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REGULATING THE IMMUNE RESPONSE: THE ROLE OF NUR77 IN T CELLS

Moriah Weir
Regis University

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A thesis submitted to
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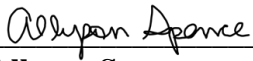
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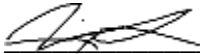
Thesis written by

Moriah Weir

Approved by

(signature: Allyson Spence)

Allyson Spence
Thesis Advisor

(signature: Bethany Lucas)

Bethany Lucas
Thesis Reader

Accepted by

Tom Howe
Director, Regis College Honors Program

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Preface and Acknowledgements

When I was a kid, I loved puzzles. It wasn't unusual to see eight-year-old me sitting alone in my room, poring over sudoku puzzles, anxious to find the perfect combination of numbers. I loved to develop logical strategies to find the answer, no matter how tedious they were. I would go through the puzzle several times, first picking off the obvious numbers, and then methodically eliminating possibilities until only one singular possibility remained for each of the 81 boxes. Logic puzzles were another one of my favorites. I could not stop thinking until I determined the implications of each clue, giving order and structure to pages and pages of brainteasers. There was nothing more satisfying to me than looking at a logic puzzle matrix or a mess of sudoku numbers and knowing that I'd come to the one, perfect, logical conclusion. I liked the tidiness of it all. I liked that the numbers all fit in their respective squares and followed the rules and that if I just spent enough time staring at the puzzle, the solution would come to me.

As a child, I knew that I wanted to solve problems when I grew up. I was confident that I could discover something amazing if I just stared at a problem long enough. What I didn't anticipate was how real-life problems don't have the same kind of satisfying answers as my books of logic puzzles. In my adult life, I usually only have a fraction of the clues I think I need to perfectly solve a problem. In my adult life, poring over a problem for hours, hoping that I'll stumble upon a flawless solution is a perfectionist's procrastination mechanism, not a viable problem-solving strategy.

My thesis has been a lesson on the messy reality of science. Studying immunology, or anything in biology, for that matter, is kind of like solving a series of puzzles. But instead of the puzzles having the same set of rules and parameters, they are constantly changing. I can't develop a perfect, methodical strategy for finding solutions when hundreds of moving parts

suddenly produce a different outcome in a different cellular context. I took immunology with Dr. Spence my junior year and decided that I absolutely loved the intricacies of the tiny parts in the immune system. The number of microscopic processes our bodies carry out to protect us from illness daily and how they do their jobs without any conscious input from us fascinated me. I asked Dr. Spence about some of her research interests and I became involved in studying a protein called Nur77. As far as we know so far, this miniscule protein is part of the process that our bodies use to prevent autoimmunity. Autoimmune disorders occur frequently, despite our body's efforts, so understanding the intricacies of the immune system and how Nur77 could be involved became very interesting to me.

I had a perfect plan for writing my thesis. I began meeting with Dr. Spence, my thesis advisor, right on schedule. We discussed laboratory research we could do and dove headlong into the literature review process. I had everything scheduled out in orderly lists and I knew that if I put the time in, I would end up with a product I was proud of. I knew that laboratory research could be messy and that things usually do not go according to plan. But I could never have anticipated that a pandemic would sweep across the globe during my junior and senior year of college, forcing all of us to adapt to living day-by-day. This was the ultimate rule change. Not only was the science confusing and difficult to pin down, but the pandemic threw my entire neat and tidy life plan into disarray.

Studying science has taught me that learning is never quite as tidy and orderly as I thought it would be as a kid. The pandemic has taught me that life isn't a matter of following the logic to some perfect conclusion. I have learned that it is essential to stay engaged in the process and to find meaning in whatever challenges present themselves to me.

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Introduction

Bacteria cover our skin, reside in our digestive tracts, and colonize our noses and mouths. The average human body actually contains more bacteria than human cells. In fact, the bacteria in the human body outnumber human cells 10 to 1 (*NIH Human Microbiome Project Defines Normal Bacterial Makeup of the Body*, 2015). Many of these bacteria are helpful to the human body, helping us digest plant matter, absorb nutrients and break down toxins. Usually, these bacteria do not hurt us. However, there are about 1400 species of pathogenic bacteria, viruses, fungi, and other organisms known to infect humans (*Microbiology by numbers,* 2011). The human immune system protects the body from infection from these more dangerous pathogenic varieties of microbes and fights off infections if the body does get invaded.

The immune system is powerful and multifaceted (Brodin 2020). There is one division of the immune system called the innate immune system that is always ready to defend the body from any unexpected foreign objects or microbes (Alberts et al. 2002) (Table 1). If you've ever accidentally cut yourself, you've noticed the swelling and redness that ensues almost immediately. When the skin is broken, immune cells rush to the point of injury and induce inflammation and redness as they recruit more immune cells to neutralize any toxins or foreign invaders (Kolaczkowska and Kubes 2013).

The other facet of the immune system is the adaptive immune system (Table 1). This branch of immunity takes a lot longer to react than the innate immune response but is much stronger and is what gives you long-term immunity (Alberts 2002). The adaptive immune system is composed of B and T cells, which have extremely specific cell-surface receptors (Marrack and Kapler 1987). When B or T cells recognize a pathogen, they become activated and make many more of themselves, creating many immune cells that can neutralize an infection in the body

(Langhoff and Steinman 1989). Because the body must make many copies of the cell that recognized the pathogen, it takes longer for this part of the immune system to be ramped up (Chaplin 2006). However, once the adaptive immune system is active, infections are cleared relatively fast in healthy people (Chaplin 2006). This immune response is also what gives people long term immunity either through vaccination or through infection (Crotty and Ahmed 2004). For example, following a measles vaccine, the body expands all the measles-fighting lymphocytes. After all the measles particles are cleared, most of the measles-specific immune cells die. However, a few of them remain in the body. The body can activate and expand these few memory cells much more rapidly than if the immune system had never seen the infection before (Crotty and Ahmed 2004). This activation and expansion is usually so rapid that the person does not even notice any symptoms of illness (Crotty and Ahmed 2004).

The immune system is constantly working hard to keep the body healthy. However, sometimes the immune system turns against the body's own cells. In the case of Type 1 diabetes mellitus, the immune system attacks the beta cells in the pancreatic islets. These cells help the body maintain stead blood glucose levels. If the immune system damages too many of the beta cells, the body can no longer maintain blood glucose levels and begins to have symptoms of diabetes. Other autoimmune disorders include rheumatoid arthritis, where the immune system attacks and damages joint tissue, Crohn's disease, where the body mistakenly attacks the digestive tract and multiple sclerosis, where the body attacks the protective coating surrounding brain cells. Systemic lupus erythematosus, often shortened to lupus, is a condition where the body attacks the joints, kidneys, brain, and heart in a more systemic manner. These diseases are characterized by inflammation and tissue damage. Autoimmune diseases have been on the rise in

Westernized countries throughout recent decades, and they are a significant burden on global health (Lerner at al. 2015).

There is a lot of interest in figuring out why autoimmune diseases happen. There seems to be some element of genetics as well as environmental influences (Khan and Ghazanfar 2018). This is what originally got me interested in the topic of my thesis: Nur77. Nur77 is a protein involved in negative selection (Thompson and Winoto 2008). Negative selection is a process that deletes T cells that could cause autoimmunity during T cell development (Klein et al. 2014). Negative selection involves complicated protein signaling that tells self-reactive T cells to undergo apoptosis, or cell death (Klein et al. 2014). The role of Nur77 in this process is known to some degree, but no one has identified what signal specifically triggers Nur77 to carry out its part in the negative selection process (Thompson and Winoto 2008).

Of particular interest to this thesis is what role Nur77 plays in regulatory T cells. Regulatory T cells are a class of T cells that help regulate the immune response and keep it from protect against autoimmunity (Shevach 2000). Regulatory T cells express Nur77, but it is yet unknown if and how Nur77 is involved in suppressing the immune system (Fasset et al. 2012; Sekiya 2013). Although science is likely a long way off from actually identifying what Nur77 does exactly and is certainly a long way off from using Nur77 as a clinical target, it is a valuable research target. Not only is it involved in autoimmunity, but it has also been implicated in several cancers, Alzheimer's, stress responses, and more (Campos-Melo et al. 2013; Kurakula et al. 2015; Jiang et al. 2016; Palumbo-Zerr et al. 2015; Qin et al. 2013; Zeng et al. 2017;).

This thesis will begin with a broad explanation of the immune system and then go into depth about the adaptive branch of the immune system. Then, it will give an overview of the process of T cell development and activation, providing context for understanding the specific

role of Nur77 in negative selection. Finally, it will provide relevant literature for what is known about Nur77 and propose some hypotheses about protein-protein interactions that Nur77 might have in regulatory T cells. This extensive literature review and the development of hypotheses sets up future research into the role that Nur77 plays in regulatory T cells.

Chapter 1: The Immune System

The purpose of this chapter is to give a broad overview of the immune system and autoimmunity. Included in this literature review are explanations of the components of the immune system, including the innate and adaptive immune system. Focus is placed on the function and development of T cells and how they can cause autoimmunity, as well as regulatory systems that the body uses to reduce autoimmune disease-causing T cells. The chapter ends with a brief overview of regulatory T cells, which are the cell type of specific interest for this thesis.

Overview of the Immune System

The immune system is the complex set of mechanisms and cells that the human body uses to defend itself against environmental pathogens that threaten it (Chaplin 2006). Before the body can defend itself against pathogens, the immune system must have ways to recognize invaders by distinguishing between self and non-self. There are two main branches of the immune system that can distinguish between cells of the body and invading pathogens (Chaplin 2006). The first branch is a set of mechanisms that respond quickly to infection or injury with inflammation, known as the innate immune system (Mechnikov 1908). The second branch, called the adaptive immune system, includes the B and T cells, which are white blood cells with intricately specific pathogen-recognizing components (Chaplin 2006). The two branches of the immune system and the components discussed in this introduction are summarized in Table 1. Table 2 summarizes each cell type discussed in the introduction and several more that will be touched on later.

The innate immune system is fully developed at the time of birth and does not change significantly throughout the life span (Zhang and Liang 2016). The broadest definition of the innate immune system is any defense mechanisms that the host directly encodes in genetic material (Chaplin 2006). Innate immune system defense mechanisms include physical barriers

against infection, cells and biomolecules constitutively expressed in the body, and cell-surface receptors that can detect the presence of pathogens (Nicholson 2016). Physical barriers against invasion include the skin, which protects against pathogens by both blocking their entry into the body, and by secreting host defense proteins (Walker et al. 2008). Other physically protective barriers to infection are mucus creating surfaces like the inside of the nose, which can catch and sweep away invading organisms before they can infect the body (Nicholson 2016).

If an invader makes it inside the body, the next line of innate defense are usually neutrophils, mast cells, and macrophages, which are cell types that can recognize that something is wrong and signal the rest of the immune system to mount a response (Portau et al. 2015). Macrophages or neutrophils know that a pathogen is present because of cell-surface receptors that can recognize molecules on the surface of pathogens (Zhang and Liang 2016). If an immune cell-surface receptor binds to a pathogen, it immediately releases chemical signals that tell cells nearby to prepare for infection by mounting defenses and recruit additional pathogen-fighting immune cells (Zhang and Liang 2016). One of the pathogen-fighting cells in the innate immune system is the natural killer (NK) cell, which can recognize and kill virally infected cells and cancer cells (Vivier et al., 2008). Macrophages and neutrophils can phagocytose, or physically envelop and decompose dead cells and pathogens (Nicholson 2016). One of the products of this process is small bits of pathogen protein, which are important in the activation of the adaptive immune system, the slower but more powerful component of the immune system.

While the innate immune system is busy fighting during the early part of the infection, the cells of the adaptive immune system are becoming activated. An antigen-presenting cell brings a chopped-up part of a pathogen or pathogen protein, called an antigen to a secondary lymphoid organ (Pennock et al. 2013). Secondary lymphoid organs are the part of the lymph system are

primarily responsible for responding to foreign invasion (Ruddle and Akirav 2009). The adaptive immune system is activated in secondary lymphoid organs (Pennock et al. 2013). Each B or T cell possesses an extremely specific cell-surface receptor (Germain 2002). If the cell surface receptor of a B or T cell recognizes antigen, it can become activated and a process called clonal expansion occurs, producing a large pool of duplicates of the activated cell (Pennock et al., 2013). Then, the clones can differentiate into effector versions of the cell, which can actually fight the infection through several different mechanisms (Germain 2002).

Table 1: The parts of the innate and adaptive immune system are summarized along with a short description of the action that the components take against foreign invaders like bacteria, viruses, and fungi.

Innate Immune System (Generalized)				Adaptive Immune System (Specific)	
Physical Barriers		**Cellular Defenses**		**Cellular Defenses**	
Skin	Blocks entry of pathogens	Macrophages, Neutrophils	Phagocytose or "eat" foreign bodies	B cells	Make antibodies against specific pathogens
Mucosal surfaces	Sweep away foreign particles	Natural killer cells	Recognize and kill virally infected cells	T cells	Effector functions defend the body
Secretions (i.e. sweat, tears)	Sweep away or neutralize bacteria and viruses	Cytokines	Chemical signals that can recruit more immune cells, induce inflammation, and cause fever	Memory cells	Recognize pathogens that the body has already fought off and neutralize infection

Activation and differentiation take a significant amount of time, which is why the innate immune system responds while the adaptive immune system mounts a response. Within a few days, the adaptive immune system is able to respond to infection. The adaptive immune system is particularly powerful because it also generates memory cells after clearing an infection

(Pennock et al., 2013). Most of the cloned cells die off after infection, but a few cells remain, flowing through the lymph system or resident in bone marrow and/or tissues (Mackay 1993). If the body becomes infected a second time, those cells respond much more quickly and are more easily reactivated (Mackay 1993). Generally, this is referred to as memory, where if a second infection occurs, the person may not even notice an infection because the body can respond swiftly and effectively.

T Cell Function

T cells play a large role in the activity of the adaptive immune system. These cells originate from hematopoietic stem cells, develop into lymphoid precursor cells, and then eventually become mature T cells that commit to different effector functions (Germain 2002). T cells develop in the thymus, which is a gland located deep to the sternum, between the lungs (Spits 2002). There are two main T cell types, named after cell-surface proteins that they express. The first type is CD4+ T cells, which are also known as helper T cells. They strengthen and control the adaptive immune response (Kohlhapp and Zloza 2017). The second type of T cells are CD8+ cytotoxic T cells. These T cells can determine if another cell is virally or intracellularly infected and kill it to stop the spread of the infection (Bakshi et al. 2014). T cells have a highly specific T cell receptor (TCR) that is important for their strong response to infection (Yanagi et al. 1984). Because T cells can react so strongly, another subclass of T cells also arise during the development process, known as regulatory T cells. These T cells can regulate the immune response and keep T cells from attacking so strongly that they hurt the body, preventing autoimmunity (Sakaguchi et al. 1995).

T Cell Receptors (TCRs) are the reason that T cells can respond so effectively to infection. TCRs recognize a cell-surface protein called major histocompatibility complex (MHC) that is

present on other host cells (Zinkernagel 1996). MHC molecules are expressed on almost all cells of the body, and their function is to present small parts of proteins, called peptides. The process of the TCR binding to an MHC that is presenting a peptide controls the specificity of the adaptive immune response (Zinkernagel 1996). Two main types of MHC molecules interact with T cells. MHC class I typically carries healthy proteins that come from inside the host cell, functioning like an identification badge. Any T cells that come in contact with it know that it is healthy, and that they should not kill the cell (Weiczorek et al., 2017). MHC molecules also communicate when there is an invasion in the body (Zinkernagel 1996). MHC class II molecules are usually found on antigen presenting cells (APCs), which present bits of pathogen peptide to T cells. APCs can phagocytose or "eat" extracellular pathogens, degrade them, and display them to T cells, which is important as part of activating T cells to fight the infection (Weiczorek et al., 2017). The interaction between the MHC molecule and the TCR is essential for T cells to be able to respond effectively to infection (Zinkernagel 1996). However, for the MHC and protein combination to be recognized successfully, there must be a T cell with a TCR that is specific to MHC and the protein being presented. This requires T cells to develop a wide variety of TCRs. This variety includes CD4+ T cells, which can interact with MHC class II, and CD8+ T cells that can interact with MHC class I (Weiczorek et al., 2017).

T Cell Development

T cell development is quite complex but results in powerfully specific T cell receptors that can effectively target any infection (Germain 2002). T cells develop from hematopoietic stem cells in the bone marrow, which differentiate into several different types of cell precursors (Germain 2002). T and B cells develop from the lymphoid precursor. Lymphoid precursors migrate from the bone marrow into the thymus. Once in the thymus, the precursors become

committed to becoming a T cell and must create unique TCRs (Germain 2002). This gives T cells their characteristic specificity and power to fight infection. T cells begin in the Double Negative (DN) stage, where they undergo a kind of shuffling process called VDJ recombination to make the first part of their TCR (Roth 2016). VDJ recombination is a process that randomly assembles three different gene segments, generating an incredible diversity of TCR structures. If the first half of the TCR is able to fold correctly, it can proceed into the Double Positive (DP) stage, where it tries to finish the TCR creation process (Germain 2002). The cell does another round of random gene shuffling for the second half of the TCR and tries to express a correctly folded TCR protein structure (Germain 2002). If the cell can do this, then it can proceed to the selective stages of development. If the T cell fails to express a functional T cell receptor at any point, it either shuffles the genes again, or it dies (Germain 2002). Throughout this process, the cells are kept alive or killed by chemical signals, proteins called cytokines (Klein et. al 2016).

Because of the incredibly random nature of VDJ recombination, there are many T cells that will not bind to anything and many that will bind to proteins that are present in the body, such as insulin (Roth 2016). T cells are powerful immune cells, and if they escaped into the rest of the body without any selection, they could be incredibly damaging. To prevent this, there are selective developmental processes to promote the survival of the T cells that are best equipped to fight infection and not cause autoimmune disease. The first selective process that the T cells go through is positive selection (Germain 2002). During this stage, the new TCRs are tested to see if they can bind to MHC, and they are differentiated into the two T cell types depending on which MHC class binds the TCR (Pennock et al., 2013). If they bind to MHC II, they become CD4+ T cells, which have a helper effector function when they fully develop (Pennock et al., 2013). If they bind to MHC I, they become CD8+ T cells, which have a cytotoxic effector function

(Pennock et al., 2013). During this stage, the most important thing is that the cells bind to MHC, and the body gives them survival signals if they bind correctly (Klein et al., 2016). The cells that do not bind to MHC strongly enough, which is roughly 90% of them, die during this stage (Germain 2002).

The next stage is negative selection, which is the process that this paper is most interested in. In this stage, the body tests the developing T cells to see if they bind too strongly to MHC. If they bind too strongly, they go through a process called apoptosis (Germain 2002). Apoptosis is a process of cell death that is context dependent, meaning that it occurs differently depending on the type of cell and the cell environment. The T cells that die during negative selection make up about 5% of the T cells, and if they entered the rest of the body at this point, they would bind to the body's own tissues and have a potentially negative effect (Germain 2002). If cells escape selection mechanisms, they can cause autoimmunity, which is an attack on the body perpetrated by the body's own defense mechanisms (Anaya et al. 2013). The process of negative selection is incredibly important for the prevention of autoimmunity, because if self-reactive T cells escape, they can cause debilitating disease, including Crohn's disease, type 1 diabetes, rheumatoid arthritis, and more (Anaya et al. 2013). A small percentage of T cells make it through the whole development process, and they leave the thymus as mature naïve T cells.

The T cell remains naïve until its TCR recognizes its antigen, which begins activation and differentiation and an immune response against the infection (Pennock et al., 2013). A TCR can recognize its antigen when an APC presents the antigen on an MHC molecule on its cell surface (Chen and Flies 2013). The interaction between a TCR and an MHC-protein complex begins T cell activation. For activation to occur fully, further signaling called co-stimulation is required (Chen and Flies 2013). The APC presents an additional cell surface protein signal called B7,

which associates with CD28 protein on the surface of the T cell, enhancing the T cell activation response (Lenschow et al. 1996).

The Role of T Cells in Autoimmunity

Autoimmunity is an immune response against an antigen that exists naturally within the body or an antigen that is not harmful, such as gluten in celiac disease (Anaya 2012). In chronic immune conditions, the body loses immunological self-tolerance, either in one organ system or in multiple organ systems (Anaya 2012; Caio 2019). T cells play a large role in the development of many chronic autoimmune disorders (Khan and Ghazanfar 2018). T cells undergo the normal negative selection process to delete self-reactive cells, which is part of central tolerance. Central tolerance is the mechanism the body uses to delete autoreactive cells before they escape into circulation. However, sometimes these cells do escape and can pose a danger to the body (Anaya 2012). The body has several other defense mechanisms outside the lymphoid organs to stop autoreactive T cells from causing an autoimmune disease, known as peripheral tolerance. The body uses several modes of peripheral tolerance to either delete self-reactive T cells or stop them from hurting the body (Khan and Ghazanfar 2018).

One mode of peripheral tolerance is anergy. Anergy is a state where T cells are functionally unresponsive (Khan and Ghazanfar 2018). In this state, T cells cannot become activated or mount an immune response. If the T cell is autoreactive, a state of anergy prevents it from causing damage to the body. The body can induce anergy either if co-stimulation fails or when a normal antigenic stimulus is too prolonged (Choi and Schwartz 2007). Clonal anergy is one of the modes of peripheral tolerance that can help prevent autoimmunity if an autoreactive T cell escapes the control of central tolerance mechanisms.

Some researchers think that clonal ignorance is another mode of peripheral tolerance. In this model, autoreactive T cells ignore the self-antigen. T cells could ignore the antigen because they cannot access the tissues containing the self-antigen, like if the self-antigen were a protein in the eye. The lack of T cell response might also be there is not enough self-antigen in these cases to induce a full-blown autoimmune response (Akkaraju et al. 1997). Another mode of immunological peripheral tolerance is clonal deletion via Fas-FasL induced apoptosis (Suda et al. 1993). Instead of activation, some self-reactive T cells undergo Fas mediated apoptosis upon TCR stimulation (Khan and Ghazanfar 2018). Another mechanism of peripheral tolerance is through inhibitory receptors like CTLA-4 and PD-1. CTLA-4 stands for cytotoxic T lymphocyte-associated antigen-4 and is present on T cells. It is structurally similar to CD28, which is one half of the co-stimulation signal (Leach et al. 1996). CTLA-4 can bind to B7 with more affinity than CD28 itself, helping to decrease co-stimulation and therefore reduce T cell activation (Leach et al. 1996). This can help the body maintain self-tolerance because it can stop self-reactive T cells from causing autoimmunity. Another inhibitory receptor, PD-1, stands for programmed cell death protein 1. PD-1 sets off a signaling cascade that results in downregulation of TCR signaling (Schildberg et al. 2016). These methods of peripheral tolerance help the body to maintain self-tolerance. However, if an autoreactive T cell escapes all the methods of maintaining self-tolerance, it can cause autoimmunity.

The last line of defense against autoimmunity are regulatory T cells, which have strong inhibitory effects on the immune system. These effects will be discussed in more detail in the next section. However, if those fail to regulate autoreactive T cells as well, then autoimmune disease can develop.

Failures in central and peripheral tolerance can lead to the development of an autoimmune disease. We do not fully understand the factors that go into the development of an autoimmune disease. It is known that infections, environmental factors and genetic alterations can play significant roles in autoimmune diseases (Khan and Ghazanfar 2018). Some alterations that are correlated to chronic autoimmune disease are changes in TCR's and TCR signaling pathways. Unusual geometric properties in TCRs can keep them from deletion, which can sometimes be detrimental (Wucherpfennig et al. 2009). Changes in several important signaling pathways such as the MAPK pathway and the extracellular signal-related kinase signaling pathway can also cause alterations in the amount of IL-2 produced (Moulton et al. 2015). IL-2 production is important in making regulatory T cells and the clonal expansion of T cells (Khan and Ghazanfar 2018). Some autoimmune diseases can be treated with low-dose IL-2, demonstrating how important it is for maintaining self-tolerance through these mechanisms (Boyman and Sprent 2012; He et al. 2020).

A specific example of the role of T cells in autoimmunity is in diabetes. Diabetes Mellitus Type 1 is immune-mediated. This disease occurs when T cells are reactive to self-antigen expressed by the beta cells of the pancreas. Beta cells produce insulin, which is the hormone that controls blood glucose levels. When too many of the beta cells in the pancreatic islets are destroyed, the body cannot control blood glucose (Concannon et al. 2009). Some genetic risk factors for Type 1 diabetes mellitus are mutations in IL-2 and CTLA-4 (Cooper et al. 2008). In the case of diabetes mellitus Type 1, there is likely a failure of tolerance and an inadequate Treg response, which leads to pancreatic beta cell injury.

Regulatory T Cell Function

The greatest interest of this project is the function of Nur77 in regulatory T cells. Regulatory T cells are a type of T cells that differentiate during the thymic selection process. These cells help to main peripheral tolerance, which means that they are another regulatory force that suppresses chronic inflammation and help protect against potential autoimmunity (Corthay 2009). Regulatory T cells are different from other types of T cells because they express higher levels of FOXP3. FOXP3 is a transcription factor that controls the development and suppressive activity of Treg cells (Fontenot et al. 2003; Rudensky 2011). Patients that do not have functioning FOXP3 develop a severe multisystem autoimmune disease in infancy, demonstrating the importance of FOXP3 and functioning regulatory T cells in preventing autoimmunity (Bennett et al. 2001). Regulatory T cells are important to maintaining a balance between a robust immune response and autoimmunity. Understanding how regulatory T cells suppress the immune system is important to finding potential therapeutic targets for both autoimmunity and cancer. The main targets of regulatory T cells are general inflammation and overactive T cells.

Regulatory T cells target generalized inflammation to help prevent unnecessary stress on the body. One way they prevent inflammation is by secreting inhibitory chemical signals called cytokines. These cytokines include interleukin-10, transforming growth factor-B, and interleukin 35 (Vignali et al. 2008). Inhibitory cytokines slow the immune response and reduce inflammation. Another way that regulatory T cells control the immune response is by mediating macrophages. Macrophages are a major part of the inflammatory response, so when regulatory T cells slow down macrophages, overall inflammatory responses are limited (Koenis et al. 2018). Although the mechanism that regulatory T cells use to suppress macrophage responses is still unknown, it is thought that they reprogram the metabolism of the macrophage's mitochondria

(Koenis et al. 2018). Regulatory T cells may control their target cells by depriving them of interleukin-2, a pro-survival cytokine, inducing apoptosis (Pandiyan et al. 2007). Changing macrophage metabolism would limit inflammatory responses by stopping macrophages from sending out inflammatory chemical signals.

The second major target of regulatory T cells are overactive or self-reactive T cells. Overactive T cell responses can cause a state of inflammation that can be damaging to the body. Self-reactive T cells are a primary cause of autoimmune tissue damage. Regulatory T cells prevent other T cells from causing damage to the body using a variety of mechanisms. A surprising way regulatory T cells can delete autoreactive T cells is through lysis. Lysis is a form of cell death that CD8+ T cells typically use to kill virally infected cells. Previously, researchers did not think CD4+ cells, like regulatory T cells, exhibit cytotoxic activity, but recent studies have shown that in humans, regulatory T cells express granzyme A and can mediate lysis (Grossman 2007). Directly killing autoreactive T cells is an effective way to prevent autoimmunity, but regulatory T cells have several other methods that they use to control overactive T cell responses and reduce inflammation.

One way that regulatory T cells can stop T cells from causing widespread inflammation is through controlling their activation. Regulatory T cells can downregulate the expression of costimulatory molecules that T cells require to be activated (Cedarbom et al. 2000). If fewer T cells become activated, the immune response will be weaker. Another mode that regulatory T cells use to control the immune response is by controlling dendritic cells. Effector T cells need dendritic cells to activate their T cell effector functions, so controlling dendritic cells is an effective way of stopping T cells from activating. Regulatory T cells affect dendritic cells through LAG3, which is homologous to CD4 (Liang et al. 2003). LAG3 can bind to MHC class

II molecules and suppress dendritic cell maturation by preventing the typically CD4-MHCII association. Fewer dendritic cells limit the number of T cells that become activated, therefore suppressing the immune response (Liang et al. 2003). Another way regulatory T cells may be able to control the function of dendritic cells is by inducing dendritic cells to produce immunosuppressive molecules (Fallarino et al. 2003). The suppressive effects of regulatory T cells seem to be contact-dependent in most cases (Vignali et al. 2008).

Regulatory T cells are particularly interesting for answering broader questions about autoimmunity because they are one of the modes of peripheral tolerance that may fail when self-reactive T cells cause autoimmunity. The negative selection process as described above must fail to delete at least a few self-reactive T cells for autoimmune disease to arise. However, regulatory T cells are another mode of peripheral tolerance that aim to reduce inflammation and curb the immune response of T cells (Vignali et al. 2008). In autoimmunity, something must become messed up in central tolerance (negative selection), peripheral tolerance, or both processes. Also of particular interest is a protein called Nur77 that is expressed in regulatory T cells and T cells in general (Fasset et al. 2012; Sekiya 2013). Nur77 is an orphan nuclear receptor, which means that its ligand has yet to be identified (Wu and Chen 2018). When Nur77 and other related family members are knocked down in research studies, mice subjects develop widespread multisystem autoimmunity (Sekiya et al. 2013). Nur77 is essential to maintaining self-tolerance, but it is still unknown exactly what it does, making Nur77 a particularly interesting research target. Chapter 2 will summarize what is currently known about Nur77.

Table 2: The immune cell types most often discussed in this paper are summarized along with a short summary of their main functions within the immune system.

Cell Type	Innate or Adaptive	Action
Macrophages	Innate	Phagocytose foreign invaders, release pro-inflammatory cytokines to recruit immune cells, can act as an antigen presenting cell (APC)
Neutrophils	Innate	Resident in most tissues, first responders to infection, phagocytose foreign invaders, release pro-inflammatory cytokines to recruit immune cells
Natural killer cells	Innate	Recognize and kill virally infected or cancer cells
Dendritic cells	Innate	Phagocytose foreign invaders, release pro-inflammatory cytokines to recruit immune cells, can act as an antigen presenting cell (APC), most important as an adaptive immune system activator
T cells	Adaptive	Mature T cells recognize antigen presented on MHC molecules and carry out effector function based off T cell lineage
CD8+ T cells	Adaptive	Cytotoxic T cells recognize and kill virally infected cells using lysis
CD4+ T cells	Adaptive	Helper T cells coordinate the immune response by recruiting macrophages, B cells, and CD8+ T cells
Memory cells	Adaptive	These cells can be either B or T cells that remain in the body after an infection; can be quickly reactivated upon reinfection
Regulatory T cells	Adaptive	T cell lineage that inhibits overactive T cell responses, protects against autoimmunity
Thymocytes	Adaptive	Refers to immature T cells developing their T cell receptors in the thymus

Chapter 2: Overview of Nur77

In order to understand what is known about Nur77 and its functions, it is important to understand the details of the classifications of Nur77, the structure of Nur77 and its typical localization. These details bring clarity to the processes by which Nur77 can modulate growth, development, differentiation, and apoptosis.

Nuclear Orphan Receptors

Nur77 was first identified in 1988 along with several other immediate early genes during the early development of mice (Hazel et al. 1988). Immediate early genes are activated immediately after cellular stimuli and they encode cellular machinery that is necessary for further gene expression. Nur77 is part of a class of immediate early genes that respond after a T cell receptor gets stimulated (Kelly 1995). Immediate early genes that respond after T cell receptor stimulation mostly encode transcription factors or DNA-binding proteins (Kelly 1995). Nur77 is an immediate early gene that can act as a transcription factor but is also seemingly involved in several other biological processes, including direct protein-protein interactions (Thompson and Winoto 2008).

Nur77 is classified as an orphan receptor, which means that its structural features and genetic sequence make Nur77 similar to other proteins that function as receptors. The term "orphan" refers to the fact that the endogenous ligand for Nur77 has not been identified (Wu and Chen 2018). Its other classification is that of a nuclear receptor. Nuclear receptors are cellular receptors made up of proteins on the nuclear membrane (Sever and Glass 2013). Nuclear receptors bind lipid-soluble steroid and thyroid hormones that can cross the cell membrane on their own (Sever and Glass 2013). When these lipid-soluble signals associate with nuclear receptors, the nuclear receptors start signaling pathways that exert transcriptional control over

regulatory processes within the cell (Evans 1988). One example of a nuclear receptor is an estrogen receptor, which binds estrogen and exerts transcriptional control on gene expression within the cell (Carruth and Shahbazi 2015). There are three main amino acid regions of nuclear receptors: the N-terminal, DNA-binding domain, and the C-terminal ligand binding domain (Mangelsdorf et al. 1995). Nur77 proteins typically exist as monomers, homodimers, or heterodimers, and can be grouped into types based on their location and mode of action (Sever and Glass 2013). Nuclear receptors can be categorized based on their location in the nucleus or whether they are anchored by chaperone proteins in the cytoplasm (Sever and Glass 2013). Nuclear receptors mediate essential biological functions and dysfunction has been associated with many diseases, including Alzheimer's, cancer, and heart disease (Giguére 1990). One example is estrogen, which is important for diagnosing and treating breast cancer. Patients respond differently to endocrine targeted breast cancer treatments depending on their estrogen receptor status (Harvey et al. 1999).

NR4A Family

The nuclear receptor superfamily is a large family of receptors that regulate processes like the actions of thyroid hormones, other steroid hormones and some vitamins (Chawla 2001). Within the nuclear receptor superfamily is the NR4A family, which has three homologous members, including Nur77/NR4A1, Nurr1/NR4A2 and Nor1/NR4A3 (Maxwell & Muscat 2006). Each member of the NR4A family has similar structural domains (Saucedo-Cardenas 1997). These structural domains include an N-terminal region, a central DNA-binding domain, and a ligand binding domain (Ocabe et al. 1995). Because the structure of a protein determines its function, understanding these binding domains are an essential vein of research to understand and processes Nur77 is involved in. There is significant evidence to suggest that Nur77 has

overlapping functions with the other members of the NR4A family because of structural homology (Kurakula et al. 2014).

Structure

Nur77 has the same regions as the rest of the family of nuclear receptors. It has four main domains: A/B, C, D, and E (Figure 1). The A/B region includes the ligand-independent activation function 1 domain (AF-1) (Wu and Chen 2018), which is important for the protein-protein interactions that will be described in subsequent chapters. The C domain contains the DNA-binding domain (DBD), which is the part of Nur77 that can interact with the DNA of other genes. This region of Nur77 recognizes the NGF1-B response element (NBRE; sequence: AAAGGTCA) and can control the expression of target genes through binding directly to DNA within the nucleus (Maira et al. 1999). Some of these transcriptional activities will be described later. The D domain is a smaller hinge-like domain that also contains a nuclear-localization sequence (NLS), which controls localization of Nur77 within the nucleus (Wu and Chen 2018). The E domain contains the ligand-binding domain (LBD), which is the part of Nur77 that would bind its ligand. The LBD is of particular interest because the ligand of Nur77 has not been identified yet. (Wu and Chen 2018). Structural analysis of this region of Nur77 have shown that is hydrophobic residues partially block the LBD (Flaig et al. 2005). This is particularly true in helix 12, which seems to be the differentiating factor between the LBD of Nur77 and its family members (Flaig et al. 2005).
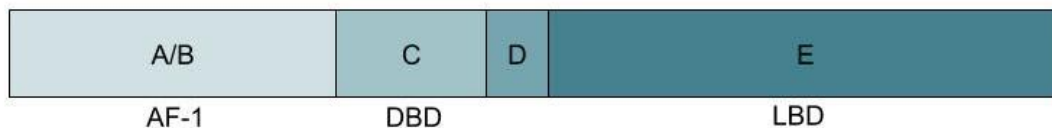
A/B	C	D	E
AF-1	DBD		LBD

Figure 1: Nur77 protein structure is divided into four segments: A/B, C, D, and E. Included in the diagram are the AF-1 region, the DNA binding domain (DBD) and the ligand binding domain (LBD).

Localization

The position of Nur77 within cells tends to be context dependent. Nur77 is expressed in many tissue types, including nervous tissue, endocrine tissue, cardiovascular tissue, and the digestive tract (Uhlén et al. 2015; NR4A1 Cell Type Data, Protein Atlas). In these tissues, Nur77 is usually present to the greatest degree in the nucleus and nuclear membrane and present in much smaller amounts in the cytosol (Uhlén et al. 2015; NR4A1 Cell Data, Protein Atlas). In developing T cells, when Nur77 localizes to the mitochondria, it promotes cell death during negative selection (Thompson and Winoto 2008). It appears that localization of Nur77 to the mitochondria requires a directed protein-protein interaction with another nuclear receptor, retinoid X receptor (RXR) (Cao et al. 2004). Nur77 protein heterodimerizes with RXR protein via their DNA-binding domains(Cao et al. 2004). RXR protein has a nuclear export sequence in its carboxyl-terminal region, which allows the whole complex to be exported to the mitochondria (Cao et al. 2004). RXR protein ligands stop the heterodimerization process and can block Nur77 localization (Cao et al. 2004).

Nuclear Receptors as Transcription Factors

Nuclear receptors can also regulate different genes in various cell types. This makes sense in the context of estrogen, where an estrogen receptor would affect transcription differentially in breast cells compared to uterus cells or neurons (Carruth and Shahbazi 2015). Nuclear receptors can act in distinct ways depending on the action of multiple coactivator proteins that work together to enhance certain functions depending on the cell type (Bulynko and

O/Malley 2011). Nur77 may work in this capacity because it is expressed in so many tissues within the body and is likely not doing the same thing in brain tissue that it does in an immune context, for example. Another role of nuclear receptors is mediating commitment to various cell lineages and provoking their development into those lineages (Evans 1988). For example, Nur77 can exert transcriptional control of T cell differentiation by suppressing a gene called Runx3, resulting in more developing T cells joining the CD8+ subgroup (Nowyhed et al. 2015). Although Nur77 can function as a transcription factor to control T cell differentiation, it is yet unknown how Nur77 could function as a transcription factor in regulatory T cells.

Nur77 Functions

The cell signaling pathways that underlie negative selection are complicated. One receptor, Nur77, plays a part in negative selection, but its role is not fully known. Researchers understand one pathway of cell death that Nur77 mediates, but the full implications of this pathway are not completely understood. Nur77 transcriptional activity has a direct correlation to its function as a cell-death mediator (Thompson and Winoto 2008). The gene that encodes the Nur77 protein is called N4Ar1 (Wu and Chen 2018). While in the nucleus, the N4Ar1 gene is transcribed to a pre-mRNA, which travels to nucleus to be modified into a mature mRNA and then is translated to the Nur77 protein. Then, the Nur77 protein can localize into the mitochondria of the cell (Thompson and Winoto 2008; Lin et al., 2004; Rajpal 2003). It is still unknown which signals direct localization of Nur77 to the mitochondria. However, upon arrival to the mitochondria, Nur77 can associate with another protein called Bcl-2. The Bcl-2 protein changes conformation, exposing a pro-apoptotic face called BH3 (Thompson and Winoto 2008; Lin et al., 2004; Rajpal 2003). Normally, Bcl-2 prevents apoptosis by inhibiting the release of proapoptotic factors from the mitochondria (Tsujimoto 1998). In this case, the Bcl-2 protein changes the way it folds in

response to Nur77. The folding change exposes a face of the protein which attaches to other pro-apoptotic proteins from the Bcl-2 family, forming a group of bonded proteins called a heterodimer (Tsujimoto 1998). These groups of BH3 dimers promote the formation small polymers made up of Bax and Bak protein effectors that pierce the outer membrane of the mitochondria, allowing proteins from the mitochondria to spill into the cytosol (Westphal et al. 2011; Burger et al. 2014). This activates proteases that stop cell function and kill the cell (Westphal et al. 2011). This whole process is important because it stops autoreactive T cells from escaping the thymus and causing autoimmunity during T cell development.

Nur77 has a specific role in mediating negative selection in T cells. However, are fewer definitive conclusions on other processes involving Nur77. It is known that Nur77 is a part of negative selection in development, but it is also present in other pathways (Nowyhed et al., 2015). Because of this, researchers have identified several ways that Nur77 mediates self-tolerance (Suzuki 2003; Hu et al. 2017). One of the ways that Nur77 can mediate self-tolerance is to act as a transcription factor that activates several target pathways (Fassett et al. 2012). Transcriptional analysis of Nur77 revealed that it helps to induce Bcl-2 expression after a TCR signals, which is another route of apoptosis regulation in immature thymocytes (Fassett et al. 2012). Nur77 also appears to be one of the factors that controls thymocyte differentiation into regulatory T cells during development (Fassett et al. 2012; Sekiya 2013). This is particularly interesting research because this group found that deletion of Nur77 receptors causes early death in mice due to autoimmunity (Sekiya 2013). These findings demonstrate that Nur77 is important for preventing autoimmunity in multiple ways: both helping to kill autoreactive T cells and promoting the development of regulatory T cells that mediate the T cell response.

A protein called TWIST2 appears to regulate Nur77 expression (Oh et al. 2016). TWIST2 also regulates how cells interpret how strongly a TCR binds to MHC, which decides whether it dies by apoptosis via negative selection or not (Oh et al., 2016). Several papers confirm the importance of Nur77, finding its presence in a variety of cell types to be important to preventing inflammatory disease in the colon, in diabetes and in cancer (Lin et al 2004; Hu et al., 2017; Burger et al., 2014; Liebmann et al., 2018; Tao 2008). For example, Nur77 deficient mice had increased colon inflammation compared to their wild type counterparts, suggesting that Nur77 helps to control inflammation by promoting regulatory T cell differentiation (Hamers et al. 2015). A search for protein-protein interactions of Nur77 in protein interaction databases yields many results, but the physiological relevance of these protein interactions with Nur77 is largely unknown (NR4A1 Result Summary | BioGRID, n.d.).

A further complication to understanding the physiological relevance of Nur77 protein-protein interactions is how context dependent the roles of Nur77 are. In immature T cells, as described by Thomspon and Winoto, Nur77 upregulation is associated with apoptosis (2008). In mature T cells and in regulatory T cells, upregulation of Nur77 no longer induces apoptosis, but cell division (Cunningham et al. 2006). Because the role of Nur77 changes even depending on maturity and lineage of T cells, Nur77 likely has many different roles, depending on the cellular context. This is one of the main challenges of hypothesizing the role of Nur77 in regulatory T cells.

Chapter 3: Nur77 Interaction Maps

The maps presented here are a consolidation of the current literature about Nur77 interactions. The actions of Nur77 are heavily context dependent. In some instances, a Nur77 interaction may promote cell survival and in other instances promote cell death. By sequestering Nur77 in the nucleus, some interactions promote Nur77 transcriptional activity and prevent pro-apoptotic interactions. Many of the interactions Nur77 has with other proteins represent a highly complicated, delicate balancing act of proteins that differs extensively given the context. The purpose of these maps is to make predictions about what kind of protein-protein interactions might happen in the regulatory T cell context. Protein-protein interactions between Nur77 and other factors are the main exploratory interest of this thesis.

Given the variety of contexts that Nur77 can be involved in, it is challenging to predict exactly what Nur77 could interact with directly. Here, the map interactions exclude the numerous Nur77-DNA interactions in the nucleus, focusing on protein-protein interactions in DNA-protein interactions that could be relevant in the context of regulatory T cells. The studies mentioned here had different research goals and a variety of methods, so definitive conclusions about how these proteins work in regulatory T cells cannot be drawn solely from these sources. In Protein Atla,s regulatory T cells express all of the proteins shown below. Because regulatory T cells express these proteins, it is theoretically possible that any of these interactions could occur in the regulatory T cell context. I paid particular attention to interactions that were specific to the typical T cell environment because the proteins and context are somewhat similar to regulatory T cells and could be plausible interactors.

Bcl-2-Nur77 Pathway

The proapoptotic Bcl-2-Nur77 pathway is perhaps the most well-documented and best

understood processes involving Nur77. I described this pathway in detail in Chapter 1. Here, the

I have expanded the pathway to include several interactors that may be involved in how Nur77

gets to the mitochondria to induce an apoptotic cell response. I have included a group of protein

kinases upstream of Nur77 in the Bcl-2 pathway in Figure 2. Although the literature does not

explicitly attach these protein kinases and phosphorylation events to the pro-apoptotic Bcl-2

pathway, several of the interactions are essential for nuclear export. The Bcl-2 pathway only

induces apoptosis in developing thymocytes, so it is yet unknown what it could do in the context

of regulatory T cells. Regulatory T cells express each of the protein kinases shown in the map, so

it is of particular interest what their function could be. One key piece of evidence is that heavily

phosphorylated Nur77 proteins can be found distributed both in the nucleus and cytoplasm, but

underphosphorylated Nur77 is sequestered in the nucleus (Fahrner et al. 1990). Because Nur77

must get out of the nucleus and translocate to the mitochondria to associate with BCL-2 and

become a pro-apoptotic factor, a number of these phosphorylation events are likely necessary to

allow for BCL-2/Nur77 mediated apoptosis.

MEK 1/2-ERK 1/2 MAPK cascade results in Nur77 phosphorylation

One of the pathways that results in phosphorylation is through mitogen-activated protein

kinases (MAP kinases). MAP kinases are a family of protein kinases that are involved in

regulating a diverse set of processes like cell differentiation and have a role in apoptosis (Pearson

et al. 2001). MAP kinases need to be activated via a three part phosphorylation cascade. Each of

the three parts of MAP kinase activation involves MAP kinase family members, such as

extracellular signal-related kinase (ERK1/2) and MAP/ERK kinase 1 (MEK1/2) (Pearson et al. 2001). MAP kinases typically have a three-tiered phosphorylation cascade that results in an activated, multifunctional MAP kinase that can then regulate other processes (Pearson et al. 2001). One of these three-tiered cascades called the MEK1/2-ERK1/2 MAPK cascade is known to phosphorylate Nur77 and regulate Nur77 translocation (Fuji et al. 2008). Ribosomal s6 kinase 1 and 2 (RSK1/2) are downstream effectors of the MEK1/2-ERK1/2 pathway and are required for Nur77 nuclear export and TCR activation-induced apoptosis in mature T cells (Wang et al. 2009; Wingate et al. 2006). Figure 1 depicts the RSK1/2-Nur77 phosphorylation event. In this case, I have not included the upstream activators of RSK1/2 because these interactions do not directly involve Nur77.

JNK and JNK-inhibitors balance survival and death signals

Another pathway depicted in Figure 2 is phosphorylation by Jun N-terminal kinase (JNK). JNKs (Jun N-terminal kinases) are a family of kinases in the MAP-kinase family that are known to be involved in cell death, both through extrinsic and mitochondrial intrinsic pathways (Dhanasekaran and Reddy 2008). JNKs can play a role in apoptosis either through activating transcription factors or by phosphorylating proteins that either promote or block apoptosis (Dhanasekaran and Reddy 2008). Another study found that when JNK phosphorylated Nur77 at the N-terminus region, Nur77 could not bind to DNA as often (Kolluri et al. 2003). This is significant because Nur77 must detach from DNA to be able to leave the nucleus. JNK phosphorylation of Nur77 is essential for nuclear export, but alone is not sufficient for Nur77 to leave the nucleus (Han et al. 2006). JNK inhibitor c-Jun inhibited this phosphorylation event in Leydig cells (Lee et al 2009). Leydig cells are part of testicular tissue and are sensitive to

hormone and steroid signals (Zirkin and Papadopoulos 2018). Because the Lee et al. research group used Leydig cells, the results are not directly related to my main investigative question about Nur77 in regulatory T cells. However, because regulatory T cells express c-Jun, it could be an inhibitor for this Nur77-JNK phosphorylation interaction (Uhlén et al. 2015; Regulatory T cell Cell Data, Protein Atlas). Because JNK phosphorylation facilitates the detachment of Nur77 from DNA, c-Jun inhibiting this detachment would likely keep Nur77 sequestered in the nucleus, bound to DNA. My hypothesis is that if c-Jun inhibits Nur77-JNK interaction, then c-Jun inhibits any action of Nur77 outside the nucleus.

Figure 1 depicts protein kinase B (Akt) inhibiting phosphorylation of Nur77. Akt is a cell-survival signal when it interacts with Nur77 (Masuyama et al. 2001). When Akt phosphorylates Nur77, it keeps Nur77 from leaving the nucleus and inducing apoptosis (Han et al. 2006). JNK phosphorylates Nur77 but induces a pro-apoptotic response, suggesting a balance between pro-survival and pro-apoptotic phosphorylation events in Nur77 (Sunayama et al. 2005).

PKC phosphorylation is essential for Nur77-mediated apoptosis in T cells

PKC (Protein kinase C) phosphorylates Nur77 to induce translocation to the mitochondria to signal for apoptosis (Thompson et al. 2010). Thompson et al. found that PKC phosphorylates Nur77 and PKC inhibitors block apoptosis in developing T cells. This demonstrates that this phosphorylation interaction is important for TCR-induced apoptosis during development. The interaction between PKC and Nur77 is different from other interactions described here because PKC is important for mitochondrial targeting, rather than nuclear export. PKC-Nur77 interaction is significant because Nur77 does need to leave the nucleus, but factors

inducing mitochondrial targeting must also be present for Nur77 to cross the mitochondrial membrane to interact with BCL-2 and induce apoptosis.

NGF interacts with Nur77-RXRa heterodimer to facilitate nuclear export

Retinoid X receptor (RXR) commonly acts as a heterodimerization partner for nuclear receptors, thereby modulating a variety of cellular processes. Typically when RXR binds to retinoic acid which is its ligand, or other nuclear receptors, it can act as a transcription factor, controlling cell metabolism (Dawson and Xia 2012). When RXR heterodimerizes with Nur77, however, RXR can act in a cytoplasmic shuttle rather than as a transcription factor (Dawson and Xia 2012). The DNA-binding domain of RXR binds to the DNA-binding domain of Nur77 (Cao et al. 2004). The Nur77-RXR interaction blocks Nur77's ability to bind to DNA, helping it to release from DNA and be targeted for nuclear export. In cells undergoing apoptosis, RXRa and Nur77 localized in the mitochondria (Cao et al. 2004). When the Cao et al. research group introduced RXR ligands into this system, Nur77 was no longer targeted to the mitochondria, suggesting that RXRa-Nur77 heterodimerization is essential for nuclear export and subsequent mitochondrial targeting (Cao et al. 2004).

Nerve growth factor (NGF) is a growth factor typically involved in the maintenance and growth of neurons, but it is also expressed in T cells (Lambiase et al. 1997). It T cells, it may help to regulate apoptosis via the Nur77 pathway. NGF is involved in Nur77-RXRa heterodimer nuclear export (Katagiri 2002). NGF treatment of PC12 cells, which are derived from the rat adrenal gland, induced nuclear export of Nur77-RXRa heterodimers. The phosphorylation interaction was found in the DNA-binding domain (DBD) of Nur77 at Ser105 (Katagiri 2000). This study was carried out in rat cells, so its physiological relevance to humans could be

questioned. However, the human equivalents are both expressed in regulatory T cells and rat and murine models are commonly used to understand the human immune system. Although this study focused more closely on how the heterodimerization and subsequent translocation of Nur77 and RXRa affected the roles that RXRa could play in cell metabolism, the fact that NGF mediates nuclear export suggests that it plays a part in Nur77 translocation to the mitochondria.
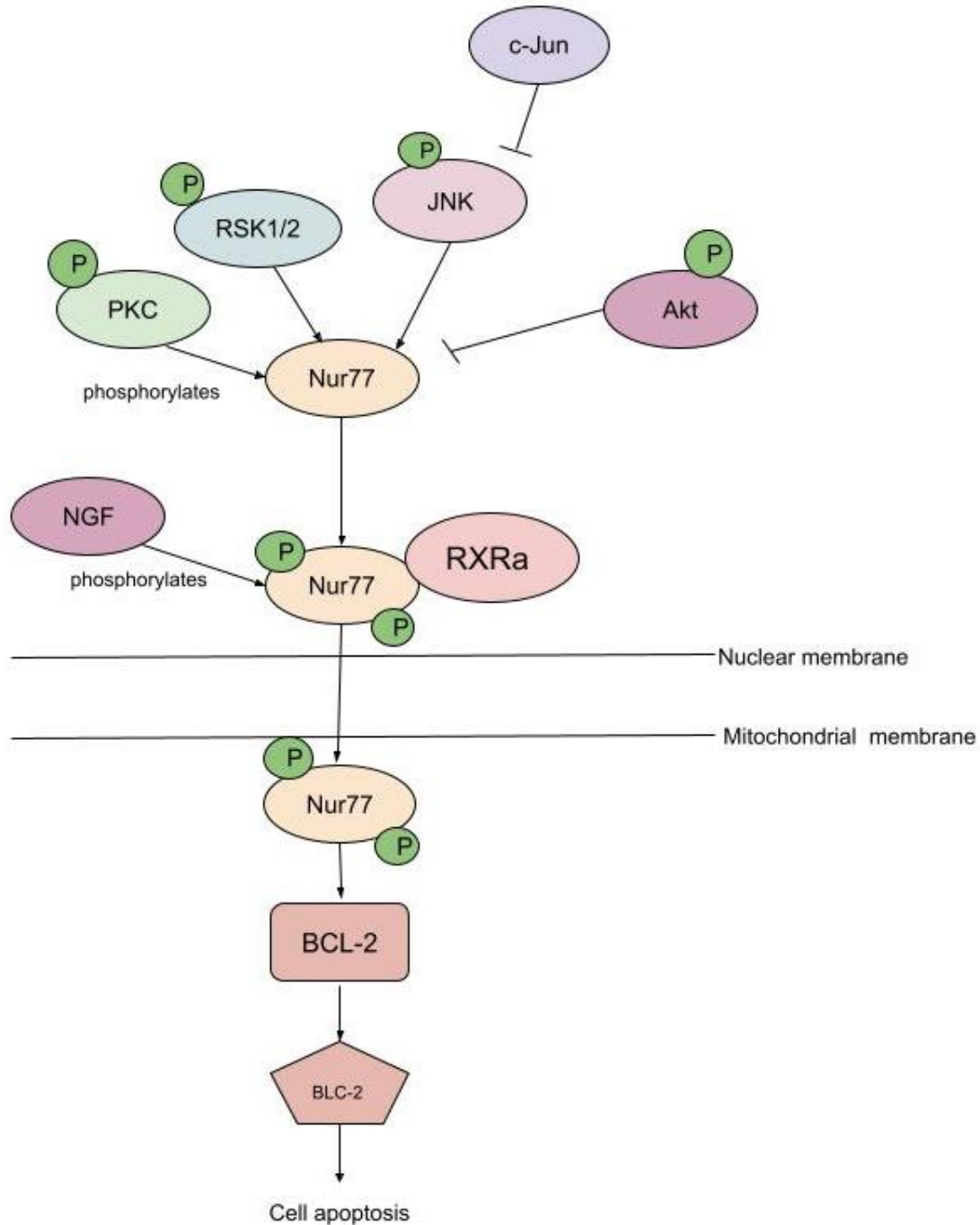
Figure 2: Proposed modes of action of Nur77 in the BCL-2 pro-apoptotic pathway. Protein kinases JNK, PKC, RSK1, and RSK2 have been shown to phosphorylate Nur77 prior to its nuclear export. Akt has been shown to inhibit the JNK pathway, and c-Jun can inhibit JNK phosphorylation of Nur77. Inhibitory interactions are denoted with a cross. Nur77-RXRa heterodimer is shown leaving the nuclear membrane with phosphorylation by NGF and once in the mitochondria, Nur77 interacts with BCL-2, eventually causing apoptosis.

Nur77 Regulates the HPA Axis

The hypothalamic-pituitary-adrenal (HPA axis) is a hormonal pathway that results in the release of glucocorticoid hormones. Glucocorticoid hormones regulate all physiological systems and are highly sensitive to environmental factors like psychological and physiological stress (Tsigos and Chrousos 1994; Spencer and Deak 2016). Under stress, the body releases corticotropin releasing factor (CRF) from part of the hypothalamus called the paraventricular nucleus (Murphy and Conneely 1997). CRF is transported to the anterior pituitary, which is a small gland in the head, where it induces the anterior pituitary to produce more pro-opiomelanicortin (POMC). POMC is a precursor to adrenocorticotropic hormone (ACTH), which regulates the synthesis of glucocorticoids (GCs) in the adrenal cortex (Murphy and Conneely 1997). Generally, more POMC leads to an increase in ACTH and an increase in glucocorticoids. Glucocorticoids inhibit the synthesis of CRF and of POMC in a negative feedback loop to maintain a homeostatic balance of cortisol and other glucocorticoids in the body (Murphy and Conneely 1997). The balance of glucocorticoids and cortisol in the body is important because stress hormones effect every other physiological system (De Kloet, et al. 1998; SDSmith and Vale 2006). Under stress, certain body functions including digestion and reproduction shut down to divert energy to the body parts responsible for responding to stress such as the brain, heart, and muscles (Smith and Vale 2006).

Glucocorticoids are a treatment option for inflammatory conditions because they reduce inflammation in autoimmune disease and allergic inflammation disorders (Kim et al. 2020). Recent research has shown that regulatory T cells play an essential role in glucocorticoid suppression of inflammation (Kim et al. 2020). When regulatory T cells are not present to an adequate degree in mice, the have an increased incidence of autoimmune disease (Adeegbe et al.

2009). Typically, autoimmune diseases and inflammation can be treated with glucocorticoids because glucocorticoids suppress inflammation (Barnes 1998). However, when mice that lacked regulatory T cells were treated with Dexamethasone (a glucocorticoid) for their multi-system inflammation, the Dexamethasone made no difference (Kim et al. 2020). To figure out why this happened, the research group deleted just the glucocorticoid receptor in regulatory T cells and found that losing this receptor completely prevented Dexamethasone from treating inflammation (Kim et al. 2020). These results suggest that the glucocorticoid receptor in regulatory T cells is essential for glucocorticoid-mediated anti-inflammatory effects. The glucocorticoids induce expression of a microRNA miR-349-3p in regulatory T cells, which may target other proteins involved in T cell metabolism (Kim et al. 2020). The same research group found that expression of miR-349-3p enhanced the anti-inflammatory effects of regulatory T cells in vivo (Kim et al. 2020). This suggests that glucocorticoids work therapeutically by binding to the glucocorticoid receptors on regulatory T cells and induce miR-349-3p expression, which enhances the suppressive effects that regulatory T cells have on inflammation in the body. This study suggests that regulatory T cells are essential to the mode of action of glucocorticoids.

Because regulatory T cells are such an important part of the anti-inflammatory response to glucocorticoids and also express high levels of Nur77, there is a real possibility that Nur77 plays some sort of role in this pathway. Although we do not yet understand exactly the mechanism by which regulatory T cells suppress the immune response, Nur77 may play a role. There is evidence that Nur77 interacts with the HPA axis and glucocorticoid receptor (Drouin et al. 1998). This evidence suggests that Nur77 influences the glucocorticoid-mediated anti-inflammatory process in regulatory T cells.

Nur77 is a transcriptional regulator in the HPA axis

Nur77 expression is induced in the PVN following stressful stimuli. Nur77 is also expressed in the anterior pituitary and its expression in the adrenal cortex is strongly induced by stress (Conneely 1996), suggesting that Nur77 plays a role in the stress response. Nur77 likely plays a role as a transcription factor in the regulation of POMC and CRF expression because there are binding sites for Nur77 and Nurr1 (another NR4A family member) in the promoter region of both these genes (Murphy et al. 1996). A later study confirmed this finding by demonstrating that Nur77 and Nurr1 enhance production of POMC through binding to the Nurr response element in the POMC gene promoter (Murphy and Conneely 1997). Nur77 enhances POMC protein production, which would result in more glucocorticoids and an anti-inflammatory effect. One complicating factor is that the Nurr response element overlaps with the response element for glucocorticoids in the POMC promoter (Murphy and Conneely 1997). The overlap between the response elements for the glucocorticoid receptor and Nur77 suggests that some sort of antagonistic relationship between the two exists (Murphy and Conneely 1997). The nature of this antagonistic relationship will be discussed in more detail later.

Nur77 associates with CREB and STAT1-3 at the POMC promoter

Nur77 acts as a transcription factor when it regulates POMC transcription (Phillips et al. 1997). Although the upstream signals that tell Nur77 whether or not to act as a transcription factor in the HPA axis have yet to be identified, the mode of action of Nur77 on the POMC promoter is partly elucidated (Mynard et al. 2004). Mynard et al. identified a composite binding site in the POMC promoter called NurRE, composed of two NBRE's (Mynard et al. 2004). NurRe overlaps with a binding site for signal transducer and activator of transcription 1-3

35

(STAT1-3), which Nur77 and STAT1-3 can bind to for an optimal synergistic effect on transcription (Mynard et al. 2004). Additionally the Nur77-STAT1-3 complex recruits cAMP response element-binding protein (CREB), which increases the transcription of POMC (Mynard et al. 2004). CREB, STAT1-3 and Nur77 can bind to the POMC promoter (Figure 3B), enhancing the production of more POMC protein, resulting in more glucocorticoids and less inflammation.

In summary, Nur77 exerts an enhancing transcriptional effect over POMC production in endocrine cell lines, which could be physiologically relevant in vivo. Although the relevance of this interaction has yet to be confirmed in animal models, the importance of regulatory T cells in mediating the anti-inflammatory effects of glucocorticoids and Nur77 transcriptional control of POMC production suggest that Nur77 is involved in the HPA axis. Figure 3A depicts a simplified version of the HPA axis and the negative feedback loop between glucocorticoids coming from the adrenal cortex to POMC and CRF production.

Antagonistic relationship between GR and Nur77

The balance of glucocorticoid receptors (GRs) and glucocorticoids is essential for regulation of the stress response. Physiological stress can decrease the effects of the immune system through glucocorticoids, and as mentioned above, the glucocorticoid receptors in regulatory T cells are an essential part of maintaining homeostatic balance of pro-inflammatory and anti-inflammatory signals as the body needs them. There is a negative feedback loop between glucocorticoids and glucocorticoid precursors, which makes sense because there has to be some sort of control that keeps glucocorticoids in check. Because Nur77 enhances

glucocorticoid production by increasing POMC transcription as described above, it is likely involved in the negative feedback loop that controls glucocorticoid transcription.

An antagonistic binding relationship between glucocorticoid receptor (GR) and Nur77 is one of the ways that Nur77 is involved in balancing pro-inflammatory and anti-inflammatory signals. A protein-protein titration demonstrated that GR progressively antagonizes Nur77 by direct binding (Philips et al. 1997; Drouin et al. 1998). Nur77 and GR antagonize each other via a direct protein-protein interaction in the nucleus (Drouin et al. 1998). GR represses Nur77-dependent transcription by direct binding, so that Nur77 cannot act as a transcription factor (Drouin et al. 1998). The antagonism between GR and Nur77 due to the direct protein-protein interaction could account for the glucocorticoid repression of POMC transcription in the HPA axis (Drouin et al. 1998). Given the fact that regulatory T cells express GR (Uhlén et al. 2015; Regulatory T Cell Data, Protein Atlas), it is possible that this GR interaction mediates the anti-inflammatory actions that glucocorticoid treatment stimulates in regulatory T cells.

More recent research suggests that Nur77-GR binding, which antagonizes further glucocorticoid production, occurs at the POMC promoter, perhaps physically blocking the NBRE enhancer region in the promoter (Bilodeau et al. 2006). An essential part of the Nur77-GR protein-protein interaction is a stabilizing factor called protein brahma homolog 1 (BRG1), which is constitutively active at the POMC promoter (Bilodeau et al. 2006). Coimmunoprecipitation assays show that BRG1 binds to Nur77 and to GR at the POMC promoter (Bilodeau et al. 2006) (Figure 3B). The interaction of Nur77, GR, and BRG1 at the POMC promoter provides additional evidence for the hypothesis that GR-Nur77 binding blocks Nur77's anti-inflammatory effect on systemic circulation as part of the negative feedback loop that keeps glucocorticoid production under homeostatic control.
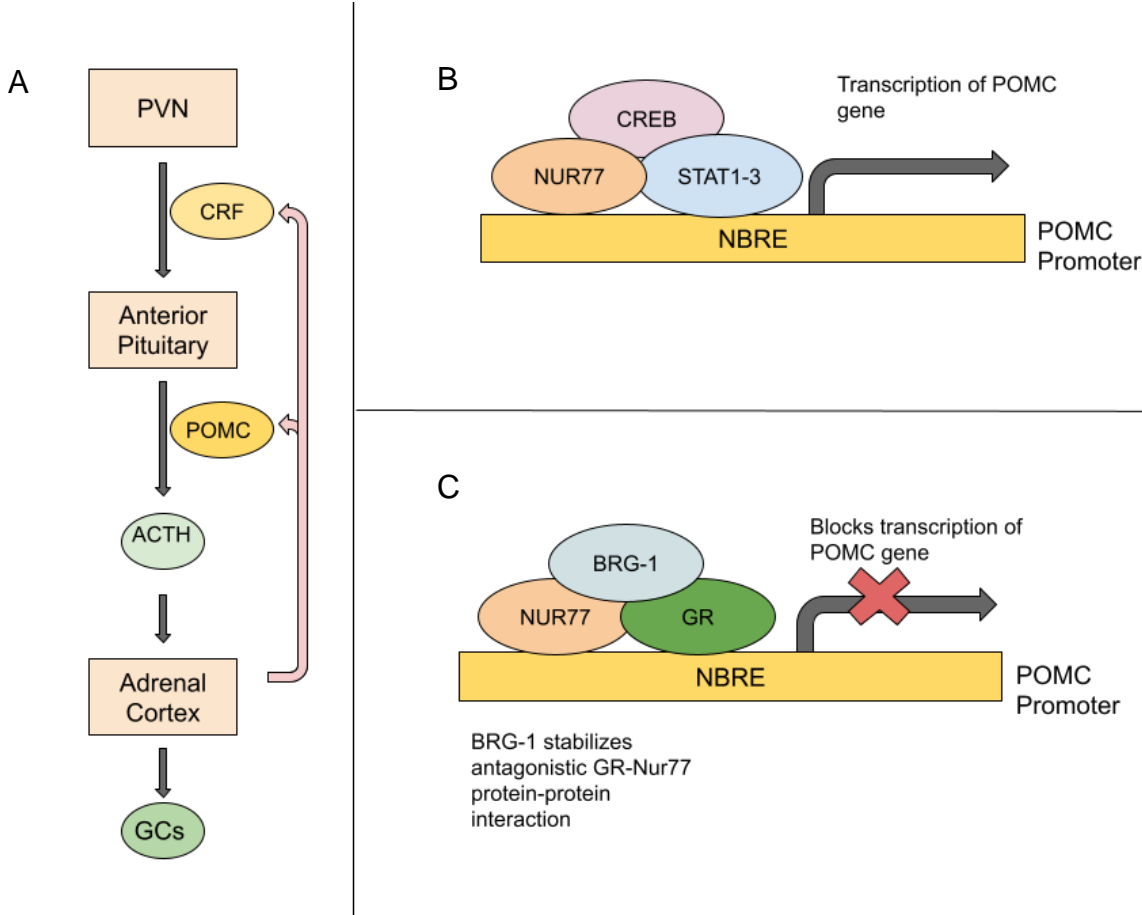
Figure 3: Possible modes of action for Nur77 involvement in HPA axis. Corticotropin releasing factor (CRF) is released from the hypothalamic paraventricular nucleus (PVN) (Figure 3A). CRF stimulates the anterior pituitary gland to release pro-opiomelanocortin (POMC), which travels to the adrenal cortex to produce ACTH and then glucocorticoids (GCs). Nur77 associates with STAT1-3 at the Nur77 binding response element (NBRE) of the POMC promoter and recruits CREB to promote transcription (Figure 3B). Nur77 engages in an antagonistic relationship with glucocorticoid receptor (GR) and is stabilized by BRG-1 in the negative feedback loop that controls glucocorticoid production (Figure 3C).

Chapter 4: Justification for Nur77 Research

Nur77 and its interactions are extremely complicated. This singular protein can interact with coactivators, coregulators, transcriptional coregulators, protein kinases, and more. Not only does Nur77 interact with a large variety of other biological molecules, but its role is highly context dependent. Nur77 is involved in autoimmunity, cancer, lung disease, chronic stress and addiction and more. Through this extensive literature review and hypothesis about the role of Nur77 in the context of regulatory T cells, it has become clear that although many different interactions between Nur77 and other proteins exist, there may only be a small number that are physiologically relevant in the context of the research questions discussed. It is impossible to address all the possibilities within the scope of an undergraduate thesis. Scientists may not elucidate the roles that Nur77 has in all its biological contexts for a long time. As a result, clinical applications of Nur77 are also probably a long way off in humans. Despite the amount of time that it may take to reach some sort of pharmaceutical application of this research, it is incredibly important to find out how Nur77 is involved in disease pathogenesis.

Role of Nur77 in preventing autoimmune disease

The focus of this paper has been on identifying the role that Nur77 plays in preventing autoimmunity. There is a large pool of evidence in animal models that demonstrates how essential Nur77 is to preventing widespread autoimmunity. Nur77's role in the prevention of autoimmune diseases has been shown through a variety of study designs.

Nur77 as a transcription factor controlling cell metabolism

Apart from apoptosis, one regulatory role that Nur77 may operate in is controlling gene transcription of metabolic pathways. Nur77 may control metabolic activity in T cells as another mode of preventing autoimmunity (Liebmann et al. 2018). In order to test this, transcriptional

networks were analyzed and it was found that that Nur77 helps to control gene expression of metabolic genes. These findings identify Nur77 as an important gene transcription factor of energy metabolism because it restricts mitochondrial function and glycolysis as well as switching between different energy pathways. This finding is significant because it demonstrates that Nur77 restricts T cell activation and proliferation through these metabolic pathways. Nur77's metabolic operation is another facet of how it prevents autoimmunity and inflammation. Potentially, these findings pinpoint Nur77 as a potential therapeutic approach. Liebmann et al.'s findings demonstrate the importance of looking at multiple facets of Nur77's operations in promoting self-tolerance. Nur77 restricts T cell activation in animal models of central nervous system autoimmunity, contact dermatitis, and arthritis.

The complexity of Nur77's interaction with Bcl-2

Although the interaction between Nur77 and Bcl-2 is the most well understood Nur77 interaction, there is likely additional complexity to the interaction that is still unknown. Nur77 regulates T cell development and differentiation aside from its known pro-apoptotic role. A research group examined the role of Nur77 in the presence and absence of Bcl-2 in the development of T cell development (Hu et al. 2017). Because Nur77 associates with Bcl-2 to expose its proapoptotic BH3 face, the research team could remove Bcl-2. In doing so, they could remove Nur77's ability to promote apoptosis and examine its role apart from that known role. Mice that were deficient in Nur77 and Bcl-2, mice deficient in just Bcl-2, mice deficient in just Nur77, and normal wild-type mice were all examined (Hu et al. 2017). Combined Bcl-2 and Nur77 deficiency altered self-tolerance through a method independent of the clonal deletion pathway that is typically associated with Nur77. Only double deficient mice developed diabetes,

which indicates that the Nur77 helps to regulate self-tolerance not just through clonal deletion, but through other possible mechanisms.

This study contributed to answering the question of whether there is functional redundancy between Bcl-2 and Nur77. The results suggested that Bcl-2 deficient mice had impaired negative selection systems, but Nur77 deficient mice did not have impaired thymocyte death, possibly due to functional redundancy in the NR4A family. This finding suggests that Nur77 may not always act in a pro-apoptotic manner. One possibility to explain this discrepancy is that NR4A proteins promote regulatory T cell development by inducing FOXP3 expression in thymocytes with stronger T cell receptor signals (Hu et al. 2017). The results of this study suggest that although the Nur77-Bcl-2 interaction is well studied, Nur77 likely has a role in T cell development outside of the pro-apoptotic signaling pathway.

The relevance of this study to the project is that it suggests there is still more to be discovered about Nur77 functions in T cell development and autoimmunity. It is important to note that Nur77 associating with Bcl-2 is not the only role that Nur77 plays in the immune system and that Nur77 is distinct from other NR4A family members. This is a key piece of evidence that suggests that further study and collecting all of the known Nur77 interactions would be valuable. If there are more pathways that Nur77 acts in during negative selection or in other parts of the T cell development process, it would be an important contribution to summarize what is currently known about Nur77 interacting partners.

Nur77 involvement in non-autoimmune disease

These key studies demonstrate the importance of Nur77 research to eventual clinical research and potential therapeutic targets in the realm of autoimmunity. However, autoimmunity

is just one of the types of disease in which Nur77 may play a role. Nur77 has been implicated in chronic inflammation, cancer, and addiction and chronic stress.

Nur77 in chronic inflammatory disease

One example of interest is in chronic inflammatory lung disease, where research has identified Nur77 as an attractive target for inflammatory disease therapies (Banno et al. 2018). Nur77 is expressed in the lungs and it has demonstrated a protective effect against inflammatory tissue damage in a rat model (Jiang et al. 2016). Statistical data has identified that Nur77 is associated with chronic obstructive pulmonary disease as well as other chronic lung inflammatory disorders (Hamers et al. 2016). Nur77 may be a potential therapeutic target in circumstances of lung inflammation (2018). In asthma, ozone exposure activated Nur77 mediated apoptosis and this led to tissue damage and lung injury (Murphy et al. 2014). However, contrasting research demonstrated that in a mouse model of allergic airway disease, Nur77 limited inflammatory responses (Kurakula et al. 2015). Likewise, in acute respiratory distress, Nur77 is known to decrease inflammatory responses to lung injury (Jiang et al. 2016). Other studies have shown that Nur77 may control wound healing in pulmonary fibrosis through a negative feedback loop and represent a therapeutic target because of its role (Palumbo-Zerr et al. 2015).

Nur77 in cancer

Nur77 has also been implicated in cancer and identified as a possible therapeutic target in the future. One study identified the role that Nur77 plays in liver cancer angiogenesis, which is the process by which tumors divert blood supply to pathogenic tissues (Zeng et al. 2017). This research team looked at the expression of Nur77 in human primary hepatic cancer specimens and

other liver disease specimens. Then, they did statistical analysis to figure out the relevance of the Nur77 expression data that they gathered. They found that Nur77 is highly expressed in multiple cancers, especially in hepatocellular carcinoma. These results suggest that Nur77 plays an important role in the progression of these liver cancers. Nur77 likely plays a role in angiogenesis in tumor growth because Nur77 is a transcription factor for several proteins that control pathologic angiogenesis (Qin et al. 2013). Nur77's involvement in the progression of tumors would be very important to uncover, especially in Nur77 could be a potential therapeutic target to slow tumor growth in cancer.

Nur77 in addiction and chronic stress

Nur77 and its other NR4A family members may play a role in chronic stress and patterns of addiction because of their role in the hypothalamic-pituitary axis (Conneely 1996). There is evidence that Nur transcription factors are associated with the chemical pathways that lead to dopamine transmission (Campos-Melo et al. 2013). Nur77 knockout mice reveal that Nur77 plays a role in adapting to changes in the dopamine neurotransmission pathways (Campos-Melo et al. 2013). There is also evidence that Nur transcription factors play a role in the stress response by regulating the expression of corticotropin releasing factor (CRF) and its precursor (POMC) in the brain (Murphy and Conneely, 1997). In this way, Nur77 could be involved in the biochemical balance in the brain.

There are several more examples of Nur77 being involved in disease pathogenesis, but these are a few key areas that might be important in the future as more is discovered about Nur77 and its role in inflammation.

Conclusion

This paper has explored the immune system broadly and delved deep into what we know about how Nur77 functions in the body. Although clinical applications are still a long way off, it is important to consider the value of basic research into Nur77. Medical treatments for autoimmune conditions exist already, but they do not address the root cause of autoimmunity. Autoimmunity, at its root, is a problem with the immune system. Therapeutic treatments can block inflammatory cytokines and reduce inflammatory signals to prevent damage to the body, but they do not address why the body sends inflammatory signals in the first place. The only way to begin to address the root cause of these inflammatory signals is basic research into the parts of the immune system. As I discovered during the process of sorting through the literature, many of the existing studies are very exploratory and do not have direct clinical applications yet. There is an immense amount of research left to go until scientists can begin to formulate ideas for therapies for autoimmune diseases. The purpose of this paper is to promote the importance of this basic research and highlight Nur77 as a key molecule for further study. Nur77 is involved in autoimmunity and inflammation, but it also plays a role in cancer, addiction, and more. Because the body expresses Nur77 in so many cellular contexts and it is involved in so many diseases, understanding Nur77's binding partners would be important research for researchers working on a myriad of diseases.

I hoped to begin the process of figuring out what Nur77 binds to. I planned to design an experiment that would look at which proteins Nur77 could bind directly to, and then investigate how they could be physiologically relevant. However, the pandemic made it incredibly difficult to begin a research project on a variety of levels. Not only were all non-essential labs closed, but it was difficult to get materials and communicate with other scientists. As a result, my thesis has

developed into a much deeper literature review than I intended. Even though this project ended up looking different that I had originally envisioned, I am so pleased with the product I have created. To my knowledge, no one has ever tried to develop a hypothesis for protein-protein interactions involving Nur77 in regulatory T cells before. This was my first experience developing a project of this scope that could have real implications for future research, and I have learned an incredible amount about immunology, but also about the process of researching and writing scientific literature. This learning will be invaluable as I move forward to graduate school.

My goal is that this literature review can provide a broader understanding of what Nur77 might bind to, and how different findings could be important to the broader context of autoimmune disease. As of this writing, I have been able to get into the research lab at Regis University to begin some preliminary research setup, in the hope that future students may be interested in taking on the lab research component of this project. So far, I have learned how to do cell culture work, which has been complicated, but incredibly fun. I have grown A549 cells, a lung cancer line, and Phoenix Eco cells, human embryonic kidney cells. Before I graduate, I am hoping to see if I can get them to express Nur77, paving the way for Dr. Spence's future students to investigate Nur77 in the lab. Even though the pandemic changed the scope of my project, I am endlessly grateful for everything I have learned in this process.

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