADRB2*. Nebivolol inhibited IL-17A in all samples tested regardless of *ADRB2* polymorphisms. Our novel results provide important new information about genetic factors in the terbutaline IL-17A response, and indicate that nebivolol may be a useful immuno-suppressive drug in Th1 and Th17-mediated inflammatory diseases or disorders.

2. Materials and methods

2.1. PBMC activation and drug treatment

For activation of peripheral blood mononuclear cells (PBMCs), venous blood was drawn from 61 healthy volunteers after an informed, signed consent was obtained. Participants were healthy as assessed by self-reporting of their health condition. Up to ten heparinized vacutainer tubes (BD, Franklin Lakes, NJ, USA) were drawn and processed using ficoll-hypaque (GE healthcare, Mississauga, ON, Canada) density centrifugation techniques to isolate the PBMC as previously described [25]. Experiments were done on the same day of the blood draw using the fresh PBMC, or the PBMC were cryopreserved and used after thawing. Post hoc analysis showed that drug effects were equivalent when comparing fresh versus cryopreserved samples, although we observed that the IL-17A cytokine level was higher over all in the fresh samples (Figure S1). The PBMCs were cryopreserved in FBS with 10% DMSO according to published protocols [26]. The viability was between 95–99% alive after thawing.

PBMCs were incubated in media which contained 10% heat-inactivated fetal bovine serum (FBS) in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 1 mM penicillin with streptomycin, and 2 mM Glutamine (Wisent Inc. QC, Canada). PBMCs were incubated at 0.5×10^6 cells in 200 μL media per well, in a round bottom 96 well culture plate (VWR, Mississauga, ON, Canada). Samples were activated with cell culture grade anti-CD3 (clone OKT3) and anti-CD28 antibodies (clone CD28.2) (eBioscience, San Diego, CA, USA) in a soluble format at 0.1 μg/mL each. For experiments with purified CD4 Th cells, PBMC were put through the CD4 T cell negative selection procedure using negative selection column (Stemcell Technologies, Vancouver Canada). The range of purity was 94–97% $CD3^+CD4^+$ cells as determined by flow cytometry staining. To activate purified Th cells, dynabeads with fixed anti-CD3 and CD28 were used at a 1:1 ratio (Thermo Fisher Scientific, Mississauga, ON, Canada). The *in vitro* drug treatments included terbutaline 10^{-5} M (Terbutaline hemisulfate salt, T2528, Sigma Aldrich, Mississauga, ON, Canada) ICI 118,551 β2AR antagonist 100 nM (Sigma Aldrich, Mississauga, ON, Canada) and nebivolol 10^{-5} M (Nebivolol hydrochloride, N1915, Sigma Aldrich). To measure cytokines, cell culture supernatants were collected after four days of incubation. The cytokines were measured with enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions for human IFNγ (BD Bioscience, San Jose, CA, USA) and human IL-17A (eBioscience, San Diego, CA, USA).

2.2. Flow cytometry

Proliferation was measured using 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE) dye (Sigma Aldrich, ON, Canada), as previously described [11]. In brief, the cells were first incubated RPMI with 10% fetal bovine serum (FBS), with CFDA-SE at a final concentration of 5 μM incubated for 5 min and washed three times with 10% FBS in PBS to remove excess dye, counted, and put into cell culture. At the time point, cells were harvested and stained with CD3-PerCP (UCHT1) and CD4-APC (RPA-t4) antibodies (BD Bioscience, Mississauga, ON, Canada). Samples were analyzed by flow cytometry and FlowJo software using the cell proliferation tool. The cell viability was counted by trypan dye exclusion.

2.3. DNA isolation and sequencing

For DNA isolation, approximately 2 × 10^6 cells of the PBMCs were taken for DNA isolation using QIAamp spin column according to manufacturer's instructions (QIAGEN, Toronto, ON, Canada). The DNA was eluted and purity checked based on the absorbance ratio 260/280 of between 1.8 to 2, on the nanodrop (NanoDrop 2000c, Thermo Scientific). Unphased sequencing was done by Sanger's method. Unphased means that maternal and paternal alleles from the genomic DNA were both present, resulting in two possible nucleotides appearing in heterozygous samples, or just one nucleotide in homozygous samples. The primer sets used are shown in Table 3.

Table 3. Primers used to sequence *ADRB2*.

Region of *ADRB2*	Primer
Upstream	5'-TCCAGATAAAATCCAAAGGGTAAA-3' (Forward)
	5'-CTCTTCTGTGGCCGCTACCT-3'(Reverse)
Upstream and open reading frame	5'-TGTATTTGTGCCTGTATGTGC-3' (Forward)
	5'-CGCGCAGTCTGGCAGGT-3' (Reverse)
Coding	5'-CGCTGAATGAGGCTTCCAG-3' (Forward)
	5'-TCTGAATGGGCAAGAAGGAG-3' (Reverse)
Coding	5'-ATCGCAGTGGATCGCTACTT-3'(Forward)
	5'-GGGGATTGAAACCAGAATTG-3'(Reverse)
Coding	5'-TCTGCTGGCTGCCCTTCT-3' (Forward)
	5'-TGCCCTTCCTTCTGCATATC-3'(Reverse)

2.4. Polymorphism and haplotype determination

Nucleotide polymorphisms at positions 46 and 79 in the *ADRB2* coding region were identified in 61 samples. Haplotypes were only determined for 56 samples due to five samples with insufficient DNA, partial sequencing failure, or one case of a previously unknown haplotype. To analyze the DNA sequence, polymorphisms were determined by the analysis package (Finch TV, Geospiza, Inc., Seattle, USA) and by inspecting the nucleotide chromatogram for the presence of two equally represented signals at the position if it was heterozygous, or one clear signal for homozygous. The polymorphisms were known SNP positions according to Drysdale et al.: -1023, -709, -654, -468, -406, -367, -47, -20, 46, 79, 252, 491 and 523 (Table 2). Using this method, haplotypes could be identified for most of the samples. However, six of the samples could have been either 4/6 or 8/11, where in either case position 16 is heterozygous Arg/Gly, and position 27 is homozygous Gln/Gln. We assigned 4/6 to these samples based on probability, because the frequency of 4/6 is approximately 30%, whereas the frequency of 8/11 is less than 1% in Drysdale's study [19]. The 4/6 or 8/11 samples were not included in the haplotype analyses focusing on haplotypes 4 and 2.

2.5. Statistics

For SNP and haplotype analysis, the cytokine concentrations obtained from ELISA for IL-17A and IFNγ were fit in a linear mixed-effects model with maximum likelihood in R (packages: lme4, lmerTest). The fixed variables are the treatment, polymorphisms at SNP 16 and polymorphisms at

SNP27, whereas the random variable was the human subject. This was followed by type III Analysis of Variance (ANOVA) using Satterthwaite's method and a significance level of 5% to evaluate the effects of the treatment or SNP on the cytokine response in cells. The cytokine concentrations were normalized using the removal of within-subject variance procedure [27] and used for graphing and performing multiple t-test with correction for multiple comparisons using the Holm–Sidak method and a significance level of 5%. Excel (Microsoft version 16.28) and Prism Graphpad 8.4.1 (GraphPad Software Inc. San Diego, California, USA) were used for the normalization and graphing respectively. For analysis, one way ANOVA by Tukey's test was performed with $p < 0.05$. For experiments with two groups, a student's t-test was performed. The figure captions specify the test and p value ranges.

2.6. Ethics approval of research

The study was approved by the Concordia University Research Ethics Committee (certificate 30009292).

3. Results

3.1. IL-17A and IFNγ response to β2AR-agonist terbutaline

To determine the effects of β2 agonist terbutaline on pro-inflammatory cytokines IL-17A and IFNγ, PBMCs from healthy participants were activated *in vitro* with T cell-stimulating antibodies anti-CD3 and anti-CD28. Terbutaline increased IL-17A but decreased IFNγ in the activated PBMCs (Figure 1). These observations are consistent with the data from our previous publication where we found that terbutaline inhibited IFNγ but increased IL-17A in most human PBMC samples [11]. We also noted that terbutaline did not raise IL-17A in some participant samples and speculated that different terbutaline responses were due to polymorphisms in β2AR.

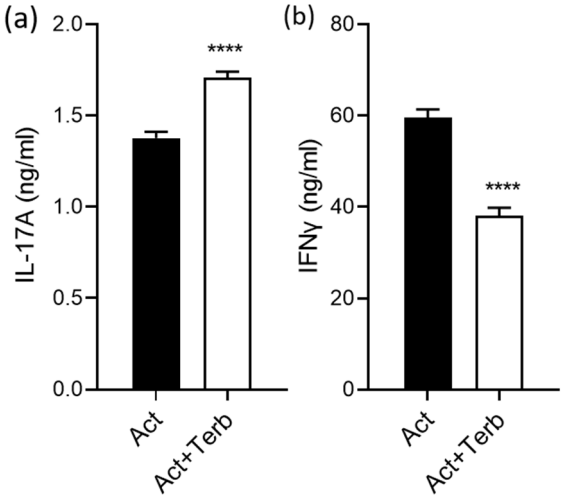

Figure 1. Modulation of IL-17A and IFNγ by β2-AR agonist-terbutaline. PBMCs were activated for 4 days with anti-CD3 and anti-CD28 antibodies (Act; filled bars), with the

addition of terbutaline (Act + Terb; open bars). (a) IL-17A and (b) IFNγ in the cell culture supernatant were measured by ELISA. Data was averaged from 61 human PBMC samples. Error bars show standard error. Student t-test (****$p < 0.0001$).

3.2. Frequencies of ADRB2 polymorphisms and how they relate to terbutaline effects on IL-17A and IFNγ

Genomic DNA from PBMCs was sequenced for *ADRB2* and its immediate upstream region. The sequences were analyzed based on SNPs at positions 46 and 79 where common, non-synonymous SNPs occur within the *ADRB2* coding region. The codon for glycine was more frequent than arginine at position 16, while glutamine was more frequent than glutamic acid at position 27 of β2AR (Table 4). Terbutaline had no effect on IL-17A in Arg16Arg homozygotes; in contrast, it increased IL-17A in Arg16Gly heterozygotes and Gly16Gly homozygotes (Figure 2a). Terbutaline suppressed IFNγ in the samples regardless of position 16 polymorphisms (Figure 2b). With respect to amino acid position 27, terbutaline augmented IL-17A and diminished IFNγ in all of the samples regardless the polymorphism (Figure 2c,d). Next, samples were classified based on the combinations of amino acid positions 16 and 27. Terbutaline augmented IL-17A in samples with Arg16Gly-Gln27Gln, Arg16Gly-Gln27Glu, and Gly16Gly-Glu27Glu, and did not significantly change IL-17A in the other combinations (Figure 2e). With respect to IFNγ, terbutaline inhibited this cytokine regardless of the position 16 and 27 combinations (Figure 2f). Thus, terbutaline consistently inhibited IFNγ in activated PBMC, but it increased IL-17A in samples homozygous or heterozygous for Gly16.

Table 4. β2AR polymorphisms occurrence in PBMC samples. Alleles at amino acid position 16 or 27 of β2AR, corresponding to nucleotide position 46 and 79 of *ADRB2*, respectively, were determined from genomic sequence from 61 healthy human PBMC samples. The occurrence was calculated for amino acid position 16 (top) and position 27 (middle), and the combinations of position 16 and position 27 (bottom).

Amino acid	Alleles	Occurrence (%)
16	Arg/Arg	13.1
	Arg/Gly	59.0
	Gly/Gly	27.9
27	Gln/Gln	47.5
	Gln/Glu	36.1
	Glu/Glu	16.4
16/27	Arg/Arg-Gln/Gln	13.1
	Arg/Gly-Gln/Gln	31.2
	Arg/Gly-Gln/Glu	27.8
	Gly/Gly-Gln/Gln	3.3
	Gly/Gly-Gln/Glu	8.2
	Gly/Gly-Glu/Glu	16.4

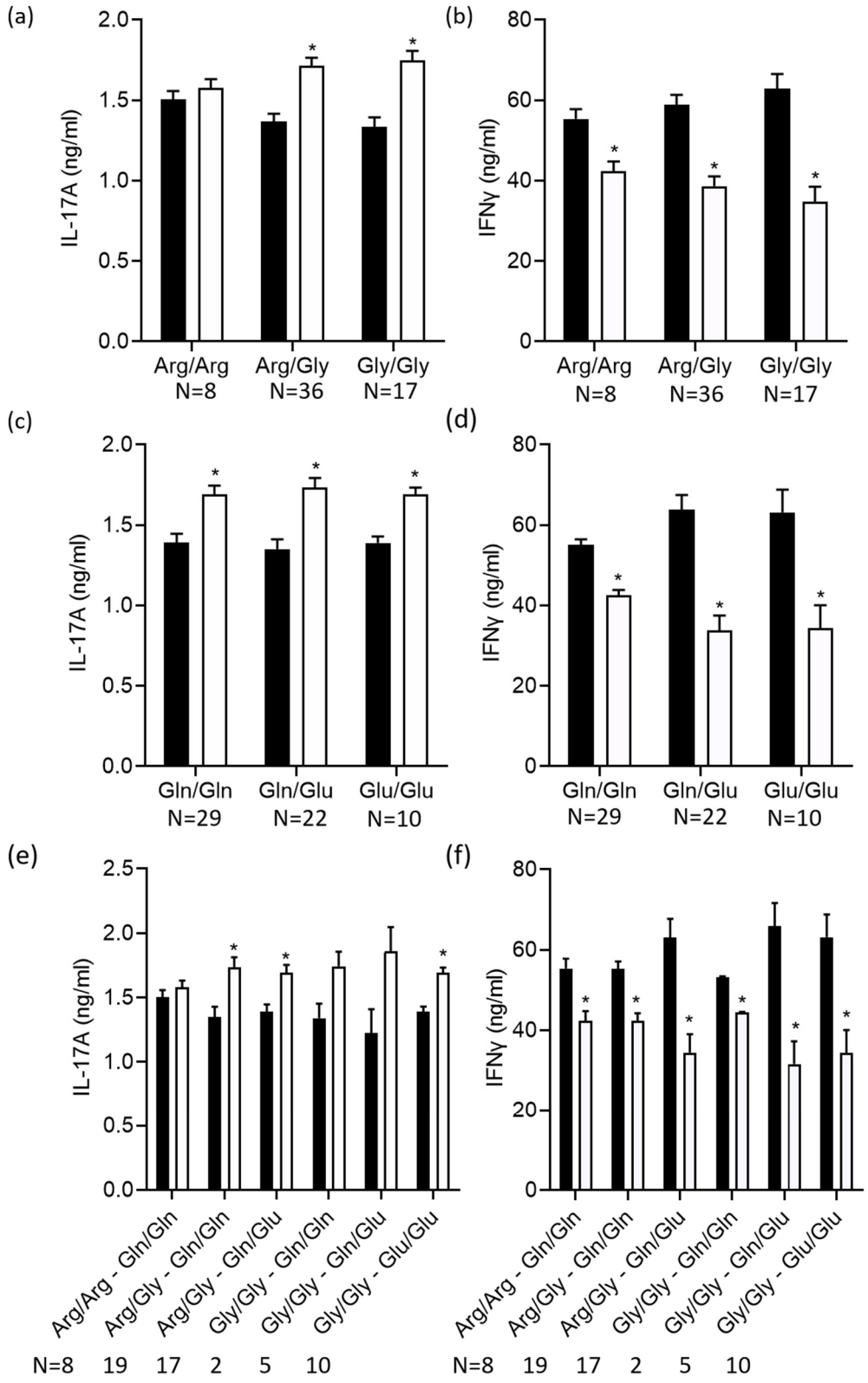

Figure 2. IL-17A and IFNγ response to terbutaline classified based on amino acid polymorphism 16 and 27 of β2AR. PBMCs were activated for 4 days with anti-CD3 and anti-CD28 antibodies (filled bars), with the addition of β2-agonist terbutaline (open bars).

The cytokine data was classified based on the identity of amino acid at position 16 (a,b), position 27 (c,d), or the combinations of 16 and 27 (e,f). Error bars show standard error. Data was derived from 61 humans PBMC samples. Multiple t-test with correction for multiple comparisons using the Holm–Sidak method (*p < 0.05). N = 61 samples. The number of participant samples in each category is listed under the x-axis labels.

3.3. Frequencies of ADRB2 haplotypes and how they relate to terbutaline effects on IL-17A and IFNγ

ADRB2 can be further classified into haplotypes based on *ADRB2* coding region and its 5' region. In 61 participants' PBMC samples, up to 11 haplotype pairs were detected, with 4 and 2 being the most prevalent. We only included these haplotype pairs in the statistical model due to their abundance in the cohort, and there were homozygous and heterozygous samples available for analysis. The 4 and 2 haplotypes represented 52% of the samples tested. The cytokine response data was reclassified based on haplotype pairing. Terbutaline had no effect on IL-17A from samples with haplotype 4/4 but augmented IL-17A in samples with haplotypes 4/2 or 2/2 (Figure 3a). Terbutaline suppressed IFNγ in all samples including 4/4, 4/2 and 2/2 (Figure 3b). Thus, at least one copy of haplotype 2, which contains Gly16, was sufficient for terbutaline to increase IL-17A. In contrast, IFNγ was inhibited by terbutaline regardless of *ADRB2* haplotype. The data from other haplotype pairs are presented in the Figure S2. Interestingly, terbutaline augmented IL-17A in samples with 4/10 haplotype, likely due to the presence of Gly16 in the 10 allele. The haplotype analysis provided more detail; however, knowing the identity of the Gly16Arg allele appears to be sufficient to predict the terbutaline effect on IL-17A.

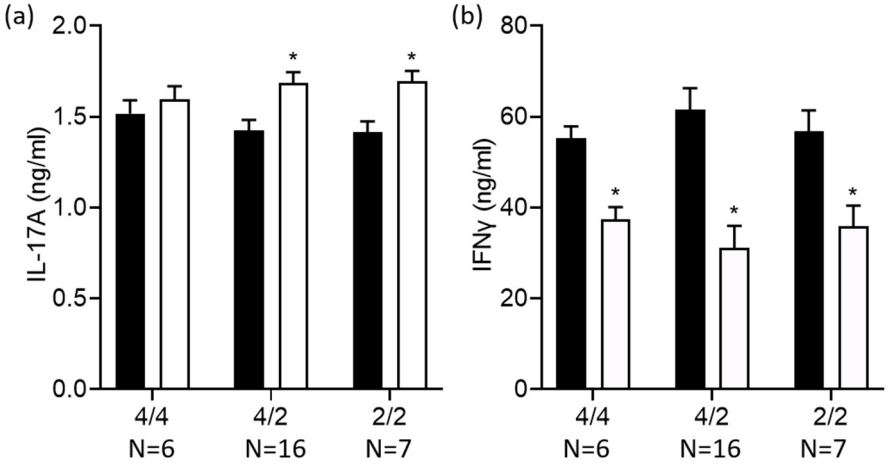

Figure 3. IL-17A and IFNγ response to terbutaline, classified by 4 and 2 haplotypes of *ADRB2*. PBMCs were activated for 4 days with anti-CD3 and anti-CD28 antibodies (filled bars) with the addition of β2-agonist terbutaline (open bars). The cytokine response data was classified based on the identity of the common haplotype 4 and 2 for (a) IL-17A, and (b) IFNγ. The error bars show standard error. Data was from 29 samples of human PBMCs. The statistic was multiple t-test with correction for multiple comparisons Holm–Sidak method (*p < 0.05). The number of participant samples in each category is listed under the x-axis labels.

3.4. The effects of nebivolol on IL-17A and IFNγ from PBMC or purified Th cells

An important goal of this study was to discover an adrenergic drug that suppresses both IL-17A and IFNγ, which are both implicated in pro-inflammatory diseases. Although terbutaline effectively suppressed IFNγ, it augmented IL-17A in samples with the common Gly16 polymorphism of *ADRB2*. We reasoned that if an agonist augmented IL-17A, then an inverse-agonist of β2AR might suppress IL-17A. Nebivolol is reportedly a β1AR agonist and β2AR-inverse agonist [9], but its effects on the immune system remain largely unexplored. We first characterized the functional effects of nebivolol on activated PBMC. Nebivolol significantly inhibited IL-17A from activated PBMC (Figure 4a). Nebivolol did not significantly inhibit IFNγ, although a trend was observed (Figure 4b). Since PBMC might have other cells that produce these cytokines, we next tested for the cellular specificity of the drug using purified CD4 T cells. Nebivolol significantly inhibited IL-17A from activated Th cells (Figure 4c). Nebivolol inhibited IFNγ from activated Th cells in two of the three samples tested (Figure 4d). To determine the specificity of nebivolol, a β2AR-specific antagonist ICI 118,551 was included in the cell culture. ICI 118,551 attenuated the suppressive effect of nebivolol on IL-17A (Figure 5). Cellular proliferation was not significantly changed by nebivolol (Figure 6a–e). No significant change in cell viability was found after nebivolol treatment (Figure 6f). Thus, nebivolol has variable effects on IFNγ. However, it robustly inhibits IL-17A in PBMC and Th cells in a β2AR-specific manner.

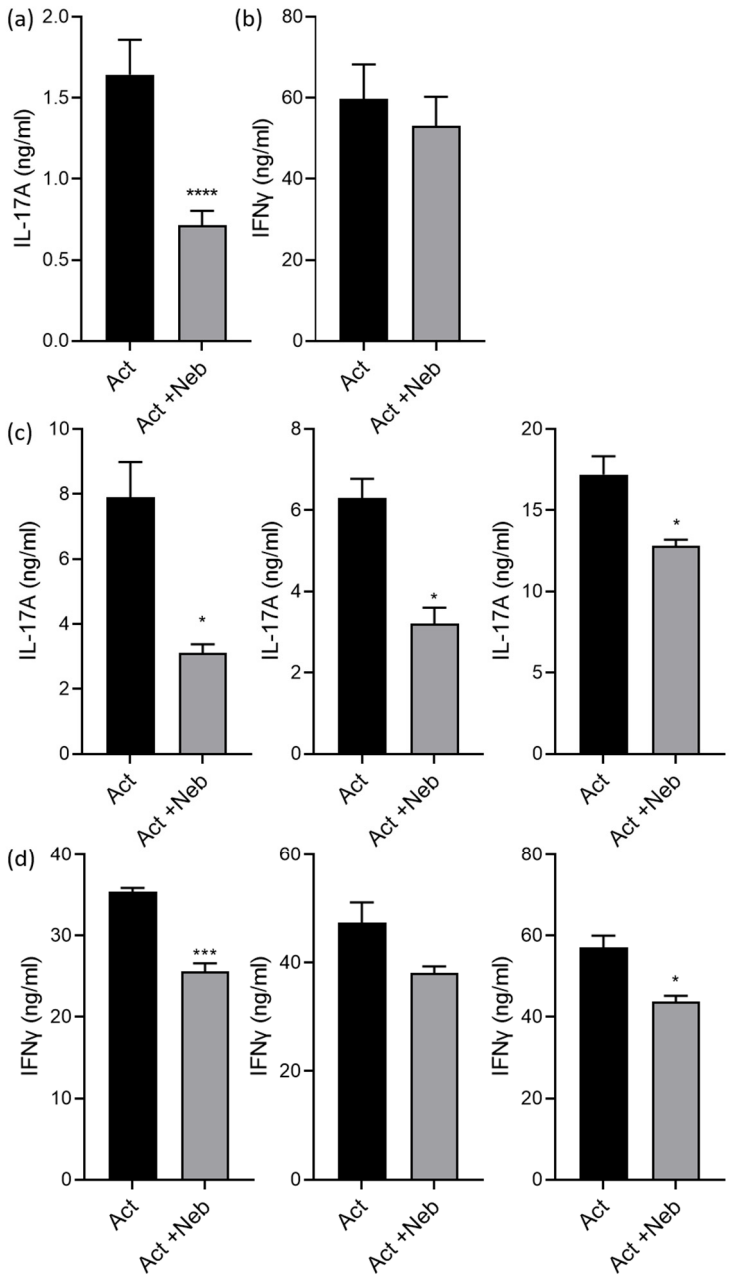

Figure 4. Effects of nebivolol on IL-17A and IFNγ secretion from activated PBMC and activated Th cells. PBMCs were activated with anti-CD3 and anti-CD28 antibodies (Act) with the addition of nebivolol (Act + Neb) for 4 days. (a) IL-17A and (b) IFNγ were measured from cell culture supernatants with ELISA. The data shown in (a) and (b) was averaged from 39 participant samples. Data analysis was done by one-way ANOVA (****$p < 0.0001$). (c,d) Purified CD4 Th cells were obtained from three participant samples using columns. CD4 T cells were activated with anti-CD3 and anti-CD28 antibody-dynabeads (Act; black bars), with the addition of nebivolol (Act + Neb; grey bars) for 4 days. (c) IL-17A and (d) IFNγ were measured from cell culture supernatants with ELISA. For the CD4 cell data, individual data is shown from the three participant samples, where the error bars are SE of the technical replicates. Paired t-test (*$p < 0.05$ **$p < 0.01$).

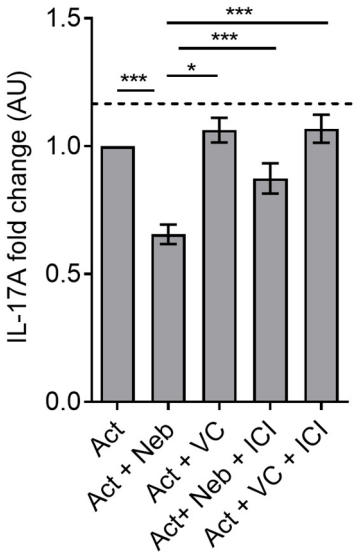

Figure 5. Nebivolol suppressed IL-17A in a β2AR-dependent manner. PBMCs were activated with anti-CD3 and anti-CD28 for 4 days (Act) with the addition of nebivolol (Act + Neb), ICI 118, 551 (ICI), or vehicle control (VC). IL-17A was measured in cell culture supernatant by ELISA and expressed as fold change. Data was averaged from four different participant samples. One way ANOVA with Tukey's multiple comparisons test (*$p < 0.05$, ***$p < 0.001$).

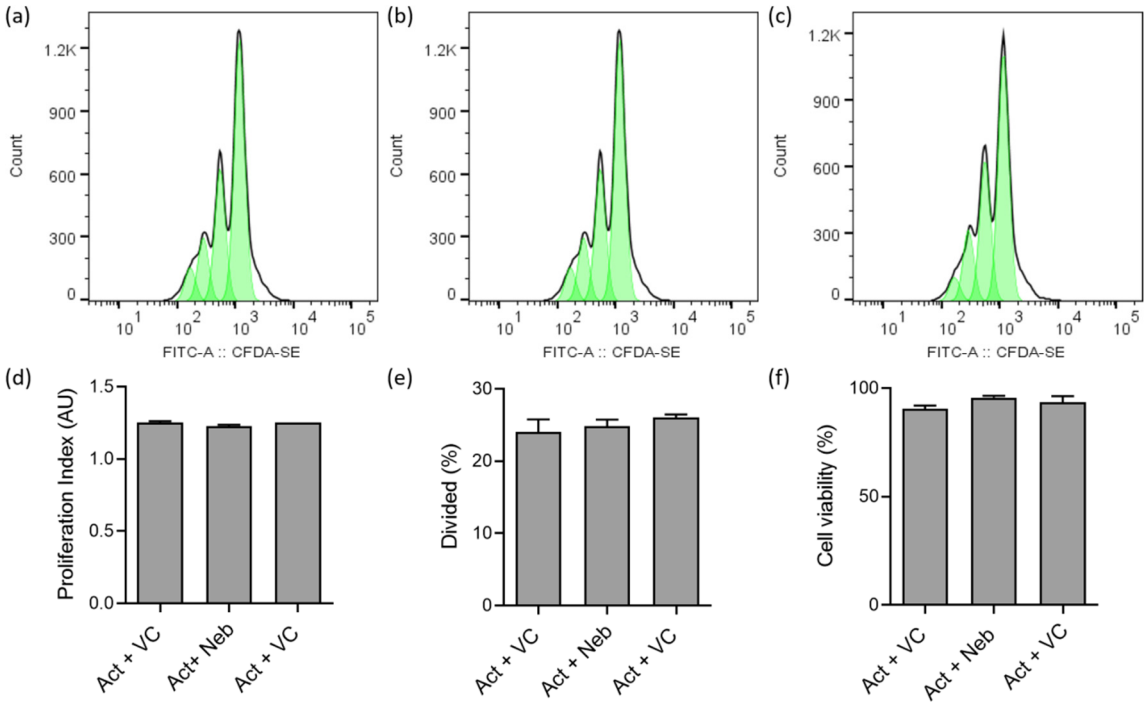

Figure 6. Nebivolol did not change the proliferation or viability of PBMC. PBMC were labelled with CFDA-SE, and the cell division peaks was calculated by the proliferation

tool on FlowJo. The treatment was with (a) activation, (b) activation plus nebivolol, or (c) activation plus vehicle control. (d) The average cell divisions of the cells that responded were calculated as a proliferation index. (e) The cells that divided at least once or more were expressed as percentage. (f) The viability of the cells was determined by trypan counting, where 100% represents maximum viability. The data shown is from one participant, which is representative of three different participant samples.

3.5. Testing the effect of nebivolol on IL-17A and its relation to ADRB2 polymorphisms

Next, terbutaline was compared to nebivolol, at equivalent doses, on the cytokine response of PBMCs *in vitro*. As expected, terbutaline had no significant effect on IL-17A in samples homozygous for Arg16, and augmented IL-17A in samples homozygous or heterozygous for Gly16 (Figure 7a,b). Nebivolol suppressed IL-17A in all of the samples regardless of the position 16 polymorphisms in β2AR (Figure 7a). Nebivolol did not significantly suppress IFNγ in the PBMC samples regardless of Gly/Arg16 polymorphisms (Figure 7b).

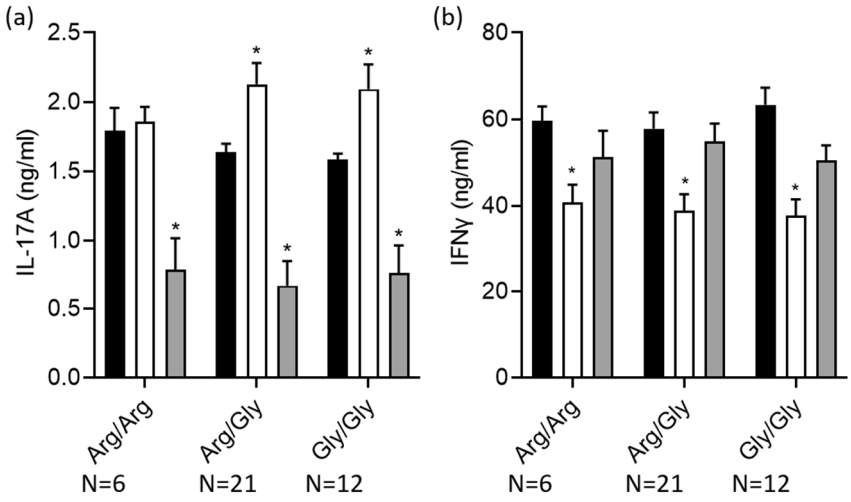

Figure 7. Modulation of IL-17A in activated PBMC by terbutaline as compared to nebivolol. PBMCs were activated with anti- CD3 and anti-CD28 for 4 days (black bars), with the addition of terbutaline (white bars), or nebivolol (grey bars) for 4 days. The samples were classified based on position 16 polymorphisms of β2AR. (a) The IL-17A was measured in the cell culture supernatant by ELISA. (b) The IFNγ was measured in the cell culture supernatant by ELISA. Data was averaged from 39 human PBMCs. Error bars show standard error. Multiple t-test with correction for multiple comparisons using the Holm–Sidak method was performed (*$p < 0.05$). The number of participant samples in each category is listed under the x-axis labels.

4. Discussion and conclusions

Common *ADRB2* polymorphisms are known to alter the response of β2AR drugs. We recently showed that a β2AR-specific agonist terbutaline suppressed IFNγ but augmented IL-17A in human

PBMC samples. In this report, we confirmed that finding with a larger sample size. Oostendorp et al. reported that IFNγ was suppressed by isoproterenol, a non-selective β-adrenergic agonist, regardless of *ADRB2* polymorphisms [24]. We demonstrated that terbutaline augmented IL-17A in samples that were homozygous or heterozygous for Gly16. In contrast, terbutaline had no significant effect on IL-17A in samples that were homozygous for Arg16 polymorphism of *ADRB2*.

Samples were analyzed in more detail using the haplotypes reported by Drysdale et al. The data showed that haplotype 2, which contains Gly16, corresponded to the drug response whether it was homozygous or heterozygous. Thus, Gly16 was the predominant feature that predicted response to terbutaline. Future studies could use just one primer set to obtain the sequence of the Arg/Gly16 polymorphism instead of sequencing the entire *ADRB2* gene and upstream region.

The dose of terbutaline used throughout this study was 10^{-5} M. This value is in the range of previous reports. For example, Ramer-Quinn et al. tested from 10^{-8} M up to 10^{-5} M terbutaline on Th1 lines, they found that 10^{-6} M and 10^{-5} M inhibited IL-2 secretion by at ~60% [16]. In a similar study, they saw significant effects on Th1 clones at the higher doses 10^{-6} M and 10^{-5} M of terbutaline [28]. Another group, Riether et al., tested 10^{-9} M up to 10^{-5} M of terbutaline, they found 10^{-5} M inhibited ~50% of IL-2 and IFNγ from mouse splenocytes [29]. The 10^{-5} M dose of terbutaline was not toxic as shown in our previous study on human PBMC [11].

The fact that terbutaline might raise IL-17A levels could be detrimental in some circumstances. It has been reported that IL-17A is pro-inflammatory in asthma and chronic obstructive pulmonary disease which are in themselves heterogeneous diseases [30,31]. Autoimmune diseases are also associated with higher IL-17A and Th17 cells. For example, higher IL-17A expression was observed in isolated Th cells from patients with juvenile systemic lupus erythematosus as compared to healthy individuals [32]. Th17 cells are elevated in multiple sclerosis patients and can be attenuated with IFNβ [33], or autologous stem cell transplantation [34]. Our results suggest that terbutaline could raise IL-17A in patients with the *ADRB2* common Gly16 polymorphism and exacerbate inflammation. In other contexts, raising IL-17A levels might be beneficial. For example, IL-17A is essential for immunity against *Candida albicans*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* infections [35,36] suggesting that terbutaline could be beneficial by augmenting IL-17A from Th17 cells to fight these pathogens. Since our experiments were done *in vitro* on healthy human participants, further experiments and pre-clinical testing would be required to explore the impact of terbutaline on Th cells *in vivo*.

The Gly16 polymorphism was the strongest determining factor for the terbutaline effect on IL-17A. It is not known how Arg or Gly at codon position 16 influences the response of β2AR to β-agonists. A study showed that Arg16 polymorphism had greater desensitization by isoproterenol in transfected cells with a luciferase reporter [24]. This suggests that Arg16 may be desensitized and thus looses its effect as compared to Gly16. Computational studies suggested that the Gly16 variant of β2AR has a 15 residue coil present in the secondary structure that is not present with the Arg16 variant. With Arg16, the vestibule formed by the transmembrane (TM) 5, 6, 7 has an open configuration and the ligand-binding site is enlarged, which enhanced the binding of albuterol [37]. The interactions that are associated with ligand binding of the β2AR (Asp113, Ser203, 204 and 207, Asn293 and Asn 312) did not change within the Gln or Glu variant [38]. Thus, Arg16 variant of *ADRB2* is likely to be more desensitized due to altered ligand binding domains.

To our knowledge, the effects of nebivolol on the immune system has not been widely explored. We chose 10^{-5} M of nebivolol based on Erickson et al. who studied the *in vitro* effects on mouse

embryonic fibroblasts (HEK) and a muscle cardiac (HL-1) cell line [8]. They showed various biological outcomes of nebivolol treatment at doses ranging from 200 nM up to 10^{-5} M, without toxicity to the cells. Similarly, our data showed no toxicity at 10^{-5} M of nebivolol on the human PBMC samples.

We discovered that nebivolol inhibited IL-17A and IFNγ from purified helper T cells that were activated with dynabeads coated with anti-CD3 and anti-CD28. Dynabeads are required to activate Th cells because adding soluble antibodies is not effective when other types of immune cells are removed. Similar experiments were performed on PBMC samples using soluble anti-CD3 and anti-CD28 antibodies. Nebivolol significantly inhibited IL-17A from the activated PBMC but did not significantly inhibit IFNγ. PBMCs contain other cell types that may produce IFNγ such as natural killer cells [39]. Thus, our data demonstrate that nebivolol attenuates IL-17A and IFNγ from Th cells but may not attenuate IFNγ in the more complex PBMC samples. Since nebivolol had not been previously studied in immune cells, we further tested its specificity and mechanism of action. We found that a β2AR-specific antagonist prevented nebivolol from inhibiting IL-17A, which indicates that the drug effects were specific for this receptor. Nebivolol did not significantly change cell proliferation or viability which indicates that the drug was not toxic to the cells. We also demonstrated that nebivolol could inhibit IL-17A from activated T cells regardless of the position 16 polymorphism of β2AR.

The mechanism of action by which terbutaline and nebivolol transmit signals via the β2AR is not completely understood. Our results showed that terbutaline consistently inhibited IFNγ, while only nebivolol consistently inhibited IL-17A. The drugs have several key differences in their biochemistry. Terbutaline is a racemic mix when used as therapeutic for asthma patients. The R-terbutaline enantiomer was more potent than the S-terbutaline as an anti-asthmatic effect [40]. Nebivolol has up to ten stereoisomers, two of which are pharmacologically active: (+*SRRR*)-nebivolol (*d*-nebivolol) and (-*RSSS*)-nebivolol (*l*-nebivolol). The *d*- and *l*-nebivolol isomers function through distinct mechanisms to synergistically produce the pharmacological and therapeutic effects. Blood pressure reduction is attributed to both isomers, but effects on heart rate and hypotension are caused by *d*- and *l*-nebivolol respectively. The *l*-enantiomer promotes endothelial nitric oxide (NO) synthesis and inhibits endothelial NO synthase uncoupling although the exact receptors are unknown [9]. Thus, a racemic version of nebivolol might carry the immuno-suppressive activity.

In conclusion, the β2AR is emerging as a relevant therapeutic target for immunomodulation in pro-inflammatory diseases. Our findings shine light on the individual variability to β-agonists based on a common receptor polymorphism. Moreover, we provided novel evidence that nebivolol warrants further study as an experimental immunomodulatory therapeutic for autoimmune conditions where IL-17A contributes to inflammation, whereas terbutaline appears to be better suited to inhibit IFNγ.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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