# Modulation of Helper T Cells by β2 Adrenergic Receptor Ligands in a PKA-Dependent Manner

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# Abstract

Modulation of Helper T Cells by β2 Adrenergic Receptor Ligands in a PKA-Dependent Manner

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Background: T helper (Th) cells may attack self-tissues in susceptible people resulting in chronic autoimmune disease. A subtype of Th cells called Th17 cells are considered to be pro-inflammatory by secreting IL-17A cytokines. Discovering new drugs to suppress Th17 cells is a major goal for researchers. Ligands for the beta2-adrenergic receptor (β2AR, encoded by *ADRB2*) are known to suppress pro-inflammatory Th1 cells, but their effects on Th17 cells have not been widely studied. I studied the effect of β2AR ligands on Th1 and Th17 cells and determined the influence of common polymorphisms *ADRB2*. The goal was to discover a potential new immunomodulatory drug to explore as autoimmune disease treatment.

Methods: Human immune cells from healthy participants obtained after informed consent were tested *in vitro*. The samples were activated with T cell-specific activator, and cytokines were measured. The *in vitro* drug treatments included a β2AR specific agonist (terbutaline), an inverse-agonist (nebivolol), a β2AR specific antagonist (ICI 118-551), cAMP analogues that promote or inhibit the pathway, PKA inhibitor and phosphodiesterase inhibitor (which raises cAMP levels). Known polymorphisms were determined by sequencing *ADRB2* from samples.

Results: Primary human Th17 cells expressed the β2AR. Terbutaline augmented IL-17A in activated peripheral blood mononuclear cells and Th17 cells, while IFNγ was concurrently inhibited. Proliferation was not inhibited, rather, an increase was observed in the presence of terbutaline. Using PKA inhibitors and cAMP analogues, it was shown that IL-17A was augmented by terbutaline in a cAMP and PKA-dependent manner. Terbutaline promoted phosphorylation of CREB. Nebivolol inhibited both IL-17A and IFNγ in activated peripheral blood mononuclear cells and Th cells. In samples where *ADRB2* was homozygous for Arg16, terbutaline inhibited IFNγ but did not augment IL-17A. Nebivolol inhibited both cytokines regardless of the polymorphism of the *ADRB2*.

Relevance: These results are novel because the inverse-agonist of β2AR has not been widely studied as an immunomodulator. The cell signalling results demonstrated that Th17 cells respond differently than other Th cells to cAMP-PKA pathway, which suggests the use of other drugs with inverse-agonist properties. Discovering new immunomodulatory drugs will give doctors more options to treat autoimmune patients.

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## Contribution of authors

I was the principal researcher for this research project where I performed the majority of its data collection and analysis as well as writing the manuscript and this thesis. The contribution from Dr. Peter John Darlington as supervisor provided co-authorship as he assisted in advising and interpretation of the results and reviewed manuscripts in preparation and manuscripts published.

Chapter 2 of my thesis includes a published article with permission granted by the Wiley publisher of FEBS journal (© 2017 Federation of European Biochemical Societies). Mahdieh Tabatabaei Shafiei helped on data analysis, experiments and editing of the manuscript. Ashley East performed the experiments for Figure 2.1 (expression of β2AR on Th17 cells) of this thesis. Erika Martire did preliminary experiments relevant for this project. Meagane H.I Maurice-Ventouris helped in the manuscript writing and editing.

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## List of abbreviations

APC: Antigen Presenting Cells

AR: Adrenergic receptor

AC: Adenylyl cyclase

ADRB2: Gene for β2AR

CD: Cluster of determination

CCR6: Chemokine receptor 6

cAMP: cyclic adenosine monophosphate

CREB: cAMP response element-binding

CFDASE: 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester

CNS: Central Nervous System

Csk: C-terminal Src kinase

dbcAMP: Dibutyryl-cAMP

ELISA: enzyme-linked immunosorbent assay

EAE: Experimental Autoimmune Encephalomyelitis

ERK: Extracellular signal-regulation kinase

Epac: Exchange proteins activated by cAMP

FBS: Fetal bovine serum

FSC: Forward Scatter

SSC: Side Scatter

GALT: Gut-associated lymphoid tissue

GTP: Guanosine-5'-triphosphate

GRK: G protein-coupled receptor kinase

H89: N-[2-(pBromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride

ICS: Intracellular cytokine staining

IL: Interleukin

IFNγ: Interferon-gamma

ICS: Intracellular cytokine staining

ICER: Inducible cAMP early repressor

ITAMs: immunoreceptor tyrosine-based activation motif

Lck: lymphocyte-specific protein tyrosine kinase

MALT: Mucosa-associated lymphoid tissue

MAPK: Mitogen-activated protein kinase

MHC: Major Histocompatibility Complex

MS: Multiple Sclerosis

NE: Norepinephrine

PBMC: Peripheral Blood Mononuclear Cells

PKA: cAMP-dependent protein kinase A

PMA: Phorbol 12-myristate 13-acetate

PDE4: Phosphodiesterase 4

PKC: Protein kinase C

$PGE_2$: Prostaglandin E2

RORγ: Receptor-related orphan receptor gamma

RPMI: Roswell Parks memorial institute

rp-CAMP: (R)-Adenosine, cyclic 3',5'-(hydrogen phosphorothioate) triethylammonium

SNP: Single Nucleotide Polymorphism

SNS: Sympathetic nervous system

TCR: T Cell Receptor

Th: Helper T

# EXECUTIVE SUMMARY

The adaptive immune system is essential to protect the body against infectious microbes (pathogens). An important feature of the adaptive immune system is that it can distinguish the fine molecular differences between pathogens and self-tissues. In this way, it can recognize pathogens as being foreign and mount an immune response against the infection, while ignoring self-tissue. If self-tissue is mistakenly identified as a pathogen, the immune system will attack it, potentially leading to autoimmune diseases in susceptible people.

Helper T (Th) cells are circulating lymphocytes, part of the white blood cell family, that are crucial for the adaptive immune system to distinguish between pathogens and self-tissues. Through their T cell receptors (TCR), T cells scan antigens that are present in the body. An antigen is any substance that can trigger an immune response, it may be from a pathogen or from a self-tissue. Decades of research has been conducted by scientists to better understand how T cells can distinguish between pathogens and self tissue, and what goes wrong with the process in people with autoimmune diseases. One subtype of Th cells, Th17 cells, became the center of attention for researchers because they have pro-inflammatory properties and cause autoimmunity in animal models. One reason that Th17 cells are pro-inflammatory is that they secrete IL-17A cytokines which allow the immune system to enter into various body tissues, and mediate tissue damage.

My thesis explored a class of drugs that have not been widely explored for their ability to modulate the immune system. In particular, I studied adrenergic receptor ligands and their action involving one of the adrenergic receptors family members called the beta2-adrenergic receptor (β2AR). It is the only adrenergic receptor found on Th cells which is why I focused on this one. There are different kinds of ligands in pharmacological terms, including an agonist which stimulates the full signalling pathway, an inverse agonist which opposes the main signalling pathway by causing a secondary signalling pathway, and an antagonist which prevents the action of any ligand on the receptor. Ligands have different levels of specificity for adrenergic receptors which is why I chose terbutaline as a β2AR specific agonist. Terbutaline is

a β2AR-specific agonist, nebivolol as a β1 agonist and inverse agonist of the β2AR, while ICI-118,551 which is a β2AR specific antagonist (1–3). The β2AR receptor is a G-protein-coupled receptor that alters the 3',5'-cyclic adenosine monophosphate (cAMP) second messenger and PKA enzyme that activate transcription factors such as cAMP response element-binding (CREB). There are very few research groups that are studying these receptors and their effects on Th cells.

My goal was to discover an adrenergic drug that could robustly suppress Th17 cells in the majority of samples tested. In Chapter 2 my hypothesis was that a β2AR agonist drug would suppress IL-17A from Th17 cells in a PKA-dependent manner. In Chapter 3 I tested a revised hypothesis that an inverse-agonist would suppress Th17 cells. In Chapter 4 I tested the hypothesis that adrenergic drug effects would be influenced by single nucleotide polymorphisms in *ADRB2*, the gene for β2AR. These hypotheses were novel at the outset of my graduate work since no other researchers had studied the immune-modulatory properties of adrenergic drugs on Th17 cells, and no researcher to our knowledge had studies inverse agonist in any context of the immune system.

The methods used to explore these hypotheses, involved immune cells in the form of peripheral blood mononuclear cells (PBMCs) which were obtained from healthy human participants. Using healthy human samples was a first step towards determining the effectiveness of the drug in an *in vitro* system. The cells were activated *in vitro* along with chemical agonists or inhibitors of β2AR-cAMP-PKA-pathway. An enzyme-linked immunoassay was used to detect IL-17A secretion and intracellular staining with the flow cytometry for detecting IL-17A and RORγ (Th17 specific transcription factor). In some experiments, I used Th cells or Th17 cells that were purified from PBMC, in order to demonstrate that the drug was working directly on Th cells or Th17 cells and not necessarily operating through other types of lymphocytes found in PBMC. To determine genetic factors that may influence the Th17 response to adrenergic drugs, *ADRB2* was sequenced from the samples. The single nucleotide polymorphisms (SNP) were determined in *ADRB2* and compared to the *in vitro* drug responses. Together, these approaches are sufficient to determine whether or not adrenergic agonists are

capable of suppressing Th17 cells, and what the potential cell signalling pathways are involved in the process.

For my results, I discovered that Th17 cells express β2AR and that agonists of the receptor augmented IL-17A secretion, while concomitantly suppressing and IFNγ. The original hypothesis was that adrenergic drugs would suppress Th17 cells, but these results disproved that hypothesis. I demonstrated that IL-17A was augmented due to cAMP-PKA-phosphorylated CREB pathway. I discovered that an β2AR inverse-agonist drug (nebivolol) suppressed IL-17A and IFNγ. That novel finding suggests that the receptor can suppress Th17 cells, but I speculate that a different type of cell signalling pathway must be used. The effectiveness of adrenergic drugs when used therapeutically, can vary depending on the single nucleotide polymorphisms within and surrounding *ADRB2* which encodes β2AR. To determine if this might be a factor for adrenergic drugs in the context of Th17 cells, the sequence of *ADRB2* was determined and polymorphisms were compared to the effectiveness of the drug *in vitro* from healthy participant samples. I demonstrated that a single nucleotide polymorphism (Arg16) of *ADRB2* alters the function of β2AR agonist, but not the inverse-agonist.

A future direction would be to test the inverse-agonist on Th17 cells from autoimmune patient samples to determine if this drug works on clinical patient samples. Moreover, animal experiments could also be performed using an experimental autoimmune model and the inverse agonist to determine the potential efficacy in treating autoimmune symptoms *in vivo*.

The relevance of the work is that having another drug option would be beneficial to patients because not all patients respond to all drugs, and some autoimmune disease drugs can lose their effectiveness over time. Moreover, nebivolol would be an off-label use since it was already approved for another indication (e.g cardiovascular purposes). This means that the cost and the time required to validate nebivolol as a new drug for autoimmunity would be lower than for an entirely new drug.

In summary, my initial hypothesis that an β2AR agonist would suppress IL-17A was disproven. I demonstrated why this was the case because the cAMP-PKA pathway augments IL-

17A likely through phosphorylated CREB. The β2AR inverse-agonist nebivolol had the desired effect of suppressing IL-17A and IFNγ, regardless of polymorphisms in the receptor. Nebivolol is a promising immunomodulatory drug that warrants further study in the context of treating autoimmune diseases.

# Chapter 1 General introduction

## 1.1 The adaptive immune system protects the body from foreign pathogens but can cause autoimmune disease in susceptible individuals.

T cells are part of the adaptive immune system. The adaptive immune system protects the body from infections by pathogens such as viruses, bacteria, fungi and other microorganisms (4). Transplanted tissue can introduce new and different antigens that the immune system specifically recognizes as foreign and a threat (5). The adaptive immune system will consider some cancer cells to be foreign if the cancer cell expresses *de novo* (mutated) proteins (6). The basis for an adaptive immune response lies in the recognition of antigens (5). An antigen is defined as any substance that can elicit an immune response. Not all antigens are foreign, in fact the vast majority of antigens presented in the body are self-antigens from body tissues. The adaptive immune system will ignore these self-antigens in healthy people (5). Antigens are usually in the form of short polypeptides derived from digested proteins, and they are presented by the major histocompatibility complex (MHC) on an antigen-presenting cell (APC) (5).

An adaptive immune response has four main functions; specific recognition, elimination, memory, and self-regulation (4). It is called adaptive because the system has the capability of recognizing antigens it has never encountered before and then mounting an immune response against the source of the antigen (4). This is essential because foreign pathogens (and cancers) are constantly mutating and trying to avoid immune detection, so the adaptive immune response is able to keep up with the ever-changing threats to the body (6,7). There is another branch of the immune cell called the 'innate immune system' that works by rapidly containing and slowing down infections based on conserved molecules that pathogens have retained over generations (8). For example, the innate Toll-like receptors found on innate immune cells are able to detect lipopolysaccharide, a carbohydrate that forms a unique component of some bacterial cell walls (8). The innate immune system is very effective at eliminating pathogens, however, it is unable to adapt to mutated or new foreign antigens, hence the need for an adaptive immune system (4). In most people, the adaptive immune system is able to adapt to

mutated antigens effectively, however, in some people with genetic and environmental susceptibility factors, their adaptive immune system makes a mistake and considers self-antigen to be a foreign antigen (4). This may result in autoimmune disease in people with genetic and environmental susceptibility factors. An autoimmune disease is chronic because self-antigens come from vital tissues and organs of the body that are consistently present and the adaptive immune system will attempt to remove the antigen source (4). It is still not understood how the environmental and genetic factors contribute to the risk of getting an autoimmune disease. Several theories have been proposed, for example, Epstein-Barr virus may carry antigens that are very close to the structure of self-antigens, which triggers an auto-reactive adaptive response, this is called molecular mimicry (7). The genetic susceptibility is also not completely understood, genes that are linked to autoimmune diseases tend to be immunology related genes, such as the MHC gene which is a genetic risk factor for acquiring an autoimmune disease (9). Thus, T cells are essential for the immune system to properly distinguish between foreign and self-antigen and to avoid autoimmunity (4).

## 1.2 T cells development: naïve T cells mature in the thymus where potentially autoreactive T cells are deleted.

Activation of the T cells is an important feature of the adaptive immune system because it determines whether or not the response will occur. When T cell activation is properly regulated, T cells can distinguish between foreign-antigen and self-antigen (10). This process, called self-tolerance, allows the adaptive immune system to attack the source of the antigen if it is foreign (i.e. micro-organism), but ignore the source of the antigen if it from the bodies own tissues (10). If it fails to do so, then self-tolerance is broken, and autoimmune diseases may occur in people with genetic and environmental risk factors. Self-tolerance is first established when T cell precursors enter the thymus (10). Before T cells become mature effector cells, they follow many steps of creation beginning as precursors in the bone marrow, selection in the thymus, and activation in the secondary lymphoid tissues (10). The first step involves T cell

progenitor cells which originate in the bone marrow and migrate to the thymus where they undergo thymic education (10,11). The most important aspects of thymic education are called positive and negative selection, which ensure that a functional naïve T cell is created without it being autoreactive against self-antigens (10,11). Thymic education begins when the T cell precursors generate a diverse repertoire of unique TCR via somatic recombination of genomic DNA (12). Each T cell precursor produces a unique TCR based on germline rearrangement of the TCR-encoding genes (12). The TCR is composed of two polypeptides the α and β chain, each chain has variable and constant domains (12). The antigen-binding sites are made up the variable domain, which is composed of gene segments (variable, joining and diversity gene segments) that are randomly rearranged in the T cell precursor in the thymus (12). The variable and joining segments contribute to form the center of the antigen-binding site of the TCR, which has three complementarity-determining regions (CDR), these are known as hypervariable regions (12). The CDR3 region accounts for the unique components of the peptides recognized by the TCR (12). The generation of unique TCR structures in each T cell prepares the immune system for broad and flexible immune responses to unknown foreign antigens that may be encountered in the future (12). Since each T cell has a unique receptor, the total T cell population is referred to as the T cell repertoire, and it contains enough diversity such that new (foreign) antigens can be detected (12).

The T cell precursors travel to the thymus where they are screened for a TCR that is functional, but not autoreactive, these are called positive and negative selection, respectively (10). The basis for T cell activation lies with the APC, that is a specialized cell type that is constantly sampling the environment for proteins, particles, and pathogens (5). The APC digests protein into smaller fragments called peptides, processes them with an MHC molecule, and presents the peptide-MHC complex on its cell surface (5). This process, known as antigen presentation, is constantly going on whether there is self or foreign antigens (5). The TCR of a T cell must be able to scan the peptide-MHC complex and determine if it is a complementary fit (which would signify a cognate antigen) (5). The affinity of the protein-protein interaction has to be sufficiently strong, which signifies the activation of the T cells (5). For these reasons, the

thymus carefully selects T cells by eliminating those with TCR that can not bind at all to MHC, bind too strongly to MHC, or bind to MHC-self antigen (10). Positive selection, the step where the T cell must recognize the MHC at low affinity, occurs in the thymic cortex (10). The TCR has to be functional and binds to MHC molecules with a low affinity to produce a cell signal through the TCR that allows the T cell to survive (13). After positive selection, the immature T cells migrate to the thymic medulla and undergo negative selection (10). Negative selection is defined as a process whereby the T cell that reacts to self-antigens undergo apoptosis (14). If a T cell precursor binds to MHC with too high affinity, they undergo apoptosis because the cell signal is too strong, indicating that this TCR would activate against MHC molecule regardless of antigen (10). Ideally, the T cell is only activated when MHC is presenting the cognate antigen otherwise the T cell would activate on any MHC molecule including self-antigens (10). Thymic tissue can display a sampling of self-antigens that represent tissues from all over the body. This display facilitates negative selection of potentially autoreactive T cells (10). If the negative selection process is absent, there is an increase in the maturation of autoreactive T cells (cells having receptors against self-antigens) that can result in autoimmunity (14).

After thymic education, most of the T cell precursors die by apoptosis, only 1% will become naïve T cells which circulate through blood and lymph and the secondary lymphoid tissues (14). The naïve T cells are defined as T cells that have not yet encountered their cognate antigen/MHC (15). Cognate is defined as the matching MHC/peptide shape that will stimulate unique TCR (12). As naïve T cells circulate through the blood vessels and secondary lymphoid tissues that include lymph nodes, the spleen and the mucosal lymphoid tissues of the gut, nasal and respiratory tract, they are scanning over the APC for the presence of cognate antigen/MHC complexes. If the T cells encounter a foreign cognate antigen/MHC then an adaptive immune response is triggered (15).

## 1.3 T cell activation, two signal model: TCR recognises cognate MHC/antigen, CD28 binds to B7 ligands, results in cellular proliferation.

The TCR is the most important aspect of T cell activation because it can distinguish between self and non-self antigens. There are also other receptors on the T cell that are required for activation to occur which are called co-stimulatory receptors. The TCR signal is called signal 1, it occurs as APC presents the peptide-MHC complex on its surface (15). When a TCR is ligated by peptide-MHC, signalling of the TCR is initiated within the cytoplasmic regions of the CD3, which is composed of four chains (CD3γ, CD3δ, CD3ε, and CD3ζ) (15). These cytoplasmic chains are called immunoreceptor tyrosine-based activation motif (ITAMs) (15). They are phosphorylated by Src family kinases (lymphocyte-specific protein tyrosine kinase (Lck) and Fyn), which are associated with the cytoplasmic domain of the co-receptor CD4 (16). Binding of the co-receptor (CD4) with the cognate antigen MHC complex permits Lck to phosphorylate ITAMs (16). When the ITAM motifs are phosphorylated by tyrosine kinase zeta associated protein of 70 kDa (ZAP-70) (16). Subsequently, ZAP-70 phosphorylates transmembrane proteins called linker for activation of T cells (LAT) which it's linked to SH2 adaptor proteins such as SLP-76 (16). These proteins organize molecules that are necessary for activation of various signalling pathways for TCR (16). Another key signalling protein is PLCγ (15). PLCγ leads to the activation of signalling pathways; mitogen-activated protein kinase (MAPK) cascade, Jun kinase (JNK), and protein kinase C (PKC)- θ (17). These signalling pathways result in activation of transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), NFAT (nuclear factor of activated T cells), Activator Protein-1 (AP-1) and cAMP response element (CRE)-binding protein (CREB)/activation transcription factor families which are essential for T cell activation (18,19). The transcription factors culminate in changes to cytoskeleton, reorganization of cell surface proteins, proliferation by mitosis, and differentiation into T cell subtypes (15). However, for a T cell to proceed through all those stages, a second signal though costimulatory receptor is required.

The co-stimulatory signal is delivered by a receptor called CD28, this is referred to as signal 2. When CD28 is stimulated it promotes proliferation, survival and differentiation of

naïve T cell (13,20,21). The CD28 receptor, which is constitutively expressed by T cells, binds to co-stimulatory ligands which are transmembrane glycoproteins CD80 (B7.1) and CD86 (B7.2) molecules on the APC. The engagement of CD28 is necessary to avoid anergy, which is a non-responsive state of the T cells (22,23). In addition, CD28 initiates signalling events that lead to the production of cytokine IL-2 which is a growth factor and pro-survival genes such as Bcl-xl (21,22,24). IL-2 drives the proliferation of activated T cells in an autocrine fashion. Once secreted by the T cell, the T cell detects IL-2 with the IL-2 receptor which will trigger intracellular signalling pathways (e.g extracellular signal-regulation kinase (ERK)) that turn on the proliferation cycle (25,26) (Figure 1.1.).

Signal 2 (CD28) is important because it helps the body avoid immune responses when they are not needed. This concept is known as the danger theory, where the immune system is more sensitive to an antigen if the body is in a state of inflammation (27). In the absence of inflammation, self-peptide antigens are always being presented on MHC molecules. When there is no inflammation, the CD80 and CD86 levels are low on the APC, which makes it harder for T cells to become activated. This minimizes the possibility of a reaction against self-antigen. When an infection or injury is present, the associated inflammation causes the CD80 and CD86 levels to rise on the APC, which lowers the activation threshold for naïve T cells. Thus, when the body is in danger it makes T cells more likely to clonally expand against the cognate antigen/MHC (27). The danger theory is another way how the adaptive immune system can react against non-self antigens while ignoring self-antigens. It also means that inflammation could increase the chances of a mistake being made by the T cell with ensuing autoimmune disease (27).

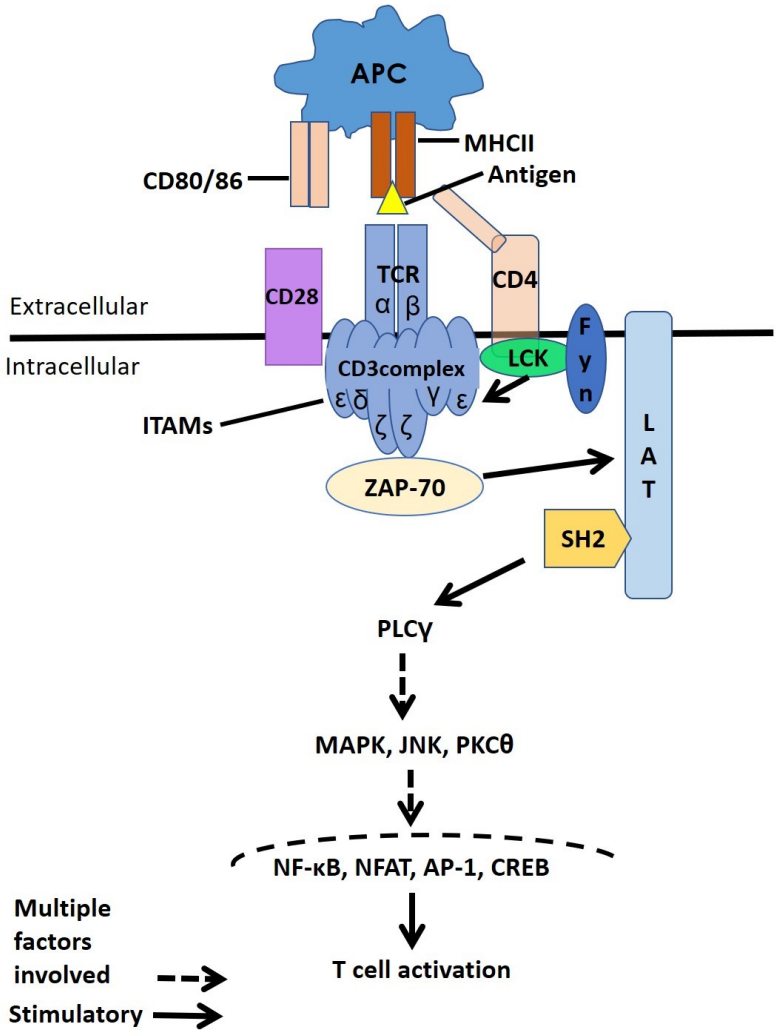



Figure 1.1. Simplified Th cell activation model of signal 1 and signal 2.
The interaction of the APC with cognate antigen-MHCII activates the T cell. When the TCR (α and β chains) recognizes the antigen and is ligated to the MHCII, the signalling is initiated. This is mediated via the cytoplasmic components of the CD3 complex (CD3γ, CD3δ, CD3ε, and CD3ζ), also referred to as ITAMs. The cytoplasmic co-receptor CD4 recognizes the MHCII, allows the Src family kinases (Lck and Fyn) to phosphorylate the ITAMs. Then ZAP-70 phosphorylates LAT and activates its adaptor SH2. Recruitment of PLCγ leads to MAPK, JNK, PCKθ pathways that culminate to transcription factors necessary for T cell activation, including NF-κB, NFAT, AP-1, CREB. For signal 2, the co-stimulatory receptor CD28 on the T cell interacts with

transmembrane glycoproteins CD80 (B7.1) and CD86 (B7.2) molecules on the APC, it lowers the levels of cAMP and allows for full T cell activation.

The second messenger cAMP is another important aspect of T cell activation. When cognate antigen-MHC complex ligates TCR, it results in several intracellular signalling pathways including adenylyl cyclase (AC) which catalyzes cAMP production (28,29). The amount of cAMP produced and the profile of the cAMP concentration within the T cell can determine if the T cell is activated or not activated. If a T cell is activated with only TCR ligation, for example with anti-CD3 antibodies that cluster and activate TCRs, then cAMP concentration will rise at a steady rate inside the cell leading to inhibition of the T cell (28). In contrast, if the TCR and CD28 are both ligated, for example with anti-CD3 and anti-CD28 antibodies, the cAMP concentration will rise and then fall creating a peak of cAMP (28). The reason this happens is that when a T cell is only activated by the TCR (without co-stimulation), the immune system interprets this as a potential auto-reactive event and therefore inhibits the T cell (Figure 1.2). When co-stimulation is present, it creates the cAMP peak which promotes T cell activation because co-stimulation signifies that inflammation is present likely due to infection or injury (Figure 1.3). Thus, cAMP is important in determining if a T cell is productively activated or inhibited.

Adenylyl cyclase is required to produce cAMP, it does so when stimulatory G-protein is present. How this works in an activated T cell is not completely understood. Lipid rafts are one important cell signalling compartment that influences many aspects of T cells (30,31). Lipid rafts are cholesterol-rich and hydrophobic which recruits signalling proteins like the TCR, co-receptors, Src kinases, adaptor proteins, and protein palmitoylation. By clustering these receptor components, lipid rafts aid in the augmentation of signal propagation of cells that are activated (31). Researchers discovered that cAMP could accumulate in lipid rafts by purifying the enriched-cholesterol and glycosphingolipid microdomains in the cell membrane of primary T cells using cell fractionation by detergent and density gradients (28). The $G_{\alpha s}$ co-purified with CD3 complex in the lipid rafts, cAMP accumulated in lipid rafts after stimulation with anti-CD3 and anti-CD28, and the increase of cAMP went back to basal levels after few minutes (2 min) creating a peak of cAMP in the lipid rafts (28). The authors also observed that cAMP

concentration increased within the first few minutes of T cell activation, and PKA was actively phosphorylating downstream substrates which could be inhibited by H89 (Abrahamsen et al. 2004). In another study, T cells that were activated after priming T cells (a previous activation) there was an increase in TCR signalling that coincided with a gradual increase in cAMP concentration over 30 minutes (29). The authors measured an increase in PKA activity with fluorescence resonance energy transfer probes, it rose within the first 2 minutes, which then was back to the basal level within 10 min (29). Lipid rafts are integral in the cAMP second messenger pathway and PKA activity in T cells. The G proteins ($G_\alpha$ and $G_i$) and AC were found in the lipid rafts (28,32). By using inhibitory antibodies for $G_{\alpha s}$, the authors could inhibit the cAMP increase that occurs when the TCR is stimulated. Conversely, using $G_i$ inhibitory antibodies the cAMP was increased (28). More evidence is that when $G_{\alpha s}$ is silenced with siRNA in a T cell line (Jurkat T cells), there was a decrease of cAMP in the stimulated TCR with plate-bound anti-CD3 and soluble anti-CD28 (19). Moreover, the cAMP increase mediated by TCR stimulation was not inhibited by a dominant-negative construct of $G_{\alpha s}$ which blocks the $G_s$PCR mediated $G_s$ activation in Jurkat T cells (19). The results from those papers indicated that the TCR stimulates $G_{\alpha s}$ that leads to cAMP pathway in lipid rafts. There are other G-protein coupled receptors capable of inducing cAMP production though a similar manner, including prostaglandin E2 ($PGE_2$) receptors, adrenergic receptors, and chemokine receptors. For example, the pro-inflammatory molecule $PGE_2$ increased cAMP levels by ~13 fold as shown on activated primary T cells (33). The total concentration and kinetics of cAMP inside a T cell is presumably influenced by the combination of TCR ligation, along with the other GPCRs that are being stimulated by their ligands.

PKA is a ubiquitous enzyme present in most cells and tissues of the body. In T cells, PKA suppressed T cell activation by phosphorylating and thus activating C-terminal Src kinase (Csk) which inhibited Lck and which prevented T cell activation (33). Thus, if Csk inhibits Lck then T cell signalling is inhibited (Figure 1.2). When PKA was inhibited with H89, there was an increase of ζ chains phosphorylation in primary T cells (28). Increasing the level of cAMP with cAMP analog or $PGE_2$, resulted in a reduction of phosphorylation of CD3ζ chains and increased Csk

activity (phosphorylation of tyrosine of lck), as it was measured in Jurkat or primary T cells with immunoprecipitation experiments (33). To determine if PKA phosphorylates and activates Csk, PKA was inhibited by H89, which resulted in Csk activity being decreased (33). Csk is regulated is by "Regulatory transmembrane molecule phosphoprotein associated with glycosphingolipid-enriched microdomains" also referred to as Csk-binding protein (CBP). When CBP is phosphorylated it associates with Csk, which allows Csk to attenuate the early steps of TCR signalling (34). An example is when CBP was overexpressed in mouse thymocytes activated with anti-CD3 and anti-CD28, the levels of IL-2 and the proliferation was inhibited (34). Thus, the cAMP-PKA-Csk pathway is inhibitory for T cell activation.

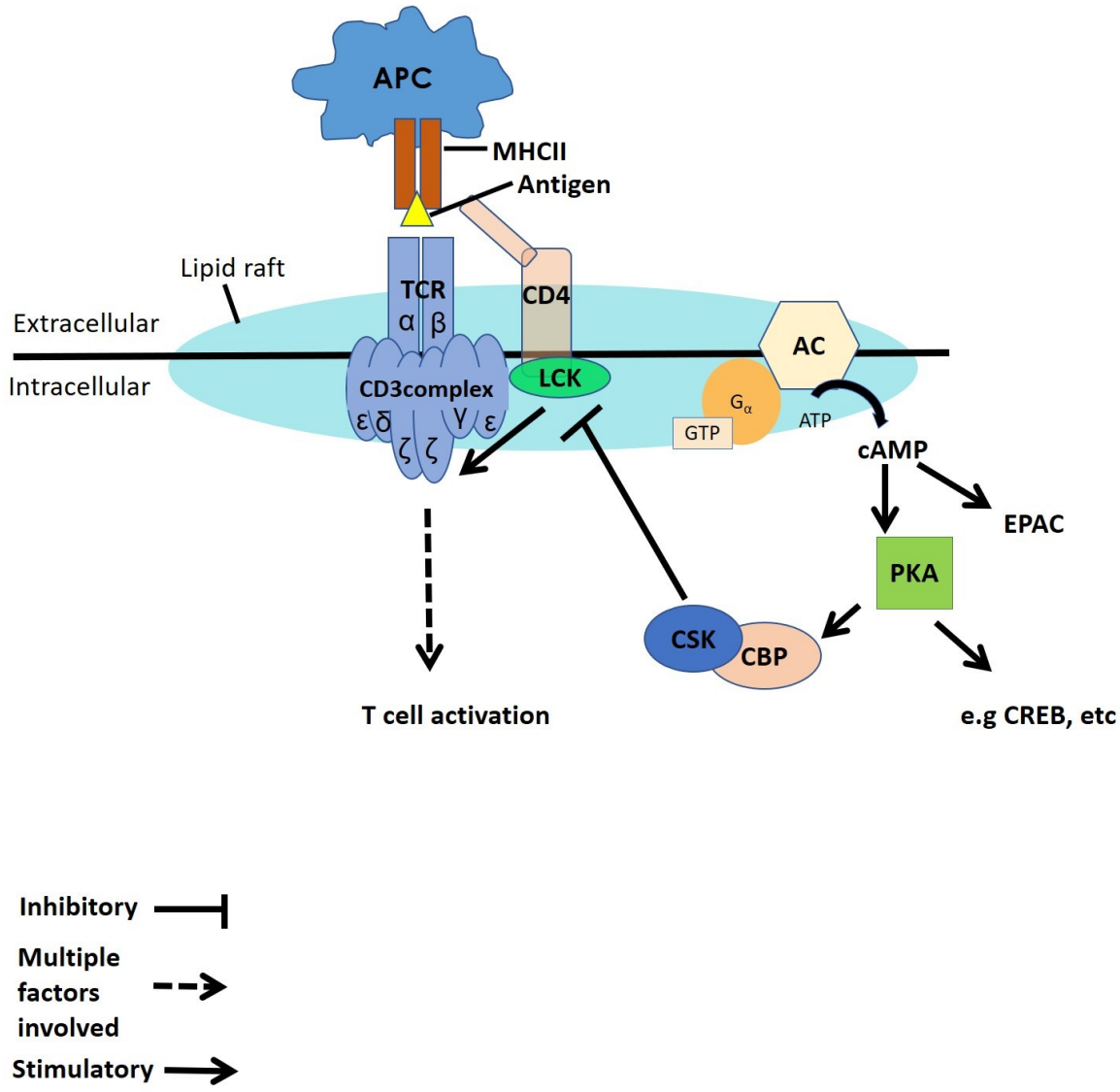



Figure 1.2 PKA-dependent inhibition of T cell activation.

The interaction of the cognate peptide-MHCII with The TCR and the CD4 leads to an increase of cAMP. This is mediated by Gαs in the lipid rafts. The cAMP activates PKA and as another example EPAC. The PKA phosphorylates and activates CBP and Csk, which inhibit Lck. Since Lck is necessary for the initiation of signalling events leading to T cell activation, Inhibiting Lck, in turn, inhibits T cell activation.

If cAMP concentrations are sustained at high levels, the inhibitory mechanism prevents full T cell activation. The co-stimulatory signal 2, mediated by CD28 receptor on the T cell, will lower cAMP levels (Figure 1.3). The mechanism of action by which CD28 lowers cAMP levels is through phosphodiesterase's (PDE), a large family of enzymes that degrades cAMP or cGMP (28). The isoform PDE4 is present in the lipid raft whether the cell is stimulated or not, as it was shown by immunoprecipitation experiments in T cells from healthy individuals (28). When T cells are only stimulated with anti-CD3, the levels of PDE4 are low, the cAMP is sustained, and T cells are not activated. Whereas, with CD28 co-stimulation added to primary T cells, there was recruitment of PDE4 and scaffold protein β-Arrestin to the lipid rafts. The PDE4 activity was measured and was at least 2 fold higher when the cells are stimulated with both anti-CD3 and anti-CD28 compared to only anti-CD3 stimulation (28). Similarly, PDE4B2, a subtype of the isoform entered lipid rafts when TCR was stimulated with antigen (Staphylococcal Enterotoxins) in Jurkat T cells transfected with PDE4B2-GFP constructs (35). Thus, CD28 lowers the levels of cAMP to facilitate T cell activation.

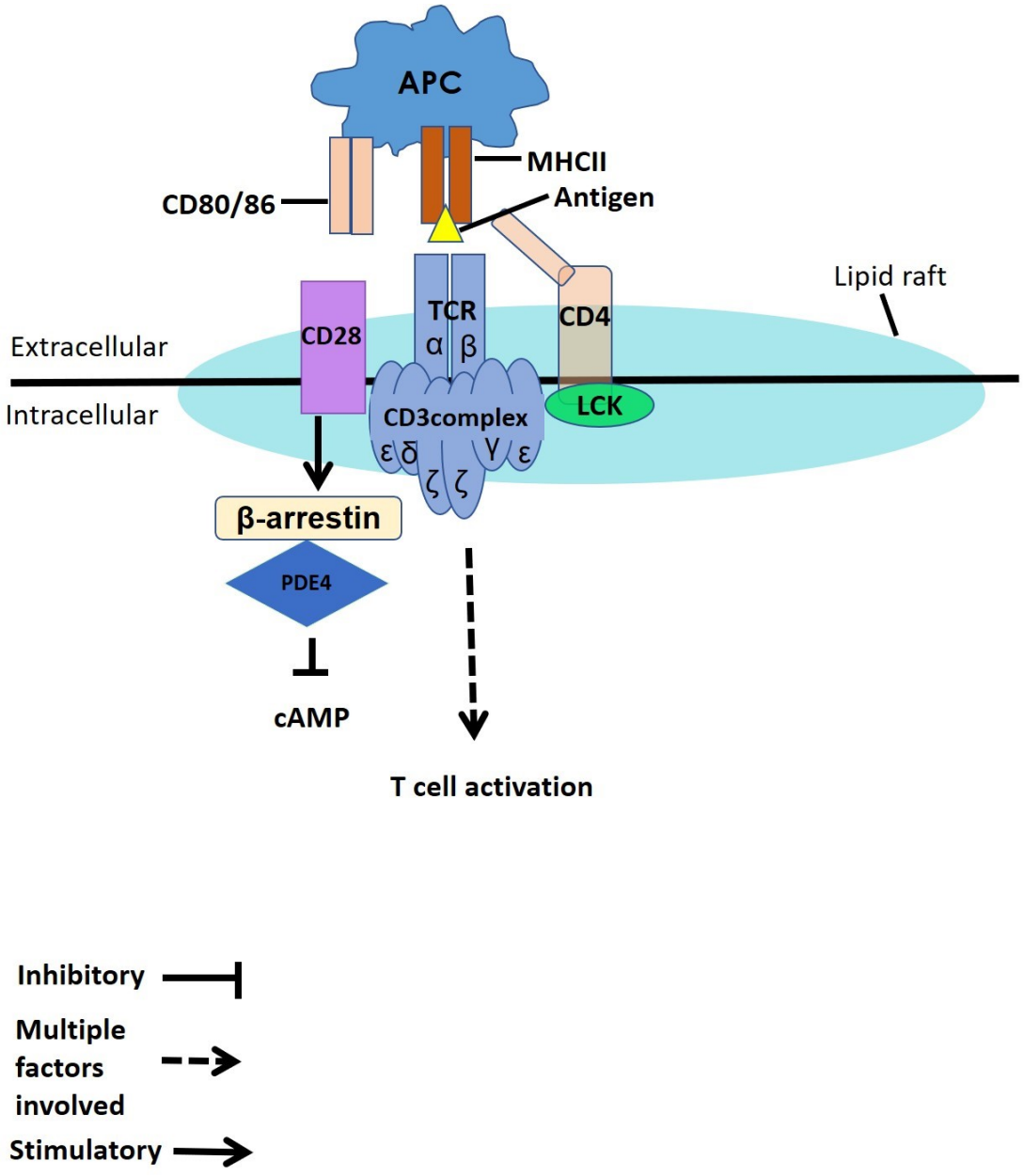



Figure 1.3. T cell activation, two signal model.

T cell activation is complete when there are interactions of the TCR (Signal 1) and the co-stimulatory signal mediated by CD28 (Signal 2). CD28 recruits scaffold protein β-arrestin to the lipid rafts. The β-arrestin, in turn, is associated with phosphodiesterase's (PDE4), whose role is to degrade cAMP. The decrease in cAMP will in turn allow full T cell activation as it would not activate PKA. Since PKA inhibits T cell activation, by interacting with kinases of TCR necessary for T cell signalling.

## 1.4 Phosphorylation of CREB and its role in T cell activation

CREB was a particular focus for my thesis due to it being part of the TCR pathway and involved in adrenergic drug pathway, making it a possible convergence point of the cell signalling pathways. CREB is a transcription factor that becomes activated when it is phosphorylated at position Ser133. Stimulation of the β2AR with fenoterol plus anti-CD3 and anti-CD28 on T cells increased cAMP and PKA activity resulting in phosphorylated CREB (36). CREB is a transcription factor that is needed for the proliferation and survival of T cells. If CREB is phosphorylated and activated at Ser133 by PKA, it interacts with a coactivator protein called CREB-binding protein (CBP) which starts transcription of CREB responsive genes. CREB has a domain at the carboxy-terminal end which binds to cAMP-responsive element (CRE) by dimerization through a leucine zipper motif (37). CRE elements are associated with promoters and enhancers of T cell-specific genes such as components of the TCR -CD3δ (a signalling component of the TCR) or IL-2 (an autocrine growth factor cytokine) (38). Mice with transcriptionally inactive CREB (Ala119) had defects in activated (conA with PMA and ionomycin or anti-CD3) T cells. T cells in these mice did not proliferate or produce IL-2 which led to G1 cell cycle arrest and apoptosis, measured using $^{3}H$ thymidine incorporation, ELISA and flow cytometry techniques respectively (39).

CREB is also required for regulating cytokines. For example, $CD4^{+}$ T cells isolated from transgenic mice with a CREB mutation (Ser133 to Ala) were stimulated with anti-CD3 or antigenic peptides, resulting in lower levels of IFNγ and IL-4 in the CREB mutants as compared to the control mice as detected by ELISA (40). Therefore, CREB is required for Th1 and Th2 cells to differentiate and secrete cytokines. CREB is also required for Th17 cells to gain their phenotype and produce IL-17A. A study showed that mutation of the CRE sites on the conserved non-coding sequence (CNS2) of the *Il17* gene locus and on the *Il17* promoter inhibited RORα and RORγ gene reporter activity in Th17 cells (41). CREB is associated with *Il17* gene promoter locus and CNS2 and synergizes with transcription factor RORγ, which are

necessary for IL-17 expression in mice, as was shown with experiments using chromatin immunoprecipitation assay and real-time PCR (41). Overexpression of negative polypeptides of the CREB (A-CREB) which inhibit pCREB, showed a reduction of IL-17 mRNA expression by murine Th17 cells (42). Inhibition of the transcriptional coactivator CBP/p300 in its bromodomain showed to reduce IL-17A on isolated CD4 cells from healthy, ankylosing spondylitis and psoriasis and psoriatic arthritis individuals which were measured with ELISA (43). In CREB-CD4 knock out mice, induced for experimental autoimmune encephalomyelitis (EAE), there were minor diseases symptoms (measured with clinical scores) compared to the control (wild type) (41). The EAE mouse models replicate some of the symptoms of multiple sclerosis (MS) by immunization of susceptible strains of mice with antigens derived from the central nervous system (44). MS is an autoimmune demyelinating disease of CNS that results in axonal transection and gradual accumulation of neurological disability (45). The $CD4^+$ cells were measured with the flow cytometer and showed to produce lower levels of IL-17 and IFNγ in the CREB-CD4 knock out mice compared to the control (wild type) (41). In another study on CREB knockout mice induced for EAE, an improvement on the EAE disease clinical scores and the IL-17 on CD4 cells that infiltrate the central nervous system was reduced, compared to wild type mice (41). Those experiments indicated that CREB promotes the expression of IL-17 and was required for the disease to occur in EAE mice.

These studies did not specifically address CREB in the context of β2AR and its ligands, which was one of my specific aims of the thesis. The literature shows that CREB can promote IL-17A in mice. In my thesis, the first hypothesis was that β2AR agonist ligands would suppress IL-17A. That hypothesis was based on the literature (section 1.16) showing that β2AR agonist ligands suppress pro-inflammatory Th1 cells by anti-proliferative effects and I reasoned that this would overcome any augmenting effects of CREB. However, as I showed in the results section of chapter 2, it became evident that β2AR agonist ligands were augmenting IL-17A and proliferation was mostly unaffected or higher contrary to the anti-proliferative effects seen with other ligands in the literature. Those results caused me to formulate a revised hypothesis in chapter 3 more in line with the concept that pCREB activity augments IL-17A.

## 1.5 Types of adaptive T cells: cytotoxic CD8 T cells, helper CD4 T cells, and rare innate T cells.

At least three different types of T cells can develop in the thymus, namely, cytotoxic CD8 T cells, helper CD4 T cells and regulatory T cell (T reg) although the latter can also be created in the periphery (outside of the thymus). My thesis focused on Th cells because of their role in distinguishing self vs non-self antigens and coordinating the adaptive immune system. Th cell dysregulation can result in autoimmune diseases and understanding how they can be modulated is useful for the development of therapies for autoimmune diseases. Here, I will also briefly review the other types, cytotoxic CD8 cells and γδ T cells.

Th cells emerge in the thymus as precursors that express CD4, CD8, and TCR in phases as they go through positive and negative selection. The CD4 molecule is a cell surface protein that binds to a type of MHC called MHCII. The Th cell also produces cytokines that will augment the antigen processing and display by the APC (Pennock et al. 2013; Smith-Garvin, Koretzky, and Jordan 2009). In this way, the Th cell will promote the activation of other immune cells such as cytotoxic T cells and innate cells, which is why Th cells are called 'helper' T cells (46). Th cells also help to enable B cell maturation, migration, and secretion of antibodies. Without Th cells, most antibodies can not be formed, although there are some exceptions called T independent antigens (26). Moreover, Th cells activate and recruit innate immune cells to the site of infection such as macrophages and natural killer cells. Thus, Th cells support the adaptive immune responses by distinguishing between self and non-self antigen, and then coordinating the responses of the other adaptive and recruiting innate immune cells (47).

Cytotoxic CD8 cells emerge in the thymus as precursors that express CD8, CD4, and TCR in phases as they go through positive and negative selection (14). There are fewer CD8 T cells as compared to CD4 T cells. The percentage of mature CD8 T cells in PBMCs from healthy individuals is 26% as compared to CD4 T cells which are at approximately 48%, as measured by flow cytometry (48). This is a ratio of 1:2 of CD8+ T cells versus CD4 T cells. The CD8 molecule is

a cell surface protein that binds to a type of MHC called MHC I (14). MHCI is expressed on virtually all nucleated cells of the body and delivers peptides from the cytosol that originate from viruses and some bacteria that can replicate in the cytosol (4). CD8 T cells are called cytotoxic because they contain internal molecules and enzymes (perforin and granzymes) that can be released onto a target cell which kills the target cells (4). This only happens if the target cell is expressing MHCI with a cognate peptide-MHC complex and foreign peptide antigens (4). A common example is a virally infected cell which will process and present viral peptides on its MHCI, this will be recognized by a cytotoxic CD8 T cell which kills the virally infected target cell (4).

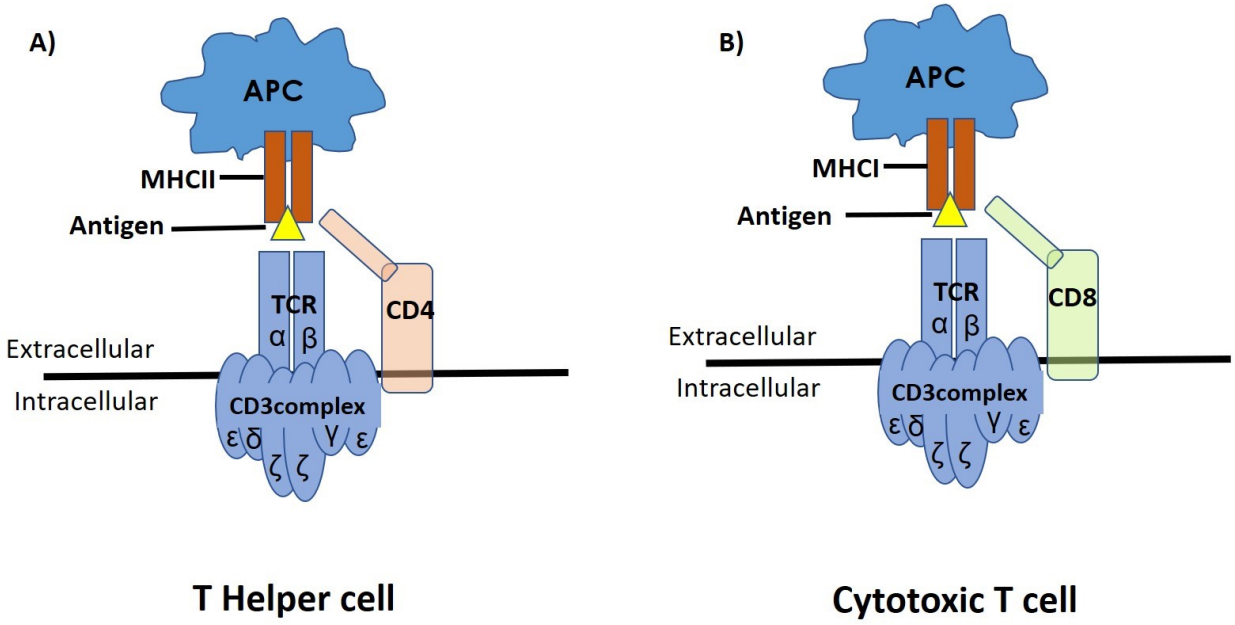



**Figure 1.4. Model Figure of T helper cells and Cytotoxic T cells.**
A) Th cells have co-receptor CD4 on their cell surface that recognizes MHC II presented by the antigen-presenting cell (APC). B) Cytotoxic T cells have co-receptor CD8 on their cell surface that recognizes the MHC I presented by the APC.

There are other types of T cells that are not considered adaptive since their TCR receptors are invariant, that is, their TCR does not undergo somatic alterations as would occur in an adaptive T cell (14). Instead, their T cell receptors are hard-wired into the genome and possess the ability to recognize conserved molecular features of pathogens (14). Non-adaptive T cells include gamma-delta (γδ) T cell that expresses a TCR variation consisting of gamma and delta chains instead of the usual alpha and beta (αβ) change (49). γδ T cells have multiple phenotypic characteristics one of them is that they can produce cytokine IL-17. When γδ T cells were isolated from PBMCS from healthy individuals and stimulated with isopentenyl pyrophosphate and irradiated dendritic cells, they produced less than 7% of IL-17, with no IFNγ produced as measured with flow cytometry techniques (50). In humans, the γδ T cells are less than 5% of white blood cells (4). γδ T cells that produce IL-17 are found in high frequencies in patients with bacterial meningitis (50). There is also a low abundance of NK-T cells, which express elements of both innate and adaptive immune molecules (51). The subset of invariant natural killer cells (iNKT) produces IL-17, as was observed in thymocytes from infants and PBMCs from adults in healthy controls and with atopic dermatitis (52). These cells also play a role in autoimmunity (53). In my thesis, experiments were performed with PBMC cells, which in theory contain small proportions of invariant T cells such as γδ and NKT cells which could contribute small amounts of IL-17A to the assays. To address this, key experiments were performed using a negative selection column that specifically removed cells that were not Th cells, including γδ and NKT cells. I also used flow cytometry staining to identify IL-17A producing Th cells, to further rule out other T cell types in the results.

## 1.6 Mechanisms of action by which T cell activation and clonal expansion are regulated.

If a naïve T cell is activated by signal 1 and signal 2, then it will expand by proliferation (mitosis) into a larger population. This is called clonal expansion because the daughter cells retain the same TCR and produce a population of clones capable of recognizing the foreign antigen (11) (Figure 1.5). Clonal expansion is an important decision-making step because the

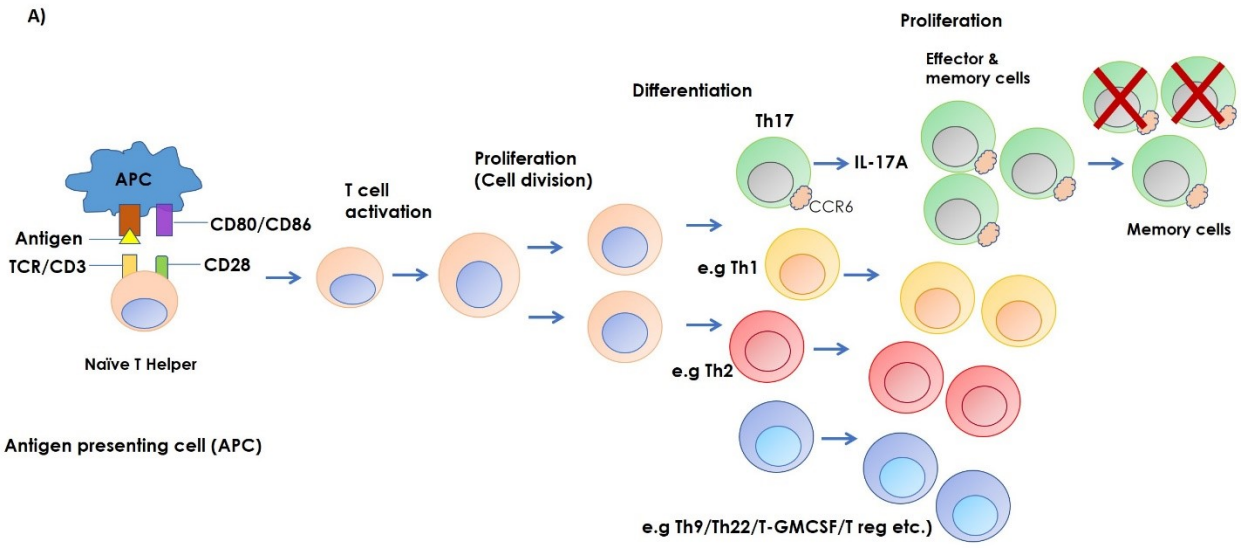
expanded number of clonal T cells all have the same cognate TCR and will create an effector response to contain, eliminate and remember the source of the antigen (11). If the antigen was a self-antigen, the clonal expanded T cells will all attack the source of the antigen leading to autoimmune disease (11). For this reason, there are many different ways that the T cell clonal expansion is are regulated (11).

Two of the best-studied regulatory mechanisms include the cell surface receptors Cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1). When the T cell is activated, CTLA-4 is induced after 24 hours, and it binds to CD80 and CD86 on APC thereby inhibiting co-stimulation and T cell activation (54). As evidence, when mice were depleted of CTLA-4, it resulted in autoimmune disease and lymphoproliferation (55–57). The severity of collagen-induced arthritis in the murine model had enhanced severity and incidence when the CTLA-4 was depleted (57). Human patients born with CTLA-4 mutations had increased CD4 T cells infiltration in various organs (e.g central nervous system, intestine), lymphadenopathy (disease of lymph nodes) and hepatosplenomegaly (swelling of liver and spleen) leading to debilitating autoimmunity (58). CTLA-4 mutations can also result in respiratory tract infections like pneumonia, gastrointestinal related like Crohn's disease, autoimmune cytopenia (lower number of mature blood cells), neurological associated diseases like autoimmune encephalitis, and malignant tumors (59). Another inhibitory receptor is PD-1, which is induced on activated T cells and binds to ligands PD-L1 and PD-L2 expressed on T cells, B cells, dendritic cells and macrophages (60,61). When PD-1 binds PD-L1, it inhibits proliferation in murine T cells activated *in vitro* with anti-CD3 (60). PD-L1 inhibits T cell activation by recruiting phosphatases to the TCR complex which dephosphorylate ITAMs thus preventing downstream signalling and proliferation (62). Recently the Nobel prize in Physiology or Medicine was awarded jointly to James P. Allison and Tasuku Honjo for their work on CTLA-4 and PD-1, respectively. Their work on these important immune-checkpoints led to the development of new therapeutic reagents that can augment T cell activation for cancer immuno-therapy (63). These results show that immunomodulation can have a major impact on health care and new treatments.

Regulatory T (T reg) cells are a T cell subset that are potent immuno-suppressors. T regs represent ~ 4-5% of $CD4^+$ T cells (64). T regs can be derived from T cell precursors, or they can be induced by cytokines TGF-β and IL-2 (65). T regs express chemokine receptor CCR8 (66) and transcription factor foxp3 (67), and $CD4^+CD25^+$ markers as observed in $CD4^+$ cells isolated from mice with quantitative PCR and western blot (67). T regs use various mechanisms to suppress activated Th cells. For example, T regs secrete IL-10 which inhibits the development and proliferation of Th17 cells, as shown in a colitis mouse model (68). Colitis can be induced by Th17 cells, and transfer of activated T regs resulted in suppression of the disease in an IL-10 dependent manner, as observed through the weight loss, endoscopic and histological colitis scores (68). Other mechanisms of action include inhibitory molecules such as CTLA-4, CD39 (nucleoside triphosphate diphosphohydrolase-1), TGF-β (69), granzymes, and perforins (70). Further evidence of the importance of T regs was performed in experiments with mice where foxp3 was deleted, which showed lymphoproliferative autoimmune syndrome (67). Moreover, mouse models (scurfy) that have mutations in the foxp3 gene develop lymphoproliferative disorder (71). Humans with mutation of Foxp3 gene (forkhead domain) are born with a fatal autoimmune disease called immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX). The IPEX patient's blood $CD4^+$ T cells showed impaired cytokine (IFNγ, IL-2, TNFα, IL-10, IL-4, IL-5) productions when activated with anti-CD3 and anti-CD28 (72). The regulatory role played by the T regs was thus shown to be linked with cytokine production by CD4 T cells. If autoreactive T cells become activated and clonally expand, they can result in autoimmunity, therefore the T regs are important in maintaining self-tolerance.

There is at least one regulatory mechanism that appears to be specific for Th17 cells, it is called the aryl hydrocarbon (AhR). AhR are cytosolic receptors that have transcription factor activities when a ligand binds. The ligands include environmental pollutants (e.g dioxins), molecules from the diet, and commensal flora or endogenous ligands tryptophan derived photoproducts 6-formylindolo[3,2-b]carbazole (FICZ) (73). When ligated, AhR, translocated into the nucleus which resulted in transcription of target genes, for example, genes for xenobiotic-metabolizing enzymes (73). Moreover, AhR can regulate T helper cells differentiation, for

example AhR is expressed on Th17 cells and the activation by ligand FICZ promotes Th17 cells differentiation (74). $CD4^+$ T cells from AHR-/- mice still had differentiated Th17 cells, however, there was no expression of IL-22 (74). The reason for this was that AhR facilitated the recruitment of the transcription factor RORγt to the promoter of IL-22 (74). IL-22 is one of the cytokines produced by Th17 cells and its pro-inflammatory, therefore AhR ligands may induce pro-inflammatory cytokine secretion by Th17 cells (74). The regulation of Th cells is important due to their roles in coordinating immune responses and if Th cells are out of control autoimmunity can happen. Understanding what can alter the regulation or what can modulate the Th cells responses is a step closer to develop therapies for autoimmune diseases.



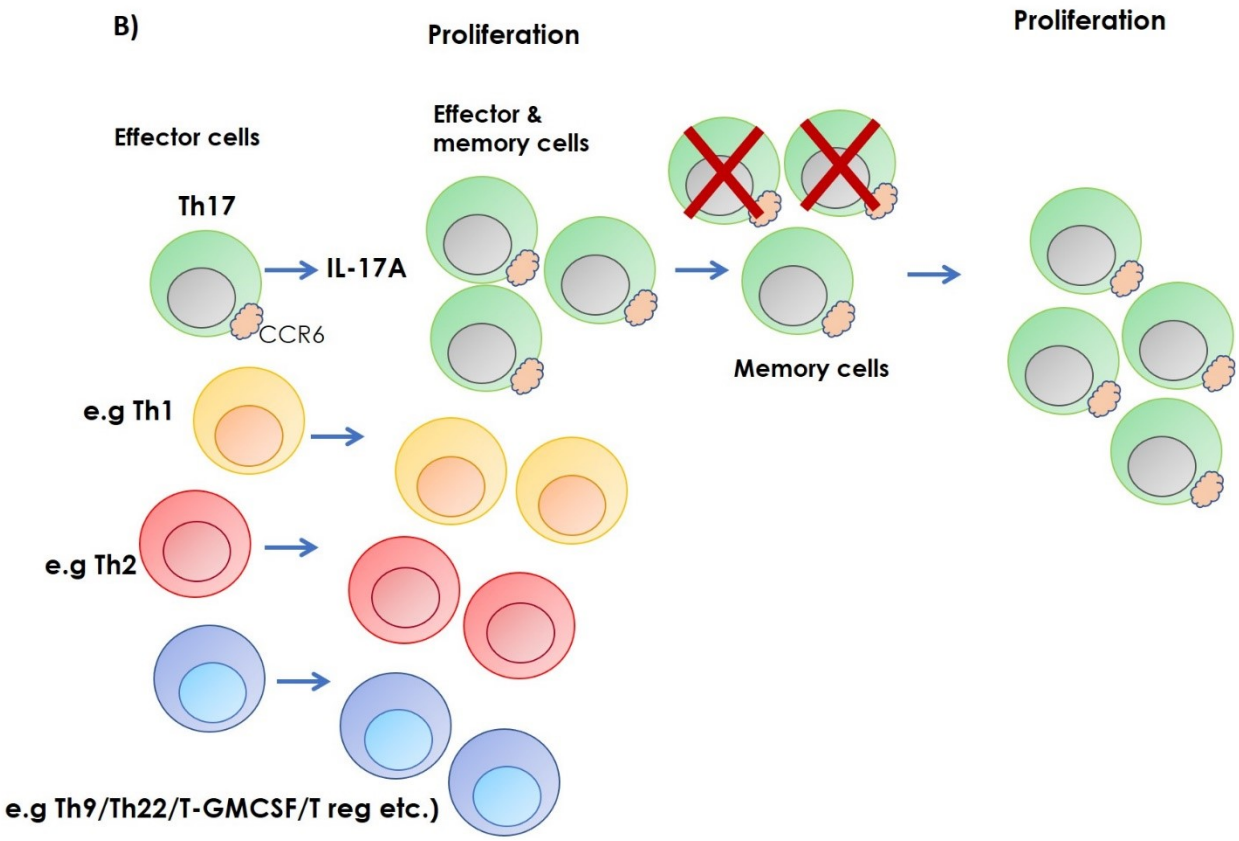



Figure 1.5. T cell activation process. A) Antigen-presenting cell (APC) presents an antigen through the MHC II to TCR of naïve Th cells. The CD4 protein helps in the recognition of the MHC II. The co-stimulatory CD28 molecule will interact with CD80/CD86 molecules present on APC which are elevated during inflammation. The stimulation of both receptors triggers T cell activation which includes proliferation and differentiation processes. Most of the effector Th cells die after their effector function is performed, but some survive and transform into memory Th cells. B) Memory cells remain for a long time in the body, and upon re-exposure of antigen, they rapidly proliferate to repel the pathogen (assuming the antigen has not mutated since the first infection). In autoimmune disease, the memory cells can be persistently activated by self-antigens leading to chronic disease. The green cells are Th17 cells, the yellow cells are Th1 cells, the pink cells are Th2, and blue cells represent the other Th cell types such as Th9/Th22/T-GMCSF/T reg etc.

## 1.7 The differentiation and function of Th cell subtypes.

Th cells have many subtypes each with a unique set of transcription factors, cytokines, and chemokine receptors. How these subtypes come about during an adaptive immune response has been the subject of intense research over the past 3 decades. When a naïve T cell emerges from the thymus and enters the circulation, it is considered naïve because it has not yet encountered its cognate peptide MHC (75). It is also called a Th0 because it has not yet differentiated. Differentiation is a process whereby an activated naïve T cell will produce different types of daughter cells, that share a common TCR, but acquire new properties depending on the circumstances of the infection (75). The new properties relate specifically to transcription factors, cytokines, and chemokines (75). As a result of differentiation, the type of adaptive immune response that occurs will change. By definition, a Th cell subset is one that has a unique transcription factor, cytokines that are produced, and chemokine receptor combinations that are commonly found on the subset. I will list the ones that fulfil most of this definition, including, Th1, Th2, Th9, Th17, Th1/17, Th22 and T-Granulocyte-macrophage colony-stimulating factor (T-GMCSF). The naming of the subsets may seem arbitrary because at first they were named in order, Th1, Th2, then after some disputes in the field regarding what Th3 would have been, researchers agreed to name subsets after the hallmark cytokine e.g. Th17 makes IL-17A, Th22 makes IL-22 and T-GMCSF makes GMCSF (76–78).

Th1 cells are defined by the production of cytokine IFNγ, transcription factor T-bet and chemokine receptor CXCR3. Evidence for this includes the observation that cells expressing CXCR3 produced IFNγ compared to CXCR3 negative that did not produce IFNγ, measured on isolated memory CD4 cells from healthy individuals and measured with the flow cytometer (79). T-bet was determined to be Th1 specific transcription factor. Northern blot analysis of RNA from clones for Th1 and Th2 showed that Th1 cells expressed T-bet when activated with anti-CD3 (TCR), compared to the Th2 clones (77). Moreover, similar results were obtained by measuring the protein of T-bet using western blot and flow cytometry techniques (77). Th1 cell

differentiation is mediated by cytokines IFNγ and IL-12. The cytokine IFNγ signals through STAT1, which activates the transcription factor T-bet the Th1 cell-specific transcription factor. T-bet leads to the expression of the IL-12 receptor complex. Stabilization of IFNγ production is mediated via IL-12 through the IL-12 receptor which signals through STAT4, leading to differentiated Th1 cells (75). The importance of STAT4 was demonstrated by Stat4-/- mice, where spleen cells had no detectable IFNγ when stimulated with anti-CD3, as measured with ELISA (80). The role of T-bet was shown by isolating murine splenocytes from transgenic TCR mouse (DO11.10) and culturing the cells under Th1 polarizing conditions. The result was that T-bet RNA expression was induced, whereas it was not induced when Th2 polarizing conditions were used (77). More proof was that when T-bet was transfected into a Th2 cell, it changes the Th2 cells into Th1 cells, as observed by retroviral transduction with GFP and T-bet on $CD4^+$ DO11.10 T cells, where the cytokines for Th1 (IFNγ and TNFα) increased and the Th2 cytokines (IL-4 and IL-5) decreased (77). These results indicated that T-bet transforms Th0 cells into Th1 cells and inhibits Th2 cells and that T-bet correlated with IFNγ expression on Th1 cells (77).

The main function of Th1 cells is the elimination of intracellular pathogens. To do this, Th1 cells produce IFNγ which interacts with the IFNγ receptor on APCs such as macrophages. The macrophages then start to consume more proteins and particles from the environment, and their antigen processing pathways are augmented (81). This means that they digest antigens into peptides, package the peptides with MHC, and transport the peptide-MHC complex to the cell surface where T cells can scan the peptide-MHC with their TCR. Moreover, some APC such as macrophages can themselves change in such a way that they promote Th1 cells. The "M1 macrophage" phenotype leads to the activation of the inflammatory process and Th1 differentiation (81,82). There are at least two possible types of macrophages, the M1 which promotes Th1 cells and M2 which promotes Th2 cells. The M1 provides anti-microbicidal properties (e.g reactive oxygen intermediates) and mediates Th1 activation by producing IL-12 (81). M1 cells can cause a sustained IFNγ in autoimmune conditions such as rheumatoid arthritis and other macrophage-associated diseases (83). In contrast, M2 macrophages promote Th2 cells and are able to reduce inflammatory responses and mediate tissue repair (81).

While Th1 cells are the major producers of IFNγ, other cells can produce it such as natural killer (NK) cells, innate-like lymphocyte cells, and CD8+ cytotoxic T cells. The role of IFNγ is primarily meant to combat intracellular pathogens. The receptor for IFNγ (IFNγR) is composed of two subunits of IFNγR1 and IFNγR2 which are expressed by most cells in the body (83). IFNγ produced by NK cells will promote the differentiation of monocytes into M1 cells which are able to promote Th1 cell differentiation and host defence against intracellular pathogens (84). IFNγ can also increase the cytotoxic capacity of cytotoxic CD8 T cells which enhanced anti-viral responses (85). To do this, IFNγ caused cytotoxic CD8 T cells to increase their content of granzymes and perforin which are cytotoxic molecules that can enter virus-infected cells and destroy the cell (86). One of the negative consequences is that sustained, high levels of IFNγ can cause chronic inflammation and autoimmune diseases with life-threatening organ failure (74,87). In high quantities, IFNγ is associated with death in patients infected with COVID19 virus, as compared to patients that recovered (88). In summary, IFNγ has many immunological functions, but it can contribute to diseases if Th1 cells are not properly regulated.

Th2 cells are defined by cytokines IL-5, IL-4, IL-13, transcription factor GATA-3, the chemokine receptor is CCR4. For Th2 cell to different from naïve T cells, IL-4 is produced by APC induces STAT6 activation, which promotes transcription factor GATA3 (89). GATA3 and c-maf promote IL-4 expression in the differentiating T cell leading to Th2 cells. Evidence for the importance of GATA-3 in Th2 cell differentiation was observed by Zheng et al. where the authors showed that GATA-3 is more expressed in Th2 clones compared to Th1 clones, as measured by RT-PCR (89). Moreover, antisense-GATA-3 construct was transfected in Th2 clones, which inhibited IL-4, IL-6, IL-5 and IL-13 (89). The authors also created GATA-3 transgenic mice where the GATA-3 was on the CD4 promoter. From the transgenic mouse, isolated naïve CD4 T cells were activated with ConA and polarized for Th1 cells (IL-2, IL-12 and anti-IL-4). Despite the expression of IFNγ in Th1 cells, Th2 cytokines were also expressed, indicating that GATA3 caused the expression of Th2 cytokines in the developing Th1 cells when GATA is overexpressed (89). The chemokine expression CCR4 by Th2 cells was seen when murine $CD4^+$ cells isolated from lymph node cells stimulated with anti-CD3 and anti-CD28 and

placed under polarizing conditions for Th2 cells (IL-4, anti-IFNγ, anti-IL12) which was measured with the flow cytometer (90). Moreover, this group also showed that IL-4-/- mice did not express CCR4 (90). Indicating that CCR4 is expressed in Th2 cells.

One of the functions of Th2 cells is to secrete cytokines (e.g IL-4, IL-5, IL-13) that promote immunoglobulin secretion by B cells (91). For example, PBMCs from allergic rhinitis and asthmatic patients showed high levels of IL-4, IL-5, and IL-13 that correlated with an increase in the antibody isotype IgE, which is produced by B cells. IgE mediates the allergies as well as the pathology of the allergic asthmas (91). Allergic reactions have eosinophilic inflammation and are mediated by IL-5 and TNFα, and asthma patients have high levels of Th2 and eosinophils in the airway mucosa (91). Thus, Th2 cells promote antibody production from B cells and are associated with inflammation in asthma and allergic reactions. Another function of Th2 cells is to eliminate parasites. Th2 cells were injected into mice inoculated with a parasite *N. brasiliensis* resulting in a significant decrease of the parasite burden compared to control mice (92). In summary, Th2 cells are defined by their cytokines IL-4, IL-5, and IL-13, chemokine receptor CCR4, transcription factor GATA-3, are associated with allergies and asthma, and protect against parasites.

Th9 cells are defined by the production of cytokine IL-9 mediated via STAT6, transcription factor GATA3, which is the same as Th2 (93). They are differentiated by TGF-β and IL-4 (94). Th9 cells co-expresses the chemokine receptor CCR3, CCR6 and CXCR3 (95). The majority of the Th9 cells express CCR6, CCR3 and CXCR3 as determined by flow cytometry (95). To demonstrate that the cytokines TGF-β and IL-4 were necessary for Th9 differentiation, activated human CD4 cells were activated with plate-bound anti-CD3, soluble anti-CD28, and cultured with TGF-β and IL-4. These conditions showed increased production of IL-9 compared to conditions without the TGF-β and IL-4 (96). The Th9 cells ($CD4^+IL\text{-}9^+$T cells ) that co-express $GATA3^+$ and $RORC^+$ did not express of IL-17A or IL-17F as measured with single-cell qt-RT-PCR (96). Memory $CD4^+$ cells induced by TFGβ and IL-4 had high levels IL-2 and IL-9 contrary to the levels of IFNγ, IL-13 and IL-17 which were significantly lower (96). One of the functions of IL-9 is to promote the production of certain antibody isotypes by B cells. When IL-9 was added to

murine B cells, the B cells produced more IgE and IgG whereas IgM isotypes were not altered as measured with ELISA (97). Another function of Th9 cells is associated with mast cell expansion. Mast cells are polymorphonuclear lymphocytes involved in allergy and asthma responses (98). For example, when ovalbumin specific Th2 and Th9 cells were adoptively transferred in mice, the Th9 cells had higher mast cells accumulation in the trachea measured by counting mast cells after being stained with toluidine blue. Furthermore, the authors observed that mast cell proteases (Mcpt1 and Mcpt2) were elevated in the lung as measured with quantitative PCR (99). Th9 cells can protect the body against parasites. In mouse models with deficient IL-9R-/-, there was increased parasites (*Strongyloides ratti)* in the intestine, and mast cell proteases that are normally required to expel the parasites were reduced (100). Thus, Th9 are defined by the cytokine IL-9 and has functions in potentiation effect on B cells antibody production and mast expansion to protect against parasites.

Th17 are defined by the production of cytokine IL-17A, transcription factor RORγ and chemokine receptor CCR6. They are differentiated from Th0 cells by cytokines IL-23, IL-6, TGFβ, IL-1 (78,101,102). IL-23 is a heterodimeric cytokine with subunit p35 and p40 (102) that signals through the IL-23 receptor. When the IL-23 receptor is stimulated by IL-23, it signals via the STAT3 pathway in T cells to promote Th17 cell differentiation (103,104). Other cytokines that are required for Th17 cell differentiation include the proinflammatory cytokines IL-6 and IL-1β, which signal through STAT3 and Akt-mTOR pathway respectively (103,105). The differentiation of Th17 cells is caused by the transcription factor Retinoic acid (RA)- related orphan receptor γ (RORγ) which is encoded by the gene *Rorc* (106,107). Th17 cells expressed the highest mRNA level of RORγ and RORα compared to Th0, Th1 and Th2 cells, as measured from $CD4^+$ T cells from mouse (OT-II) activated with OVA peptides and splenic antigen-presenting cells (78). In order for RORγ to be expressed, the cytokine IL-23 stimulates the IL-23 receptor leading to STAT3 signalling in the Th cell (78,103). To observe the relationship between STAT3 and RORγ, $CD4^+$ T cells from STAT3 knock out mice were activated with anti-CD3 and anti-CD28 and polarizing conditions for Th17 cells (IL-6, TGFβ, IL-23, anti-IL-4 and anti-IFNγ). These conditions resulted in minimal RORγ mRNA expression in the STAT3 knock out compared to the wild type

$CD4^+$ T cells (78). Similarly, RORγ -/- mice had decreased Th17 cells cytokines (IL-17, IL-17F and IL-22) and decreased IL-23 receptor mRNA levels compared to the wild type (78). Overexpression of RORα and RORγ in naïve OT-II CD4 T cells by retroviral co-infection augmented IL-17, IL-17F and IL-23R as measured with RT-PCR (78). RORγ also promoted the expression of chemokine receptor CCR6, which is one of the defining molecules expressed by Th17 cells (107). To show this, naïve T cells were derived from human cord blood from healthy newborns. They used cord blood because it contains T cell progenitor cells and is completely devoid of mature T cells, so lineage studies are not contaminated with mature T cells. The cells were activated with anti-CD3, anti-CD28, and polarizing conditions for Th17 cells, which resulted in higher IL-17A and RORγ mRNA, and higher CCR6 expression as compared to the Th1 and Th2 cells. The authors obtained similar results with memory ($CD45RO^+$) cells which had a high level of mRNA of RORγ on $CCR6^+CD45RO^+$ cells compared to Th1 and Th2 memory cells (107). *In vitro*, Th17 cells can be induced from Th0 cells with a cocktail of molecules, that includes IL-6, TGFβ, IL-23, anti-IL-4 and anti-IFNγ for murine cell cultures, however, in the human cell culture TGFβ is not added (78,108). Those culture conditions are required when differentiating naive Th0 cells into Th17 cells, however, they are not required when working with memory Th17 cells. In my thesis experiments, I did not use differentiating factors because PBMC and purified cells are memory Th17 cells that are expanded by the TCR and CD28 activating stimulus.

The principal cells that produce IL-17 and express RORC mRNA were shown to be the effector memory Th17 cells ($CD4^+CD45RO^+CCR7^-CCR6^+$), these experiments were done on isolated $CD4^+$ T cells from healthy individuals, activated with immobilized anti-CD3 and soluble anti-CD28 (109). Recently, novel molecular markers of human Th17 cells were discovered including apolipoprotein D (APOD), complement component 1, Q subcomponent-like protein 1 (C1QL1) and cathepsin L (CTSL) (110). These proteins were measured in the supernatant of the distinct isolated Th cells (Th1, Th2, T regs and Th17 cells) and the authors showed that the Th17 cells cultures had the highest amounts of APOD, C1QL1 and CTSL (110). For example, there was about ~19 fold higher APOD, ~36 fold higher C1QL1, ~6 fold higher CTSL in Th17 cells cultures

compared to Th1 cells (110). These proteins can be used in future studies to also help in the characterization of Th17 cells, as It is associated with inflammation in diseases like atherosclerosis (110).

*In vivo,* Th17 cells are produced in mucosal associate lymphoid tissues (MALT) and gastrointestinal associated lymphoid tissues (GALT) (106). In the GALT, Th17 cells are dispersed throughout the epithelium and the lamina propria which is part of the underlying layer of connective tissue (106). During infections the GALT can produce new Th17 cells and expand memory Th17 cells, resulting in potent response to bacteria or fungal infections (106). The main types of pathogens that Th17 cells help to fight against are certain bacterium (e.g *straphylococcus aureus*) and fungi (e.g *Candida albicans*) (111–114). To fight these pathogens, IL-17 recruits neutrophils and activates them at the site of infection (112). Th17 cells are essential to clear these pathogens. For example, IL-17 receptor-deficient mice were unable to recruit neutrophils to the site of infection by *Candida albicans* and failed to control the infection (112). Another example of the effect of IL-17 and neutrophils was observed by adoptively transferring Th17 cells to BALB/c mouse and exposed to ovalbumin resulted in an increase of neutrophils in the bronchoalveolar lavage fluid (115). Moreover, treating the mouse with IL-17 antibody reduced the quantity of neutrophils in the bronchoalveolar lavage fluid (115). Another function of IL-17A include the production of reactive oxygen species by endothelial cells, for instance IL-17 induced in murine brain endothelial cells reactive oxygen species (e.g superoxide) (116). These changes allow for a robust immune response at the site of infection and elimination of the pathogen by other immune cells. Thus, Th17 cells are defined by the cytokine IL-17A and has functions in recruitment of neutrophils and elimination of certain bacteria or fungi.

Th cells are the major producers of IL-17A in the adaptive immune system (117). However, there are minor producers of IL-17A. For example, $CD8^+$ T cells were purified from PBMC and stained with CCR6 markers revealing that the $CD8^+CCR6^+$ Th cells were ~ 0.26% of the Th cells as compared to the compared to $CD4^+CCR6^+$ cells which were ~5.46% of the Th cells (107). Natural killer cells from healthy humans do not express IL-17A as measured with qPCR

and IL-17 ELISA and intracellular cytokine staining (118). Regulatory T cells can also express IL-17A, although at low abundance, only 0.22% of regulatory T cells ($CD4^+Foxp3^+$), express IL-17 from healthy individuals (119). In my thesis, I used PBMCs which are mostly composed of Th cells but may also contain some of the minor subsets. To address that I also made use of purified Th Cells, purified Th17 cells, and intracellular flow cytometry in selected experiments which can pinpoint IL-17A sources. The details and limitations are expanded upon in the results section and general discussion.

Th22 cells are defined by the production of cytokine IL-22, the transcription factor RORγ, and chemokine receptors including CCR4, CCR6 and CCR10 (120). Th22 cells are differentiated from naïve T cells by the cytokines IL-6 and TNF-α (120). Naïve CD4 cells under Th22 polarizing condition from *Rorc(γt)-/-* mice had a reduction in IL-22 (121). To demonstrate Th22 chemokine expression, experiments were performed using human CD4 cells sorted by $CCR10^+CCR6^+CCR4^+$. The results showed that Th22 cells only produced the cytokine IL-22 and not other cytokines, as measured with flow cytometry (120). IL-22 is the ligand for IL-22 receptor (IL-22R) which has a STAT3 cell signalling pathway and is expressed throughout the body (122). IL-22 stimulates IL-22R on tissues including skin, digestive or respiratory tissue, where it mediates the production of antibacterial proteins (123). IL-22 also increased mucins which were associated with the amelioration of chronic colitis in the mice (124).

There is a hybrid called 'Th1/17 cells' in humans that expresses elements of both Th1 and Th17, including transcription factors T-bet and RORγ, cytokines IL-17 and IFNγ, and chemokines receptor CCXCR3 and CCR6. In a study by Boniface *et al*, human naïve T cells were isolated from PBMCs and placed under Th17 inducing conditions (IL-1β, IL-23 and $PGE_2$). This resulted in three types of cells: one that only produces $IL\text{-}17^+$, one that only produces $IFN\gamma^+$, and one that produces both cytokines $IL\text{-}17^+$ and $IFN\gamma^+$, as measured with dual cytokine staining and the flow cytometer (125). The authors also showed that the Th1/Th17 cells co-express T-bet and RORγ as measured by real-time PCR. Moreover, the percentage of Th1/17 cells was up to 15% and an average of ~7.5% amongst the human samples tested (125). Besides the co-expression of transcription factors, it was observed that Th1/Th17 cells co-express chemokine

receptor CCR6 and CXCR3 from $CD4^+$ cells from healthy individuals (126). Th1/17 cells were stimulated with anti-CD3 and IL-2 and showed to express both cytokines $IL\text{-}17^+/IFN\gamma^+$, which was measured with the flow cytometer (126). Although Th1/Th17 cells produce IL-17, the percentage of IL-17 expressed by the Th1/Th17 ($CCR6^+CRXCR3^+$) cells was four-fold lower than in Th17 ($CCR6^+$) cells, measured in PBMCs from healthy individuals by flow cytometer (127).

Th GMCSF cells are a subtype of T cells that produce GM-CSF, are induced by IL-2, and express chemokine receptor CCR10 and CCR4 and transcription factor T-bet (128). Th GMCSF populations expressing GMCSF and T-bet comprise up to 2% of the $CD4^+$ population in human PBMC (128). As mentioned, T-bet is the Th1-specific transcription factor. There are no known transcription factors for Th GMCSF cells. Th GMCSF producing cells expressed $CCR4^+$ $CCR10^+$ $CXCR3^-$ $CCR6^-$ as shown by flow cytometry (128). For a naïve T cell to differentiate into Th GMCSF cells, IL-12 cytokines are required to promote differentiation, while IL-1β, IL-6, IL-23 inhibit differentiation as was shown in naïve CD4 cells isolated from human PBMCs (128). Differentiation of Th GMCSF cells requires STAT5 since blocking STAT5 with an inhibitor in naïve cells prevented IL-12 from promoting Th GMCSF differentiation (128). Th cells can co-express GMCSF along with IL-17 and IFNγ. The percentage of healthy human $CD4^+$ cells co-expressing $GM\text{-}CSF^+IL\text{-}17^+$ are ~0.45%, $GM\text{-}CSF^+IFN\gamma^+$ are ~ 4.76% and $GM\text{-}CSF^+IL\text{-}17^+IFN\gamma^+$ are ~0.16% as measured with flow cytometry techniques (128). The function of GMCSF is to promote macrophages to kill bacteria by enhances the phagolysosomal fusion (129). GMCSF is elevated in patients with psoriasis, chronic inflammatory skin diseases, suggesting that it can also exacerbate an inflammatory condition (130).

In summary, Th17 cells represent one subset among a wide variety of Th cell subsets. The exact context of an infection or autoimmune condition will determine which Th subsets expand, and how they will contain a pathogen or exacerbate inflammatory conditions.

## 1.8 Regulatory relationship of Th subtype Th1, Th2 and Th17

There are relationships between the Th cells subtypes where factors of one subtype can affect the differentiation of the other Th cells subtypes. For example, Th2 cells inhibit Th1 cells by secreting IL-4 which inhibits IL-12 receptor on developing Th1 cells. Since IL-12 is required for T-bet and differentiation of Th1 cells, Th2 cells can repress Th1 cells in this way (76). Another molecular aspect are STAT signalling pathways. STAT4 phosphorylation is correlated with the expression of IL-12Rβ2 and it was observed on murine CD4 T cells that are treated with polarizing conditions for Th2 (IL-4 and anti-IL12) that there was lost of STAT4 phosphorylation (76), indicating that Th2 can suppress Th1 cells. Conversely, murine CD4 Th cells activated with anti-CD3 and treated with anti-IL4 (Th2 neutralizing condition) and IL-12 resulted in IFNγ producing cells (131). The cytokine IFNγ produced by Th1 cells suppresses Th2 cells differentiation via induction of transcription factor T-bet resulting in induction of transcription factor Runx3 where together enhances the production of IFNγ by binding to the IFNγ promoter, at the same time binds to the IL-4 gene (IL-4 silencer) which silences the IL-4 gene (132). There are other STAT molecules involved, for example, Th2 cells required IL-4-STAT6 pathway for their differentiation (133). IFNγ suppressed STAT6 pathway in part by inducing inhibitory protein-suppressor of cytokine signalling (SOCS1) which in term inhibited IL-4 receptor signalling (133). Murine CD4 Th cells overexpressing SOCS1 showed suppression of STAT6 and IL-4 whereas reduction of SOCS1 showed stimulation of STAT6 (133). Thus, Th1 and Th2 cell differentiation processes oppose each other, which is a way that the immune response is skewed to Th1 or Th2 but not both.

There are regulatory checkpoints between Th1, Th2 and Th17 cells. Harrington *et al*. first showed that differentiation from murine naïve CD4 cells of Th17 cells was treated with IL-23 and IFNγ neutralizing antibody, resulted in an increase of IL-17 (131). To demonstrate the regulation by IFNγ on IL-17, naïve T cells from IFNγ deficient mouse were isolated and the addition of exogenous IFNγ inhibited IL-17 producing cells (131). To show the effect of Th2 (IL-4) on Th17 cells, murine CD4 T cells were treated with exogenous IL-4 (Th2 polarizing condition)

resulting in Th17 development being blunted (131). This indicates that Th17 cell differentiation is suppressed by Th1 and Th2. IL-23 is a polarizing cytokine for Th17 cells expansion, it promoted IL-17 but diminished IFNγ production by activated anti-CD3 and anti-CD28 $CD4^+$ human T cells (134). When the IL-23 was added to the cultures the IFNγ was decreased and IL-17 increased, whereas when IL-12 was added the cell cultures the IFNγ was increased and IL-17 was decreased (134). Furthermore, murine *Il17a*-/- $CD4^+$ T cells had increased IFNγ, and IL-17A inhibited Th1 by suppressing effector genes (e.g *ifng*, *il12rb2*), STAT1- phosphorylation and T-bet (135). Thus, Th1 and Th17 cells display a down-regulatory cross talk.

## 1.9 Immunological memory is maintained by long-lasting memory T cells

After the infection is cleared by the adaptive immune system most of the effector T cells generated by the clonal expansion will die. Some of them transform into long-lasting memory T cells after the infection is cleared. Immunological memory also involves memory B cells which differentiate into plasma cells upon re-encounter of specific antigen and have a rapid and effective antibody response (136).

The memory cells can be categorized into two main subtypes, the central memory (TCM) and the effector memory T cells (TEM). The TCM has high expression of homing chemokine receptor and adhesion molecules which allows for entry into secondary lymphoid organs. TEM have lower levels of homing chemokine receptors and adhesion molecules which makes them more likely to traffic through peripheral tissues (23,137). Both types of memory cells work together as the TEM control the initial exposure of the antigen, and the TCM scan APC/antigens in the lymph nodes, spleen, and associated lymphoid tissues like GALT. Memory cells are important because they can react much faster than naïve T cells, thus providing robust immunity (23,137). They also retain their differentiated subtype, for example, memory Th17 cells having markers such as CCR6 and RORγ will be long-lived after an infection (138,139). Th17 cells display higher proliferation ability compared to Th1 and Th2 cells. There were higher levels of cyclin genes and lower levels of cyclin-dependent repressors in the Th17 cells, as

demonstrated in human memory Th17 cells (140). Moreover, Th17 cells have lower apoptosis as they express higher amounts of *BCL* family genes (e.g *BCL2* and *BCLXL*) and hypoxia-inducible factors (140). The activation of hypoxia-inducible factors is a contributor to *BCL* genes, and it mediates its signal through notch signalling that promotes the survival of Th17 cells (140). The hypoxia-inducible factors make that Th17 cells are resistant to apoptosis, especially in low-oxygen environments of inflamed tissues (140). These features of Th17 cells show that these cells are long-lived and persistent. The memory system ensures that a second infection with the same pathogen does not cause illness, it is also the basis for immunization by vaccines (141), which prompts naïve T cell activation and subsequent immunity against an actual infection.

## 1.10 Pro-inflammatory versus anti-inflammatory paradigm in autoimmune diseases. Role of Th cells.

One of the paradigms that immunologists have used to understand immunomodulation is that of a balance of pro-inflammatory versus anti-inflammatory. Inflammation is defined as heat, pain, swelling and immune cell infiltration at sites of injury or infections. Pro-inflammatory mediators are molecules and immune cells that cause these signs of inflammation in the tissues. On the other hand, anti-inflammatory mediators are cells and molecules that inhibit inflammatory responses. In the context of autoimmune disease, there are specialized cells like the T helper cells that secrete cytokines that are pro- inflammatory (e.g IL-17A) and other cells that release anti-inflammatory cytokines (e.g IL-4). Therapies target inflammatory Th cells due to their contribution to pathogenesis in autoimmune diseases (e.g multiple sclerosis). For instance, glatiramer acetate is used in MS as a therapy, it promotes anti-inflammatory responses, by promoting Th2 while it decreases pro-inflammatory Th subtypes such as the Th1 cells or Th17 cells (142). PBMCs and serum samples from MS patients taken before and after three to six months of treatment with glatiramer acetate, showed decreased of pro-inflammatory cytokines including but not limited to IL-17 and IFNγ and increased in anti-inflammatory cytokines such as IL-4 and IL-10. However, not all patients responded to the

treatment (142). The Th17 cells are considered to be pro-inflammatory because CCR6 allows Th17 cells to enter inflamed tissue including the crossing of the blood-brain barrier, and it opens up the blood-brain barrier allowing other cells to enter the central nervous system and cause tissue damage (108). Once in the tissue, IL-17 cells then recruit neutrophils and macrophages to sites of inflammation which in turn release cytotoxic molecules (143). In a patient with autoimmune disease such as MS, the presence of these immune cells in the central nervous system results in chronic inflammation. Thus, therapies for autoimmune diseases seek to rebalance the inflammation by decreasing pro-inflammatory responses and shifting towards anti-inflammatory responses.

Although Th17 cells are essential in the host defence against infections, these cells have may be a cause of symptoms in autoimmune diseases, such as MS, psoriasis and rheumatoid arthritis (108,144–146). Animal modelling studies confirmed that Th17 cells were linked with inflammatory autoimmune diseases, for example, Th17 cells could transfer disease from a mouse with EAE to a healthy mouse (147). Researchers used the EAE model to demonstrate that passive transfer of T cells from mice with EAE to healthy mice was more efficient with Th17 cells as compared to Th1 cells (147). Even though both Th17 and Th1 cells are pro-inflammatory and transfer EAE, they showed a different phenotype of the disease (148–150). Th1 cells caused classical paralytic EAE which characteristics is paralysis progressing from tail to head, whereas, Th17 mediated atypical ataxic EAE which characteristics are lack voluntary movement and unbalanced walking (150). In another study, EAE was induced by adoptive transfer of either Th17 or Th1 cells, which showed that Th17 cells induced more mononuclear cells, IL-17 secretion, and neutrophils in the CNS compared to the adoptive transfer of Th1 cells (149).

Despite the difference listed above, both Th1 and Th17 cells can cause autoimmune disease in mice models, and they have been implicated in disease severity and relapses in humans with MS. Both Th1 cells and Th17 cells were observed within the CNS of EAE, and within the active lesions of MS patients (45). Th1 cells activate microglia which promotes inflammation and lesion development (151). Th17 cells exacerbate inflammation and recruit innate immune cells by secreting IL-17A (151). Another detrimental role of Th17 cells is to open

the blood-brain barrier. The blood-brain barrier is a tight junction of endothelial cells that separates the CNS from the general circulation. It has been shown that IL-17 disrupts tight junctions of the blood-brain barrier, thus allowing Th17 cells to cross into the CNS (108,152). This increase in vascular permeability allows Th1 cells and more Th17 cells to enter the CNS, which in turn recruits and activates myeloid cells which exacerbates inflammation (151). Moreover, Th17 cells activate neural and microglia in the CNS, which amplifies neuroinflammation in EAE (153,154). Thus, Th1 and Th17 cells are pro-inflammatory and contribute to chronic illness in MS patients.

Another example of IL-17 exacerbating autoimmune disease, is in rheumatoid arthritis, where synovial fluid has high levels of IL-17A that exacerbates associated joint destruction. IL-17A mediates the increase of chemokines expression on fibroblast-like synoviocytes, by recruiting macrophages, neutrophils, dendritic cells and Th17 cells promoting the inflammation in the joints (155,156). In summary, Th17 cells and its hallmark cytokine IL-17A are important for host defense against pathogens but can exacerbate autoimmune disease if they become dysregulated.

## 1.11 Possible therapeutic potential of inhibiting Th17 cells.

Immunomodulatory therapies are of interest for treating autoimmune diseases. For example, EAE symptoms were diminished in IL-17 deficient animals, and the severity of the EAE was reduced by neutralizing IL-17 with an anti-IL-17 antibody (157,158). Similarly, in animal models for arthritis, the severity and joint damage were reduced with IL-17A neutralizing antibodies (159,160). Secukinam, an IL-17 inhibiting antibody, is being studied as a potential treatment for psoriasis (an autoimmune disease of the skin) in humans (161). This antibody is also being studied as a potential treatment for MS, but it is not yet used clinically (162). There are other approaches to inhibit Th17 cells, such molecules that target RORγ, namely digoxin, urosolic acid, SR1001 and TMP778, which cause a reduction in the severity of symptoms in EAE (163–167). Therefore, neutralizing IL-17A or directly inhibiting Th17 cells has potential benefits

for treating Th17 mediated inflammatory autoimmune diseases such as MS and rheumatoid arthritis.

Many of the established drugs and therapies that are used in the clinic or in clinical trials are known to suppress Th17 cells. For example, glatiramer acetate, fingolimod, dimethyl fumarate and IFN-β inhibited Th17 cells along with their other effects (45). Fingolimod decreased both Th1 and Th17 cytokine in MS patients (168). IFN-β, which is an antiviral cytokine, reduced IFNγ and IL-17 cytokine levels which shifted towards a protective Th2 phenotype (45,169). A type of stem cell therapy, called mesenchymal stem cells, inhibited Th1 and Th17 responses in EAE (170). Mesenchymal stem cells were tested because they are known to suppress the immune system by secreting prostaglandin E2, which can suppress T cells (171,172). However, in pre-clinical testing mesenchymal stem cells increased IL-17A from Th17 cells suggesting that the therapy might exacerbate MS (171). Another stem cell therapy called autologous hematopoietic bone marrow transplantation was tested in clinical trials, it stopped disease progression in severe MS patients, and inhibited Th17 cells (but not Th1 cells) in an immunology sub-study on clinical samples (173). In summary, inhibiting Th17 cells is a potential way to treat certain autoimmune diseases.

Off-label use of drugs means that a drug was approved for one disease but was found to be useful in another disease. Clinical trials still have to be conducted for safety and efficacy, however, the major costs associated with new drug clinical trials are avoided with off-label drug prescriptions. In MS, off-label drugs in use include Rituximab (antibody targeting CD20 surface marker on B cells) is a treatment for rheumatoid arthritis and B-cell malignancies (174–176), modafinil which is prescribed to used to treat narcolepsy (177), Amitriptyline is used to treat depression (178), and albuterol a beta-agonist which was tested in an add-on study with glatiramer acetate (brand name Copaxone) (179). These drugs have been tested or prescribed as off-label in MS patients. The disease-modifying therapies (e.g dimethyl fumarate) for MS in the United States can have annual costs of ~$50,000 or in Canada of ~ $20,000 (180). An example of the lower cost the off-label use of Rituximab in the United States compared to the

therapies already in use for MS, the range of Rituximab treatment can be lower than ~$20,000, (~ $8,000 to $16,000) (174).

In my thesis, I found evidence that nebivolol might be a useful immunomodulatory drug. The use of nebivolol as an off-label treatment for MS could, in theory, fall under the reduced cost range that can be similar or lower to Rituximab. As nebivolol tablets can be sold as low as 0.61 cents per tablet for 2.5mg (181). Aside from the cost consideration, off -label drugs can provide more options for neurologists to treat MS. Not all patients will respond to a given drug, and some drugs loose effectiveness over time, or as the disease evolves in a patient. In the conclusions of my thesis, I discuss nebivolol and its potential off-label usefulness in treating autoimmune disease such as MS.

## 1.12 SNS innervation of lymphoid organs by the sympathetic nervous system, and modulation of Th cells

In the previous sections, T cells and their role in protective immunity, and autoimmunity was discussed, as was the importance of immunomodulatory drugs in treating autoimmune patients. In my thesis, I focused on the β2AR because it is an important regulatory receptor that was first characterized in the context of the sympathetic nervous system (SNS). The SNS can modulate the immune system by releasing epinephrine (adrenaline) and nor-epinephrine (noradrenaline) into body tissues. These hormones are catecholamines synthesized from tyrosine by tyrosine hydroxylase which is the rate-limiting step. As they are produced, they can be released from SNS nerve endings, interact with adrenergic receptors, and regulate many metabolic changes and tissue responses throughout the body. For example, they increase breathing rate, blood flow to the muscle, and release of glucose from the liver which prepare the body for physical or perceived psychological danger, the so-called fight or flight response (182).

The SNS innervates almost every part of the body. With respect to the immune system, the sympathetic nerves are directly connected to lymphoid tissues (183–185). The sympathetic nerve fibers are distributed along the vasculature and areas of the cortex where T cells are found in close proximity to the SNS nerve endings (183–185). There are also SNS adrenergic nerve fibers around the splenic artery which travel along the white pulp and periarterial lymphatic sheath (183–185). The white pulp and the periarterial lymphatic sheath are locations that also have abundant T cells. In lymph nodes, there are sympathetic nerve fibers in the marginal zone and marginal sinus where T cells reside (183–186). The noradrenergic fibers run alongside the vasculature and lymphatic channels in the lymphatic channels in the medulla and are distributed in vessels of the paracortical and cortical regions (183–185). Furthermore, the MALT and GALT are innervated with sympathetic nerves fibers in the vasculature and parenchymal areas of lymphocytes, these areas contain T cells and plasma cells (183,187). This information confirms that the SNS is in close proximity to T cells in lymphoid tissue. The release of NE in the spleen and bone marrow in mice was measured using the severe combined immunodeficiency (SCID) mice that were reconstituted with clones of Th2 specific for antigen-keyhole limpet hemocyanin (KLH) and B cells specific for antigen trinitrophenyl (TNP) (188). The results showed that NE was released from nerve terminals after 8h with antigen challenge (TNP-KLH), as it was measured with [$^3$H]NE turnover analysis (188). This study also showed that using ganglionic blocker the NE induced by antigen challenge was blocked (188). The latter study showed that there was a release of NE and occurred after 8h of antigen challenge and that the B cells which express the β2AR can have increased antibody production. Thus, the release of NE from the sympathetic nerves upregulates immune cells.

## 1.13 The β2AR is a G- protein-coupled receptor with several cell signalling pathways and ligands.

The catecholamine hormones are sensed by adrenergic receptors found throughout the tissues of the body. Adrenergic receptors (AR) are divided into two main families, αAR and βAR,

each of which has additional subfamilies (189). The βARs are further divided into β1AR, β2AR and β3AR. My thesis focused on the β2AR due to its implications for immunity, which will be introduced in section 1.16. In this section, I will review the knowledge regarding β2AR signalling that was derived from non-immune cells and tissues. The β2AR belongs to a family of guanine nucleotide-binding protein (G-protein) coupled receptors (190). It has 7 transmembrane-spanning α-helices, 3 extracellular loops with amino-terminus and 3 intracellular loops with carboxy-terminus (191–193). β2AR has two pathways, the canonical GPCR pathway, and the arrestin pathway. The canonical GPCR signalling pathway of β2AR involves heterotrimeric G proteins alpha (α), beta (β) and gamma (γ) subunits which interact with the cytoplasmic tail of the receptor. When activated, the Gα subunit the GDP is exchanged for GTP (190). The $G_{\alpha}$ subunit now bound to GTP dissociates from the $G_{\beta\gamma}$ subunit and stimulates AC (190). AC catalyzes the formation of cAMP by the hydrolysis of adenosine triphosphate. The second messenger cAMP mediates its effect by effector molecules that include PKA. PKA is a holoenzyme that has two catalytic subunits are associated with the regulatory subunit dimer. When the regulatory subunit is associated together with the catalytic subunit, the enzyme is maintained in an inactive conformation. The consecutive binding of cAMP to the regulatory subunits of PKA results in conformational changes that releases the active catalytic subunits. The catalytic subunits can then phosphorylate target substrates on serine or threonine residues of sequences Arg-Arg-X-Ser/Thr, Arg-Lys-X-Ser/Thr, Lys-Arg-X-Ser/Thr, or Lys-Lys-X-Ser/Thr (18,190). One important target substrate is 'transcription factor cAMP response element-binding protein' (CREB), which once phosphorylated at ser133, can interact with coactivator protein to start CREB responsive genes (37). Moreover, PKA regulates other proteins including L-type $Ca^{2+}$ channel (194). Stimulation of β2AR with carvedilol results in cAMP/PKA signalling in local plasma membrane domains which phosphorylate and activates L-type $Ca^{2+}$ channel ($Ca_v$ 1.2) in HEK293 cells expressing FLAG-tagged β2AR, where phosphorylation was measured with western blot and $Ca_v$ 1.2 activity in murine hippocampal neurons with patch-clamp electrophysiology technique (194). When antagonist ICI and PKA inhibitor H89 were used, the $Ca_v$ 1.2 phosphorylation was blocked, indicating that phosphorylation of $Ca_v$ 1.2 was mediated via the cAMP-PKA pathway induced by β2AR stimulation (194).

The stimulation of calcium channel by PKA results in calcium influx, which stimulates nitric oxide synthase (NOS) enzymes which produce nitric oxide (NO) an important cell signalling molecule (195). NO stimulates guanylyl cyclase enzyme which converts GTP to cyclic guanosine monophosphate (cGMP) a second messenger structurally similar to cAMP but with different roles than cAMP (195). One role of the second messenger cGMP is to activate protein kinase G (PKG). In a study in mice cardiomyocytes, it was demonstrated that cGMP had an anti-hypertrophic effect on the cardiomyocytes (196). The authors demonstrated the antihypertrophic effect using nebivolol and PKG inhibitors and showed that the effect was mediated by the cGMP/PKG signalling pathway (196). In a study performed in human vascular endothelial cells, it was shown that the increase in intracellular $Ca^{2+}$ increased the eNOS phosphorylation, as it was demonstrated using drugs that remove $Ca^{2+}$ from the medium (197).

Another PKA-independent pathway is the 'exchange proteins activated by cAMP' (EPAC). Upon activation by cAMP, EPAC can phosphorylate and activates PKC which in turn phosphorylates and activates MAPK (198). Neonatal mouse cardiac fibroblasts stimulated with βAR agonist-isoproterenol produced more IL-6, mediated via EPAC/PKC/MAPK pathway (198). The authors knocked down EPAC by adenovirus, which inhibited MAPK activation, as measured by immunoblot analysis (198). The IL-6 production was also inhibited when PKC was knocked down using shRNA (198). Thus, EPAC/PKC/MAPK pathway can be stimulated with βAR-isoproterenol in neonatal mouse cardiac fibroblast.

There are also β2AR cell signalling pathways that are not caused by cAMP or PKA. For example, the arrestin cell signalling pathway is induced when the β2AR cytoplasmic carboxy-terminus tail is phosphorylated by a G protein-coupled receptor kinases (GRK). GRK enzymes have 7 members which phosphorylate β2AR tail and recruit arrestin the cytoplasmic tail of the receptor (199). Arrestin is a scaffolding protein that plays roles in degrading cAMP and desensitizing the receptor (200). For example, β-arrestin was in close proximity to β2AR as shown using bioluminescence resonance energy transfer (BRET) in transfected FLAG-tagged β2AR HEK293 (embryonic human kidney cells) cells stimulated with isoproterenol (199). Arrestin recruits phosphodiesterase enzymes (PDE) which break the phosphodiesterase bond of

cAMP (200). This action limits the duration of the Gs-stimulated cAMP signals (201). Evidence for this includes a study by Perry *et al*. who showed an increase in β-arrestin recruitment and PDE4D3 in isoproterenol-stimulated HEK293 cells. The cells used were transfected with FLAG-β2AR green fluorescent protein construct and measured with immunoprecipitation technique which was observed to recruit PDE4D3 and β-arrestin when treated with isoproterenol (202). The authors also examined mouse embryonic fibroblast-derived from a β-arrestin knockout mouse. When the fibroblasts were treated with isoproterenol, PDE4D3 did not associate with the membrane arrestin (202), indicating that arrestin is necessary for PDE4 recruitment. Knocking down β-arrestin 1 and 2 with small interfering RNAs (siRNA) in HEK293 cells resulted in cAMP accumulation, indicating that β-arrestin 2 has a role in suppressing cAMP levels (203). Arrestin also causes receptor internalization by clathrin-coated pits (190). An example of β-arrestin mediated internalization was shown by O'Hayre *et al.* which showed that β-arrestin 2 promotes β2AR endocytosis via clathrin-coated pits when stimulated with isoproterenol, it was observed by using siRNA targeting β-arrestin HEK293 cells. The latter experiments showed that the receptor did not cluster in clathrin-coated pits when the β- arrestin was knock down (204). This was demonstrated by measuring total internal reflection fluorescence microscopy on FLAG- β2AR HEK293 cells (204). Moreover, it was shown that there was an attenuation of β2AR internalization on the β-arrestin 2 KO cells with stimulation of agonist isoproterenol, which was measured in the siRNA suppressed β-arrestin 1 and 2 HEK293 cells (203). Thus, stimulation of β2AR leads to β-arrestin which can limit the cAMP pathway by recruiting PDE, internalization of receptor, and by initiating a signalling pathway-ERK.

There are numerous ligands for the β2AR that are endogenous or created as drugs. The endogenous ligands include norepinephrine and epinephrine which are part of the catecholamine family (205). There is also a wide range of pharmacological drugs that stimulate this receptor. Which pathway predominates will depend on the ligand present. An agonist ligand will promote the GPCR pathway, in contrast, an inverse-agonist will promote the arrestin pathway (2). Partial agonists do not exhibit the maximal activity of agonists but still renders the ligand-binding site active to a certain extent (193). Antagonists do not disturb the equilibrium

state but it is a competitive ligand for the binding site and prevents the binding of agonists, inverse-agonists, and partial agonists (206). Some of these drugs have similar structures as the hormones, which involve a combination of a catechol chemical group, but they have been modified for improved pharmacokinetics (205). The drugs are also modified to improve receptor-specificity since endogenous hormones react with all of the adrenergic receptor subtypes albeit with differing affinities. Terbutaline, which is a β2AR-specific agonist drug, has an N-t-butyl and a resorcinol phenyl ring, as compared to norepinephrine that has the catechol ring, β-hydroxyl group and an amine group (207). Due to its structure, terbutaline is resistant to the action of catechol O-methyltransferase, which action is to degrade catecholamines (208).

For this reason, terbutaline binds preferentially to β2AR while norepinephrine binds preferentially to αAR, although norepinephrine will also bind β2AR if hormone concentrations are high enough, or if a tissue lacks αAR. Drugs such as terbutaline were developed this way to be more specific for acting on lung tissues, where the β2AR agonist effect promotes relaxation of bronchioles and improved airflow in asthmatics (209–211).

In the absence of a ligand, there is an equilibrium state of the β2AR (212). This is where the β2AR still has a low but detectable level of GTP exchange with GDP that releases a Gαs and promotes a low level of cAMP signalling in absence of ligand (212). The ligands can then inhibit or promote this basal cAMP signalling in order to maintain homeostasis (212). When an agonist binds, there is a conformational change in the receptor that involves a rotamer toggle switch. The rotamer toggle is a structure in the intracellular domain where the receptor twists in the middle (TM6) leading to changes in the cytoplasmic tail of the receptor and changes to G protein binding (193,212). There is an ionic lock that is between the conserved E/DRY motif on the TM3 and the Asp/Glu of the TM6 (213). When an agonist binds, there is an outward movement of the TM6 cytoplasmic end where the ionic lock breaks (213). These conformational changes result in creating a cavity where G protein can bind as was demonstrated with crystallography studies (213). A study by Yao *et al.* showed when the ligand binds, the conformational changes of the β2AR can be monitored by the fluorescence emitted as the β2AR is labelled at Cysteine 265 is located in TM6 tail and is in a cavity between the TM3,

TM5 and TM6, that has a conformationally sensitive fluorescent probe, in this study, they labelled it with monobromobimane using sf9 insect cells (214). When the β2AR is active there is a tilt in the TM6 that results in activation of the receptor and thus conformational change that would displace the fluorescent chemical and the intensity and maximal emission wavelength can be measured. Isoproterenol (agonist) increased the fluorescence intensity and maximal emission wavelength (214). ICI 118-551 stabilized an inactive conformation of the β2AR, resulting in the prevention of the coupling of the β2AR and the Gs complex as it was measured by the fluorescence intensity (214). The conformational change caused a guanine nucleotide exchange factors (GEF) to exchange GTP for GDP on Gα, then Gα s is released from the heterotrimer (213). In the presence of an agonist, the equilibrium state shifts towards being active (213). The agonists, which can be hormones or drugs, typically have a positively charged amine or ethanolamine group, called the "tail", which can interact with the "head' and another anchor site on the transmembrane 5 of the receptor resulting in β2AR signalling through G proteins (193). Moreover, the ligands bind to polar and hydrophobic contact residues within the transmembrane helices (190,193). In the presence of an inverse-agonist, the equilibrium state shifts towards being less active, and arrestin pathways may come into play (215). The inverse-agonist stabilizes the inactive receptor conformation by not allowing the movement of the transmembrane 5 (TM5), as compared to the agonist where increased binding affinity is associated with TM5 (193). Using computational methods, the conformational changes of the β2AR was observed where the TM5 tilts towards the binding pocket on TM3/7 where the ethanolamine group of the ligand binds, using isoproterenol (agonist) the TM5 movement increases predicted binding affinities (2-3pKd) (~1000 fold) compared to inverse-agonist (carazolol) where the TM5 did not tilt and thus there was little change in the binding affinity (0.5 pKd) (193). When Asp113 located in the transmembrane 3 is mutated to Ser by site-directed mutagenesis, β2AR  was not activated by albuterol as compared to the wild type β2AR, the experiments were performed on Chinese hamster fibroblast (216). There are key agonist binding sites on β2AR, which include Ser 203, Ser207, Ser204 located in TM5, which interact with the hydroxyl group of the agonist (217). These studies demonstrated that there is ligand binding at transmembrane 3 and one of the binding sites is Asp113, and transmembrane 5 with

Ser 203, 204 and 207 and that conformational change of the receptor upon ligand binding can then result in intracellular signalling pathways.

There are no known endogenous inverse-agonists produced by the body. There are, however, several pharmacological drugs that have this effect on the β2AR. Carazolol and nebivolol are two examples of inverse-agonists, they are commonly used for treating cardiovascular diseases (209,210,218). Nebivolol is a selective β1AR antagonist and an inverse-agonist of the β2AR (2,219). Nebivolol promoted GRK/β- arrestin recruitment to β2AR which phosphorylated ERK and inhibited the cAMP-PKA pathway (2). Another cell signalling pathway invoked by nebivolol is that it promotes nitric oxide production, as seen in mouse thoracic epithelial cells which vasodilate in response to nebivolol (220). Nebivolol has not been explored in the context of immunomodulation which is why it is a novel aspect of my thesis. In Chapter 3 I found that nebivolol will inhibit IL-17A and IFNγ.

To summarize, β2AR is a G- protein-coupled receptor with an agonist-induced cell signalling pathway mediated via the cAMP-PKA. Inverse agonists can oppose this pathway and induce alternative pathways via arrestin or NO molecules. The results summarized in this section were mostly from non-immunological cells and tissues. The novelty of my thesis was to test β2AR agonist or inverse-agonist on Th17 cells, which may help to create new therapies for autoimmune diseases.

## 1.14 Polymorphism of the β2AR may affect the activity of adrenergic drugs

Adrenergic drugs are prescribed for respiratory, cardiovascular, and psychiatric diseases, however, their responses can be variable due to polymorphisms within or adjacent to the gene encoding the receptor (221). Pharmacogenomics is a field of research that involves the study of the effect of SNPs on drug responses (221). An SNP is defined as a variation at a single nucleotide in the coding sequence of a gene, intronic DNA or in the satellite regions upstream

or downstream. There are synonymous SNP, in which the encoded amino acid is not changed, and the nonsynonymous SNP for which the encoded amino acid is changed in the protein (221).

The β2AR is known to have common SNPs found in and surrounding its gene, *ADRB2*, which is an intronless gene on chromosome position 5q31–32 (222,223). The most frequent SNPs found in the population are Arg16Gly, Gln27Glu, Thr164Ile and Cys19Arg which is the 5'-leader cistron of the human β2AR coding block (223). The Cys19Arg polymorphism is within nucleotides (position -47 T/C) upstream of the open reading frame of the *ADRB2*, it encodes a 19 amino acid peptide, noted as β2AR upstream peptide (BUP) (224). The polymorphism at -47 of the translated peptide is at amino acid position 19 of the 5'-leader cistron. The polymorphism at position -47 in the BUP, alters the expression of the β2AR in human airway smooth muscle (225). The variant arginine at nucleotide position -47 (noted as Arg19) has lower β2AR expression (225). There was downregulation of the receptor with the arginine variant, as observed with COS-7 cells fibroblast cell lines with radioligand binding and luciferase assays (225). The uncommon (allelic frequency 1%) polymorphism Thr164Ile is in the fourth transmembrane of the β2AR, in transfected cells, the cells expressing the Ile164 showed to have diminished ligand affinity to epinephrine, norepinephrine and isoproterenol (226). It was observed that there was a diminished capacity to activate adenylate cyclase in Chinese hamster cells lines radioligand binding and AC assay (226). Studies performed on healthy individuals showed in heterozygous polymorphism isoleucine 164 have diminished heart rate and contractility after infusion of terbutaline compared to the wild type homozygous threonine (227).

In the field of asthma, there are noted differences in how a patient might respond to asthma drugs based on commonly occurring single nucleotide polymorphisms (SNPs) found in the *ADRB2* gene (221). Groups of SNP on the same chromosome that are inherited together are referred to as haplotypes, which can be more accurate than SNP in predicting drug responses (221). Arg16 is more resistant to downregulation than Gly16 where the expression of the β2AR was lost with the Gly16 variant, shown on human airway smooth muscle and in Chinese hamster fibroblast transfected with β2AR and treated with isoproterenol (228). In another

study, the level of expression of β2AR was higher in haplotype 2 compared to haplotype 4 as measured with radioligand binding, luciferase expression and mRNA. The experiments were performed in HEK293 cells transfected with β2AR constructs for haplotype 2 and 4, where haplotype 2 has Gly16 and haplotype 4 has Arg16, (223). Studies performed with asthma cohorts showed that the variability of lung capacity responses to β agonists has a correlation to the polymorphism found on the *ADRB2* (229,230). In asthmatic cohorts, Individuals with homozygous Arg16 that were treated with β-agonist albuterol or salbutamol had a lower response in lung function compared to individuals with Gly16 (230–232). Wechsler *et al.* reported that individuals with homozygous for Arg16, had an impaired response to salmeterol which is a long-acting $\beta_2$AR agonist drug in asthma cohort (233). Palmer *et al.* stated that the odds ratio for an asthma exacerbation was 3.4 for Arg16 homozygous compared to Gly16 homozygous during 6 months of follow-up (234). The difference between the Arg16 and Gly16 variants could be attributed to the tachyphylaxis effect on the β2AR. There is no tachyphylaxis effect when the Gly16 variant is present due to downregulation of the receptor at baseline, whereas Arg16 being resistant to downregulation can have tachyphylaxis effect when exposed to exogenous ligand (228,230). A study in an asthma cohort showed that haplotypes had different responses in the forced expiratory volume (an indicator of lung function) observed to treatments with albuterol, where haplotype pair 2/2 (contains Gly16) conferred most reactivity to the treatment compared to haplotype pair 4/4 (contains Arg16) (223). The Gly16 variant had a faster response to repetitive stimulation with epinephrine, more cAMP when stimulated with epinephrine, faster translocation of the β-arrestin2 to the cell membrane and more phosphorylation of GRKs (235). These results indicated that Gly16 variant had augmented functionality in which the activation kinetics became faster upon repetitive stimulation with epinephrine. In the first 15 residues of Gly16 variant of β2AR, there is a coil present in the secondary structure which is not present in the Arg variant (236). As such, the vestibule formed by TM 5, 6, and 7 is more open, such that the ligand-binding site is enlarged and ligand (e.g. albuterol) binding is enhanced., as determined through computational studies on the β2AR (236).

The polymorphism Glu at position 27 was shown to play a role in the down regulation of the receptor. Experiments by Small *et al.* created mutations of all Ser and Thr to Ala on the cytoplasmic tail so there were no longer sites for GRK phosphorylation and the desensitization of the receptor was measured. There was no loss of function with the exposure of the agonist in the modified receptor compared to the wild type β2AR (216). Patients with uncontrolled asthma were associated with Arg16 of the *ADRB2*, but there was no association with the SNP of the *ADRB2* and the Th2 mediated asthma (237).

Polymorphisms of ADRB2 can influence how asthma drugs work in ethnic groups. For example, black people responded less or failed to respond to asthma treatment with long-acting beta-agonists compared to white people (238). Some therapies for asthma with β2AR agonist salmeterol can even have adverse effects (e.g asthma-related deaths) especially in African Americans (239). There was an association between the polymorphism at the upstream region at -47 (Arg19Cys) and the bronchodilator drug response, where there was diminished response to beta-agonists when subjects had the Cys allele rather than the Arg (240). Identifying the SNP and haplotypes of individuals can thus help in predicting the outcome to the agonist on an asthma patient in ethnic groups that have higher frequencies of certain polymorphisms.

When adrenergic agonist was added to human immune cells, there was not a link between SNP of ADRB2 and the cytokine IFNγ, whereas the cytokine IL-5 was inhibited with the haplotype CysGlyGln (222). This indicates that adrenergic agonist inhibits Th1 cell IFNγ response regardless of polymorphisms. There are few other studies assessing ADRB2 polymorphisms and immune response. In my thesis, the analysis of polymorphisms and ADRB2 with respect to Th17 cell response to adrenergic ligands is novel.

## 1.15 Anti-proliferation effect PBMC and CD4 cells by agonist of β2AR

Agonists of the β2AR can inhibit proliferation of immune cells. For example, the β agonist fenoterol inhibited proliferation of immune cells from patients that were suffering from

allergies (241). The immune cells were activated in vitro with purified allergen extract and the proliferation was assessed with $^{3}H$ thymidine incorporation. Fenoterol caused an increase in cAMP levels in immune cells which accounted for the anti-proliferative effect. There was no programmed cell death (apoptosis) as measured by DNA fragmentation assay, indicating that fenoterol did not kill the cells. These results showed that stimulation of the β2AR with agonist fenoterol had an anti-proliferative effect in allergen exposed PBMCs from allergy patients. Other agonists such as norepinephrine, epinephrine and isoproterenol (a non-selective exogenous βAR agonist) were also shown to inhibit proliferation of activated T cells (242,243). Isoproterenol inhibited the proliferation of purified human Th cells, cytotoxic T cells, and memory T cells, which were obtained using negative selection and proliferation measured with $^{3}H$ thymidine incorporation (242). These results also demonstrated that stimulation with isoproterenol inhibited proliferation and was linked with IL-2 inhibition and cAMP accumulation in purified human T cells (242). IL-2 was also inhibited by norepinephrine and terbutaline in anti-CD3 activated isolated splenic naïve CD4 T cells from BALB/c mice (243). Terbutaline salbutamol, salmeterol, and fenoterol (all β-agonists) were shown to inhibit proliferation of isolated $CD4^{+}$ T cells and $CD8^{+}$ T cells from human PBMCs activated with PHA (T cell mitogen) and IL-2, proliferation was measured using $^{3}H$ thymidine incorporation (244). The authors noted that terbutaline was the weakest anti-proliferative effects, the numbers were salbutamol 59% inhibition, salmeterol 90% inhibition, fenoterol 52% inhibition and terbutaline (21% inhibition).

These studies indicate that β2AR agonists have an anti-proliferative effect on T cells. However, there are contradictory studies. Norepinephrine did not change the proliferation of Th cells from BALB/c mice activated with ConA mitogens, where proliferation was measured with thymidine incorporation techniques (245). Epinephrine and norepinephrine had no effect on the proliferation of PBMCs from healthy individuals stimulated with PHA mitogens (246). Thus, the β2AR can be anti-proliferative or have no effect on proliferation. The different results may be the agonists used, concentrations, or the details of experimental systems. In my thesis results, I report no anti-proliferative effect and an increase (10 %) in proliferation by β2AR agonists, which is further discussed in the chapter and general discussion.

## 1.16 β2AR on T helper cells, function of beta-adrenergic receptor in Th1, Th2 and Th17 cells

The density of β2AR varies amongst T cells, $CD8^+$ cells had the highest density of β2AR (1800 units) which was more than double that of $CD4^+$ cells (750 units), as measured in healthy individuals PBMCs using competitive radioligand binding (48,247). The β2AR is also a function in the adaptive immune system of mice. For example, in the BALB/c model of chronic mild stress, the stress stimulus increased β2AR expression on T cells compared to the control (BALB/c) without stress, as measured with radioligand binding techniques (245). The authors treated T cells from the chronic stress mice with norepinephrine which increased cAMP compared to the non-stressed mice, as measured with cAMP enzyme immunoassay (245). Thus, an adrenergic agonist can stimulate cAMP in murine T cells through the β2AR which inhibits the Th1 cell. Similarly, there was a reduced percentage of IFNγ in human $CD4^+$ T cells treated with β2AR agonist salmeterol and this reduction of IFNγ was reversed with the use of β2AR specific antagonist ICI 118,551 (248). The $CD4^+$T cells were obtained from healthy individuals PBMCs and the $CD4^+$ T cells were activated with superantigen staphylococcal enterotoxin B or cytomegalovirus (248). These results demonstrated that the stimulation of β2AR on the $CD4^+$ cells decreased IFNγ, and no other immune cell was likely to be involved since the Th cells were purified by magnetic-based techniques. This group also showed an indirect effect by treating antigen-presenting cells with salmeterol of and then incubating them with isolated Th cells. The salmeterol-treated APC reduced the percentage of $IFN\gamma^+CD4^+$ as compared to control APC. These results demonstrated that an adrenergic drug can have direct effects on the T cell or indirect effects via the APC that both can decrease IFNγ from Th cells. In a study by Wahle *et al.* norepinephrine and epinephrine inhibited IFNγ in human PBMCs simulated with PHA mitogens or stimulated with anti-CD3 and anti-CD28. This effect was abrogated with βAR antagonist propranolol whereas no effect seen with the antagonist for α1 receptor urapidil (246). Similar to other studies, the authors found that βAR agonist raised the concentration of cAMP in the Th

cells and $CD8^+$ T cells, where the cAMP was measured with cAMP ELISA (246). The second messenger cAMP is known to inhibit Th1 cells (28,29,33).

The expression of adrenergic receptors has been studied on Th1 cells and Th2 cells. Studies performed by Sanders *et al.* reported β2AR expression on Th1 cells and not in Th2 cells. They attempted to measure other adrenergic receptors, but β2AR was the only one present on T cells. To test if this receptor was functional, the authors stimulated β2AR with terbutaline on murine cells line of Th1 and Th2 cell, which inhibited IFNγ but did not change IL-4 levels, as measured with ELISA. Since Th1 and Th2 cells induce antibody isotypes produced by B cells (IgG2a and IgG1 respectively), the Th1 and Th2 cell-dependent antibody production was measured when β2AR was stimulated in Th1 and Th2 cells lines. The stimulation of Th1 and Th2 cells with terbutaline before activation by antigen-presenting B cells inhibited IgG2a but did not affect IgG1, respectively. The inhibition was prevented using antagonist βAR nadolol (249). In addition, β2AR ligand bindings only occurred in Th1 cells, but not Th2 cells using the β2AR antagonist ICI 118, 551 for $^{125}$I-pindolol displacement of radiolabelled ligands. These results demonstrated that Th1 express the β2AR and that adrenergic agonists inhibit Th1 cells response (IFNγ) and alter antibody isotype production by B cells (249).

To understand why Th1 cells expressed β2AR but Th2 cells did not, studies were performed on cell lines to address epigenetic modifications. Th1 cells expressed β2AR, but Th2 cells did not express β2AR due to epigenetic modification of the gene (250). The researchers demonstrated this by acquiring naïve $CD4^+$ T cells from spleens of female BALB/C mice, which were then activated with anti-CD3 and anti-CD28, with cytokines that promote Th1 (IL-2, IL-12, anti-IL-4)or Th2 (IL-2, IL-4, anti-IFNγ) differentiation for 5 days. After 5 days Th1 or Th2 cells were sorted based on the expression of IFNγ or IL-4, respectively, using fluorescent activated cell sorting techniques, which yielded highly enriched Th subsets. The levels of β2AR mRNA were measured with quantitative real-time PCR in the differentiated Th1 and Th2 subtypes, they observed that Th1 cell-expressed β2AR mRNA but Th2 cells did not. Another approach the authors used was to examine mouse T cell lines, which are cloned and transformed Th1 or Th2 cells that grow as clonal populations. Th1 cells line (AR100.9, D1.1, and HDK-1) and Th2 cells

line (CDC35, LNT-1, and LNT-4) were compared for this study. There were higher levels of β2AR mRNA expression in IFNγ producing cells compared to a low β2AR mRNA expression in IL-4 producing cells, as measured by quantitative real-time PCR. The authors discovered that there was a histone and acetylation modification in the *ADRB2* promoter in Th1 cells that facilitated transcription of β2AR mRNA, whereas Th2 cells lacked this modification and could not transcribe β2AR mRNA. Together, these results indicated that epigenetic mechanism mediates the differential β2AR mRNA expression between Th1 and Th2 cells (250). Those studies did not address Th17 cells, the reason being that Th17 cells were not discovered. That is one of the reasons for my first aim of measuring β2AR on Th17 cells.

Human Th cells have also been studied in the context of β2AR agonists. PBMCs from healthy volunteers were activated *in vitro* and stimulated with epinephrine, norepinephrine, terbutaline, or propranolol a βAR antagonist. When epinephrine, norepinephrine or terbutaline was added to cell cultures, they suppressed Th1 cells, thereby inducing a shift towards Th2 cells because Th1 cells normally suppress Th2 cells (251). The authors showed that PBMCs stimulated with the βAR agonist showed a decrease of IFNγ levels and an increase of Th2 mediated cytokines, including IL-10, IL-4 and IL-5 using ELISA (251).

In the literature, most studies focus on adding exogenous drugs and hormones to determine the impact of adrenergic signalling on the immune system. However, it is now known that lymphocytes can synthesize and secrete their own catecholamines which may work in an autocrine fashion. This was shown by blocking the rate-limiting enzyme tyrosine hydroxylase with alpha-methyl-p- tyrosine (α-MT) in lymphocytes isolated from mesenteric lymph of mice, and activated in vitro with concanavalin A, an antigen-independent mitogen (252). The result was a decrease of catecholamines produced, as measured with High-Performance Liquid Chromatography with Electrochemical Detection. The authors also observed downregulation mRNA and protein expression of Th2 cells (GATA-3 (transcription factor) and IL-4), and upregulated expression of mRNA and proteins of Th1 cells (T-bet (transcription factor) and IFNγ), as measured with real-time PCR, western blot and RT-PCR. This means that autocrine catecholamines are suppressing Th1 cells, when the suppression is

removed, the Th1 cells increase while Th2 cells decrease (252). Researchers added an inhibitor monoamine oxidase and enzyme that degrades catecholamines, to murine lymphocytes. The result was that it downregulated Th1 cells (T-bet and IFNγ) allowing for upregulated Th2 cells (GATA-3 and IL-4) (252). It was also demonstrated that epinephrine inhibited Th1 cells in PBMCs stimulated with tetanus-Toxoid and PHA which increased Th2 cell responses (IL-10, IL-4), as measured with ELISA (253).

The effects of adrenergic agonists on Th17 cells are now beginning to be understood. For example, dendritic cells are APCs that regulate the differentiation of $CD4^+$ T cells by secreting cytokines such as IL-12, and IL-23. Th1 cells are induced by IL-12, which is composed of heterodimers of p40 and p35 (254). Th17 cells are induced by IL-23 which is composed of the p35 with the p19 subunit (254). They measured p35, p19 and p40 subunits by qPCR because it allowed them to define the cytokines. The balance of IL-12 and IL-23 will influence the outcome of Th cell differentiation. For example, when the dendritic cells were pre-treated with epinephrine had a decrease of IL-12 and increase in IL-23 (254). In those experiments, the authors used LPS to stimulate the dendritic cells to cause the dendritic cells to secrete cytokines. When there were no LPS added, the dendritic cells did not make cytokines, and epinephrine had no effect. To know if the effects were dependent on the β2AR, the authors added of butoxamine, a β2AR antagonist. The antagonist abrogated the decrease in IL-12 and increase in IL-23 that was caused by epinephrine, as measured with qPCR (254). Dendritic cells pre-treated with epinephrine and co-cultured with $CD4^+$T cells increased the IL-17 but decreased the IFNγ (254). The results from that study indicated that dendritic cells stimulated by epinephrine induces Th17/IL-17 and diminishes Th1 cells response by $CD4^+$T cells. Another study on dendritic cells was done by Manni *et al*. The authors added salbutamol (β2AR agonist) to dendritic cells and activated the dendritic cells with lipopeptide (PAM) and muramyl dipeptide (MDP). The salbutamol increased IL-23 while it inhibited IL-12, measured by qPCR on cytokine subunits (255). In addition to the *in vitro* evidence of the dendritic cells favouring the Th17 cell priming, *in vivo* experiments with an injection of pre-treated dendritic cells with salbutamol and PAM and MDP showed increased IL-17 while a decrease of IFNγ in cells from

the lymph nodes, analyzed by ELISA (255). Mice injected with norepinephrine, PAM and MDP showed increased IL-17 but decreased IFNγ levels, and these effects were abrogated with ICI 118,551 (antagonist) (255). Thus, catecholamine hormones like epinephrine or norepinephrine can regulate Th cell differentiation by modulating dendritic cells, favouring Th17 cells and diminishing Th1 cells. A more recent (2016) study showed that mouse Th cells treated with norepinephrine had lower levels of IFNγ, but higher levels of IL-17A which indicated that Th17 cells may react differently than other Th1 cells (256). The study by Case *et al* used T cells ($CD4^+$) isolated from spleens of C57BL/6 mice activated with anti-CD3 and anti-CD28 and stimulated with norepinephrine. The result was a decrease in IL-2, IFNγ, TNFα and IL-10, and an increase in IL-17 and IL-6 using cytometry bead array technique. The authors linked the cytokine changes to mitochondrial reactive oxygen species. Superoxide was increased when T cells were treated with norepinephrine, as measured with flow cytometry using dihydroethidium and MitoSOX Red. An antioxidant (Mito-Tempol) restored the cytokine IL-2, IFNγ and IL-17 (256).

In 2018 there was a contradictory study performed by Liu *et al.*, showing there is a decrease of IL-17 on murine cells from a mouse model of arthritis when CD4 cells are treated with β2AR agonist. In their study, the expression of β2AR on CD4 cells from control and collagen-induced arthritis model was demonstrated with immunofluorescence of spleen sections as well as western blot performed with ankle and spleen, in which the density of β2AR was downregulated in CIA mouse model. The exposure of norepinephrine and terbutaline showed a decrease of Th17 cells phenotype (IL-17, IL-22 and RORγ) while the antagonist ICI 118,551 blocked these effects. Naïve CD4 cells isolated from spleen from collagen-induced type II induced arthritis murine model for arthritis were polarized Th17 cells cocktail (TFG-β1, IL-6, TNFα, IL-1β and IL-23) for a period of 48 hours. Subsequently, the polarized Th17 cells and activated cells with anti-CD3 and anti-CD28 were treated with norepinephrine or terbutaline for a period of 24 hours, where the cytokines IL-17 and IL-22 were measured with ELISA and real-time PCR. In addition, terbutaline inhibited the CIA induced CD4 T cell proliferation stained with CFSE measured with the flow cytometer. To identify the agonist effect on Th17 cells, the use of PKA inhibitor H89 abolished the agonist effect. To identify the signalling pathway is mediated

via the cAMP-PKA pathway, cAMP was measured with ELISA and PKA activity assay kit was performed, where terbutaline increased both cAMP and PKA in Th17 cells from CIA mice. Overall, these studies indicated that Th17 cells expressed the β2AR and the response to stimulation with norepinephrine and terbutaline inhibited Th17 cells differentiation via cAMP-PKA signalling pathway in CIA mice (257).

In summary, several studies have shown that Th1 cells express β2AR and are inhibited by agonists of this receptor, while Th2 cells do not express β2AR but can still expand when Th1 cells are inhibited. The effect of adrenergic agonists on Th17 cells was not clear at the start of my PhD thesis, which is why I focused on Th17 cells in my thesis. Together with the results of my thesis, the balance of evidence now indicates that Th17 cells are augmented by adrenergic agonist. I proposed a model by which this type of inhibition occurs via the β2AR (Figure 1.6).

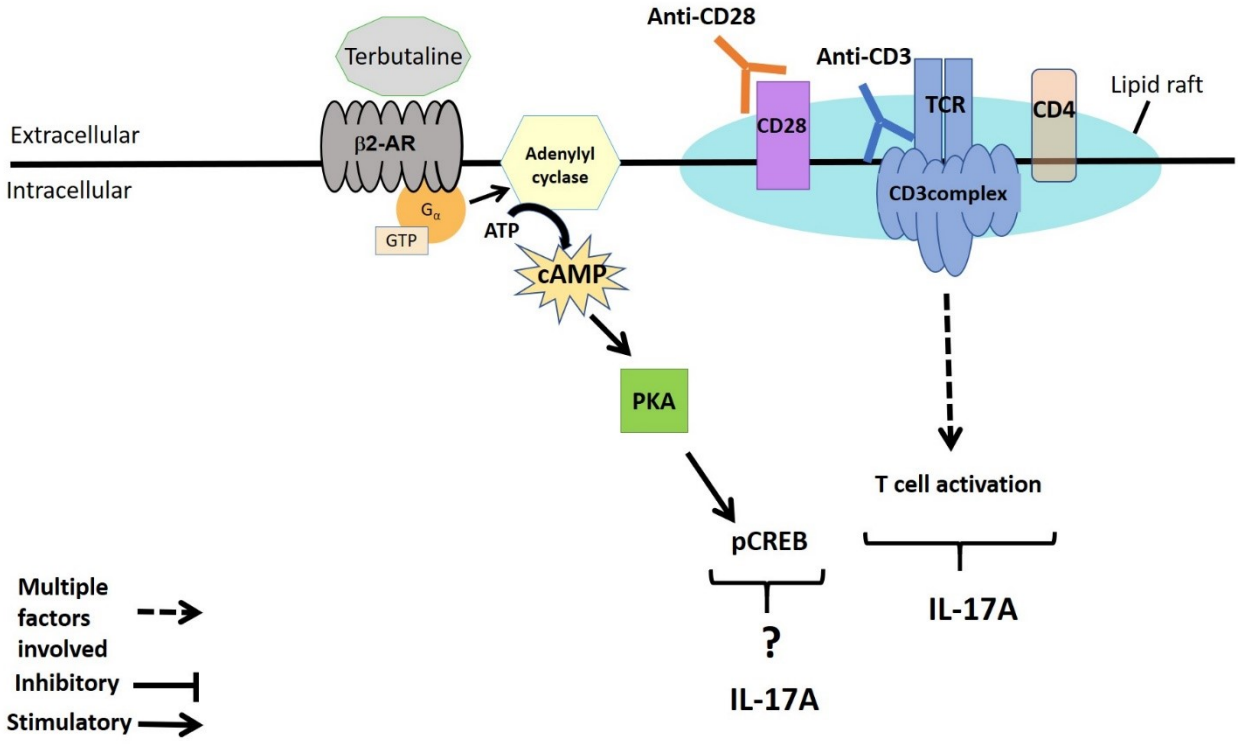


**Figure 1.6. Model figure of signalling pathway mediated by cAMP-PKA-CREB that influences the IL-17A response in Th cells.**

The stimulation of the β2AR with terbutaline leads to signalling pathway mediated by the G-coupled protein. The GTP associates with the Gα subunit that associates with the enzyme adenylyl cyclase (AC). AC converts ATP to cAMP. This second messenger (cAMP) binds and activates PKA. PKA phosphorylates and activates transcription factor CREB. CREB subsequently promotes IL-17A transcription. The T cells are activated using antibodies targeting the CD3 complex of the TCR (anti-CD3) and co-stimulatory signal CD28 (anti-CD28), which causes several signalling pathways that are not depicted in detail in the diagram.

## Hypotheses

In Chapter 2 my initial hypothesis was that a β2AR-specific agonist (terbutaline) would inhibit IL-17A produced by Th17 cells. That hypothesis was based on extensive literature showing that cAMP and PKA are suppressive for T cells, and β2AR-specific agonists inhibit Th1 cells. The literature also showed that adrenergic agonists were anti-proliferative for T cells, although terbutaline was the weakest in this respect. These facts pointed to a hypothesis that terbutaline would inhibit IL-17A. The hypothesis was partially supported by results from a minority of the samples that I tested. However, in the majority of samples, terbutaline augmented IL-17A, as did a PKA-activating drug. Thus, the hypothesis that terbutaline was suppressing IL-17A was considered to be disproven.

In Chapter 3, I revised my hypothesis from the previous chapter and demonstrated that terbutaline augmented IL-17A in a cAMP and PKA-dependent manner. A new hypothesis was that an inverse-agonist of β2AR would inhibit IL-17A from Th17 cells. The results showed that in all samples tested nebivolol suppressed IL-17A, which supported the revised hypothesis.

In chapter 4 my hypothesis was that SNP within and adjacent to *ADRB2* would modulate the effects of the β2AR drugs. The results showed that one of the specific SNPs modulated the terbutaline effect on IL-17A. This supported the hypothesis that SNP may account for the way certain β2AR ligands work on the receptor.

## Specific Aims

**Specific Aim 1** To determine if β2AR agonist inhibits IL-17A from Th17 cells in a cAMP/PKA dependent manner.

- **Specific Aim 1.1** To determine if Th17 cells express β2AR protein.
- **Specific Aim 1.2** To determine if a β2AR-specific agonist inhibits activated helper T cells.
- **Specific Aim 1.3** To determine if direct stimulation of PKA replicates the effect of β2AR agonist.

**Specific Aim 2** To determine how the β2AR agonist was augmenting IL-17A. To determine if an inverse-agonist of β2AR will diminish Th17 cells response.

- **Specific Aim 2.1** To determine if the augmented IL-17A was due to an increase in the proportion of Th17 cells in the samples. To determine if it was due to increased expression in each cell.
- **Specific Aim 2.2** To determine if β2AR agonist-induced phosphorylation of CREB, a downstream substrate of PKA.
- **Specific Aim 2.3** To determine if the β2AR-agonist augments IL-17A in a cAMP-PKA dependent manner.
- **Specific Aim 2.4** To determine if nebivolol will diminish Th17 cells responses.

**Specific Aim 3** To determine if SNP located within and adjacent to *ADRB2* are linked to β2AR agonist or inverse-agonist effects on IL-17A and IFNγ.

- **Specific Aim 3.1** To determine the complete sequence of *ADRB2* in primary human PBMC.
- **Specific Aim 3.2** To determine if the combinations of SNP correlate to differential effects of β2AR agonist or inverse-agonist on IL-17A and IFNγ.

## Chapter 2 Foreword (Bridging Text)

The immune system has to constantly adapt and adjust to the presence of new pathogens. These adaptations and adjustments, collectively, are known as immunomodulation. Studying immunomodulation is important because there are diseases such as autoimmune disease and asthma that can be treated by immunomodulatory drugs. The overall goal of my thesis was to discover if an adrenergic drug would inhibit IL-17A, which would be used in the future to treat autoimmune diseases. In this chapter, I chose to study a β2AR agonist in more detail and determine its effects on Th17 cells. At the outset of the project, no other group had studied the effect of ligands for adrenergic receptors on Th17 cells. We were the first to discover the presence of the β2AR on a proportion of Th17 cells (specific aim 1.1). The presence of the receptor on a proportion of Th17 cells did not confirm that this receptor was functional. To address this, in specific Aim 1.2 I determined if a β2AR-specific agonist terbutaline would inhibit activated Th cells. Literature on adrenergic drugs showed anti-proliferative effects on T cells, and inhibition of Th1 cells, so we predicted terbutaline would inhibit Th17 cells. In the first samples I tested, terbutaline inhibited IL-17A, however, it became apparent that in the majority of samples terbutaline augmented IL-17A which disproved the hypothesis. As those results became more clear, I read more on transcription factors and learned that phosphorylated CREB might augment IL-17A.

The source of the IL-17A, which I measured with ELISA, was not immediately obvious because the cells used were a mixed population (PBMC) that could contain a minority of other types that make IL-17A. To address that I repeated experiments on purified Th17 cells and found a similar result, that is, terbutaline augmented IL-17A. I included IFNγ in my measures (which is predominantly made by Th1 cells) as a control group where we expected adrenergic drugs to suppress IFNγ, which was indeed the case. Thus, terbutaline regulated IL-17A from Th17 differently than IFNγ. Initially, I presumed that Th17 cells would be suppressed since they are closely related to Th1 cells which were known to be inhibited by adrenergic agonists. When I added a chemical dbcAMP instead of terbutaline, it replicated the effect- IL-17A augmented,

and IFNγ inhibited, which answered Specific Aim 1.3 which was to determine if direct stimulation of PKA replicates the effect of β2AR agonist.

The results from chapter 2 disproved the hypothesis that terbutaline would suppress, rather, it augmented IL-17A levels. It was a novel discovery because Th17 cells had not been studied in the context of adrenergic drugs. Additional papers were published in the meantime, which I discuss in this chapter and the general thesis discussion. The results from chapter 2 left several unanswered questions which I addressed in later chapters. In chapter 3, I demonstrated that β2AR augmented IL-17A in a PKA-dependent manner, which led me to explore inverse agonists that would oppose that pathway such as nebivolol. Nebivolol suppressed both Il-17A and IFNγ as shown in Chapter 3, which suggested a promising new therapeutic drug. Another finding from chapter 2 was that the adrenergic agonist had no effect or even inhibited IL-17A production in a minority of samples that were tested. In chapter 4, I discovered that the variations in terbutaline responses were linked them to a specific polymorphism in the gene for the receptor.

In summary, chapter 2 demonstrated for the first time that Th17 cells express the β2AR and that terbutaline augments IL-17A. It also provided the basis for the following studies that led to the discovery of a potential new immunomodulatory drug.

# Chapter 2 Reciprocal Modulation of Helper Th1 and Th17 Cells by the β2-Adrenergic Receptor Agonist Drug Terbutaline.

The following chapter is based on the published manuscript we produced with minor formatting modification with FEBS permission, FEBS (2017), 284(18):3018-3028. © 2017 Federation of European Biochemical Societies

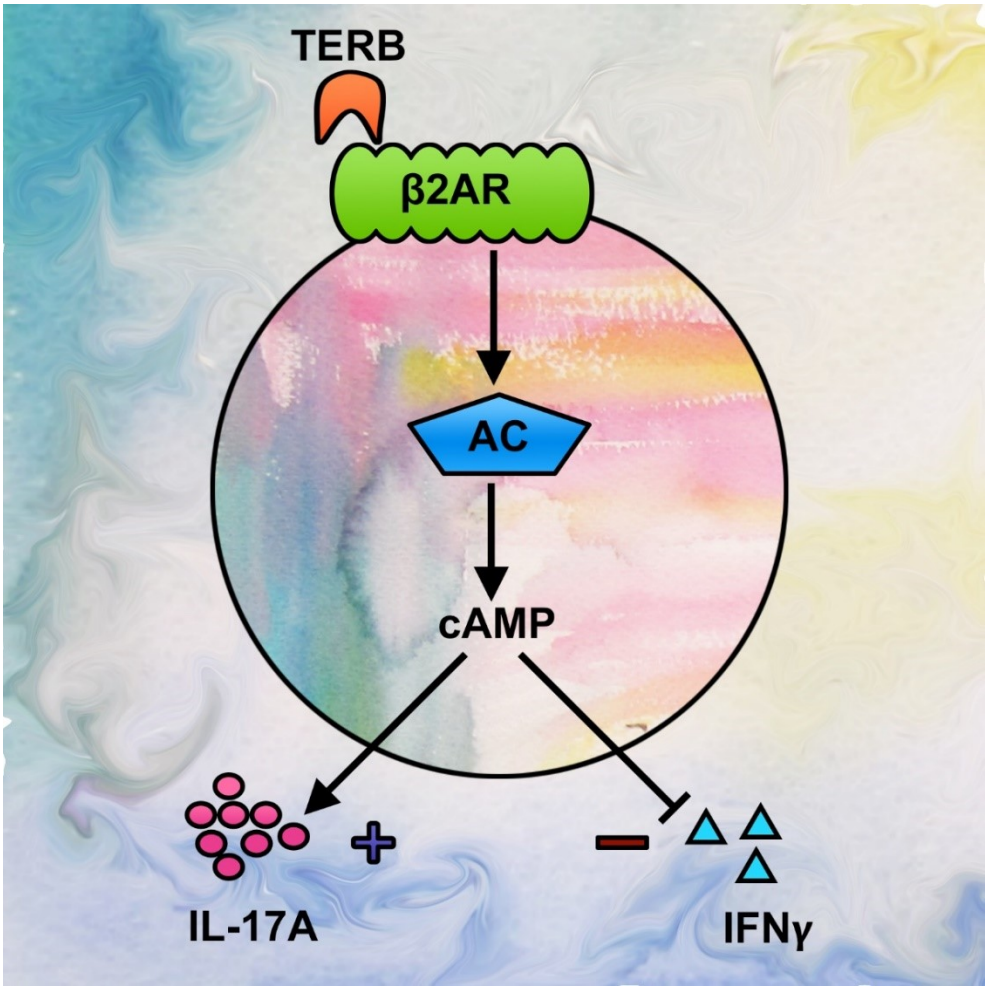



Figure 2.0 Graphical abstract Th17 cells target specific pathogens but they are also linked with autoimmune diseases. Catecholamine hormones released by the sympathetic nervous system can respond to adrenergic receptors. We demonstrated the presence of β2-adrenergic receptor on Th17 cells. Terbutaline, a β2-agonist drug, augmented IL-17A. The drug reduced IFNγ levels indicating that a reciprocal regulation between Th17 and Th1 cells occurred.

## 2.1 Abstract

Catecholamine hormones are powerful regulators of the immune system produced by the sympathetic nervous system. They regulate the adaptive immune system by altering T cell differentiation into T helper (Th) 1 and Th2 cell subsets, but the effect on Th17 cells is not known. Th17 cells, defined in part by chemokine receptor CCR6 and cytokine IL-17A, are crucial for mediating certain pathogen-specific responses, and are linked with several autoimmune diseases. We demonstrated that a proportion of human Th17 cells express β2-adrenergic receptor, the flow cytometry profile showed that 76.8% of the Th17 cells expressed β2AR, a G protein-coupled receptor that responds to catecholamines. Activation of peripheral blood mononuclear cells, which were obtained from venous blood drawn from healthy volunteers, with anti-CD3 and anti-CD28 and with a β2-agonist drug, terbutaline, augmented IL-17A levels ($p<0.01$) in the majority of samples. Terbutaline reduced IFNγ indicating that IL-17A and IFNγ are reciprocally regulated. Similar reciprocal regulation was observed with dbcAMP. Proliferation of Th cells was monitored by carboxyfluorescein diacetate N-succinimidyl ester (CFSE) labelling and flow cytometry with antibody staining for CD3 and CD4. Terbutaline increased proliferation by a small but significant margin ($p<0.001$). Next, Th17 cells ($CD4^+CXCR3^-CCR6^+$) were purified using an immunomagnetic positive selection kit, which removes all other mononuclear cells. Terbutaline increased IL-17A from purified Th17 cells, which argues that terbutaline acts directly on Th17 cells. Thus, hormone signals from the sympathetic nervous system maintain a balance of Th cells subtypes through the β2-adrenergic receptor.

## 2.2 Introduction

T cells are an essential part of the adaptive immune system; they are responsible for recognizing foreign antigens depicted by the major histocompatibility complex (MHC) on the APC such as dendritic cells (DC). T cells also maintain immunological memory of the recognized pathogen. T cell activation is the result of the T cell receptor (TCR), with the attached CD3 component (22), responding to antigen/MHC complex, with co-stimulatory signals received through CD28 (22). T helper (Th) cells are defined by the presence of CD4 marker on their surface (258). Upon activation, Th cells differentiate into distinct lineages of effector/memory subsets such as Th1, Th2, and Th17 cells that express defining cytokines IFNγ, IL-5, and IL-17A (107,131,259,260). Th17 cells can be identified by chemokine receptor CCR6, and transcription factor RORγ (107,126,259,261). Th cells diversity provides the immune system with the ability to mount an appropriate response against various classes of pathogens (262). For example, upon infections with *Candida albicans* and *Staphylococcus aureus* Th17 cells are induced (263). The cytokine IL-17A, which comes primarily from Th17 cells, mediates the production of inflammatory cytokines such as IL-6 and IL-1 and chemokines CXCL-1,2,5,8, CCL-2 and CCL-20 that in turn leads to the recruitment of neutrophils and macrophages to sites of inflammation (144–146). These cells are contributors for inflammatory autoimmune diseases, like multiple sclerosis, psoriasis, and rheumatoid arthritis (108,144–146).

The balance of Th subsets is primarily determined by the strength of TCR engagement, co-stimulation, and polarizing cytokines produced by APCs. Catecholamine hormones also influence the balance of Th subsets. Catecholamines consist of two main hormones, norepinephrine (NE) and epinephrine, which mediate metabolic changes throughout the body in a fight or flight response. This response is controlled by the SNS. The SNS releases NE through adrenergic nerves in areas where T cells reside, such as the parenchyma of the lymph nodes and white pulp of the spleen (183–185). T cells express the β2 subtype of the adrenergic receptor (AR) family, which responds to catecholamines (243,249,250,264,265). AR are a family of G protein-coupled receptors present in most tissues. The β2AR transduces signals with

stimulatory Gα subunit, AC and increases the intracellular second messenger cAMP, which in turn activates PKA. β2AR is encoded by *ADRB2*, which has common single-nucleotide polymorphisms (SNPs) that lead to missense changes in amino acid residues and influence the pharmacodynamics of the receptor (222,223). The effect of β-agonists on Th cells depends on the species of animal, subtype of T cells and type of agonist used. β-agonists such as isoproterenol and fenoterol inhibited IFNγ (266), while NE raises the level of IFNγ in Th1 cells (267). Murine Th1 cells have variable effects in response to β-agonists (243,249,265,268,269). Human Th2 cells do not express β2AR or other AR due to histone modifications that prevent the gene from being expressed (250). However, Th2 cells expand in response to β agonists, since the competing Th1 cells are supressed (243,249,264–266,269–272). Catecholamines can indirectly influence Th17 differentiation by acting on APCs. DCs provide co-stimulation and cytokines necessary for CD4 cells to function (254), and produces IL-23 cytokine, which is necessary for Th17 expansion (131,147,273). Murine DC treated with β2AR -agonist-salbutamol made more IL-23, facilitating the differentiation of effector Th17 cells (255). β2AR stimulation with NE or salbutamol on mice DC induced IL-17A, and it decreased the production of IFNγ by CD4 cells (254,255). While it is clear that catecholamines influence APCs, there is comparatively less known about how catecholamines directly influence Th17 cells. We tested the hypothesis that β-agonists modulate Th17 responses using a β2-specific agonist, terbutaline. In the majority of samples tested, terbutaline increased IL-17A production in activated PBMCs, and concomitantly decreased IFNγ production. Purified Th17 cells treated with terbutaline had elevated IL-17A levels and decreased IFNγ.

## 2.3 Materials and Methods

Activation and treatment of cells

Venous blood was drawn from healthy volunteers after obtaining informed consent. The project was approved by the Concordia University Human Research Ethics Committee. Our board of ethics is independent and conforms to the Helsinki guidelines. A venipuncture needle was used to draw up to six heparinized vacutainer tubes (BD, Franklin Lakes, NJ, USA). Whole

blood was processed through a ficoll-hypaque (GE healthcare, Mississauga, ON, Canada) density gradient centrifugation technique as previously described (274). PBMCs were washed twice and suspended at 3.125 x$10^6$ cells/ml in R10 media made of Roswell Park Memorial Institute medium RPMI 1640 supplemented with 1mM penicillin, 1mM streptomycin, 2mM glutamine and 10% heat-inactivated fetal bovine serum (Wisent Inc. QC, Canada). In a round bottom 96 well culture plate, 0.5 x $10^6$ PBMCs were placed in media with either no activation or activation with soluble antibodies against CD3 (clone OKT3, eBioscience, San Diego, CA, USA) and CD28 (clone CD28.2, eBioscience, San Diego, CA, USA), at a final concentration of 0.1 µg/ml each. Drug treatments (Sigma, ON, Canada) included terbutaline ($10^{-5}$M based on reference(243)) and ICI118,551 antagonist (100 nM). The dbcAMP was added each day at 100µM for a final estimated concentration of 500µM. Each treatment was done with at least two replicates. Culture conditions were 5% $CO_2$ and 37°C for four days in a humidified incubator. Th17 cells ($CD4^+CXCR3^-CCR6^+$) were purified using an immunomagnetic positive selection kit. PBMCs were first pre-enriched by negative selection with a human $CD4^+CXCR3^-$ T cell cocktail, followed by a positive selection using CCR6 positive selection cocktail. The kit removes any cell expressing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A, CD45RA high, and CXCR3. The $CD4^+CXCR3^-CCR6^+$ cells remained in the tube (Stemcell Technologies, Vancouver, Canada). Purified Th17 cells were activated with dynabeads human T-activator CD3/CD28, used according to the manufacturer's instructions (Thermo Fisher Scientific, Mississauga, ON, Canada).

Measuring proliferation and differentiated cells

Prior to plating, PBMCs were labeled with (6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFDASE) (Sigma Aldrich, ON, Canada) as previously described (102). In brief, cells were suspended in pre-warmed R10 media and mixed with CFDASE diluted in PBS, at a final concentration of 5µM. This remained at room temperature for five minutes and was then washed three times with PBS containing 10% FBS (275). Cells were then used for the experiments. On day four, cell staining was done according to the published protocol (171). The conjugated antibodies were CD3-PercP and CD4-APC (eBioscience, San Diego, CA, USA) at 1:10

dilution. Samples were transferred to micro-centrifuge tubes and analyzed by flow cytometry (FACS verse BD Bioscience, Mississauga, ON, Canada). In order to observe differentiation, different cytokines were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

Detection of β2AR by flow cytometry

PBMCs were stained with either the primary antibody β2AR rabbit polyclonal (1mg/ml) at a 1:50 dilution or a rabbit Ig antibody, used as a negative control (5mg/ml), at a 1:250 dilution. The secondary goat-anti-rabbit-FITC molecular was added at a 1:500 dilution. Primary antibodies for CD3-PercP, CD4-APC, and CCR6-PE were added (eBioscience, Mississauga, ON, Canada) to facilitate identification of Th cells in the PBMC mixture. Cells were washed twice with PBS and resuspended in 1% formaldehyde. Samples were transferred to microcentrifuge tubes and analyzed by the Acuri flow cytometer (BD Bioscience, Mississauga, ON, Canada).

## 2.4 Results

Expression and function of β2AR on Th17 cells

To determine if Th17 cells express this receptor, PBMCs obtained from healthy human subjects were stained with antibody specific for human β2AR. Th17 cells were gated based on expression of CD3, CD4 and the chemokine receptor CCR6 (Figure 2.1a). A proportion of Th17 cells expressed β2AR (Figure 2.1b). The CCR6-negative fraction expressed negligible amounts of β2AR (data not shown). Next, PBMCs were activated with a T cell-specific stimulus in the absence or presence of terbutaline, which is a β2AR-specific agonist. When PBMCs were exposed to only terbutaline without the activation stimulus, there were no cytokines detectable in the supernatant (data not shown). Upon activation in the presence of terbutaline, an increase of IL-17A concentration was observed in the majority of the samples (Figure 2.2a). A minority of the activated samples showed a trend of decreased IL-17A upon treatment with terbutaline (Figure 2.2a). When data was pooled together, the level of IL-17A produced by activated cells was increased by approximately 1.3-fold ($p < 0.01$) upon treatment with

terbutaline (Figure 2.2b). Terbutaline decreased IFNγ concentrations in the subjects' samples to about 0.6 fold of the activated control group (Figure 2.2c,d). The terbutaline effect on IL-17A and IFNγ was attenuated by the β2AR-specific antagonist ICI 118, 551 (Figure 2.2b,d). Together, this data demonstrates that a proportion of Th17 cells express a functional β2AR and that exposure of PBMC to a β2AR agonist tends to increase IL-17A and decrease IFNγ.

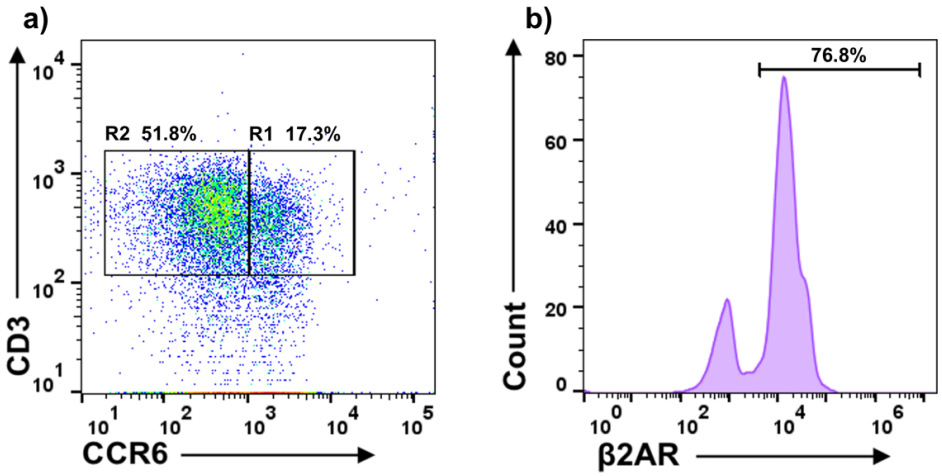



**Figure 2.1. Expression of β2AR on Th17 cells.** a) PBMCs were stained for CD3, CCR6, β2AR and analyzed by flow cytometry. (b) Histogram plot of $CD3^{+}CCR6^{+}$ cells (gate R1) with the bracketed overlay derived from isotype-control staining denoting the positive expressing cells. The peak on the left of panel B, are the Th17 cells that do not express the β2AR. The peak on the right is the Th17 cells that do express the β2AR. It was determined by evaluating the isotype control staining which shows the non-specific staining. Note that panel B is the gated cells (R1) from panel A, which are the Th17 cells. This data represents samples from 5 different human subjects.

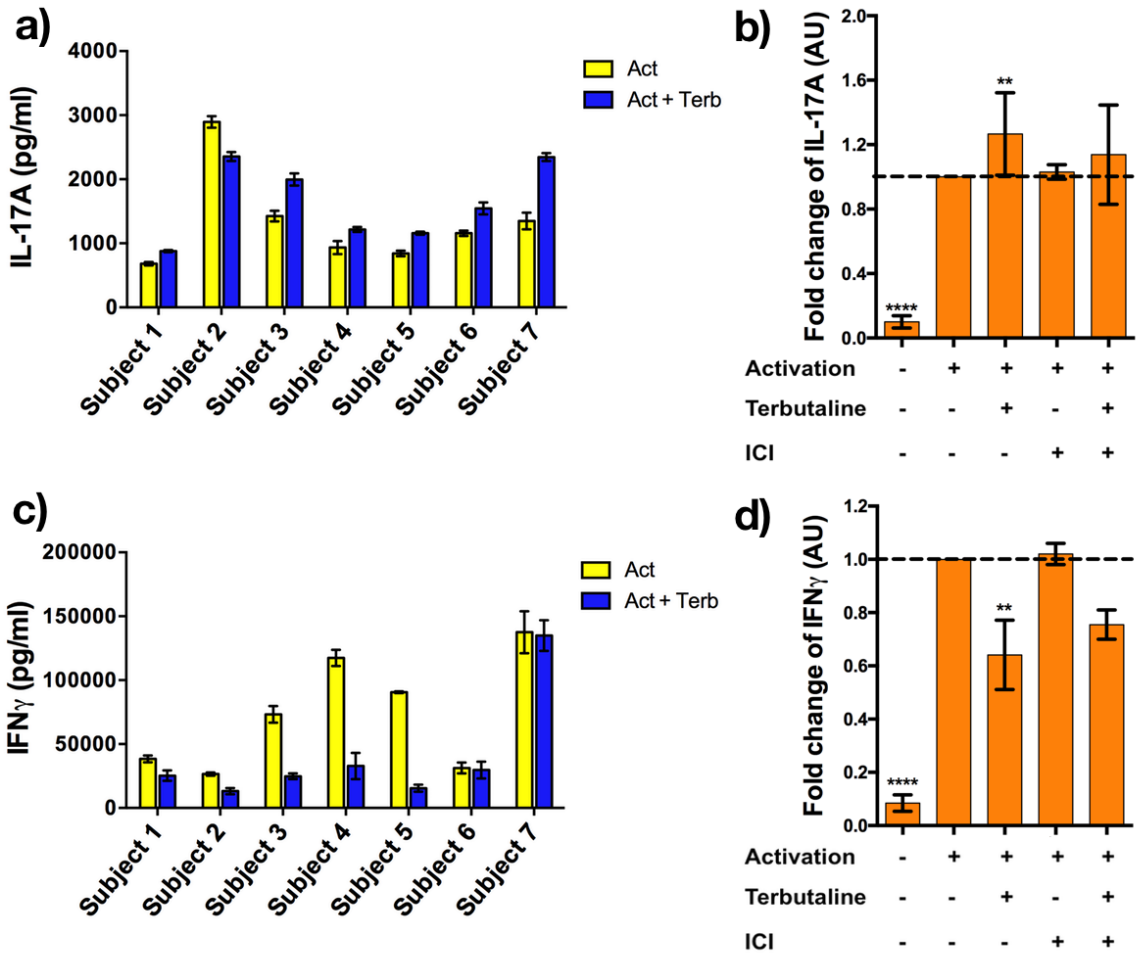



**Figure 2.2 The reciprocal effects of β2AR specific agonist on IL-17A and IFNγ**. Anti-CD3 and anti-CD28 antibody-treated PBMCs were cultured for four days, with and without the β2AR agonist terbutaline ($10^{-5}$M), and the β2AR antagonist ICI (100nM). Supernatants were collected and analyzed for IL-17A and IFNγ by ELISA. a,c) The concentrations of IL-17A and IFNγ for representative subjects are shown. b,d) Data was calculated as fold change compared to the activated group which was set to 1.0 (dotted line). (b) IL-17A fold change, pooled from 17 experiments. (d) IFNγ fold change, pooled from 10 experiments. 1-way ANOVA followed by Tukey's multiple comparison test (***<0.001, **< 0.01). Error bars show the standard deviation.

It was surprising that terbutaline did not increase IL-17A levels in all subjects' PBMC samples. IFNγ decreased in those samples indicating that they were still responsive to the drug. To evaluate the reproducibility of the results, a series of test/re-test experiments were performed. Representative subjects gave two blood samples drawn at least one week apart. For subject 1, where terbutaline augmented IL-17A in the first sample, terbutaline still augmented IL-17A in the second sample (Figure 2.3 a,b). For subject 2, where terbutaline reduced IL-17A in the first sample, terbutaline still reduced IL-17A in the second sample (Figure2.3 c,d). Thus, the pattern of the different responses was reproducible.

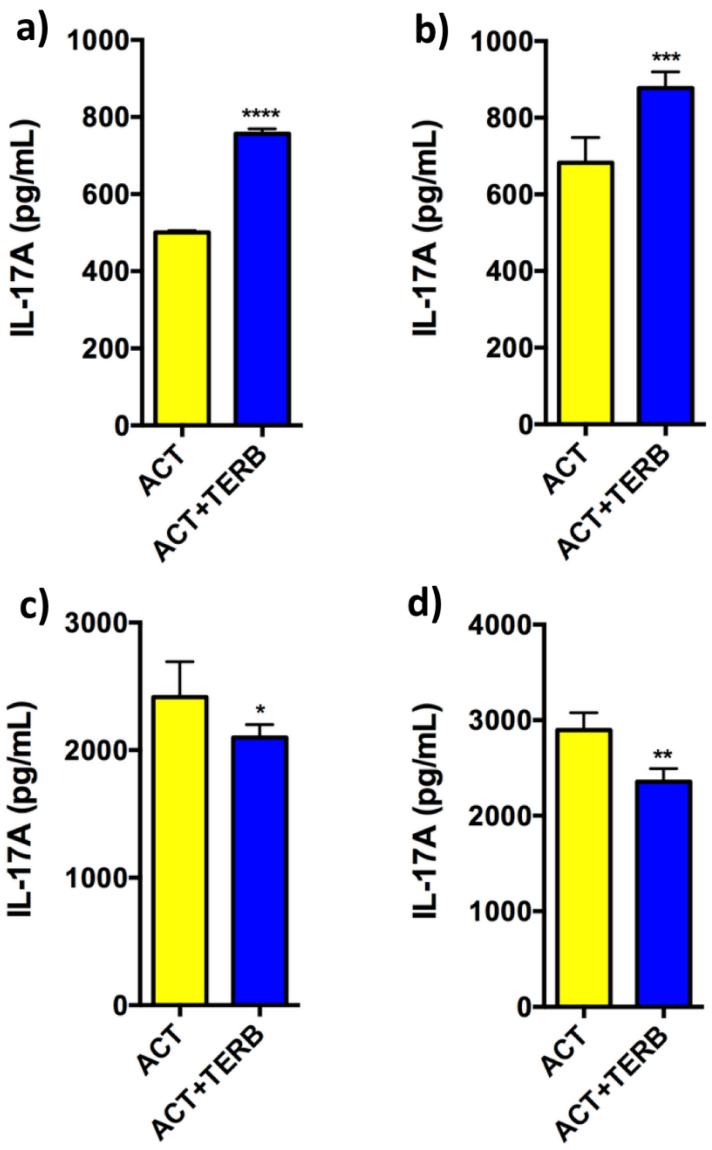



**Figure 2.3. Test/retest of IL-17A levels upon β2AR stimulation with terbutaline.** Blood was drawn and tested from the same subject at least two weeks apart. Samples were treated with terbutaline and the cytokine IL-17A was measured with ELISA. The concentration of IL-17A of subject 1 measured for (a) the first time, and (b) the second time. The concentration of IL-17A of subject 2 measured for (c) the first time, and (d) the second time. ACT= Activated samples with anti-CD3 and anti-CD28, ACT+TERB= Activated samples with terbutaline ($10^{-5}$M). Unpaired t-test*p<0.05 ** p< 0.01***p<0.001,****p<0.0001. Error bars show the standard deviation.

Effect of β2-agonist on proliferation

To determine if the effect of β2-agonist on cytokine levels was related to proliferation, PBMCs were stained with the fluorescent dye CFDASE prior to incubation with the reagents. As expected, without the activating stimulus, cells retained a high level of fluorescence signifying that they did not proliferate (Figure 2.4a). Upon activation, nearly 50% of Th cells had divided, upon activation with terbutaline, about 60% of Th cells had divided (Figure 2.4 b,c). This effect was abolished by the addition of β2 antagonist (Figure2.4d). Pooling data together from several samples revealed that terbutaline increased proliferation by approximately 1.1 fold, which is a 10% increase (Figure2.4e). All subjects PBMCs had a slightly elevated proliferation response when terbutaline was added, regardless of whether the IL-17A went up or did not change (data not shown). Cell viability, obtained from trypan blue counting of the samples, was constant through all conditions (Figure 2.4f).

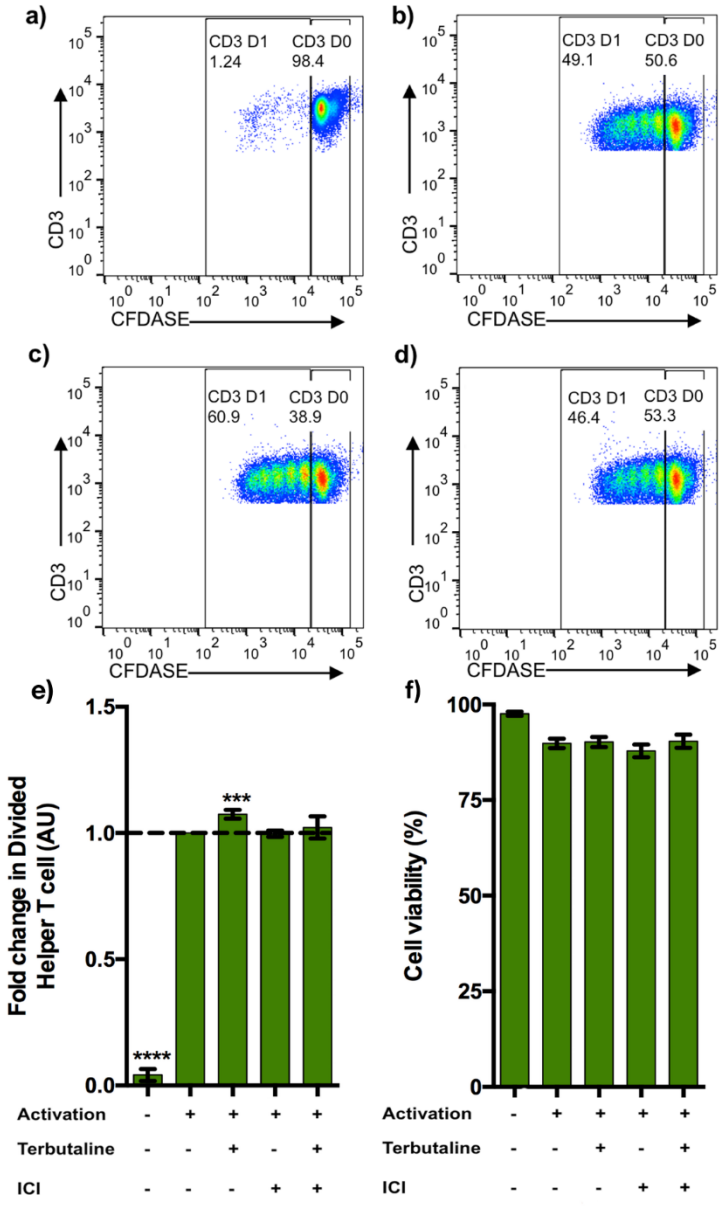



**Figure 2.4. Effect of β2AR agonist on proliferation of helper T cells.** PBMCs were stained with CFDASE, a proliferation-tracking dye, and incubated with terbutaline and/or ICI as described in figure 2.2. At four days, samples were stained with CD3 and CD4 then analyzed by flow cytometry. Data was gated on $CD3^{+}CD4^{+}$ events (not shown). The level of CFDASE was plotted against CD3 for (a) non-activated, (b) activated, (c) activated plus terbutaline, and (d) activated

plus terbutaline plus ICI. Undivided (D0) and divided (D1) events are shown. (e) Pooled data from 11 experiments calculated as fold change relative to activated group. (f) Cell viability was determined by trypan blue counting. Data pooled from 15 experiments. 1-way ANOVA followed by Tukey's multiple comparisons test. (****<0.0001, ***<0.001). Error bars show the standard deviation.

cAMP promotes IL-17A and inhibits IFNγ

The results with terbutaline, which is known to activate a G protein-coupled receptor, suggests that cAMP pathway promotes IL-17A production by Th cells while inhibiting IFNγ. To test this using an alternate approach, samples were activated in the presence of dbcAMP, which is a non-hydrolysable analogue of cAMP. A higher level of IL-17A was detected in the cell culture supernatants of activated samples treated with dbCAMP ($p<0.01$), relative to the activation control group (Figure 2.5a). The amount of IL-17A in the presence of dbcAMP had a trend to be higher than the amounts measured in the presence of terbutaline (Figure 2.5a). IFNγ levels were decreased ($p< 0.0001$) by dbcAMP in activated samples, relative to the activation group, which was similar to the effects of terbutaline (Figure 2.5b). Thus, cAMP is stimulatory for IL-17A while it has an opposite effect on IFNγ.

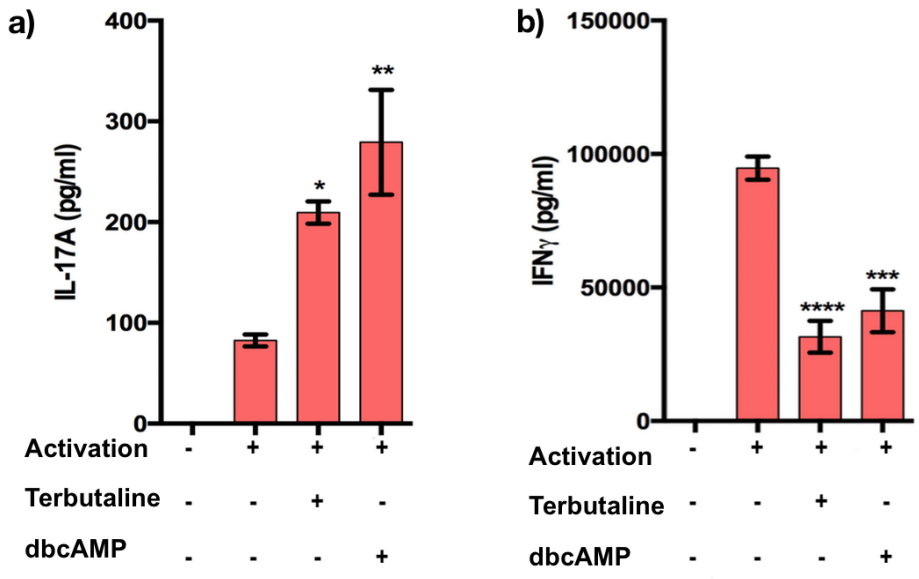



**Figure 2.5. Treatment with dbcAMP causes reciprocal regulation of IL-17A and IFNγ.** PBMC samples were activated with anti-CD3 and anti-CD28 antibodies with either terbutaline, or dbcAMP (500µM). After four days of incubation the levels of (a) IL-17A and (b) IFNγ were measured with ELISA. 1-way ANOVA followed by Tukey's multiple comparison test (*< 0.05,**< 0.01 ,***<0.001, ****< 0.0001) relative to the activated group. Representative of three experiments. Error bars show the standard deviation.

Effect of β2AR on purified Th17 cells.

The previous experiments used PBMC, which contains Th cells, and various other immune cell populations. To determine if the agonist effects were direct and specific for Th17 cells, immunomagnetic positive selection kit was used to prepare a purified fraction of Th17 cells, where live lymphocytes were 85.6% (Figure 2.6a) and resulted in 96.8% CD3+CD4+ cell population (Figure 2.6b). Cells expressing $CD3^{+}CD4^{+}$ were 93.2% positive for the CCR6 marker of Th17 cells (Figure 2.6c). The purified Th17 samples were then activated with a T cell-specific stimulus of anti-CD3 and anti-CD28 coated beads. Terbutaline increased the IL-17A levels and decreased IFNγ detected in the supernatants of purified Th17 cells (Figure 2.6 d,e). This demonstrates that the effect seen by terbutaline was likely due to a direct effect on Th17 cells and not due to other cells that could be found in the PBMC mixture. A schematic diagram that represents terbutaline and possible reciprocal regulation on IL-17A and IFNγ cytokine in Th cells is shown in Figure 2.7.

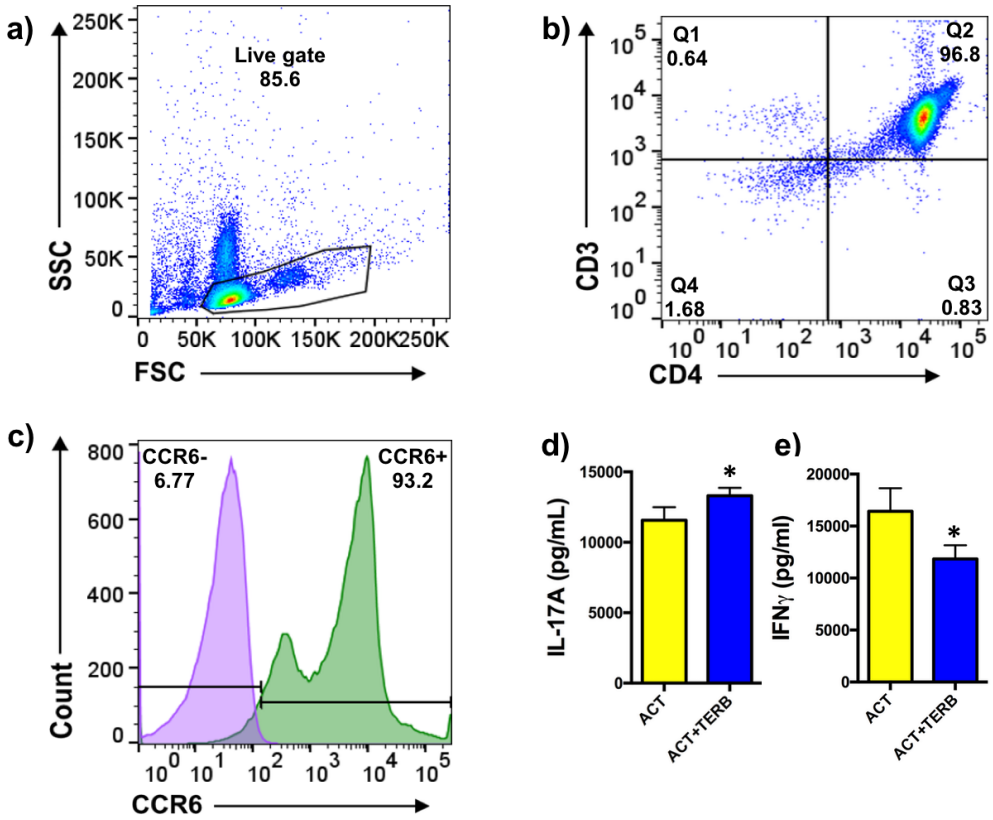



**Figure 2.6. The effect of β2AR agonist on purified Th17 cells.** Th17 cells were obtained from PBMC cells by an immunomagnetic positive selection kit that yields purified $CD3^{+}CD4^{+}CCR6^{+}$ cells. (a) Density dot plot of forward-scatter (FSC) vs side-scatter (SSC). Live cells are gated, indicating that 85.6% are live lymphocytes. (b) Purity was 96.8% selective for $CD3^{+}CD4^{+}$. (c) 93.2% of the $CD3^{+}CD4^{+}$ cells were $CCR6^{+}$ cells. (d, e) Purified $CD3^{+}CD4^{+}CCR6^{+}$ cells were incubated with dynabeads coated with anti-CD3 and anti-CD28 for four days with or without terbutaline ($10^{-5}$M). The level of d) IL-17A e) IFNγ in the supernatant was measured with ELISA. This data is representative of four experiments. Student t-test (*<0.05). Error bars show the standard error.

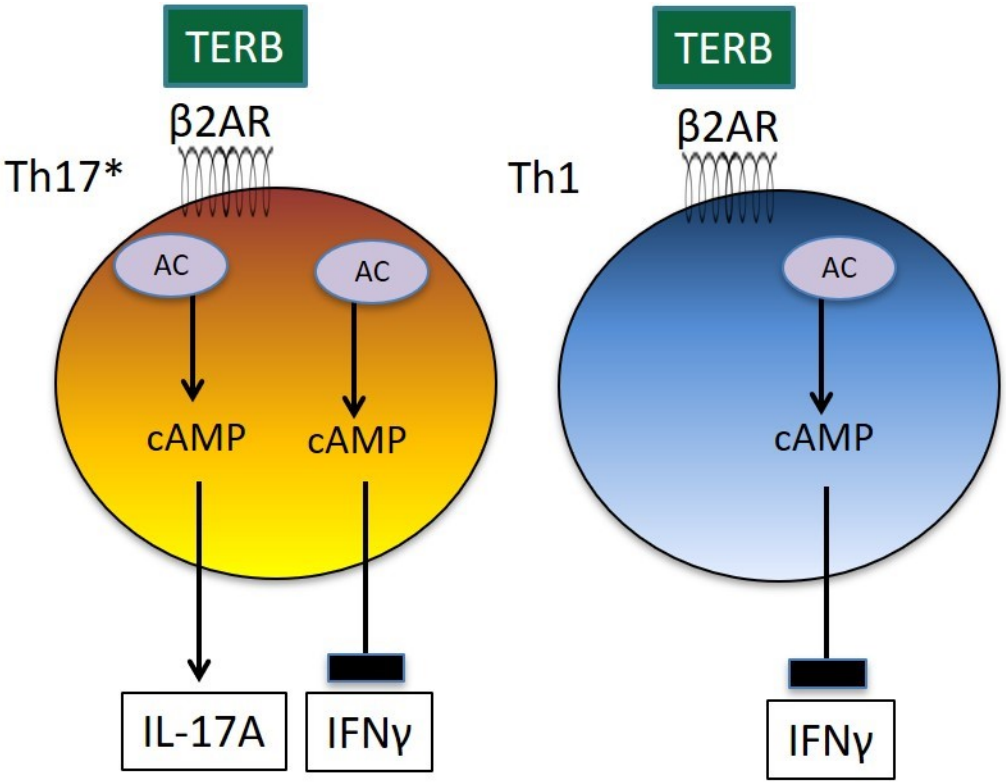



**Figure 2.7. Modulation of activated Th17 and Th1 cells by β2-AR agonist terbutaline (TERB).** Upon the treatment of activated Th17 cells with terbutaline, IL-17A secretion was promoted (->) possibly through the effect of adenylyl cyclase (AC). The IFNγ is inhibited by terbutaline in the Th17 cells and likely inhibited in the Th1 cells. * In a minority of samples from people terbutaline inhibited IL-17A.

## 2.5 Discussion

### Expression and function of β2AR on Th17 cells

T cell differentiation is determined by a number of factors including the strength of TCR-signalling, co-stimulation, and polarizing cytokines. Catecholamine hormones also influence this process by acting through adrenergic receptors. We demonstrated that a proportion of Th17 cells express the β2AR and that a β2-specific agonist augments IL-17A production. The cells that expressed β2AR were likely to be previously activated *in vivo* due to the expression of CCR6, a chemokine receptor present on effector and memory Th17 cells (261). These results indicate that a proportion of Th17 cells express β2AR and should therefore respond to β2AR agonists. We found that terbutaline increased IL-17A in PBMCs and purified Th17 cells, but inhibited IFNγ. The effect on IFNγ is consistent with previous reports where Th1 cells responses are inhibited by adrenergic agonists (246,266,268). However, there is a controversy in the literature in results related to adrenergic agonists and Th1 cells. Reports have shown that treatment with adrenergic agonist increase Th1 cytokines (265,267), or inhibit Th1 cells (254). The experimental systems can explain the difference in Th1 cytokine levels obtained by our study and other reports; The timing and order of the addition of the adrenergic agonist and activation stimulus might alter the responses (243,276).

Another example of reciprocal regulation was seen with Prostaglandin E2 ($PGE_2$), a proinflammatory chemical that signals through a G protein-coupled receptor. PBMCs treated with $PGE_2$ produced more IL-17A and less IFNγ (172). Reciprocal regulation also occurred when Th17 cells were cultured with mesenchymal stem cells that produced and secreted $PGE_2$ (171,172). Thus, agents that cause G protein signalling and elevate cAMP tend to promote IL-17A responses while inhibiting IFNγ. The theoretical summary mechanism of action of how the IL-17A and IFNγ can be influenced by terbutaline is shown on figure 2.7.

Terbutaline augmented IL-17A in the majority of PBMC samples tested, however, there were a minority of subjects' PBMC that showed no change in IL-17A or a downwards trend in IL-17A in response to terbutaline. All of the samples tested had more IL-17A with dbcAMP exposure which points to differences in the upstream β2AR receptor pathway, or the receptor

itself. Common polymorphisms exist for the *ADRB2* (223). In asthma cohorts, the variability of lung capacity responses to β agonists has been correlated to polymorphisms in the *ADRB2*, which supports the idea that there is a link between gene and function for adrenergic drugs (229,230,277). A recent study did not find a correlation between the polymorphisms of *ADRB2* and IFNγ responses to isoproterenol in T cells (222). To date, there is no adequate explanation as to why *ADRB2* polymorphisms are related to pharmacodynamics of the drug-receptor interaction. We are currently investigating the link between *ADRB2* polymorphisms and the IL-17A terbutaline phenotype.

Effect of β2-agonist on proliferation

We observed a small but significant proliferative effect on Th cells activated and treated with terbutaline. An anti-proliferation effect on T cells has been reported with various adrenergic agonists such as isoproterenol, fenoterol, NE, and the inflammatory mediator $PGE_2$, which was among the strongest proliferation inhibitors (241,242,278–283). However, terbutaline is relatively weak in comparison with these agonists (244). Co-stimulation also partially reverses the anti-proliferative effect (284). The activation mix contained anti-CD28 co-stimulatory antibodies, which may account for why we did not see an anti-proliferative effect.

cAMP pathway promotes IL-17A

We demonstrated that dbcAMP augments IL-17A levels in activated PBMCs. Generally, cAMP is thought inhibit T cell activation and proliferation (285). For Th1 cells, the cAMP pathway has been shown to be inhibitory (249). The fact that dbcAMP augmented IL-17A suggest that the signalling pathways in Th17 cells is different than other T cells. It has been shown that upstream signalling compartments are notably different in Th17 cells than other subsets(286). Moreover, knockouts of the stimulatory Gα subunit decreased cAMP accumulation in Th cells, which in turn reduced the differentiation of Th17 cells (287). Thus, Th17 cells appear to require the cAMP-PKA pathway for their development.

Effect of β2AR on purified Th17 cells.

Performing the experiment on highly purified Th17 cells revealed that terbutaline augmented IL-17A levels to a similar extent as seen in the PBMC mix. β2AR agonist augmented the levels of IL-17A on highly purified Th17 cells. There was detectable IFNγ in these cells, and treatment with β2AR agonist decreased the level of IFNγ. The source of the IFNγ in the purified Th17 cells could be from Th17 cells that can produce IFNγ as well, or residual Th1 cells (263,288,289). The fact that terbutaline modulated purified Th17 responses rules out the involvement of APCs in the observed effects, since the magnetic columns removes myeloid cells.

Potential physiological relevance

Sustained activity of the SNS could result in NE secretion into the T cell areas of the secondary lymphoid tissues which primes DCs to favour Th17 differentiation after the naïve cells turn to effectors (254,255). In addition, lymphocytes are also capable of synthesizing catecholamines through the action of tyrosine hydroxylase, which may play a part in auto-regulating the balance of Th cell subsets (252,290–295). There is a balance of subtypes of Th cells that is maintained within the body to effectively eliminate pathogens and keep a healthy immune system. The differential production of cytokines showed that stimulation of the β2AR with terbutaline augments the Th17 cell population. The parasympathetic nervous system, which generally opposes the function of the sympathetic nervous system, will inhibit Th17 cells via the neurotransmitter acetylcholine acting through the α7nAChR, a nicotinic receptor that responds to acetylcholine from cholinergic nerves (296). Thus, sympathetic signals boost Th17 responses in most people, while parasympathetic signals inhibit Th17 responses.

## Chapter 3 foreword (bridging text)

In chapter 2, I demonstrated that terbutaline, a β2AR-specific agonist, augmented IL-17A which is one of the major cytokines made by Th17 cells. That finding disproved the initial hypothesis that β2AR agonists would inhibit Th17 cell responses. The β2AR has a cell signalling pathway involving second messenger cAMP and PKA enzyme which are known to be suppressive towards Th cells such as Th1 cells, which is why it was hypothesized that Th17 cells would be inhibited. Upon further reading, I discovered that phosphorylated CREB might augment IL-17A, although no researcher had tested this idea in the context of adrenergic drugs. The fact that Th17 cells were reacting differently than Th1 cells to terbutaline suggested that there was either an alternative signalling pathway in Th17 cells or, Th17 cells were augmented by the cAMP-PKA pathway. In the previous chapter, I tested the cAMP analogue dbcAMP which activates PKA and showed that IL-17A was augmented. That finding supported a new hypothesis that a β2AR-specific agonist augmented IL-17A through a cAMP-PKA dependent pathway.

There were some key unanswered questions remaining from Chapter 2. For example, I knew that terbutaline augmented IL-17A in PBMC samples, and in purified Th17 cells, however, it was not clear why there was more IL-17A. Using ELISA to measure IL-17A in cell culture supernatants is quantitative, but does not yield information on which cells, or how many cells have IL-17A. If IL-17A was augmented, it could be due to each cell making more cytokine, perhaps due to elevated IL-17A transcription, translation or secretion. Or, it could be due to more Th17 cells being present due to enhanced proliferation or differentiation and RORγ expression. Specific aim 2.1 was meant to address this. I determined if the augmented IL-17A was due to an increase in the proportion of Th17 cells in the samples, or if it was due to increased expression of Il-17A in each cell. To make the determination I used intracellular cytokine staining that measures IL-17A within each cell of the sample and provides intensity of staining in each cell. The Th17 transcription factor RORγ was also increased by terbutaline further arguing that it was Th17 cells that increased. These experiments supported the

conclusion that terbutaline was causing more cells to produce IL-17A, because there was a higher proportion of Th17 cells, but the amount of IL-17A per cell was unchanged. It was important to know this because it suggested that terbutaline was inducing proliferation of Th17 cells which might explain why I saw a small increase in proliferation. There are some limitations to this interpretation that are discussed in the chapter discussion.

For specific aim 2.2, I sought to determine if the β2AR agonist-induced the phosphorylation of the transcription factor CREB, which is a downstream substrate of PKA. Based on my readings phospho-CREB could augment IL-17A, but that was not known in the context of adrenergic drugs and Th17 cells. Using a flow-cytometry based approach I concluded that terbutaline increases phospho-CREB in the Th17 cells which supported the notion that terbutaline augments PKA activity which phosphorylates CREB. However, it does not demonstrate the involvement of PKA. To do that, I created specific aim 2.3 to determine if terbutaline augments IL-17A thought a cAMP-PKA dependent pathway. Using PKA inhibitors, and a cAMP activator, it was clear that terbutaline was inducing the cAMP/PKA pathway via β2AR which augmented IL-17A.

Now that the mechanism of action by which terbutaline augmented IL-17A was more clear, I decided to test a drug that may oppose that pathway. Specific aim 2.4. was to determine if an inverse-agonist of β2AR, nebivolol, would diminish IL-17A levels. Since nebivolol is also a β1 agonist I included a β2AR-antagonist in the experiments to ensure specificity of the response. I also measured nebivolol effect on IFNγ since the goal of this project was to ideally find a new drug that would inhibit both IL-17A and IFNγ since they are both detrimental to autoimmune disease. The results showed that both IL-17A and IFNγ were inhibited by nebivolol in a β2AR-specific manner. In the discussion, I speculate on why the two drugs tested have differing effects on these two cytokines.

Nebivolol is best known as a drug used to treat cardiovascular conditions. It had not been explored as an immunomodulator by immunology researchers. Therefore, my research indicates that a β2AR inverse-agonist has the potential to be a novel immunomodulatory drug.

# Chapter 3 The effects of β2- adrenergic agonist terbutaline and inverse-agonist nebivolol on IL-17A production by Th17 cells.

## 3.1 Abstract

**Background**

Immunomodulatory drugs that are used to treat cell-mediated autoimmune diseases typically suppress Th1 cells and augment Th2 cells. Drugs from the family of the β2-adrenergic receptor (β2AR) agonists have been tested as immunomodulators. They can inhibit Th1 cells and promote Th2 cells in human samples and animal models of autoimmunity. However, the effect of these drugs on Th17 cells is not completely understood. Recent results showed that β2AR-agonist terbutaline augmented IL-17A secretion. In the context of autoimmunity, Th17 cells and IL-17A are considered to be pro-inflammatory, thus, augmenting them would not be beneficial in theory. In this report, we determined the cell signalling pathway responsible for the β2AR-agonist effect on Th17 cells and showed that an inverse- β2AR-agonist nebivolol could suppress both IFNγ and IL-17A in human immune cell samples.

**Methods**

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were collected from venous blood using ficoll techniques and activated *in vitro* with soluble anti-CD3 and anti-CD28 antibodies, or anti-CD3/CD28 dynabeads. Cellular proliferation was measured with CFDASE with the flow cytometer. Intracellular cytokine staining was used to measure the proportion of IL-17A and RORγ expressing cells, Ser133phospho-CREB by flow cytometer. Secreted cytokines IL-17A and IFNγ were measured with ELISA. Chemicals used were terbutaline, H89 and rpcAMP which are PKA inhibitors, rolipram a phosphodiesterase inhibitor, and nebivolol a β2AR-inverse-agonist.

**Results**

Terbutaline augmented IL-17A ($p<0.001$) and diminished IFNγ ($p<0.01$) cytokine secretion from activated PBMC, while Th cells proliferation increased upon terbutaline treatment. The

proportion of Th17 ($p<0.05$) cells were augmented by terbutaline as determined by intracellular staining for IL-17A and RORγ. Terbutaline increased Ser133p-CREB in activated PBMC ($p<0.05$). The PKA inhibitors H89 and rpcAMP prevented terbutaline from augmenting IL-17A ($p<0.05$). Rolipram augmented IL-17A similar to the effect of terbutaline. In contrast, nebivolol suppressed both cytokines IL-17A and IFNγ.

**Conclusion**

We demonstrated that a β2AR-specific agonist terbutaline augmented Th17 cells through a cAMP and PKA-dependent pathway. Thus, terbutaline could exacerbate Th17-mediated autoimmune disease. In contrast, nebivolol diminished IL-17A and IFNγ and warrants further testing in Th17 mediated autoimmune diseases.

## 3.2 Introduction

The beta 2-adrenergic receptor (β2AR), is one of the only adrenergic subtype expressed on T lymphocytes (264). Th1 cells express β2AR, while Th2 cells do not express this receptor as shown with radioligand binding and a specific β2AR antagonist ICI 118,551 for $^{125}$I-pindolol displacement (249). The reason that Th2 cells do not express β2AR is due to epigenetic modification oh histones on *ADRB2* the gene encoding β2AR (250). The ligands for β2AR include endogenous catecholamines epinephrine and norepinephrine, and a variety of adrenergic drugs used to treat asthma and cardiovascular diseases. Catecholamines and other β2AR agonists have potent modulatory effects on the function of adaptive T helper (Th) cells (297,298). For example, the βAR agonist isoproterenol decreased IL-2 and increase of cAMP in human memory T cells (242). Murine naïve CD4 cells treated with norepinephrine or terbutaline had lower levels of IL-2 (243). Murine Th1 cells treated with terbutaline had lower IFNγ but there was no change in the IL-4 from Th2 cells (249). Th1 cells treated with terbutaline had an increase of cAMP which correlated to the suppression of IFNγ (249). The βAR agonist salmeterol decreased IFNγ in human isolated $CD4^+$ T cells activated with superantigen staphylococcal enterotoxin B or cytomegalovirus, and the effects were β2AR-specific since they were blocked

by the antagonist ICI 118,551 (248). Norepinephrine or epinephrine decreased IFNγ but had no effect on IL-4 in PBMCs that were stimulated with PHA, IL-2, tetanus toxin or anti-CD3, or plate-bound anti-CD3 and anti-CD28 (246). The authors also showed that norepinephrine and epinephrine inhibited IFNγ from $CD4^+$ T cells and $CD8^+$ T cells, and this observation coincided with an elevation of cAMP in the cells (246). Epinephrine, norepinephrine, and terbutaline also inhibited IFNγ from PBMCs that were stimulated with lipopolysaccharide, an innate component of bacterial cell wall (251). The authors found that the adrenergic agonists increased Th2 cytokines IL-10, IL-4 and IL-5 which may have been due to Th1 inhibition (251). When catecholamines suppressed Th1 cells in murine T cells, the Th1 transcription factor T-bet was decreased, which may have facilitated expansion of Th2 cells since the Th2 transcription factor was increased (252). This would account for how Th2 cells expand despite the fact that they do not express adrenergic receptors.

Less is known about how adrenergic agonists modulate Th17 cells. Th17 cells are pro-inflammatory Th cells that produce the cytokine IL-17A which mediates the recruitment of neutrophils to sites of infection (144). They are also identified by the transcription factor RORγ, and chemokine receptor CCR6 (107). The natural function of Th17 cells is to protect against bacteria (e.g *straphylococcus aureus*) and fungus (e.g *Candida albicans)* (113,299). Th17 cells can exacerbate autoimmune diseases such as multiple sclerosis or rheumatoid arthritis in people who have genetic and environmental susceptibility (108,300–302). In recent studies, norepinephrine caused higher levels of IL-17A and lower levels of IFNγ in murine T cells (256). Similarly, our group showed that human Th17 cells are augmented by the β2AR-specific agonist-terbutaline, as measured by elevated IL-17A levels, while Th1 cells responses were inhibited (303).

We showed previously that dbCAMP, a non-hydrolysable version of cAMP reproduced the effect of terbutaline on Th17 cells, suggesting that cAMP signalling pathways augmented Th17 cells (303). The main signalling pathway induced by full agonists of the β2AR is a G-protein-coupled receptor mechanism (Figure 3.1) where Gα stimulatory subunit activates AC (191). Conformational changes occur to accommodate the ligand in the β2AR binding pocket

which will then trigger an intracellular pathway response (193,304). AC catalyses the conversion of adenosine triphosphate to cAMP which will bind and activate the PKA (265,304). The activation of PKA leads to several intracellular changes in the cell, including the phosphorylation of the transcription factor cAMP response element-binding (CREB) (37,38). In the context of T cells, CREB promotes cell proliferation, survival, cell differentiation and T cell activation (37,38,40,41,305–307). Furthermore, CREB is required for the expression of IL-17A cytokines in T cells, which indicates that the cAMP pathway may promote Th17 cell phenotype (41,308,309). PKA can activate CREB by phosphorylation at Ser133 site which is prevented when the site is mutated (37,39,310,311). PKA inhibitors, such as H89 or rpcAMP, strongly inhibited the downstream phosphorylation and activity of CREB (37,38,312). Another regulator of cAMP in T cells is phosphodiesterase 4 (PDE4) which catabolizes cAMP thereby inactivating PKA (200,202,313). The chemical rolipram can selectively inhibit PDE4 which results in elevated cAMP in T cells (35,314).

Depending on the stabilization of the receptor, the G protein-coupled or the β-arrestin signalling pathway can be triggered (201,315). Phosphorylation of the cytoplasmic tail of the β2AR by GRK recruits' scaffold β-arrestin protein (199). The β-arrestin plays roles include the recruitment of phosphodiesterase enzymes which degrade cAMP molecules, thus counteracts the G protein-coupled signalling, also plays role in desensitizing and internalizing the receptor (199,204). Thus, the β2AR has the capacity to either raise or lower cAMP depending on the type of ligand encountered. For example, an agonist will activate the receptor while antagonist will oppose the g protein pathway and lower cAMP levels (191). Nebivolol is an inverse-agonists for the β2AR and it can promote the arrestin pathway over the cAMP-PKA pathway (2). Nebivolol is prescribed for cardiovascular diseases treatments because it releases nitric oxide from vascular cells leading to vasodilation (209). Nebivolol has not been studied as an immunomodulatory drug.

In this study, we demonstrated the β2AR agonist terbutaline augments IL-17A cells and diminishes IFNγ cells in a cAMP and PKA-dependent manner. This suggests that terbutaline would exacerbate symptoms in a Th17 cell-mediated autoimmune disease. We discovered that

the inverse-agonist nebivolol diminished IL-17A and IFNγ in samples from healthy participants. Thus, nebivolol warrants further study as a possible new immunomodulatory drug to treat Th1 and Th17-mediated autoimmune diseases.

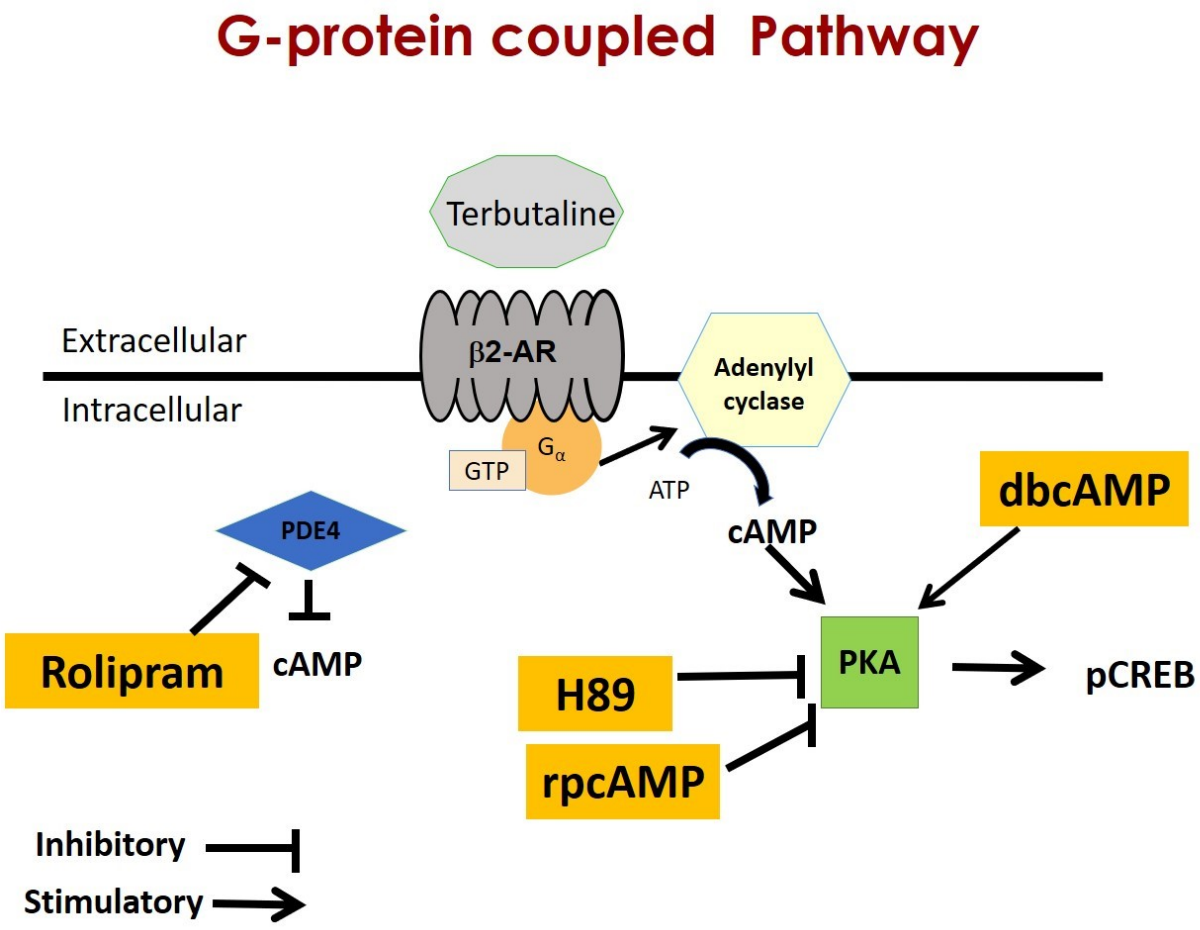



Figure 3.1. Signalling model of β2AR Function. Classic G protein-coupled receptor signalling where GαS activates AC, which elevates cAMP. cAMP activates PKA which phosphorylates a series of kinases, which inhibits T cell activation. PKA phosphorylate CREB. Phosphodiesterase 4 (PDE4) recruited by the β2AR stimulation, which degrades cAMP. Several chemicals were used to interrogate the pathway including dbcAMP (Chapter 2), rolipram, H89, rpcAMP (Chapter 3). The arrows indicate : ---] Inhibition → activation

## 3.3 Material and Methods

**Obtaining cells**

The study was approved by the Concordia University Human Research Ethics Committee (certificate 30009292). The Exclusion factors included cardiovascular disorders, autoimmune disease, neurological disorders, metabolic diseases, pregnancy, regular smokers, beta-blockers, beta-agonists, under the age of 18, or recent recreational drug use or nicotine within 24 hours of the study. Informed, signed consent was obtained prior to the blood donation. Venous blood collected in sodium heparin-coated vacutainers were processed by ficoll techniques to obtain PBMC (303). For the indicated experiments, PBMC were applied to Th cell negative selection by CD4/CD3 using EasySep™ Human CD4 Negative Selection Kit (Stemcells technologies Vancouver, BC, Canada). The purity was assessed with CD3-PercP (UCHT1) (1:10), CD4-APC (RPA-t4) (1:10) (BD Bioscience, Mississauga, ON, Canada) antibodies, and analyzed by flow cytometry (BD Bioscience, Mississauga, ON, Canada) with FlowJo software (Treestar Inc., AZ, USA). For flow cytometry analysis, the data was first gated on live cells using the forward-scatter (FSC) vs side-scatter (SSC) to exclude small debris. Helper T cells were then gated on CD3-PercP vs CD4-APC. At least 50,000 events were obtained on the flow cytometer (FACS Verse, BD Bioscience).

**Cell culture conditions**

PBMC were cultured in RPMI with 10% fetal bovine serum (FBS), 1mM penicillin/streptomycin, 2mM L-glutamine in an incubator maintained at 37$^{o}$C and 5% $CO_2$. Activation was done *in vitro* in 96 well plates using between 0.4 - 0.5x$10^6$ PBMC cells per well. The total number of cells per well was the same for each condition, but the value had to be adjusted for experiments depending on yield. To activate, soluble antibodies CD3 (clone OKT3, eBioscience, San Diego, CA, USA) and anti- CD28 (clone CD28.2, eBioscience, San Diego, CA, USA), were added at a final concentration of 0.1 μg/ml each. Where indicated, dynabeads were used to stimulate the purified T cells, they are pre-coated micro-particles with anti-CD3 and CD28 were used at a 1:1 ratio (Thermo Fisher Scientific, Mississauga, ON, Canada). The dynabeads were also used in the CREB experiment so they could be removed prior to the

staining to avoid cross-reaction with the other antibodies used for staining. The various drugs added were terbutaline ($10^{-5}$M) (Sigma, ON, Canada), H89 (1µM, 2µM, 10µM) (Sigma, ON, Canada), rpCAMP (10µM) (Sigma, ON, Canada), and rolipram (1µM) (Sigma, ON, Canada). These chemicals are water-soluble, so they did not require a vehicle control. However, rpcAMP was soluble in DMSO so a vehicle control was used with matched dilution to what was used for the rpcAMP. Nebivolol was used at $10^{-5}$M (Nebivolol hydrochloride, N1915, Sigma Aldrich) and a DMSO vehicle control was included at a matching dilution.

**Proliferation and cytokine measurement**

Proliferation was measured using 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFDASE) dye (Sigma Aldrich, ON, Canada), as previously described(275,303). Before the cells were treated with the drugs, they were labelled with CFDASE. In brief, the cells were resuspended in RPMI with 10% fetal bovine serum (FBS), with CFDASE at a final concentration of 5µM incubated for 5 minutes and washed three times with 10% FBS in PBS to remove excess dye, counted, and put into cell culture. At the timepoint, cells were harvested and stained with CD3-PercP (UCHT1) (1:10), CD4-APC (RPA-t4) (1:10) (BD Bioscience, Mississauga, ON, Canada), and analyzed by flow cytometry and FlowJo software using the cell proliferation tool. Cytokines were measured from the cell culture supernatants using ELISA according to the manufacturer's instructions for human IFNγ (BD Bioscience, Mississauga, ON, Canada), or human IL-17A (eBioscience, Mississauga, ON, Canada).

**Intracellular staining**

Intracellular IL-17A and RORγ were measured using established intracellular cytokine staining (ICS) procedures (173). In brief, the cells were treated with a mixture of ionomycin (0.5µg/ml), brefeldin A (5µg/ml) and phorbol 12-myristate 13-acetate (PMA)(0.02µg/ml) for a five-hour incubation at 37$^{o}$C at 5% $CO_2$. The cells were fixed and permeabilized with BD fixation/permeabilization solution (BD Bioscience, Mississauga, ON, Canada). Fluorescently labelled antibodies were then added. Surface markers targeting helper T cells (CD3-PercP (1:10) and CD4-APC (1:10)) (BD Bioscience, Mississauga, ON, Canada) in addition of IL-17-PE (1:10), or RORγ-PE (1:10) (BD Bioscience, Mississauga, ON, Canada). Samples were incubated for 45 min,

on ice in the dark. Two washes (493 x g 5 min) with staining buffer (1% FBS in 1X PBS) was done and resuspended in 200 µl staining buffer in microcentrifuge tubes and then analyzed by flow cytometry, BD FACS verse (BD Bioscience, Mississauga, ON, Canada). At least 50,000 cells were obtained by flow cytometry.

**Measuring pCREB**

In order to ensure precise timing, cryopreserved PBMCs were thawed and used for the CREB experiments. The freeze/thaw procedures were optimized to result in a 99%+ viability after thawing, as published elsewhere (173). PBMC were rested in the incubator (37$^{o}$C in 5 % $CO_2$) for one hour. T cell blast was made by adding low doses of PMA (1ng/ml) and Ionomycin (1ng/ml) and incubating for 24h. The cells were then washed and incubated in media for an additional 24h rest period. The cells were then washed cultured in 0.01% FBS RPMI and activated with dynabeads and the indicated drug treatments for 15 min in a sterile 96 well plate. The cells were then placed on a magnet and washed to remove dynabeads, and fixed in 4% formaldehyde, for 15 min at room temperature, washed two times in staining buffer (1% FBS in 1XPBS), and resuspended in 10µl of staining buffer. Then True-Phos™ Perm Buffer (Biolegend, San Diego, CA, USA) was added for one hour at -20$^{o}$ C. Following steps are on ice otherwise indicated. Cells were washed two times with staining buffer and human serum IgG was added for 15 min to block nonspecific bindings. Antibodies were added for 30 min include: CD3-Percp, CD4-PeCy7, (BD Bioscience, Mississauga, ON, Canada), total CREB-PE (48H2) (Abcam, Toronto, ON, Canada), phospho- Ser133 CREB- A488 (87G3) (Abcam, Toronto, ON, Canada), and CCR6-APC (1:10) (BD Bioscience, Mississauga, ON, Canada). All experiments were done in triplicate wells. Cells were resuspended in staining buffer and analyzed by the flow cytometer (BD FACS verse). Sequential gating was done for characterisation of the cell population of the flow cytometry data. The gates and quadrants of the experiments are placed based on the controls, which include no stain control and single stain control (data not shown). The population characteristics, that is the percentages and fluorescence intensity were analyzed depending on the marker of interest.

## 3.4 Results

To determine if the cAMP-PKA pathway was involved in β2-agonist effects on T cells, a series of cell signalling experiments were performed on human PBMC. As we previously showed, the selective β2 agonist terbutaline augmented IL-17A in PBMC samples activated with anti-CD3 and anti-CD28 (Figure 3.2A). In contrast, terbutaline diminished IFNγ in the same samples (Figure 3.2B). Since PBMC contain a mix of cell types, we tested the effects of terbutaline on purified helper T cells. The purified, activated helper T cells showed a similar pattern where terbutaline augmented IL-17A but diminished IFNγ (Figure 3.2C, D). The proliferation of Th cells increased upon terbutaline treatment (Figure 3.3).

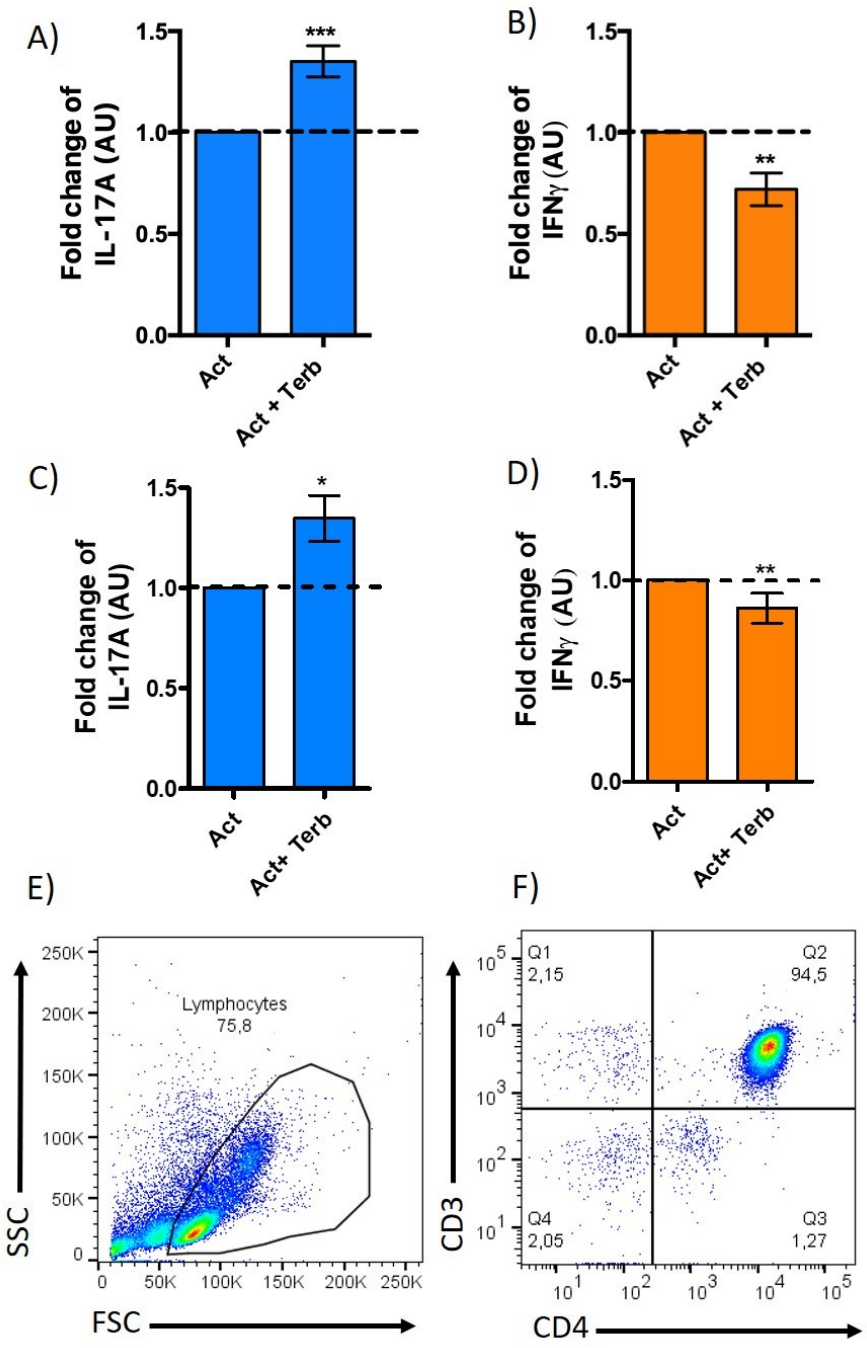



**Figure 3.2. IL-17A and IFNγ response to β2AR specific agonist terbutaline**. A, B) PBMC were obtained from healthy humans, and the cells were exposed *in vitro* to activation (anti-CD3 and anti-CD28), or activation plus terbutaline for four days, then supernatants were collected. lL-

17A and IFNγ were measured by ELISA. The data is calculated as fold change compared to the activated group which was set to 1.0, where the dotted line is indicated (A) IL-17A (B) IFNγ. 1-way ANOVA followed by Tukey's multiple comparisons test (**<0.01 ***<0.001). Error bars show the standard error. Data are pooled from 42 PBMCs samples, with at least two replicates of each experimental group. The data is calculated as fold change compared to the activated group which was set to 1.0, where the dotted line is indicated C, D) Isolated helper T cells were activated with dynabeads (with anti -CD3 and anti-CD28) with or without terbutaline and measured by ELISA for (C) IL-17A (D) IFNγ. A comparison relative to the activated group was made, Student T-test (*<0.05 **<0.01). Error bars show the standard error. A representative of 3 independent experiments. At least two replicates of each experimental group E) density dot plot of forward-scatter (FSC) vs side-scatter (SSC) for PBMCs, live cells are gated and named lymphocytes showing 75.8% live cells F) Purity check of isolated helper T cells stained with CD3 and CD4, showing 94.5% purity.

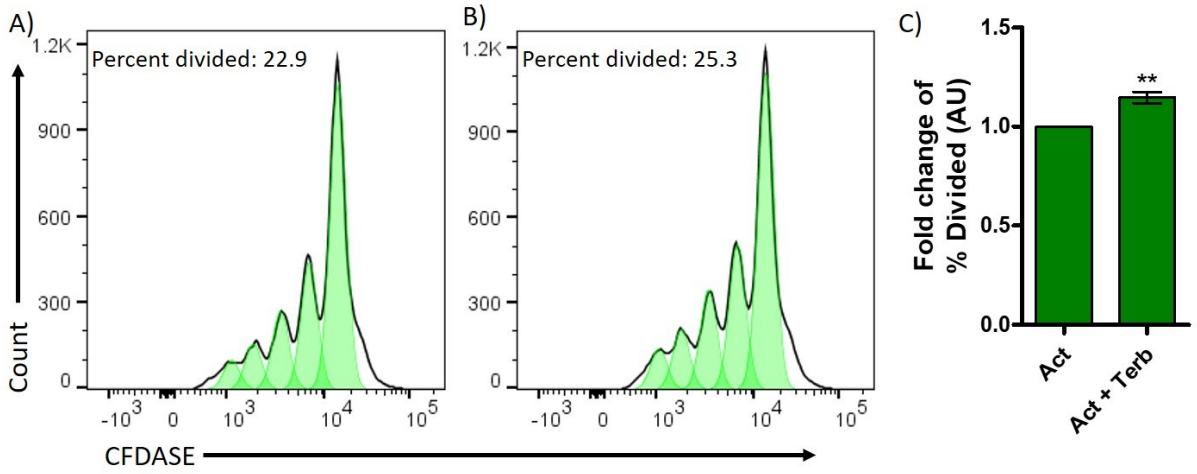



**Figure 3.3. Proliferation of Th cells treated with Terbutaline.** Flow cytometer analysis of a representative PBMC stained with CFDASE dye, then treated with activation mixture anti-CD3 and anti-CD28 and terbutaline for 4 days. Samples were gated on Th cells (CD3 vs CD4), data not shown. Histogram plots for CFDASE are shown, for A) activated cells or B) activated cells plus terbutaline. On the y-axis is the number of cells and the x-axis is the fluorescence of CFDASE. C) Proliferation was analyzed using the flowjo proliferation tool, results of the fold change of percent divided relative to the activated group. Data pooled from 8 independent experiments. Two replicates of each experimental group. Error bars are made with standard errors. Paired T-test (**<0.01).

To determine if β2AR agonist augmented IL-17A and inhibited IFNγ in a PKA-dependent manner, the PKA inhibitors H89 and rpcAMP were used in cell culture experiments. H89 abrogated the ability of terbutaline to inhibit IL-17A in comparison to the activated cells plus terbutaline group (Figure 3.4A). H89 abrogated the ability of terbutaline to inhibit IFNγ in comparison to the activated cells plus terbutaline group (Figure 3.4B). The dose of H89 used (2μM) was selected because a higher dose had shown inhibition of IL-17A and the same effect of IFNγ at 10μM compared to the 2μM of H89 (Figure 3.4 C, D) but the 10 μM significantly decreased proliferation (Figure 3.4E). The viability was not changed for the H89 doses tested

(Figure 3.4F). Thus, H89 blocks the terbutaline effects at the lower dose where it does not alter proliferation or viability.

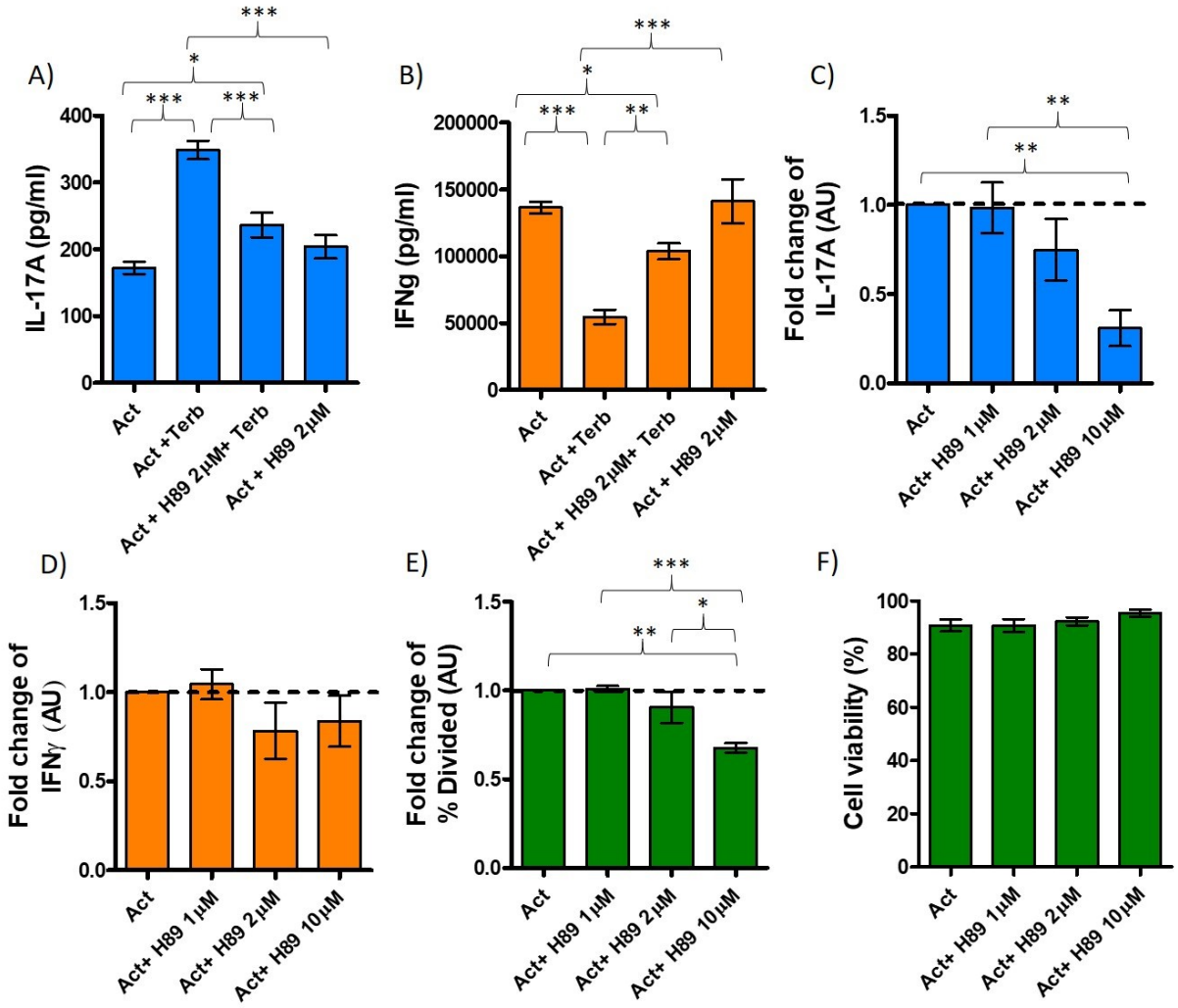



**Figure 3.4. The effect of IL-17A and IFNγ of immune cells treated with H89.** To determine the involvement of the cAMP pathway in IL-17A and IFNγ production, PBMCs were activated and treated with terbutaline and/or H89. Supernatants were collected and measured with ELISA. Act: activated (anti-CD3 and anti-CD28) plus Terb (Terbutaline). Addition of both H89 (2µM) and terbutaline was done and supernatants were measured for A) IL-17A (pg/ml) and B) IFNγ (pg/ml), data is representative of 3 independent experiments. C, D, E) The data was calculated

as fold change compared to the activated group which was set to 1.0, where the dotted line is indicated. Concentrations of H89 (1µM, 2µM, 10µM) C) Shows the IL-17A levels, Data pooled from 6 experiments. D) shows the IFNγ levels, data pooled from 6 experiments. E) Proliferation of cells was measured by staining with CFDASE. Data are pooled from 8 experiments. F) The cell viability was measured by trypan dye exclusion counting, data pooled from 6 experiments. Two replicates of each experimental group for A-F. Error bars show the standard error. One-way ANOVA followed by Tukey's multiple comparisons test (*<0.05 **<0.01***<0.001).

Since H89 may have off-target effects at the higher doses, another PKA inhibitor was tested (rpcAMP). Terbutaline augmented IL-17A secretion from activated PBMC. However, when it was combined with rpcAMP IL-17A levels were inhibited. Even without terbutaline, rpcAMP inhibited IL-17A secretion (Figure 3.5). These results demonstrated that terbutaline augmented IL-17A and in a cAMP-PKA dependent manner.

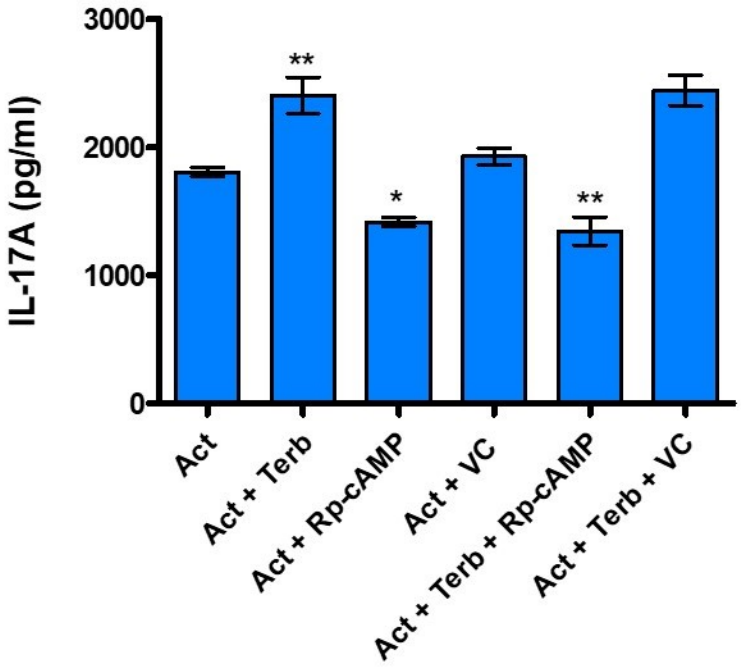



**Figure 3.5. The effect of IL-17A of PBMCs treated with rpcAMP**. To determine the involvement of the cAMP pathway in IL-17A production, PBMCs were activated and treated with rpcAMP. Supernatants were collected and measured with ELISA. Act: activated (anti-CD3 and anti-CD28) plus rpcAMP (10µM) and its vehicle control (VC). The concentration of A) IL-17A pg/ml, representative of 4 independent experiments. Two replicates of each experimental group. Data is compared relative to the activated group. Error bars show the standard error. One-way ANOVA followed by Tukey's multiple comparisons test. (*<0.05**<0.01).

Since previous experiments inhibited the pathway, we next promoted the pathway using rolipram a phosphodiesterase inhibitor. The addition of rolipram to the activated PBMC augmented IL-17A secretion (Figure 3.6A), whereas it diminished IFNγ (Figure 3.6B). The cell viability of these cells was not changed in the addition of rolipram indicating a lack of toxicity from this chemical (Figure 3.6C). These results showed that rolipram has similar effects as dbcAMP as we previously showed, and terbutaline, that is, it augmented IL-17A and inhibited

IFNγ. Together, these results demonstrate that the cAMP-PKA pathway augments IL-17A secretion.

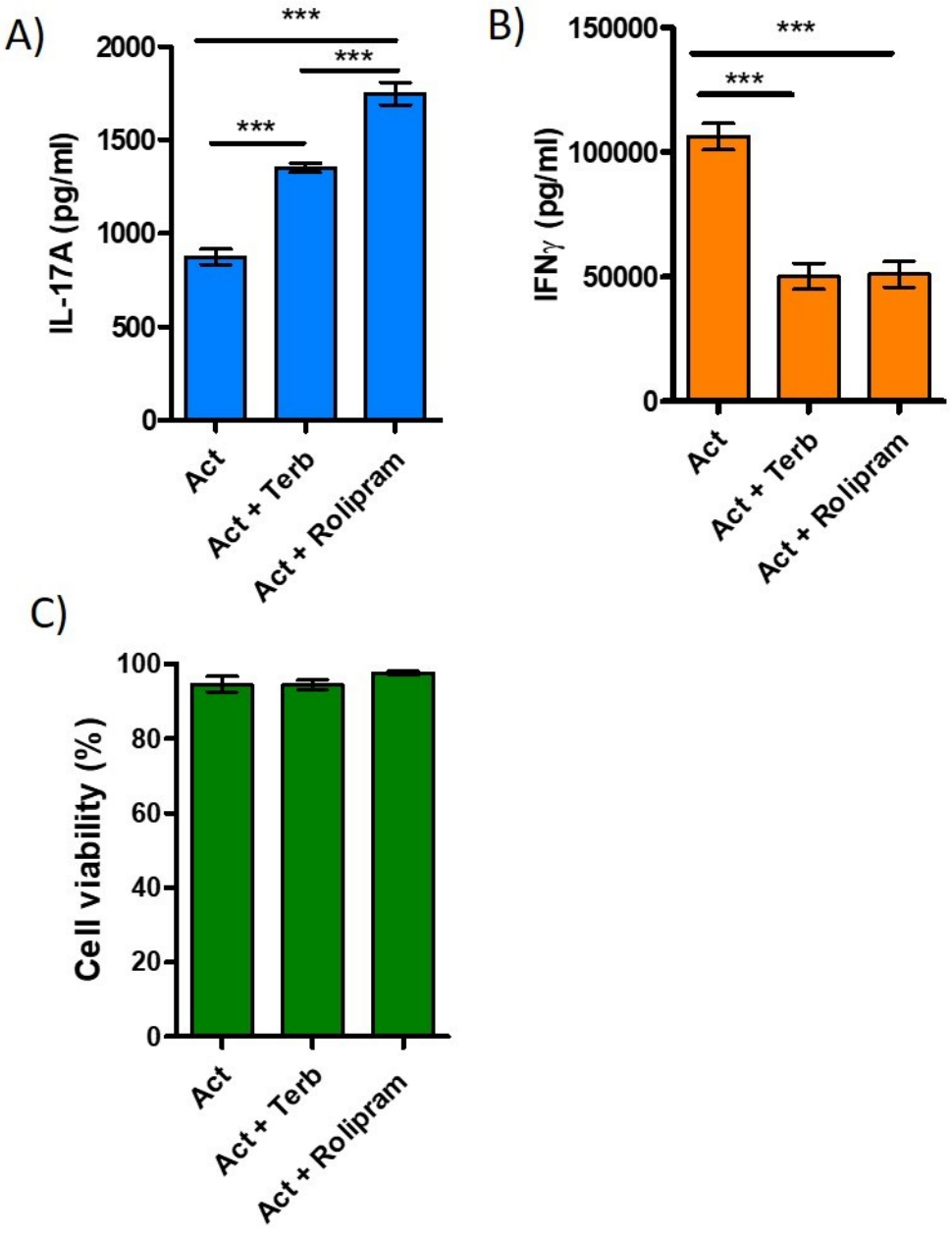



**Figure 3.6. The effect of IL-17A and IFNγ of immune cells treated with rolipram**. To determine the cAMP-PKA pathway involvement with IL-17A and IFNγ production, rolipram was added.

Supernatants of activated (anti-CD3 and anti-CD28) PBMCs in addition with rolipram (1μM) were measured for IL-17A and IFNγ with ELISA. A) Shows the IL-17A levels, a representative from 6 experiments. B) shows the IFNγ levels, a representative from 6 experiments. C) The cell viability was measured by trypan dye exclusion counting, data pooled from 3 experiments. Two replicates of each experimental group. Error bars show the standard error. One-way ANOVA followed by Tukey's multiple comparisons test (***<0.001).

Next, intracellular CREB and phospho-CREB was measured since it is a downstream substrate of PKA activity. A representative of the live lymphocytes density dot plots is shown in Figure 3.7A. Th cells ($CD3^{+}CD4^{+}$) were gated from the live lymphocyte's population (Figure 3.7B). In order to determine the effect in Th17 cells, the cells were also stained with CCR6. The gated Th17 ($CCR6^{+}CD4^{+}$) cells are shown in Figure 3.7C. There was an augmentation of pCREB in the gated Th17 cells when terbutaline was added compared to the activated group (Figure 3.7 D, E). The fold change of the pooled experiments showed an augmentation in pCREB with the addition of terbutaline (Figure 3.7F). This demonstrates directly that terbutaline raises pCREB inside of Th17 cells.

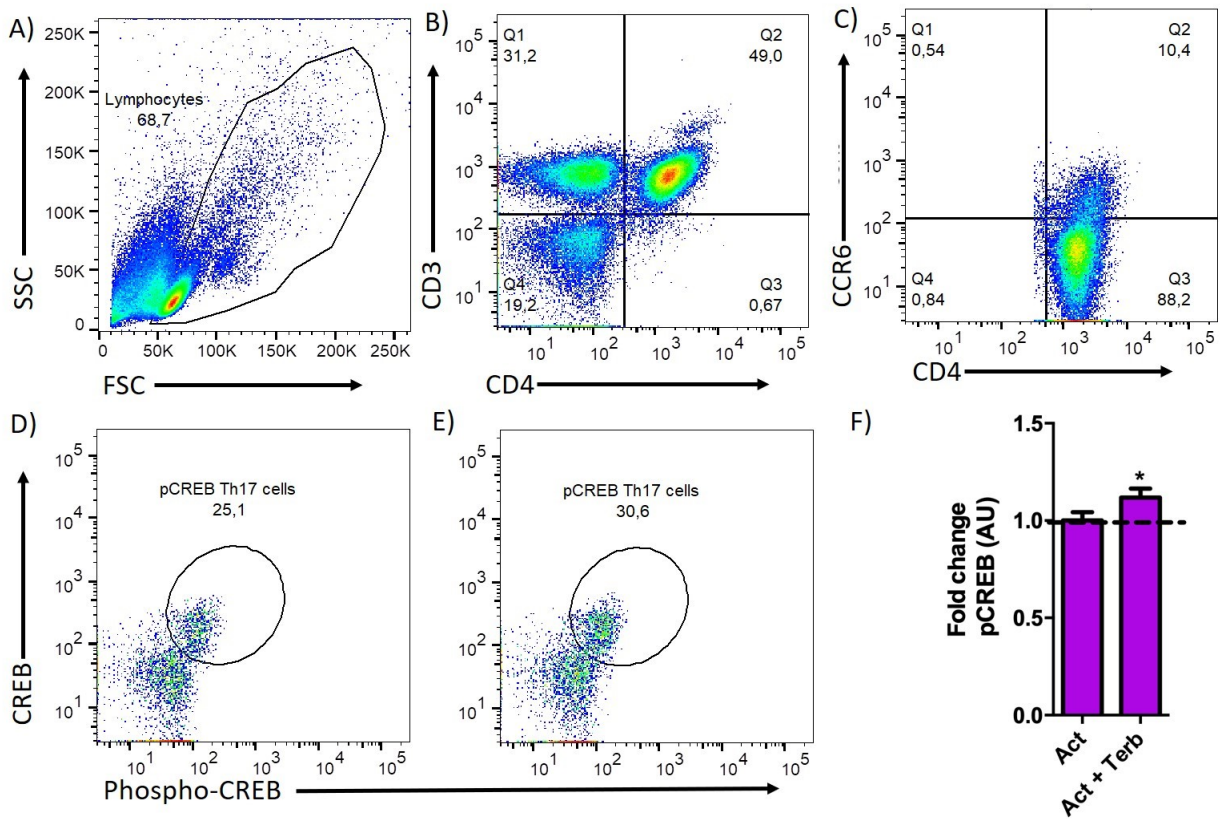



**Figure 3.7. Percentage of phosphorylated CREB in PBMC.** PBMCs were activated (Dynabeads anti-CD3 and anti-CD28) and treated with terbutaline and p133 CREB was measured by flow cytometry. Sequential gating was performed to identify specific cell populations. A) the population of lymphocytes is gated based on the side-scatter (SSC) and forward-scatter (FSC) plot, the gate drawn identifies the live lymphocytes from the sample (66.7%). B) From the gated lymphocytes, the population is analyzed based on the markers identifying the Th cells, that is CD3 for TCR and CD4 for Th cells. On quadrant 2 of panel B, there is the cell population containing double-positive stains $CD3^{+}CD4^{+}$ (49%), identifying the Th cell subpopulations. C) From the Th subpopulation of panel B, the Th17 cell population was identified by markers CCR6 and CD4. The Th17 cell population is in quadrant 2, which is the $CCR6^{+}CD4^{+}$ cell (10.4%) that is considered the Th17 cells. The quadrants were placed based on the controls, which include the non-stain and single stain controls (Data not shown). D, E) from the Th17 cell subpopulation of panel C, the pCREB population was identified by observing the markers CREB and pCREB, where the pCREB population was gated “pCREB Th17 cells”. Panel D is the activated samples (25.1%)

and panel E is the activated samples also with terbutaline (30.6%). F) pooled data of the pCREB of the Th17 cells. Three individual experiments were pooled. Three replicates of each experimental group. Error bars show standard error. Student T-test (*<0.05).

To determine if terbutaline affected the proportions of Th17, a single-cell analysis was performed using intracellular staining of cytokines (ICS) for IL-17A. There was a higher percentage of Th17 cells with activation and terbutaline treatment as compared to activating the samples without terbutaline (Figure 3.8A-C). The mean fluorescence of IL-17A inside of the cells was unchanged, indicating that terbutaline did not change the amount of IL-17A on a per-cell basis (Figure 3.8D). The amount of IL-17A in the cell culture supernatants from the single-cell analysis experiments was analyzed for IL-17A. As expected, IL-17A was higher in the presence of terbutaline (Figure 3.8E). Thus, terbutaline augments IL-17A in the cell culture supernatants by boosting the proportion of Th17 cells.

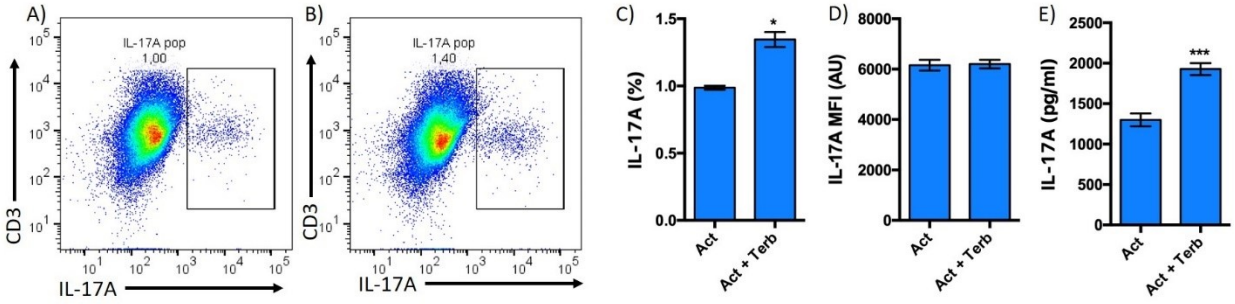



**Figure 3.8. IL-17A Intracellular cytokine staining in PBMCs treated with terbutaline.** PBMCs were treated with activation mixture anti-CD3 and anti-CD28, and terbutaline. The cells were fixed, permeabilized and stained with anti-IL-17A and analyzed with the flow cytometer. A, B) Representative dot plots are shown for IL-17A on lymphocytes gated on CD3 vs IL-17A, A) activated cells B) activated cells plus terbutaline. C) The percentage of Th cells expressing IL-17A is shown. D) The mean fluorescence intensity (MFI) of IL-17A in the cells from the flow

cytometry data is plotted in arbitrary units (AU). E) IL-17A cytokine from the supernatant of these experiments was measured with ELISA. There is an increase in IL-17A when adding terbutaline. The data from this Figure are representative of 4 independent experiments. Two replicates of each experimental group. Unpaired T-test (*<0.05 **<0.01), error bars are standard error.

To verify the finding that terbutaline expands the proportion of Th17 cells, the Th17-specific transcription factor RORγ was analyzed in Th cells. Terbutaline augmented the proportion of Th cell expressing intracellular RORγ in activated Th cells gated from PBMC (Figure 3.9A-C). The control CD4 stain was included to show that the population gate from panels A and B is the population positively stained for RORγ (Figure 3.9 D). The fluorescence intensity of RORγ was not changed (Figure 3.9E). Cell culture supernatants from these experiments confirmed that terbutaline treatment resulted in higher levels of IL-17A (Figure 3.9F). Thus, terbutaline increased the number of $CD3^{+}CD4^{+}ROR\gamma^{+}$ and $IL\text{-}17A^{+}$ T cells which is consistent with the phenotype of Th17 cells.

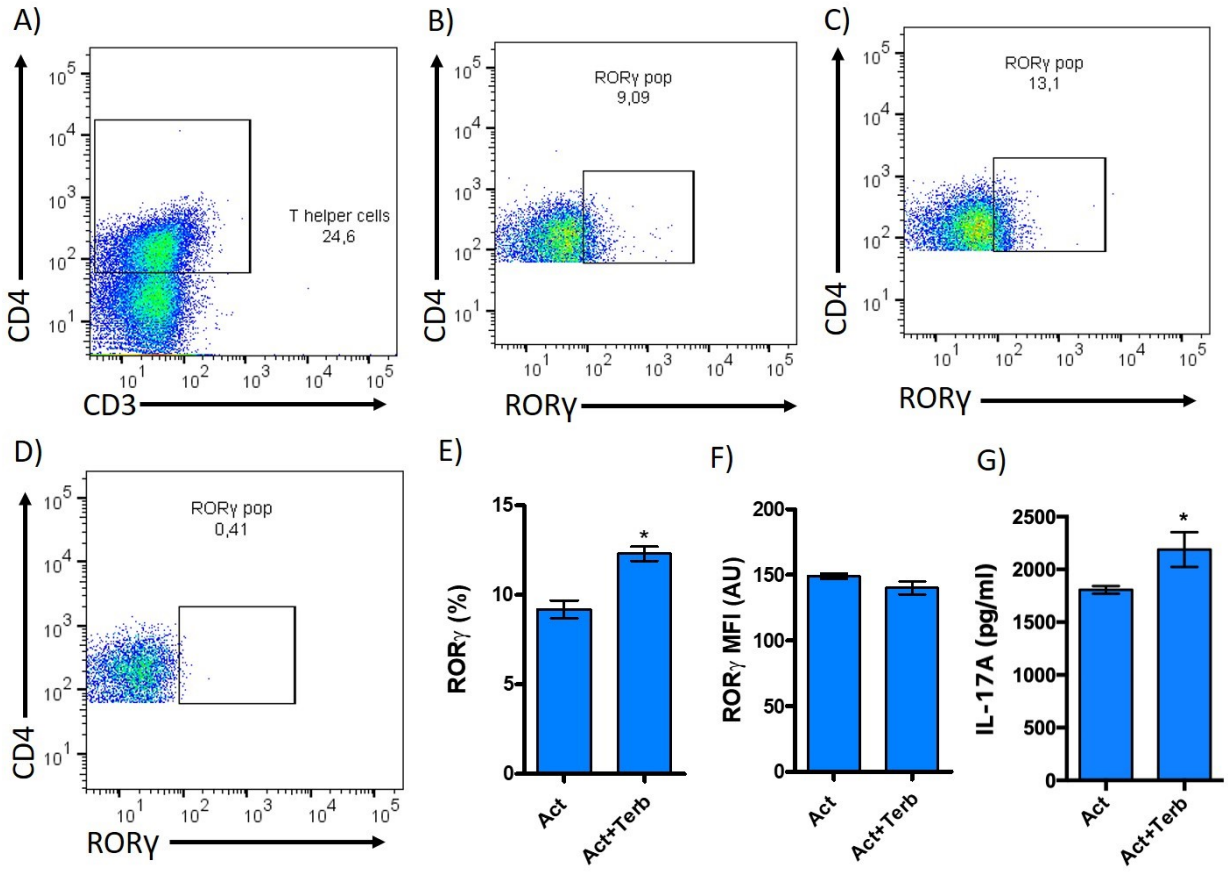



**Figure 3.9. RORγ Intracellular staining in PBMCs treated with terbutaline.**

A) $CD3^{+}CD4^{+}$ gated cells on PBMCs population, RORγ expression in Th cells from B) activated (anti-CD3 and anti-CD28) C) activated cells plus terbutaline samples. D) The $CD4^{+}$ only stain control showed 0.41% on the RORγ population gated, which was subtracted from the RORγ percentage that is pooled and showed in panel E), where less than 1% is background. The no stain control and isotype control showed 0% on the gate drawn for the RORγ population. Data not shown E) The percentage of cells expressing RORγ was background subtracted and then pooled together. There was a higher percentage of cells expressing RORγ when treated with terbutaline. F) The mean fluorescence intensity (MFI) of RORγ is shown, the flow cytometry data is plotted in arbitrary units (AU). G) Secreted IL-17A was measured in the supernatant of these experiments by ELISA. The data of this figure is representative of two independent

experiments. Two replicates of each experimental group. Unpaired T-test (*<0.05), error bars are made with standard error.

The finding that terbutaline augmented IL-17A in the majority of samples tested is not a desirable quality for potential new treatments for Th17-mediated autoimmune diseases. For this reason, we tested nebivolol which is considered an inverse-agonist of the β2AR. Its effects on T cells had not been previously researched. We conducted *in vitro* experiments to determine if this drug acted directly in the cells of interest. First, we applied nebivolol to purified Th cells that were activated *in vitro* and found that the inverse-agonist nebivolol suppressed IL-17A and IFNγ secretion in purified Th cells (Figure 3.10). Next, we showed that nebivolol suppressed IL-17A in a β2AR-dependent manner using the specific antagonist ICI 118, 551 (Figure 3.11A) and confirmed that nebivolol was not altering cellular proliferation or viability (Figure 3.11B, C). The results showed that nebivolol inhibited both IL-17A and IFNγ in Th cells and PBMC samples, in a β2AR-dependent manner.

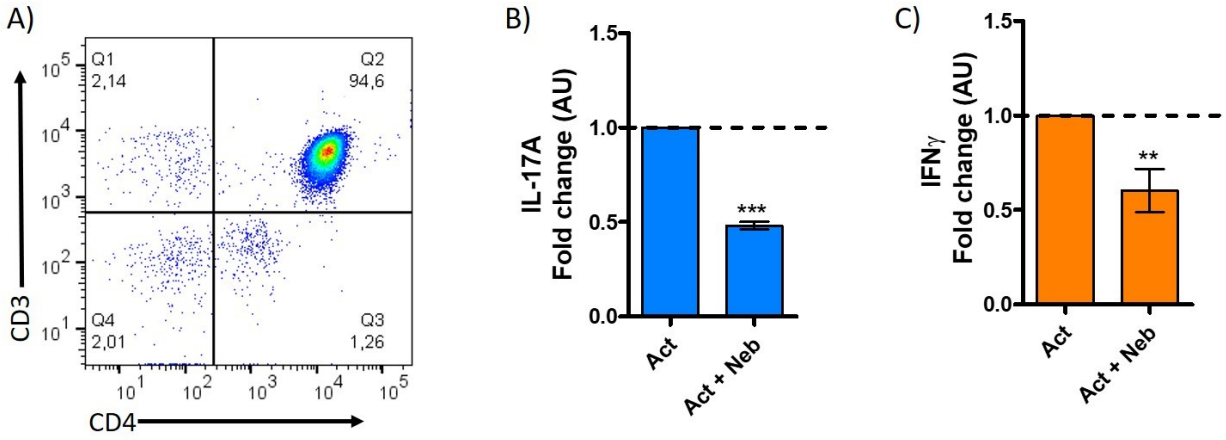



**Figure 3.10. Inverse-agonist nebivolol suppresses IL-17A and IFNγ secretion in purified Th cells.** Th cells were obtained from 3 healthy participant PBMC samples and activated in vitro with dynabeads pre-coated with anti -CD3 and anti-CD28, without or with nebivolol. A) An

example of the purified Th cells prior to activation, stained with CD3 (y-axis) and CD4 (x-axis) and analyzed by flow cytometry. B, C) IL-17A and IFNγ cytokines were measured in supernatants after 4 days activation, the raw data was calculated as fold change relative to the activated group, which was set to 1.0, where the dotted line is indicated. ELISA for IL-17A (B) and IFNγ (C) were performed, they showed significant decrease of both cytokines measured. Pooled data from 3 individual experiments. Two replicates of each experimental group. Error bars are shown from standard error. Student T-test (**<0.01, ***<0.001).

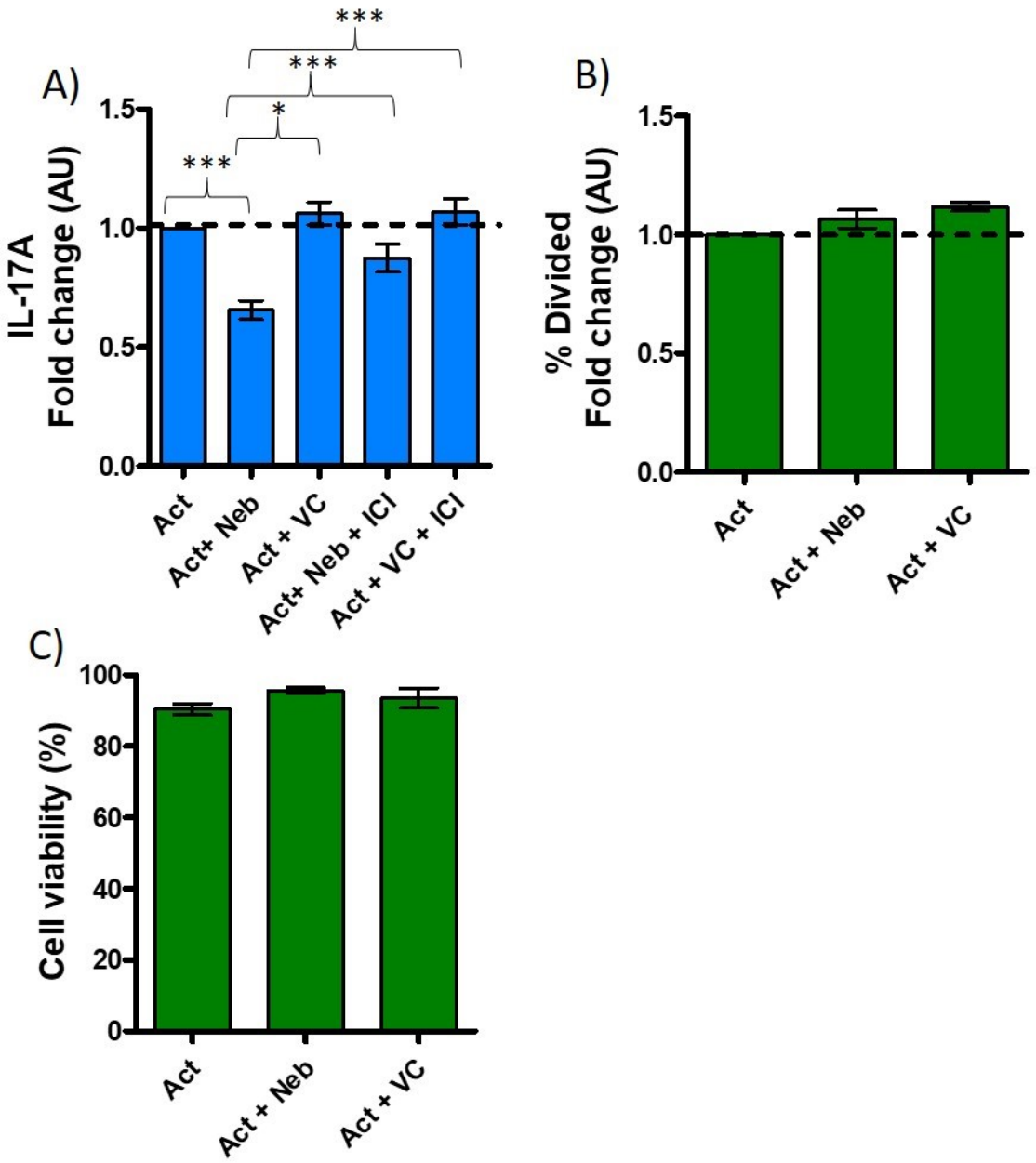



**Figure 3.11. Inverse-agonist nebivolol suppresses IL-17A in a β2AR-dependent manner**. PBMC samples were activated with anti-CD3 anti-CD28 for 4 days and the IL-17A levels in supernatants were measured and expressed as fold change compared to activated control,

which was set to 1.0, where the dotted line is indicated. Nebivolol which required a vehicle DMSO was compared to just the vehicle added as a control (VC), and the β2AR-specific antagonist ICI 118, 551 was added to determine if the effects were via this receptor. A) Nebivolol suppresses IL-17A in a β2AR-dependent manner. Pooled data from 4 individual experiments. Two replicates of each experimental group. B) To determine if nebivolol altered cellular proliferation, PBMC were labelled with CFDASE, and the number of cell divisions (expressed as percentage of cells that divided at least once) is shown. There was no significant effects observed on proliferation. Three replicates of one experimental group. C) To determine if nebivolol altered cellular viability, PBMC were counted using trypan exclusion at the end of the incubation time. There was no significant effects observed on cell viability. Data pooled from 3 experiments. Two replicates of each experimental group. One-way ANOVA followed by Tukey's multiple comparisons test. (*<0.05, **<0.01,***<0.001).

## 3.5 Discussion

Adrenergic agonists are emerging as immunomodulatory compounds, however, their mechanism of action on the adaptive immune system is not completely understood. Recently, we demonstrated that the β2 agonist terbutaline augmented IL-17A and diminished IFNγ cytokine secretion in primary human T lymphocytes (303). One possible explanation for the decreased IFNγ was that the drug was altering proliferation of T cell. A previous study showed that the adrenergic agonists norepinephrine or terbutaline decreased proliferation on murine and human $CD4^+$ T cells (243,244). However, we did not detect anti-proliferative effect of terbutaline, rather, we observed a small but significant increase in activated human T cells treated with terbutaline. Other studies have not found anti-proliferative effect of adrenergic agonists in T cells (245,246). Thus, it is possible that adrenergic agonists inhibit Th1 cells through various mechanisms of action depending on the type of agonist or source of the T cells. For example, Th1 cells were inhibited by adrenergic agonists via the cAMP second messenger pathway (264,265,316), and we showed that cAMP analogue (dbCAMP) inhibited IFNγ (303). Similarly, dbcAMP and Forskolin (which increases endogenous cAMP) reduced the IFNγ levels as

measured with cAMP enzyme-immunoassay, ELISA and western blot (317). The PKA inhibitor (Rp-cAMP) reversed the cAMP inhibitory effect on IFNγ, whereas the EPAC inhibitor had no effect (317). Those experiments were done on PBMCs from latent tuberculosis patients with no clinical or radiological evidence of active tuberculosis stimulated with heat-killed M. tuberculosis. The authors did not address β2AR effects in those studies. In another study, CREB inhibited the IFNγ promoter by competitive binding of Jun/ATF2 which these are proteins that positively regulate IFNγ production as shown with CREB overexpression plasmids and IFNγ promoter-luciferase reporter in T cells (318). Thus, cAMP inhibits IFNγ due in part by CREB suppressing IFNγ transcription.

We further explored the mechanism of action by which terbutaline augmented IL-17A. The proportion of Th17 cells expressing intracellular IL-17A was increased even though the intensity of intracellular IL-17A on a per-cell basis was unchanged. This indicated that terbutaline was causing more Th17 cells in the activated cultures which accounted for the augmented IL-17A cytokines measured by ELISA. The augmentation of IL-17A was due to the acting of second messenger cAMP which plays a different role in Th17 cells as compared to Th1 cells. For example, dbcAMP promotes a Th17 phenotype in human naïve T cells, PBMCs and murine $CD4^+$ T cell (303,319,320). Moreover, we found that rolipram augmented IL-17A secretion while inhibiting IFNγ. This was similar to the literature where rolipram augmented IL-17A in activated murine Th cells (321), and rolipram inhibited IFNγ in activated T cells from human samples (322). By inhibiting phosphodiesterase's, rolipram is known to significantly elevate cAMP in T cells (323). Thus, the cAMP pathway augmented IL-17A while inhibiting IFNγ.

The fact that dbCAMP augmented IL-17A does not directly demonstrate that the β2AR uses this pathway. To test the hypothesis that β2AR augmented IL-17A via a cAMP and PKA-dependent manner, we included the PKA inhibitors H89 and rpcAMP. H89 is a widely used PKA inhibitor but it can have off-target effects at high doses, while rpcAMP a more specific PKA inhibitor (324). Both inhibitors prevented terbutaline from augmenting IL-17A in human lymphocytes, which confirms that β2AR augmented IL-17A via a cAMP and PKA-dependent manner. A previous study showed that H89 treatment of activated murine $CD4^+$ T cell resulted

in diminished IL-17, however, adrenergic receptors were not studied (320). A similar conclusion was drawn by Tsai *et al.* who showed that inhibition of PKA by rp-Br-cAMP in activated CD3/CD28 human T cells inhibited the production of IL-17A (309). As further evidence that β2AR augmented IL-17A via PKA-dependent manner, we measured one of the PKA substrates CREB. Terbutaline increased phosphorylation of CREB at Ser133 in activated human T cells. Phosphorylated Ser133-CREB binds to the IL-17A promoter region and promotes transcription (41,309), and pCREB collaborates with RORγ in promoting *Il17* transcription (41). Inhibition of CREB using negative polypeptides overexpression resulted in a reduction of IL-17 mRNA levels which further supports the role of pCREB in augmenting IL-17A (42). Other evidence that cAMP is stimulatory for IL-17 was observed in mouse models where the Gα subunit was knockout on the Th cells, there was cAMP reduction and the Th17 cells differentiation was diminished (Li et al. 2012).

The main goal for this research was to discover an immunomodulatory adrenergic agonist that may of use for treating Th1 and Th17-mediated autoimmune disease. Terbutaline was a potent inhibitor of IFNγ, however, it augmented IL-17A in most of the samples tested which suggest a detrimental outcome for a Th17-mediated autoimmune disease. Since the PKA-cAMP-CREB cell signalling pathway augments IL-17A, we tested an inverse-agonist of the β2AR which is known to oppose the PKA-cAMP-CREB cell signalling pathway in other cell types (2). Nebivolol is an inverse-agonist of the β2AR used for cardiovascular disease (209), and it has not been studied as an immunomodulator. Nebivolol inhibited both IL-17A and IFNγ *in vitro*. This was observed in PBMC samples, and in purified Th cell samples. The nebivolol effect on IL-17A was β2AR-specific as shown with the β2AR-inhibitor, and it did not alter the proliferation or viability of T cells. Future experiments could be done to address the underlying mechanism of action by which nebivolol might inhibit both IFNγ and IL-17A. One possibility is the cell signalling molecule nitric oxide, which is explained in more detail in the general discussion section of this PhD thesis.

In conclusion, β2AR drugs are emerging as immunomodulatory drugs. Terbutaline, an agonist of the β2AR suppressed Th1 cell cytokine IFNγ, however, it augmented Th17 cytokine IL-

17A which could be detrimental in autoimmune disease. Nebivolol, an inverse-agonist, inhibited both cytokines *in vitro*, indicating that it warrants further study as an immunomodulator.

## Chapter 4 foreword (bridging text)

Throughout the previous two chapters, I demonstrated that terbutaline, a β2AR agonist, inhibited IFNγ but augmented IL-17A in most of the human lymphocyte samples that I tested. These effects were due to the cAMP-PKA-pCREB cell signalling pathway, as indicated by my results, together with the recent literature that was cited. A major goal of the thesis was to discover an adrenergic drug that would be a potentially useful immunomodulator for treating autoimmune disease. In my experiments, terbutaline inhibited IL-17A in a minority of the samples that I tested, but the majority resulted in augmented IL-17A. If terbutaline were to be used as an inhibitor of IL-17A, I predict that it would only be effective in a small proportion of people. Ideally, a drug should be effective on all samples tested and have a regular effect. When I considered this problem, and read the recent literature confirming that the cAMP-PKA-pCREB promotes transcription of *IL-17A*, I decided to test an inverse-agonist (nebivolol) which opposed that pathway. Nebivolol inhibited both IFNγ and IL-17A in all of the samples tested although the sample size was small (N=4). In chapter 4 I tested nebivolol on a larger sample size and determined one of the factors that might influence drug response. Specific Aim 3.1 was to determine the complete sequence of *ADRB2* in primary human PBMC. There SNPs in the immediate upstream region, and within the coding segment of *ADRB2* the gene that encodes the β2AR, which I was able to determine in the blood samples. Specific Aim 3.2 was to determine if the combinations of SNP correlate to differential effects of β2AR agonist or inverse-agonist on IL-17A and IFNγ. I speculated that such single nucleotide polymorphisms that are located within, or nearby *ADRB2*, could have accounted for inter-individual variations. I found one polymorphism Arg16 that was homozygous in the samples where IL-17A did not respond or were inhibited by terbutaline. That suggests that genotyping might be needed to know which patient would benefit from terbutaline. However, in chapter 3 I identified nebivolol as a better candidate. Nebivolol suppressed IL-17A and IFNγ in all of the samples I tested, regardless of polymorphisms. In the chapter 4 discussion, I speculate why the two drugs are affected differently by polymorphisms.

In summary, nebivolol is a promising new immunomodulatory drug that warrants further study as a potential clinical treatment for Th1 and Th17-mediated autoimmune diseases. The next steps to reach that goal are described in the chapter 4 discussion and expanded on in the general discussion of the thesis.

# Chapter 4 The influence of *ADRB2* single nucleotide polymorphisms on immunomodulation of IL-17A by β2-adrenergic receptor agonist terbutaline and inverse-agonist nebivolol

**Abbreviated title:** Influence of *ADRB2* polymorphisms on IL-17A

## 4.1 Abstract

**Background:** Adrenergic drugs are emerging as immunomodulatory agents for pro-inflammatory autoimmune diseases. Helper T (Th) cells coordinate immune responses with IL-17A and IFNγ cytokines, but these cytokines may also exacerbate autoimmune diseases. β2-adrenergic receptor (β2AR) agonists suppressed IFNγ, but their influence on IL-17A is not fully understood. We assessed β2AR agonist and inverse-agonist effects on IL-17A and IFNγ, and the influence of β2AR gene (*ADRB2*) polymorphisms.

**Methods:** Mononuclear cells from venous blood of healthy human participants was cultured with T cell activators anti-CD3 and anti-CD28 antibodies, with either terbutaline (β2AR agonist) or nebivolol (β2AR inverse-agonist). IL-17A and IFNγ were measured using enzyme-linked immunosorbent assay. Genomic *ADRB2* and its immediate upstream region were sequenced using Sanger's method. Cytokine response to drug was analyzed based on polymorphisms.

**Results:** Terbutaline augmented IL-17A and inhibited IFNγ in the majority of samples tested. Terbutaline failed to augment IL-17A in samples where *ADRB2* encoded homozygous Arg16, although it still inhibited IFNγ. Nebivolol inhibited IL-17A in a β2AR-dependent manner, regardless of *ADRB2* polymorphisms.

**Conclusions:** Terbutaline augmented IL-17A except with Arg16 variation, and inhibited IFNγ. Nebivolol inhibited both IL-17A and IFNγ cytokines regardless of polymorphisms. Thus, the inverse-agonist nebivolol has more desirable properties for further exploration as a putative therapeutic for Th cell-mediated pro-inflammatory diseases since it inhibits both IL-17A and IFNγ regardless of *ADRB2* polymorphisms.

## 4.2 Introduction

T helper (Th) cells coordinate adaptive immunity but they can also exacerbate proinflammatory autoimmune diseases. In particular, Th1 and Th17 cells are considered to be the main driver of autoimmune pathology in many inflammatory and autoimmune disorders, and researchers have worked on new immunomodulatory therapeutics to treat autoimmune diseases (325). The distinct Th1 subsets play a role in protective immunity partly by secreting cytokines. Th1 cells initiate immune responses against intracellular pathogens by secreting IFNγ, while Th17 cells initiate immunes response against fungi such as *Candida albicans* and extracellular pathogens such as *Streptococcus pneumoniae*, and *Staphylococcus aureus* by secreting IL-17A and other cytokines. In contrast, Th2 cells secrete humoral cytokines, promote antibody responses, and are considered to be anti-inflammatory (111,112,326,327). Accumulated evidence demonstrates that Th17 cells can become dysregulated in pro-inflammatory autoimmune diseases, for example in MS and rheumatoid arthritis (328–331).

Adrenergic drugs are used to treat cardiovascular disease or asthma, but their role as immunomodulatory agents is not fully understood (179). Adrenergic drugs interact with adrenergic receptors (ARs) divided into α and β with differing functions and tissue distribution. On T cells, the β2AR is the only AR family member known to be expressed. In particular, Th1 cells and Th17 cells express β2AR, while Th2 cells do not express β2AR because the gene is repressed (249,250,268,270,303,316). When adrenergic agonist is added, Th1 cells tend to be suppressed leading to less IFNγ and expansion of Th2 cells which are normally suppressed by Th1 cells (249,251). Our group showed that β2AR-specific agonist terbutaline decreased IFNγ from Th1 cells, but augmented IL-17A in most of the samples. We noted that terbutaline had no effect on IL-17A or even suppressed IL-17A in ~15% of samples, which suggested that gene polymorphisms in *ADRB2* were causing inter-individual variability to drug response *in vitro* (303).

*ADRB2*, which is the gene encoding β2AR, is a relatively short, intronless gene located on chromosome 5q31-q32 that is translated into a 413 amino acid protein product forming a

classic seven-transmembrane G-protein coupled receptor (332). Within nucleotides -102 to -42 upstream of the open reading frame of the *ADRB2*, there is a region that is transcribed and translated into 19 amino acid peptide called 'β2AR upstream peptide' (BUP). The BUP contains one non-synonymous polymorphism at nucleotide position -47 T/C which alters amino acid Cys19Arg (224). The other three common nonsynonymous polymorphisms within the *ADRB2* include nucleotide 46 G/A in the coding region which alters Gly16Arg, nucleotide 79 C/G which alters Gln27Glu, and nucleotide 491 C/T which alters Thr164Ile (191,223,333). The allelic frequencies of Cys19Arg, Gly16Arg, Gln27Glu, and Thr164Ile are 37%, 65%, 55%, and 1% respectively (191,225,334). The SNP at position 164 is located in the fourth transmembrane of the β2AR. The variant Ile164 had diminished ligand affinity to the agonist such as epinephrine, norepinephrine and isoproterenol and had diminished capacity to activate adenylyl cyclase enzyme (226).

Researchers have evaluated *ADRB2* polymorphisms and how asthma patients respond to β-adrenergic drugs. It was shown in asthmatic cohorts that the subjects with homozygous Arg16 treated with β-agonist albuterol or salbutamol had diminished responses in lung function as compared to the subjects with Gly16 (230–232). In a study on an asthma cohort, Drysdale *et al*. identified 12 haplotypes based on *ADRB2* genotyping. The 4/6 and 2/2 haplotype pairs were found to confer the highest reactivity to the β2AR agonist albuterol, while 2/4 and 2/6 were intermediate, and 4/4 conferred the lowest reactivity to the drug when considering the improvement of airflow in asthma patients (219,223). Where haplotype 2/2 contains Gly16 and Glu27 and haplotype 4/4 contains Arg16 and Gln27. These findings suggest that the Arg16 polymorphism corresponding to haplotype 4 will have a reduced response to adrenergic ligands.

In the present study, we tested *ADRB2* polymorphisms as they relate to immunomodulatory properties of two adrenergic drugs, terbutaline and nebivolol. Terbutaline is an adrenergic agonist commonly used for treating asthma, while nebivolol is an inverse adrenergic inverse-agonist used to treat cardiovascular disease (209,210,277,335). The receptor specificity of these drugs appears to be terbutaline as a β2-specific agonist (336,337). Nebivolol

is a β2AR-specific inverse-agonist and a β1AR-specific antagonist (2,219). The effects of nebivolol on T cell cytokine production has not yet been studied, nor has the interaction with receptor polymorphisms. We found that terbutaline augmented IL-17A, except in samples where Arg16 polymorphism is homozygous in *ADRB2* (haplotype pair 4/4). Nebivolol inhibited IL-17A and IFNγ in the samples regardless of receptor polymorphisms. Therefore, nebivolol suppresses both of the cytokines tested in healthy human samples and warrants further study as a potential treatment for Th17-mediated autoimmune diseases.

## 4.3 Materials and Methods

### *4.3.1 PBMC Activation and Drug Treatment*

For activation of human lymphocytes, venous blood was drawn from 61 healthy volunteers after an informed, signed consent was obtained. The study was approved by the Concordia University Research Ethics committee (certificate 30009292). From 63 participants, 2 participants were excluded due to not meeting the inclusion criterion of healthy condition, which was assessed by self-reporting of their health condition. Chronic illness, disease, or disorder was excluded from the study. Up to six heparinized vacutainer tubes (BD, Franklin Lakes, NJ, USA) were drawn and processed using ficoll-hypaque (GE healthcare, Mississauga, ON, Canada) density centrifugation techniques to isolate the peripheral blood mononuclear cell (PBMCs) fraction as previously described (274). PBMCs were suspended 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). 0.5 x $10^6$ PBMCs were incubated in media in a round bottom 96 well culture plate (VWR, Mississauga, ON, Canada). Samples were activated with cell culture grade anti-CD3 (clone OKT3) and anti-CD28 antibodies (clone CD28.2) respectively (eBioscience, San Diego, CA, USA) in a soluble format at 0.1 μg/mL each. Where indicated, dynabeads were used to stimulate the cells, they are pre-coated with anti-CD3 and CD28 were used at a 1:1 ratio (Thermo Fisher Scientific, Mississauga, ON, Canada). The *in vitro* drug treatments included terbutaline $10^{-5}$M (Terbutaline hemisulfate salt, T2528, Sigma

Aldrich, Mississauga, ON, Canada) and ICI 118,551 antagonist used at 100 nM (Sigma Aldrich, Mississauga, ON, Canada) which both terbutaline and ICI118,551 are water-soluble and do not require an organic solvent control group. Nebivolol was used at $10^{-5}$M (Nebivolol hydrochloride, N1915, Sigma Aldrich) and a DMSO vehicle control was included at a matching dilution. A randomly selected subset of 39 samples was tested with nebivolol compared to terbutaline.

*4.3.2 Enzyme-linked Immunosorbent Assay (ELISA)*

To measure cytokines, cell culture supernatants were collected after four days of incubation. The cytokines were measured with enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions for human IFNγ (BD Bioscience, San Jose, CA, USA) and human IL-17A (eBioscience, San Diego, CA, USA).

*4.3.3 Flow Cytometry*

Proliferation was measured using 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFDASE) dye (Sigma Aldrich, ON, Canada), as previously described (275,336). Before the cells were treated with the drugs, they were labelled with CFDASE. In brief, the cells were resuspended in RPMI with 10% fetal bovine serum (FBS), with CFDASE at a final concentration of 5µM incubated for 5 minutes and washed three times with 10% FBS in PBS to remove excess dye, counted, and put into cell culture. At the timepoint, cells were harvested and stained with CD3-PercP (UCHT1) (1:10), CD4-APC (RPA-t4) (1:10) (BD Bioscience, Mississauga, ON, Canada), and analyzed by flow cytometry and FlowJo software using the cell proliferation tool. The cell viability was measured by trypan dye exclusion counting.

*4.3.4 DNA Isolation and Sequencing*

For DNA isolation, approximately $2x10^6$ cells of the PBMCs were taken for DNA isolation using QIAamp spin column according to manufacturer's instructions (QIAGEN, Toronto, ON, Canada). The DNA was eluted and purity checked based on the absorbance ratio 260/280 of between 1.8 to 2, on the nanodrop (NanoDrop 2000c, Thermo Scientific). Sequencing was done by Sanger's method. The primer sets used to sequence the upstream and ORF regions of *ADRB2*

were the following; (1) *ADRB2* I01 5'- TCCAGATAAAATCCAAAGGGTAAA-3' (Forward), 5'-CTCTTCTGTGGCCGCTACCT-3'(Reverse); (2) *ADRB2* I02 5'-TGTATTTGTGCCTGTATGTGC-3'(Forward), 5'- CGCGCAGTCTGGCAGGT-3' (Reverse). Three sets of primers were used for the coding/exon in the region of *ADRB2*:

(1) *ADRB2* E01a 5'- CGCTGAATGAGGCTTCCAG-3'(Forward),5'- TCTGAATGGGCAAGAAGGAG-3'(Reverse); (2) *ADRB2* E01b 5'- ATCGCAGTGGATCGCTACTT-3'(Forward), 5'-GGGGATTGAAACCAGAATTG-3'(Reverse); (3) *ADRB2* E01c 5'- TCTGCTGGCTGCCCTTCT-3'(Forward), 5'- TGCCCTTCCTTCTGCATATC-3'(Reverse); obtaining product size of ~2441bp.

*4.3.5 Polymorphism and Haplotype Determination*

Individual SNPs at nucleotide positions 46 and 79 in the *ADRB2* coding region were identified in 61 samples. Haplotype was determined for 56 samples due to five samples with insufficient DNA, partial sequencing failure, or one case of a previously unknown haplotype. To analyze the DNA sequence, polymorphisms were determined by the analysis package (Finch TV, Geospiza, Inc., Seattle, USA) and by inspecting the nucleotide chromatogram for the presence of two equally represented signals at the position if it was heterozygous, or one clear signal for homozygous. The polymorphisms were known SNP positions according to Drysdale *et al*.: -1023, -709, -654, -468, -406, -367, -47, -20, 46, 79, 252, 491 and 523 (Table 4.1). Using this method (which is unphased), haplotypes could be unambiguously identified for most of the samples. However, six of the samples could have been either 4/6 or 8/11. We assigned 4/6 to these samples because the frequency of 4/6 is approximately 30%, whereas the frequency of 8/11 is less than 1%(223). The main haplotype analysis presented in the paper was for haplotypes 2 and 4, which were unambiguously identified with our method.

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

**Table 4.1 SNP localization and haplotype classification of the *ADRB2*.** The number of the nucleotide is determined based on the first nucleotide of the start codon which is the +1. The 5' indicates 5' upstream of *ADRB2* ORF; syn, synonymous SNP. BUP is the 19 amino acid peptide translated in the upstream region of the *ADRB2,* it can be Cys or Arg depending on the nucleotide, C at position -47 results in amino acid Cys and T in amino acid Arg. AA16 is the amino acid 16 can be either Gly if the nucleotide is G or Arg if the nucleotide is A, AA27 is the amino acid 27 can be either Gln if the nucleotide is C or Glu if the nucleotide is G, AA164 is the amino acid 164 can be either Thr if the nucleotide is C or Ile if the nucleotide is T of the *ADRB2*. The combination of the SNPs of the nucleotide positions shown on the first row of the table allowed to characterize the corresponding haplotypes. This table shows the haplotype 2 and 4 because it was the most prevalent in our samples. The other haplotypes characterizations can be found in Drysdale *et al.* 2000. This table was based from Drysdale *et al.* (223).

### *4.3.6 Statistics*

The cytokine concentrations obtained from ELISA for IL-17A and IFNγ were fit in a Linear Mixed-Effects Model with maximum likelihood in R (packages: lme4, lmerTest). Fixed variables included: the treatment, polymorphisms at SNP 16 and polymorphisms at SNP27, whereas the random variable was the human subject. This was followed by type III Analysis of Variance (ANOVA) using Satterthwaite's method and a significance level of 5% to evaluate the effects of the treatment or SNP on the cytokine response in cells. Furthermore, the cytokine concentrations were normalized using the removal of within-subject variance procedure (338) and used for graphing and performing multiple T-test with correction for multiple comparisons using the Holm-Sidak method and a significance level of 5%. Excel (Microsoft version 16.28) and Prism Graphpad 8.4.1 (GraphPad Software Inc. San Diego, California, USA) were used for the normalization and graphing respectively. The terminology and the reasoning behind the tests are expanded in the general thesis discussion.

## 4.4 Results

### *4.4.1. IL-17A and IFNγ Response to β2AR Agonist Terbutaline*

Human PBMC samples from healthy participants were activated *in vitro* with T cell-stimulating antibodies anti-CD3 and anti-CD28 in the presence of a β2 agonist, terbutaline. Mean values from the group showed that terbutaline augmented IL-17A (Figure 4.1A), and simultaneously diminished IFNγ (Figure 4.1B). There were 14 samples that had no change or diminished IL-17A levels out of the 61 samples treated with terbutaline (23%). This was similar to our previous study where a minority of samples (~15%) tested had no change or diminished IL-17A with terbutaline (336). We speculated that these outlier samples were due to common β2AR polymorphisms.

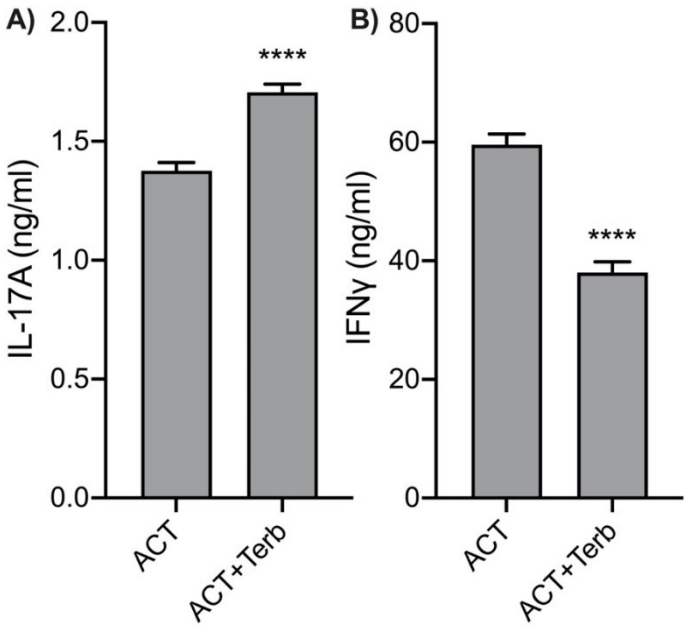



**Figure 4.1. Modulation of IL-17A and IFNγ by β2-AR agonist-terbutaline as shown in pooled samples.** PBMCs were activated *in vitro* for 4 days (ACT = anti- CD3 and anti-CD28) without or with β2-agonist terbutaline $10^{-5}$M (ACT + Terb). The amount of A) IL-17A and B) IFNγ in the cell culture supernatant was measured by ELISA. All of the samples were pooled to show the group

effect prior to taking into account SNP. Data pooled from 61 human PBMCs samples. Two replicates of each experimental group. Error bars show standard error. Student T-test. (**** $p<0.0001$).

### 4.4.2 *Frequencies of ADRB2 Polymorphisms and how they Relate to Terbutaline Effects on IL-17A and IFNγ*

Genomic DNA from the PBMC samples was sequenced for *ADRB2*, a relatively short, intron-less gene that encodes β2AR. The sequences were analyzed based on SNPs at positions 46 and 79 where common, non-synonymous SNPs are known to occur within the coding region (Table 4.2). We found that the glycine codon was more frequent than arginine at SNP 46, while glutamine was more frequent than glutamic acid codon found at SNP 79 (Table 4.3). To determine the influence of SNPs on IL-17A and IFNγ responses after terbutaline treatment, the cytokine response was reclassified based on SNP identities. Terbutaline augmented IL-17A in samples heterozygous for Arg/Gly or homozygous for Gly/Gly at nucleotide position 46. In contrast, terbutaline had no effect on IL-17A in samples homozygous for Arg/Arg (Figure 4.2A). Terbutaline suppressed IFNγ in the samples regardless of SNP at position 46 (Figure 4.2B). Next, the samples were grouped by SNP 79. Terbutaline augmented IL-17A (Figure 4.2C), and it diminished IFNγ (Figure 4.2D) in all of the samples regardless of SNP 79 alleles. Next, the samples were classified based on the combinations of SNPs at positions 46 and 79. Terbutaline augmented IL-17A in samples with Gly/Arg-Gln/Glu, Gly/Arg-Gln/Gln, Gly/Gly-Glu/Glu polymorphisms. A trend of augmentation of IL-17A in Gly/Gly-Gln/Gln and Gly/Gly-Gln/Glu was observed. In contrast, terbutaline did not affect cytokines in samples grouped as Arg/Arg-Gln/Gln (Figure 4.2E). Terbutaline suppressed IFNγ in all of the 46 and 79 SNP combination groups (Figure 4.2F). This data demonstrates that arginine at position 46 prevented terbutaline from augmenting IL-17A levels. The other polymorphisms had no influence on the two cytokines that we measured.

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

**Table 4.2. SNP nucleotides and amino acid positions and the relation to haplotype designations.** Modified from Drysdale *et al*. (223). The nucleotide column refers to the SNP identity as positions 46 and 79 codons. The amino acid column refers to the corresponding amino acid position in the β2AR protein. The haplotype column indicates all of the possible haplotypes that contain the SNP.

| Amino acid | Allele | Percentage occurence (%) |
|---|---|---|
| 16 | Arg/Arg | 13.1 |
| | Arg/Gly | 59.0 |
| | Gly/Gly | 27.9 |
| 27 | Gln/Gln | 47.5 |
| | Gln/Glu | 36.1 |
| | Glu/Glu | 16.4 |
| 16/27 | Gly/Arg -Gln/Glu | 27.8 |
| | Gly/Arg -Gln/Gln | 31.2 |
| | Gly/Gly -Glu/Glu | 16.4 |
| | Gly/Gly -Gln/Gln | 3.3 |
| | Arg/Arg -Gln/Gln | 13.11 |
| | Gly/Gly -Gln/Glu | 8.2 |

**Table 4.3. *ADRB2* polymorphisms occurrence of the human samples tested.** Alleles at amino acid position 16 or 27 of β2AR (corresponding to nucleotide position 46 and 79 of *ADRB2*, respectively) were determined from genomic sequence from 61 healthy human PBMC samples. The occurrence percentage was calculated for amino acid position 16 and position 27 separately (top two rows), and when considering the combinations of position 16 and position

27 (bottom row). Based on our data, and the data presented by Drysdale *et al* (223) where the most prevalent haplotypes were studied, Arg/Arg at position 46 was only found to be paired with Gln/Gln at position 79, other combinations were not observed for *ADRB2* SNP or haplotypes (only haplotype 2 that has Glu is paired with Gly).

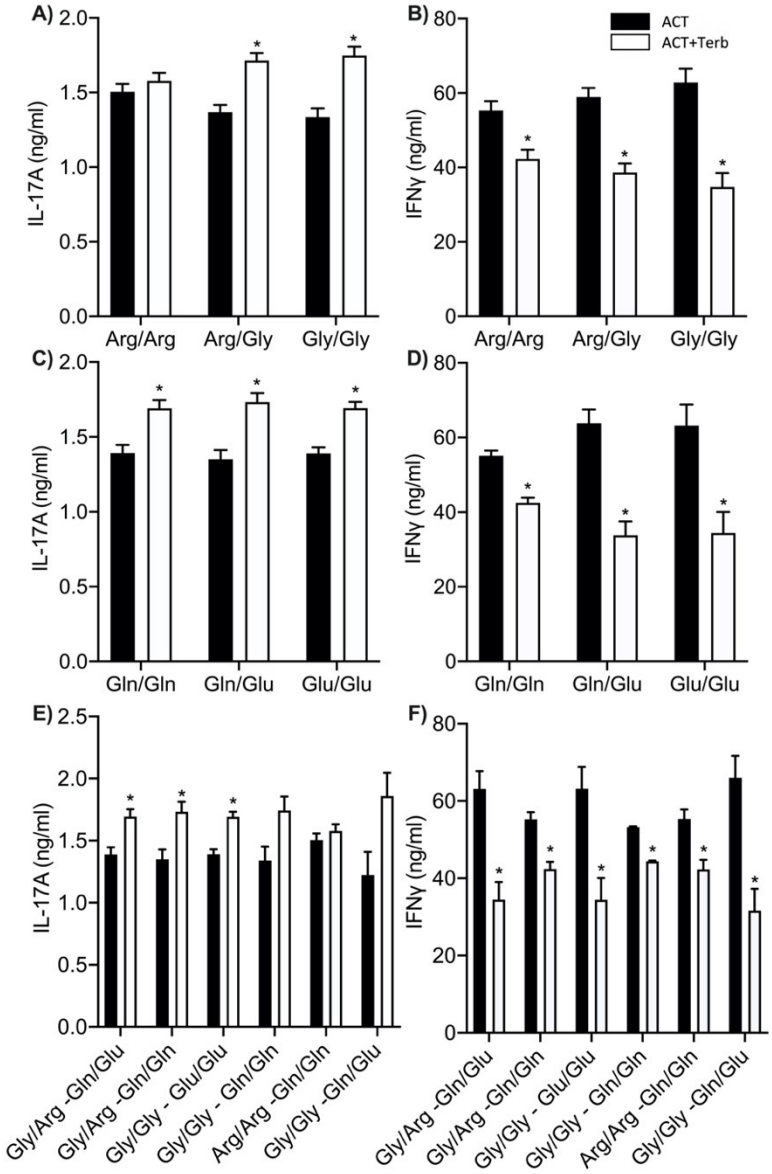


**Figure 4.2. IL-17A and IFNγ response to terbutaline classified based on SNP position 46 and 79 of *ADRB2*.** The cytokine response data presented in figure 4.1 was classified based on the identity of SNPs at position 46 (A, B), position 79 (C, D), or the combinations of 46 and 79 (E, F). With respect to IL-17A, the samples homozygous for arginine (due to SNP 46) failed to respond to terbutaline. Error bars show standard error. Data from 61 humans PBMCs samples. At least two replicates of each sample were done on the experiments. Multiple T-test with correction for multiple comparisons using the Holm-Sidak method (* $p < 0.05$). N=61 samples.

### *4.4.3 Frequencies of ADRB2 haplotypes and how they relate to terbutaline effects on IL-17A and IFNγ*

To determine haplotypes, further sequencing of the *ADRB2* coding region and upstream BUP and untranslated region (~1000bp) was performed from genomic DNA in 61 PBMC samples (Figure 4.1). Up to 11 haplotype pairs were detected in the samples with 4 and 2 being the most prevalent; 16 samples were 2/4 heterozygous, 7 samples were homozygous for 2, and 6 samples were homozygous for 4. We only included these haplotype pairs in the statistical model due to their abundance in the cohort, they represented 52% of the samples tested. To determine the influence of these haplotypes on IL-17A and IFNγ responses after terbutaline treatment, the cytokine response data shown in Figure 4.1 was reclassified based on haplotype identity. Terbutaline had no effect on IL-17A from samples with haplotype 4/4 and augmented IL-17A in samples with haplotypes 4/2 or 2/2 (Figure 4.3A). Terbutaline suppressed IFNγ in all samples including 4/4, 4/2 and 2/2 (Figure 4.3B). Thus, at least one copy of haplotype 2 was sufficient to confer terbutaline-induced IL-17A augmentation, while IFNγ was modulated regardless of *ADRB2* haplotype. Of the samples in our study, only one had the rare polymorphism at position 491 (Thr164Ile) corresponding to haplotype 7 region. The data from the less abundant haplotype pairs are available in the supplemental data file (Figure S4.1).

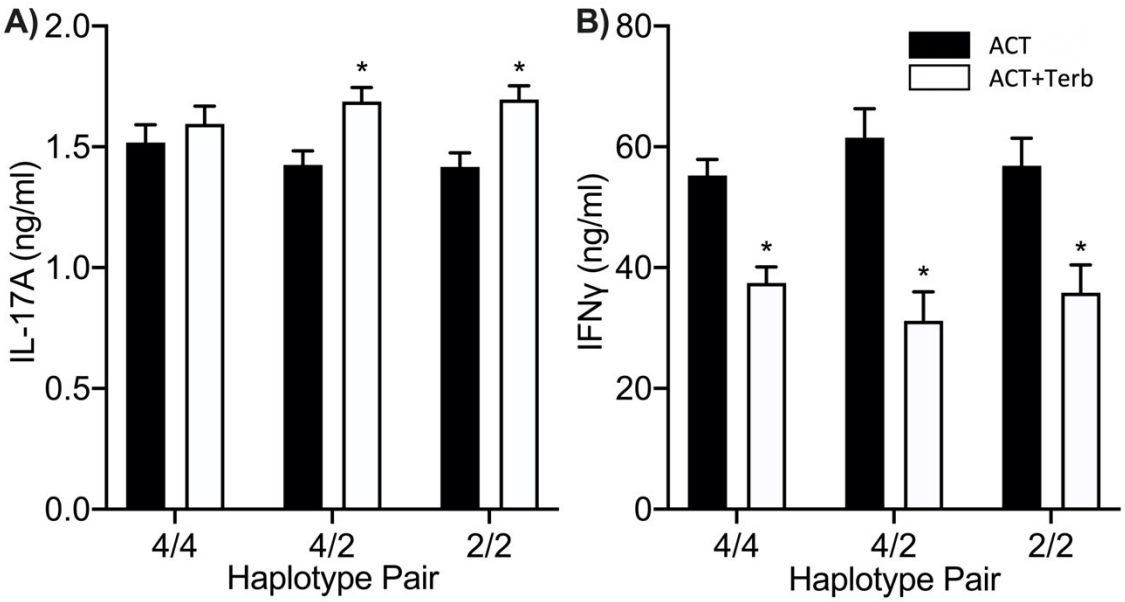



**Figure 4.3. IL-17A and IFNγ response to terbutaline classified based on common haplotypes of *ADRB2***. The cytokine response data presented in figure 4.1 was classified based on the identity of the common haplotype 2 and 4 for A) IL-17A, and B) IFNγ. With respect to IL-17A, the samples homozygous for 4 failed to respond to terbutaline, the other samples showed reciprocal modulation of IL-17A and IFNγ. Error bars show standard error. Data of 29 samples of human PBMCs samples. At least two replicates of each experimental group. Multiple T-test with correction for multiple comparisons using the Holm-Sidak method (* $p < 0.05$).

*4.4.4 Testing the effect of nebivolol inverse-agonist of β2-AR and its relation to ADRB2 polymorphisms.*

We compared the effects of terbutaline to nebivolol at equivalent doses on the cytokine response of PBMCs in 39 of the samples where the DNA sequence of *ADRB2* was known. As expected, terbutaline augmented IL-17A in samples with one or two copies of the glycine 16

codon and had no effect on IL-17A in samples homozygous for arginine 16 codon (Figure 4.4). Nebivolol suppressed IL-17A in all of the samples that were tested regardless of *ADRB2* polymorphisms at position 46 (Figure 4.4). In conclusion, nebivolol inhibits IL-17A in all of the samples tested regardless of polymorphisms of ADRB2.

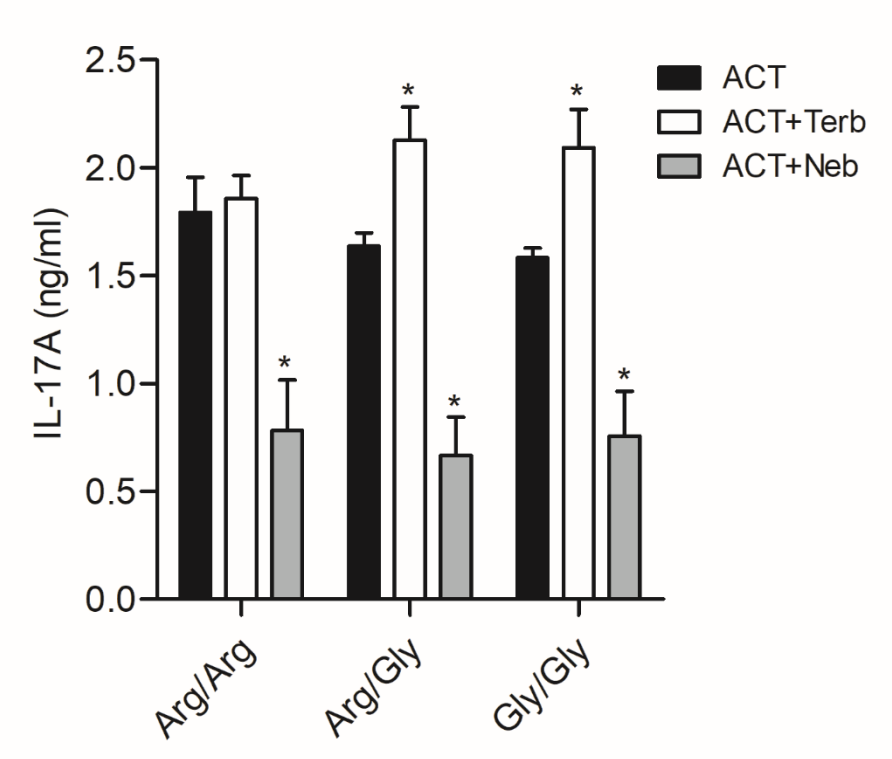



**Figure 4.4 Modulation of IL-17A in activated PBMC by terbutaline as compared to the inverse-agonist nebivolol.** PBMCs were activated *in vitro* for four days (ACT = anti- CD3 and anti-CD28) without or with terbutaline (Terb, $10^{-5}$M) or nebivolol (Neb, $10^{-5}$M). The amount of IL-17A measured in the cell culture supernatant by ELISA. The samples were classified based on amino acid 16 (nucleotide position 46). Error bars show standard error. Data of 39 human PBMCs samples, with at least two replicates of each experimental group. Multiple T-test with correction for multiple comparisons using the Holm-Sidak method (* $p < 0.05$).

## 4.5 Discussion

Common polymorphisms located within or immediately upstream of *ADRB2* are known to alter the response of β2AR ligands. We recently showed that a β2AR-specific agonist terbutaline suppressed IFNγ and augmented IL-17A in human PBMC samples (336). In the present study, we demonstrated that terbutaline, a β2AR-specific agonist, inhibited IFNγ in all of the samples tested. That finding was similar to Oostendorp *et al.* who reported that IFNγ was suppressed by isoproterenol, a non-selective β-adrenergic agonist. The suppression occurred regardless of *ADRB2* polymorphisms (222). We also demonstrated that terbutaline augmented IL-17A in the majority of the samples. In theory, that was not a desirable outcome when considering the immunomodulatory properties in the context of autoimmune disease. Autoimmune diseases can be exacerbated by IL-17A and Th17 cells, for example in multiple sclerosis or rheumatoid arthritis (45,331,339). Terbutaline augmented IL-17A in samples that were homozygous or heterozygous for Gly16 in the β2AR. In contrast, terbutaline inhibited or had no effect on IL-17A in samples that were homozygous for Arg16 in the β2AR. Individuals homozygous for 4/4 (Contains Arg16) had no significant response to terbutaline, while 2/2 homozygous (contains Gly16) or 2/4 heterozygous showed the expected augmentation in IL-17A with terbutaline. This finding emphasizes the fact that one copy of Gly16 in haplotype 2 is sufficient for the drug response when the unresponsive haplotype 4 (Arg16) is present. Thus, in a minority of samples with the Arg16 codon, terbutaline may lower IL-17A which in theory would be beneficial for treating a Th17 autoimmune disease.

This study is the first to address *ADRB2* polymorphisms with respect to IL-17A modulation. There are other contexts where it could be beneficial. For example, Th17 cells and IL-17A is essential for immunity against *Candida albicans*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* infections (111,340). Terbutaline could be beneficial by augmenting Th17 cells and IL-17A in those scenarios, however, adrenergic agonists suppress T cell proliferation in some studies (242–244). More pre-clinical research is required to determine the efficacy of adrenergic agonists as immunomodulators in the context of infections.

The Arg/Gly 16 codon was the strongest determining factor for terbutaline effect on IL-17A. It is not completely known how Arg or Gly at codon position 16 influences the response of β2AR to β-agonists. Another study showed that Arg19Gly16Glu27 which is haplotype 2 had less cAMP desensitization by isoproterenol in PBMCs than haplotype Cys19Gly16Glu27 and Cys19Arg16Gln27, which could be haplotype 5, 6, 7, 10, 11 and haplotype 1, 3, 4, 8, 9, 12, respectively (222). This suggests that in our study, the Arg16 receptor variant may have been more desensitized and thus lost its effect on IL-17A as compared to the Gly16 variants which would retain full activity. Moreover, the polymorphism at position 27, did not show a differential response in respect with IL-17A or IFNγ. Thus, indicating that the effect of the SNP at position 16 may be the one that influences the response of these cytokines.

The immunomodulatory profile of terbutaline in the healthy samples we tested suggested that IFNγ is suppressed and IL-17A is augmented. Ideally, a drug would suppress both of these cytokines to be beneficial in the context of autoimmune diseases that are mediated by Th1 and Th17 cells. Previously we showed that stimulation of PKA by dbcAMP augmented IL-17A (336), indicating that a drug that opposes this pathway may suppress IL-17A. Nebivolol is an inverse-agonist of β2AR prescribed for high blood pressure or heart failure due to its vasodilatory properties which improve oxygen delivery to stressed cardiac tissue (210,341). Nebivolol is called an inverse- (or partial-, biased-) agonist because it favours an alternative cell signalling pathway that opposes the classic G-protein pathway. To our knowledge, the effects of nebivolol on immune system cytokines has not been previously explored. We found that nebivolol diminished IL-17A and IFNγ from activated PBMCs *in vitro*. Nebivolol mediated its effects *in vitro* regardless of *ADRB2* polymorphisms, which suggests that putative therapeutic effects would be more consistent than terbutaline which only inhibited IL-17A in a small proportion of samples. Further studies on autoimmune patient blood samples, animal modelling studies, and clinical trials would have to be carefully conducted before nebivolol could be considered for approval in patients. Off label use of nebivolol would have effects on blood flow to cardiac tissues, which would have to be monitored.

The mechanism of action by which terbutaline and nebivolol transmit signals via the β2AR is not completely understood. Our results showed that both of these drugs inhibited IFNγ, while only nebivolol consistently inhibited IL-17A. The drugs have several key differences in their biochemistry. Terbutaline is a racemic mix when used as therapeutic for asthma patients (3). The R-terbutaline enantiomer was more potent than the S-Terbutaline as an anti-asthmatic effect (3). Nebivolol has up to ten stereoisomers, two of which are pharmacologically active: (+*SRRR*)-nebivolol (*d*-nebivolol) and (-*RSSS*)-nebivolol (*l*-nebivolol) (219). The *d*- and *l*-nebivolol isomers function through distinct mechanisms to synergistically produce the pharmacological and therapeutic effects (219). Blood pressure reduction is attributed to both isomers, but effects on heart rate and hypotension are caused by *d*- and *l*-nebivolol respectively (219). The *l*-enantiomer promotes endothelial nitric oxide (NO) synthesis and inhibits endothelial NO synthase uncoupling (219). Thus, the racemic version of nebivolol could induce NO which suppressed both IL-17A and IFNγ.

The cell signalling pathways triggered by the two drugs can differ. Terbutaline mediates its signal via the cAMP pathway. For instance, Th1 cell lines when treated with terbutaline showed an increase of cAMP, which showed to have an inhibitory effect as IFNγ was decreased (249). Similarly, isolated $CD4^+$ and $CD8^+$ T cells treated with terbutaline increased the levels of cAMP, and the cytokine IFNγ was decreased (246). We demonstrated that dbcAMP which activated PKA and decreased IFNγ, which was similar effects as terbutaline (303). In contrast, nebivolol has functional selectivity. It mediated its signal via the GRK/ β-arrestin pathway and not the cAMP pathway, as shown in mouse embryonic fibroblasts (2). However, in the context of immunomodulation of cytokines IL-17A and IFNγ, nebivolol has not been studied. Another feature of nebivolol is that it could increase NO synthesis by promoting nitric oxide synthase activity in endothelial cells (209,219). Experiments were published using a chemical compound that releases NO in cell culture. When the compound was added at 100µM, the resulting NO inhibited IL-17A and IFNγ in $CD4^+$ T cells (342). More research is required to determine if nebivolol induces NO in T cells and what are the effects of IL-17A. The general discussion of the thesis includes detailed proposed experiments.

The evidence indicates that the β2AR has different signalling pathways depending on the nature of the ligand. My results showed that the Gly/Arg SNP at position 16 abrogated part of the terbutaline effects. There may be a biochemical explanation for this phenomenon. Computational studies suggested that the Gly16 variant of β2AR had a 15 residues coil present in the secondary structure that is not present with the Arg16 variant. With Arg16, the vestibule formed by the transmembrane (TM) 5, 6, 7 is more of an open configuration and the ligand-binding site is enlarged, which enhanced the binding of albuterol (236). The SNP Glu27 variant resulted in better binding of the agonists including terbutaline, albuterol and isoproterenol as compared to the Gln27 variant (343). The interactions that are associated with ligand binding of the β2AR (Asp113, Ser203,204 and 207, Asn293 and Asn 312) did not change within the Gln or Glu variant (343).

The transmembrane portion of β2AR may also account for which cell signalling pathways are engaged. When an agonist binds, it leads to conformational changes in transmembrane domain 6 that change the cytoplasmic tail conformation and modulates G-protein binding (213). For example, isoproterenol is a full agonist of β2AR and binds the transmembrane domain 6 which results in Gs coupling as measured with fluorescent probes and labelled Cys 265 in sf9 insect cells (214).

It is not known why the Arg/Arg *ADRB2* variant does not respond well to terbutaline, but it responds to the inverse agonist nebivolol. I speculate that SNP Gly16 allowed terbutaline to induce G-protein coupling to the cytoplasmic tail, which induced downstream cAMP/PKA and augmentation of IL-17A. To test this, I suggest that an immunoprecipitation could be performed where β2AR is pulled down with antibodies specific for the receptor attached to dense mico-beads, and the resulting bound products would be western blotted for G-protein. Doing immunoprecipitations requires a large number of cells in the millions, so a model cell line may be used such as Jurkat T cells which are a mature T cell with intact signalling pathways commonly used to research T cell signalling (344). The Jurkat cells could be transfected with overexpression constructs with *ADRB2,* comparing the ones with Arg or Gly 16 codons. Another experiment could be to obtain the crystal structure or computational study comparing β2AR

interaction with either Arg16 or Gly16 to determine how the ligand-binding differs. In a published study, the SNP at position 16 and 27 of the *ADRB2* did not correlate to ligand binding, however, the authors did not express downstream G protein binding (223,345).

To explain why nebivolol was not sensitive to the Gly/Arg 16 SNP, further information on how this drug binds to the receptor would be required. There is a crystal structure for nebivolol and β2AR (346). The structured showed key amino acids that are essential interactions in the binding cavity of the β2AR, for example, Asp113 (TM3) and Ser204 (TM5) (347). Nebivolol was shown with computational studies to have the hydrogen bonding pattern with those key amino acids and thus docked in the active site of β2AR. There was an interaction with Phe194 (situated in the middle of extracellular loop 2) and Tyr308, Ile309 (extracellular ends of helices 6 and 7) which are binding sites of nebivolol in β2AR (346). Computational studies showed that terbutaline interacted with amino acids that are agonist binding sites, which include the Asp 113 (transmembrane 3), the 3,5 hydroxyl groups which have hydrogen bonding interaction with Ser 203, 207(transmembrane 5) and Asn 312 (transmembrane 7), there is also increased interaction of the tert-butyl group with Phe193 (Extracellular loop 2) (343,348). Thus, there could be some differences between the way the two ligands interact with the receptor suggesting that SNP could have different effects on their function.

In this report, the sex, age or ethnicity were not analyzed. In published asthma studies there was no association of age or sex with the differential responses observed in the SNP of the *ADRB2* (222,223,230,231,234,349). There can be differences in immune cells percentages between female and male, as one study showed that females had higher $CD4^+$ T cells (350). There are differences in age groups, for example, there was a positive association with $CD4^+$ T cell with older age. Moreover, the was no change in the frequency of Th17 cells with age, sex or ethnicity (350). Ethnicity could be associated with the responses and the SNP of the *ADRB2*. The frequency of Caucasians having haplotype 2 was higher (~8 times) compared for instance with African Americans (223). African Americans responded less, or not at all, to β-agonist treatments for asthma, as compared to whites (238,239). Thus, the sex, age and ethnicity of participants should be part of future studies of the SNP of the *ADRB2* and the IL-17A responses.

In conclusion, β2AR is emerging as a relevant therapeutic target for immunomodulation. Our findings will help to predict individual variability to β-agonists based on polymorphisms, and we identified for the first time an inverse-agonist that warrants more study as an experimental immunomodulatory therapeutic.

**4.6 Acknowledgements**

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**Supplementary data**

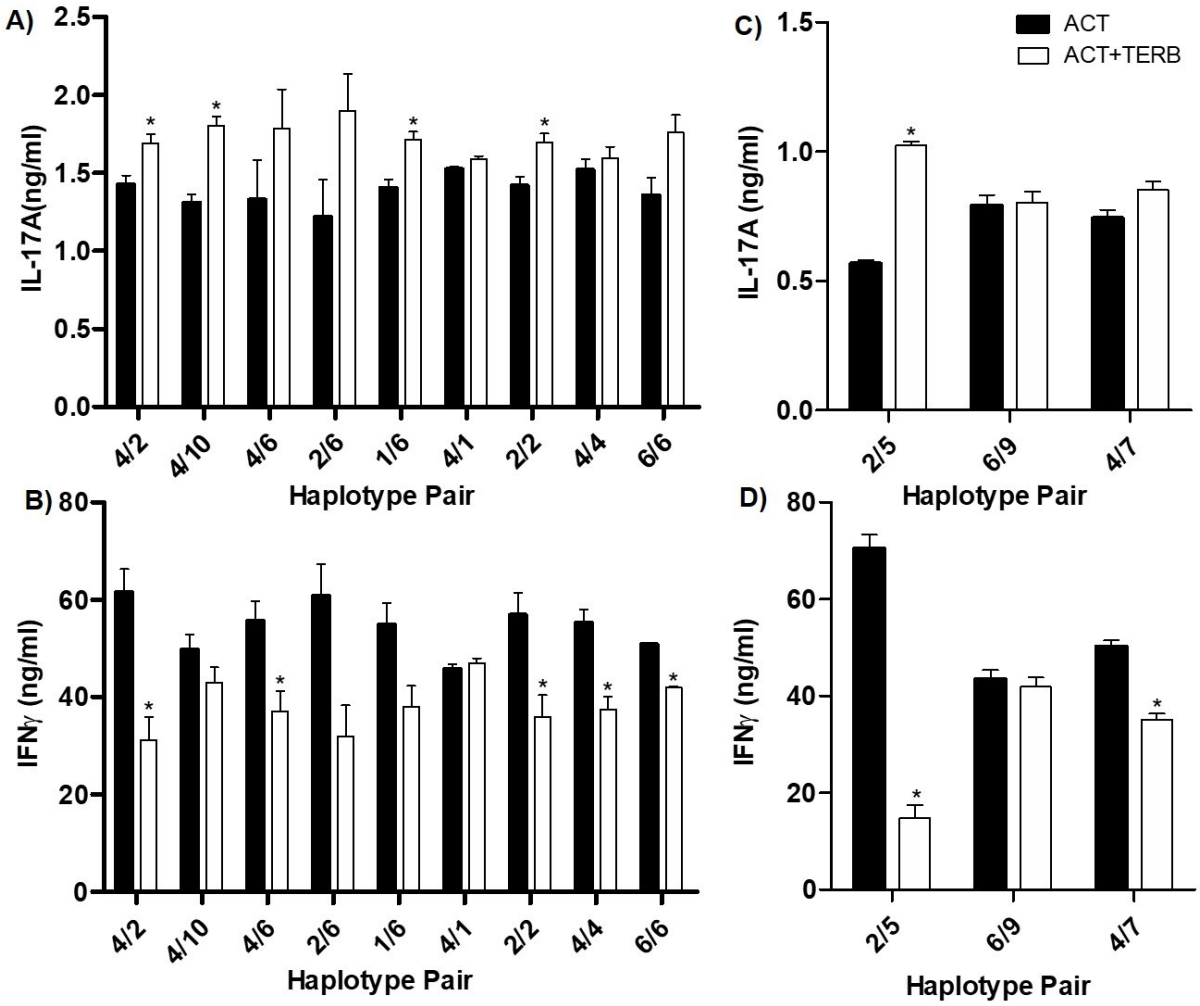


**Figure S4.1. IL-17A and IFNγ response in haplotype pair of *ADRB2* for all of the haplotypes detected in the entire 56 samples.** PBMCs were activated *in vitro* for four days (ACT = anti- CD3 and anti-CD28 at 0.1µg/ml) without or with β2-agonist terbutaline $10^{-5}$M (ACT + TERB). The cytokines IL-17A and IFNγ were measured in cell culture supernatants using ELISA. The *ADRB2* sequence was categorized as haplotype pairs. The amount of A) IL-17A and B) IFNγ are shown for haplotypes that were represented in at least 2 or more samples. The amount of C) IL-17A and D) IFNγ are shown for haplotypes that were only represented in one sample. Data of 56

human PBMCs samples, with at least two replicates in each experimental group. Error bars show standard error Multiple T-test with correction for multiple comparisons using the Holm-Sidak method (* $p < 0.05$).

# Chapter 5 General discussion

## 5.1 The effect of β2AR agonist on IL-17A from Th17 cells and IFNγ from Th1 cells.

The goal of my thesis was to determine if adrenergic drugs could suppress Th17 cells. Researchers have been discovering new drug treatments to suppress these T cell subtypes because they are detrimental in patients with autoimmune diseases. In MS patients, Th17 cells are considered pro-inflammatory because they secrete IL-17A which opens the blood-brain barrier and causes tissue damage, thus, IL-17A is often used as a measure of a detrimental Th17 response *in vitro* (108). IL-17A can also recruit and activate neutrophils that can cause tissue damage (351). It had already been shown that adrenergic drugs could suppress Th1 cells, however, little was known about how adrenergic drugs would modulate IL-17A from Th17 cells. The β2AR is the only adrenergic receptor expressed on Th cells (268). For these reasons, I explored β2AR -specific adrenergic drugs as a possible way to suppress both IFNγ and IL-17A from human T cells.

In Chapter 2, the *specific aim 1 was to determine if β2AR agonist inhibits IL-17A from Th17 cells in a cAMP/PKA dependent manner*. *Aim 1.1 was to determine if Th17 cells express β2AR protein*. This was important to address because there was no literature on the topic, and I knew from other Th cells that the expression of β2AR could be promoted or suppressed by histone modifications (250). I found that a proportion of Th17 cells expressed β2AR protein on the cell surface using a specific antibody and flow cytometry. The proportion of Th17 cells that expressed β2AR could represent a new subset of Th17 cells, or it could represent Th17 cells that are at different phases of activation. More detailed subset analysis would be required for example using single-cell RNA sequencing which can discover subsets based on gene clustering. The next step, *specific Aim 1.2 was to determine if a β2AR-specific agonist inhibited activated helper T cells.* The initial hypothesis was that β2AR agonist would suppress IL-17A from Th17 cells. This hypothesis came from the NSERC operating grant that funded research program at its outset. It was based upon literature showing that Th1 cells are suppressed, and proliferation of T cells was inhibited by β2-agonists (250,268,270). Moreover, it was hypothesized that the

β2AR agonist would inhibit Th17 cells because cAMP has been extensively studied as an immunosuppressant in T cells due to the cAMP pathway being inhibitory for T cell activation and proliferation (19,28,33,352–354). I demonstrated that Th17 cells express β2AR, that β2AR-agonist augmented IL-17A, and that β2AR-antagonist prevented the augmentation. This was also observed in purified Th17 human cells. Furthermore, the β2AR-agonist suppressed IFNγ as expected (303). The finding that a β2AR-specific agonist augmented IL-17A disproved the initial hypothesis was that β2AR agonist would suppress Il-17A from Th17 cells. I discovered in the literature, that cAMP and phosphorylation of CREB by PKA might augment IL-17A, which could explain my discrepant findings. To conclude the first chapter I tested dbcAMP which directly activates PKA and found that it augmented IL-17A (303). I did not see any anti-proliferative effect of the agonist on T cells. The proliferation results (repeated in chapter 2 and 3) are in contrast to other studies which showed an anti-proliferation effect on T cells with different adrenergic agonists (e.g. Isoprotenerol) (241,242,278–283). Among the various agonists, terbutaline is weaker in comparison to the other agonists in its anti-proliferative effects (244). In other studies, there was no change in proliferation when murine CD4 cells were treated with norepinephrine and activated with ConA (245). Human PBMCs there was no change in proliferation treated with norepinephrine or epinephrine and activated with PHA (246). Thus, the apparent discrepancy is likely because the literature used different agonists than used in our study.

Our initial hypothesis that Th17 cells would be suppressed by adrenergic agonist was not supported by the data. Even though Th1 were suppressed, and the literature showed anti-proliferative effects of β2AR-agonists, my results suggested that cAMP, PKA, and CREB augmented IL-17A. A paper published by Case *et al*. also found mouse Th cells treated with norepinephrine had lower levels of IFNγ, but higher levels of IL-17A which was similar to what I found in human cells (256). In contrast, Liu Y *et al.* showed that Th17 cells responses were inhibited by the stimulation of β2AR with norepinephrine and terbutaline in a PKA-dependent manner, in a mouse model of rheumatoid arthritis (257). The inhibitory effect on Th17 cells that Liu *et al*. reported was only seen in the mice that were immunized with adjuvant and collagen antigens and not the control group, indicating that in a healthy mouse the adrenergic agonist is

not inhibitory. In my study, and in the Case *et al.* study the cells were from healthy T cells from human and mice, respectively. More research is required to know how immunizations or rheumatoid arthritis might alter the function of adrenergic agonists on Th17 cells.

## 5.2 Mechanisms of action of β2AR agonist-terbutaline on IL-17A and IFNγ cytokine levels

To understand why β2AR-agonist augmented IL-17A from Th17 cells I explored the cell signalling pathways in more detail*. In specific aim 1.3, I determined if direct stimulation of PKA replicates the effect of β2AR agonist,* that was done using dbCAMP which is an analogue of cAMP that stimulates PKA. It also augmented IL-17A in activated PBMC samples, indicating that the PKA pathway was not suppressing IL-17A-producing cells rather, it appeared to augment IL-17A (303). That result did not demonstrate that β2AR-agonist was using the PKA pathway however since the dbcAMP was added directly to activated cells. To confirm how the β2AR-agonist was working, and to find a drug that would suppress IL-17A, I developed *specific Aim 2 which was to determine how the β2AR agonist was augmenting IL-17A and to determine if an inverse-agonist of β2AR will diminish Th17 cells response*.

There were remaining questions about what the drug was doing to the IL-17A producing cells. In the Aim 1 experiments, most of the results used ELISA, which is excellent for quantifying the concentration of cytokines that cells secrete into cells culture supernatant, but it does not tell us which cells made the cytokine. As outlined in the introduction, Th17 cells are the major producers of IL-17A but some minor subsets could make IL-17A. *In specific aim 2.1, I determined if the augmented IL-17A was due to an increase in the proportion of Th17 cells in the samples, or, if it was due to increased expression of IL-17A in each cell.* For this reason, I established an intracellular staining protocol which allowed me to measure cytokines inside of cells along with other markers of Th cells using flow cytometry. This approach allowed me to determine if the proportion or numbers of Th17 cells changed when treated with β2AR agonist-terbutaline. I showed a higher percentage of cells producing IL-17A and RORγ when treated with terbutaline. This suggests that terbutaline is stimulatory for the proliferation of

differentiation of Th17 cells which may explain why I observed a small but significant increase in proliferation.

To know if Th17 cells proliferated more experiments would be required such as obtaining pure Th17 cells and attempting the proliferation assay. Since the intensity of IL-17A (on a per-cell basis) was unchanged, I concluded that terbutaline was not increasing expression of IL-17A in the cells, rather, it was increasing the proportion of cells that express IL-17A. In other words, the augmented IL-17A I measured by ELISA was due to more Th17 cells making the cytokine.

*For specific aim 2.2, I determined if β2AR agonist-induced phosphorylation of CREB, a downstream substrate of PKA.* My Chapter 2 results indicated that a PKA activator would cause more IL-17A. Based on my readings I knew that CREB could be responsible for augmenting IL-17A. For example, a study in murine cells showed that dbcAMP would increase IL-17mRNA in murine Th17 cells (355). A study in human Th17 cells showed that dbcAMP increased $CD4^+$ $IL-17^+$ T cells, RORc and secreted IL-17A, as it was determined using flow cytometry, real-time RT-PCR and ELISA respectively (356). Furthermore, PKA phosphorylated CREB which augmented the IL-17A gene promoter, thus inducing L-17A production (309). Thus, it was plausible that β2-agonists augmented IL-17A by inducing PKA and CREB activity. I measured phosphorylated-ser133 CREB using intracellular staining and flow cytometry. Terbutaline caused a small but significant increase in the phospho-ser133 CREB in Th cells which indicates that CREB was more active. This supports the hypothesis that Th17 cells response (IL-17A) mediated by β2AR stimulation is in a PKA-dependent manner. Usually, this pathway is inhibitory for T cells, even in my experiments I say that dbcAMP inhibited Th1 cells. Other researchers confirmed that Th1 cells are suppressed by the PKA-CREB pathway. For example, IFNγ was decreased when dbcAMP was added to PBMCs stimulated with heat-killed *M. tuberculosis*, IFNγ was inhibited (317). The addition of dbcAMP to anti-CD3 and anti-CD28 activated PBMCs cultures, inhibited IFNγ as compared to the activated only samples as measured with ELISA (357). In umbilical cord blood CD4 cell samples, dbcAMP resulted in increased cAMP and diminished cytokines including IFNγ (358). These results indicate that Th17 cells are somewhat unique in that the PKA-CREB pathway augments Th17 cells. In summary, Th17 cells require the cAMP-PKA-pCREB

signalling pathway to express IL-17A. In contrast, IFNγ is inhibited by this pathway in Th1 cells. Thus, adrenergic drugs are immunomodulators of Th cells, but their effects are dependent on which cell type and which gene is being studied.

### 5.2.1 cAMP and PKA signalling pathway is stimulatory for IL-17A

The experiments described in the previous section relied on adding dbcAMP which directly activated PKA, but it does not demonstrate that β2AR-agonist activates PKA. Since CREB was phosphorylated I assumed that PKA had been activated by the agonist. To test this, *specific aim 2.3 was to determine if the β2AR-agonist augments IL-17A in a cAMP-PKA dependent manner.* I blocked the PKA pathway with H89 and rpcAMP which are PKA inhibitors and promoted the PKA pathway with a phosphodiesterase inhibitor (rolipram). My results showed that H89 and rpCAMP diminished IL-17A compared to the activated plus terbutaline treated group. These results demonstrated that blocking the PKA pathway can prevent β2AR from augmenting IL-17A. My results are in agreement with Tsai *et al.* who showed that using PKA inhibitors prevented IL-17A expression (309). My results also showed that the PKA inhibitors prevented terbutaline from inhibiting IFNγ, which confirms that this cell signalling pathway can be suppressive towards cells that make IFNγ including Th1 cells.

To further confirm that the cAMP pathway augmented IL-17A I tested rolipram which inhibits phosphodiesterases thereby elevating cAMP (35). When added to activated PBMC, rolipram augmented IL-17A and inhibited IFNγ, which was similar to what I observed with terbutaline. These findings are similar to other studies, where an increase of IL-17A was observed on purified T helper cells when treated with rolipram, in a mouse model for multiple sclerosis (321). Another example of how rolipram modulates T cells responses has been seen where Th1 cytokines are suppressed favouring the shifts of cytokine balance toward Th2 cells profile (359,360).

My results showing that the cAMP, PKA, CREB pathway augments Th17 cells is contrary to the paradigm that this pathway is an immunosuppressant in T cells (33,352,353). I do not

dispute the paradigm with its supporting papers because I also observed that the pathway inhibits Th1 cytokines. It seems that Th17 cells respond differently to numerous stimuli that either promote or inhibit the cAMP, PKA, CREB pathway (Figure 5.1). Future studies should directly measure the cAMP levels upon β2AR stimulation in addition of the PKA inhibitors and activator. This could aid to further demonstrate the cAMP-PKA pathway being stimulatory for IL-17A in the context of β2AR.

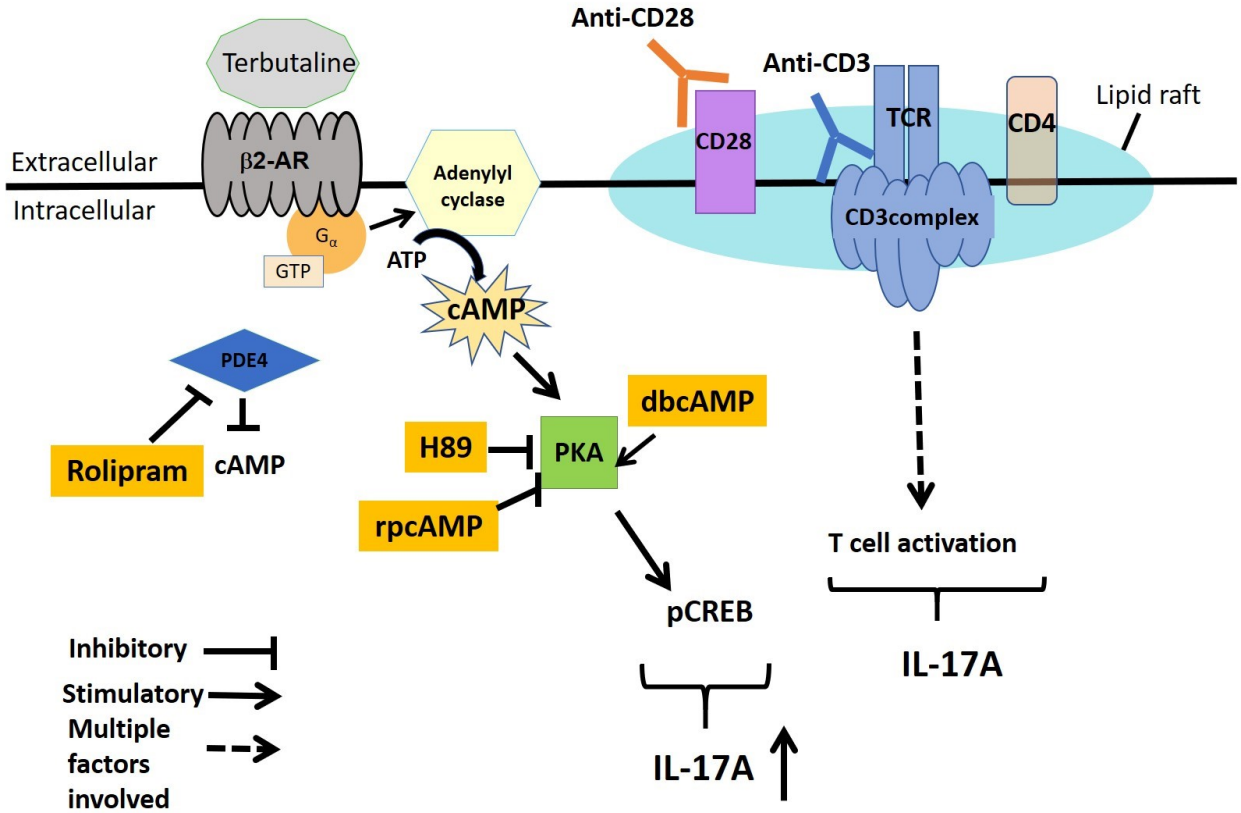


**Figure 5.1 Summary of results of the thesis incorporated with the literature: The β2AR signalling model for stimulation of IL-17A response in Th cells**. Terbutaline ligates β2AR, which releases Gs, thereby activating cAMP production by adenylyl cyclase. Second messenger cAMP then activates PKA, pCREB, which augments gene expression of RORγ which causes the T cell to

produce IL-17A. My results indicated that more T cells produce IL-17A in response to terbutaline in a PKA and cAMP-dependent manner. The experiments used pharmacological agents that increased the cAMP (dbCAMP or rolipram), PKA inhibitor (H89 and rp-cAMP), the phosphorylated CREB was measured, the use of anti-CD3 and anti-CD28 antibodies to activate the cells and the cytokine in the supernatant and intracellular cytokine staining for IL-17A.

My discovery that terbutaline augments IL-17A via the β2AR is novel because other researchers had not addressed Th17 cells and adrenergic drugs, and no researcher has done similar experiments in humans. There was a recently published finding with another G protein-coupled receptor system. $PGE_2$ is an example of a ligand that interacts with a G-protein coupled receptor and augments IL-17 via the cAMP-PKA pathway. $PGE_2$ is a proinflammatory molecule that is secreted by damaged tissues, or directly from APC which can ligate the $PGE_2$ receptor EP2 and EP4 on T cells (320). These receptors are G-coupled protein receptors associated with Gαs and induce AC that increases cAMP that leads to activation of PKA, which then initiates activation (phosphorylation) of transcription factor CREB (361). A study by Yao *et al.* showed the $PGE_2$ pathway was mediated via cAMP-PKA and augmented IL-17 (320). The authors also included the PKA inhibitor H89 in combination with $PGE_2$ or other agonists EP2 or EP4, which prevented the augmentation of IL-17. More evidence that $PGE_2$ was augmenting IL-17 was in a study by Napolitani *et al*, who showed that IL-17 was augmented in the addition of $PGE_2$ in memory $CD4^+$T cells activated with anti-CD3 and anti-CD28 antibodies. The authors obtained T cells from healthy individuals and measured cytokines (IL-17) with ELISA (362). Another piece of evidence that stimulatory Gα is capable of augmenting IL-17 was found in a knockout mouse. In a murine model, the stimulatory Gα subunit was knockout on Th cells, and the cAMP was decreased in T cells, and differentiation of Th17 cells was reduced (287). Together with my results on β2AR and IL-17A, these data demonstrate that at least two different G-protein coupled receptors will augment IL-17A in T cells.

### 5.2.2 Inverse agonist nebivolol inhibits IL-17A and IFNγ, has therapeutic potential

The initial hypothesis from the was that terbutaline would suppress Th17 cells, which would be useful to treat autoimmune disease. My results disproved the hypothesis and showed that the cAMP, PKA, CREB pathway augments Th17 cells. Upon reading the literature I found that nebivolol is a β2AR-specific inverse-agonist, and a β1AR-specific antagonist (2,219). Since T cells do not express β1AR, where mRNA expression of β1AR, β2AR, β3AR was measured in murine naïve $CD4^+$ T cells and only the β2AR was expressed (265). Nebivolol would, in theory, be β2AR-specific towards T cells. *My specific Aim 2.4 was to determine if nebivolol will diminish Th17 cells responses.* I observed that adding nebivolol to activated PBMCs, or isolated Th cells, suppressed both cytokines IL-17A and IFNγ. The effects were confirmed to be β2AR-specific since ICI 118 551 attenuated the suppressive effects of nebivolol (ICI 118 551 is β2AR-specific antagonist). It is not known why terbutaline augmented IL-17A while nebivolol suppressed IL-17A. In other cell types, nebivolol recruits β-arrestin to the cytoplasmic portion of β2AR, for example, nebivolol stimulated the β-arrestin -ERK pathway in mouse embryonic fibroblast (2). Nebivolol can also induce NO synthase (NOS) and NO in cardiac tissues (209). NO is known to be a suppressor of IL-17A, as was shown with a chemical that produces NO *in vitro* (342). More research is required to know if nebivolol induces arrestin recruitment or NO production in T cells causing suppression of IL-17A and IFNγ. I tested another inverse-agonist called carazolol, but its effects were inconsistent (shown on appendix Figure A3).

Nebivolol has therapeutic potential to be used as an immunomodulator by diminishing both pro-inflammatory cytokines (IL-17A and IFNγ) that are involved in autoimmune diseases. There are existing treatments used in the autoimmune disease MS such as dimethyl fumarate, which decreases both Th1 and Th17 cells and promotes an anti-inflammatory Th2 response (363). Another example is IFN-β, which is an antiviral cytokine also used as a drug. IFN-β reduced both IFNγ and IL-17 cytokine levels which shifted towards a protective Th2 phenotype (45,169,364). In my results, the β2AR specific agonist terbutaline augmented IL-17A while inhibiting IFNγ, which could be overall detrimental for Th17 mediated diseases like MS.

Nebivolol decreased both IFNγ and IL-17 which would be beneficial, although more research would be done before it could be used in patients (see section 5.6). Thus, adrenergic drugs such as inverse-agonists of the β2AR are candidates for further exploration as immune-modulatory drugs for treating autoimmune diseases.

### 5.2.3 Why can cAMP-PKA CREB signalling pathway be stimulatory for one cytokine but inhibitory for the other?

Inducible cAMP early repressor (ICER) is part of the CREB/CREM family of transcription factors. pCREB will induce the expression of ICER in cells. Then, ICER will repress transcription of other genes that are mediated by CREB by binding to CRE-like motifs, and by interfering with the recruitment of CBP/p300 (365). In this way, ICER is a negative feedback signal since it is induced by pCREB, and then inhibits other genes induced by pCREB. With respect to cytokines, ICER inhibits IL-2, as demonstrated in thymocytes from humans (366). Moreover, the β2AR agonist fenoterol caused $CD4^+$ T cells to express higher levels of ICER in the hyperreactive SNS model, and this inhibited IFNγ (367). In contrast, ICER is required for IL-17 expression in $CD4^+$ cells as shown in ICER/CREM $^{-/-}$ murine CD4 T cells. T cells from those genetically modified mice expressed less Th17 related genes including *Il23r, Il17a and Il17f* (368). The authors could rescue the Th17 phenotype by transfection with vectors containing ICER, which demonstrates the requirement of this transcription factor in Th17 cell differentiation (368). Besides the murine experiments, significantly higher amounts of ICER was expressed in memory sorted Th17 activated by anti-CD3 and anti-CD28 from healthy individuals (368). These papers show that ICER is stimulatory for IL-17A cells whereas inhibitory for IFNγ cells, indicating that the same pathway can have the opposite outcome. As another example that cAMP response element modulator (CREM) α is associated with the IL-17A, as it was observed in Isolated $CD4^+$ T cells from patients with autoimmune disease juvenile systemic lupus erythematosus showed higher IL-17A expression CREMα expression levels and lower IL-2 expression, as compared to healthy individuals (369). It was associated with the higher disease activity of the patients. That is, the high diseases activity had increased IL-17A, CREMα and reduced IL-2 (369). Thus, the ICER and CREM which are mediated by CREB are stimulators for the IL-17A. The same pathway

can be stimulatory for IL-17A whereas inhibitory for IFNγ. The stimulation of the β2AR is mediated via the cAMP-PKA pathway that results in being stimulatory for IL-17A but inhibitory for IFNγ as shown in this thesis.

### 5.2.4. Nitric oxide may account for some of the effects of β2AR agonists on T cells.

To discover new pathways that might be differentially induced by terbutaline and nebivolol in activated Th17 cells, I did a pilot experiment using single-cell RNA drop sequencing revealed some interesting genetic patterns suggesting a wider difference between terbutaline and nebivolol than just the cytokine effects (Appendix Figure A2). One possible explanation for these discrepancies effects is nitric oxide, a potent cell signalling molecule that is rapidly produced in T cells by an enzyme called inducible nitric oxide synthase (iNOS) from L-arginine, NADPH and oxygen (195). Nebivolol is known to cause smooth muscle cells to induce NO, which in turn causes vasodilation when used as a cardiac drug (341). NO binds to the $Fe^{+2}$ heme group of soluble guanylate cyclase (sGC), an enzyme that converts GTP to cGMP. This second messenger binds to three things including protein kinase G (PKG), cGMP-gated channels, and cGMP regulated phosphodiesterase (195,370). Broeders *et al*. showed that nebivolol increased NO levels in mouse aorta, and when coadministration with butoxamine (a β2AR antagonist) the increase of NO was prevented. The authors measured NO using Griess method (220). Terbutaline induced NO on murine urothelial cells and was abrogated with antagonist ICI 118 551 (371). The NO release was mediated via the adenylyl cyclase-cAMP pathway, as demonstrated using GTP analogue, cAMP analogues, forskolin, phosphodiesterase inhibitor and measuring cAMP levels (371). Thus, adrenergic receptors can induce NO synthesis and cGMP second messenger signalling in other cell types but this has not been demonstrated in T cells.

With respect to T cells, iNOS and NO are potent inhibitory factors that suppress T cell activation (372). The concentration of NO is a key variable when predicting how it may affect cytokines. This was demonstrated by Obermajer *et al*, who added a NO donor chemical called (DETA-NONOate) to $CD4^+$ T cells and measured the cytokines after T cell activation (342). With NO at concentrations of ≥100μM, the authors observed that IL-17A and IFNγ were suppressed

on $CD4^+$ T cells (342). These results indicated that the concentration of NO higher than 100µM is inhibitory for IL-17A and IFNγ by $CD4^+$ T cells. However, at lower concentrations than 100 µM it is stimulatory for IL-17A. The cytokines IL-17A, IL17F and IL-23 were suppressed when NOS and NOS2 were inhibited, as it was measured in $CD4^+$ T cells (342). The second messenger cGMP is stimulatory for IL-17A as it was showed that cGMP analogue increased IL-17A and inhibitor of cGMP decreased IL-17A (342). The latter study showed that depending on the concentration of NO it would result in inhibition of IL-17A and IFNγ, but NO is also stimulatory for IL-17A at a lower concentration than 100µM and is mediated via the cGMP. Similarly, to the latter study, in a study by Niedbala *et al*. the IL-17A was inhibited at a concentration of exogenous NO (NOC-18) of ≥100µM, as measured with ELISA, mRNA expression and intracellular cytokine staining. Additionally, the mRNA expression of IL-21, IL-22 and IL-23R was inhibited with the addition of NO (373). The authors showed that IL-17A and IL-22 was inhibited with exogenous NO at ≥100µM in isolated $CD4^+$T cells stimulated with bead coated anti-CD3 and anti-CD28 from healthy individuals as it was measured with ELISA and intracellular cytokine staining by the flow cytometer (373).

As a future direction, I hypothesize that the addition of nebivolol to the PBMCs or CD4 cells in my study can lead to increase NO (≥100 µM) in the cells. NO could inhibit IL-17A and IFNγ. Future experiments can consist in the measurement of the NO levels to determine if the stimulation of the β2AR with the different agonist will raise or lower the levels of NO and how much it will change the levels of NO. If the stimulation of the β2AR with nebivolol would increase NO levels in the cells by ≥100 µM, that could indicate that nebivolol triggers the synthesis of NO in immune cells. Nebivolol has not been studied in this context. The NOS2 expression can be measured to determine if its functioning and if there is an increase or decrease when the agonist of the β2AR are added. Additionally, the cGMP signalling pathway that is stimulatory for IL-17A. Therefore, adding a cGMP analogue and cGMP inhibitor concomitantly to the agonist of the β2AR to determine if its synergistic or inhibitory effect with the agonist added, respectively. Techniques would include NO assay, flow cytometry for characterization the NOS, intracellular cytokine staining for IL-17A, RORγ and IFNγ cells as well

as CFSE to measure the proliferation, all these performed on PBMCs, $CD4^+$ T cells and Th17 cells from healthy individuals.

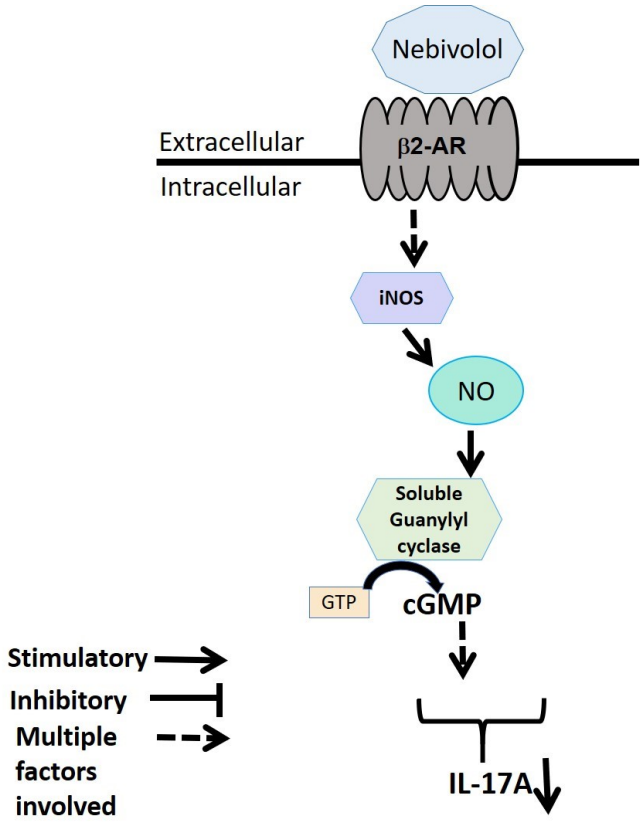


**Figure 5.2, Model of theoretical inhibitory signalling pathway of the stimulation of β2AR with inverse-agonist nebivolol**. Nebivolol could mediate signalling by increasing nitric oxide (NO) by the Inducible nitric oxide synthase (iNOS). The NO binds to soluble guanylyl cyclase that converts GTP to cGMP. This pathway may be inhibitory for IL-17A at concentrations higher than 100μM, however, the cGMP signalling pathway is stimulatory for IL-17A at lower concentrations of NO. This model is a speculation of what may have happened in our samples tested with nebivolol which showed a decrease of IL-17A. Future studies can dissect this pathway in details to determine if this is how nebivolol reduced the IL-17A.

## 5.3 Link of polymorphisms of *ADRB2* and IL-17A and IFNγ cytokine response to adrenergic drugs.

Human variability is a factor to consider when exploring new adrenergic drugs. Not all samples had the same Th17 cell response when β2AR is stimulated by terbutaline-the vast majority showed the augmentation, but about 15%-20% showed no effect or suppression of IL-17A by terbutaline. *Specific Aim 3 was to determine if SNP located within and adjacent to ADRB2 are linked to β2AR agonist or inverse-agonist effects on IL-17A and IFNγ*. Asthma studies, discussed in the introduction, showed attenuated adrenergic drug response in lung function of patients, so we reasoned that such polymorphisms could explain our results on T cells. *My specific Aim 3.1 was to determine the complete sequence of ADRB2 in primary human PBMC.* The complete sequence of *ADRB2* and its upstream region was performed from blood samples using sanger sequencing. There are up to 13 SNP known to be in the *ADRB2* and its upstream region. *ADRB2* has no introns so all of the SNP after the start codon were in the coding regions. Upstream, there is a short translated peptide (BUP) that has one known SNP, and in the upstream untranslated region, there are additional known SNP according to Drysdale *et al*. (223). I did not discover new SNP since they were already extensively characterized in the literature. Next, my *specific Aim 3.2 was to determine if the combinations of SNP correlate to differential effects of β2AR agonist or inverse-agonist on IL-17A and IFNγ*. We demonstrated that there is a link between the SNPs of the *ADRB2* and the IL-17A. Individuals having the nonsynonymous SNPs at position 46 (Arg16Gly), had diminished or no change in IL-17A levels upon treatment of activated cells *in vitro* with terbutaline. In contrast, there was no link with the IFNγ and SNPs of the *ADRB2*. Similarly, Oostendorp *et al.* found no connection between β-agonist and Th1 responses, but the authors did not address Th17 responses (222). The reasons explaining the link between *ADRB2* polymorphism and the terbutaline response are not understood, it may be related to receptor desensitization as mentioned in chapter 4 of my thesis. Asthma studies on the SNP and haplotypes of the β2AR did not report an association between the response of the treatment with β2AR agonists and sex or age of participants (222,223,230,231,374), however, there could be an association of haplotypes and ethnicity. For instance, Caucasian showed to

have higher frequency percentage of haplotype 2 (~48%) as to compare for instance with African Americans (~6%) (223). We did not record ethnicities of participants and thus should be considered for future studies to determine if there would be an association of the haplotypes, ethnicity and the IL-17A response. In Chapter 4 I confirmed that the inverse-agonist nebivolol inhibited IL-17A from activated lymphocytes in a larger sample size that chapter 3. All of the samples tested were inhibited by nebivolol, and there was no link with the various SNP within or surrounding *ADRB2.*

In summary, my results show that terbutaline is most likely to augment IL-17A in human immune cells which is not a desirable outcome if the end goal is treating autoimmune diseases. However, nebivolol suppressed IL-17A and was not affected by gene SNP, indicating that it warrants further study as a therapeutic (discussed in section 5.6).

## 5.4 Strengths and limitations of experimental approaches

One of the features of my thesis was that all of the research was done on human samples. This provides better interpretation of the biological relevance to humans, as compared to rodent research which is common in immunology. I also employed the appropriate technology for my studies. Many experiments I used the PBMC mix (all white blood cells). PBMCs contains up to 70% T cells and is also easier to obtain in higher quantities to perform experiments, compared to isolated subtypes of immune cells like the Th or Th17 cells. Isolated Th or Th17 cell experiments are used to measure the direct effect of the treatments given, but the yields are typically much lower and the cost per experiment higher as compared to using PBMC. The amount of isolated Th cells and Th17 cells is more limited to perform a series of experiments, therefore PBMCs are a preference for experiments that require many test conditions. Th17 cells can be as low as 5% of the total T cells in a human sample, which means even less since T cells are only about 40% of a blood draw. Very low yields of Th17 cells means that a Western blot is not possible and using a cruder fraction will mostly include Th1 cells which can be as high as 50% of the T cells. Where indicated, I performed limited

experiments on the purified Th17 cells to validate the key point of β2AR modulating the Th17 cell directly.

Flow cytometry is one of the few ways to deal with the characterization of subtypes of Th cells amongst a mixed immune cell sample, I used it to pinpoint the molecules of interest like the cytokine IL-17A, the transcription factor RORγ, and the signalling molecules CREB and the phosphorylated counterpart. If would have purified Th17 cells, the phosphorylated CREB can be measured using the western blot techniques. For instance, in THP1 cells the phosphorylated ( Ser 133) CREB was measured with western blot techniques on the effects of a metabolite of arachidonic acid on the IL-17A production (375). Moreover, the phosphorylated CREB was measured with western blot in murine T cells polarized for Th1 and Th2 cells, that were treated with fenoterol or $PGE_2$ plus anti-CD3 and anti-CD28 (36). These papers showed that phosphorylated CREB can be measured with western blot techniques. However, Th17 cells low yield obtained from isolated PBMCs from healthy individuals would not be able to perform western blot to measure the phosphorylated CREB on these cells. The experimental approach in human cells provides a high standard of evidence that brings us one step closer to having a drug that may warrant further testing in humans.

There are some limitations when studying human samples, for example, humans have a wide variety of genetic variations. If a mouse model system were used, the mice are usually from an inbred colony with very similar genetics between mice which makes the mouse experimental results more reproducible and robust. To overcome the human variability that is encountered in studies using primary cells, I repeated the experiments using a relatively large number of samples for such a study. For example, I tested up to 61 blood samples for the SNP study and took into account the most relevant SNP that were within or adjacent to the *ADRB2*. An advantage to mouse studies is that genetic knockout or insertional mutants can be created to dissect the function of receptors and enzymes. This can not be easily done in primary human lymphocyte samples, although transfection, transduction, suppression, and gene-editing technology is now making it possible to do reliable studies in primary cells *in vitro*.

Immunology experiments typically use polyclonal stimulation of the T cell which may be considered a limitation. In a natural immune response, an APC presents antigen in the form of a peptide bound to MHC molecules- this provides a dense surface of receptors that cause TCR and CD28 to cluster and induce cell signalling. It is not practical to do this *in vitro* because only a very small proportion of T cells will react to a given peptide/MHC. Polyclonal activation means that the majority of T cells get activated regardless of their antigen specificity. For my thesis, I used different types of polyclonal stimulators depending on the source of the immune cells. In the majority of experiments, I studied PBMCs which are mixtures of immune cells. PBMCs were activated with two antibodies including anti-CD3 and anti-CD28 in a soluble format. CD3 antibodies stimulate the TCR complex by binding to the extracellular portion of CD3 and cross-linking it with other TCR complexes. The anti-CD28 binds to CD28 and stimulates it. The antibodies were used in soluble format meaning that they were suspended in a buffer and not attached to any surface. They are used this way because it is less expensive than substrate-bound options. The soluble antibodies will adhere to the Fc receptors on monocytes in the PBMC, which effectively creates a surface where the anti-CD3 and anti-CD28 bind to, which further increases the crosslinking. Cross-linking means that the antibodies are causing TCR complex and CD28 to cluster together, which promotes downstream cell signalling. In some experiments, I used highly purified Th cells that were devoid of monocytes, so the soluble antibodies would not work properly. To activate purified Th cells, I used anti-CD3 and anti-CD28 antibodies that were pre-coated onto a nano-beads (Dynabeads). The nano-bead is considered a surrogate APC, it clusters the antibodies on a solid cell surface and induces TCR and CD28 cross-linking.

Another factor that may influence the different T cells responses observed between studies on adrenergic agonists is the timing of the addition of the drug. For example, when β2AR agonist-norepinephrine or terbutaline was added to murine naïve CD4 cells at the same time as the activation and differentiation stimulus (anti-CD3/anti-CD28 and IL-12), there was increased levels of IFNγ when compared to naïve CD4 cells that were activated without the drug, the naïve cells were priming into Th1 cells when agonist added (265). When

norepinephrine or terbutaline was added to murine Th1 cells after they had been activated with ovalbumin and IL-2 and re-stimulated with anti-CD3 and anti-CD28 there was no change in IFNγ (243). Thus, when the adrenergic agonist was added after the T cell was activated it lost its effect. In my experiments, I added terbutaline at the same time as the T cells were being activated, resulting in suppression. That is in line with the literature on how Th1 are affected. There was a decrease of IFNγ in PBMCs stimulated at the same time with tetanus and epinephrine or terbutaline (251). Since those papers were separate studies using different agonists, it would be useful in future studies, to add adrenergic agonist at different time points, either before, during, or after the activation of T cells. If the adrenergic agonist is added at the same time the T cell is being activated, then I expect to observe Th1 suppression.

When conducting *in vitro* experiments, the use of growth media can be viewed as a limitation because it is different from the normal growth environment of lymphocytes in blood and lymph fluid in the body. I used a media which contains FBS which contains high levels of vitamins, minerals, glucose and hormones including low levels of catecholamines that could alter the responses. There are synthetic growth medias now available however they are costly and do not outperform the use of FBS. Another issue that is inherent to *in vitro* studies is the oxygen levels in the incubator, which is the same as atmospheric oxygen (~18%), which is considerably higher than in blood (~6%) (376). There are nitrogen-infused incubators that lower the oxygen to physiological levels, and there are serum-free media that can be employed. It would be useful to include this in the budget of future projects in the laboratory to improve the apparatus.

There were some limitations regarding the flow cytometry approach. To measure single-cell Th17 responses I used intracellular staining and flow cytometry. This approach is commonly used in immunology to determine how subsets of immune cells are affected in a complex mixture like PBMC or tissues. One limitation of that approach is the number of fluorescent-antibodies that you can combine in a single test sample, which will determine how deep the analysis goes into the subtype, is limited in number. In mid to late 1990's it was common to use 2 or 4 colour flow cytometry which allowed basic phenotyping since 2000's the number has

steadily increased, but the technology appears to be limited when the number of colour parameters gets over 10. The flow cytometer I used, FACSVerse, has three lasers and can measure up to 8 colour parameters. I used in my experiments up to 5 fluorochromes, an example was a combination of CD3 (For TCR), CD4 (For Th cells), CCR6 (for Th17 cells), CREB and pCREB. It gets more complicated when using secondary antibodies that cross-react with other primary antibodies. That can limit the options since each antibody would have to be of a unique species or isotype. One way to overcome these limitations is to use a new technology called single-cell RNA sequencing. During my PhD, I collaborated with Dr. Dumeaux and Dr. Hallet to learn and implement single-cell RNA analysis using the drop sequencing method. The mRNA of a single cell is isolated in a droplet with a bead that has unique barcoded primers. It forms single-cell transcriptomes attached to microparticles (STAMPs), which are then reverse transcribed followed by amplification which is then sequenced (377). Because of the STAMPs that were barcoded the transcript of each cell can be identified. This technique allows to have the expression profile of individual cells. As seen in the appendix (Figure A2), I was able to perform drop-sequencing on purified Th17 cells that were activated and treated with terbutaline or nebivolol. The results suggested that the adrenergic drugs are associated with a wider range of gene changes that we previously thought. My preliminary results suggest that it is feasible, and also revealed an interesting hypothesis of new gene expression pathways. Now, this technology combines the sequencing with RNA-coded antibodies, making it possible to gain information on single-cell RNA expression protein-based expression. In the future such technology will likely make flow cytometry less relevant, however, the cost of drop sequencing and the reagents would need to be included in a research budget with the appropriate justification that the methods are valid and informative.

There was a limitation in the sample sizes available for study. The highest sample size was in chapter 4 where up to 61 participant samples were analyzed. We did not perform a power calculation because this was not a clinical study with just one dependent variable. In a previous clinical project in multiple sclerosis, between 5 and 10 samples were statistically sufficient to detect differences in IL-17A and IFNγ secretion in patient samples measured by

enzyme-linked assay similar to what was done in my thesis (173). For our genetic analysis, we tested 61 because it resulted in sufficient numbers in the main SNP categories, for example, the N value for the most common haplotype 2/4 had N=16, 2/2 N=7, and 4/4 N=6. These N values were in the range of the published clinical study (173). In a study by Israel *et al.* the number of samples analyzed for Arg16 were 37 and for Gly16 were 41. The authors observed the association between the SNP and use of albuterol in forced expiratory volume (231). Another example from Wechsler *et al*. the sample size for Arg16 was 42 and for Gly16 was 45, where they observed the effect of SNP of *ADRB2* and salmeterol on morning expiratory flow (349). In the paper by Oostendorp *et al*. were they did not observe a difference in IFNγ and the haplotypes of *ADRB2*, the authors had a size sample of 25 (222). Drysdale *et al.* had 125 Caucasian patients with asthma to observe the haplotype association with the agonist drug response (223). Taylor *et al*. had a sample size of N=17 for Arg16 and N=51 for Gly16 and N=40 for Arg/Gly, where they observed the asthma exacerbations and the association with the SNP16 of *ADRB2* (232). Our study falls within the range of those studies, however, some of those studies had double or more sample size. The sample size limitations can be overcome in future studies with a change in study design and a larger operating budget. With each blood draw and experiment, there is an associated reagent and sequencing cost. Despite some budgetary considerations, the sample sizes in this thesis were appropriate and similar to the normal sample sizes used in comparable immunology studies as cited above.

In my experiments where various drugs were compared and different measures were obtained, I considered the minimum standard of acceptability to be that an experiment was done at least 3 times on different human participants. Each experiment was defined by a unique participant, with internal replicates on the *in vitro* test plate. One exception was the experiment for RORγ that was done 2 times. If the data from the experiments had a representative finding that was significant, then we showed one representative experiment. In some cases, the data could be pooled together from the three experiments, which is indicated in the captions. The pooling of data is considered a stronger way to show data, however, in the immunology field it is common practice to show representative data.

A final limitation to discuss is the use of *in vitro* experiments. The question arises: how can I interpret the biological relevance of the magnitude of changes that I observed *in vitro?* When statistically significant differences are observed *in vitro*, they do not necessarily translate into a biologically significant (impactful) difference *in vivo*. The body has many homeostasis and metabolism systems that would potentially alter the pharmacokinetics of a drug administered. In my *in vitro* experiments, I regularly observed a 20-30% magnitude increase in IL-17A response to terbutaline or a similar-sized decrease with nebivolol. A question is whether or not such a change would be impactful in a real physiological situation where IL-17A is involved. The only way to directly test this *in vivo* in humans would be to administer adrenergic drugs to a participant that was undergoing an immunological stimulus such as a vaccine, or to do a mouse study where Th17 activation is administered with terbutaline. Those type of experiments have not been reported in the literature, and they were not within the scope of the operating grant or research ethics for the thesis studies. The question is whether or not a ~30% change in IL-17A would be impactful in a physiological situation *in vivo*. One study looked at the effects of cold exposure ($0^{o}$ C environment), which increased the levels of norepinephrine in human participants. The authors' measured immune system factors such as pro-and anti-inflammatory cytokines (e.g IL-6, IL-10, IL-12, IL-17A and more) during exercise that found a similar level of increase (~25%) in IL-17A (378). In a study with asthma cohorts, children having asthma attacks had 30% more IL-17A compared to healthy control groups (379). Theses situations demonstrate that in physiological settings IL-17A fluctuates by ~25%. Thus, the fluctuations I observed in my *in vitro* experiments are in line with physiological fluctuations of IL-17A reported in the literature. Actual *in vivo* experiments would have to be performed to determine if drug pharmacokinetics would alter the responses *in vivo*, for example, the doses I used *in vitro* may be too low since the liver typically metabolizes a proportion of ingested drugs which would require a higher *in vivo* dose.

## 5.5 Demographic considerations for immunology studies

One of the ways that immunology research can be improved is to take into account demographics such as age, sex, ethnicity, and physical attributes of the participants. In my research, I did not control the results for human variations such as age, sex, demographics, drug use, and health status, just to name a few. One way to control for these would be to obtain a detailed survey of their demographics, and then perform an adjustment technique where the measured drug response is adjusted based on age for example.

Demographics have not been widely studied by immunologists; however, it is an important and growing area of research. New research on COVID-19 by Takahashi *et al*. showed that men had a stronger innate response (e.g IL-8, IL-18) to SARS CoV-2 infection while females had a stronger adaptive response (380). There were higher amounts of activated T cells (e.g markers for activated T cells: HLA-DR and CD38) as compared to men. With respect to age, the authors showed that the older men had a worse outcome than young men, whereas the outcome was not affected by age for women. This indicates that sex and age may influence how innate and adaptive immunity occurs in patients infected with SARS CoV-2. The underlying mechanisms of action by which age and sex alter T cell responses require more research to fully understand (380).

A study compared the percentage of $CD4^+$T cells between female. The authors showed that female had higher $CD4^+$T cells than male, and the percentage of CD8+T cells and NK cells was lower in females than males (350). The frequencies of $CD4^+$T cells, $CD4^+/CD8^+$ T cell ratio, Th1 and Treg were positively associated with older age, where five age groups were studied. The ranges of age studied started with 20-30 years old and the last group age was a range of 61 -70 years old (350). The group of 20-30 years old ~ % 4 of Th1 cells and the group of 61-70 years old ~% 42 of Th1 cells. Whereas the proportion of Th2 was the opposite with age, that is there are less Th2 cells as the age of individuals increases (350). Thus, according to recent research, there could be an influence of sex and age with certain subtypes of immune cells.

To address demographics in future studies, a multifactor regression would be another approach to find groups of variables that might be linked to the dependent variable (i.e. the effect of a drug on IL-17A). It is uncommon to control for such human variable in an immunology study, primarily due to sample size. Immunology studies can be found with as few as 1 sample per figure, but less than 10 samples are common due to the cost and complexity of the measures. The reason I was unable to conduct a demographic study was because it was not part of the initial study design. Future studies should include a demographic questionnaire for the participants to fill out and have sufficient sample size to address the possible confounding variables related to demographics. Another option is to include age and sex-matched healthy controls in the event that MS patient samples are being tested. That is a more common way to control for major demographic variables. The demographic of sex may not be relevant in the context of Th17 cells, since in a published study, the proportion Th17 cells were not correlated with sex or age in a sample of healthy Chinese individuals (350). The authors observed a higher proportion of Th17 cells in Italian individuals compared to Chinese individuals from the Shanxi district, indicating that ethnicity may be related to the proportion of Th cells (350).

Even though demographics were not part of the study design, I took several measures to minimize the impact of confounding variables by using an exclusion factor list. We excluded any person by asking them if they had any medical conditions diagnosed or were taking prescription or recreational drugs. Some exceptions included birth control for example since it would not, in theory, affect the results. The age of the blood donors was not recorded since we were not statistically powered to stratify data by age or conduct a regression or multi-factor analysis, in our inclusion criteria it was 18 years and older. It is uncommon to do so in a primary human immunology manuscript due to limitations in cost and time of studying human samples. Another limitation is that we did not formally record or analyze the sex of the participant, again, this would be very uncommon in immunology manuscripts, but we recognize its importance. We observed that blood samples acquired from females and males had no obvious difference, by reviewing the participants' names (which is not an ideal way to assess sex) we saw no instance where the terbutaline response was linked to sex. The SNP variations in their

*ADRB2* genes were significantly related to terbutaline response, which argues against some other variate being involved. We acknowledge that a larger sample size with a more extensive demographic analysis would be of value for future studies.

## 5.6 Future direction: pre-clinical study on nebivolol as an immunomodulatory drug for treatment of Th17-mediated autoimmune disease.

Conducting *in vitro* experiments on human immune cell samples facilitates rapid screening of potential new therapeutic compounds. My *in vitro* results on healthy human immune cell samples suggests that nebivolol the inverse β2AR agonist has potential as a suppressor of Th1 and Th17 cells. The next steps of research could include *in vitro* testing on blood samples acquired from MS patients to determine if the drugs have the effects that I documented in healthy participants. Age and sex-matched MS patient samples and healthy human participants would be compared using the *in vitro* test of activated PBMC with the nebivolol drugs and solvent ((vehicle) controls). In theory, if those studies showed that nebivolol suppressed Th1 and Th17 cells in MS patients to the same or greater extent as the healthy sample, it is promising for further exploration. Clinician-scientists might consider further testing in patients in the form of phase 1 clinical trial. A clinical trial was published where β-agonist salbutamol was administered to healthy participants *in vivo*, and their cytokine responses in blood or lung tissues were measured. Salbutamol resulted in inhibition of cytokines necessary for Th1 cells development (IL-12) and augmented cytokines necessary for Th2 cell development (IL-4) (381). To my knowledge, that type of study focusing on Th17 cells has not been done. Another study compared albuterol combined with the approved drug Copaxone in MS patients. The safety profile was acceptable and the data suggested efficacy although the limitations were that the phase 1 trial is not designed for efficacy, and Th17 cells were not measured at the time (179). Another example is patients with secondary progressive MS who took oral salbutamol. There was a decrease of IL-12 in activated monocytes and increase of anti-inflammatory cytokines (IL-10, IL-4, IL-5) from PBMCs taken at various points

during three weeks of the study (382). Moreover, MS patients who took oral terbutaline alongside other treatments (e.g Interferons, Copaxone etc.) and the other group were MS patients without any pharmacological treatment (383). The cytokines measured after oral terbutaline on the whole blood showed on MS patients that have other treatments alongside, a slight increase in anti-inflammatory cytokine IL-10 and untreated MS patients a decrease of IL-10, whereas IL-12 had the opposite reaction for the MS groups. IFNγ was slightly increased for both MS groups contrary to the healthy controls samples that showed a decrease of IFNγ response (383). My research indicates that nebivolol warrants further testing and could be perhaps considered for a clinical trial by doctors in the future. The studies that have been published on adrenergic drugs and MS consistently find that pro-inflammatory Th1 is suppressed while anti-inflammatory Th2 is augmented, but none of the studies to date addressed Th17 cells. I speculate that adrenergic agonists would augment pro-inflammatory Th17 cells in MS patients which is not a desirable effect given the ability for Th17 cells to infiltrate CNS and exacerbate MS tissue damage. Nebivolol in theory would suppress both Th1 and Th17 which is a better indicator than I observed with terbutaline which suppressed Th1 but augmented Th17 cytokine. To my knowledge, there are no studies published to date that perform clinical testing of inverse-β2 agonists as immunomodulators for treatment of Th17-mediated autoimmune disease.

To get health Canada approval of a new drug, or for off-label use of approved drugs, there has to be some evidence of efficacy in laboratory studies. My studies on healthy human samples support the efficacy of nebivolol. Another common approach to testing new compounds is to use an animal model typically primate or rodent. For MS, there are many suitable models, the most common being the murine EAE which involves immunization with adjuvant and antigen from the central nervous system (44). The suggested experiments that can be carried out in the future to investigate how the adrenergic agonist (e.g nebivolol) would modulate the response of Th17 cells and affect the symptoms of the EAE model, would first consist in having a control animal which can be the wild type mice (C57BL/6J) and then the EAE induced mice (44). The experiments with the mice models would consist in measuring the β2AR

expression on the isolated Th cells and Th17 cells. The latter would be accomplished with flow cytometry techniques as it would be able to provide single-cell characteristics. The animal models would then be administered with the agonist drugs, terbutaline, nebivolol and ICI 118,551. When the animals would be administered the drugs, there should be monitoring of the cardiovascular parameters since nebivolol is a drug used for cardiovascular diseases. The clinical scores of the EAE mouse models should be noted to observe if there is improvement or exacerbation upon administration of the adrenergic compounds. Moreover, the Th cell and Th17 cells from mice models should be isolated before and after administration of the drugs. Where cytokines responses should be measured with ELISA, including the IL-17A and the IFNγ. With these measurements and comparing to the clinical scores, it can be observed if the treatment would cause exacerbation of symptoms or it would be diminishing the symptoms. I predict that nebivolol would diminish symptoms since in the *in vitro* study done in this thesis showed to have decreased both pro-inflammatory cytokines, IL-17A and IFNγ, that are contributors to the exacerbation of the chronic inflammation that occurs in MS. However, the treatment with terbutaline I predict would exacerbate the symptoms in the EAE model, as the *in vitro* study showed there was an augmentation of IL-17A which is pro-inflammatory and therefore not ideal for autoimmune diseases like MS.

## 5.7 The immunomodulatory properties of adrenergic drugs may have relevance for asthma treatment

While the main focus of my thesis was on potential new drugs for Th17-mediated autoimmune disease, there are other fields where my thesis has relevance such as asthma. Asthma is a disease that causes airway inflammation; this results in obstruction of the airflow and airway hyper-responsiveness (384,385). There are different phenotypes of asthma, one of which is Th17 mediated airway inflammation (384). The IL-17A released by Th17 cells promotes the recruitment of inflammatory cells, such as neutrophils or monocytes to sites where airway inflammation occurs (384,386,387). In patients with moderate and severe asthma, increased

levels of IL-17A are found in bronchoalveolar lavage fluids and bronchial biopsies; this is associated with airway reactivity and disease severity (384). The combination of IL-17 mediated effects on airway epithelial cells are contributors to the inflammation in asthma. To target airway diseases such as asthma, treatments include a combination of bronchodilators (commonly β-agonists are used for short-term and long-term therapy) and other drugs with anti-inflammatory properties. The β2AR ligand used for this research is terbutaline, which is currently given as a treatment for asthma (335). The use of terbutaline for the treatment of asthma can be beneficial for the bronchodilation and impact the underlying inflammation that occurs in this respiratory disease (388). My data suggests that terbutaline could increase IL-17A levels, which could, in theory, worsen the inflammation and make the disease worse. It can also suggest that individuals with Arg16 (haplotype 4) homozygous, will have diminished bronchodilation, but may also have less IL-17A increase which could be beneficial to the underlying inflammation. The underlying inflammation present in asthma could be modulated in a cell-dependent manner with the stimulation of the β2AR on immune cells. Indeed, anti-inflammatory effects were observed in macrophage cells upon addition of salbutamol and terbutaline, as those two β2-receptor agonists inhibited the expression of the macrophage inflammatory chemokine (388). Therefore, the same drug that helps improve airway function could also worsen the underlying inflammation making the disease worse in the long run. More research would be needed to address terbutaline effects on IL-17A in asthmatic patients.

## 5.8. The immunomodulatory properties of adrenergic drugs may have relevance for immunity to pathogens

Another area of relevance for my research is the immunity to pathogens and how it is altered by endogenous stress hormones (epinephrine and norepinephrine). My discovery that beta2-agonist increases Th17 cells as observed in this thesis challenges the paradigm which states that catecholamines hormones are regarded as an immunosuppressant. For instance, there has been an association between psychological stress and the susceptibility to the

common cold, where the rate of infection was increased suggesting that stress inhibits the immune system (389). Immunomodulation can occur *in vivo* when the endogenous hormones are released by the sympathetic nervous system upon stressors like endurance exercise (e.g Marathon runners) or psychological stressors (e.g tests). For instance, in a study where they measured catecholamines levels in the blood before and right after participation to a marathon race, there was a three-fold increase in both epinephrine and norepinephrine, indicating that there was an activation of the sympathetic nervous system (390). After seven days of the marathon, the catecholamine levels showed to return to the baseline (390). This shows that stressor like running a marathon (endurance exercise training) activates the sympathetic nervous system and thus increases catecholamines levels in the blood. The increase in catecholamines can in turn modulate the immune responses. The levels of effector T cells measured in marathon trained individuals showed lower levels of IFNγ T cells and an increase of IL-4 T cells and Th1/Th2 ratio compared to controls, as measured with flow cytometry techniques from PBMCs (391). This study showed that there are more Th2 cells in response to endurance exercise training, which as demonstrated by Chmura *et al.* (390) there is an increase of catecholamines in marathon runners. Thus, showing that there is an immunomodulatory effect *in vivo* that appears to have higher Th2 cells responses. However, there should be concomitant measurements of Th17 cells responses to observe the immunomodulatory effect that increased catecholamines like marathon runners can have. Another example of endogenous hormones released by the activation of the sympathetic nervous system can be through psychological stressors, which in turn can have immunomodulatory effects *in vivo*. For instance, subjects performed physiological stress test (Trier Social Stress Test) to observe the effect of immune responses, focusing on Th1 and Th2 cells responses (392). The Th1 response (IFNγ) was increased two hours after the test but returned to baseline after 24h. On the other hand, Th2 cells response (IL-4) decreased two hours after the test. The effect of the physiological stress test did not show a shift towards the anti-inflammatory responses (392). The mRNA levels of β2AR increased at six and 24h, whereas the levels of glucocorticoid receptors were unchanged. It was measured in PBMCs from healthy individuals, cytokines were measured with the flow cytometer and mRNA with PCR (392). The latter study showed that

there is a change in cytokine responses to stressors, which can activate both the sympathetic nervous system and the hypothalamic-pituitary-adrenal axes. However, the levels of catecholamines and glucocorticoids were not measured in that study. That study by Xiang *et al.* does appear to be contradictory to the observations made *in vitro*, where the Th1 response (IFNγ) were decreased when exposed to adrenergic agonists, as observed in my published article (303) or others (393,394). However, the timing of the measurements on the cells were only until one day, compared to the *in vitro* study as the one presented in this thesis which was for four days and using an agonist (terbutaline) that is not degraded by the enzymes that normally degrade catecholamines (Catechol-O-methyltransferase) (208), thus the possible effect of the agonist in my study is longer. The stimulation of the receptor can be speculated to be longer and thus increasing the levels of cAMP which results to be inhibitory for IFNγ and stimulatory for IL-17A in this thesis.

My research focused on Th1 and Th17 cells, however, previous researchers have been focused on the Th1 and Th2 response to catecholamines or βAR-agonist. Cells may react differently depending on their interaction with hormones and their specific environment; this can potentially influence the host's ability to defend and protect itself against pathogens. The other studies also observed an increase in Th2 cells response to catecholamines (250,290,395,396). Th1 cells are known to suppress Th2 cells, so when adrenergic drugs inhibit Th1 cells it allows Th2 cells to expand. Thus, the paradigm that the adrenergic drugs are immunosuppressant applies to Th1 cells in certain circumstances, but Th2 cells may expand as a result of Th1 suppression, and IL-17A may increase with adrenergic drugs. It is better to view adrenergic drugs as immunomodulators with context-specific effects. Immunomodulation is not necessarily beneficial for infections. Suppressing Th1 cells could make an individual be susceptible to intracellular pathogens, but it would also shift the response to Th2 which protects against certain viruses and parasites. As each subtype of Th cell has a function in the body, altering the Th cell balance with the stimulation of β2AR by ligands or endogenous hormones can be beneficial or disadvantageous for certain pathogens encountered. Th17 cells target extracellular fungus and bacteria. Since each immune cell is different, understanding

how Th17 cells respond upon stimulation of the β2AR could provide valuable insight into the potential effect of hormones like norepinephrine have in the host protection against infections. For instance, an increase in Th17 cells response would be beneficial when the host encounters pathogens like *Staphylococcus aureus*, as Th17 cells can provide the optimal protection against it (113). Therefore, the host protection provided by Th1 or Th17 cells could be modulated by the release of the hormones, which can alter the host protection provided. It is more correct to view adrenergic drugs as modulators, rather than suppressors.

## 5.9 Explanation and justification of statistical methods used

In this thesis, I worked with samples from human subjects where the cytokines levels were measured after activating the samples for 4 days. The level of cytokine differed between subjects with just activation, for example, subject A could have 100 units of IL-17A, subject B could have 5000 units of IL-17A. If the drug caused subject A to drop to 50 units, and subject B to 2500 units, they would both have a 50% decrease, but the data could not be combined as such. To correct for the baseline cytokine level, the fold change was calculated in chapters 2 and 3 figures. The fold change calculation normalizes the activated control sample to a value of one, and the treatment groups become relative to one. For example, if a treatment group was 0.5 it would be inhibited by 50%, and if it was 1.5 it would be augmented by 50%. In doing this calculation, each experiment has an activated control value of 1, and the data can be pooled with other experiments and analyzed by one-way ANOVA to determine if there is a difference amongst the groups. If the ANOVA is significant to <0.05, then a post hoc test, in this case Tukey's multiple comparison test, is performed in order to determine if the null hypothesis is rejected for the between-group means. In other words, if two groups were significantly different, the Tukey's post hoc would be below 0.05. There are 3 key assumptions that must be met for Tukey's test to be valid, which include; normal distribution, independent variable and homoscedasticity (i.e homogeneity of variance). By reporting the fold change the data became normalized, thus making the data set eligible for the statistical test chosen. To test the

assumption of homoscedasticity (i.e homogeneity of variance), F test was used. Excel was used for the calculation and the F value reported was smaller than the F critical, thus accepting the null hypothesis, which indicated the population had equal variances.

In chapter 4, to study the significance of different drug treatments or different SNPs, the data was normalized through the removal of within-subject variance procedure (338). This method was used because it brings the baseline values closer by removing the variance between subjects which enables more consistent figures, improves the ANOVA and allows data to be pooled. The removal of within-subject variance procedure involves calculating the *grand mean* which is the average of all values in the data set, and then calculating the *subject mean* for every participant which is the average of all values coming from the same participant. The *subject deviation from the grand mean* is then calculated for every participant by subtracting the *grand mean* from the *subject mean*. Subsequently, a new data set is generated by subtracting the *subject deviation from the grand mean* for a certain participant from each value of that participant. This procedure is applied to bring the participant's data closer to the average of the overall data set, thereby reducing variance between groups. After the removal of within-subject variance was performed, the data was used for graphing and performing multiple T-test with correction for multiple comparisons using the Holm-Sidak method and a significance level of 5%. The Holm-Sidak method is an extension of the Bonferroni-method which controls false-negative discoveries or type 1 errors when performing various hypotheses. It tests the hypotheses that are not negatively dependent which results in a more powerful test than the Bonferroni-method. In the Holm-Sidak method the p-values are arranged from smallest to largest after the multiple T-test has been performed. There is the significance level $\alpha$ set to 5%. The number of comparisons is defined (i). The smallest p-value is compared to $1-(1-\alpha)^{(1/i)}$ (397,398). This method was chosen because it is powerful and reduces the chances in a non-rejection of a false null hypothesis (type II error). In experiments where I was comparing mean values between just two treatments, a student T-test was used, and significance was considered at $p \leq 0.05$. The tests were indicated in figure captions.

The N value indicated in figure captions of the results in chapter 2, 3 and 4, indicates the number of participant's sample. For instance, n=3 indicates that it is data from 3 participants' cell samples. After the primary cells are isolated from the participants, these cells with the corresponding cell culture medium and corresponding treatment are placed in culture plates wells with replicates of each condition. Under the caption of the figures, the number of replicates is indicated as experimental groups. That is for each condition (e.g terbutaline, ICI 118-551) from one participant's sample, there are at least two replicates of each experimental group as indicated in figure captions.

To study the effects of the drug treatment and SNPs on the cytokine concentrations while accounting for the human variation, the concentrations of the IL-17A and IFNγ cytokines obtained from enzyme-linked immunosorbent assays (ELISA) were fit in a linear Mixed-Effects Model with maximum likelihood. The Linear Mixed Models assumes the dependent variable (cytokine concentrations) to be linearly related to the other variables and coming from a normal distribution. To meet the normality assumption, a LOG10 transformation of the data was required and the normal distribution was verified visually in a bar plot. The linearity assumption was met and verified in a Q-Q plot. The treatment, genotype at SNP16 and genotype at SNP27 were the fixed variables, whereas the random variable was the human subject. This enabled us to test the significance of the treatment or SNP on the cytokine response while taking human variation into consideration. The model was analyzed by type III Analysis of Variance (ANOVA) using Satterthwaite's method and a significance level of 5% to evaluate the effects of the treatment or SNP on the cytokine response in cells. The Satterthwaite's t-test or also known as Welch's t-test, makes the assumption that the distributions of the populations are normal. The ANOVA demonstrates the fixed variables that significantly explain the variance observed in the dependent variable that is the cytokine concentration. Type 3 ANOVA was used because the number of replicates per subject was varying (399).

## 5.10 Conclusion

In conclusion, previous studies have focused on the Th1/Th2 balance but have not investigated the Th17 cells response and their regulation by β2AR. In my thesis, I showed the *in vitro* fine-tuning of immune response that can be provided by the stimulation of β2AR, shifting the response towards Th17 cells, or away from Th17 cells depending on the agonist used. I identified a precise genetic element in the *ADRB2* that confers reactivity to the β-agonist. I discovered an inverse-agonist that has promising aspects for treating autoimmune disease. A better understanding of how adrenergic drugs modulate Th cell subsets will help in the tailoring of therapies for specific infections or associated diseases where cytokine balance is important.

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# Appendix

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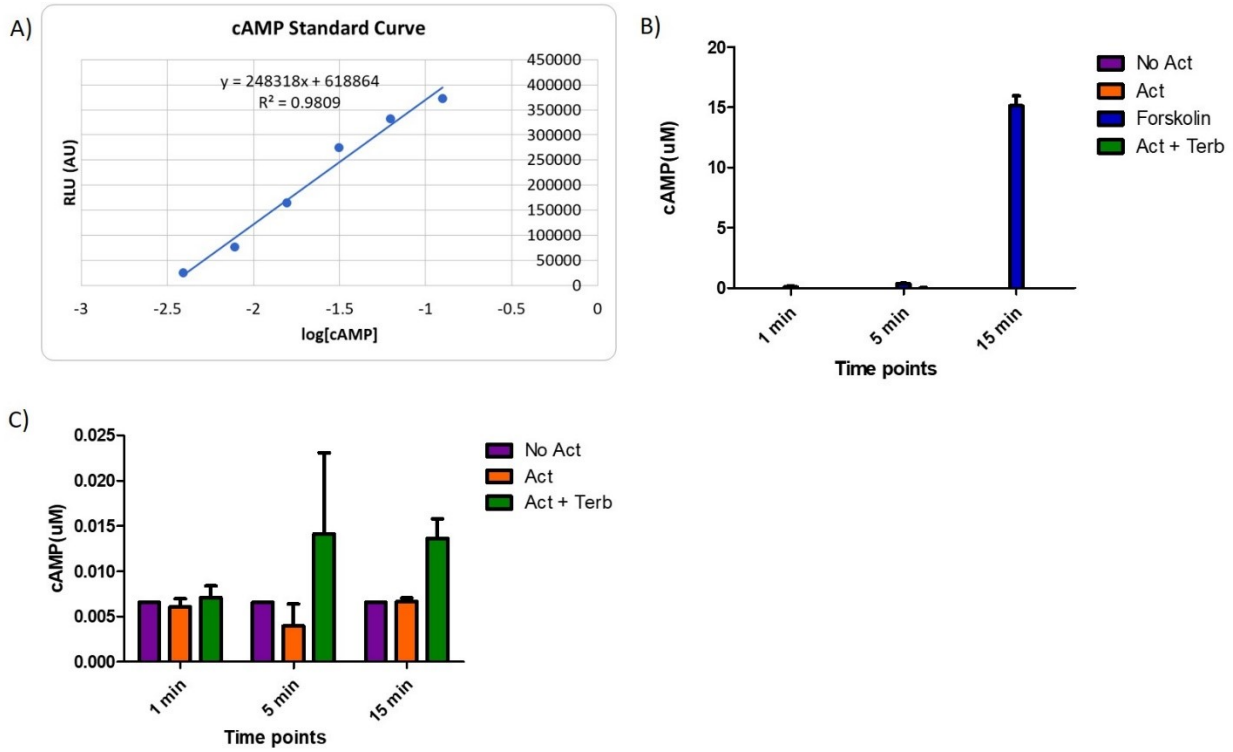


Figure A1. cAMP levels upon β2AR agonist terbutaline, or the positive control forskolin, on Jurkat T cells.

The levels of cAMP produced were measured using a cAMP glo assay. This assay relies on a cell lysate, which releases cAMP, which will then react in a plate containing PKA dependent substrates that produce luminescence. The luminescence is inversely proportional to the levels of cAMP in the sample. Manufacturer's instructions were followed for the cAMP glo assay. Forskolin was used as a positive control since it stimulates adenylyl cyclase enzyme which increases cAMP.

The standard curve is shown in Figure A1, A) Standard curve of cAMP glo assay. The cAMP was measured at 3-time points (1, 5 and 15 min) (Figure A1, B, C). Jurkat T-cells were incubated in different treatments to determine their effects on cAMP levels. Non activated (No Act), which do not contain activation mixture. Activated (Act) which contain 2ng/mL phorbol 12-myristate

13-acetate (PMA) and 0.1µg/mL anti-CD3, both of which activate the T cell pathways. Activated cells plus β2AR agonist terbutaline ($10^{-5}$M) (Act+ Terb) and Forskolin (62.5 mM)

Jurkat T cells, is a Th cell line that has been used extensively to study T cell activation. The advantages of using this cell line (Jurkat T cells) are that they continuously proliferate, they are large cells with high protein content, they are clonal from the same person, and they use a classical cAMP-PKA pathway (400). Using these cells allows to establish the techniques with a minimum of human variation since the cells are clonal. Pilot data from our lab indicates that β2AR are expressed on Jurkat T cells (401). Therefore, the levels of cAMP was expected to change upon addition of β2AR agonist.

The control for cAMP glo assay worked, as forskolin cAMP levels were consistently higher than the treatments (Figure A1, B). However, the cAMP levels upon different treatments were not consistent after replication of assay. Due to inconsistency of the response of cAMP to the treatments given the measurements of cAMP were not carried on to primary human T cells.

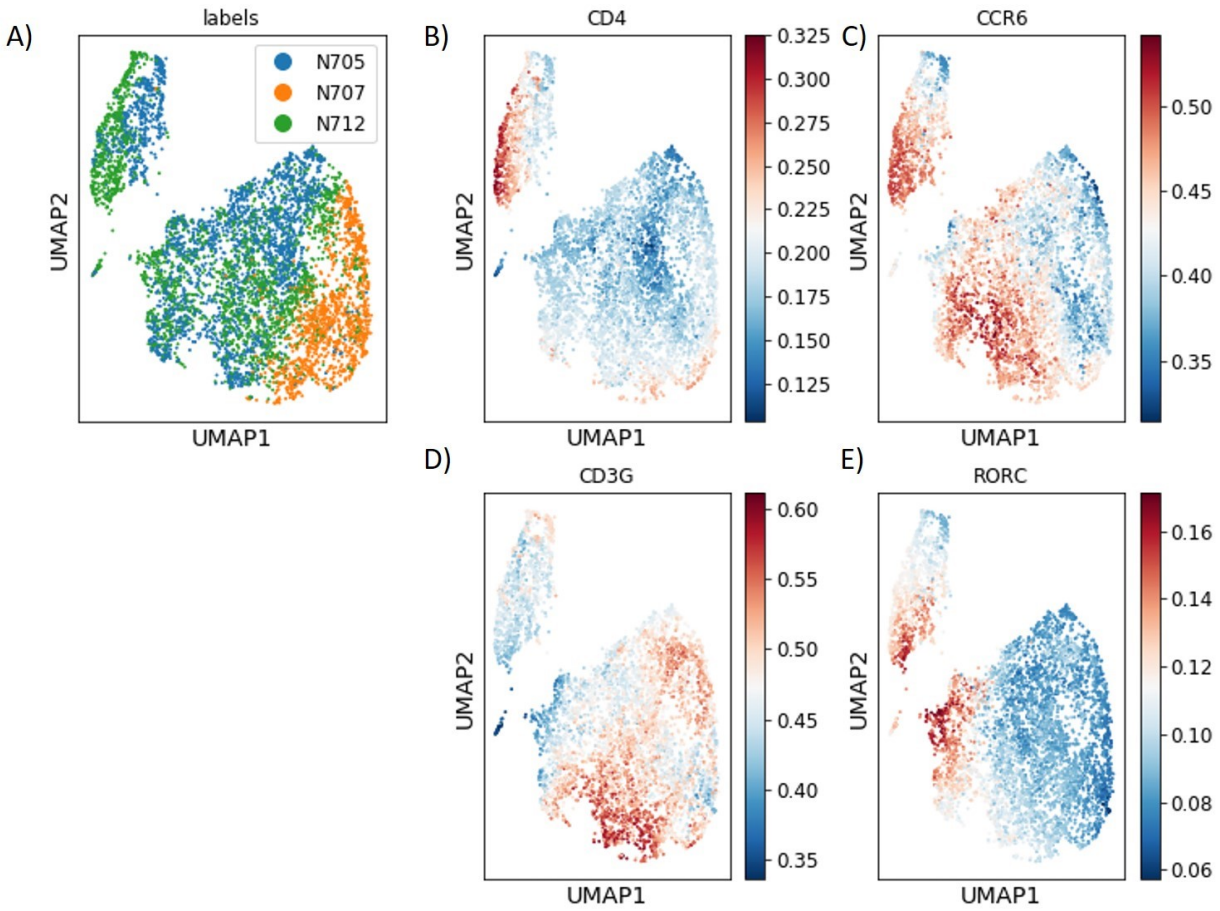


Figure A2. Th17 cells single-cell characteristics using high throughput drop sequencing reveals possible new gene clusters altered by adrenergic drugs.

The detailed protocol is written below this caption. In brief, purified Th17 cells from human PBMC were activated with dynabeads human T-activator CD3/CD28, along with either terbutaline or nebivolol treatment. Samples were run through a custom made drop sequence device to apply cell-specific probe, then libraries of cDNA made with the group tagmentation. Samples were then pooled and sent for high throughput sequencing at IRIC Genomics Center. In the Figure, UMAP is designed to conserve relationships between data points: in the case of scRNA-seq data, two cells are mapped close to each other if they have similar gene expression profiles. UMAP is used to reduce the scRNA-seq data for combined samples N705, N707 and N712 to two dimensions.N705 refer to activated cells with dynabeads human T-activator

CD3/CD28, N707 refers to activated cells + Terbutaline ($10^{-5}$M), N712 refers to activated cells + Nebivolol (10uM). A) each cell has been coloured by its sample of origin. The algorithm discovered two distinct clusters, a small cluster (cluster A) consisting of cells from activated samples (N705) and activated + nebivolol samples (N712) and a larger cluster (cluster B) consisting of cells from each samples (N705, N712 and N707). Each of these clusters are characterized by distinct global gene expression profiles. In order to visualize particular genes whose expression distinguishes the two clusters, the normalized expression values for genes CD3, CD4, CCR6, RORγ were projected onto the Figure. In B-E, cells are coloured by the relative magnitude of the expression value of the gene in question. CD4 and CD3G are differentially expressed between cluster A and cluster B, and act as potential markers for two cell states comprising the population of activated + nebivolol samples (N712). CD4 is a co-receptor identifying T helper cells, while CD3G is a T cell receptor. The activated cells + Terbutaline samples (N707) show very low expression for each of the four marker genes, while activated samples (N705) show no distinct patterns of expression which could be used to distinguish cell types or states.

In summary, nebivolol appears to act on a gene cluster that defines helper T cells (CD3 and CD4), along with a signalling molecule of the T cell receptor. This can lead to future studies on the mechanism of action of this drug. We now know that the effects of adrenergic drugs can extend beyond just cytokines and transcription factors.

**Detailed Procedure of Drop Sequencing**

Informed, signed consent was obtained prior to the blood donation as described in chapter 2. Venous blood collected in sodium heparin-coated vacutainers were processed by ficoll techniques to obtain PBMC (303). Th17 cells were isolated as previously described (303). The Purified Th17 cells were activated with dynabeads human T-activator CD3/CD28, used according to the manufacturer's instructions (Thermo Fisher Scientific, Mississauga, ON, Canada). The Th17 cells were treated with Terbutaline ($10^{-5}$M) or nebivolol (10uM) in addition of the dynabeads. Culture conditions were incubated at 5% $CO_2$ and 37°C for 24h in a

humidified incubator. The cells were then used for the single-cell RNA isolation using the dropseq.

Droplets of ~1 nl in size made with microfuildics device (Dropseq) built-in Dr Halett laboratory at Concordia University. Microparticles (Beads) containing barcode are suspended in lysis buffer that is ran in the microfluidics device at an equal rate to create single-cell suspension. A generated droplet has a single cell and a barcoded bead. The cells are lysed as they are generating the droplets. The cell's mRNAs is captured on its barcoded bead. The droplets are broken with perfluorooctanol that is in 6x saline-sodium citrate buffer (SSC). The beads are washed with 6x SSC and reverse transcriptase buffer and resuspended in a reverse transcriptase mixture, then is treated with exonuclease I to remove the RNA that was not captured by the bead primers. The beads were washed with water and counted with the hemocytometer to be aliquoted into PCR tubes, which are then amplified. After the amplification, the PCR reactions are purified using Ampure XP beads and pooled. The amplified cDNA was quantified with Agilent 2200 TapeStation system. Tagmentation of the groups and cDNA fragments are amplified and using the Nextera XT kit (Illumina). The samples are sent to IRIC centre for NextSeq 500. The procedures were done following Macosko *et al.* (377).

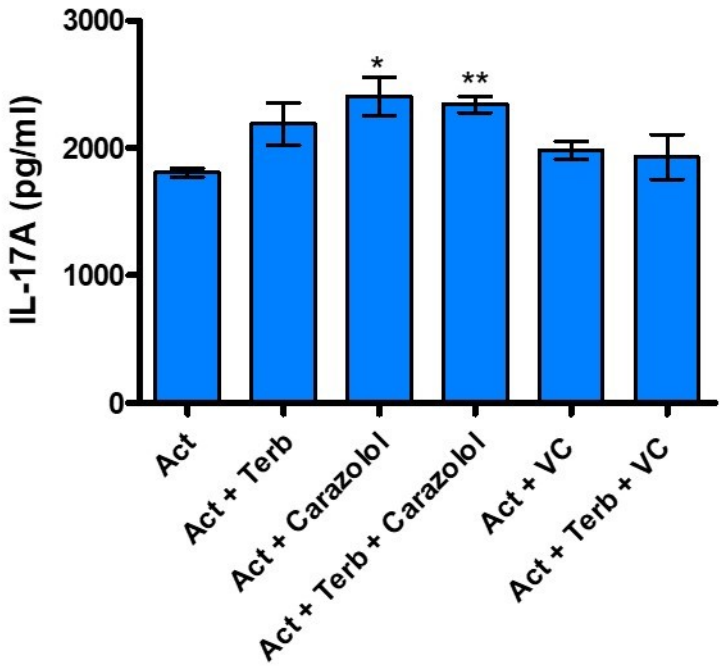


Figure A3. PBMCs effect of carazolol in IL-17A.

Isolated PBMCs were activated with anti-CD3 and anti-CD28 and with carazolol 10uM. The supernatants were collected after four days of incubation. ELISA was performed for IL-17A, data is representative of 5 individual experiments. Error bars are shown from standard error. One-way ANOVA followed by Tukeys post test. (*<0.05 **<0.01).

Carazolol is an inverse-agonist of the β-adrenergic receptor. It showed inconsistent results and it was not studied further.

## Recruitment Ad for participants on my study

**Study Participants Wanted**

The Darlington lab in the Department of Exercise Science at Concordia University is seeking participants for the "HORMONAL REGULATION OF T CELL GENOMICS" study. The study consists of one 15 minute visit.

You will be asked to give a small amount of blood.

Refreshments will be provided.

Participants should:

- Anyone above the age of 18 years old
- Be comfortable with blood draws and needles
- Not be taking any prescriptions medications that affect blood pressure or immune function
- Have no chronic medical conditions (autoimmune disease, cancer, lung or heart disease)
- Not have had any vaccinations (including flu shots) within the past 2 months

This information was advertised on the PERFORM center website.