The Regulation of T Helper Cells by Adrenergic Agonists and The Role of Nitric Oxide

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Abstract

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We have previously found that nebivolol inhibits the cytokines of T helper 1 and T helper 17 cells which are linked to autoimmunity and in turn supports nebivolol's candidacy as a therapeutic of autoimmune diseases such as multiple sclerosis. In this thesis, I aimed to confirm the inhibitory role of nebivolol on T helper cells and explore whether these effects are mediated by nitric oxide induction. The findings of this thesis show that nebivolol inhibited the cytokine production of T helper 17 and T helper 1 cells in samples of human peripheral mononuclear cells. I also found that these effects were not mediated by nitric oxide and its predominant signaling pathway. Instead, a low level of nitric oxide was produced during adrenergic signaling and helped to stabilize T helper 17 and T helper 1 cells. Finally, nebivolol, which has not been investigated before on human immune cells, represents a promising immunomodulatory adrenergic drug that can help to mitigate the symptoms of autoimmune diseases without inducing cell death or altering nitric oxide levels.

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List of Abbreviations

ADAP: adaptor protein

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

AP-1: Activator protein 1

APC: antigen-presenting cells

AR: adrenergic receptor

cAMP: cyclic adenosine monophosphate

CD: cluster of differentiation

cGMP: cyclic guanosine monophosphate

CREB: cAMP response element-binding protein

DAG: diacylglycerol

DC: dendritic cells

DN: double negative

DP: double positive

Elk-1: ETS family-like protein

ERK1/2: extracellular signal-regulated kinase 1/2 also known as MAPK3

GPCR: G protein-coupled receptor

IFN: interferon

IL: interleukin

ILC: innate lymphoid cells

IP3: inositol 1,4,5-trisphosphate

ITAM: immunoreceptor tyrosine-based activation motif

LAT: linker for activation of T cells

MAPK: Mitogen-activated protein kinases

MHC: major histocompatibility complex

MS: multiple sclerosis

NFAT: Nuclear factor of activated T-cells

NFκB: nuclear factor-κB

NK: natural killer cells

NO: nitric oxide

NOS: nitric oxide synthase

PBMCs: peripheral blood mononuclear cells

PDE: phosphodiesterase

PI-3k: phosphoinositide 3-kinase

PIP2: phosphatidylinositol (4,5)-bisphosphate

PIP3: phosphatidylinositol (3,4,5)-trisphosphate

PKC-θ: protein kinase C-θ

PKG: protein kinase G

PLC-γ: phospholipase C-γ

sGC: soluble guanylyl cyclase

SLP76: domain-containing leukocyte protein of 76 kDa, ,

SP: single positive

STAT: transducer and activator of transcription

TCR: T cell receptor

TGF-β: transforming growth factor-β

Th: T helper cells

WBC: white blood cell

ZAP-70: ζ-chain-associated protein kinase 70

CHAPTER I: Introduction

Preamble

The regulation of T helper (Th) cell responses has been the focus of researchers aiming to manage autoimmune diseases and abnormal immune reaction. Studies on human and mice Th cells showed that the dysregulation of different subsets of Th cells is associated with various diseases such as atopic dermatitis, rheumatoid arthritis and multiple sclerosis (MS) (Fletcher et al. 2010; Raphael et al. 2015; Esaki et al. 2016; Yasuda et al. 2019; Leipe et al. 2020). Different regulatory mechanisms occur in the body to ensure that Th cells are functional and self-tolerant including the two-signal activation and transcriptional networks of Th cells. However, some Th cells with aberrant responses escape the central and peripheral checkpoints and proliferate to cause autoimmune disorders. Therefore, to prevent autoimmunity or mitigate the symptoms, we need to investigate other regulatory mechanisms to manage the immune responses of Th cell subsets. In this thesis, I explored two regulatory mechanisms: one lies in the intersection between the sympathetic nervous system and adaptive immunity and the other depends on nitric oxide (NO) gas. These mechanisms are poorly understood in Th cells especially in human samples. However, they could provide promising therapeutics when better explored and understood.

1.1. T Cells Development, Activation and Differentiation

The main cellular components of adaptive immunity include T cells and B cells. These cells are unique for having antigen receptors which are essential for the specificity of immune responses (Dzierzak and Bigas 2018). The nomenclature of these cells comes from their site of development where T cells develop in the thymus and B cells develop in the bone marrow (Crisan and Dzierzak 2016). While B cells differentiate into antibody-producing plasma cells, T cells differentiate into different effector cells with various functions that direct and regulate the adaptive immune responses (Crisan and Dzierzak 2016). This has made T cells the focus of the research aiming to investigate and examine the regulation of the adaptive immune system which is the core of this thesis.

Naïve Th cells emerge from the thymus having TCR complexes and CD4 coreceptors spanning their plasma membrane (Ma et al. 2016). The activation of Th cells requires coordinated interactions between molecules occurring on Th cells and the antigen presented on a major histocompatibility complex class II (MHC-II) molecule of an antigen presenting cell (APC) (Santamaria et al. 2018). The presentation of an antigen on an MHC-II molecule is necessary for the TCR complex to recognize the pathogen, and this process is carried out by APCs (Santamaria et al. 2018). Theoretically, all cell types can serve as APCs. However, few types such as macrophages, B cells and dendritic cells, function primarily to present antigens to Th cells and are referred to as specialized APCs (Kashem et al. 2017). After recognizing the antigens and internalizing them, APCs present those antigens on MHC-II molecules for the Th cells. From this point forward, the TCR complex of Th cells will carry over the activation process. Notably, a TCR alone is not able to initiate the activation of Th cells (Call et al. 2002; Kuhns and Davis 2012). Instead, an assembly of many parts besides TCR is needed for the signal transduction across the plasma membrane of Th cells and are called collectively as TCR complex (Kuhns and Davis 2012). In addition, for Th cells to become fully activated, a ligation between costimulatory molecules on APC (CD80/CD86) and Th cells (CD28) is needed and without it the activation will be stalled (Harding et al. 1992). The requirement of binding CD3/CD4 and CD28 with molecules on APCs ensures that Th cells are fully activated only when there is a non-self-antigen and this process is referred to as two-signal activation. The consequences of CD3 and CD28 ligation explain the rationale behind using anti-CD3 and anti-CD28 antibodies to activate Th cells *in vitro* as was followed in this thesis.

Despite the inherent endogenous central and peripheral mechanisms to induce self-tolerance, some autoreactive T cells manage to escape all checkpoints. The failure of the central and peripheral immunogenic tolerance mechanisms and the following release of self-reactive T-cells to the periphery results in unwanted proinflammatory responses and T-cell mediated autoimmune disorders, such as rheumatoid arthritis and MS (Cheng and Anderson 2018). Autoimmune diseases are a diverse group of chronic disorders caused by autoreactive T cells that recognize self-antigens and initiate

abnormal immune responses targeting a specific organ or many organ systems within the host (Anaya 2012). Several autoimmune disorders are linked to the dysregulation of Th cells which requires a better understanding of their activation, differentiation and regulation in order to discover new ways to treat the patients. There are also numerous immuno-modulatory drugs on the market that suppress autoimmunity and improve quality of life of the patients. One of the main findings from my thesis is a drug that may be useful for suppressing Th cell activation.

Activated Th cells differentiate into different effector cells with various functions depending on the type of antigen and the cytokine present in the milieu of Th cells. The differentiation of Th cells is directed by two signals: the first comes from the TCR ligation by the compatible antigen, and the second results from the binding of milieu cytokines to their receptors and their downstream signalling (Schmitt and Ueno 2015; Gagliani and Huber 2017). These signals upregulate the expression of genes needed for Th cells to commit to a certain subset lineage and to proliferate and perform the unique effector functions of this subset (Illustration 1) (Gagliani and Huber 2017; Saravia et al. 2019). A main highlight of Th cell differentiation is the activation of transcription factors whose activities are linked to specific subsets of Th cells and therefore named major transcription factors. These factors are necessary for lineage commitment as they induce survival and morphological changes and upregulate the expression of genes and coactivators specific to a certain lineage, while repressing the genes associated with alternate lineages (Hwang 2005; Usui et al. 2006; Fang and Zhu 2017). In addition, they can upregulate the production and release of cytokines necessary for the immune responses of the associated Th cell subset (Jogdand et al. 2016; Tripathi et al. 2017; Saravia et al. 2019). There are still some questions to be answered regarding the transcriptional networks of different Th subsets where it was shown that they could be regulated by other factors besides the antigen type and the milieu cytokines.

1.2. T Helper Cell Subsets

Following the differentiation of Th cells into different subsets, they perform unique effector functions such as the generation of cytokines and chemokines specifically suited to face the invading pathogen (Saravia et al. 2019). Although we have a better understanding of Th cell subsets than any time before, some concepts such as the factors that regulate the functions of Th cells or make them dysregulated, still need to be studied and clarified. It is immensely beneficial to study the different Th cell subsets given their significant contribution to the immune responses in different contexts. However, I will focus on Th1 and Th17 cell subsets because these are the most prominent subsets whose dysregulation is associated with autoimmunity and immunoregulation and autoimmunity are the focus of this thesis

1.2.1. Th1 cell subset and IFNγ

Th1 was the first subset to be identified based on cytokine production in 1986 (Mosmann et al. 1986). Th1 cells, in a healthy state, initiate immune responses against intracellular viral and bacterial pathogens (Szabo et al. 2000; Zhang et al. 2014a; Zhu 2015), and were shown to be involved in diabetes Type I, Crohn's disease and several autoimmune disorders (Abbas et al. 1996; Romagnani 2000; Hoyer et al. 2009). For a naïve Th cell to differentiate into Th1 subset, IL-12 and IFNγ cytokines must be present in the immediate milieu (Illustration 1) (Frucht et al. 2001; Lighvani et al. 2001; Afkarian et al. 2002). Downstream of cytokine receptors, transducer and activator of transcription (STAT) family proteins become activated to induce the major transcription factors. In the case of Th1, IL-12 and IFNγ activate STAT4 and STAT1 proteins, respectively which, in turn, activate T-bet; the major transcription factor of Th1 cells (Szabo et al. 2000; Afkarian et al. 2002; Zhang et al. 2014b). Following the activation of T-bet, the cells differentiate into Th1 cell subset and produce IFNγ and tumor necrosis factor-α (TNFα) cytokines to face viral and bacterial pathogens in addition to the proliferative IL-2 cytokine (Afkarian et al. 2002; Christie and Zhu 2014).

IFNγ cytokine regulates the differentiation of Th1 cells in an autocrine manner because it is released by Th1 cells and direct their differentiation and proliferation

(Afkarian et al. 2002). IFNγ is a type II interferon cytokine which is predominantly produced by Th1 subset, and its downstream signalling leads to transcriptional regulation of the genes involved in the immune response (Schroder et al. 2004; Pennock et al. 2013). This cytokine binds to CD119 and IFNγR2 receptors and results in the augmentation of macrophage activation and the expression of MHC molecules and antigen processing components in addition to supressing the Th17 and Th2 cell subsets (Bach et al. 1997; Frucht et al. 2001; Swanson et al. 2001; Usui et al. 2006; Murphy and Weaver 2016). Although released by different cell subsets such as CD8+ cytotoxic T cells, and natural killer (NK) cells (Young 1996; Bach et al. 1997), Th1 cells remain the paramount producers of IFNγ in the context of adaptive immune response (Frucht et al. 2001; Sen 2001). Therefore, measuring the levels of IFNγ in cell cultures has been a commonly used *in vitro* technique to infer the degree of activation and function of Th1 subset as was followed in this thesis.

1.2.2. Th17 cell subset and IL-17A

In 2005, the Th17 cell subset was identified as a subset distinct from Th1 and Th2 subsets and associated with autoimmunity (Harrington et al. 2005). The differentiation of Th17 cells was found to be suppressed by IFNγ and IL-4 the major cytokines of Th1 and Th2 cell subsets, respectively. However, committed Th17 cells were unaffected by the cytokines of Th1 and Th2 (Harrington et al. 2005). Th17 cells initiate immune responses against fungi and extracellular pathogens (Ivanov et al. 2006a; Zielinski et al. 2012), and their dysregulation can result in proinflammatory autoimmune diseases, such as MS (Kotake et al. 1999; Tzartos et al. 2008; Brucklacher-Waldert et al. 2009; Durelli et al. 2009). Cytokines such as IL-6, IL-23, and transforming growth factor-β (TGF-β) in the immediate milieu of naïve Th cells are required for Th17 lineage commitment (Illustration 1) (Ivanov et al. 2006a; Zielinski et al. 2012). The downstream signaling of IL-6 and IL-23 cytokines results in the activation of STAT3 proteins which in turn activate the major transcription factor of Th17 namely the receptor-related orphan receptor-γt (ROR-γt) (Yosef et al. 2013; Jogdand et al. 2016; Tripathi et al. 2017). TGF-β signaling leads to the activation of SMAD2 and SMAD3 proteins which also transduce the activation signal to ROR-γt transcription factor

(Ivanov et al. 2006a; Zhou et al. 2008; Yosef et al. 2013). Interestingly, TGF-β could lead to the upregulation of Foxp3, the major transcription factor of T_{regs}, which antagonizes ROR-γt in the absence of IL-6, and IL-23 cytokines that inhibit Foxp3 (Zhou et al. 2008). These findings demonstrate the intricacy of Th cell differentiation which is necessary for specific immune responses.

The transcription factor ROR-γt directs the differentiation into Th17 lineage and induces the expression of IL-17A IL-17F and IL-22 which are the signature cytokines of Th17 and play a key role to combat fungal, intracellular and extracellular bacterial infections (Ivanov et al. 2006a; Yosef et al. 2013). In murine T cell, ROR-γt deficiency resulted in the loss of Th17 cells and weakened autoimmunity (Ivanov et al. 2006b). This finding not only shows the significance of Th17 cells in autoimmunity and the role of ROR-γt in Th17 differentiation, but also features the transcriptional network of Th17 cells as a therapeutic target to treat autoimmunity (Stadhouders et al. 2018). This direction is still under investigation and requires more clinical research under physiological conditions besides *in vitro* experiments. Although minor amounts of IL-17A is produced by $CD8^+$ T cells, neutrophils, and innate lymphoid cells 3s (ILC3s), Th17 cells are its predominant producers making the levels of this cytokine an indicator of Th17 cells' occurrence and function (Liang et al. 2007; Tesmer et al. 2008; Matsuzaki and Umemura 2018). IL-17A is a proinflammatory cytokine which binds to IL-17AR receptor to induce the expression of antimicrobial peptides and other proinflammatory cytokines to kill pathogens and initiate inflammation (Abbas et al. 1996; Gu et al. 2013; Murphy and Weaver 2016; Matsuzaki and Umemura 2018). A recent study shows that IL-17A exerts a negative feedback on Th17 cells by inducing the production of IL-24 cytokine which in turn inhibits the activity of Th17 cell (Chong et al. 2020). Nonetheless, measuring IL-17A in cell culture supernatants remains a standard method to evaluate the occurrence and function of Th17 cells given that it's mainly produced by this cell subset.

The prominent proinflammatory features of Th1 and Th17 cell subsets and their main cytokines have made them the focus of preventative health research (Raphael et

al. 2015). Despite the active investigation of Th cell subsets, we still need to know more about their regulation, which is essential to manage the morbidities associated with their dysregulation such as in autoimmune diseases. In this thesis, I was aiming to discover novel regulatory mechanisms of Th cell subsets in order to suggest efficient therapeutic approaches. Moreover, since IFNγ and IL-17A cytokines have pathogenic roles in autoimmune diseases and can be expressed by different immune cells, regulating the levels and signaling pathways of these cytokines regardless of the producing cells is equally important for therapeutic developments. For example, inhibiting the production or signaling of IL-17A cytokine using antibodies reduced the symptoms of autoimmune diseases such as psoriasis, rheumatoid arthritis and MS (van den Berg and McInnes 2013; Patel et al. 2013; Burkett and Kuchroo 2016). The samples of peripheral blood mononuclear cells (PBMCs) used in this study contain many immune cell types that could produce IFNγ and IL-17A such as NK cells and $CD8^+$ cells (Kleiveland 2015). This could help us investigate the regulation of these cytokines at the level of peripheral white blood cells (WBCs) in the blood besides the regulation of Th1 and Th17 cell subsets since Th cells form 50% or more of PBMCs (Kleiveland 2015).

1.3. Immunoregulation by adrenergic signaling

The first immunomodulatory scheme that I will discuss is the adrenergic signaling in immune cells. This scheme represents the interaction between the immune system and the autonomous sympathetic nervous system which releases catecholamines that are sensed by adrenergic receptors (also known as adrenoceptors ARs) (Kohm and Sanders 2001; Eisenhofer and Lenders 2018). Catecholamines are neurotransmitters and hormones such as dopamine, epinephrine, and norepinephrine that function to maintain homeostasis in the nervous system and the body (Eisenhofer and Lenders 2018). The actions of catecholamines result in the activation of the sympathetic nervous system which is responsible for the “fight or flight” reaction of the body.

ARs belong to the guanine nucleotide-binding G protein–coupled receptor (GPCR) superfamily and are divided into α1, α2, β1, β2, and β3 subtypes. While α1 ARs are coupled to stimulatory Gq proteins that activate the phospholipase C enzyme, α2

ARs are coupled to inhibitory Gi proteins which inactivate adenylyl cyclase (Wang 2012; Bylund 2013). However, α1 and α2 ARs are mainly expressed in the smooth muscle cells and central nervous cells, respectively, suggesting that they do not contribute to the regulation of T cells (Wang 2012; Bylund 2013). On the other hand, the β subtypes differ from α ARs in their signaling and occurrence. β1 and β2 ARs activate the Gs-adenylyl cyclase which stimulates the cyclic adenosine monophosphate (cAMP) leading to the activation of protein kinase A (PKA) (Illustration 2) (Hieble 2009; Bylund 2013; Ciccarelli et al. 2017). β1 ARs are mainly found in the heart and kidney, whereas β2 are distributed throughout the body specifically in the smooth muscle cells of the lungs. Given that β3 ARs were discovered recently as compared to β1 and β2 ARs, there have been a debate on their functions and occurrence. However, our current knowledge of β3 ARs is that they are expressed in the adipose cells and can couple to both inhibitory and stimulatory G proteins (Moens et al. 2010).

There is evidence from human and murine models that β2 ARs are expressed in the immune cells and predominantly in T cells (Kohm and Sanders 2001; Ross et al. 2018; Wu et al. 2018). Interestingly, studies shows that β2 ARs are expressed in Th1 and Th17 cells which pose many questions regarding their role in regulating Th cell functions (Ramer-Quinn et al. 1997; Sanders et al. 1997a; Kohm and Sanders 2001; McAlees et al. 2011; Sanders 2012; Carvajal Gonczi et al. 2017). If these receptors are capable of initiating a specific and differential regulation of Th cells, many agonistic and antagonistic adrenergic drugs which are readily available and cleared for safety and side effects could be tested in clinical trials and used as immunomodulatory drugs.

Early studies deduced some functions of β2 ARs on T cells before having the technology to measure the expression of these receptors on T cells. These studies showed that the activation of adenylyl cyclase and production of cAMP in T cells were elevated by using a β2 adrenergic agonists such is isoproterenol and this effect was reversed using an antagonist such as propranolol (Makman 1971; Melmon et al. 1974; Bach 1975). β2-adrenergic signaling was shown to induce murine DCs to produce IL-6

cytokine promoting Th17 differentiation (Manni et al. 2011), and inhibit the production of IL-12 cytokines to decrease the differentiation into Th1 cells (Sonnenfeld et al. 1992).

The standard method to study the adrenergic signaling in immune cells has been to use ligands such as agonists or antagonists and observe the alterations in cytokine production and gene expression. There is a dichotomic understanding of the adrenergic ligands being considered either agonists or antagonists. By definition, β AR-antagonists block the signaling from ARs and do not affect cAMP levels or the internalization of β AR. In contrast, β AR-agonists trigger the adrenergic signaling to increase cAMP levels in the cells and β AR internalization. This conviction was challenged after revealing that there is a specific type of agonism called the biased agonism. GPCRs signal through two pathways: the first through the Gα and Gβγ proteins and the second through G protein-coupled receptor kinase (GRK)/β-arrestin pathway (Illustration 2) (Lefkowitz and Shenoy 2005). Full β2- adrenergic agonists simultaneously trigger the two aforementioned pathways whereas biased or inverse agonists only activate one pathway (T. Andresen 2011). The G-protein-mediated signaling results in the activation and elevation of cAMP and PKA whereas the β-arrestin-mediated signaling leads to the activation of MAPKs and extracellular signal–regulated kinases (ERKs) (Barlic et al. 2000; DeFea et al. 2000). In addition, the β-arrestin-mediated signaling regulates the cAMP levels in the cytosol by desensitization the G-protein-mediated signaling and initiates antiapoptotic signaling (Revankar et al. 2004; Lefkowitz and Shenoy 2005). This paradigm of β2 AR signaling adds a level of complexity to adrenergic immunoregulation and paves the way for new adrenergic agonists to be investigated as immunomodulatory drugs.

Although our current knowledge implies that adrenergic signaling could indirectly regulate the immune responses of different Th cell subsets by acting on other cells including APCs such as macrophages and dendritic cells, the direct effects of adrenergic signalling on Th cells is poorly understood. There have been few studies that assessed the direct effect of adrenergic signaling on Th cells and their findings were contradicting. For example: cytokine production of Th1 was shown to be significantly inhibited by triggering β2-adrenergic signaling using norepinephrine (NE) (Ramer-Quinn

et al. 1997; Sanders et al. 1997b; McAlees et al. 2011). In two publications, Huang et al, induced catecholamines production by lymphocytes and found that lymphocyte-derived catecholamines inhibit Th1 cell differentiation (Huang et al. 2015a; Huang et al. 2015b). In murine cell cultures of Th1/Th2, terbutaline, a specific β2-adrerenrgic agonist, suppressed Th1 cells, leading to less IFNγ (Sanders et al. 1997a; Agarwal and Marshall 2000). In contrast, in another study, NE increased the IFNγ production by differentiated Th1 cells (Swanson et al. 2001). Similarly, the β2-adrenergic blocker, propranolol, used in experimental autoimmune encephalomyelitis (EAE) rats, resulted in an increased IL-17 and Th17 cell frequency in male rats (Vujnović et al. 2019). In contrast, propranolol led to a decrease in the number of Th17 cells in EAE rats (Pilipović et al. 2019). Moreover, our lab found that terbutaline increased IL-17A cytokine levels in samples of PBMCs (Carvajal Gonczi et al. 2017). Nonetheless, there is a general conviction that the adrenergic signaling in immune cells leads to exacerbating the inflammation and increases the production of IL-17A cytokines although it may still inhibit IFNγ.

1.4. Nebivolol as an immunomodulatory drug

The immunomodulatory roles of adrenergic drugs used to treat cardiovascular diseases or asthma were investigated by researchers in order to use them to treat autoimmunity. These drugs represent interesting candidates for immunotherapy given that their β AR signaling is well tested and understood in various tissues, and their safety is confirmed since they are already approved for clinical use. For example: albuterol, an asthma drug, was found to improve the clinical outcome of glatiramer acetate-based therapy of MS (Khoury et al. 2010). However, more research is needed to study different candidates belonging to different types of agonists and antagonists given that most ligands used in *in-vitro* experiments are full agonists or full antagonists such as isoproterenol and propranolol, respectively. Here, we are studying nebivolol which is an adrenergic drug used to treat hypertension (Ignarro 2008). Nebivolol mitigates hypertension symptoms by inducing a vasodilation in blood vessels in a nitric oxide (NO)-dependent mechanism (Broeders et al. 2000; Angulo et al. 2010). It was considered to be a selective β1 and β2 AR blocker or antagonist given that it does not increase the levels of cAMP in the cytosol. However, nebivolol was shown to increase

the β AR internalization similarly to β AR agonists (Pauwels et al. 1989; Pauwels et al. 1991; Frazier et al. 2011). In a study evaluating its signaling, nebivolol was revealed as an inverse or biased agonist of β2 AR, and likely β1 AR, that activates the GRK/β-arrestin pathway only without inducing the G-protein-mediated signaling (Erickson et al. 2013). Given this, we would expect nebivolol to be different from other agonists as a result of its unique signaling pathway which could provide a novel mechanism to regulate the immune cells. To our knowledge, nebivolol has never been studied as an immunomodulatory drug or explored as a possible autoimmunity therapeutic drug which is one reason I studied it more in the thesis.

Interestingly, our group recently discovered for the first time that nebivolol inhibits the production of IL-17A and IFNγ in samples of human PBMCs (Figure S 9 unpublished data). An adrenergic agonist capable of suppressing two prominent proinflammatory cytokines being IL-17A and IFNγ is precisely what is needed to treat autoimmune diseases characterized by elevated levels of these cytokines. Therefore, in my thesis I performed a study to further understand how nebivolol is regulating the immune cells and whether its regulatory mechanism is mediated by NO. It is well known that NO is an important immunomodulatory agent as I will present in the next section.

1.5. Nitric oxide regulation of Th cells

Considering that nebivolol is known to increase nitric oxide signalling in cardiac cells, the second immunomodulatory scheme to be discussed is the NO signaling in immune cells. Autoimmunity and pathogenic inflammation are associated with elevated NO levels (MacMicking et al. 1997; Sarchielli et al. 1997; Bogdan 2001). Furthermore, during inflammation, high levels of NO are produced (Bogdan et al. 2000; Coleman 2001) and contribute to the progression of many diseases such as asthma (Kharitonov et al. 1994), rheumatoid arthritis (McInnes et al. 1996) and MS (Sarchielli et al. 1997). In the following section, I will describe NO, its synthesis and signaling in addition to its relationship with Th cells.

NO is a highly reactive, short-lived free radical that is the smallest signaling molecule known and has various molecular targets (Bogdan 2001). Moreover, NO was shown to be a key messenger and regulatory molecule in neurotransmission (O'Dell et al. 1991; Schuman and Madison 1991), vascular contractility (Rapoport et al. 1983; Förstermann et al. 1986), gene transcription (Khan et al. 1996; Gudi et al. 1999) as well as in pathogenesis and inflammation (MacMicking et al. 1997; Bogdan 2001).

NO is synthesized in different cell types by a family of enzyme isoforms called the nitric oxide synthases (NOSs). There are three isoforms that have been identified: neuronal NOS (nNOS/NOS1), inducible (iNOS/NOS2) and endothelial NOS (eNOS/NOS3). All NOS isoforms bind the calcium-modulated protein calmodulin and contain haem. The generation of NO by all NOS requires a substrate of L-arginine, co-substrates of molecular oxygen (O^2) and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), and cofactors of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydro- L-biopterin (BH4). (Crane et al. 1998; Alderton et al. 2001). Notably, the generation of NO from L-arginine occurs in two steps where NOS hydroxylates L-arginine to produce N^{ω} -hydroxy-L-arginine which remains bound to NOS which then oxidizes it to produce NO and L-arginine (Illustration 3) (Noble et al. 1999; Stuehr et al. 2001). This explains the use of N^{G}-Monomethyl-L-arginine acetate salt (L-NMMA) as a general NOS inhibitor in many studies aiming to block the generation of NO and it will be used in my thesis as well.

The different NOS isoforms are expressed in different cell types and at different rates under different conditions of health and disease. iNOS only was showed to be expressed in the immune cells such as macrophages and T cells and to play a role in inflammation and septic shock (Förstermann and Sessa 2012; Bogdan 2015). Given that iNOS is involved in inflammation and regulates the immune system through multiple intracellular mechanisms (Förstermann 2000; Salvemini et al. 2003), it is necessary to understand the regulation of different Th cell subsets by iNOS.

The NO formed by NOS isoforms can modulate the cellular signaling by targeting many proteins and enzymes in the producing cells or neighbouring cells (reviewed in

(Guzik et al. 2003)). The predominant signaling pathway of NO occurs through the activation of soluble guanylyl cyclase (sGC) and the following generation of cyclic guanosine monophosphate (cGMP) (Rapoport et al. 1983; Furchgott et al. 1984; Förstermann et al. 1986; Knowles et al. 1989; Garthwaite 1991; Fischer et al. 2001). The synthesis of the second messenger cGMP leads to the recruitment and activation of protein kinase G (PKG), phosphodiesterases (PDEs), and cyclic nucleotide-gated channels (Illustration 3) (Derbyshire and Marletta 2012). Other nonclassical signaling mechanisms of NO includes the induction of significant post-translational protein modification through the nitration of Tyr or S-nitrosylation of Cys thiol groups (Martínez-Ruiz et al. 2011). Furthermore, NO-mediated S-nitrosylation was shown to modulate the activation and function of sGC which highlights a remarkable relationship that is still under investigation (Beuve et al. 2016). S-nitrosylation was shown to negatively regulate the development of T cells. For example: a lymphocyte deficiency, lymphopenia, was developed in mice lacking the denitrosylase glutathione/S-nitrosoglutathione reductase (GSH/ GSNOR) which reverses S-nitrosylation, and the condition was improved by deleting the iNOS gene (Yang et al. 2010; Anand et al. 2014). In addition, NO becomes inactivated when it reacts with superoxide anion (O_2^-) and this reaction generates oxidant peroxynitrite ($ONOO^-$) which results in oxidative damage and DNA breakage (Illustration 3) (Lee et al. 2003; Mikkelsen and Wardman 2003; Ridnour et al. 2004). Thus, NO can modulate the immune cells besides other cell types and in the following section, I will explore how Th cells affect NOS expression and NO release as well as how NO regulate the Th cells and their cytokines.

1.5.1. Reciprocal Modulation between NO and Th cells

A reciprocal modulation between Th cells and NO has been observed in various *in vitro* studies using mouse or human samples (Pahan and Mondal 2012). The modulation occurs in one direction when cytokines released by Th cells regulate the expression of NOS genes or NO production, and in another direction when the released NO by Th cells or other immune cells modulate the proliferation and cytokine production of Th cells.

A- Th cells regulate NO

The IFNγ cytokine was shown to be sufficient to effectively induce the transcription of iNOS gene in many cell types including Th cells, macrophages and glial cells (Stuehr and Marletta 1987; Xiong et al. 1996; Jovanovic et al. 1998; Saha and Pahan 2006). IFNγ is believed to induce the iNOS gene transcription and hence the NO production through STAT1 transcription factor which becomes activated following the binding of IFNγ to its receptor and phosphorylating Janus kinases (JAK2) (Xie et al. 1993; Kitamura et al. 1996; Dell'Albani et al. 2001). On the other hand, the effect of IL-17A cytokine on the transcription of NOS genes and NO production is still under debate. Jovanovic *et al.* found that IL-17 alone has no effect on the levels of NO and iNOS gene in human PBMCs and macrophages (Jovanovic et al. 1998). However, IL-17 was shown to induce the transcription of iNOS in murine chondrocytes, endothelial cells and fibroblasts (Miljkovic et al. 2003; Miljkovic et al. 2005). Furthermore, a positive correlation between IL-17A and NO was observed in the PBMCs of patients with Inflammatory bowel diseases (Rafa et al. 2013). It is suggested that IL-17 induces the expression of iNOS by inducing a phosphorylation of Src, Ras and TRAF6 proteins which activate NF-κB and MAPKs which in turn activate AP-1 (Miljkovic and Trajkovic 2004; Yang and Yuan 2018). In addition, binding IL-17 to its receptors activates JAK1/2 which leads to iNOS upregulation through STAT1 (Miljkovic and Trajkovic 2004). Nonetheless, in a study evaluating the effects of circulating IL-17 in plasma on cerebral endothelial cells, IL-17 was shown to suppress NO production in a Rho-kinase-dependent inhibitory phosphorylation of eNOS (Faraco et al. 2018). The regulation of NO production and iNOS transcription by Th cells is a complex process as it depends on the cell type and occurs on the transcriptional levels. Therefore, more studies are needed to address this topic in various cell types and under nonpathological conditions especially that NO is a potent mediator of inflammation and central biological processes.

B- NO regulates Th cells

The NO production in the immune system alters the function and stability of Th cells, which consequently modulates the immune responses (Liew 1995; Kolb and Kolb-

Bachofen 1998; van der Veen 2001). However, there are divergent findings regarding the role of NO on different Th cell subsets (Bauer, Jung, Tsikas, Stichtenoth, C. Frölich, et al. 1997; van der Veen et al. 1999) suggesting that NO can both enhance and suppress Th cell functions (Liew 1995). There are few studies discussing the effect on NO on Th1 and Th17 cells. Instead, most studies evaluate the NO- mediated regulation of other cell types which will eventually modulate the development of Th1 and Th17 cells (Bogdan 2015). Although this approach provides a valuable knowledge regarding the immunomodulatory mechanisms of NO on Th cell development, understanding the direct effects of NO on Th cells will help to develop therapeutics to combat morbidities associated with T cell dysregulation. In the following section I will review the current knowledge regarding the direct as well as the indirect NO-mediated regulation of Th cells.

Exogenous NO applied by the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) treatment on naïve murine Th cells enhanced the development of Th1 cells, whereas fully committed Th cells remained unaffected (Niedbala et al. 1999). These types of donor chemicals are used for research because they slowly release NO, since NO can not be used directly as a reagent due to its short half-life. The Th1 master transcription factor, T-bet, was suppressed in T cells using gemfibrozil which is a drug that inhibits iNOS expression and NO production meaning that NO induces the development of Th1 cells (Pahan et al. 2002; Xu et al. 2006; Dasgupta et al. 2007; Jana et al. 2007). There is more evidence suggesting an inhibitory role of NO on Th1 cells. NO was shown to regulate the development of murine Th1 cells indirectly by suppressing the synthesis of their driving cytokine IL-12 in APCs such as macrophages and dendritic cells (Huang et al. 1998; Xiong et al. 2004). Moreover, In cultures of human PBMCs, NO directly reduced the proliferation of Th1 cells and IFNγ release in a time-dependent manner (Macphail et al. 2003). The reduction of IFNγ cytokine following the NO treatment was linked to the inhibition of Th1 cells proliferation regardless of the cytokine (Xiong et al. 1996). Accordingly, the number of murine cells expressing T-bet transcription factor were reduced by NO under mycobacterial infection (Pearl et al. 2012). In contrast, the treatment of human Th cells with exogenous NO inhibited the cytokine secretion of

activated Th1 cells especially IFNγ (Bauer, Jung, Tsikas, Stichtenoth, J.C. Frölich, et al. 1997). NO donor reduced IFNγ cytokine in human Th cells in a dose-dependent manner where it had no effects on IFNγ cytokine levels at low doses (Obermajer et al. 2013) whereas the NO donor GSNO at intermediate levels had no effect on IFNγ (Singh et al. 2018). While there are divergent findings regarding the role of NO on Th1 cells and IFNγ cytokine, the findings regarding Th17 cells seem to be more consistent.

In human and murine samples, exogeneous NO suppressed the proliferation and function of Th17 cells (Niedbala et al. 2011). This suppression was shown to be due to the NO-mediated inhibition of the expression of the transcription factor aryl hydrocarbon receptor (AHR) which enhances the development Th17 cells (Niedbala et al. 2011). Accordingly, EAE mice lacking iNOS had higher levels of AHR expression and developed more severe MS conditions as a result of the increase in Th17 cells and IL-17A cytokine levels (Niedbala et al. 2011). However, the AHR-dependent explanation of the NO-mediated inhibition of Th17 cells and IL-17 cytokine was challenged when the aforementioned findings could not be replicated and the AHR expression was not significantly different between wild type and iNOS-knockout mice (Xue et al. 2018). The alterations in the expression of iNOS and AHR were found to be synchronous but there is not a causal relationship between them (Wheeler et al. 2013).

An alternative explanation for the NO-mediated inhibition of Th17 cells and IL-17 cytokine depends on the tyrosine nitration by NO of many proteins especially the ROR-γt (Xiong et al. 2004; Yang et al. 2013). The NO donor, S-nitrosoglutathione (GSNO), improved the MS conditions in EAE mice by suppressing the release of IL-17 from Th17 cells and this suppression was shown to be due to the inhibition of the phosphorylation of STAT3 and the expression ROR-γt in Th cells (Nath et al. 2010; Singh et al. 2018). Accordingly, the increased expression of iNOS in naïve T cells, negatively regulates the differentiation of Th17 cells by nitrating ROR-γt (Yang et al. 2013; Zhang et al. 2013). Moreover, the NO donor SNAP inhibited IL-17 cytokine production and release in Th17 cells in a dose-dependent manner (Yang et al. 2013), and this inhibition was due to reduced ROR-γt binding and activation of IL-17 promoter (Xiong et al. 2004; Yang et al.

2013). The nitration of specific tyrosine residues affects the structure and function of many proteins and transcription factors in Th cells which in turn modulates the immune response (Ji et al. 2006; Prasad et al. 2007; Cheng et al. 2018). For example the nitration of tyrosine residue in IKBα resulted in its dissociation from NF-κB (Yakovlev et al. 2007), and the nitration of the p65 protein inhibited its binding and activation of NF-κB (Khan et al. 2006)

Despite the inhibitory role of NO on Th17 cells and IL-17 cytokine, NO was shown to affect Th17 cells differently depending on the dose. In cultures of human Th cells, NO reduced the release of IL-17A at high concentrations, whereas it augmented IL-17A at lower concentrations, where the concentrations refer to the donor chemical (Obermajer et al. 2013). Thus, low NO levels induce the stability and function of Th17 cells in human Th cells (Obermajer et al. 2013). In contrast, high NO levels induce cell death in isolated mice Th cells (Brüne et al. 1999; Kiang et al. 2008) and result in modifications of endogenous proteins (de Vera et al. 1996; Thomas et al. 2004). Notably, there is not a universal definition of low and high NO doses to be used *in vitro*. Instead, researchers opt to use a gradient of NO donor concentrations to infer the different effects of varying NO levels. Although this concept complicates the translation of the *in vitro* findings into clinical studies using human patients, these studies still provide interesting insights into the effects of different NO levels on the immune cells.

Most studies evaluating the effects of NO on the immune cells were performed *in vitro* on murine cells (Liew 1995; van der Veen 2001). Notably, there are profound differences in the regulation and function of NO between mice and humans (Schneemann and Schoedon 2002; Fang 2004; Schneemann and Schoeden 2007). Although researchers have been using murine and rodent models to study different aspects of the immune systems, humans have evolved to tolerate lower levels of NO as compared to mice and rats which forms a barrier to translate NO experimental findings in mice and rats into human studies (García-Ortiz and Serrador 2018). Moreover, the main enzyme responsible for NO production is iNOS which releases large amounts of NO, whereas human cells mainly depend on the constitutive NOS enzymes given the low-level of NO they tolerate (García-Ortiz and Serrador 2018). All in all, it is necessary

to study the immunomodulatory roles of NO in human T cells in order to recruit it to face autoimmunity.

1.6. The adrenergic signaling and the role of NO

Given the significance of the adrenergic signalling and NO in Th cell regulation, the involvement of NO in Th cell adrenergic signaling can strengthen our understanding of Th cell regulation by adrenergic drugs and support their candidacy as immunomodulatory agents. A relationship between adrenergic signaling and NO was described in different tissues and experimental models. For example, propranolol, a β2-adrenergic antagonist, reduced the NO generation in embryonic stem cells (Sharifpanah et al. 2014), whereas isoproterenol, a β2-adrenergic agonist, increased the iNOS activity and NO release in human umbilical vein endothelial cells (Yao et al. 2003). Accordingly, the therapeutic effects of nebivolol are associated with the induction of NO release, increased expression of NOS2 mRNA and activation of the NO pathway in endothelial cells (Kuroedov et al. 2006; Ladage et al. 2006; Angulo et al. 2010; Chien et al. 2013; Mose et al. 2015). These findings suggest that the stimulation or blocking of adrenergic receptors can modulate the NO levels. However, the adrenergic-mediated regulation of NO in Th cells is poorly understood. In murine macrophages and human monocytes, isoproterenol was found to regulate Th cells and NO production differently depending on the inflammatory stimulus (Szelenyi et al. 2006). The full β2-adrenergic agonist increased the expression of NO and proinflammatory cytokines such as TNFα and IL-12 with protein kinase C (PKC)-activating phorbol myristyl acetate (PMA), whereas it decreased their expression with lipopolysaccharide (LPS) (Szelenyi et al. 2006). This dual modulation by isoproterenol was explained using the kinase pathway where it increases MAPK phosphorylation in cells treated with PMA, and decreases ERK1/2 and p38 activation in LPS-treated cells (Szelenyi et al. 2006). nebivolol's biased agonism leads to the activation of MAPKs and ERK1/2 as well as the desensitization of cAMP and PKC (Lefkowitz and Shenoy 2005; Erickson et al. 2013). Therefore, I hypothesized that nebivolol can increase the production of NO in human PBMCs by its unique signaling pathway. Moreover, the nebivolol-mediated NO

production is expected to explain the inhibition of IL-17A and IFNγ cytokine production in activated PBMCs.

1.7. Conclusion

Despite the significance of NO and β2ARs in modulating the immune responses, the effects of β2-adrenergic biased agonists on the expression and release of NO in Th cells are yet to be discovered. In this thesis, I aimed to confirm the unpublished findings regarding the effects of nebivolol on the function and stability of Th1 and Th17 cell subsets. I also investigated the effects of different NO donor concentrations on the functions of Th1 and Th17 in samples of human PBMCs. The evaluation of Th1 and Th17 cell stability and function will be performed by measuring their hallmark cytokines, IFNγ and IL-17A, as well as the gene expression of their master transcription factors, T-bet and ROR-γt, all respectively. Finally, I evaluated the NO-inducing ability of nebivolol and whether its effects on Th cells are mediated by the NO pathway. This was achieved by measuring the Th cells' main cytokines following the inhibition of the NOS enzymes and the blocking or induction of the cGMP pathway which is the main signaling pathway of NO. In addition, the NOS2 gene expression was measured following the nebivolol treatment.

My thesis was the first to investigate the link between β2ARs and NO in Th1 and Th17 cells in samples of human PBMCs. The modulation of NO expression in Th cells by nebivolol will be immensely valuable to manage autoimmunity and inflammation especially that these pathological conditions are associated with increased proinflammatory cytokines and NO production. Nebivolol is already approved for safe use for cardiovascular disease, suggesting that it can be further explored for possible off-label use in autoimmunity.

Illustrations

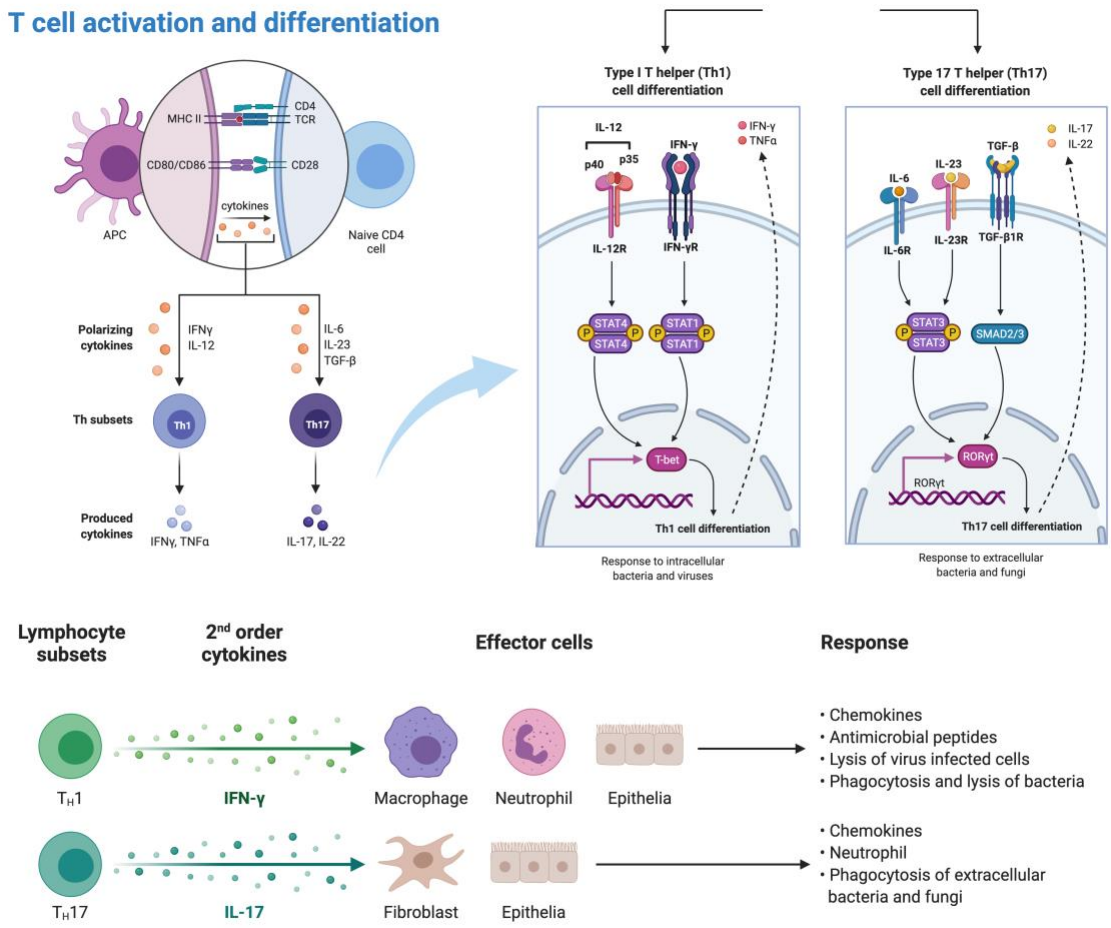

Illustration 1: **T helper 1 and T helper 17 cell activation and differentiation**: The differentiation of Th cells is initiated by two routes: 1) The T cell receptor (TCR) and coreceptor (CD4) recognition of a peptide antigen presented on a major histocompatibility complex MHC II of an antigen presented cell (APC). 2) The binding of milieu cytokines to their receptors induces a signaling pathway to activate specific transducer and activator of transcription (STAT) proteins besides other protein in order to upregulate master transcription factors of the corresponding Th cell subset, T-bet for Th1 cells and RORγt for Th17 cells. Following the differentiation of Th cells, they release cytokines characteristic of the Th cell subset. The cytokines target many cell types and induce different proinflammatory responses (Pennock et al. 2013; Huang et al. 2015b; Murphy and Weaver 2016). Created using Biorender with the acknowledgement of Anna Lazaratos.

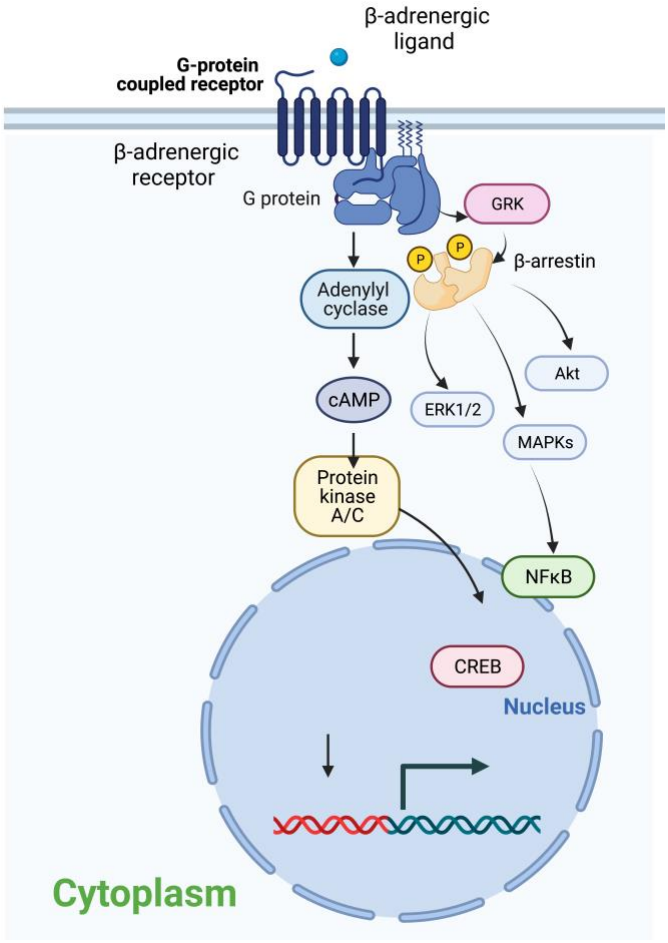

Illustration 2: **Adrenergic signaling:** the adrenergic signaling occurs through two intracellular pathways namely the cAMP pathway and the GRK/β-arrestin pathway. Abbreviations used include: NFκB: nuclear factor-κB, CREB: cAMP response element-binding protein, (Pennock et al. 2013; Murphy and Weaver 2016). Created using Biorender with the acknowledgement of Akiko Iwasaki, PhD (content expert) Ruslan Medzhitov, PhD (content expert).

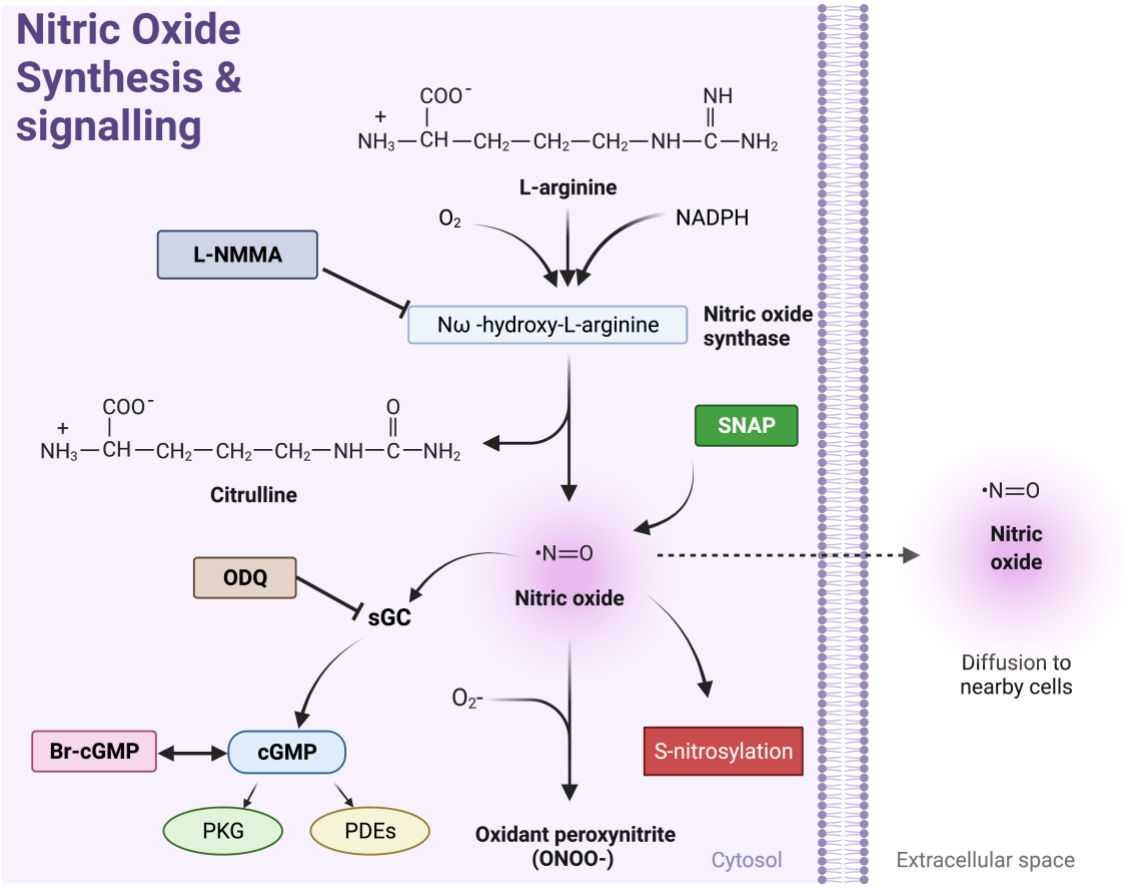

Illustration 3: **Nitric oxide synthesis and signalling**. Nitric oxide synthases generate nitric oxide from a substrate of L-arginine and co-substrates of molecular oxygen (O^2) and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH). The generation of nitric oxide involves the creation of an intermediate product of Nω-hydroxy-L-arginine which remains bound to the NOS enzyme before being converted into nitric oxide and citrulline. NO signals predominantly though the soluble guanylyl cyclase (sGC) which activated cyclic guanosine monophosphate (cGMP). cGMP activated protein kinase G (PKG) and multiple phosphodiesterase. Nitric oxide can also bind anion superoxide to form oxidant peroxynitrite ($ONOO^-$) which leads to DNA breaks and cell death. Finally, through S-nitrosylation, nitric oxide can induce modifications of multiple targets within the cell. The illustrations demonstrate the drugs that were used throughout this thesis to investigate the role of nitric oxide in immunoregulation. The drugs included: nitric oxide donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP), general nitric oxide synthase inhibitor: NG-Methyl-L-arginine acetate (L-NMMA), cGMP analogue 8-Bromoguanosine 3′,5′-cyclic monophosphate sodium (Br-cGMP) and cGMP inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Created using Biorender.

CHAPTER II: Materials and Methods

2.1. PBMCs Activation and Drug Treatment

Peripheral blood mononuclear cells (PBMCs) which contain T cells and other white blood cells, were isolated from venous blood according to a standard protocol (Freundlich and Avdalovic 1983). The blood was drawn from six healthy human participants by a licenced phlebotomist after an informed, signed consent was obtained. The participants were interviewed prior to drawing blood to confirm the criterion of healthy state by self-reporting of their health condition. The exclusion criteria included chronic illness, recent vaccination, hormonal treatment or autoimmune diseases. The study was approved by the Concordia University Research Ethics committee (certificate 30009292). Up to twelve heparinized vacutainer tubes (BD, Franklin Lakes, NJ, USA) were drawn and processed using ficoll-hypaque (GE healthcare, Mississauga, ON, Canada) density gradient centrifugation techniques to isolate the layer of PBMCs (Tabatabaei Shafiei et al. 2014).

PBMCs were incubated for 24 hours at 37˚ in media which contained 10% heat-inactivated fetal bovine serum (FBS; Wisent Inc., Montreal, QC, Canada) in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 1mM penicillin with streptomycin, and 2mM glutamine (Wisent Inc. QC, Canada). Following the 24-hour incubation time, 0.5 x 10^6 PBMCs were cultured in RPMI 1640 medium supplemented with 10% FBS in a round bottom 96 well culture plate (VWR, Mississauga, ON, Canada) at a concentration of 2.5 x 10^6 cell/ml. The viability check and count of PBMCs was performed before culturing the cells using trypan (Trypan Blue Solution, 15250061, Gibco™, Waltham, MA, US) and an automated cell counter (Countess™ 3 FL, Invitrogen™, Carlsbad, CA, USA). The activation of PBMCs was achieved by adding cell culture grade anti-CD3 (clone OKT3) and anti-CD28 antibodies (clone CD28.2) (eBioscience, San Diego, CA, USA) in soluble format at 0.1 mg/mL each. This activation technique was followed by studies using PBMCs as a model as well as our recent work at Dr. Darlington's lab (Macphail et al. 2003: 1; Wahle et al. 2005; Carvajal Gonczi et al. 2017)

The *in vitro* drug treatments included the adrenergic agonist **nebivolol** at 10^{-5}M (Nebivolol hydrochloride, N1915, Sigma Aldrich), the NO donor **SNAP** used at concentrations of 10µM, 25µM, 50µM, 100µM and 250µM (S-Nitroso-N-acetyl-DL-penicillamine, N3398, Sigma Aldrich, Mississauga, ON, Canada), the NOS2 inhibitor **1400W** at 0.2µM (1400W dihydrochloride, W4262, Sigma Aldrich, Mississauga, ON, Canada), and the selective inhibitor of NO-sensitive soluble guanylyl cyclase (sGC) **ODQ** at 10µM (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, O3636, Sigma Aldrich, Mississauga, ON, Canada). Nebivolol, SNAP, 1400W, and ODQ are dimethyl sulfoxide (DMSO)-soluble and require an organic solvent control group which was included at a matching dilution and referred to afterwards as DMSO vehicle control. Other *in vitro* drug treatments included the cell permeable analog of cyclic guanosine 3′:5′-monophosphate (cGMP) **Br-cGMP** at a concentration of 100µM (8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt, B1381, Sigma Aldrich, Mississauga, ON, Canada), and the general NOS inhibitor **L-NMMA** at a final concentration of 500µM (NG-Methyl-L-arginine acetate salt, M7033, Sigma Aldrich, Mississauga, ON, Canada). Both Br-cGMP and L-NMMA are water-soluble and do not require an organic solvent control group.

2.2. Enzyme-linked Immunosorbent Assay (ELISA)

To measure cytokines, cell culture supernatants were collected after four days of incubation at 96-hour time point. The cytokines were measured with a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions for human IFNγ (BD Bioscience, San Jose, CA, USA) and human IL-17A (Invitrogen™, Carlsbad, CA, USA).

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

After six days of incubation, a total RNA extraction was performed on cells collected from four biological replicates of each condition (approximately $2x10^6$ cells) using spin columns according to the manufacturer's protocol (PureLink™ RNA Mini Kit, 12183018A Invitrogen™, Carlsbad, CA, USA). The total RNA was measured and verified for chemical and protein contamination using a spectrophotometer (NanoDrop™

2000c, ThermoScientific™), and then used to create a cDNA library using reverse transcription kit according to the manufacturer's protocol (iScript™ Reverse Transcription Supermix, 1708841, BioRad, Hercules, CA, US). The cDNA was then used in a TaqMan analysis of mRNA expression using TaqMan-recommended inventoried assays for the NOS2 gene, the major transcription factors of Th1 (TBX2 or T-bet) (Szabo et al. 2000) and Th17 (RAR Related Orphan Receptor C, Rorc) (Ivanov et al. 2006a) and the reference gene (HPRT1) [Gene IDs: NOS2: Hs01075527, TBX2: Hs00203436-m1, Rorc: Hs01076112, HPRT1: Hs02800695_m1, respectively] (Applied Biosystems, Foster City, CA, US). The gene expression of NOS2, TBX2, and Rorc was normalized to the expression of the reference gene HPRT1 and expressed as a relative expression where the fold increase was denoted as $(2^{-\Delta CT})$ and ΔCT was calculated using the formula: $\Delta CT = CT_{(target\ gene)} - CT_{HPRT1}$.

2.4. Statistical Analysis:

Due to the small sample size and the differential response of different subjects and given the importance of interindividual variability in immune regulation, we found that it will be more informative to present the figures of key phenomena in two formats, pooled and individual figures. The pooled figures were plotted using the aggregate data from different subjects whose cells were treated for the same condition and expressed as means + SEM (the number of subjects or participants (n) was provided in the individual figure panel and associated legend). On the other hand, the individual figures were plotted using the data from the three biological replicates for every subject and expressed as means + SEM (the participant code (LCR) was provided in the individual figure panel and associated legend). In cases of contradictory responses from different subjects, only the individual figures were provided as the pooled figure doesn't depict the observed phenomenon. Microsoft® Excel for Mac Version 16.49 was used to calculate the means and SEM and normalize the cytokine concentrations from the ELISA plates' fluorescence readings as well as the gene expression levels from qRT-PCR plates' fluorescence readings. GraphPad Prism Version 9 software was used to plot the figures and analyze the data using Student's t test (two-tailed) and 1-way and 2-way analysis of variance (ANOVA) with correction for multiple comparisons using

Bonferroni method, where appropriate, with $P < 0.05$ considered as significant ($P \geq 0.05$ (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****)).

CHAPTER III: Results

3.1. Nebivolol downregulates the IL-17A and IFNγ cytokines in samples of human PBMCs

To determine the *in vitro* effects of the biased adrenergic agonist, nebivolol, on the function of Th1 and Th17 subsets within samples of human PBMCs, the levels of IL-17A and IFNγ cytokines were measured using sandwich ELISA on the culture supernatants collected after 4 days of incubation with the drug. In terms of the IL-17A cytokine response, the nebivolol treatment of activated human PBMCs resulted in a reduction of 61.40% ($P<0.0001$) in the pooled data as compared to the positive control of activated PBMCs, (Figure 1). The nebivolol-induced inhibition of IL-17A cytokine was consistent across the different subjects that were included in the study where I observed a decrease of 54.56% ($P<0.0001$) in LCR288, 51.56% ($P<0.0001$) in LCR289, 72.24% ($P<0.0001$) in LCR293, and 60.41% ($P<0.0001$) in LCR294, all compared to the positive control of activated PBMCs (Figure 2 A-D). Moreover, the observed effect of nebivolol was specific to the drug since the DMSO vehicle control of nebivolol did not cause a change in the levels of IL-17A cytokine as compared to the positive control in the pooled data ($P=0.7991$) (Figure 1) nor in all four subjects ($P>0.9999$, $P=0.7237$, $P=0.0797$, $P>0.9999$ for LCR288, LCR289, LCR293 and LCR294,respectively) (Figure 2 A- D). Thus, nebivolol considerably downregulates IL-17A cytokine levels in all samples of human of PBMCs which supports its candidacy as an immunomodulator agent to mitigate the symptoms of autoimmune diseases characterized by high IL-17 cytokine profile.

On the other hand, the levels of the IFNγ cytokine were reduced in the pooled data as compared to the positive control upon treatment with nebivolol ($P<0.0001$) (Figure 3). However, the subjects responded differently to nebivolol (Figure 4). While LCR288 and LCR289 had a reduction in IFNγ levels compared to the positive control of 46.2% and 50.3% respectively (Figure 4 A, B), LCR294 had a slight increase of 23.5% ($P=0.0273$) in IFNγ levels (Figure 4 D), whereas the IFNγ cytokine levels were unchanged for LCR293 ($P=0.7051$) (Figure 4 C). The DMSO vehicle control of nebivolol did not change the levels of IFNγ.

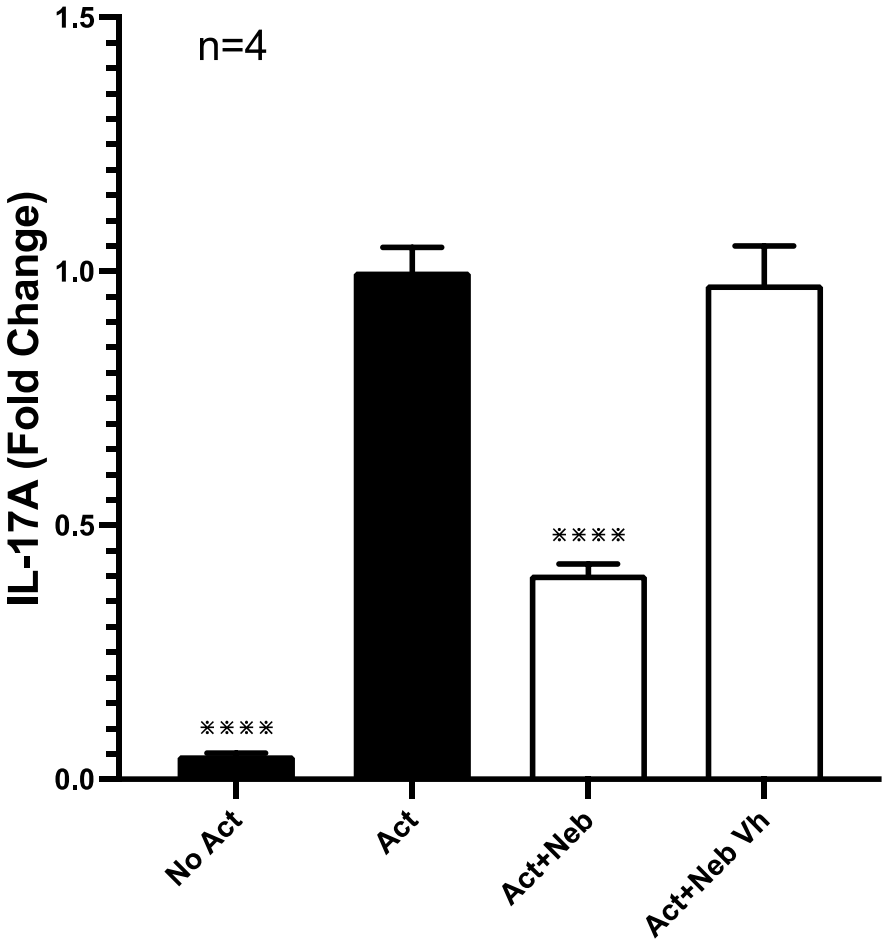

Figure 1: The fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the pooled data from all subjects (n=4). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

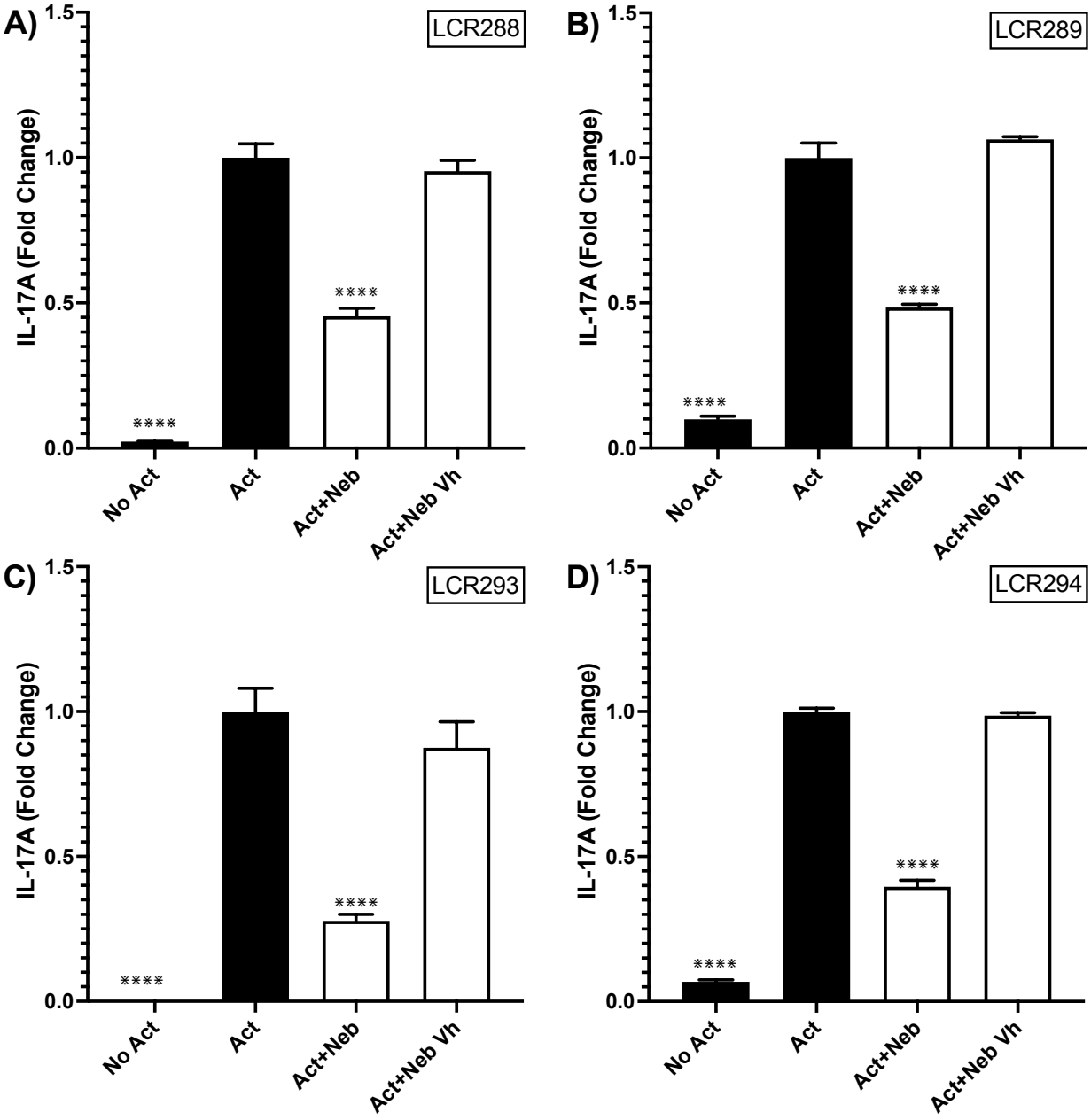

Figure 2: The individual data fold change from Figure 1 data, of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

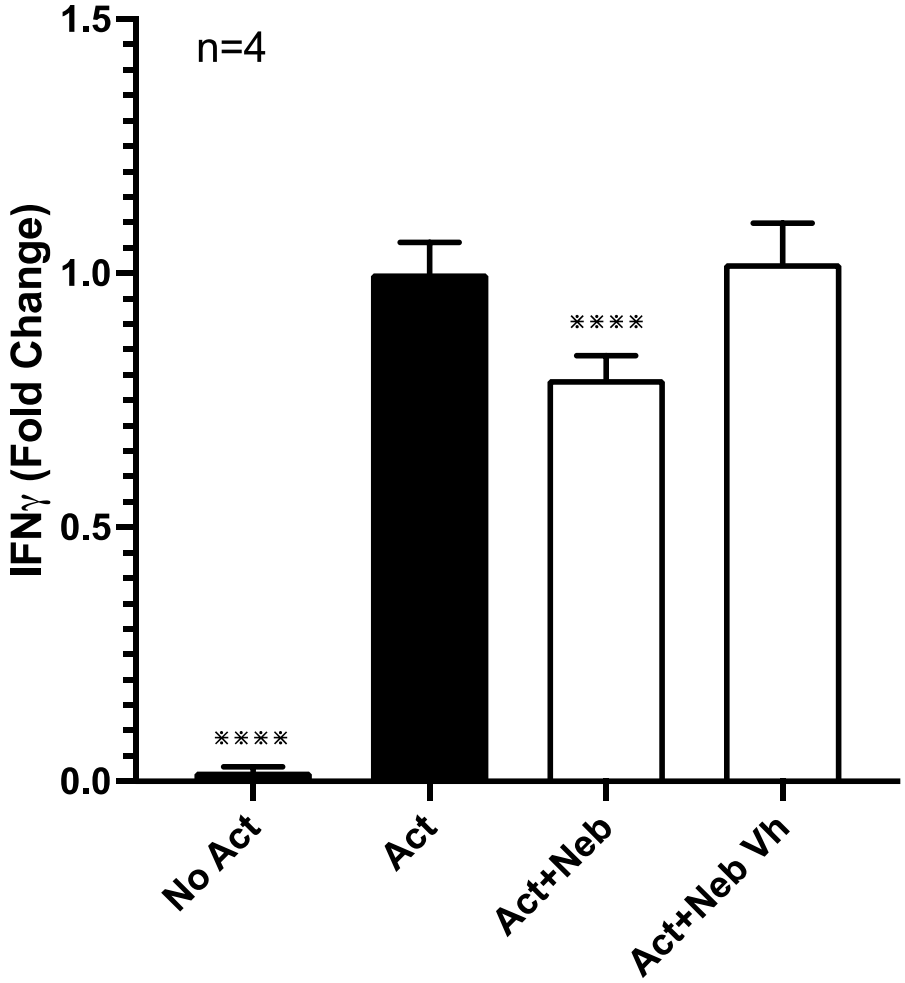

Figure 3: The fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the pooled data from all subjects (n=4). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

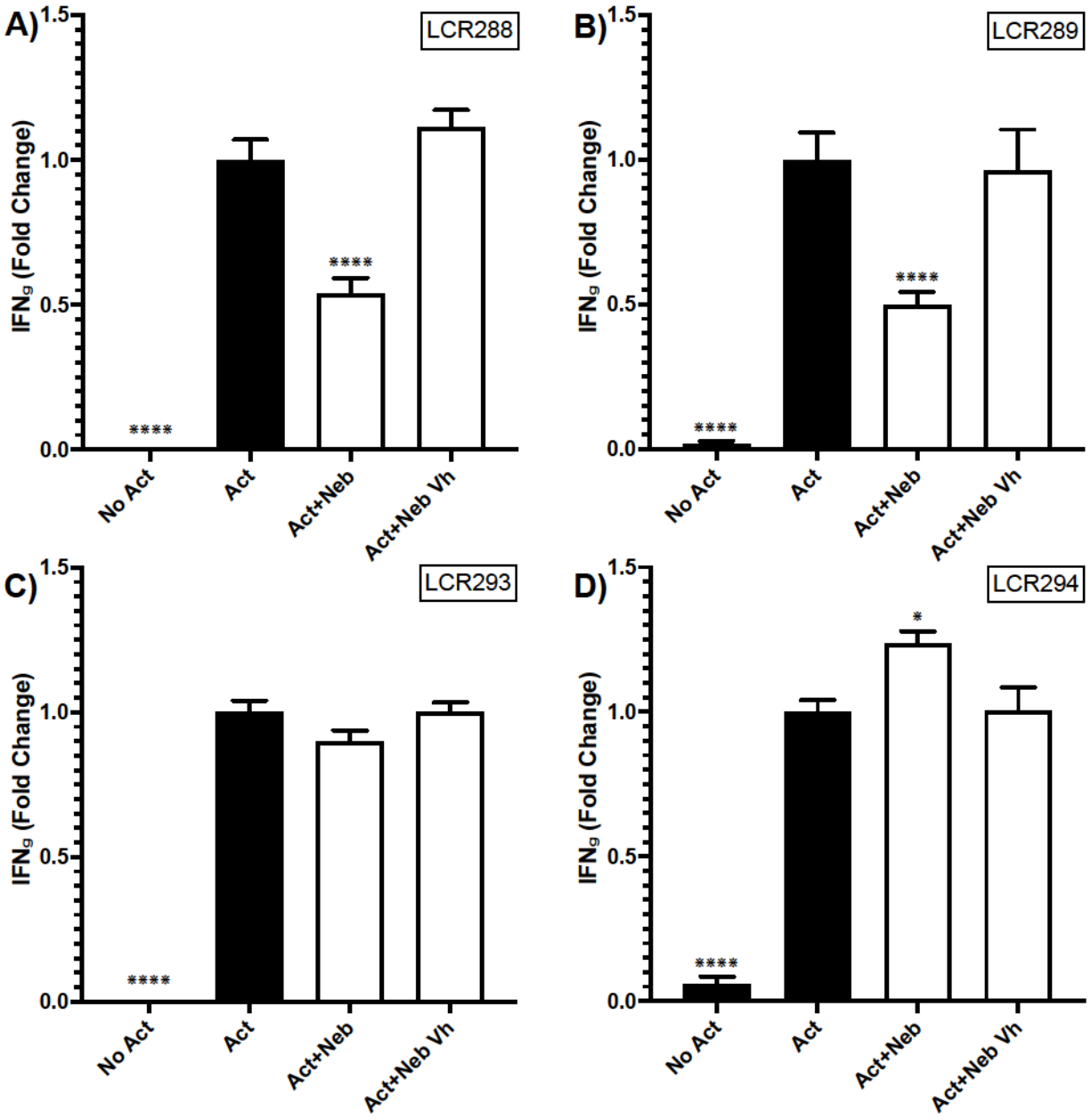

Figure 4: The individual data fold change from Figure 3, of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include No Act: negative control, Act = positive control, Act+Neb= activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh= activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

all subjects which makes the effects on IFNγ levels that were mentioned above specific to nebivolol. In a previous data set that I worked on with a larger sample size, nebivolol significantly decreased IFNγ (Figure S 9), thus the general conclusion is that nebivolol can suppress the cytokine in most participants. Therefore, nebivolol's biased agonism downregulates IFNγ and IL-17A cytokine levels in human PBMCs which further supports the investigation of nebivolol as an immunomodulatory agent.

3.2. NO donor regulates the concentrations of IL-17A and IFNγ in a dose-dependent manner

Nebivolol has been used as an adrenergic drug to lessen hypertension through dilating the blood vessels in a NO-dependent mechanism (Angulo et al. 2010; Erickson et al. 2013). Therefore, to determine whether the immunomodulatory effects of nebivolol are mediated by NO, I investigated the *in vitro* effects of NO on the regulation of Th cells and the production of IL-17A and IFNγ in human PBMCs. Different concentrations of the NO donor SNAP were added to samples of PBMCs from three subjects (n=3) and incubated for four days. Following the incubation time, the cell culture supernatant was collected and used to measure the concentrations of IL-17A and IFNγ cytokines using Sandwich ELISA. Overall, a gradual decrease in IL-17A and IFNγ cytokines was observed with increased concentration of SNAP across all individual subjects and in the pooled data (Figure 5Figure **6**,Figure **7**Figure 8). In the rest of this section, the effects of the different SNAP concentrations will be described.

SNAP concentration of 10μM: the lowest SNAP concentration (10μM) did not change the levels of the IL-17A cytokine in the pooled data as compared to the positive control of activated PBMCs (Figure 5). However, this dose of SNAP resulted in varied IL-17A responses across different subjects (Figure 6). The levels of IL-17A cytokine were unchanged for LCR293 and LCR289. However, LCR294 showed an increase of 18.7% in IL-17A cytokine levels as compared to the positive control ($P<0.05$) (Figure 6 A, B, C). Similarly, the pooled data for IFNγ cytokine showed that IFNγ cytokine was unchanged compared to the positive control ($P>0.05$) following the treatment with 10μM of SNAP (Figure 7). The IFNγ response to the lowest SNAP dose was consistent across

all subjects where no change was observed as compared to the positive control in LCR289, LCR293 and LCR294. Overall, the data shows that the lowest dose of SNAP used in this thesis does not affect the levels of IFNγ and IL-17A cytokines and demonstrates that PBMCs can tolerate low amounts of exogenous NO.

SNAP concentration of 25μM: Following the treatment with a higher dose of SNAP, the levels of IL-17A cytokines in the pooled data were reduced by 12.1% as compared to the positive control ($P<0.05$) (Figure 5). However, this dosage of SNAP, resulted in no change of IL-17A cytokine levels in LCR289 and LCR293, whereas only LCR294 showed a reduction of 10.1% in IL-17A levels as compared to the positive control ($P=0.0239$) (Figure 6 A, B, C). For the IFNγ cytokine levels, no change was observed following the SNAP (25μM) treatment in the pooled data as compared to the positive control (Figure 7). However, one subject, LCR289, had a reduction of 35.9% ($P=0.0014$) in IFNγ cytokine levels (Figure 8 A) as compared to the positive control whereas the IFNγ cytokine levels for the other subjects, LCR293 and LCR294, remained unchanged following the same treatment (Figure 8 B, C). This data shows that with higher SNAP dosage, the levels of IL-17A and IFNγ are starting to decrease which suggests an immunoinhibitory role of NO.

SNAP concentration of 50μM: When the SNAP concentration increased to become 50μM, the levels of IL-17A and IFNγ cytokines were reduced in the pooled data where I observed a reduction of 23% ($P<0.0001$), and 32% ($P<0.0001$) for IL-17A and IFNγ as compared to the positive control, respectively (Figure 5Figure 7). However, the inhibition of IL-17A and IFNγ was not consistent across all individual subjects. In terms of IL-17A cytokine levels, a reduction of 21% ($P<0.05$) for LCR293 and 31% ($P<0.001$) for LCR294 was observed as compared to the positive control whereas there was a trend of decrease of 17% in LCR289, (Figure 6 A, B, C). Similarly for IFNγ cytokine, LCR289 had a reduction of 40%, ($P=0.0004$), and LCR294 had a reduction of 34.98% ($P=0.0019$) whereas LCR293 had a trend of decrease of 22% (Figure 8 A, B, C). This data further suggests an inhibitory role of higher NO dosages on Th cells within samples of human PBMCs. Given the observed reduction in IL-17A and IFNγ cytokine levels

following the nebivolol treatment, a NO-mediated mechanism would require a significant induction of NO synthesis and signaling.

SNAP concentrations of 100μM and 250μM: Given the aforementioned observations with increasing SNAP dosage, it is expected that the highest SNAP concentrations of 100μM and 200μM will bring about a significant reduction in IL-17A and IFNγ cytokine levels, as compared to the positive control of activated PBMCs. Following the treatment with 100μM of SNAP, I observed a decrease of 46% ($P<0.0001$) and 53% ($P<0.0001$) in the pooled averaged data for IL-17A and IFNγ cytokines, respectively (Figure 5Figure 7). The SNAP-induced inhibition for IL-17A and IFNγ cytokines was consistent across all individual subjects. LCR293 showed a reduction of 60.86% ($P<0.0001$) for IL-17A cytokine and 43% ($P=0.0001$) for IFNγ cytokine (Figure 6BFigure 8B). LCR294 had a decrease of 59% ($P<0.0001$) for IL-17A cytokine and 64.72% ($P<0.0001$) for IFNγ cytokine (Figure 6CFigure 8C). Lastly, LCR289 showed a reduction of 19.83% ($P=0.0265$) for IL-17A cytokine and 51% ($P<0.0001$) for IFNγ cytokine. Similarly, following the treatment with 250μM of SNAP, a reduction of IL-17A and IFNγ cytokine levels was observed in the pooled data and across all individual subjects. The levels of IL-17A cytokine dropped by 78% in the pooled averaged data of all subjects as compared to the positive control ($P<0.0001$) (Figure 5). There was a reduction in the IL-17A cytokine levels of 67% in LCR289 ($P<0.0001$) (Figure 6 A), 81.88% in LCR293 ($P<0.0001$) (Figure 6 B), and 86% in LCR294 ($P<0.0001$) all compared to the positive control of activated PBMCs (Figure 6 C). The levels of IFNγ cytokine, on the other hand, were reduced in the averaged pooled data by 69% ($P<0.0001$) (Figure 7) and across all subjects by 70% ($P<0.0001$) in LCR 289 (Figure 8 A),74% ($P<0.0001$) in LCR293 (Figure 8 B), and 64% ($P<0.0001$) in LCR294 all compared to the positive control (Figure 8 C). Thus, high levels of exogenous NO result in a considerably significant reduction of IL-17A and IFNγ cytokines which suggests a cytotoxic effect of high NO levels on immune cells in samples of human PBMCs.

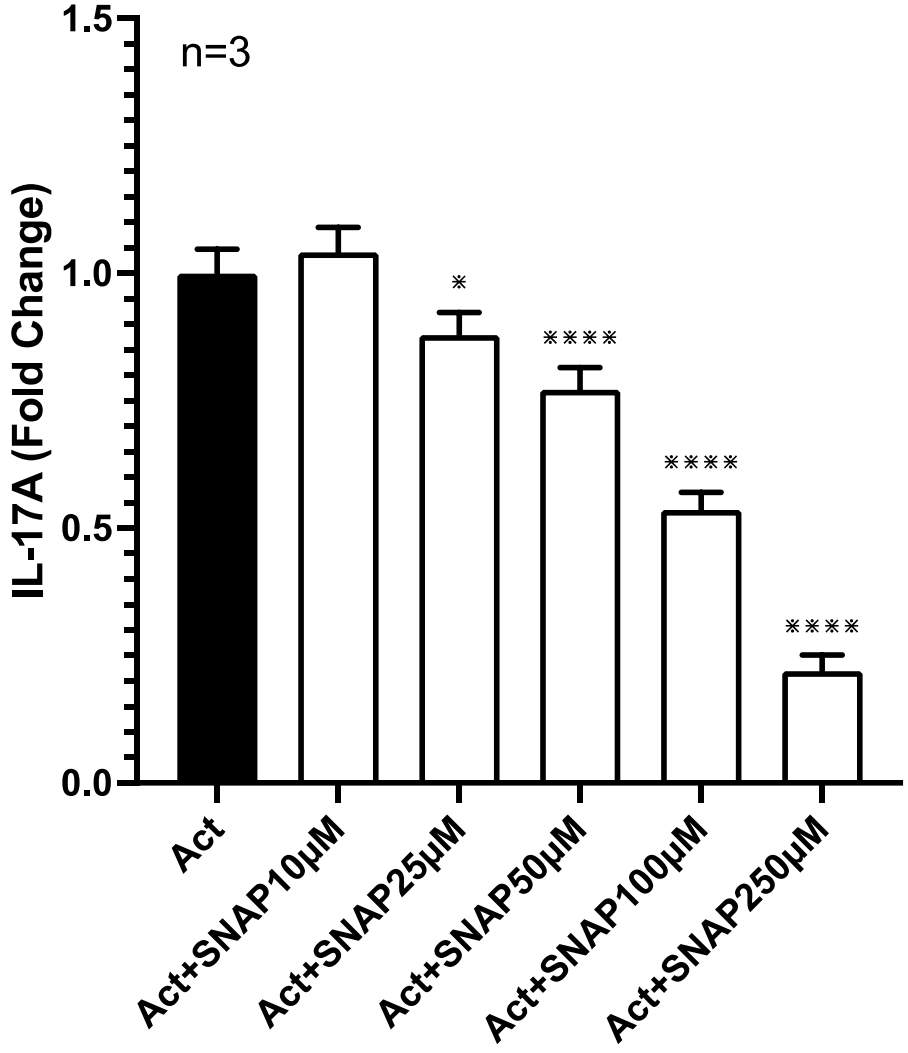

Figure 5: The fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include Act: positive control, and the NO-donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) that is used at final concentrations of 10μM, 25μM, 50μM, 100μM and 250μM. The DMSO vehicle control of SNAP are insignificant and negated from this figure to better depict the effects of the drug. The significance was calculated using the pooled data from all subjects (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

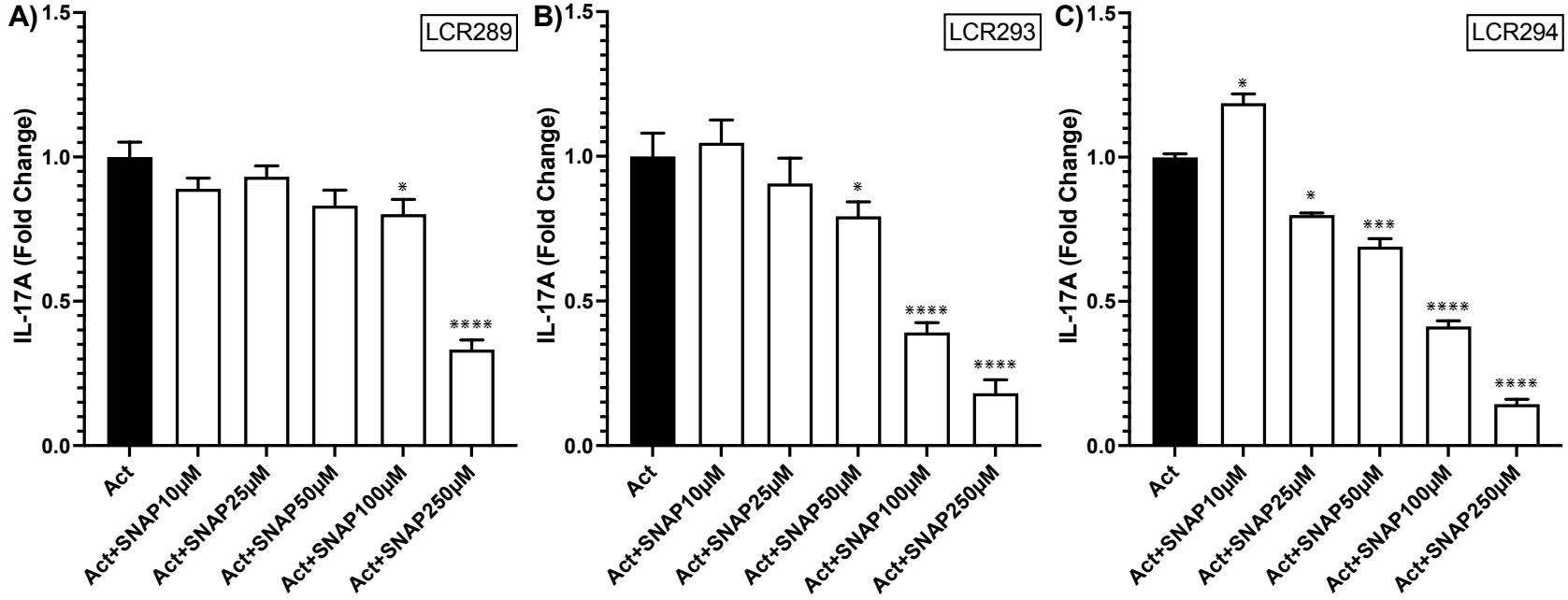

Figure 6: The individual data fold change from figure 5 data of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, and the NO-donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) that is used at final concentrations of 10μM, 25μM, 50μM, 100μM and 250μM. The DMSO vehicle control of SNAP are insignificant and negated from this figure to better depict the effects of the drug. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

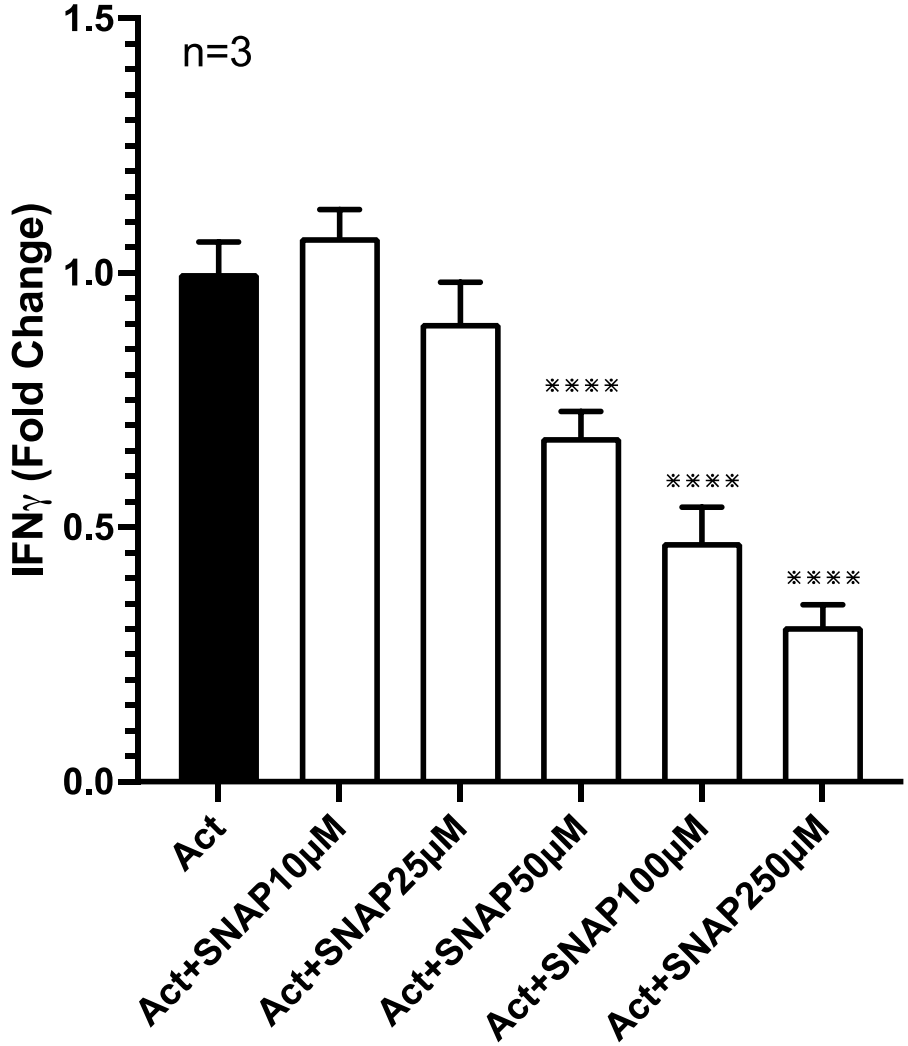

Figure 7: The fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include Act: positive control, and the NO-donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) that is used at final concentrations of 10μM, 25μM, 50μM, 100μM and 250μM. The DMSO vehicle control of SNAP are insignificant and negated from this figure to better depict the effects of the drug. The significance was calculated using the pooled data from all subjects (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****)

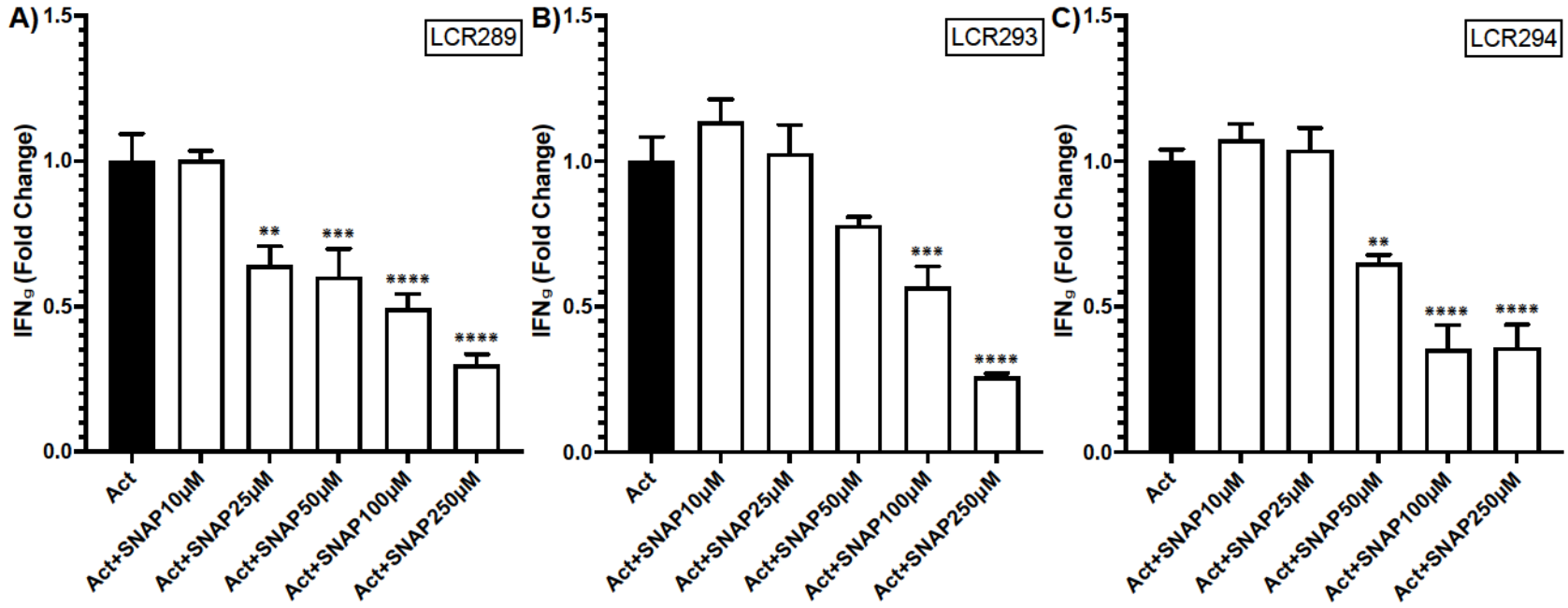

Figure 8: The individual data fold change from figure 7 of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, and the NO-donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) that is used at final concentrations of 10μM, 25μM, 50μM, 100μM and 250μM. The DMSO vehicle control of SNAP are insignificant and negated from this figure to better depict the effects of the drug. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

3.3. Different NOS isoforms affect the nebivolol-induced regulation of IL-17A and IFNγ cytokines

The inhibitory effects of nebivolol are similar to those of high exogenous NO dosage. Therefore, to investigate whether the effects of nebivolol are mediated by NO induction, I evaluated the NO-inducing ability of nebivolol. First, I evaluated the effects of NOS enzymes on activated PBMCs as well as on nebivolol-treated PBMCs by using two NOS inhibitors: L-NMMA which is a general inhibitor of all NOS isoforms and 1400W which is a selective NOS2 inhibitor. The use of different inhibitors could help to understand whether other isoforms of NOS are involved in Th regulation.

The treatment of activated PBMCs with L-NMMA resulted in a reduction of 18% in the levels of IL-17A cytokine as compared to positive control of activated PBMCs (P=0.0003) (Figure 9). The L-NMMA-induced reduction of IL-17A cytokine levels was variable across individual subjects. While the level of IL-17A was not changed in LCR289 following the L-NMMA treatment of activated PBMCs (Figure 10 A), a reduction of 18% (P=0.0362) in LCR293 (Figure 10 B), and 23% (P=0.0052) in LCR294 (Figure 10 C) was observed. Upon supplementing nebivolol-treated PBMCs with L-NMMA, further inhibition of 11% was observed in IL-17A cytokine level in the pooled averaged data (P=0.0279) (Figure 9). This effect was consistent with the individual data of LCR293 and LCR294 where a reduction of 12% (P=0.0264) (Figure 10 B) and 10% (P=0.0314) (Figure 10 C) was observed in comparison with nebivolol-treated PBMCs. However, treating nebivolol-treated PBMCs with L-NMMA did not change the levels of IL-17A in LCR289 (Figure 10 A). This data suggests that blocking NO production does not counteract the nebivolol-induced inhibition of IL-17A cytokine and therefore NO cannot explain the nebivolol's effects on IL-17A cytokine.

The IFNγ cytokine response of LCR289 was excluded because the DMSO vehicle control of nebivolol added to L-NMMA-treated cells resulted in a significant change of 10.95% (P=0.0158) of IFNγ cytokine levels as compared to PBMCs treated with L-NMMA only (Figure S 1) which impaired our ability to deduce the effects of L-NMMA on PBMCs treated with nebivolol. The two participants, LCR293 and LCR294, whose cells

were treated with L-NMMA, had contrasting responses in terms of IFNγ cytokine level which made the presentation of pooled data uninformative as mentioned above. Treating activated PBMCs with L-NMMA led to a trend of decrease in IFNγ cytokine levels by 28% in LCR293 (Figure 11 A) whereas no change was observed in IFNγ levels in LCR294 (Figure 11 B). Similarly. treating nebivolol-treated activated PBMCs with L-NMMA led to varying effects on individual subjects. The levels of IFNγ cytokine following the treatment of nebivolol-treated PBMCs with L-NMMA were unchanged in LCR293 and increased by 44% in LCR294 (P=0.0024) (Figure 11 A, B). In conclusion, due to the small sample size and the varying observations regarding the IFNγ response, I could not draw a conclusion about the outcome of blocking all NOS isoforms. Lastly, the nebivolol vehicle control of DMSO did not change the levels of IL-17A and IFNγ cytokines which validates that the effect of L-NMMA with nebivolol were due to the drugs only.

Although the use of a general NOS inhibitor such as L-NMMA helps to understand the role of NO on Th cells and adrenergic signalling, a better understanding of the specific contribution of NOS2 isoform was needed. NOS2 enzyme is the only isoform that leads to the synthesis of large amounts of NO as compared with other isoforms. Given that nebivolol leads to an inhibition of IL-17A and IFNγ cytokines that is comparable with the inhibition induced by high doses of exogenous NO, NOS2 activity could explain the suggested NO-mediated immunoregulation of nebivolol. Because the IL-17A cytokine response of PBMCs treated with 1400W was opposing in different subjects, I did not aggregate the data from different participants to better depict the effects of 1400W in individual subjects. LCR294 was excluded from this analysis because the DMSO vehicle control of 1400W resulted in a significant change in IL-17A levels as compared to the positive control of activated PBMCs (P=0.0002) (Figure S **2**). In LCR289, the levels of IL-17A cytokine were unchanged following the treatment of activated PBMCs with 1400W (Figure 12 A) as well as following the 1400W treatment of nebivolol-treated PBMCs. Contrarily, in LCR293, 1400W led to an increase of 35.37% (P=0.0047) in IL-17A cytokine (Figure 12 B),

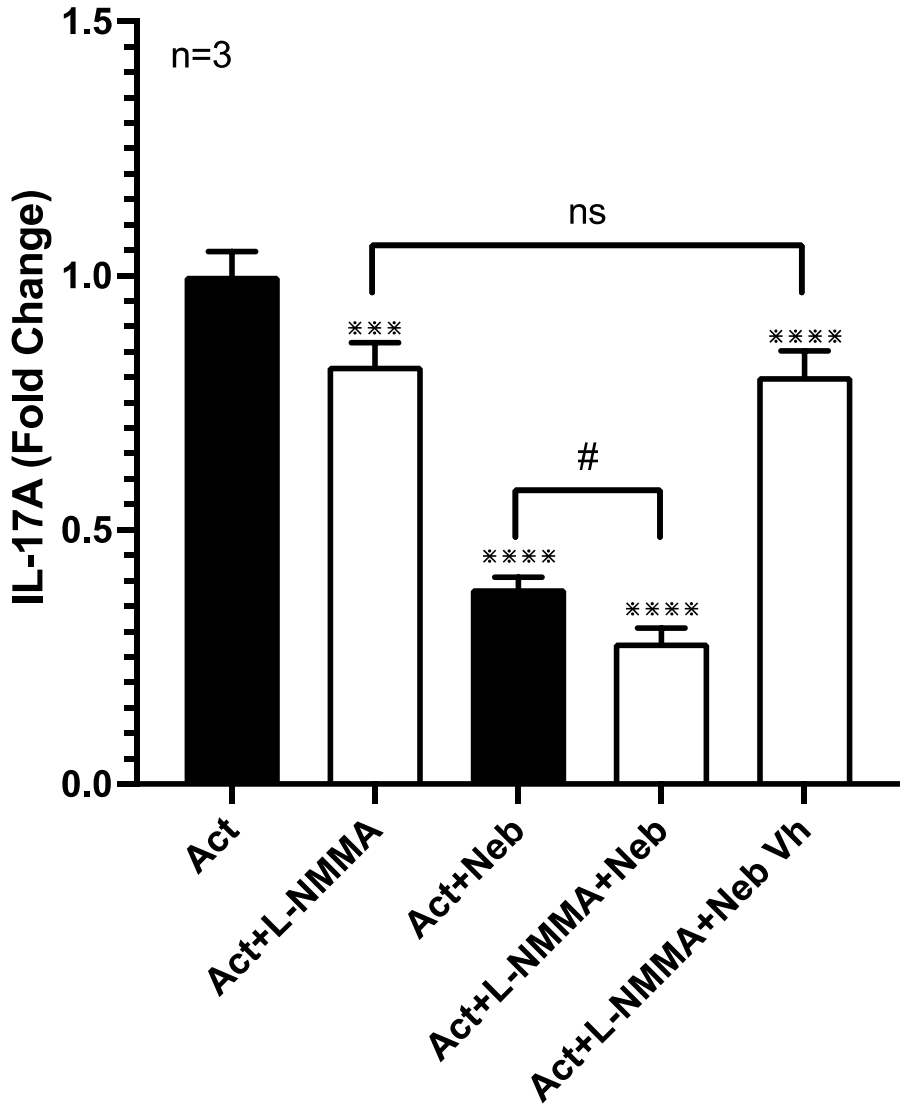

Figure 9: The fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include Act: positive control, Act+L-NMMA: activated PBMCs supplemented with 500μM of NOS inhibitor N^G-Monomethyl-L-arginine acetate salt (L-NMMA), Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+L-NMMA+Neb: activated PBMCs treated with 500μM of L-NMMA and 10μM of nebivolol, and Act+L-NMMA+Neb Vh: activated PBMCs treated with 500μM of L-NMMA and the DMSO vehicle control of nebivolol. The significance was calculated using the pooled data from all subjects (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎/#), 0.0021 (⁎⁎/##), 0.0002 (⁎⁎⁎/###), <0.0001 (⁎⁎⁎⁎/#####).

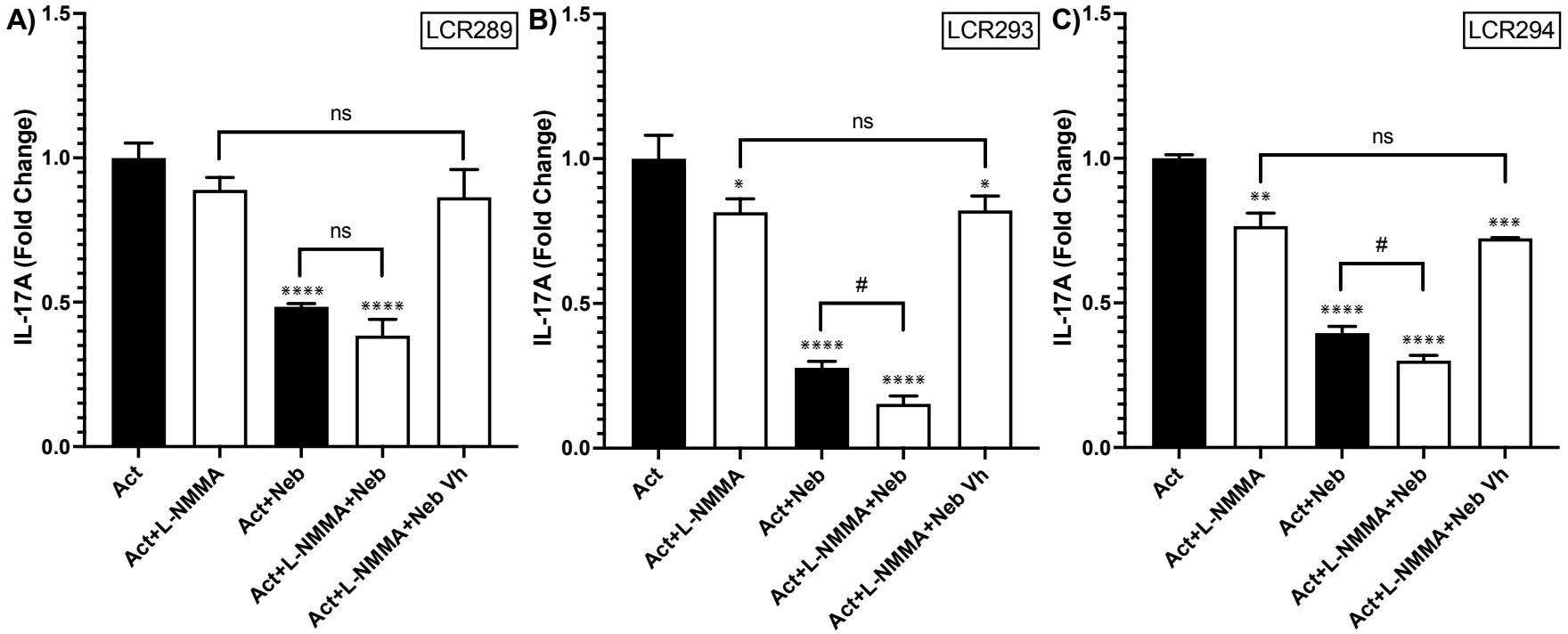

Figure 10: The individual data fold change from Figure 9, of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, Act+L-NMMA: activated PBMCs supplemented with 500μM of NOS inhibitor N^G-Monomethyl-L-arginine acetate salt (L-NMMA), Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+L-NMMA+Neb: activated PBMCs treated with 500μM of L-NMMA and 10μM of nebivolol, and Act+L-NMMA+Neb Vh: activated PBMCs treated with 500μM of L-NMMA and the DMSO vehicle control of nebivolol. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎/#), 0.0021 (⁎⁎/##), 0.0002 (⁎⁎⁎/###), <0.0001 (⁎⁎⁎⁎/####).

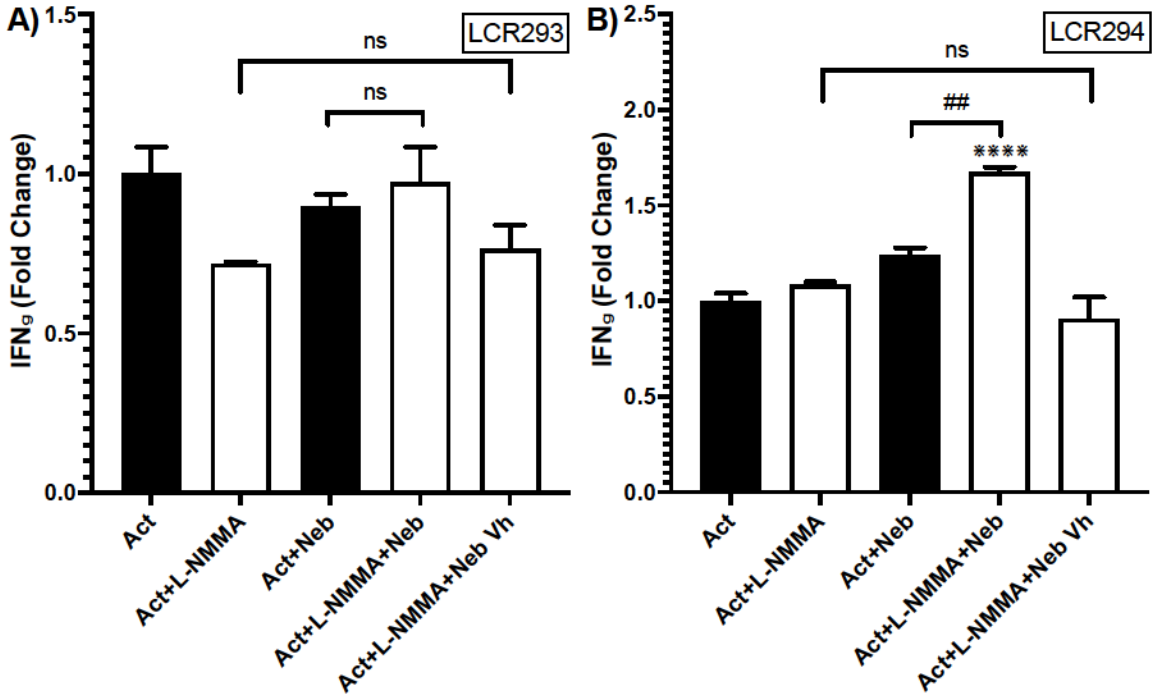

Figure 11: The individual data fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, Act+L-NMMA: activated PBMCs supplemented with 500μM of NOS inhibitor N^G-Monomethyl-L-arginine acetate salt (L-NMMA), Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+L-NMMA+Neb: activated PBMCs treated with 500μM of L-NMMA and 10μM of nebivolol, and Act+L-NMMA+Neb Vh: activated PBMCs treated with 500μM of L-NMMA and the DMSO vehicle control of nebivolol. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*/#), 0.0021 (**/##), 0.0002 (***/###), <0.0001 (****/#####).

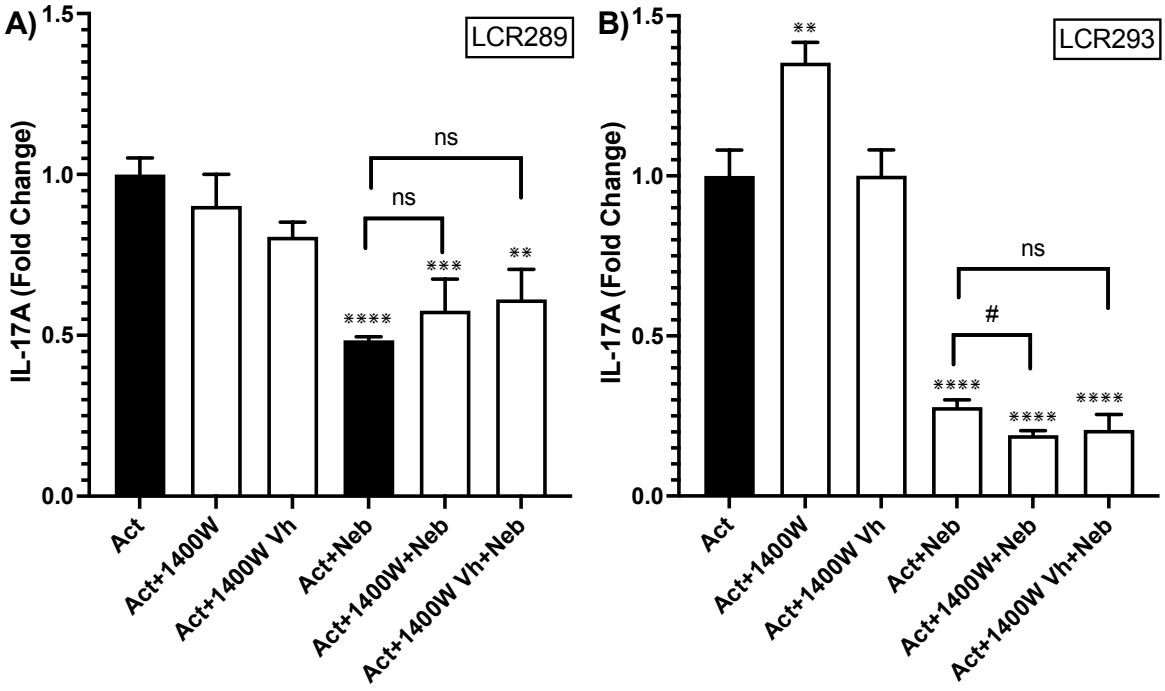

Figure 12: The individual data fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, Act+1400W: activated PBMCs supplemented with 0.2μM of NOS2 inhibitor 1400W dihydrochloride (1400W), Act+1400W Vh: activated PBMCs treated with the DMSO vehicle control of 1400W. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+1400W+Neb: activated PBMCs treated with 0.2μM of 1400W and 10μM of nebivolol, and Act+1400W Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of 1400W. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎/#), 0.0021 (⁎⁎/##), 0.0002 (⁎⁎⁎/###), <0.0001 (⁎⁎⁎⁎/#####).

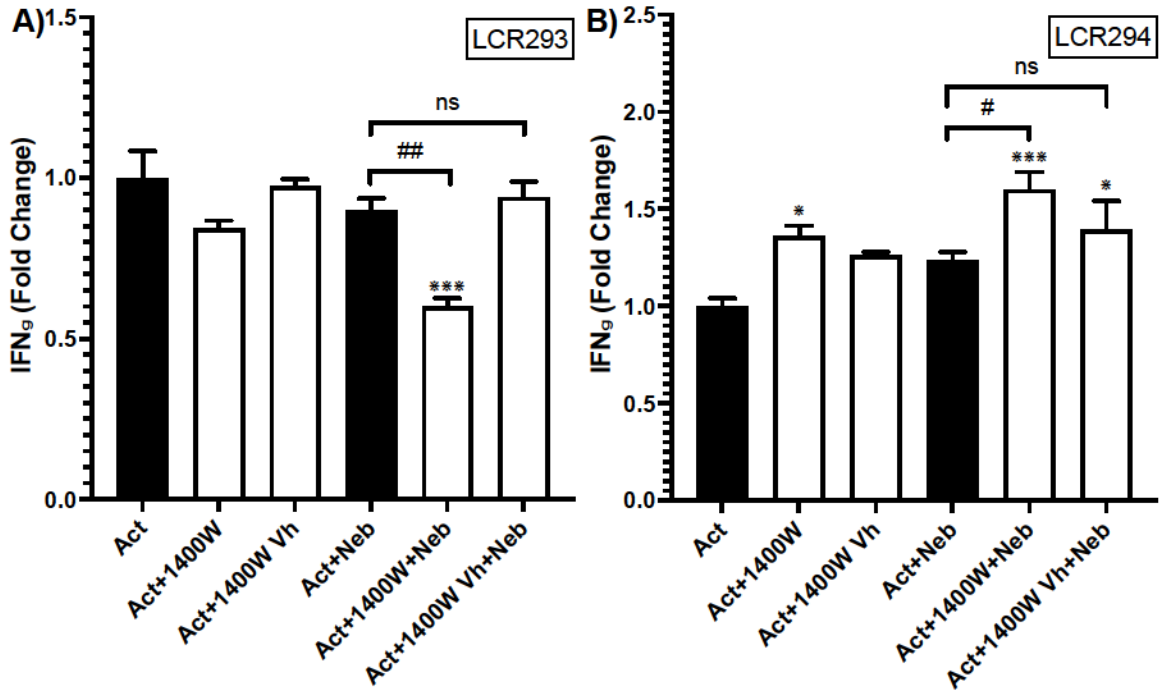

Figure 13: The individual data fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, Act+1400W: activated PBMCs supplemented with 0.2μM of NOS2 inhibitor 1400W dihydrochloride (1400W), Act+1400W Vh: activated PBMCs treated with the DMSO vehicle control of 1400W. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+1400W+Neb: activated PBMCs treated with 0.2μM of 1400W and 10μM of nebivolol, and Act+1400W Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of 1400W. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*/#), 0.0021 (**/##), 0.0002 (***/###), <0.0001 (****/#####).

whereas the added 1400W to nebivolol-treated PBMCs resulted in a decrease of 8.76% ($P=0.0378<0.05$) in IL-17A cytokine level. For LCR289 and LCR293, the DMSO vehicle control of 1400W and nebivolol did not cause a significant change in IL-17A cytokine levels (Figure 12 A, B) which relates the changes seen previously are the due to the drugs only. Due to the small sample size and varied IL-17A response to 1400W, I could now draw a conclusion regarding the role of NOS2 enzyme in IL-17A regulation.

For the IFNγ cytokine response to 1400W, I observed varied responses of different subjects. LCR289 was excluded because the DMSO vehicle control of 1400W led to a significant change in IFNγ cytokine level ($P=0.0298$) as compared to the positive control of activated PBMCs (Figure S 3). The contrasting responses of LCR293 and LCR294 necessitated the presentation of figures from individual subjects. While the treatment with 1400W did not change the IFNγ cytokine levels in LCR293 (Figure 13 A), LCR294 had an increase in IFNγ cytokine level of 36.39% ($P=0.0328<0.05$) (Figure 13 B). Similarly, when adding 1400W to nebivolol-treated PBMCs, the IFNγ cytokine levels dropped by 29.79% ($P=0.0048<0.05$) in LCR293 (Figure 13 A) but increased by 36.50% ($P=0.0377<0.05$) in LCR294 (Figure 13 B). The vehicle DMSO control of 1400W and nebivolol for LCR293 and LCR294 did not result in a significant change in IFNγ cytokine levels (Figure 13 A, B) which means that results discussed above are specific to the drugs used. Nonetheless, the varied IFNγ response to 1400W prevented me from drawing a conclusion regarding the role of NOS2 enzyme in IFNγ regulation. In conclusion, the data resulting from blocking NOS isoforms does not support my hypothesis of NO-mediated regulation of Th cells by nebivolol. Instead, it shows that the NO produced during adrenergic signaling is required to stabilize Th17 cells similarly to the findings of Obermajer et al. (Obermajer et al. 2013).

3.4. Nebivolol regulates IL-17A and IFNγ cytokine levels independently from the cGMP-cGK signalling pathway

To explore the role of the cGMP-cGK pathway in the nebivolol-induced effects in Th cells, an analogue and inhibitor of the cGMP pathway were used to treat activated PBMCs and nebivolol-treated activated PBMCs. The cGMP analogue, Br-cGMP, did not

change the IL-17A cytokine levels in activated PBMCs as compared to the positive control in the pooled data (Figure 14). This effect of Br-cGMP on IL-17A cytokine levels was consistent across all individual subjects where no change was observed in LCR289 (Figure 15 A), LCR293 (Figure 15 B), or LCR294 (Figure 15 C). Moreover, treating PBMCs with nebivolol and Br-cGMP did not change the levels of IL-17A cytokine levels as compared to nebivolol-treated PBMCs in the pooled data (Figure 14). Similarly for individual subjects, supplementing nebivolol-treated PBMCs with Br-cGMP did not change the levels of IL-17A in LCR289 (Figure 15 A), LCR293 (Figure 15 B) or LCR294 (Figure 15 C). Although the effects of Br-cGMP on IL-17A cytokine levels were similar increasing trends across all participants, none of these responses reached statistical significance which hinders my ability to state the role of cGMP signaling in IL-17A regulation.

Regarding the IFNγ cytokine response to Br-cGMP, we observed that different subjects had contrasting responses. While LCR289 and LCR293 had a decreased IFNγ cytokine levels of 30% and 35% ($P=0.0185<0.05$), respectively, upon treating their PBMCs with Br-cGMP as compared to the positive control of only activated PBMCs (Figure 16 A, B), the levels of IFNγ cytokine did not change for LCR294 (Figure 16 C). Moreover, adding Br-cGMP to nebivolol-treated PBMCs did not change the levels of IFNγ in LCR289, LCR293 and LCR294 as compared to nebivolol-treated PBMCs (Figure 16 A, B, C). This data shows that cGMP signaling pathway does not explain the nebivolol-mediated regulation of IL-17A and IFNγ cytokine levels nor does it support the involvement of NO in this regulation, contrarily to my hypothesis.

In terms of the IL-17A cytokine response to ODQ, two participants were excluded from the analysis because the DMSO vehicle control of ODQ resulted in a significant change in IL-17A cytokine levels in LCR289 ($P=0.0003$) (Figure S **4** A), and LCR294 ($P=0.0005$) (Figure S **4** B). Treating activated PBMCs from LCR293 with ODQ had no effect on levels of IL-17A as compared to the positive control of activated PBMCs . Similarly, adding ODQ to nebivolol-treated PBMCs did not change the levels of IL-17A cytokine as compared to activated PBMCs treated with nebivolol (Figure 17). This data

confirms that the cGMP signaling pathway does not explain IL-17A cytokine response to nebivolol.

For IFNγ response to ODQ, the different subjects included in the study had considerably different responses which necessitates the presentation of their individual data. The IFNγ cytokine response of LCR289 was excluded given that the vehicle control of ODQ caused a significant change in IFNγ cytokine levels (P=0.0456) (Figure S 5). When treating their PBMCs with ODQ, LCR293 was unaffected (Figure 18, A) whereas LCR294 had an increase of 124.88% (P=0.0004) (Figure 18, B), all compared to the positive control of activated PBMCs. Adding ODQ to nebivolol-treated PBMCs did not change the levels of IFNγ cytokine as compared to PBMCs treated with nebivolol only in LCR293 (Figure 18, A), and LCR294 (Figure 18, B).

3.5. The Gene Expression Results from the RT-qPCR

The RT-qPCR TaqMan gene expression assay is a novel technique in our lab which needed a long time of optimization and troubleshooting. Given the short amount of time, we were able to obtain gene expression results from 5 participants. Unfortunately, not all the data obtained was reliable. Therefore, we established a set of criteria for exclusion including: 1- a standard error of the mean higher than 25% for the positive control of activated PBMCs or the nebivolol-treated PBMCs, 2- a significant change of gene expression in the DMSO vehicle control of nebivolol as compared to the positive control. These criteria were in line with our ELISA criteria and other literature using gene expression assays.

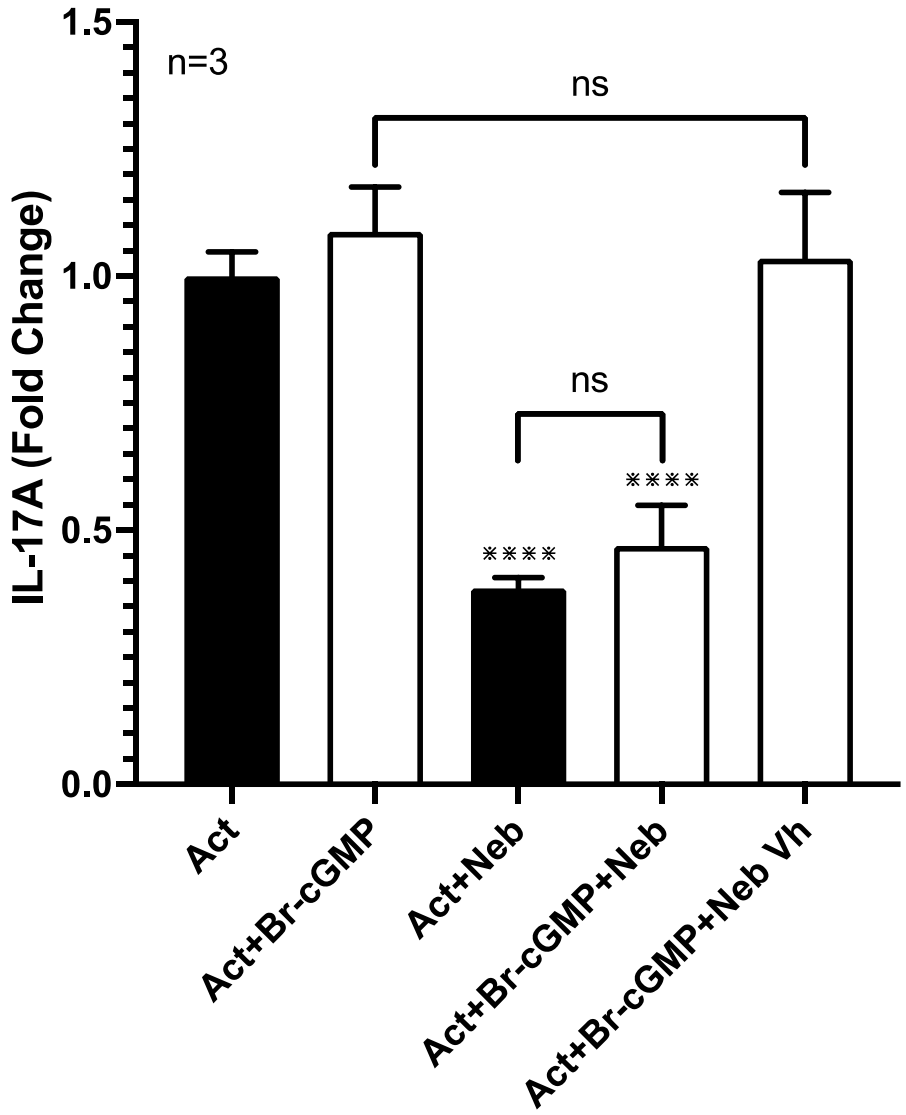

Figure 14: The fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include Act: positive control, Act+Br-cGMP: activated PBMCs supplemented with 100μM of 8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt (Br-cGMP), Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Br-cGMP+Neb: activated PBMCs treated with 100μM of Br-cGMP and 10μM of nebivolol, and Act+Br-cGMP+Neb Vh: activated PBMCs treated with 100μM of Br-cGMP and the DMSO vehicle control of nebivolol. The significance was calculated using the pooled data from all subjects (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

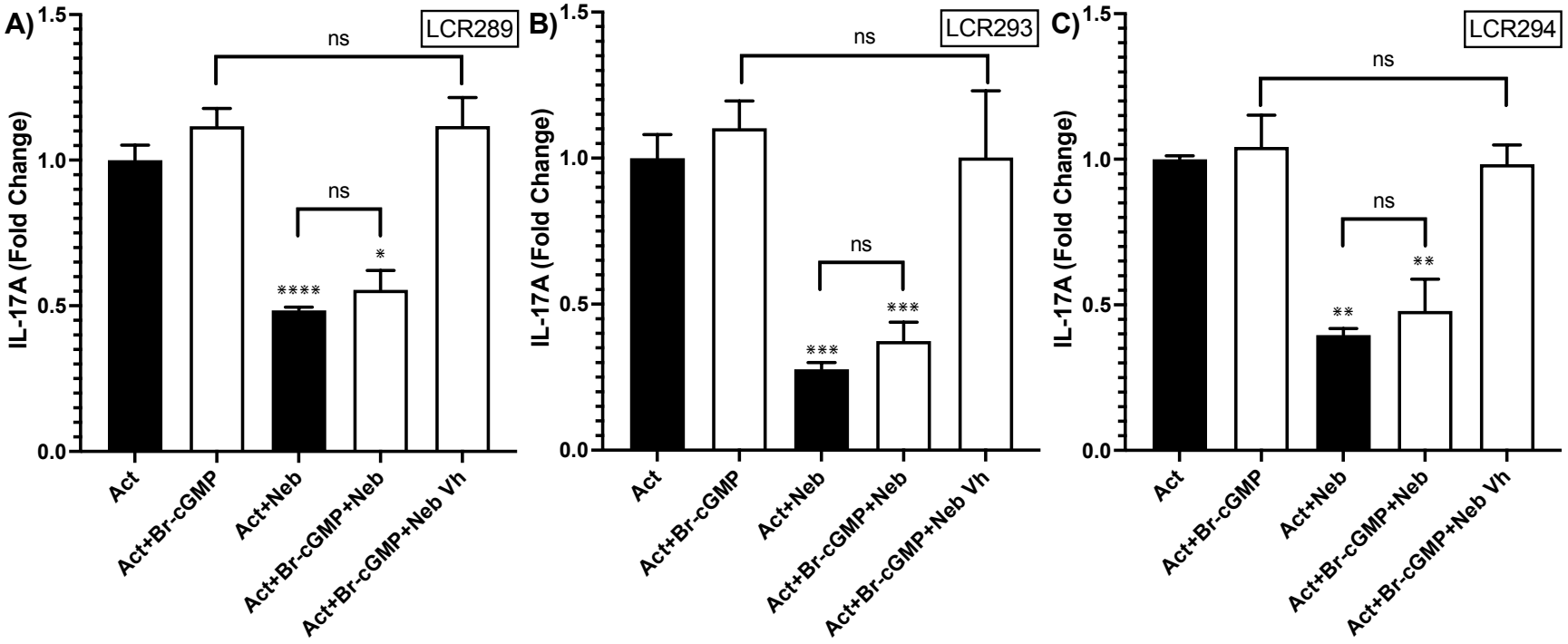

Figure 15: The individual data fold change from Figure 14, of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, Act+Br-cGMP: activated PBMCs supplemented with 100μM of 8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt (Br-cGMP), Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Br-cGMP+Neb: activated PBMCs treated with 100μM of Br-cGMP and 10μM of nebivolol, and Act+Br-cGMP+Neb Vh: activated PBMCs treated with 100μM of Br-cGMP and the DMSO vehicle control of nebivolol. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

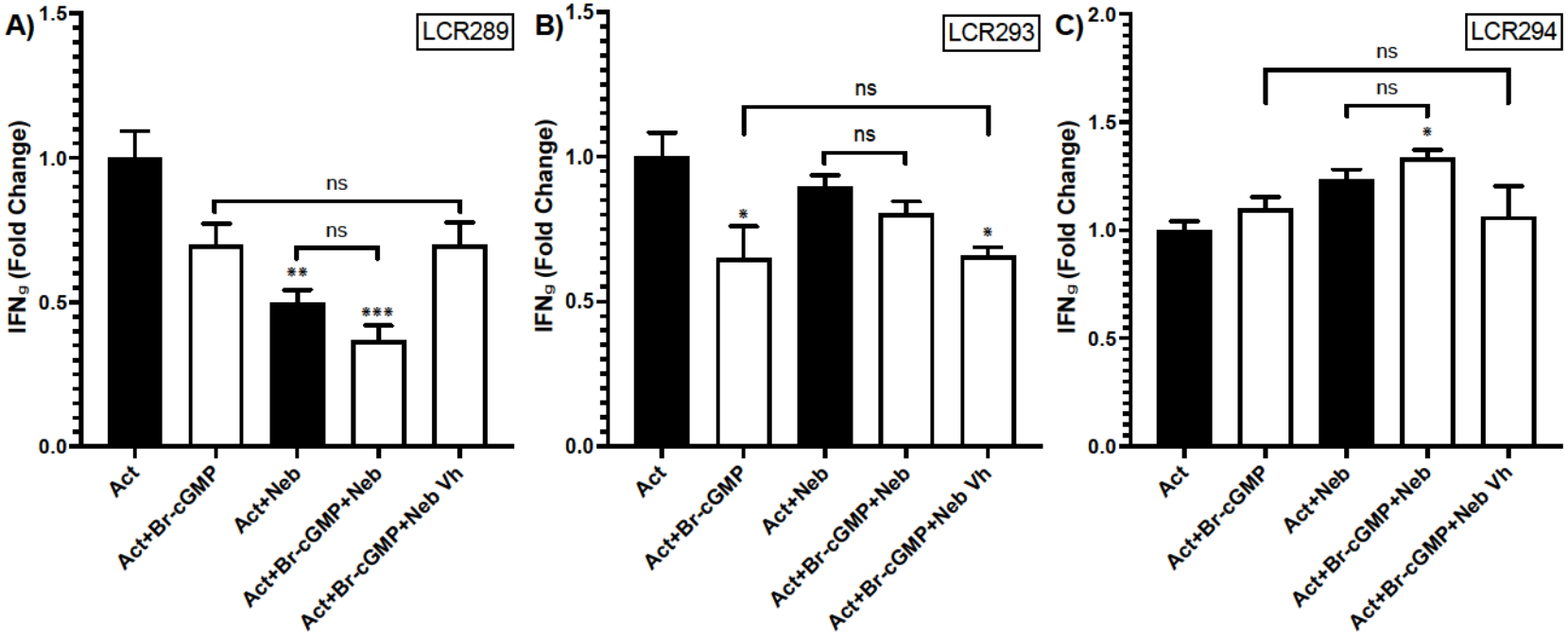

Figure 16*:* The individual data fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by an LCR code. The conditions include Act: positive control, Act+Br-cGMP: activated PBMCs supplemented with 100μM of the cGMP analogue 8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt (Br-cGMP), Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Br-cGMP+Neb: activated PBMCs treated with 100μM of Br-cGMP and 10μM of nebivolol, and Act+Br-cGMP+Neb Vh: activated PBMCs treated with 100μM of Br-cGMP and the DMSO vehicle control of nebivolol. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

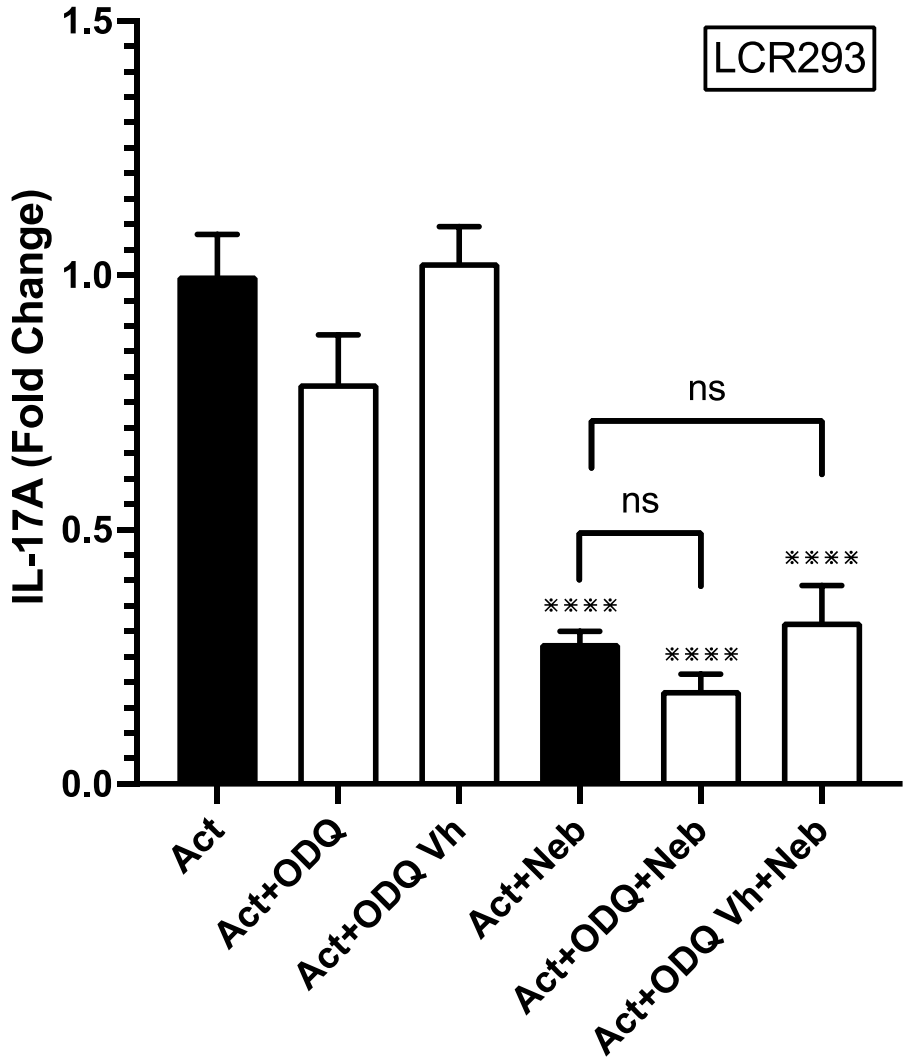

Figure 17: The individual data fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in an individual subject encoded by LCR293. The conditions include Act: positive control, Act+ODQ: activated PBMCs supplemented with 10μM of 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), Act+ODQ Vh: activated PBMCs treated with the DMSO vehicle control of ODQ. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+ODQ+Neb: activated PBMCs treated with 10μM of ODQ and 10μM of nebivolol, and Act+ODQ Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of ODQ. The significance was calculated using the data from the biological replicates of the subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

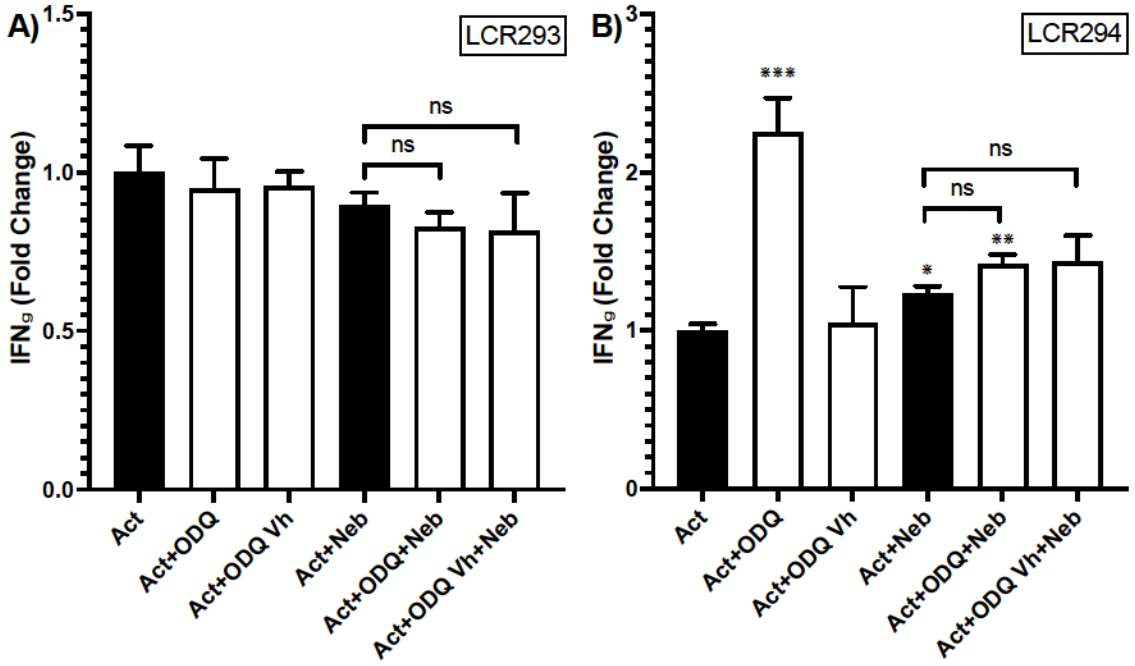

Figure 18*:* The individual data fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in an individual subjects encoded by LCR codes. The conditions include Act: positive control, Act+ODQ: activated PBMCs supplemented with 10μM of 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), Act+ODQ Vh: activated PBMCs treated with the DMSO vehicle control of ODQ. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+ODQ+Neb: activated PBMCs treated with 10μM of ODQ and 10μM of nebivolol, and Act+ODQ Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of ODQ. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

For the TBX2 gene expression, which is the major transcription factor of Th1, the experiment was performed on the cDNA isolated from four participants, LCR178, LCR288, LCR289 and LCR294. None of the results of these experiment made it to the final stages. The DMSO vehicle control of nebivolol in LCR178 resulted in significant decrease of TBX2 gene expression of 38.35% (P=0.0054<0.05) as compared to the positive control of activated PBMCs (Figure S 6 A). In LCR289, the SEM of the positive control and nebivolol-treated PBMCs reached 45.45% and 34.07%, respectively (Figure S 6 B). In LCR288, the SEM for the positive control condition was 40.39% and the SEM

for the DMSO vehicle control of nebivolol was 41.15%. Although the vehicle control of nebivolol caused 113.26% increase TBX2 gene expression, the large SEM values for the (Act) and (Act+Neb Vh) prevented the effect of the vehicle control from reaching statistical significance (Figure S 6 C). Finally, in LCR294, the SEM of the positive control and nebivolol-treated cells was 61.65% and 57.32%, respectively. Given these findings, the results of TBX2 gene expression for all subjects were excluded from the study.

The major transcription factor of Th17, Rorc, was also measured in the DNA isolated from five subjects' PBMCs. Only one subject's data, LCR253, was considered for not meeting any exclusion criterion mentioned above. In LCR253, treating activated PBMCs with nebivolol resulted in 35% in Rorc gene expression as compared to the positive control (Figure 19). The Rorc gene expression results for LCR178 were excluded because the vehicle control of nebivolol caused a significant decrease in gene expression of 56% (P=0.0004) (Figure S 7 A). LCR289 was also excluded because the positive control and nebivolol-treated PBMCs had a SEM of 57.96% and 26.04%, respectively (Figure S 7 B). Lastly, LCR288's positive control had SEM of 29.19% (Figure S 7 C), and LCR294 had a SEM of 46.03% for the positive control and 81.81% for the nebivolol condition (Figure S 7 D) which led to their exclusion from the study.

The NOS2 gene expression was measured in the cDNA of five subjects. Two subjects only, LCR178 and LCR253, were considered given that they did not meet any of the criteria mentioned above. Upon treatment with nebivolol, we observed a decrease in NOS2 gene expression by 49% (P=0.0246) in LCR178 (Figure 20 A), and by 1% in LCR253 (Figure 20 B) as compared to the positive control of activated PBMCs. In both subjects, the DMSO vehicle control of nebivolol did not cause a significant change in NOS2 gene expression (Figure 20 A, B). The excluded subjects included LCR289, LCR294, and LCR288 (Figure S 8 A-C). The positive control had a SEM of 67.58% in LCR289 (Figure S 8 A), 58% in LCR294 (Figure S 8 B), and 28% in LCR288 (Figure S 8 C). In addition, LCR294 and LCR288 had a SEM for the nebivolol-treated condition of 64% and 51% respectively (Figure S 8 B, C). Even though the nebivolol treatment in

LCR288 led to an increase of 268% ($P=0.0183<0.05$) of NOS2 gene expression and the SEM of the positive control and nebivolol conditions cannot undermine the effect seen, we opted for following our exclusion criteria and listing the figure of this subject under supplementary figures (Figure S 8 C). However, due to its importance, I used the findings from this subject's experiment in the discussion.

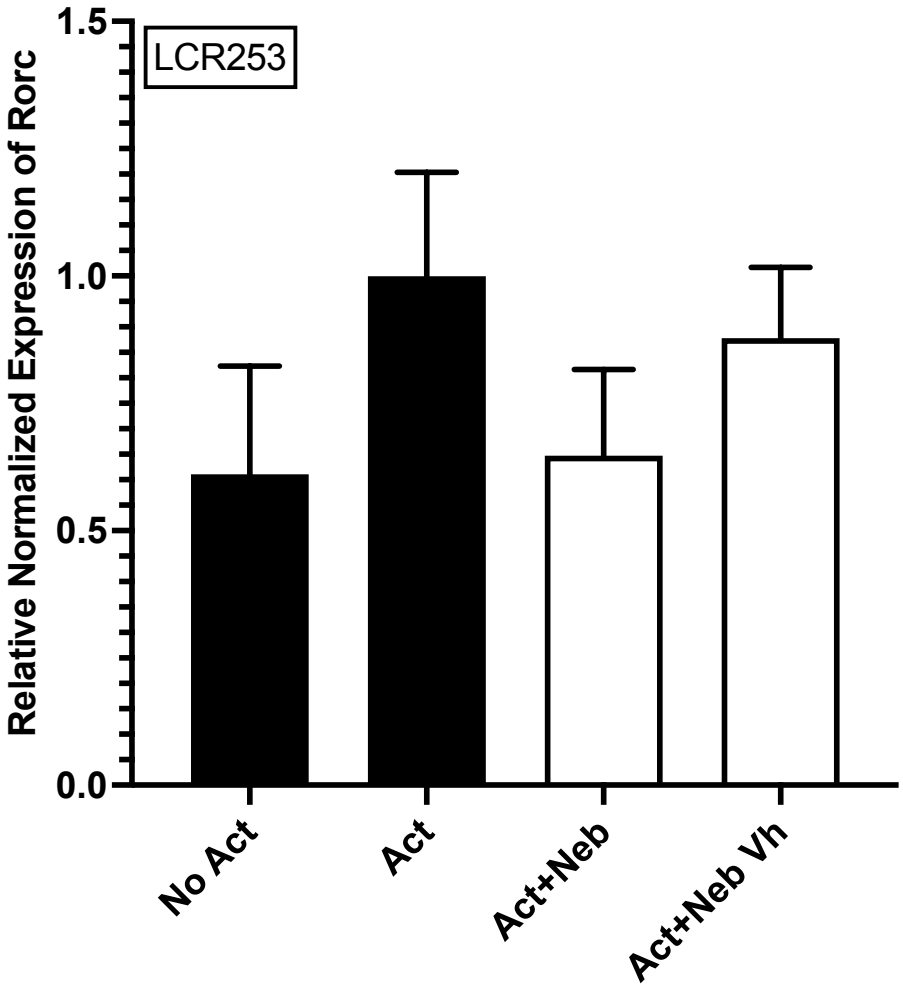

Figure 19: The gene expression of Rorc gene normalized by the expression of the reference gene HPRT1 as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the technical replicates (n=3) of the biological replicate (n=1) from every subject. P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

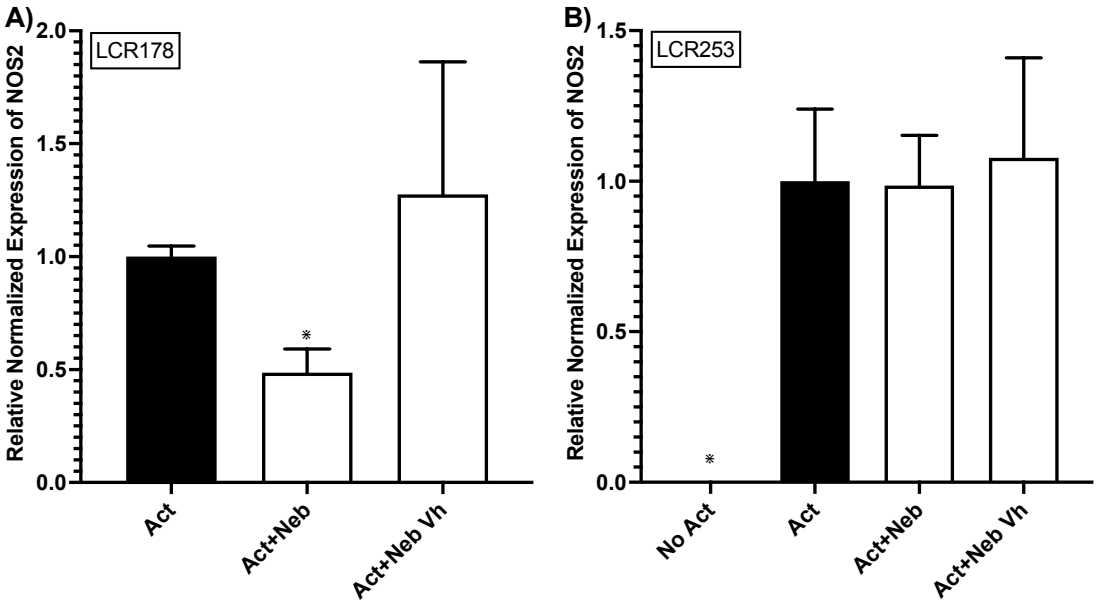

Figure 20: The gene expression of NOS2 gene normalized by the expression of the reference gene HPRT1 as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act). The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the technical replicates (n=3) of the biological replicate (n=1) from every subject. P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

CHAPTER IV: Discussion

Human PBMCs were adopted for this study because the inclusion of other cell types besides $CD4^+$ T cells in the cell culture is more relatable to the physiological state where multiple white blood cell types are present in the bloodstream. Since the study aims to evaluate the β2AR-mediated NO release in human cells, PBMCs will serve as a better model than isolated Th cells because they contain lymphocytes, monocytes, and dendritic cells which respond to NO (Kleiveland 2015; García-Ortiz and Serrador 2018) and β2-adrenergic agonists (Szelenyi et al. 2006; García-Ortiz and Serrador 2018; Gao et al. 2019) and mediate the modulation of Th cells. This will take our *in vitro* study a step forward to better elaborate the outcomes of adrenergic drugs and NO on human Th cells.

Nebivolol was found to significantly inhibit IL-17A cytokine production in all samples without reducing the levels of the master transcription factor of Th17 cells ROR-γt (**Figure 1**, **Figure 2**, **Figure 19**). In addition, the trypan counts performed on nebivolol-treated PBMCs showed that the cells viability was unaffected. This shows that nebivolol negatively regulates the function of Th17 cells in a mechanism independent from the transcriptional regulation. Interestingly, the biased agonism of nebivolol activates the GRK/β-arrestin pathway which desensitizes the cAMP-mediated signaling (Lefkowitz and Shenoy 2005). Previous findings show that the stimulatory effect of selective β2 AR agonists such as terbutaline were cAMP-mediated (Carvajal Gonczi et al. 2017). Given that the use of β2 AR blockers such as propranolol reduced the viability of Th17 cells (Pilipović et al. 2019), nebivolol provides a new tool to reduce the levels of IL-17A cytokines like a β2 AR blocker while maintaining the stability of Th17 cells through the induction of the antiapoptotic β-arrestin signaling. To further confirm this conclusion, a blockage of the β-arrestin pathway needs to be performed on nebivolol-treated PBMCs to reveal whether the nebivolol-mediated inhibition of Th17 is occurring through this pathway.

On the other hand, the effects of nebivolol on the IFNγ cytokine production showed an interindividual variability. While two subjects had a significant inhibition of IFNγ cytokine following the nebivolol treatment (**Figure 4** A, B), one subject had an increased IFNγ levels. (**Figure 4** D), and another subject was unaffected (**Figure 4** C). Despite the small sample size, the interindividual variability alludes to a potential role of the genetic background specific to each subject since all other experimental variables were fixed. In a previous study I worked on, a large sample size confirmed that nebivolol significantly inhibited IFNγ (**Figure S 9**). The nebivolol-mediated inhibition of IFNγ can be explained with the desensitization of the cAMP pathway through the β-arrestin signaling. However, the contrasting results of the other subjects shows that there are other confounding variables contributing to the nebivolol-mediated regulation of Th1 cells. In conclusion, in samples of human PBMCs, the nebivolol-mediated β2 AR biased agonism has inhibitory effects on Th17 cells and variable or suppressive effects on Th1 cells.

Another question that remains to be answered is related to the involvement of the NO signaling pathway in nebivolol-mediated signaling, especially that the activation of ERK1/2 through β-arresting increases NO production (Erickson et al. 2013). The results of the gene expression experiments showed that the NOS2 gene expression was reduced following the nebivolol treatment in one subject (**Figure 20** A) and unaffected in another (**Figure 20** B). In contrast, a subject whose PBMCs had a significant decrease of both IL-17A and IFNγ cytokines following the nebivolol treatment showed a significant increase of approximately 2 folds in NOS2 gene expression (Figure S 8 C). Due to the differential results of NOS2 gene expression and the possibility of other NOS isoforms such as NOS3 involvement in nebivolol-mediated Th cell regulation, I explored the role of NO using compounds inhibiting NOS isoforms or the cGMP signaling pathway.

Although the effects of NO donors on human PBMCs as well as in other cell types were examined in other studies (Korhonen et al. 2008; Obermajer et al. 2013). I needed to evaluate the effects of exogenous NO on human PBMCs in our experimental settings which will make our discussion of the NO-mediated regulation of Th cells more

consistent. The lowest dose of the NO donor SNAP (10μM) resulted in a slight insignificant increase in IFNγ and IL-17A cytokine levels (**Figure 5**,**Figure 6**, **Figure 7**,**Figure 8**). Moreover, an increasing inhibition of IFNγ and IL-17A cytokine was observed with higher NO donor doses suggesting a negative relationship between exogenous NO and IL-17A and IFNγ cytokine production (**Figure 5**,**Figure 6**, **Figure 7**,**Figure 8**). While these findings are in agreement with recent studies (Obermajer et al. 2013), they challenge the dogma that NO suppresses Th17 cell (Niedbala et al. 2011; Wheeler et al. 2013; Xue et al. 2018). The high exogenous NO is suggested to induce apoptotic oxidative damage and DNA breakage through the generation of ($ONOO^-$) (Lee et al. 2003; Mikkelsen and Wardman 2003; Ridnour et al. 2004). However, lower doses of exogenous NO could induce the c-GMP-PKG signaling pathway leading to enhanced IL-17A and IFNγ cytokine production. Although the induction of IL-17A and IFNγ cytokines was not evident in my data, a lower NO donor dosage could show a pronounced enhancement of these cytokines.

NO plays opposite roles during the nebivolol-mediated regulation of Th1 and Th17 cells. The nebivolol-mediated inhibition of IL-17A was exacerbated by blocking NOS2 (**Figure 12** B) as well as all NOS isoforms (**Figure 9**, **Figure 10** B, C). Thus, the NO released in samples of human PBMCs during adrenergic signaling induced the stability of Th17 cells. Although both inhibitors similarly reduced the level of IL-17A in nebivolol-treated PBMCs which suggests a main contribution of the NOS2 isoform, blocking all NOS isoforms reduced the levels of IL-17A significantly in activated PBMCs (**Figure 9**, **Figure 10** B, C) whereas, NOS2 blockage had variable outcomes from no change to induction of IL-17A (**Figure 12** A, B). While this confirms the stabilizing role of NO on Th17 cells, the NOS isoform responsible for NO production is still ambiguous because of the varied response to NOS2 inhibitor and the small sample size. Moreover, since high NO levels inhibit Th17 cells and reduce IL-17A cytokine levels, the NO levels released during adrenergic signaling are suggested to be low. Measuring the NO output would also be informative to know how much relative NO was released by the cells, this can be achieved with a Greiss assay or measuring nitrite and nitrates.

In contrast, the effects of NOS blockade on the IFNγ cytokine responses of nebivolol-treated cells were varied depending on the subject and its unique response to nebivolol. Unlike IL-17A cytokine response, treating PBMCs with NOS inhibitors had no effect on the IFNγ levels except for one subject that had an increased IFNγ cytokine following the inhibition of NOS2 (**Figure 11**, **Figure 13**). Furthermore, blocking all NOS isoforms resulted in no change in samples unaffected by nebivolol (**Figure 11** A), whereas the samples that had a nebivolol-mediated increase in IFNγ had a significant increase in IFNγ cytokine levels following NOS inhibition (**Figure 11** B). However, using NOS2 inhibitors on nebivolol-treated cells had different outcomes. In samples that were unaffected with nebivolol, NOS2 inhibition resulted in a significant reduction in IFNγ (**Figure 13** A), whereas samples that had higher IFNγ following nebivolol treatment experienced a significant increase in IFNγ (**Figure 13** B). This suggests that the NO produced by NOS2 enzyme had a bidirectional effect on Th1 cells depending on their response to adrenergic signaling. Despite the observed significance of NOS enzymes in nebivolol-mediated regulation of Th1 cells, the small sample size prevented me from drawing a conclusion regarding the exact role of NOS enzyme isoforms during adrenergic signaling.

To better understand the signaling pathway through which NO is modulating Th cells and their adrenergic signaling, I investigated the cGMP-cGK signaling pathway which is the predominant pathway for NO. The cGMP signaling was induced using 8-bromoguanosine 3′,5′-cyclic monophosphate (Br-cGMP) which is cell-permeable analogue of cGMP that activates cGMP-dependent protein kinase. This analogue is reported to be more resistant to hydrolysis than cGMP and to mimic NO donors in its effects (Zimmerman et al. 1985). Additionally, a partial blockage of the cGMP pathway was performed using ODQ which is a selective cell-permeable inhibitor of sGC which is responsive to NO. While it would have been optimal to use knockout mice to evaluate this pathway, inhibitors drugs can still reveal interesting trends even if they don’t reach statistical significance.

The induction of cGMP signaling did not change the levels of IL-17A cytokine in activated PBMCs as well as in nebivolol-treated PBMCs of all subjects (**Figure 14**, **Figure 15**). However, I observed a slight increase of IL-17A cytokine that did not reach statistical significance following the treatment with cGMP analogue in all subjects. This resembles the stimulatory effects on IL-17A cytokines that were observed with the lowest does of the NO donor SNAP (**Figure 5**) and suggests a stimulatory role of cGMP signaling in Th17 cells. Nonetheless, a higher dose of cGMP analogue needs to be used in order to validate this conclusion. Accordingly, blocking cGMP signaling did not change the levels of IL-17A cytokine in activated PBMCs as well as in nebivolol-treated PBMCs (**Figure 17**). In addition, the cGMP inhibition led to a slight decrease of IL-17A cytokine that did not reach statistical significance which is in accordance with the findings obtained by inducing cGMP signaling (**Figure 17**).

The induction of cGMP signaling did not change the levels of IFNγ cytokine levels in nebivolol treated PBMCs (**Figure 16**). However, it reduced the level of IFNγ cytokine in the activated PBMCs of one subject only (**Figure 16** B). cGMP inhibition did not affect the level of IFNγ cytokine in nebivolol-treated PBMCs (**Figure 18** A, B) and increased the level IFNγ cytokine in the activated PBMCs of one subject only (**Figure 18** B). Although these findings show that the cGMP signaling inhibits Th1 cells, a larger sample size can help to confirm this conclusion. Nonetheless, based on my findings and the observed trends, I suggest that there is a reciprocal modulation of Th1 and Th17 cells by the cGMP signaling pathway. This means that the cGMP signaling pathway induces Th17 cells yet inhibits Th1 cells. Interestingly, if this modulation was shown to be evident in a larger sample size with varying doses of cGMP analogue and inhibitor, the cGMP signaling pathway can be recruited to finetune Th cell subsets during autoimmune diseases characterized Th1 or Th17 aberrant responses.

Notably, ODQ blocks the generation of cGMP without inactivating the NO present in the samples which does not impair NO from inducing alternative signaling. Therefore, the insignificant findings of this thesis following the use of cGMP inhibitor and analogue allude to an alternative pathway induced by NO. The findings of this thesis, shows that

NO is produced samples of PBMCs during adrenergic signaling, and the produced NO signals in cGMP-independent pathway. Moreover, the nebivolol-mediated modulation of Th1 and Th17 cells is occurring through a mechanism independent from NO. However, NO is produced at low levels during biased adrenergic signaling and acts on Th cells to stabilize or inhibit them.

Finally, it is noteworthy that most studies discussing the NO-mediated regulation of Th cells were performed in murine cells such as mice, which nictitates more studies in human samples. The contribution of NOS isoforms as well as the roles of NO released are different between humans and mice suggesting that the constitutive eNOS in humans is the isoform responsible for the NO functions observed with iNOS in mice (Reiling et al. 1996; Nagy et al. 2003; Ibiza et al. 2006; García-Ortiz and Serrador 2018). This thesis examined a small sample size which might be insufficient to draw enough conclusions regarding the NO-mediated regulation of Th cells by adrenergic signaling. Therefore, I recommend examining the gene expression of eNOS and considering a larger sample size in future studies.

In conclusion, the biased β2 AR agonist, nebivolol, inhibits the production of the proinflammatory cytokine IL-17 in all samples of human PBMCs whereas it inhibits IFNγ cytokine release in most samples. While this thesis does not confirm a direct relationship between NO release and the biased adrenergic agonism, it shows that the level of NO released during adrenergic signaling is minimal and it helps to stabilize Th1 and Th17 cells. Therefore, nebivolol can be explored further to be used as an immunomodulatory therapeutic to mitigate the symptoms of MS and other T cell-mediated autoimmune diseases which are characterized by high IL-17A and IFNγ cytokine levels (van den Berg and McInnes 2013; Patel et al. 2013; Burkett and Kuchroo 2016).

Appendix A

Supplementary Figures

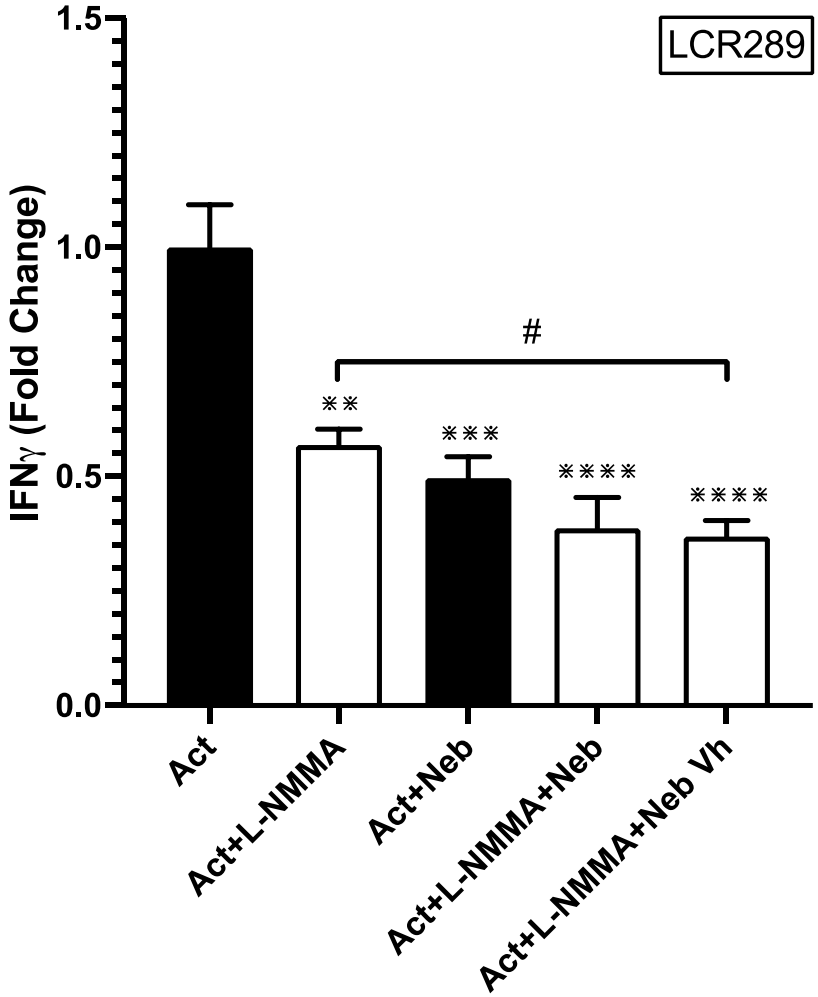

Figure S 1: The fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in an individual subject encoded by LCR289. The conditions include Act: positive control, Act+L-NMMA: activated PBMCs supplemented with 500μM of NOS inhibitor N^G-Monomethyl-L-arginine acetate salt (L-NMMA), Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+L-NMMA+Neb: activated PBMCs treated with 500μM of L-NMMA and 10μM of nebivolol, and Act+L-NMMA+Neb Vh: activated PBMCs treated with 500μM of L-NMMA and the DMSO vehicle control of nebivolol. The significance was calculated using the data from the biological replicates of the subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*/#), 0.0021 (**/##), 0.0002 (***/###), <0.0001 (****/#####).

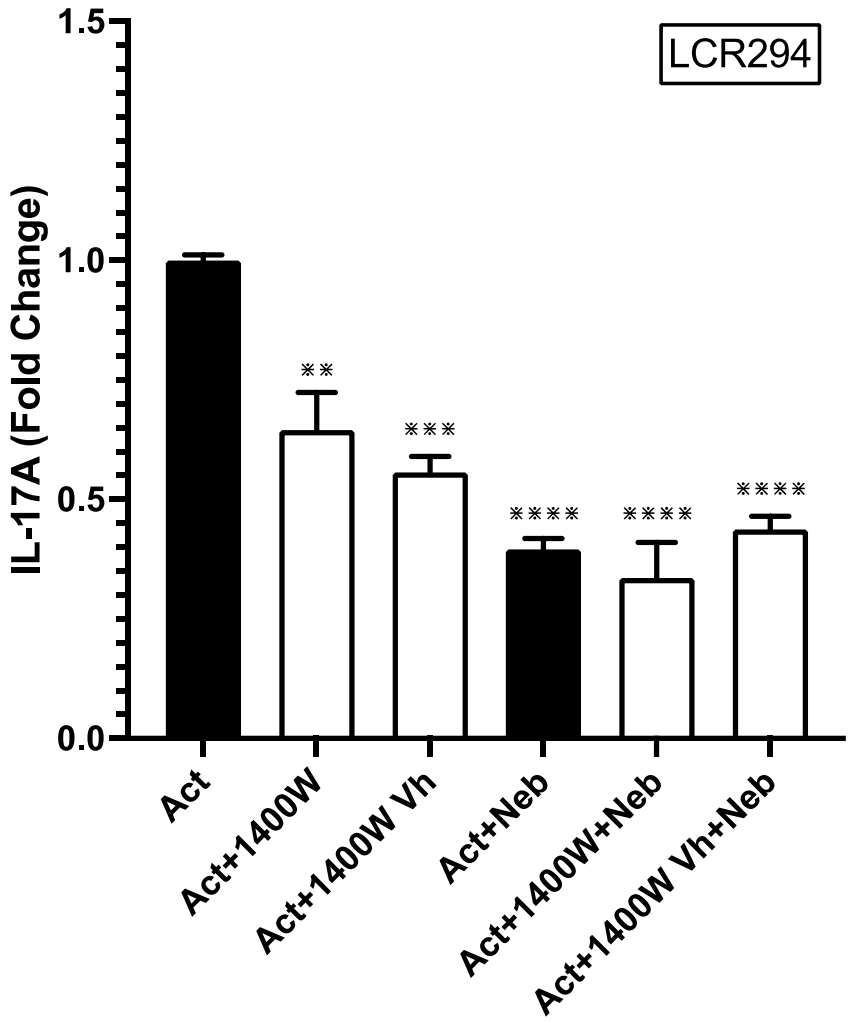

Figure S 2: The fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in an individual subject encoded by LCR294. The conditions include Act: positive control, Act+1400W: activated PBMCs supplemented with 0.2μM of NOS2 inhibitor 1400W dihydrochloride (1400W), Act+1400W Vh: activated PBMCs treated with the DMSO vehicle control of 1400W. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+1400W+Neb: activated PBMCs treated with 0.2μM of 1400W and 10μM of nebivolol, and Act+1400W Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of 1400W. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

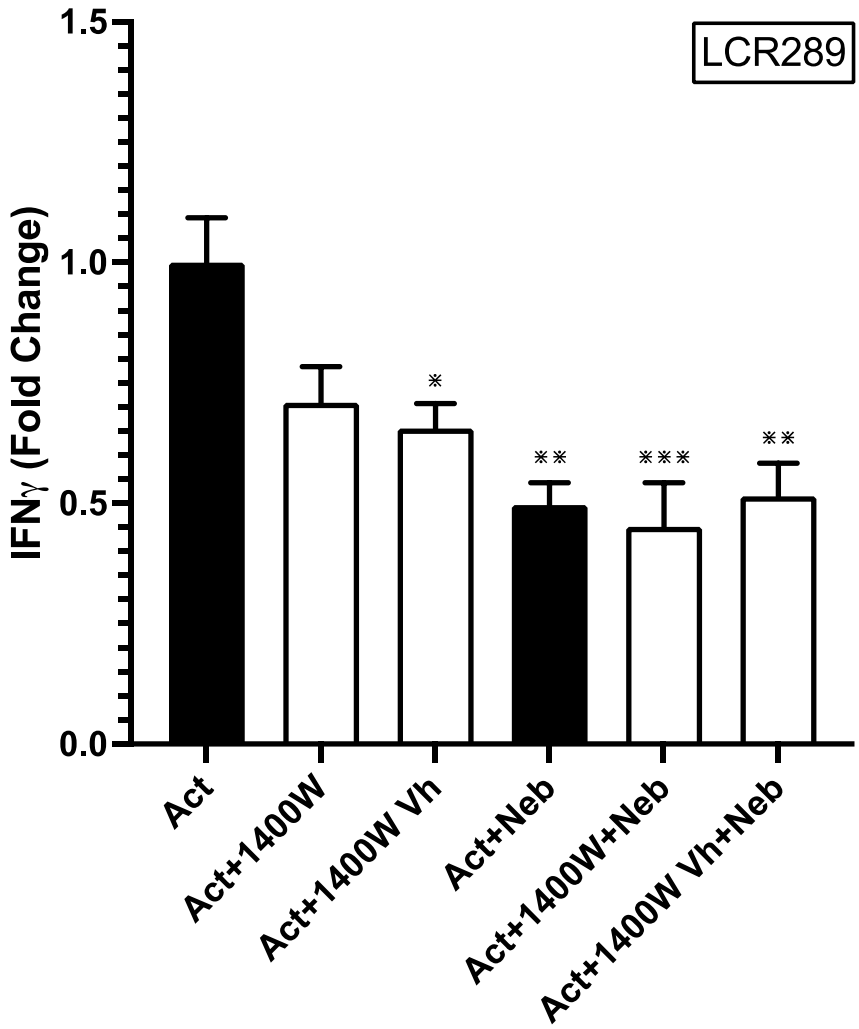

Figure S 3: The fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in an individual subject encoded by LCR289. The conditions include Act: positive control, Act+1400W: activated PBMCs supplemented with 0.2μM of NOS2 inhibitor 1400W dihydrochloride (1400W), Act+1400W Vh: activated PBMCs treated with the DMSO vehicle control of 1400W. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+1400W+Neb: activated PBMCs treated with 0.2μM of 1400W and 10μM of nebivolol, and Act+1400W Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of 1400W. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

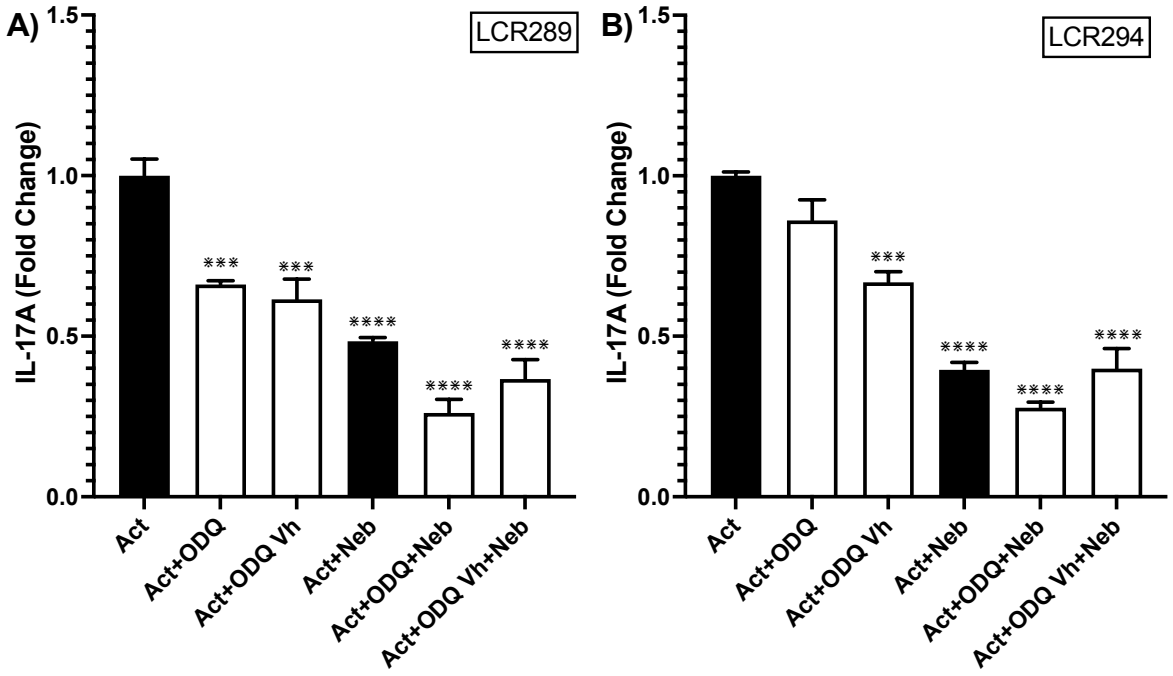

Figure S 4*:* The fold change of IL-17A cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by LCR codes. The conditions include Act: positive control, Act+ODQ: activated PBMCs supplemented with 10μM of 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), Act+ODQ Vh: activated PBMCs treated with the DMSO vehicle control of ODQ. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+ODQ+Neb: activated PBMCs treated with 10μM of ODQ and 10μM of nebivolol, and Act+ODQ Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of ODQ. The significance was calculated using the data from the biological replicates of the subject (n=3). P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

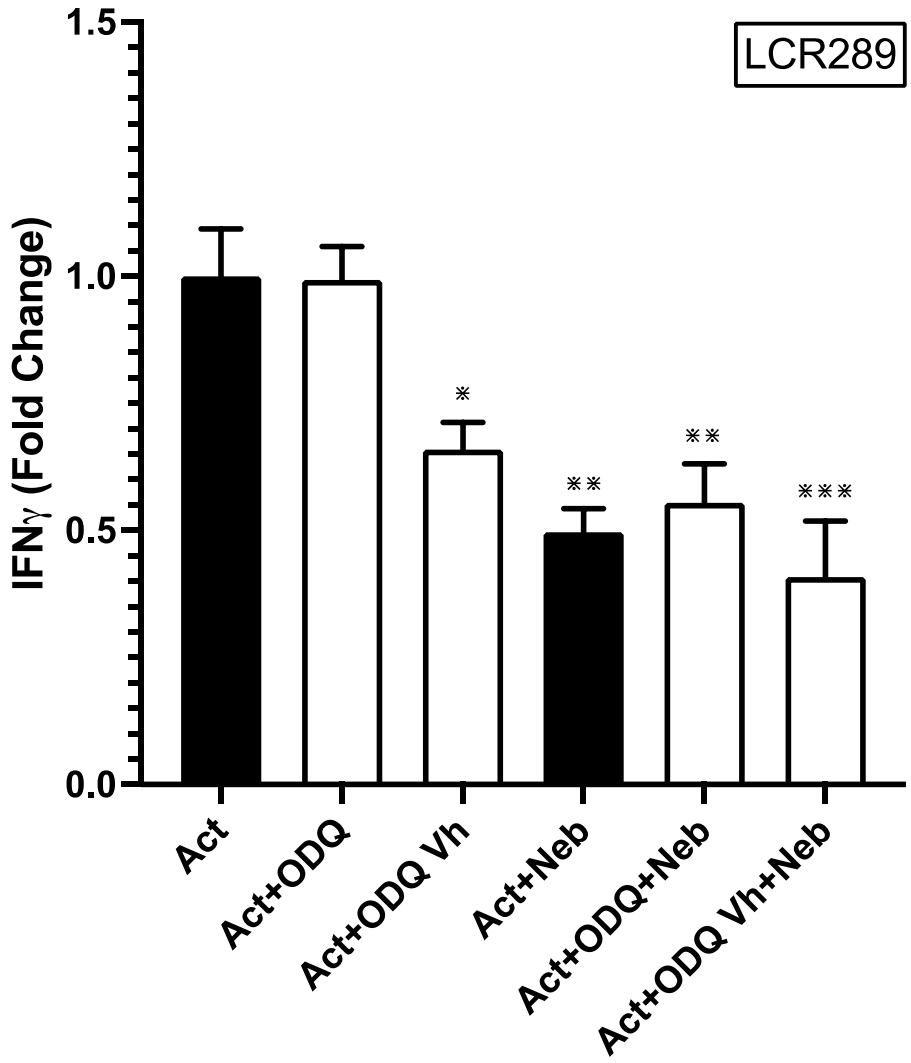

Figure S 5*:* The fold change of IFNγ cytokine concentration as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in an individual subject encoded by LCR289. The conditions include Act: positive control, Act+ODQ: activated PBMCs supplemented with 10μM of 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), Act+ODQ Vh: activated PBMCs treated with the DMSO vehicle control of ODQ. Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+ODQ+Neb: activated PBMCs treated with 10μM of ODQ and 10μM of nebivolol, and Act+ODQ Vh+Neb: activated PBMCs treated with 10μM of nebivolol and the DMSO vehicle control of ODQ. The significance was calculated using the data from the biological replicates of every subject (n=3). P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

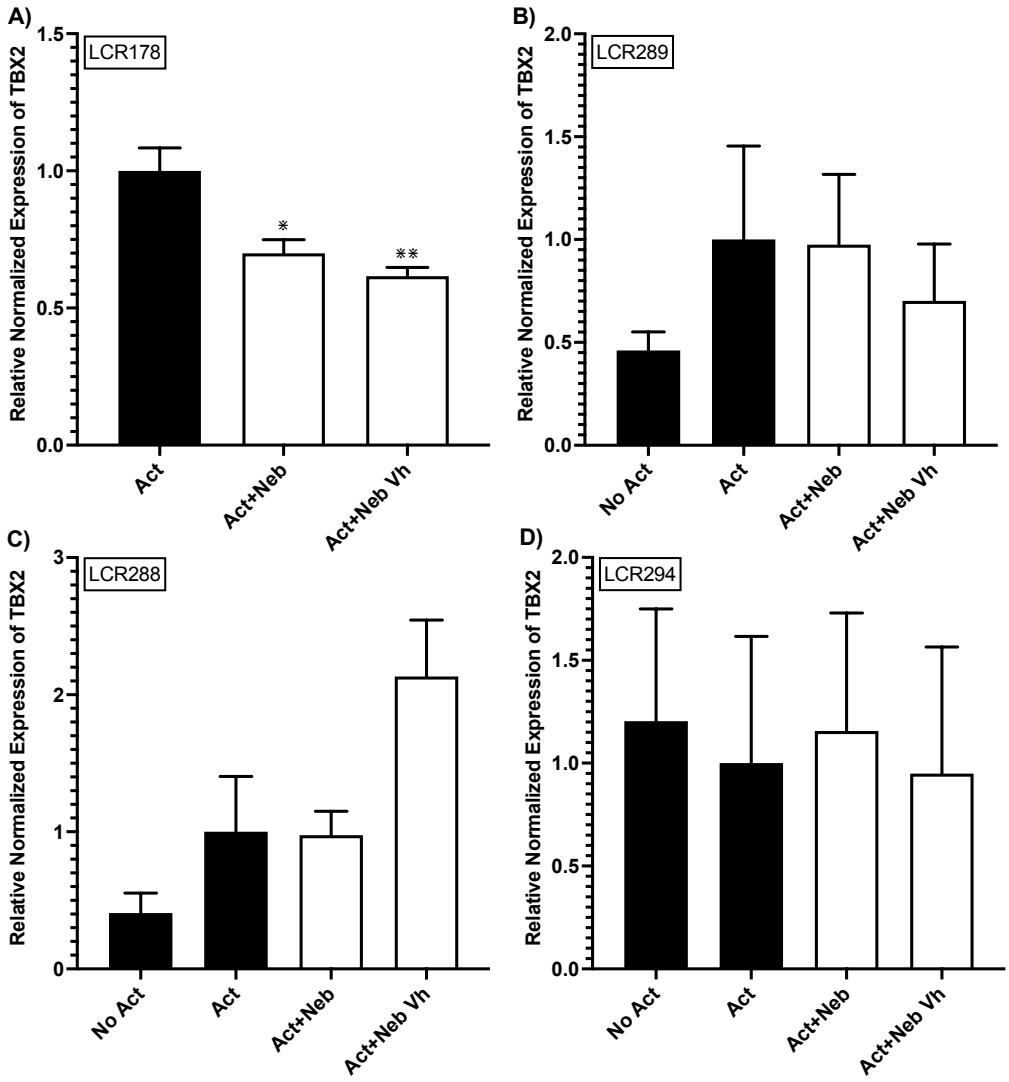

Figure S 6: The gene expression of TBX2 gene normalized by the expression of the reference gene HPRT1 as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by LCR codes. The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the technical replicates (n=3) of the biological replicate (n=1) from every subject. P≥ 0.05 (no symbol), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

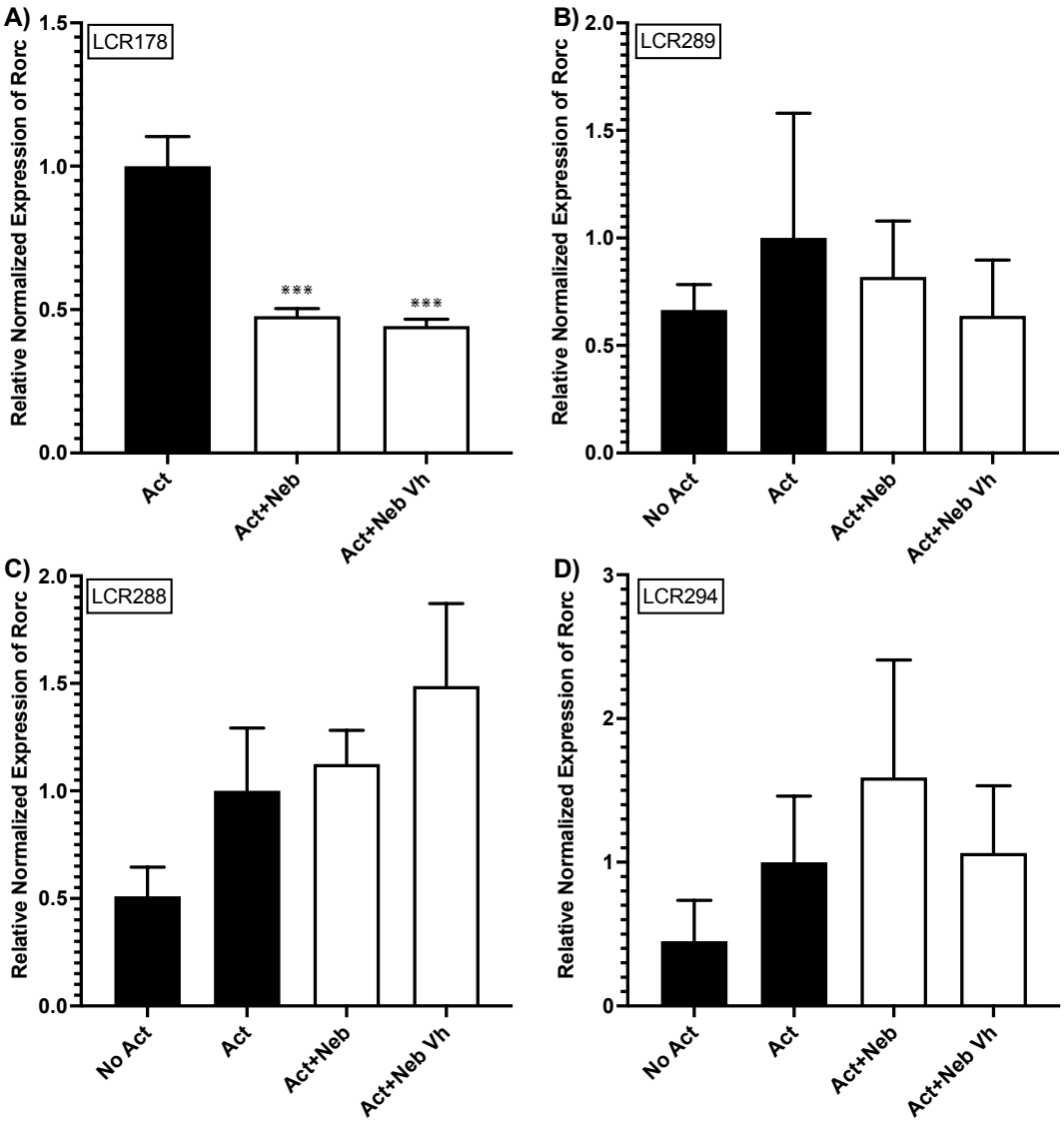

Figure S 7: The gene expression of Rorc gene normalized by the expression of the reference gene HPRT1 as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by LCR codes. The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the technical replicates (n=3) of the biological replicate (n=1) from every subject. P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

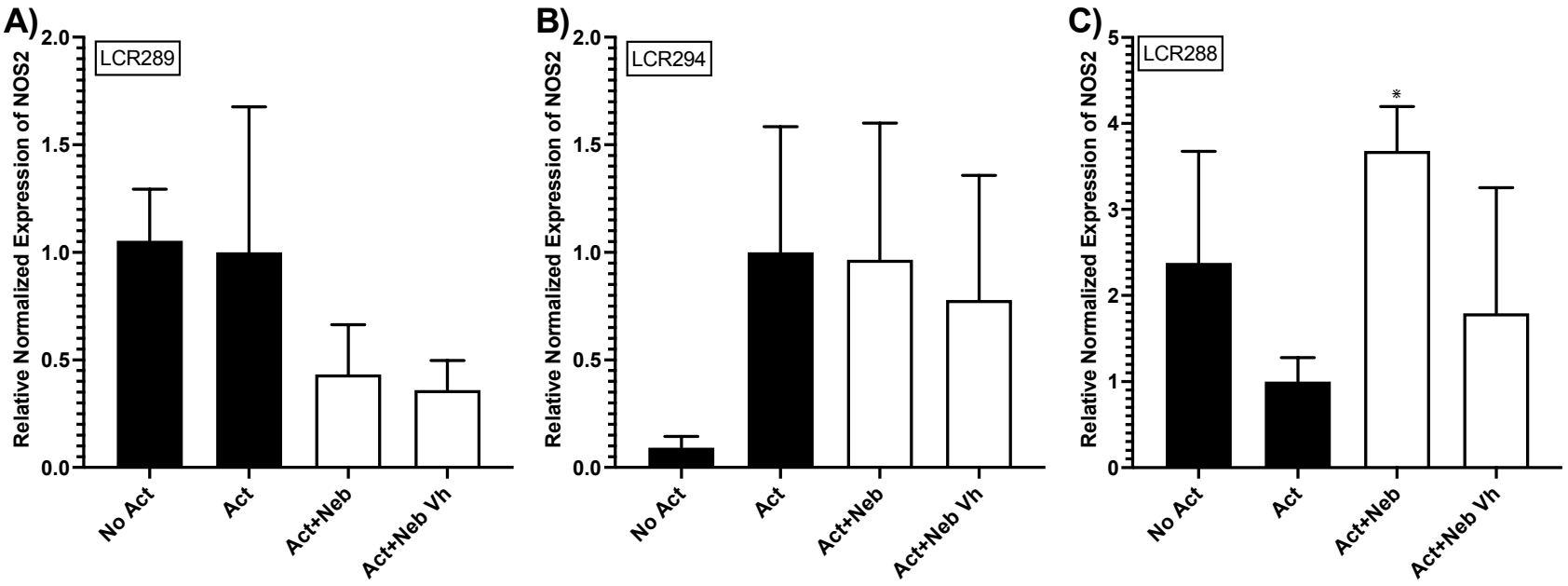

Figure S 8: The gene expression of NOS2 gene normalized by the expression of the reference gene HPRT1 as compared to the positive control of activated PBMCs with anti-CD3 and anti-CD28 antibodies (Act) in individual subjects encoded by LCR codes. The conditions include No Act: negative control, Act: positive control, Act+Neb: activated PBMCs treated with 10μM of nebivolol, Act+Neb Vh: activated PBMCs treated with the vehicle control of nebivolol which is DMSO. The significance was calculated using the technical replicates (n=3) of the biological replicate (n=1) from every subject. P≥ 0.05 (no symbol), 0.0332 (⁎), 0.0021 (⁎⁎), 0.0002 (⁎⁎⁎), <0.0001 (⁎⁎⁎⁎).

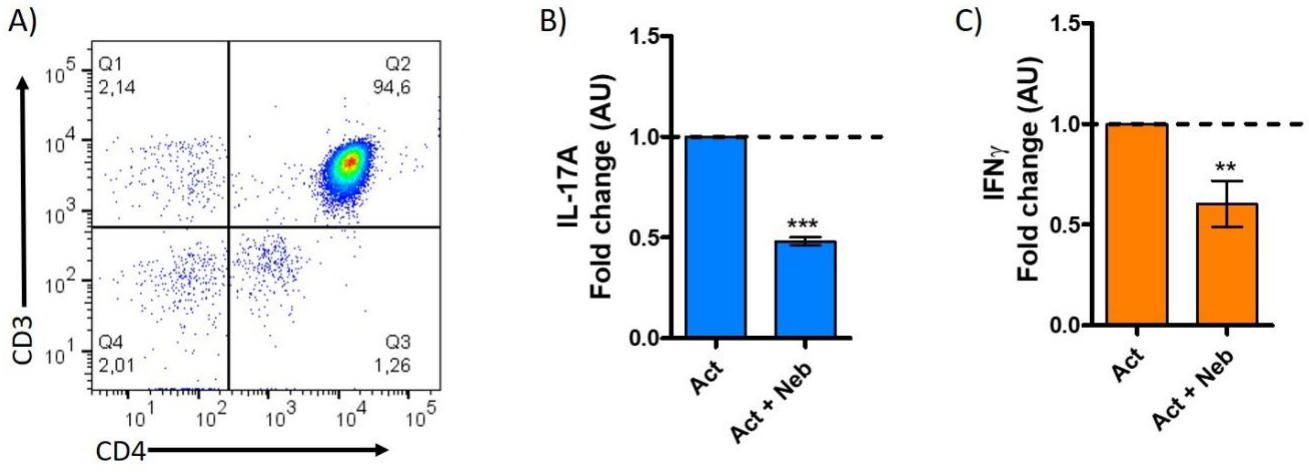

Figure S 9: Nebivolol suppressed IL-17A and IFNγ secretion in purified Th cells. Th cells were obtained from human PBMC samples and activated in vitro with dynabeads pre-coated with anti -CD3 and anti-CD28, without or with nebivolol. A) An example of the purified Th cells prior to activation, stained with CD3 (y-axis) and CD4 (x-axis) and analyzed by flow cytometry. B) IL-17A and C) IFNγ cytokines were measured in supernatants after 4 days activation and expressed as fold change relative to the activated group (dotted line). Data was pooled from 3 experiments. Error bars are shown for standard error. Data from 39 participants tested. Student T-test (**<0.01, ***<0.001).

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