The Effect of Ligands of the β2 Adrenergic Receptor on Interleukin-2 Production in T lymphocytes

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ABSTRACT

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The immune system protects the body from infections and diseases. Helper T cells play an essential role in coordinating immune responses. In adaptive immunity, helper T cells are arguably the most important, as they are required for all adaptive immune responses. T cells activate and produce interleukin-2 (IL-2), a growth factor that causes T cells to proliferate rapidly when the body faces pathogen invasion. T cells trigger pro-inflammatory cytokine release that fights infection. However, constant T cell activation leads to an uncontrolled high amount of pro-inflammatory cytokines, causing tissue damage, resulting in chronic inflammation and, eventually, autoimmune disorders.

We previously found that nebivolol suppressed the pro-inflammatory cytokine, interleukin-17A which is implicated in the pathophysiology of inflammatory diseases and autoimmunity. Currently, there is little known about the effect of nebivolol on T cell activation regarding IL-2 production. In this thesis, I assess whether the immunomodulatory role of nebivolol extends to the IL-2 levels in T cells. My findings demonstrate that nebivolol suppressed IL-2 production in Jurkat T cells, and an inhibitory trend was observed in peripheral blood mononuclear cells (PBMCs) on activated T cells. I also found that nebivolol suppressive activity was β2-adrenergic receptor-dependent, which also implicated nuclear factor kappa B (NFκB) activity, as observed by its inhibition on phosphorylation levels.

These results are novel because there has not been a study on how nebivolol modulates IL-2 levels through the β2-adrenergic receptor. In particular, these findings add to our previous study demonstrating nebivolol immunomodulatory activity on T cells.

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LIST OF ABBREVIATIONS & ACRONYMS

Akt - serine/threonine protein kinase involved in cell growth and survival

APC - antigen-presenting cells

AR - adrenergic receptor

BCL10 - B cell lymphoma/leukemia 10

CARMA1 - caspase recruitment domain-containing membrane-associated guanylate kinase protein-1

cAMP - cyclic adenosine monophosphate

CD - cluster of differentiation

$CD4^+$ - helper T cell

$CD8^+$ - cytotoxic T cell

CNS - central nervous system

DC - dendritic cells

GPCR - G protein-coupled receptor

GRK - G protein-coupled receptor kinase

IFN - interferon

IKK - IκB kinase

IκB - nuclear factor kappa B inhibitor

IL - interleukin

ITAM - immunoreceptor tyrosine-based activation motif

LAT - linker for activation of T cells

MALT1 - mucosa-associated lymphoid tissue translocation protein-1

MAPK - Mitogen-activated protein kinases

MHC - major histocompatibility complex

MS - multiple sclerosis

NEMO - nuclear factor kappa B essential modulator

NFκB - nuclear factor kappa B

NIK - nuclear factor kappa B inducing kinase

PBMCs - peripheral blood mononuclear cells

PKA - protein kinase A

PKC-θ - protein kinase C-θ

PMA – phorbol 12-myristate 13-acetate

RA - rheumatoid arthritis

SNS - sympathetic nervous system

TAD - transcription activation domains

TBST – Tris-buffered saline with 0.1% Tween 20 detergent

TCR - T cell receptor

Th - T helper cells

CHAPTER I: INTRODUCTION

1. T Cells and Adaptive Immunity

T cells, a significant component of adaptive immunity, mount a specific host response against foreign antigens from various microorganisms (parasites, fungi, bacteria), viruses, cancer cells, and toxins (Chaplin, 2010). Adaptive immunity facilitates pathogen-specific responses to combat the extensive variation of antigen structures in pathogens. A key feature of adaptive immunity is the generation of immunological memory after pathogen encounters. Memory T cells are long-lived cells acting as librarians storing information, where a second encounter with the same pathogen would yield a quicker and more robust response for elimination (Bonilla & Oettgen, 2010). An antigen is a substance that triggers an immune response. Not all antigens are foreign, and T cells recognize and tolerate self-antigens procured by healthy cells. Antigens are short peptides that are processed and presented on the major histocompatibility complex (MHC) proteins by antigen-presenting cells (APCs) (Janeway et al., 2001). Upon antigen encounter, T cells clonally expand and differentiate into specialized T cells to destroy the foreign invaders.

There are two types of T cells: cytotoxic T cells ($CD8^+$) and helper T cells ($CD4^+$). Our lab is researching only the $CD4^+$, but both T cells are crucial in achieving a regulated immune response against pathogens. $CD8^+$ mediates the killing of infected cells and intracellular viruses such as HIV (Laidlaw et al., 2016). $CD4^+$ are responsible for immune cell activation, including $CD8^+$, are essential regulator of immune cell homeostasis. The importance of T cells in controlling viral infections was demonstrated in a study of lymphocytic choriomeningitis virus (LCMV) in $CD8^+$ and $CD4^+$ knockout mice (Matloubian et al., 1994). Usually, mice infected with LCMV show acute viral infection. However, in $CD8^+$ KO mice, the infection was persistent as the infection was unable to be cleared. Interestingly, in $CD4^+$ KO mice, despite the presence of $CD8^+$, the infection developed into a chronic infection (Matloubian et al., 1994). The absence of $CD4^+$ results in no activation of $CD8^+$, priming it to function, outlining the vital capacity of $CD4^+$ in regulating immune cell activation and activity.

$CD4^+$ can differentiate into different subsets called helper T cells (Th) with an identifying number of subscripts, for example Th1, Th2, and Th17 cells. Each exhibits distinct cytokine profiles against invading pathogens (Luckheeram et al., 2012). Cytokines are small proteins that control immune cell activity. The different Th subsets produce pro-inflammatory or anti-

inflammatory cytokines, depending on the host invaders. Pro-inflammatory cytokines such as interleukin-17A (IL-17A) produced by Th17 or interferon-gamma (IFNγ) by Th1 are involved in the pathological pain processes. Anti-inflammatory cytokines such as IL-4 produced by Th2 act as pro-inflammatory regulators (Zhang & An, 2007). Pro-inflammatory cytokines are released to stimulate and recruit more immune cells to the sites of infections. Once the pathogen is cleared, the anti-inflammatory cytokines are released and bind to the activated immune cells' inhibitory receptors, downregulating the pro-inflammatory cytokine receptors, eventually bringing the immune cells back to equilibrium.

Each T cell subtype releases specific pro-inflammatory cytokines that target particular pathogens for elimination. Th17 releases pro-inflammatory IL-17A that plays a role in clearing fungal and extracellular bacterial infections. Mice that lack Il-17A genes, *il17A-/-* were found to be more susceptible to *Staphylococcus aureus* infection, an opportunistic bacteria (Ishigame et al., 2009). Unfortunately, excessive pro-inflammatory cytokine production leads to chronic inflammatory diseases such as inflammatory bowel disease (IBD), comprised of Crohn's disease and ulcerative colitis caused inflammation of the colon and small intestine, respectively (Zwicky et al., 2019). There is a high expression of pro-inflammatory cytokines such as IL-17A in the inflamed mucosa of IBD patients, compared to the control group (Fujino et al., 2003). The involvement of pro-inflammatory cytokines in inflammatory disease pathophysiology makes them an attractive therapeutic target. Targeting and reducing pro-inflammatory cytokines would 1) diminish chronic inflammation and tissue damage, which would halt disease progression (Biggioggero et al., 2018; Smolen et al., 2008) and 2) prevent the development of other diseases as chronic inflammation increased susceptibility to comorbidities (Don-Doncow et al., 2019; Mercurio et al., 2019; Newcombe et al., 2018).

2. T Cell Activation and Interleukin-2 Production

In order to eliminate pathogens, T cells require two stimulatory signals to be activated to initialize proliferation and differentiation. These signals are provided by antigen-presenting cells (APCs). APCs have a major histocompatibility complex (MHC), a surface protein that presents foreign antigen peptides to T cells. Signal one occurs via the antigen presentation by the MHC complex of the APCs binding to the T cell receptors (TCR) of T cells. The second signal thought to amplify signal one is costimulatory. Signal two occurs by binding CD80 and CD86 proteins of

APCs to the CD28, the co-receptor protein on the T cells. Signal two is required for T cell activation, without it, T cells undergo apoptosis (Fig 1) (Noel et al., 1996).

The activation of T cells results in the production of IL-2 and IL2 receptors (IL-2R). IL-2 activates the AKT pathway which provides an antiapoptotic signal for survival and proliferation (Cantrell & Smith, 1983; Jones et al., 2000; Kelly et al., 2002). IL-2 binds to immune cells that express IL-2Rs in an autocrine manner, which means IL-2 binds to the same cell that secretes it, or in a paracrine manner, meaning that IL-2 binds to neighboring cells (Pol et al., 2019). IL-2R may contain any of three subunits: IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132), forming weak, intermediate, and strong affinities, respectively, depending on the receptor's combination (Mitra & Leonard, 2018). An independent receptor makes for a weak affinity (Kd: ~10^{-8} – 10^{-7}M), a βγ dimer forms an intermediate affinity (Kd: ~10^{-9}M), and the trimeric αβγ creates a strong affinity (Kd: ~10^{-11}M) (Ross & Cantrell, 2018). Each immune cell has a different expression of IL-2R, giving rise to different sensitivities based on their distinct roles.

IL-2 is vital growth factor, however, a high amount of IL-2 produced by overactive T cells was associated with autoimmunity (Wood et al., 1988). Early investigators showed high levels of IL-2 and IL-2R in autoimmune inflammatory disease models, including increased numbers of T cells in the inflammatory sites in EAE (experimental autoimmune encephalomyelitis) mice brain and spinal cords (Merrill et al., 1992) and in autoimmune patients (Wood et al., 1988). High levels of IL-2 increased other pro-inflammatory cytokine levels, and decreased anti-inflammatory cytokines in inflammatory diseases (Fallahzadeh et al., 2011) and autoimmune patients (Brynskov & Tvede, 1990; Huan et al., 2022; Li et al., 2020). These findings suggest that IL-2 production is a good indicator of T cell activation and can be used as a reference for T cell overactivity when produced excessively (Murakami et al., 2019). My thesis assessed whether terbutaline or nebivolol inhibited the production of IL-2.

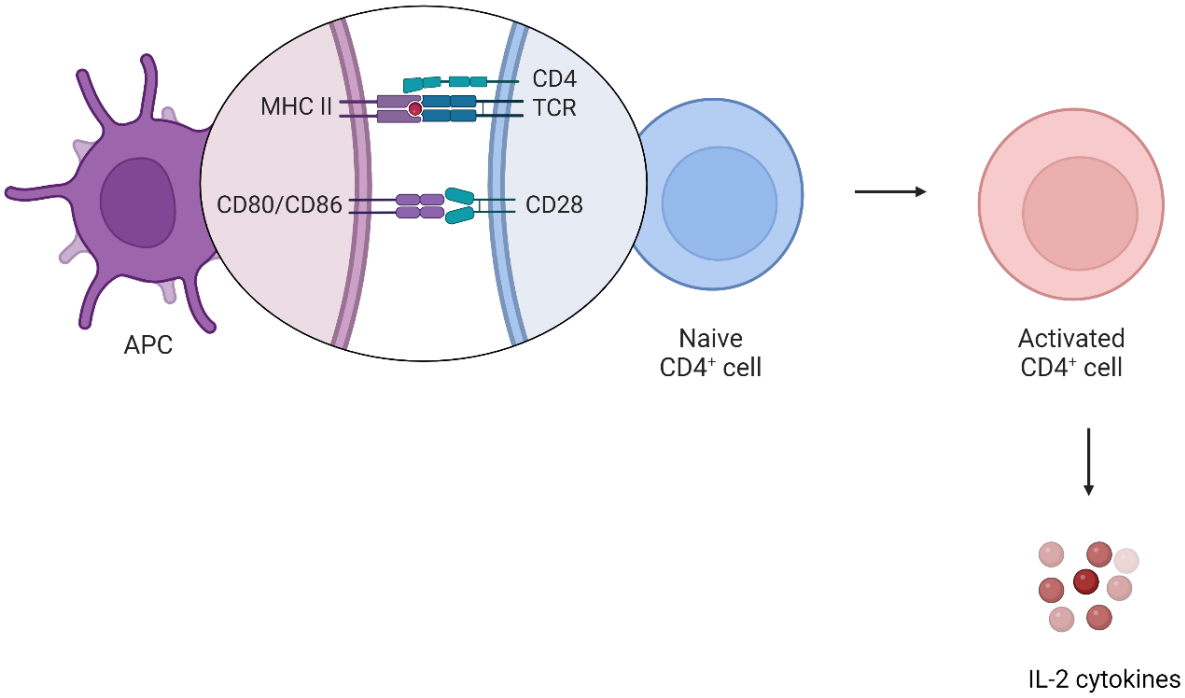


Figure 1. Antigen-presenting cell and T cell activation

The interaction between naive T cell TCR and its cognate antigen presented on the MHC by APC and the TCR:MHC complexes. The second co-stimulatory signaling between CD80 and CD86 with CD28 compliments the intracellular signaling process triggered by the TCR:MCH signaling complex, leading to an activated T cell. The activated $CD4^+$ secretes IL-2 cytokines, which bind its IL-2R in autocrine and paracrine fashions, causing T cell proliferation and clonal expansion. Created with BioRender with acknowledgment to Anna Lazaratos.

3. β2AR: One Receptor with Two Signaling Pathways

There is communication between the central nervous system (CNS) and the immune system that plays a vital role in maintaining physiological homeostasis and immune responses (Kenney & Ganta, 2014). Neuroimmune communication occurs through the sympathetic nerve fibers innervated into lymphoid tissues including bone marrow, thymus, spleen, and lymph nodes (Chhatar & Lal, 2021). The sympathetic nervous system (SNS) releases neurotransmitters such as epinephrine in the adrenal glands, and norepinephrine throughout various tissues of the body that affect the immune system's function. The SNS richly innervates the thymus, which is the maturation location for T cells (Besedovsky et al., 1987). The mature T cells reside in the thymus's corticomedullary junction, which contain a high density of SNS nerves, particularly found to have a high expression of β2AR in both humans and rats (Bellinger et al., 2008; Marchetti et al., 1994). At this location, the SNS is in close proximity to T cells and the nerve ending can release norepinephrine directly onto the T cell (Felten et al., 1992). Since T cells express the β2AR, they can respond to norepinephrine. Despite this being the paradigm for many decades, it is still not completely understood how β2AR signaling regulates T cells. In my thesis I have addressed the role of β2AR using T cell culture experiments.

The adrenergic receptors (AR) belong to a superfamily of 7-transmembrane receptors, also known as G protein-coupled receptors (GPCRs), consisting of α1-, α2-, β1-, β2- and β3AR (Graham, 1990). Both α1AR and α2AR are mainly expressed in smooth muscle and the central nervous cells. While β1AR is primarily found in the heart and kidney, β2AR is distributed to the heart, vascular and airway smooth muscle, while β3AR is in adipose cells (Bylund, 2013; Graham, 1990; Madamanchi, 2007). Adrenergic GPCRs are widely distributed in cells and tissues that regulate various physiological processes, and their dysfunction can contribute to disease (Johnson, 2006). Adrenergic GPCRs have a wide variety of ligands, which makes them well-studied for drug targets and discovery.

In T cells, the β2AR is expressed predominantly (Wu et al., 2018), which poses many questions about their involvement in immune cell regulation. The relationship between β2AR and T cells through interactions with epinephrine or norepinephrine in modulating cytokines production needs to be explored. Epinephrine and norepinephrine inhibited IFNγ in T cells (Swanson et al., 2001; Wahle et al., 2005). These immunomodulatory activities were also observed with the exogenous β-adrenergic drug isoproterenol in reducing pro-inflammatory cytokines in mice myocardium (Chandrasekar et al., 2004), while terbutaline increased IL-17A

(Carvajal Gonczi et al., 2023). Where the terbutaline finding was interesting as it was the first to show increased inflammatory activities in T cells.

There are three different classes of ligands: agonists, antagonists, and inverse-agonists. Ligands, for example, drugs or hormones, are molecules that can bind to their target receptors (Miller & Lappin, 2023). An agonist is a ligand that binds to a receptor and activates it to create a biological response. Antagonist binds to a receptor and inactivates it by preventing the agonist from binding, thus nullifying any biological response (Salahudeen & Nishtala, 2017). Inverse agonism is a recently recognized phenomenon where the inverse agonist occupies the same receptor site as an agonist, but activates an alternative signaling pathway (Berg & Clarke, 2018). The alternative pathway is thought to oppose the classical agonist pathway, thus ligands described as inverse agonists are referred to as antagonists in some publications.

The β2AR has two signaling pathways: the classical heterotrimeric G protein pathway and the GRK/β-arrestin pathway. The classical pathway is activated by binding of agonists, such as catecholamines. This results in the dissociation and interaction of heterotrimeric G protein subunits Gα with their downstream effector proteins, such as adenylyl cyclase activation, leading to increased cAMP production (Weis & Kobilka, 2018). The cAMP activates protein kinase A (PKA), which inhibits and suppress T cell activation (Ramstad et al., 2000). Unlike the classic pathway, the GRK/β-arrestin pathway does not involve heterotrimeric G protein activation. Instead, its activation leads to the phosphorylation of β2AR c-terminal tails by GRK5 and GRK6, which are GPCR protein kinases. Phosphorylation increases the binding affinity of the multifunctional adaptor protein β-arrestins towards the receptor. The interaction between β-arrestins and the β2AR creates a molecular arrangement that hinders the heterotrimeric G protein engagement, blocking the classical signaling pathway (Shukla et al., 2014). The bound β-arrestins on the receptor serve as docking proteins for the formation of clathrin-coated pits by interacting with AP-2 and clathrin (Oakley et al., 2000), which orchestrates the MAPK activation that regulates T cell early development and maturation (Lefkowitz et al., 2006; Rincón, 2001). The β-arrestins also recruit cAMP-specific phosphodiesterases 4 (PDE4), which degrade cAMP (Abrahamsen et al., 2004), another way the alternative pathway opposes the classical pathway.

4. The Agonist and Inverse Agonists: Terbutaline, Carvedilol, and Nebivolol

All β-adrenergic agonist drugs are designed to mimic endogenous catecholamine activities, and are mainly used to treat respiratory disorders such as asthma and COPD (Abosamak &

Shahin, 2023). Agonist drugs take advantage of the high expression of β2AR in airway smooth muscle and lung tissues (epithelial, endothelial, and type II cells) (Larocca et al., 2011). The β2AR activation produces cAMP that relaxes the smooth muscle fibers, resulting in bronchodilation. Different β2AR agonists including terbutaline are on the market for respiratory disorders. Most agonist experiment were carried out in peripheral blood mononuclear cells (PBMCs), which are primary cells containing all the white blood cell types (lymphocytes, monocytes, and dendritic cells (DCs)) allowing the assessment of different immune cell interactions (Kleiveland, 2015), isolated T cells ($CD4^+$) and *in vivo* animal models such as mice and rat.

I chose terbutaline as the agonist for my study because it is a selective β2AR agonist (Hsu & Bajaj, 2023). Terbutaline increased anti-inflammatory cytokine production in PBMCs (Goodman et al., 1996; Heesen et al., 2002) and in rats by reducing the T cell population (Wiegmann et al., 1995). Surprisingly, terbutaline increased pro-inflammatory cytokine, IL-17A in PBMCs through the β2AR stimulation (Carvajal Gonczi et al., 2017). The effect of agonists on IL-2 production has been shown with different drugs, namely, isoproterenol, albuterol, and salmeterol in PBMCs, isolated $CD4^+$, and mice (Averill et al., 1988; Bartik et al., 1993; Sekut et al., 1995), which all showed reduced IL-2 levels. There has not been a study done on terbutaline's effects on IL-2 production. I proposed that terbutaline will also decrease IL-2, as seen with other agonists (Averill et al., 1988; Bartik et al., 1993; Sekut et al., 1995).

All β-blockers work as antagonist by blocking the agonist from activating the classical β2AR pathway (Wang et al., 2004), resulting in less calcium influx and vasodilation, lowering blood pressure and heart rate (Clarke et al., 2010). The majority of beta blockers are antagonists, however, there are a few such as carvedilol and nebivolol that exhibit inverse-agonism activity (Wisler et al., 2007). There are several effects such as downregulation of cAMP production, recruitment of β-arrestins, β2AR-c termini tail phosphorylation, and ERK1/2 activation, that an antagonist must produce to be considered an inverse-agonist (Michel et al., 2020; Wisler et al., 2007). These biochemical effects have important clinical relevance. Physicians commonly prescribe β-blockers to treat various medical problems. For example, bisoprolol and nadolol reduce inflammation in hypertensive patients (Toyoda et al., 2020), carvedilol minimizes the force of heartbeat in chronic heart failure (CHF) (Shaw et al., 2009), and propranolol reduces inflammation in kidney failure in a mouse model (Tsai et al., 2020). Most research on inverse-agonist drugs is in the field of cardiovascular diseases, but their effects on T cell activation and IL-2 production have not yet been established.

The inverse-agonist I chose to study is nebivolol as our previous study demonstrated its anti-inflammatory activities in PBMCs in reducing IL-17A (Carvajal Gonczi et al., 2021), but its mechanism of action is not elucidated. IL-2 is a T cell growth factor that promotes T cell proliferation in autoimmune disorders (Raza et al., 2006). Nebivolol's activity on IL-2 production has not been assessed in PBMC and Jurkat T cells. Investigating nebivolol's mechanism of action on IL-2 could help understand nebivolol's inverse-agonism activity in downregulating pro-inflammatory cytokine. I also explored carvedilol as it was the first established inverse-agonist shown to modulate IL-2 in PBMC (Erickson et al., 2013; Wisler et al., 2007). Nebivolol is the latest generation beta-blocker for hypertension, angina, and congestive heart failure treatment (Weiss, 2006). Nebivolol is a β1AR antagonist and β2AR inverse agonist (Priyadarshni & Curry, 2023). Similarly, carvedilol is also a β1AR antagonist, β2AR inverse agonist, and α1AR antagonist. Carvedilol treats heart failure, hypertension, and myocardial infarction (Singh & Preuss, 2023). As the newest beta-blocker on the market, nebivolol has better tolerability and less contraindication, allowing broader usage in patients than older beta-blockers (Weiss, 2006).

Nebivolol has been shown to decrease T cell density and activity, including atherosclerosis plaque size in the atherosclerotic mouse model (Pyka-Fosciak et al., 2013). Nebivolol also was shown to decrease pro-inflammatory cytokines in hypertensive patients (Hussain et al., 2017), and in healthy patient PBMCs (Carvajal Gonczi et al., 2021). Carvedilol also showed anti-inflammatory activity observed in rats with autoimmune myocarditis (Yuan et al., 2004) and liver injury (Júnior et al., 2016). As mentioned, carvedilol reduced IL-2 in healthy PBMCs (Yang et al., 2003), and in myocardial infarction (Tepliakov et al., 2004). Currently, there is only nebivolol and carvedilol that has been studied as the inverse-agonists of β2AR (Erickson et al., 2013; Wisler et al., 2007), and shown to be anti-inflammatory in T cells (Carvajal Gonczi et al., 2021; Yang et al., 2003). However, the effect of nebivolol on IL-2 production in T cells is yet to be investigated. Since carvedilol is an inverse-agonist and demonstrated inhibitory effect on IL-2, I hypothesized that nebivolol would also reduce IL-2 production in T cells.

5. Nuclear Factor kappa B (NFκB) Regulates T Cell Growth

When studying immunomodulatory drugs, it is important to consider the role of nuclear factor kappa B (NFκB) because this transcription factor is involved in immune and inflammatory responses. The activation of T cells leads to the initiation of NFκB signal transduction events essential in their differentiation, survival, and effector function (Daniels et al., 2023; Los et al.,

1995). There is inflammatory crosstalk between β2AR-NFκB signaling. The β2AR agonists, isoproterenol showed upregulation of NFκB activity and pro-inflammatory cytokine production in mice myocardium (Chandrasekar et al., 2004) and in mice aortic endothelial cells (Ciccarelli et al., 2011). On the other hand, the inverse-agonist, carvedilol, downregulated IL-2 production and NFκB activity in PBMCs (Yang et al., 2003). To understand the interplay between β2AR-NFκB, we must understand their signaling cascades in T cells.

T cell activation initiates NFκB signaling cascades through the activation of PKCθ, a protein kinase C, which belongs to a family of serine/threonine kinases that regulate cellular functions (Shah et al., 2021; Sun, 2012). PKCθ activates the CBM complex, consisting of cytoplasm adaptor proteins composed of the caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1), B cell lymphoma/leukemia 10 (BCL10), and mucosa-associated lymphoid tissue translocation protein-1 (MALT1) (Shah et al., 2021). PKCθ phosphorylates and activates the CBM complex that recruits tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase, which then proceeds to polyubiquitinate the IκB kinase (IKK). IKK is a protein complex consisting of two kinase subunits (IKKα and IKKβ) and a non-catalytic subunit NEMO (IKKγ) (Zhou et al., 2004). The polyubiquitination degrades NEMO, activating IKK and leading it to phosphorylate IκB, a protein that sequesters NFκB in the cytoplasm. The phosphorylation of IκB causes its degradation by the proteosome, which then frees and activates NFκB (Fig 2) (Israël, 2010). NFκB consists of five transcription factors: p50, p52, p65 (RelA), c-Rel, and RelB. RelB, c-Rel, and p65 contain C-terminal transcription activation domains (TADs), enabling co-activator recruitment and target gene expression. On the other hand, p50 and p52 lack TADs, thus forming homo- and heterodimers with c-Rel, RelB, or p65. The dimerized form would bind to target κB sites such as *Il-2* expressions in T cells to modulate gene expression (Hayden & Ghosh, 2011).

As mentioned, NFκB is a critical regulator in immune responses to inflammation, infection, and stress. There has been an implication of NFκB involvement in autoimmune diseases, such as Crohn's disease (Gelbmann et al., 2003) and rheumatoid arthritis (RA) (Handel et al., 1995; Marok et al., 1996). Chronic inflammation, such as autoimmunity, has been shown to increase comorbidities associated with myocardial infarction (heart attack) and heart failure in RA patients (Panoulas et al., 2007). The findings also show that the majority of older RA patients have undiagnosed and uncontrolled hypertension. A chronic activated NFκB would lead to hyperactive T cells. Thus, drugs designed are targeted towards reducing pro-inflammatory cytokines by putting a brake on the NFκB activation (McDaniel et al., 2016). Nevertheless, a

better model of pro-inflammatory control via the NFκB pathway is reducing its overactivity, not completely blocking the basal NFκB activity. The basal activity of NFκB is crucial to maintaining T cell viability, keeping our immune system defense undisrupted, and minimizing the risk of immunodeficiencies (Makarov, 2001; Miller et al., 2014).

There is a therapeutic potential that could be harnessed from β2AR-NFκB crosstalk, as there is an involvement of the β-arrestins in modulating NFκB activity. Witherow et al. showed a direct interaction between β-arrestins and IKKα and IKKβ of the IKK complex through immunoprecipitation in activated Jurkat cells. They also reported reduced NFκB activity when β-arrestins were overexpressed and, in contrast, showed increased NFκB activity when they knocked down β-arrestins with siRNA (Witherow et al., 2004). Moreover, Gao et al. demonstrated that β-arrestins knocked down with siRNA also decrease pro-inflammatory cytokines associated with NFκB vascular inflammatory activities in THP1 cells, a human monocyte cell line (Gao et al., 2004). Nebivolol's anti-inflammatory properties via the alternate GRK/β-arrestins pathway could provide anti-inflammatory therapeutic potential in modulating T cell activity in inflammatory disorders. Nebivolol anti-inflammatory properties would provide several benefits, including 1) prevention and reduction of inflammation associated with cardiovascular risk, 2) its safety profile is established, enabling the possibility of combination treatments such as diabetes, which is linked to hypertension, and 3) cost-effective treatment for poor socioeconomic patients in developing countries for management of autoimmunity disorders linked with hypertension. Therefore, more research regarding the anti-inflammatory properties of nebivolol is important to help tailor its prescription for each individual.

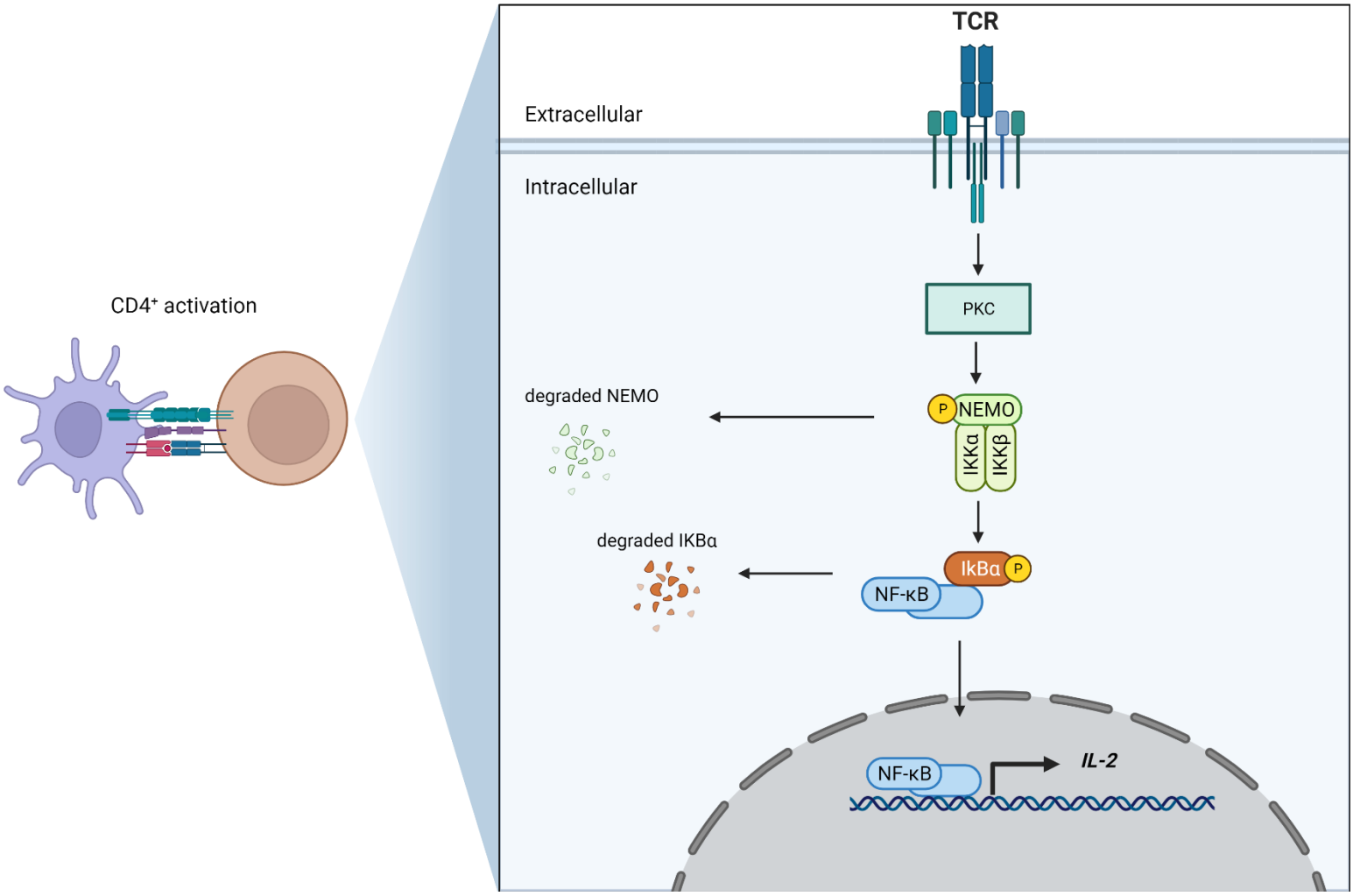


Figure 2. Simplified NFκB signaling pathway

The activation of $CD4^+$ leads to the NFκB signaling pathway initiation. PKC signals the downstream events of NEMO phosphorylation of the IKK complex (IKKα, IKKβ, NEMO) leading to its degradation. The switched on IKK complex phosphorylated IκBα resulting in its degradation to release NFκB. NFκB moves into the nucleus and turn on the *Il2* expression. Created with BioRender.

CHAPTER II: HYPOTHESES AND AIMS

1. Hypothesis

The β2AR agonist terbutaline and the inverse-agonists nebivolol and carvedilol decrease IL-2 production in T cells. Nebivolol suppression of IL-2 is caused by NFκB phosphorylation inhibition.

2. Aims

2.1.1. To investigate the effect of terbutaline, carvedilol, and nebivolol on IL-2 production in activated Jurkat T cells.

2.1.2. To investigate the effect of nebivolol on IL-2 production in PBMCs.

2.1.3. To determine if the impact on IL-2 levels by nebivolol occurs through the β2AR pathway.

2.1.4. To determine by assessing the phosphorylation levels of NFκB in the presence of nebivolol if the downregulation of IL-2 production occurred via the NFκB pathway.

CHAPTER III: MATERIALS AND METHODS

1. Jurkat T Cells Activation and Drug Treatment

Jurkat T cells were a generous gift from Dr. Shih of Concordia University, were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM Glutamine, 1 mM penicillin, and streptomycin (Wisent Inc. QC, Canada). Cells were incubated at 45,000 cells in 200 µL media per well in a round bottom 96 well culture plate (VWR, Mississauga, ON, Canada). Cells were activated with 0.1 µg/mL of anti-CD3 (OKT3) and 20 ng/mL phorbol myristate acetate (PMA) (eBioscience, San Diego, CA, USA). The *in vitro* drug treatments include nebivolol 10^{-5} – 10^{-7}M (Nebivolol hydrochloride, N1915, Sigma Aldrich), carvedilol 10^{-5} – 10^{-7}M (Carvedilol, C3993, Sigma Aldrich), β2AR antagonist, ICI-118-550 (100nM), and vegetable glycerin (Sigma Aldrich, Mississauga, ON, Canada) as drug vehicle (alternative to dimethyl sulfoxide (DMSO)) (Van de Water et al., 1988). Cell culture supernatants were collected after 48h of incubation to measure cytokines.

2. PBMC Activation and Drug Treatment

PBMCs was collected from venous blood drawn from healthy participants after collecting inform consent. The project was approved by the Concordia University Human Research Ethics Committee according to the Helsinki guidelines. 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 PBMC as previously described (Tabatabaei Shafiei et al., 2014). The PBMCs were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM penicillin, and streptomycin (Wisent Inc. QC, Canada). Cells were incubated at $0.5x10^6$ cells in 200 µL media per well in a round bottom 96 wells culture plate (VWR, Mississauga, ON, Canada). Cells were activated with 0.1 µg/mL of anti-CD3 (OKT3) and 0.1 µg/mL of anti-CD28 (eBioscience, San Diego, CA, USA). Cell culture supernatants were collected after 48h of incubation to measure cytokines.

3. Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the IL-2 cytokines, both PBMCs and Jurkat T cell culture supernatants were collected after 48h of incubations. The IL-2 cytokines were then determined by ELISA (1:1 dilutions), according to the manufacturer's instructions for human IL-2 (BD Bioscience, San Jose, CA, USA). To measure the IL-17A cytokines, PBMCs culture supernatants were collected after four days of incubations. The IL-17A cytokines were determined by human IL-17A (Invitrogen™, Carlsbad, CA, USA) ELISA kit (1:6 dilutions) according to the manufacturer's protocol. The sample's absorbance was measured at 450 nm, with a wavelength correction at 570 nm with SYNERGY H1 microplate reader (BIO-TEK).

4. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The protocol for *β2AR* expression measurement was based on our recent study (Carvajal Gonczi et al., 2023). In brief, $2x10^6$ Jurkat T cells were either non-activated (No Act) or activated (Act) with 0.1 µg/mL of anti-CD3 (OKT3) and 20 ng/mL phorbol myristate acetate (PMA) (eBioscience, San Diego, CA, USA). Total RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity and the concentration of the RNA samples were measured using a spectrophotometer (NanoDrop™ 2000c, ThermoScientific™). The RNA purity was determined by the 260/280 ratio, where a ratio of 2.0 was used to create a cDNA library using iScript™ Reverse Transcription Supermix for RT-qPRC (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The mRNA expression analysis was then performed using a TaqMan™ Fast Advanced Master Mix (Applied Biosystems™, Foster City, CA, USA) according to the manufacturer's protocol. TaqMan™ gene probes were used to measure the gene expression for *ADRB2* (Kit number: Hs00240532_s1) and the housekeeping gene *PPIA* (Kit numbers: Hs99999904_m1; Applied Biosystems™). Using the CFX Maestro Software (Bio-Rad Laboratories). The expression of the gene was normalized to the housekeeping gene, the data was further normalized based on Act, and then calculated and shown as a fold increase in relative expression ($2^{-\Delta\Delta CT}$), where $\Delta\Delta CT = \Delta CT_{(target\ gene)} - \Delta CT_{PPIA}$, and $\Delta CT = CT_{(target\ gene)} - CT_{PPIA}$.

5. Western Blot

Jurkat T cell lysates were prepared based on our recent protocol (Carvajal Gonczi et al., 2023). Cells were activated with 0.1 µg/mL of anti-CD3 (OKT3) and 20 ng/mL phorbol myristate acetate (PMA) (eBioscience, San Diego, CA, USA) for 15, 30, and 60 minutes with and without 10^{-5}M nebivolol (Nebivolol hydrochloride, N1915, Sigma Aldrich), and 0 minutes for the unstimulated (No act) control. 10 million cells per conditions were pelleted (2-3minutes, 500xg, 4°C). The cells were then washed with 1X cold PBS and pelleted again (5 minutes, 500xg, 4°C), then added 250ul of cold lysis buffer and left on ice for 30 minutes. Afterward, the cells were centrifuged at a max speed of 9.3g, 4°C, 5 minutes, the supernatant was collected, and protein concentration was determined. For immunoblotting, membranes were probed with a primary mouse monoclonal for human phospho-Ser365 NFκB/p65 (2ug/mL, R&D Systems, Canada) with secondary goat anti-mouse IgG HRP (1:3000, BioRad) in 2% skim milk in TBST. The primary goat anti-human NFκB/p65 (1:1000, Santa Cruz Biotechnology Inc., USA) with secondary rabbit anti-goat IgG HRPP (1:10,000, ElabScience) in 5% skim milk in TBST was used for the total NFκB control. The primary mouse anti-human α-tubulin (1:1500, Santa Cruz Biotechnology Inc., USA) with secondary goat anti-mouse IgG HRPP (1:3000, BioRad) in 5% skim milk in TBST was used for the loading control. The membranes were developed with enhanced chemiluminescence using Bio-Rad ClarityTM Western ECL Substrate (BioRad) and analyzed on gel imager chemidoc XRS+ system (BioRad).

6. Statistical Analysis

Raw data were processed in triplicate using Microsoft Excel Version 2308 to determine means and viability percentages. IL-2 cytokines concentrations for each ELISA well and calculated by the Gen5 Software. The pooled data were plotted using the aggregate individual data of different participants (LCR) for PBMCs and individual plates with the same number of cells (45,000 per well) for Jurkat T cells. The data were aggregated based on the same treatment conditions and expressed as means (Fold Change) ± SEM. The individual figures were plotted based on three technical replicates for every experiment for Jurkat T cells and PBMCs and expressed as means ± SEM. GraphPad Prism Version 10 was used to plot and analyze all the data. Statistical analysis with multiple comparisons were done using a one-way ANOVA ($p<0.05$). *Post hoc* Tukey's multiple comparison test was used to determine the

differences between means ($p \geq 0.05$ (ns), *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). A paired Student's t-test was performed for statistical analysis between two groups.

CHAPTER IV: RESULTS

1. The Expression of β2AR and β1AR in Jurkat T cells

To study how classical agonists and inverse-agonists affected the immune system, I established a cell model of T cells based on Jurkat T cells. These are, a commonly used cell line that is an effective way to study biochemistry and function of T cells. To determine if β2AR and β1AR were present in Jurkat T cells, their gene expression, *ADRB2* (Fig 3A) and *ADRB1* (Fig 3B) in both non-activated and activated was measured with qPCR and expressed relative to a housekeeping gene. Jurkat T cells expressed β2AR in both activated and non-activated states. The *ADRB2* (Fig 3A) expressions was increased in the activated state compared to the non-activated state in Jurkat T cells. Jurkat T cells also expressed β1AR (Fig 3B) in both activated and non-activated states. In contrast to *ADRB2*, the *ADRB1* expressions was decreased in the activated state compared to the non-activated state.

2. The Effect of Terbutaline, Nebivolol, and Carvedilol on The Production of IL-2 and Cells' Viability in Jurkat T Cells

To determine agonist and inverse-agonist effects on IL-2 production, Jurkat T cells were activated and incubated for 48h with or without the adrenergic drugs, and IL-2 levels were measured with ELISA. Terbutaline (Fig 4A and Fig S1A-D) showed no effect on IL-2 in all three concentrations (10^{-5}, 10^{-6}, 10^{-7}M) tested as compared to activated cells. Due to the lack of an effect of terbutaline on IL-2, the drug's efficacy was evaluated by measuring its effect on IL-17A in PBMC. Our previous study showed that terbutaline increased IL-17A in PBMC (Carvajal Gonczi et al., 2023). Therefore, terbutaline's effect on IL-17A was evaluated as a control for its efficacy. Terbutaline increased IL-17A (Fig 4B) compared to activated cells. Cells viability (Fig 4C) was also assessed with or without terbutaline at different concentrations (10^{-5}, 10^{-6}, 10^{-7}M), which showed viability of >85% in all concentrations.

The inverse agonists, carvedilol (Fig 5A and Fig S2A-D) and nebivolol (Fig 6A and Fig S3A-D) showed significant suppression of IL-2 compared to activated cells. No significant impact on IL-2 was observed at 10^{-6} – 10^{-7}M concentrations for neither carvedilol (Fig 5A) nor nebivolol (Fig 6A). Vegetable glycerin as the solvent for both carvedilol and nebivolol were also assessed for any discrepancy. The solvent control showed no suppressive activity in both carvedilol (Fig

5A) and nebivolol (Fig 6A). Cells viability was also assessed, with carvedilol showed cell viability (Fig 5B) of >81% in all concentrations, and >85% in nebivolol (Fig 6B) at all concentrations, indicated no toxicity for both drugs and their solvent controls.

3. The Effect of Nebivolol on The Production of IL-2 and Cell Viability in PBMCs

To assess other immune cell interactions and individual variabilities, Nebivolol's inhibitory effect on IL-2 production was further explored in PBMCs. PBMCs were activated with or without nebivolol and incubated for 48h. Nebivolol (Fig 7A-D) at 10^{-5}M downregulated IL-2 in two out of four participants. However, nebivolol (Fig 7A-D) showed no effect in other concentrations (10^{-6} – 10^{-7}M) in all the participants. Out of four participants, nebivolol demonstrated IL-2 suppression in HRT1 (Fig 7A) and in HRT34 (Fig 7C) at 10^{-5}M. However, nebivolol did not affect IL-2 levels in HRT4 (Fig 7B) and HRT35 (Fig 7D) at 10^{-5}M, suggesting individual variations. The solvent control (Fig 7A-D) assessment showed no effect on IL-2 at all concentrations. Cell viabilities (Fig S4A-D) averaged >92% for all concentrations tested, indicating no toxicity exerted on the cells by either nebivolol or its solvents.

4. The Inhibition of IL-2 by Nebivolol in a β2AR-Dependent Manner

The receptor specificity of nebivolol's suppressive activitiy on IL-2 was evaluated with ICI-118-550 (ICI), a β2AR specific inhibitor. Activated Jurkat T cells treated with or without ICI, and incubated for 48h. Nebivolol (Fig 8 and Fig S6A-D) (10^{-5} M) suppressed IL-2 production, and this suppression was attenuated by the ICI. This indicated that nebivolol inhibited IL-2 in a β2AR specific manner in T cells.

5. The Downregulation of NFκB Phosphorylation by Nebivolol in Suppressing IL-2 Production

Nebivolol's downregulatory mechanism on IL-2 was further explored by assessing NFκB phosphorylation activity. Jurkat T cells were activated for 15, 30, and 60 minutes with or without nebivolol, lysed, and immunoblotted for detection of phosphorylation and compared to α-tubulin as the loading control. NFκB phosphorylation (Fig 9A) was increased in activated cells

compared to non-activated cells. Nebivolol (Fig 9A - B) decreased NFκB phosphorylation in 15 minutes by compared to the activated cells. NFκB phosphorylation diminished (Fig 9B) significantly from 30 – 60 minutes for both activated cells with and without nebivolol, suggesting no more phosphorylation occurred. Nebivolol (Fig 9A – B) showed no inhibitory effect on NFκB at 30 or 60 minutes. At 60 minutes (Fig 9B), the phosphorylation activity dropped to the same level as non-activated cells, indicating that NFκB phosphorylation returned to the basal level.

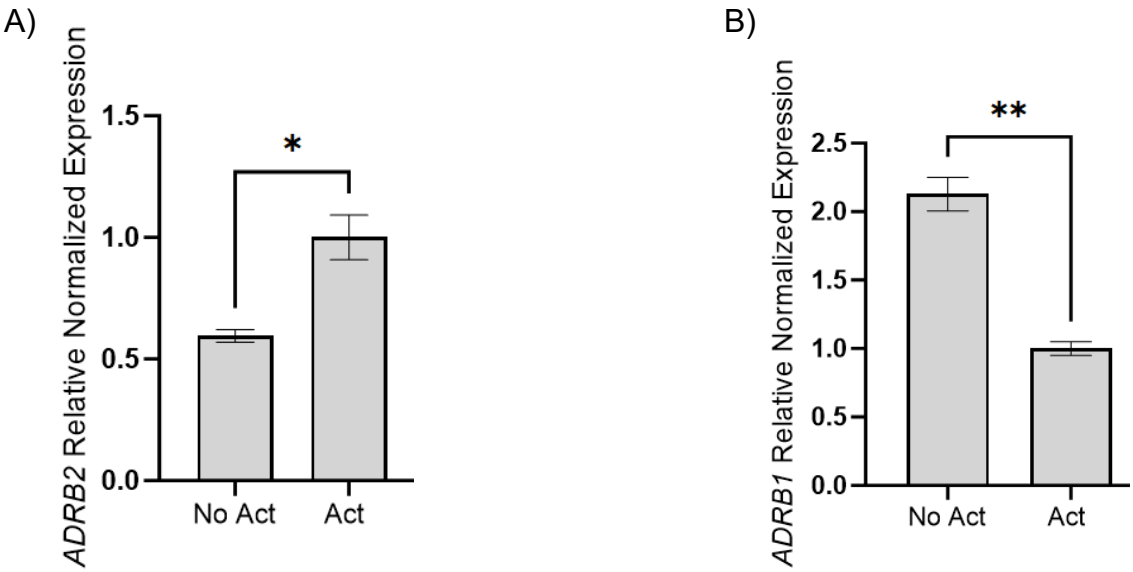


Figure 3. Expression of β2AR and β1AR in Jurkat T cells.
Jurkat T cells was incubated for 48h non-activated (No Act), or activated with PMA and anti-CD3 antibody (Act). **A) *ADRB2* expression and B) *ADRB1* expression** were measured by qPCR and expressed relative to a housekeeping gene of one experiment. Statistical significance was determined using Student's t-test (*p<0.05, **p<0.01).

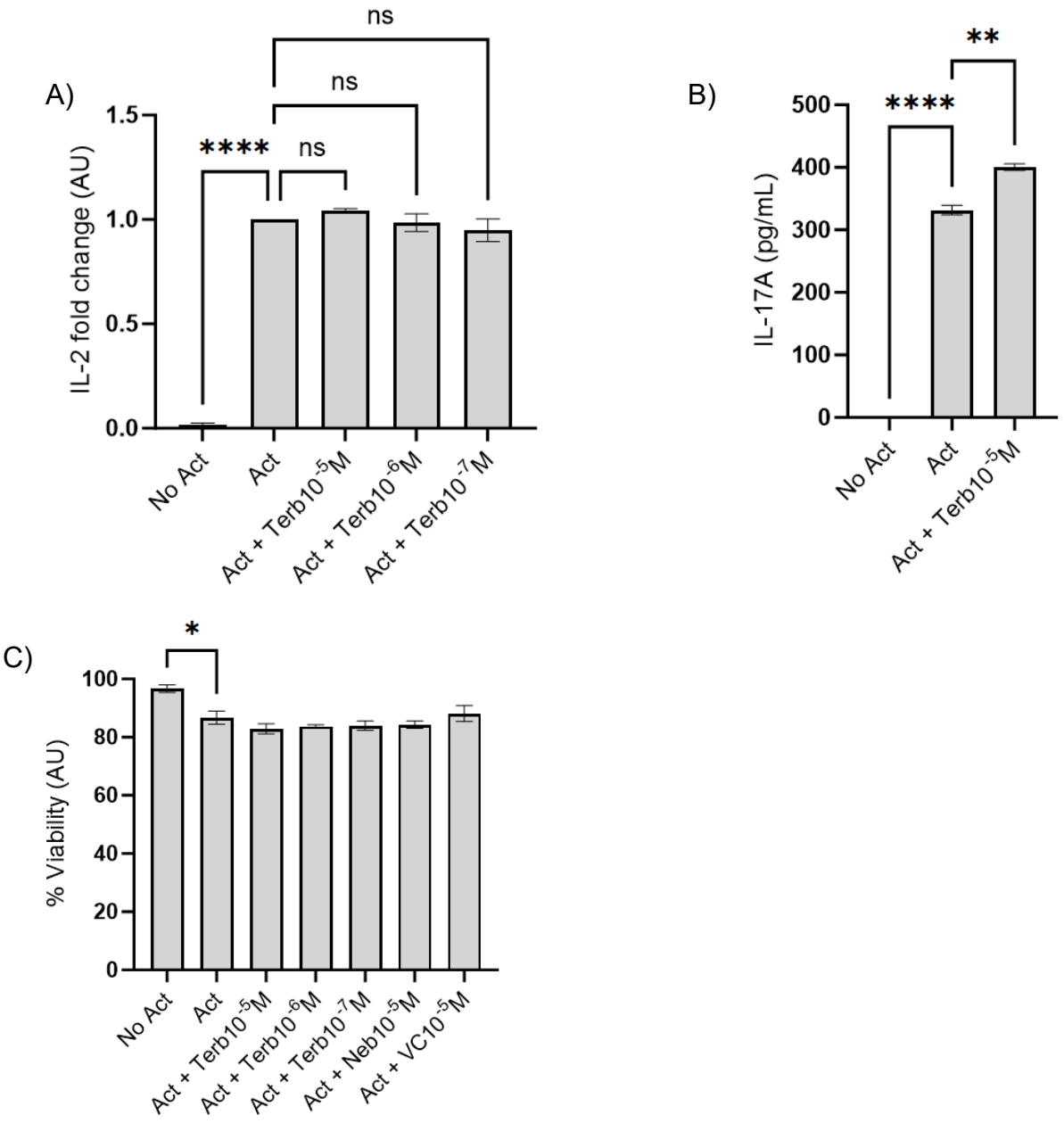


Figure 4. Terbutaline's effect on IL-2 production in Jurkat T cells and IL-17A in PBMC. Jurkat T cells were activated for 48h with PMA and anti-CD3 antibody (Act), and PBMC was activated for 4 days with anti-CD3 and anti-CD28 antibodies (Act), with or without terbutaline (10^{-5}, 10^{-6}, 10^{-7}M) (Act + Terb. **A) IL-2 production in Jurkat T cell B) IL-17A production in PBMC C) Cell viability percentage.** Results are presented as the mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, *p<0.05, **p<0.01, ****p<0.0001).

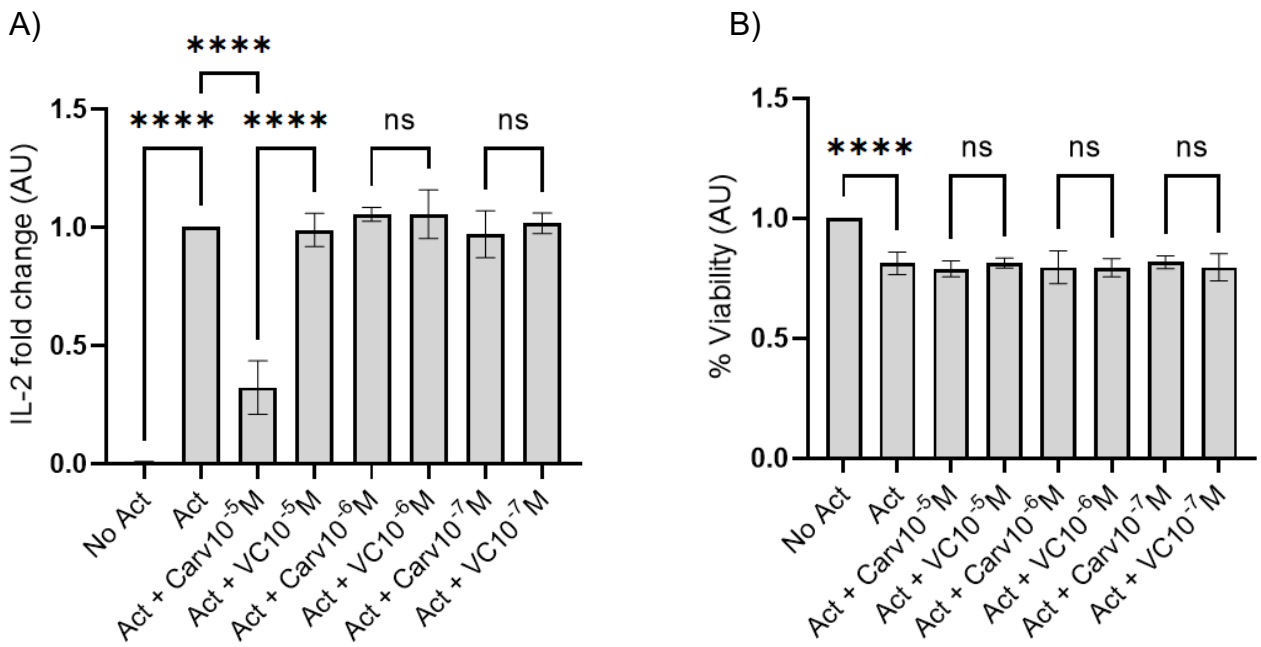


Figure 5. Carvedilol's effect on IL-2 production in Jurkat T cells.
Jurkat T cells were incubated for 48h non-activated (No Act), activated with PMA and anti-CD3 antibody (Act), with or without carvedilol (10^{-5}, 10^{-6}, 10^{-7}M) (Act + Carv), and with vehicle control (Act + VC). **A) IL-2 production B) Cell viability percentage.** Results are presented as the mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, ****p<0.0001).

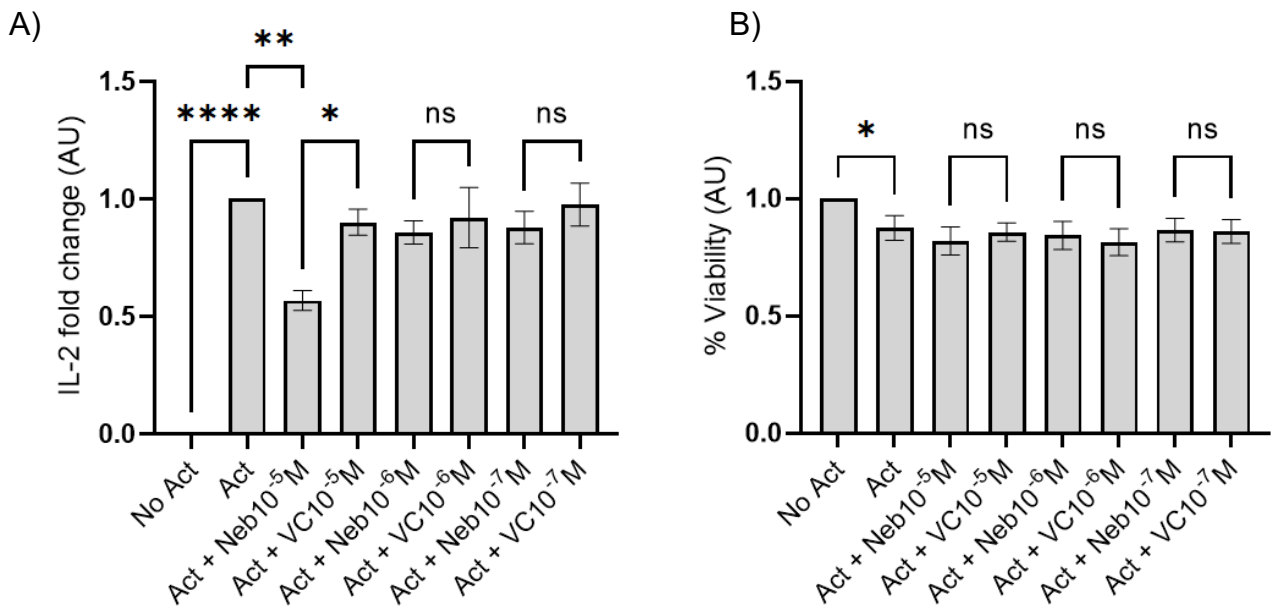
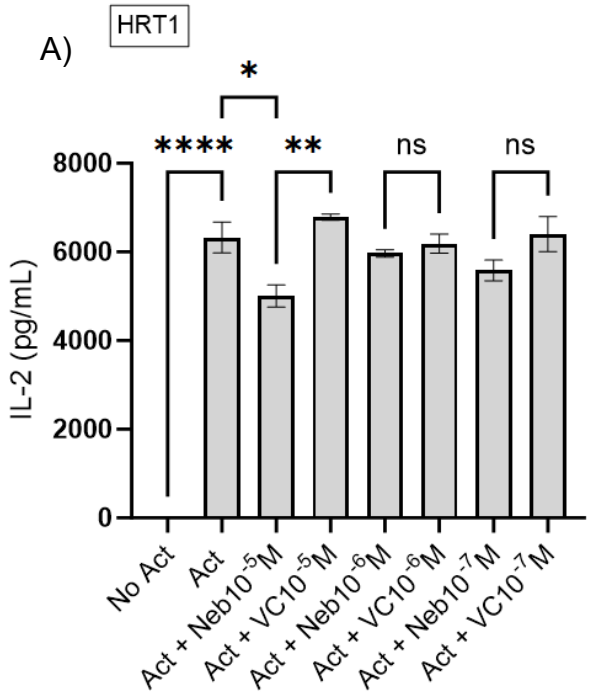


Figure 6. Nebivolol's effect on IL-2 production in Jurkat T cells.
Jurkat T cells were incubated for 48h non-activated (No Act), activated with PMA and anti-CD3 antibody (Act), with or without nebivolol (10^{-5}, 10^{-6}, 10^{-7}M) (Act + Neb), and with vehicle control (Act + VC). **A) IL-2 production B) Cells' viability percentage**. Results are presented as the mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, *p<0.05, **p<0.01, ****p<0.0001).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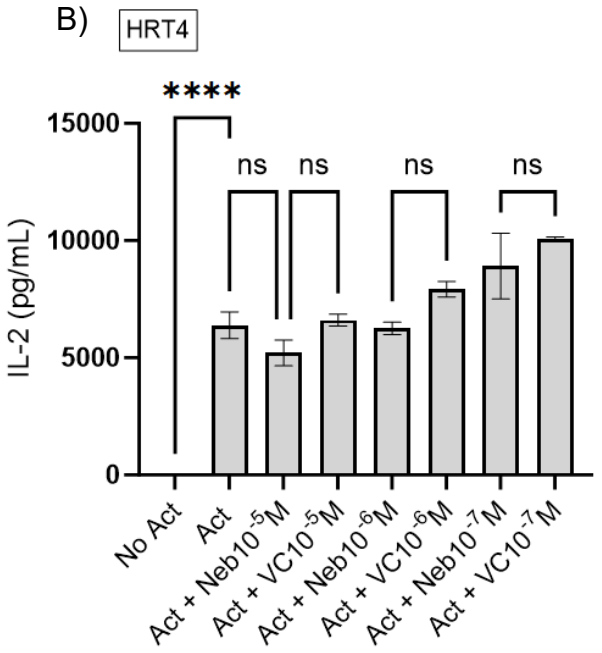
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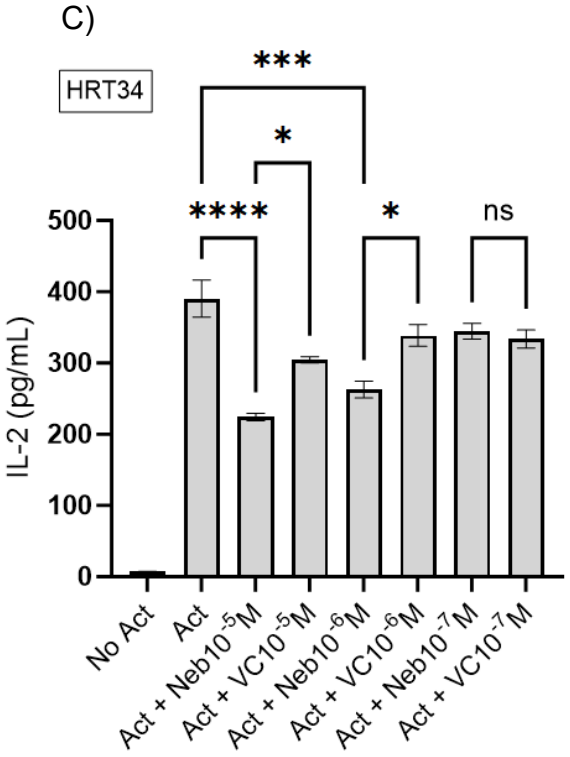
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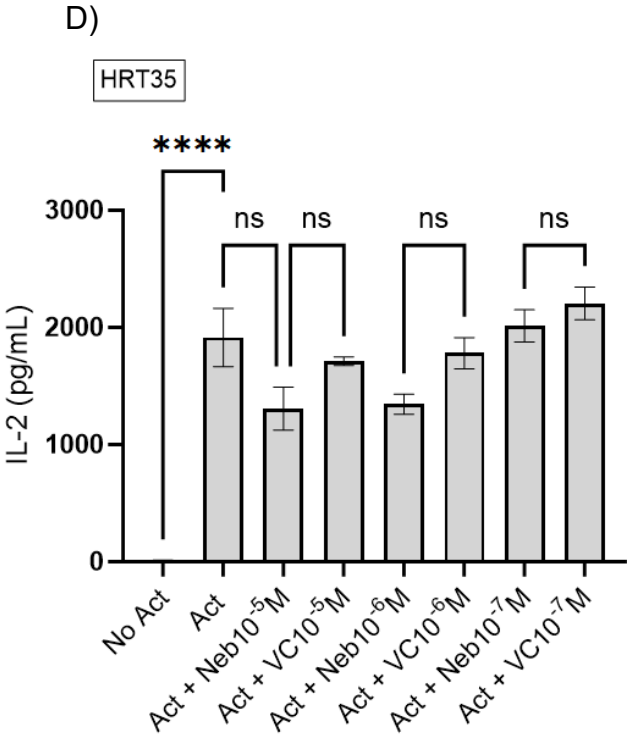
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Figure 7. Nebivolol's effect on IL-2 production in PBMCs.
PBMCs were incubated for 48h non-activated (No Act), activated with anti-CD28 and anti-CD3 antibody (Act), with or without nebivolol (10^{-5}, 10^{-6}, 10^{-7}M) (Act + Neb), vehicle control (Act + VC). **IL-2 production in different participants A) HRT1, B) HRT4, C) HRT34, D) HRT35.** Results are presented as the mean ± SEM of four different participants. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

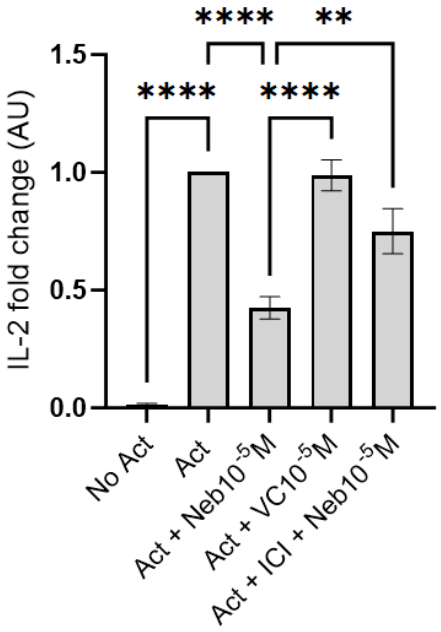

Figure 8. Nebivolol suppressed IL-2 in a β2AR-dependent manner.
Jurkat T cells were activated for 48h with PMA and anti-CD3 antibody (Act), with the addition of nebivolol (Act + Neb), ICI-118-550 (ICI) 10^{-7}M, or vehicle control (Act + VC). Results are presented as the mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (**p<0.01, ****p<0.0001).

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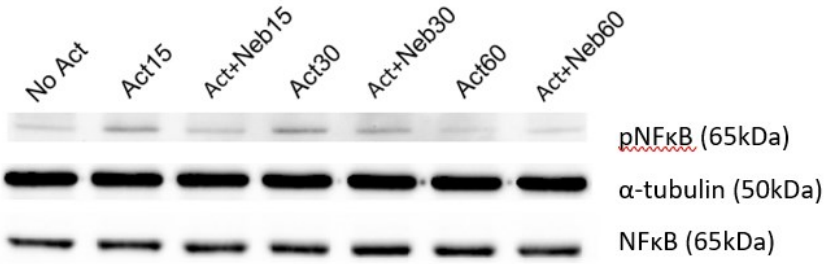

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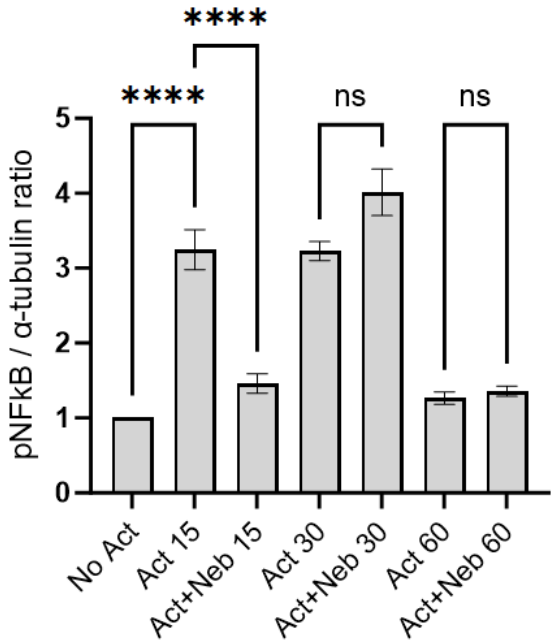


Figure 9. Nebivolol's effect on NFκB phosphorylation levels in Jurkat T cells.
Jurkat T cells were activated for 15, 30, and 60 minutes with PMA and anti-CD3 antibody (Act), with and without nebivolol (Act + Neb). pNFκB and α-tubulin levels were measured with immunoblotting. **A) pNFκB phosphorylation in Jurkat T cell B) ratio of pNFκB p65:α-tubulin.** Results are presented as the mean ± SEM of six independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, ****p<0.0001).

CHAPTER IV: DISCUSSION

Here, I investigated the effects of β2AR agonist and inverse-agonist ligands in modulating T cell activation in Jurkat T cell and PBMC. My findings shown that terbutaline have no effect on IL-2 in Jurkat T cell, while both carvedilol and nebivolol have a significant IL-2 suppression in both Jurkat T cell and PBMCs. I primarily utilized the Jurkat T cell line because it is one of the best *in vitro* model systems for characterizing T cell activation signaling pathways (Goldsmith et al., 1989). The advantages of using an *in vitro* model are that Jurkat T cells provide a controlled environment that lacks other immune cell interactions present *in vivo,* and being a clonal cell line, Jurkat T cells are less variable than primary immune cells from human participants (Brodin & Davis, 2017; Kaur & Dufour, 2012). My data showed a trend that nebivolol inhibited IL-2 in PBMCs, which are primary lymphocytes, but it was only statistically significant in two of the four participant's samples. Such variation is to be expected in different people when the effect size of the drug is small.

Another advantage to Jurkat T cells is its availability, low cost, and the minimization of ethical concerns are advantages compared to primary human T cells or animal models (Levy, 2012). There are also disadvantages to using Jurkat T cells, namely, they are cancer cells that lack PTEN (Wang et al., 2000), the enzyme that normally shuts off AKT. By losing this tumor suppressor, Jurkat T cells will continually proliferate even without T cell activation signals. Thus, it is not a valid model to study T cell proliferation unless the goal is to understand cancer pathways, which was not the focus of my thesis. For those reasons, I also tested the drug's effect on primary human cells derived from healthy human participants.

Among the β-AR family, T cells were only known to express the β2AR (Fan & Wang, 2009; Sanders, 2012; Williams et al., 1976), but my findings showed that β1AR was also expressed. That β2AR is expressed in Jurkat T cells (Liao et al., 2015; Takemura et al., 1995), was confirmed in my findings on *ADRB2* expression in mRNA from the Jurkat T cells. I further showed that its expression was higher in the activated state compared to the non-activated cells, which is a novel discovery. It suggests that T cells increase their expression upon activation, which enhances the immunomodulatory properties of this receptor. Since β1AR is the preferred receptor for nebivolol, I also assessed *ADRB1* gene expression in Jurkat T cells. Unlike β2AR, β1AR expression was decreased when activated compared to non-activated. There are no studies on the β1AR effect on T cell activity, as its expression on Jurkat T cells is a novel finding. Nebivolol is a β1AR antagonist and the preferred target receptor (Baker, 2010).

β1AR is highly expressed in cardiomyocytes and when stimulated by an agonist activates through the Gs pathway. The β1AR Gs pathway activates adenylyl cyclase, which generates cAMP production, resulting in PKA activation (Madamanchi, 2007), similar to that of β2AR-agonist activated. Since nebivolol is an antagonist, there is a possibility that it would block this receptor, resulting in no cAMP generated, a shared similarity to its inverse-agonism activity on β2AR in T cells. However, this is hypothetical as it needs to be assessed with β1AR agonist on T cells and measure cAMP to ascertain if the same effect on cardiomyocytes is also seen in T cells. Having only shown the expression by qPCR, these results would have to be confirmed with a Western blot to verify protein levels of both β2AR and β1AR in Jurkat T cells.

My first aim was to assess any difference between agonist and inverse-agonist in modulating IL-2 levels in T cells. Based on the literature, the agonist terbutaline was expected to suppress IL-2 from T cells. However, it showed no effect on IL-2 in T cells in all dosages tested. This result was surprising because other studies with different agonists for example, isoproterenol in PBMCs (Averill et al., 1988) and salmeterol in both isolated $CD4^+$ and murine T cells (Sekut et al., 1995) showed an inhibitory effect on IL-2.

To verify that terbutaline stock used in these experiments was effective, I replicated a finding from a previous study showing that terbutaline increased IL-17A from PBMCs (Carvajal Gonczi et al., 2017), and showed that terbutaline was effective and increased IL-17A production. These results suggest that terbutaline does not inhibit IL-2 production from Jurkat T cells. Although agonists like isoproterenol and salmeterol inhibited IL-2 in previous studies (Bartik et al., 1993; Sekut et al., 1995), other agonists have shown no effect on IL-2, for example, albuterol in both isolated murine $CD4^+$ (Sekut et al., 1995) and epinephrine on PBMCs and HT-29, a human colon cancer cell line (Bessler et al., 2020). Although those studies were carried out with different cell types *in vitro*, Bessler et al. used a lower concentration of $5x10^{-10}$ M of epinephrine, which could be the reason for no effect on IL-2. At the same time, Sekut et al. used $6x10^{-6}$ M albuterol for *in vitro* isolated murine $CD4^+$. Holen & Elsayed did an agonist comparisons study with 10^{-6} – 10^{-7}M concentrations and found that terbutaline was the weakest β2AR agonist in inducing cytokine production in PBMCs, isolated $CD4^+$, and cell lines (Holen & Elsayed, 1998). Furthermore, terbutaline was previously shown to have various effect on IFNγ production in different lymphoid organ, where it increased IFNγ in DLN (lymph nodes that drains) cells, inhibited IFNγ in MLN (mesenteric lymph nodes) cells, and shown no effect on IFNγ in splenocytes in arthritic model rats (Lubahn et al., 2014). The lack of effect suggested that terbutaline also does not work on IL-2 in Jurkat T cells.

The first aim of this study was to assess the inverse-agonists, nebivolol and carvedilol, and their effect on IL-2 in Jurkat T cells. Nebivolol suppressed IL-2 at 10^{-5} M, which was a novel finding. Carvedilol also inhibited IL-2 at 10^{-5} M in the Jurkat T cell. Previous research has shown that carvedilol suppressed IL-2 production in PBMCs at 10^{-5}M (Yang et al., 2003). My experiment with nebivolol and carvedilol supported the results of Yang et al. in the PBMCs study. Moreover, the fact that these two inverse-agonists inhibited IL-2, but terbutaline did not, suggests that an alternative signaling pathway is responsible for the inhibitory effect. However, how nebivolol inhibited IL-2 is unknown, leading me to explore the transcription factor NFκB that regulates IL-2 production in T cells.

As mentioned, some agonists, such as salmeterol, have been shown to decrease IL-2 (Sekut et al., 1995). Studies have demonstrated that the agonist's inhibitory effect occurred through the classical pathway, where the agonist binds to β2AR and activates G protein signaling, resulting in cAMP formation and activation of PKA, leading to IL-2 inhibition (Ramstad et al., 2000). In these previous studies, the inhibitory effects on IL-2 observed were caused by the downstream PKA activity, which phosphorylates and activates C-terminal Src kinase (Csk), which in turn inhibited Lck, resulting in the suppression of T cell activation (Ramstad et al., 2000; Wehbi & Taskén, 2016). On the other hand, alternative pathways inhibit cAMP formation, which suggests that the inverse-agonist inhibition of IL-2 is unique compared to the classical pathway. The alternative pathway involves the GRK5/6 phosphorylation and β-arrestin recruitment. Studies showed that β-arrestins were involved in the NFκB pathway inhibition by binding with IκBα (Gao et al., 2004; Luan et al., 2005; Witherow et al., 2004). The interaction of β-arrestins with IκBα prevented its degradation, which hindered NFκB phosphorylation and kept NFκB inactivated in the cytosol (Shih et al., 2011). In agreement with the literature, my result showed that nebivolol downregulated NFκB activity in T cells. My results also aligned with Yang et al.'s findings, where carvedilol stopped IκBα degradation in PBMCs, inhibiting NFκB activation (Yang et al., 2003). Moreover, a defect in the Rel family of NFκB gene, *Rel* in mice displayed a ~50-fold lower production of IL-2, resulting in diminished proliferation of T cells compared to control (Köntgen et al., 1995), indicating the direct link of NFκB in regulating IL-2 production in T cells.

After analyzing the data, I observed that nebivolol and carvedilol only suppressed IL-2 at the highest concentrations (10^{-5}M), tested across the range of concentrations (10^{-5} – 10^{-7}M). The range used for nebivolol and carvedilol was based on previous studies (Brehm et al., 2001; Carvajal Gonczi et al., 2021; Yang et al., 2003). In those earlier studies, Carvajal Gonczi et al.

demonstrated that nebivolol only affected IL-17A at 10^{-5}M in PBMCs, while Brehm et al. showed that nebivolol reduced cell proliferation of human coronary smooth muscle cells (haCSMCs) only at 10^{-5} – 10^{-6}M, with no effect at 10^{-7}M concentrations (Brehm et al., 2001; Carvajal Gonczi et al., 2021). Since I also did not observe a dose response, which is a gradual reduction in the effect at lower doses, there must be some reason why the drug needs to be at a higher dose. There are two possibilities to explain the lack of dose response, one, lipophilic properties, and two, receptor binding selectivity.

Drug lipophilicities are a vital characteristic in drug design as they contribute to drug solubility and permeability through the membrane, which essentially determines the absorption, distribution, metabolism, excretion, and toxicity (ADMET) (Arnott & Planey, 2012). Most beta-blockers are lipophilic. High lipophilicity leads to non-selective binding (Testa et al., 2000), which can potentially increase toxicity (Price et al., 2009). On the other hand, low lipophilicity results in poor ADMET properties. Nebivolol exhibited a moderate level of lipophilicity (Poirier & Tobe, 2014); as we observed, no toxicity was displayed in all concentrations. It must pass through the lipid membranes to access the transmembrane β2AR active site. Due to nebivolol's lipophilic nature, 98% of it was bound to the blood plasma protein albumin (Fongemie & Felix-Getzik, 2015). To address this issue, experiments would need to be done in albumin-free media, or an alternative mode of nebivolol, such as in liposomes, would be explored to deliver the full dose directly to the cells.

The second characteristic of nebivolol that may explain the lack of dose response is receptor binding selectivity. Nebivolol has a higher selectivity towards β1AR than β2AR (S. Gupta & Wright, 2008), a feature also observed in other β-blockers (Lertora et al., 1975). The high dose of nebivolol could have caused off-target effects, such as with β1AR or other unknown receptor activities. For this reason, I combined nebivolol with ICI-118-551 (ICI) to assess the β2AR receptor selectivity. ICI is a highly β2AR-specific antagonist (Arnold et al., 1985; Zalli et al., 2015). To determine if IL-2 suppression was β2AR specific, I employed 10^{-7}M of ICI based on previous studies (Carvajal Gonczi et al., 2021; Marmary et al., 1989). The addition of ICI partially attenuated the inhibitory effect of nebivolol on IL-2 in T cells. Because the effect was only partial, I can not entirely rule out off-target effects, such as with β1AR binding, but the ICI data confirmed partial dependence on the β2AR.

The majority of my study was on Jurkat T cells, which have many experimental advantages for studying the T cell receptor but are also the product of a leukemia patient from

decades ago and are thus not biologically relevant in areas outside of cancer research. To address this, I tested nebivolol on PBMC to determine if it inhibited IL-2 in primary cells. Our previous paper showed that nebivolol could inhibit IL-17A but not IFNγ from PBMC (Carvajal Gonczi et al., 2017). My data on PBMC showed that IL-2 downregulation was only observed at HRT1 (Fig 5A) and HRT34 (Fig 5C), which indicated individual variabilities of primary cells. This variability in PBMC results could be linked to the β2AR gene, *ADRB2* polymorphism, which accounted for various results on IL-17A levels in the presence of terbutaline. In a previous study from the lab, they showed no polymorphism effect was observed under nebivolol, although the terbutaline effect on PBMC was dependent on polymorphisms within the coding region of *ADRB2* (Carvajal Gonczi et al., 2021). *ADRB2* polymorphism was associated with how effective beta-blocker treatment was in acute coronary syndrome (Lanfear et al., 2005) and congestive heart failure (Kaye et al., 2003), splitting patients into good and non-responders. There is yet a study to be conducted on the effect of *ADRB2* polymorphism on IL-2 levels, and given our sample size of n=4, more samples and genotyping are needed to draw conclusions.

My study's limitations are that most of the experimentations were carried out with *in vitro* cell lines as it could not imitate the complexity of *in vivo* systems. This could be improved by substitution with PBMCs, isolated $CD4^+$, or with mice *in vivo,* which is common in immunology studies. Although I used PBMCs to check for nebivolol's effect on IL-2, it would be difficult to do western blot replicates as we would need the same participants, which would not be feasible. These PBMC limitations also applied to isolated $CD4^+$, as the yield per individual would be much smaller than that of PBMCs. Another limitation was the small sample size for the PBMC studies, as observed, there are variabilities among individuals. Thus, a bigger sample size would better represent the general population. I did not directly determine whether there was an interplay between nebivolol and β-arrestins. I suggest more exploration of the alternate pathway in future studies. For example, overexpression of GRK5/6 or β-arrestins and measuring their interactions with the β2AR in the presence of nebivolol using immunoprecipitation would be an informative experiment. I would expect that nebivolol induces an association between β-arrestins, GRKs, and the β2AR.

In conclusion, the inverse-agonist, nebivolol, downregulated IL-2 production in a β2AR-dependent manner. The effect was not seen with an agonist, terbutaline, but was seen with another inverse agonist, carvedilol. Nebivolol's inhibitory effect on IL-2 production occurred by downregulating the NFκB phosphorylation. NFκB is central in regulating inflammation and immune responses (Hilliard et al., 2002; Miagkov et al., 1998; Tomita et al., 1999). Thus, the

downregulation of NFκB activity underscores nebivolol's immunomodulatory potential. Lastly, this study also sheds some light on beta-blocker impacts on the immune systems. IL-2 production is crucial for early T cell growth and proliferation; hence, the anti-inflammatory effect of nebivolol as an inverse-agonist offers prospect of targeting the pro-inflammatory cytokines through the NFκB signaling in a G-protein independent pathway.

SUPPLEMENTARY FIGURES

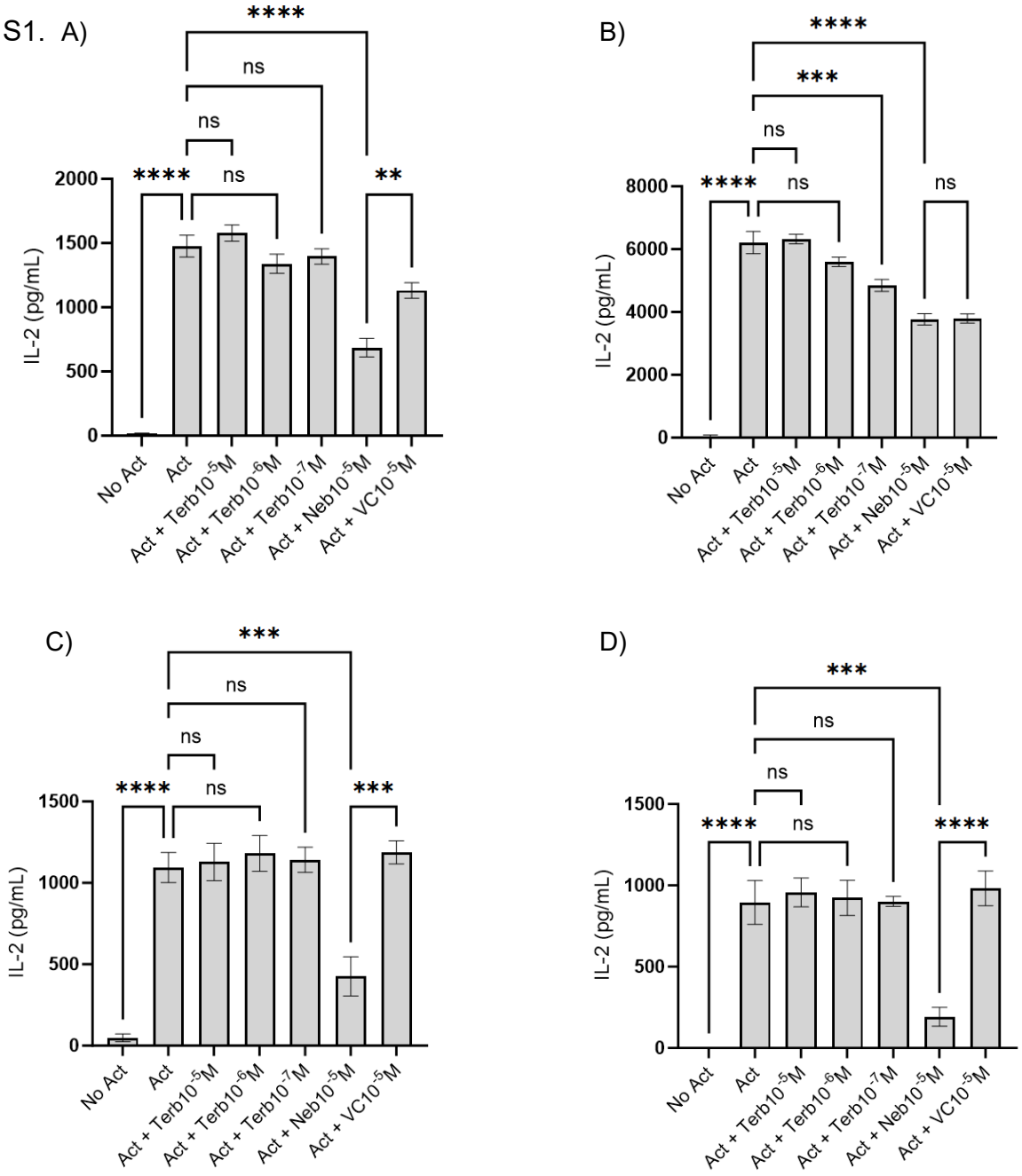
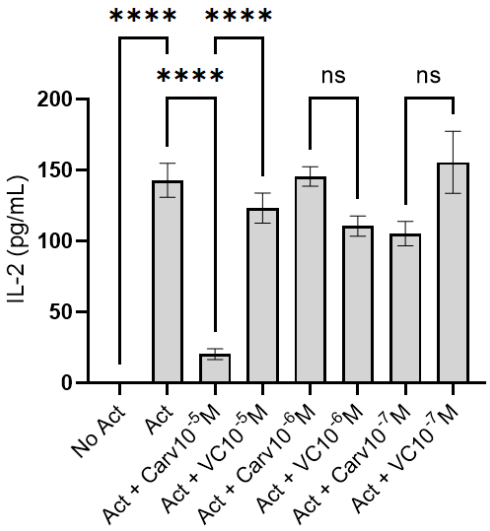
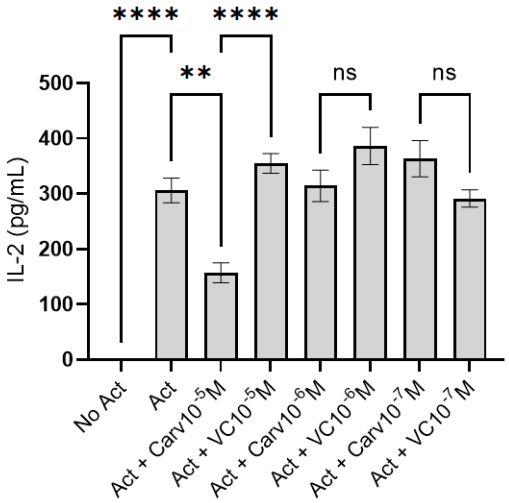


Figure S1. Individual data on terbutaline's effect on IL-2 production in Jurkat T cells. Jurkat T cells were incubated for 48h non-activated (No Act), activated with PMA and anti-CD3 antibody (Act), with or without terbutaline (10^{-5}, 10^{-6}, 10^{-7}M) (Act + Teb). Nebivolol ((10^{-5}) and its VC act as positive control to assess terbutaline's drug efficacy. Results are presented as the mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, **p<0.01, ***p<0.001, ****p<0.0001).

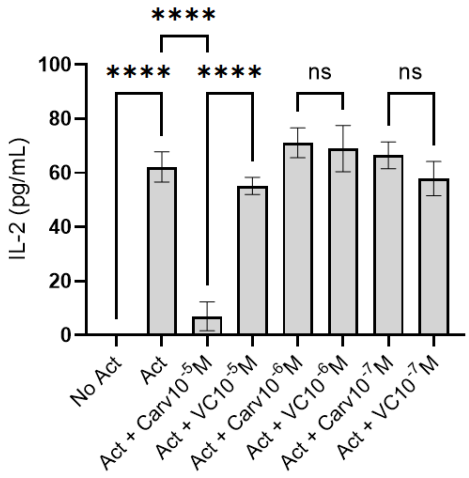
S2. A)



B)



C)



D)

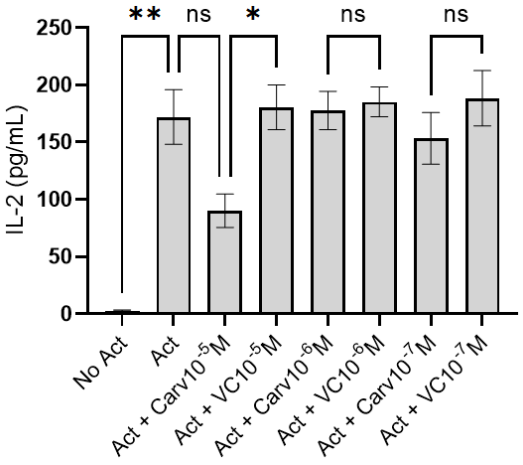
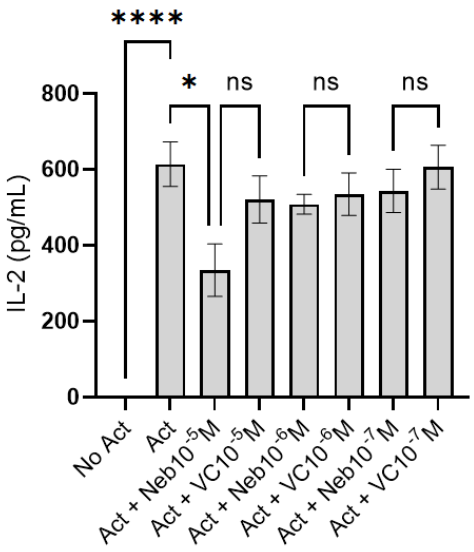


Figure S2. Individual data carvedilol's effect on IL-2 production in Jurkat T cells.
Jurkat T cells were incubated for 48h non-activated (No Act), activated with PMA and anti-CD3 antibody (Act), with or without carvedilol (10^{-5}, 10^{-6}, 10^{-7}M) (Act + Carv), and with vehicle control (Act + VC). Results are presented as the mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, *p<0.05, **p<0.01, ****p<0.0001).

S3. A)



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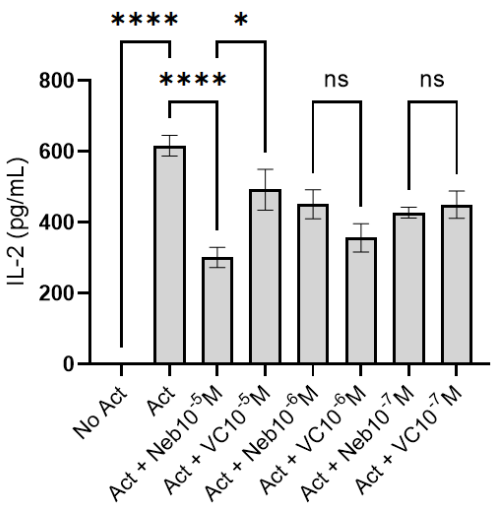

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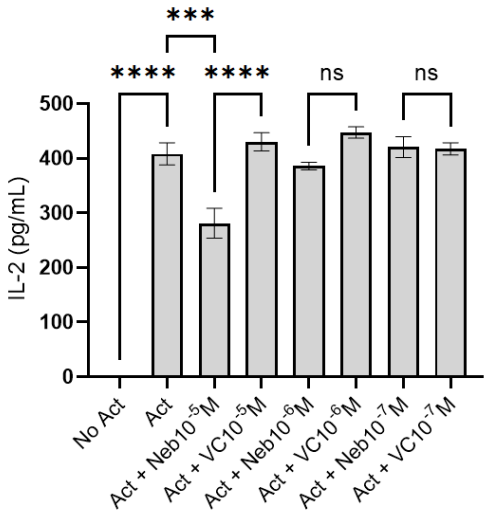

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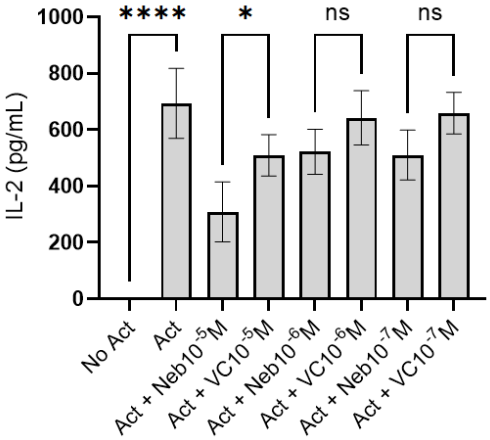


Figure S3. Individual data nebivolol's effect on IL-2 production in Jurkat T cells.
Jurkat T cells. Cells were incubated for 48h non-activated (No Act), activated with PMA and anti-CD3 antibody (Act), with or without nebivolol (Act + Neb) (10^{-5}, 10^{-6}, 10^{-7}M), and vehicle control (Act + VC). Results are presented as the mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, *p<0.05, ***p<0.001, ****p<0.0001).

S4. A)

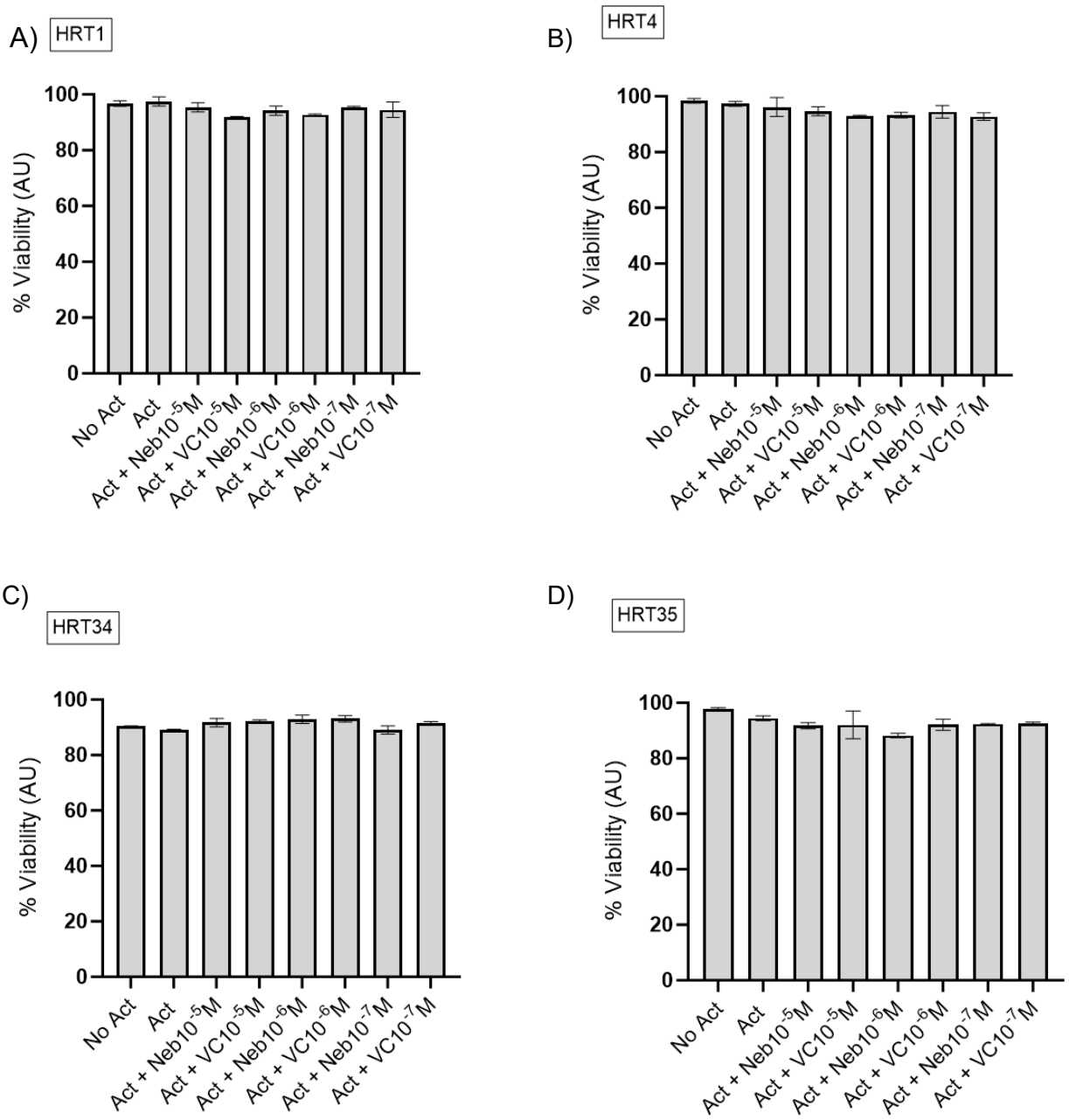
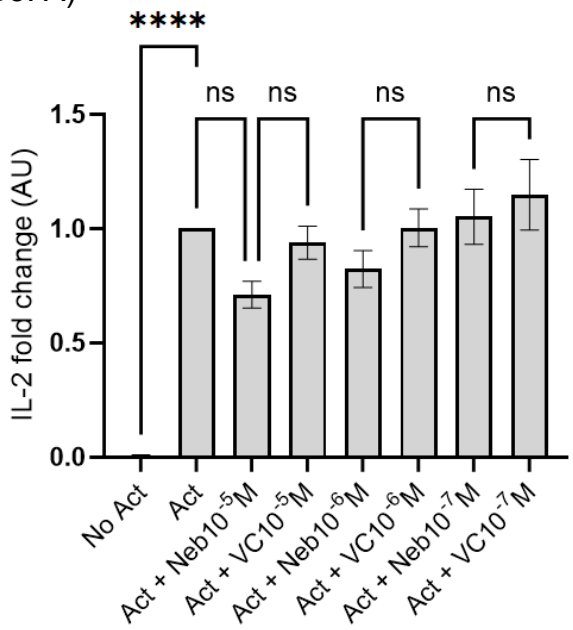


Figure S4. Individual data on nebivolol's effect on the percentage viability in PBMC. PBMCs' were incubated for 48h non-activated (No Act), activated with anti-CD28 and anti-CD3 antibody (Act), with or without nebivolol (Act + Neb) (10^{-5}, 10^{-6}, 10^{-7}M), and its vehicle control (Act + VC). Results are presented as the mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant)

S5. A)



B)

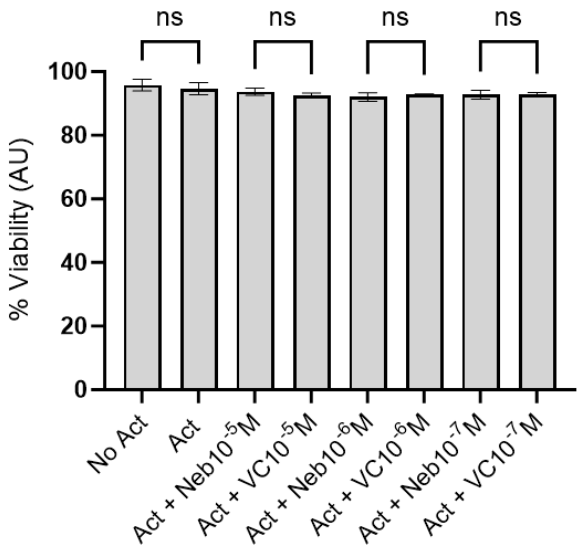


Figure S5. Pooled data on nebivolol's effect on IL-2 production in PBMC.
PBMCs' were incubated for 48h non-activated (No Act), activated with anti-CD28 and anti-CD3 antibodies (Act), with or without nebivolol (Act + Neb) (10^{-5}, 10^{-6}, 10^{-7}M), and vehicle control (Act + VC). **A) IL-2 production, B) Cell viability percentage.** Results are presented as the mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, ****p<0.0001).

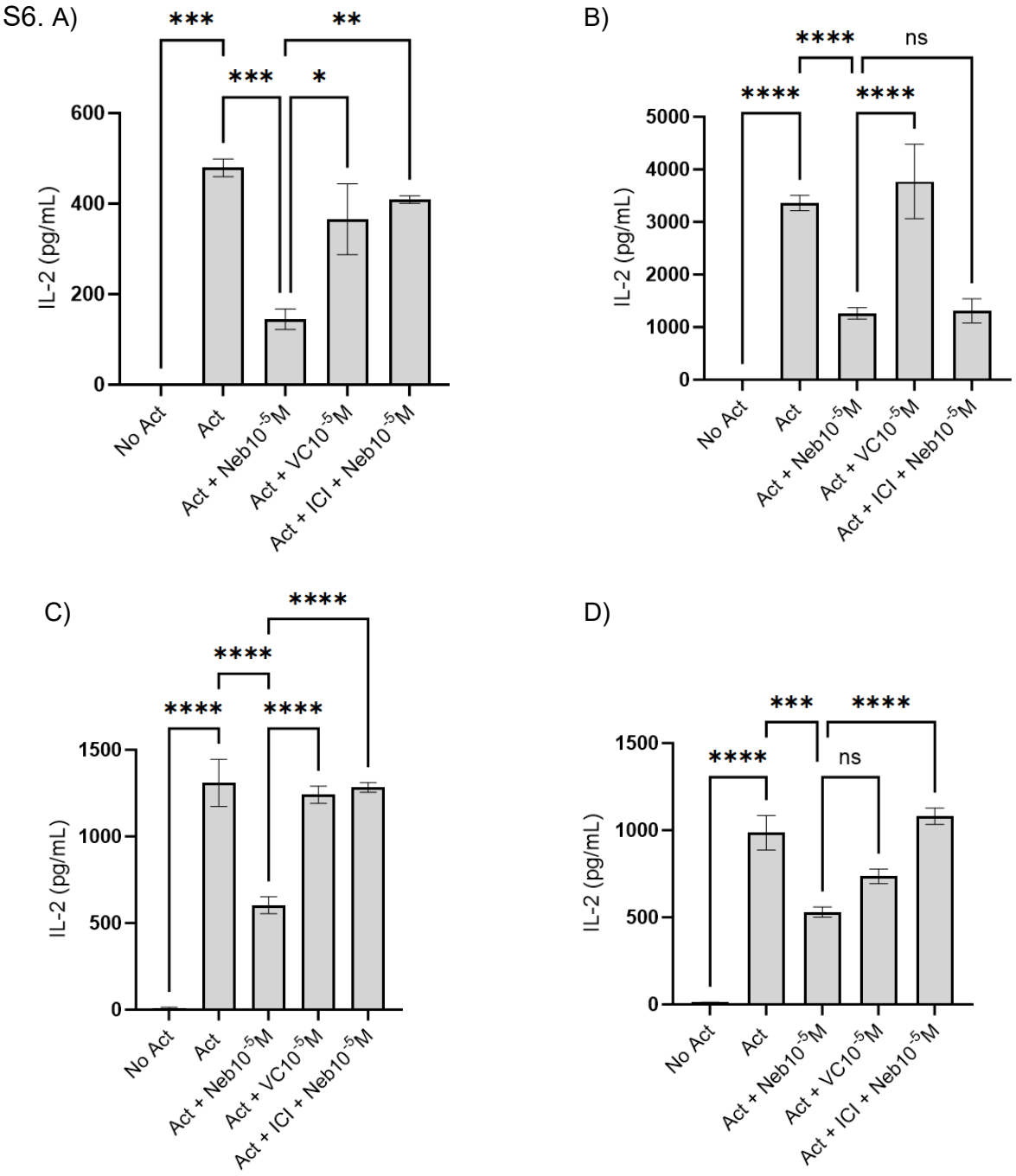


Figure S6. Individual data on the effect of ICI-118-551 on IL-2 production in Jurkat T cell. Jurkat T cells were incubated for 48h non-activated (No Act), activated with anti-CD28 and anti-CD3 antibody (Act), with the addition of nebivolol (Act + Neb), ICI-118-550 (ICI) 10^{-7}M, or vehicle control (Act + VC). Results are presented as the mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test (ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

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