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Opportunities to Model Autoimmunity Using Human Induced Pluripotent Stem Cells

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DEDICATION

To

Mis padres, que llegaron sin nada y me lo dieron todo, su amor constante, aliento y sacrificios han sido la base de mi camino. Su fe en mí ha sido una fuente constante de fuerza, y no estaría aquí hoy sin su apoyo.

My sisters, my greatest cheerleaders, whose encouragement has been a constant source of strength.

Those who paved the way before me and to those who will follow.

This achievement is as much yours as it is mine.

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ABSTRACT OF THE THESIS

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Human induced pluripotent stem cells (iPSCs) have emerged as innovative tools and revolutionized the study of autoimmune disorders by providing a versatile platform for modeling the immune system. By deriving iPSCs directly from patients, researchers are able to replicate their distinct cellular environments to understand individual immune responses for personalized treatments. These models are instrumental in understanding disease mechanisms, identifying novel therapeutic targets, and developing personalized treatment strategies.

These findings underscore the potential of human iPSCs to bridge the gap between basic research and clinical applications, leading to improved patient outcomes. Future directions include improved reprogramming techniques, refining differentiation protocols, and integrating multi-omics approaches which enhance the fidelity and applicability of human iPSC-based models. This paper reviews the current efforts to utilize iPSCs to model autoimmune diseases, highlighting the impact of genetic, epigenetic, and hormonal variations which contributes to autoimmune pathophysiology. By reviewing relevant studies, this thesis discusses how sex chromosomes, hormonal influences, and immune cell dynamics contribute to disease susceptibility and severity, offering a promising avenue for developing patient-specific therapies for autoimmune disorders.

INTRODUCTION

Overview of Autoimmunity

There are over one hundred different autoimmune diseases that have been identified and affect over twenty-four million people in the United States alone. The immune system creates antibodies to protect from harmful viruses and toxins that can make people ill and can be activated when the body fails to recognize antigens as their own prompting an immune response [1]. The immune system consists of the innate and adaptive immune systems. The adaptive immune system is responsible for creating targeted responses against pathogens and can store information about the invader and how to fight it. Additionally, the adaptive immune system makes use of B-lymphocytes and T-lymphocytes to produce this highly specialized response [1] allowing it to attack faster when faced with a past antigen. In contrast the innate immune system lacks a memory and is non-specific to pathogens.

When the immune system is overactive, it can begin to attack and damage the tissues and organs. This can then lead to the development of autoimmune diseases because the body cannot differentiate between invader cells and healthy cells producing autoantibodies that produce an abnormal immune response. The exact mechanism by which this occurs is not widely understood, however not all autoimmune diseases are caused by the creation of autoantibodies [1]. Macrophages, innate immune cells distributed throughout the immune system, play a central role in the development of autoimmune diseases when dysfunction occurs. Macrophage dysfunction can lead to the secretion of pro-inflammatory cytokines and chemokines which are thought to underlie many diseases. The exact etiology of autoimmune diseases is not known but it is associated with gene expression, hormonal fluctuations, or environmental influences. Current beliefs are that environmental and genetic risk factors interact in a way that induces

disease evolution to immune activation to early clinical signs and symptoms and results in a phenotype fitting classification and diagnostic criteria [2]. Molecular mimicry may explain why the immune system attacks itself: a foreign antigen bears structural similarities to host cells, triggering self-destructive attacks that can range from minor to fatal [3]. However, there is insufficient research to support this hypothesis due to the complex nature of autoimmune disorders [4]. Therefore, no single theory can fully explain the onset and progression of these diseases[4]. Given the complexities and uncertainties surrounding the mechanisms of autoimmunity, treatment strategies must be multifaceted and tailored to the individual needs of patients.

Treatments of Autoimmune Disorders

Effective treatment of autoimmune disorders often involves a combination of immunosuppressive therapies and emerging biologic treatments aimed at modulating specific aspects of the immune response. However, they do not identify the mechanisms that are responsible for the initiation and progression of these diseases [5]. To properly isolate these diseases at the source, there must be a fundamental understanding of abnormal immune reactions: how they arise, sustain themselves, and the intrinsic mechanisms used to suppress immune responses in healthy populations [5]. Current therapies often emphasize immunosuppression [6], which can lead to increased morbidity and mortality among patients. Additionally, accurately addressing changes in the incidence and prevalence of autoimmunity is challenging due to the absence of universal standards for diagnosing these diseases. Since there are a multitude of autoimmune diseases, many are underdiagnosed due to being rare and having heterogeneous conditions [2]. Autoimmune disorders exhibit significant variation in

pathogenesis, ranging from organ-specific conditions, notably characterized by lesions confined to a single organ, to systemic, which involves pathological damage to multiple organs and tissues. A standardized national and international database does not exist and the little information that does exist is subject to referral bias [2]. Referral bias occurs when patients with severe symptoms are more likely to benefit from treatment in specialized clinics or included in studies compared to those with milder symptoms which can lead to skewed data and misleading conclusions about the prevalence and outcomes of autoimmune diseases. Addressing referral bias and establishing a database for autoimmunity patients that is representative of a larger population free of sex bias is crucial for ensuring that treatment plans and studies are valid and applicable to a wider audience. Autoimmune diseases affect patient livelihood due to physical limitations and disabilities and are costly since they are chronic in nature. Therefore, there is a need for more research into autoimmunity and the underlying mechanisms at play.

The Landscape of Autoimmunity

According to the National Institute of Health, autoimmune diseases affect roughly fifty million people in the US alone and are the third most prevalent disease after cancer and heart diseases [7]. Autoimmune disorders are challenging to treat and categorize due to the significant variation in their presentation and progression. This variability complicates the accurate reporting of incidence rates for rarer autoimmune diseases and hinders efforts to standardize treatments. For this reason, much of what is known about autoimmunity is heavily influenced by autoimmune disorders that are more prevalent and have a higher incidence such as those outlined below.

Type 1 diabetes destroys insulin-producing pancreatic β-cells which leads to insulin deficiency subjecting patients to lifelong exogenous insulin replacement and can develop at any age with the incidence around puberty [8]. Autoantibodies are not believed to be the cause of the disease but rather genetics influencing the susceptibility of type 1 diabetes. Autoantibodies can serve as markers for ongoing β-cell destruction although it is not particularly useful for diagnosing type 1 diabetes. Contrary to other autoimmune diseases, type 1 diabetes is more prevalent in males than females however, the opposite is true in populations that have a lower prevalence of the disease [9]. Researchers propose that the male prevalence observed is likely due to differences in susceptibility and response to infection, which are influenced by sex hormones.

This understanding of sex-based differences in immune response is particularly relevant when considering the impact of such factors in autoimmune conditions, such as multiple sclerosis, a chronic inflammatory and neurodegenerative disorder of the central nervous system affecting 2.8 million people worldwide [10]. Its prevalence is continuously increasing, particularly affecting older adults. It is also predominantly affecting women more frequently than men at a rate of 2:1 to 3:1 [10], [11] depending on geographic location. Researchers theorize that this female prevalence occurs due to the complex interplay between biology, genetics, and environment. While multiple sclerosis is primarily a neurodegenerative disease, it has autoimmune components as the adaptive immune system has been shown to drive focal inflammation and relapses contributing to episodes of neurological disability.

Rheumatoid arthritis, like most autoimmune diseases, causes the body's immune system to attack itself resulting in joint inflammation and pain for the sufferer. Depending on the severity, rheumatoid arthritis can damage the skin, eyes, lungs, heart, and blood vessels. Its cause

is unknown although there is a genetic component that influences its development. Roughly 1.3 million people suffer from rheumatoid arthritis in the U.S. alone and biological females are three times more likely to be affected [12]. Rheumatologists have reported that rheumatic diseases have a higher incidence in females than males as well as links between biological sex and the severity of autoimmune rheumatic diseases [13]. Menarche, pregnancy, and menopause, periods of hormonal shifts, all influence disease activity.

Systemic lupus erythematosus (SLE), the most common form of lupus, affects multiple tissues and organ systems because it is a chronic autoimmune inflammatory disease. Current etiology on SLE remains unclear but is thought to be influenced by environmental, genetic, epigenetic, and hormonal factors [14]. Similar to the diseases covered above, SLE affects more females than men (roughly at a rate of 8:1) [14] and its prevalence is seen in young females during peak reproductive age. Symptoms or flares in SLE have been observed to worsen during periods of hormonal shifts i.e. menopause, menstruation, etc. therefore considerable evidence supports that sex hormones as well as sex chromosome genes are major factors in the development of SLE. Significant hormonal fluctuations, particularly those experienced by females during their reproductive years, have been identified as factors influencing disease activity in SLE, RA, and MS, suggesting that female reproductive hormones play a role in the development of these disorders. In contrast, this may indicate that type 1 diabetes is less affected by sex hormones, such as estrogen, which are more prevalent in women.

There is a great need for improved avenues to study autoimmunity initiation, progression, and treatments as pathogenesis varies between disorders as described in this section. Our review shows that iPSC technology has been applied the most to RA and MS whereas there are fewer examples for lupus and T1D.

Methodological Approaches

Having explored the landscape of common autoimmune disorders and their multifaceted nature, there are several methodological approaches researchers can employ to model these complex conditions. Animal models such as mice have long been used to complement and enrich data from human studies [15]. Research on mice has demonstrated that females are more susceptible to autoimmune diseases consistent with human patterns. These models allow for genetic manipulation, enabling researchers to investigate disease mechanisms that would be challenging to study otherwise. Additionally, animal models facilitate the examination of interventions and their effects on the whole organism, including complex immune responses [15]. For this reason, much of what is known about autoimmunity has been obtained from animal models.

However, there are genomic and immune system differences between species, suggesting that animal data is not directly transferable to humans and therapeutic success in animals does not guarantee clinical efficacy in humans [16]. Animal models often face ethical scrutiny, highlighting the increasing need for alternative approaches [17]. However, their ability to offer valuable insights into disease mechanisms and treatment effects continues to make them a critical tool in autoimmune research [17]. While animal models have traditionally been used in the study of cell therapies, it is now essential to explore alternative methods.

Pluripotent stem cells are characterized by their ability to self-renew and differentiate into specialized cell types from one of the three primary germ layers: meso-, endo-, and ecto-derm [18]. Induced pluripotent stem cells (iPSC) and embryonic stem cells (ESC) are the two more common types of PSCs used in research. Human induced pluripotent stem cell technology offers a promising avenue for developing safe treatments and studying these diseases in vitro[17],

facilitating the exploration of new therapies compared to established cell lines that are typically derived from specific tissues. Established cell lines can provide a controlled environment and reduce variability which can be replicated due to the stable nature of cell lines and are suitable for large-scale screening and genetic studies. However, they lack the complexity of a whole organism and miss interactions between different cell types and tissues. Over time, cell lines can accumulate genetic mutations leading to altered behavior.

iPSCs can be derived from adult somatic tissues that are plentiful and have the potential to be reprogrammed to differentiate into the desired tissue-specific cells. Embryonic stem cells are isolated from developing embryos and are taken from the inner mass during early development. They were the first type of stem cell to be applied in research since it contains many potential therapeutic applications like gene therapy and drug screening. To the date, they are commonly used in clinical trials however, using hESCs remains controversial and is constantly under ethical scrutiny [18]. iPSCs generally have a unique expression signature however, with extended culture tends to resemble that of ESCs, so it is important to not subject iPSCs to prolonged culture. Whereas human induced pluripotent stem cells are advantageous because they retain the same genetic background as the patient, they were derived from allowing recurrent studies on genetic effects on cellular function, making iPSCs ideal for disease modeling and drug studies [18].

Purpose of the review

Although animal models have shown potential for curative treatments, translating these findings into clinical applications requires further research due to species differences in biological responses and a lack of understanding of which patient population might benefit from a given treatment with respect to the disease endotype. Human studies provide the most direct

information about human disease processes and capture genetic and environmental diversity seen in human populations. However, human studies are subjected to ethical considerations and regulatory approvals that can limit research scope. Therefore, more representative preclinical models are needed to properly assess human disease pathogenesis. Understanding why immune diseases develop, how they progress, and how to cure them is often hindered by the inability to apply relevant human tissues and isolate the onset of autoimmunity. As of now there are many limitations that prevent researchers from properly identifying the underlying mechanisms of autoimmunity. This can be attributed to the lack of knowledge available for most autoimmune disorders and limited treatment options. Current biological approaches for treating autoimmune diseases show limited efficacy and oftentimes lead to systemic side effects. iPSC technology offers a highly sage clinical treatment option for autoimmunity and serves as an effective platform for investigating and developing new therapies in the laboratory.

Traditional in vitro methods for modeling autoimmune diseases need to be improved upon; however, these methods must also address sex-based differences. A female in her childbearing years is more likely to suffer from autoimmunity yet, very little is known on how autoimmunity affects females. Research suggests that these sex differences arise from sex hormones, genetic disposition, and environmental influences [3]. Furthermore, responses to therapies and medication safety can vary by sex, yet pharmacogenomic factors are often inadequately considered in clinical trials [3]. iPSC technology is a promising avenue for developing safe treatments, studying autoimmunity, and establishing the need for sex-based research. This thesis aims to enhance the modeling of the immune system by refining existing techniques using iPSCs.

Chapter 1: Applications for iPSCs for Autoimmune Diseases

1.1 iPSCs in Autoimmune Disease Modeling

iPSCs are at the forefront of modeling and increasing understanding of many autoimmune disorders because they can be directly taken from a patient. Additionally, they can model the specific genetic and cellular characteristics of the human immune system that contribute to the specific autoimmune disease. Researchers can examine autoimmunity progression and highlight immune cell behavior and interaction in a controlled environment. Furthermore, iPSC technology can be used for drug studies and assess their efficacy and safety in a personalized manner.

In particular iPSCs can be employed for immune reconstitution, allowing for the replacement of cells lost to an autoimmune disease, through immune modulation of an autoimmune disease in vivo, and for increasing understanding of underlying mechanisms in vitro [16]. For example, iPSC technology is constantly evolving and recent efforts are actively being employed to differentiate into innate immune cells like macrophages since macrophages play a pivotal role in controlling host homeostasis [19]. Researchers believe that cells from autologous iPSCs will help to mitigate immune rejection and relieve diseases in combination with other technologies, i.e. gene-editing techniques, genome engineering, etc. without compromising patient safety.

Notable research includes investigations into multiple sclerosis and rheumatoid arthritis using iPSCs and iPSC-derived cells. These studies have provided valuable insights into the pathogenesis of autoimmune diseases and have paved the way for novel therapeutic strategies.

1.2 iPSC Models of Rheumatoid Arthritis

As mentioned in the previous chapter, rheumatoid arthritis (RA) is a chronic inflammatory condition that primarily affects the joints. Fibroblast-like synoviocytes are often used for iPSC reprogramming because they are specialized cells located in the joints that produce proinflammatory cytokines, like IL-6 and contribute to bone destruction. Healthy fibroblast-like synoviocytes can be implanted during total knee surgery to help maintain joint homeostasis [20]. In RA, fibroblast-like synoviocytes become hyperplastic and invasive, which contributes to joint inflammation, cartilage degradation, and bone erosion. The somatic cells of RA patients were isolated, transduced, and reprogrammed into iPSCs [17]. These iPSCs exhibited varying degrees of abnormal properties compared to healthy controls, including higher proliferation and energy consumption in the fibroblast-like synoviocytes [20]. These abnormal properties can be counteracted by inhibiting NMNAT3 using tannic acid [20] indicating that tannic acid may be a potential treatment for rheumatoid arthritis. Overall, iPSCs derived from RA fibroblast-like synviocytes can serve as a valuable tool for probing the pathogenic roles of synoviocytes.

1.3 iPSC Disease Models in Multiple Sclerosis

Somatic cells from patients with multiple sclerosis can be used to generate iPSCs through cellular reprogramming and differentiate into neural stem/progenitor cells, neurons, mature astrocytes, oligodendrocyte progenitor cells and oligodendrocytes [21]. One study showed that neural progenitor cells from multiple sclerosis cases have reduced ability to maintain their stem cell properties, displayed signs of aging, and prevented the maturation of oligodendrocyte

precursor cells into oligodendrocytes [19]. Thus, lowering the potential for repairing myelin which lead to more inflammation and nerve damage [22]. However, it remains unclear whether the myelination defects cause primary progressive multiple sclerosis progression or are a consequence of it.

Another study [20] demonstrated that oligodendrocyte progenitor cells and oligodendrocytes can be produced using iPSCs from multiple sclerosis patients. However, it is not well established that any differences exist between healthy controls and multiple sclerosis patients [23]. Starost et al. showed that the behavior of iPSC-derived oligodendrocytes from patients with relapsing-remitting multiple sclerosis was like controls in growth, migration, differentiation, and stress response [24]. Nonetheless, Lopez-Caraballo et al. showed that conditioned media from multiple sclerosis-iPSCs-derived oligodendrocyte precursor cells lacked certain proteins that reduced their migration capacity [23]. It is believed that adding activators like laminin and bFGF could help to improve the migration of these cells and suggests a potential treatment target [23]. Another study on primary progressive multiple sclerosis oligodendrocytes exhibited differences in genes related to cell adhesion, apoptosis, and inflammation and identified the inflammasome component, Nlrp2 as a biomarker for disease progression [25]. These findings highlight the potential of iPSC-derived oligodendrocytes in understanding and treating multiple sclerosis. Although further research is necessary to explore the difference between healthy and diseased cells, and the development of effective therapeutic strategies targeting these cells.

Ghirotto et al. found that multiple sclerosis-astrocytes impaired uptake and increased proton lead which is linked to disease pathology [26]. iPSCs-derived astrocytes can produce inflammatory cytokines and make them targets for CD8+ T cells and affect the oligodendrocyte

progenitor cells differentiation by down-regulating myelin genes [27]. These astrocytes both harm and help: they induce toxicity through increased NF-κB activation but can protect the central nervous system tissue by secreting growth factors like neurefulin-1β in inflammatory environments [28]. Overall, while iPSCs-derived astrocytes offer insights into their roles, their diversity limits their use as a therapeutic target. These diverse findings underscore the complexity of using iPSC-derived cells to model multiple sclerosis, with mixed results observed for astrocytes, where some studies reported no significant differences between those derived from MS patients and healthy controls.

1.4 Cell Therapy and Drug Discovery in Rheumatoid Arthritis

iPSC immunotherapy for rheumatoid arthritis involves regulatory T cells, Tregs, due to its ability to infiltrate into the joints and inhibit further arthritis development in animal models of RA [29]. Additionally, Tregs plays a crucial role in maintaining immune tolerance and preventing autoimmune responses. FoxP3, a key anti-apoptotic gene, was transfected in combination with Bcl-xL into iPSCs to promote the survival and optimize differentiation of Tregs when implanted in mice. The transduced Tregs resulted in a lower arthritis score than FoxP3 alone [29]. Another study found that the transfection of Ag-specific T cell (TCR) was not necessary to prevent inflammation in iPSCs-derived Tregs [30]. However, antigen-specific iPSCs-derived Tregs, a subset of T cells, were more effective in relieving arthritis in an antigen induced RA model [31]. By using iPSCs to generate Tregs, researchers can potentially develop personalized therapies that enhance the body's natural ability to control autoimmune responses, leading to more effective and targeted treatments for RA. It is noteworthy to further investigate

iPSCs-derived Tregs to treat rheumatoid arthritis in addition to other autoimmune diseases while addressing tumorigenicity risk, immune rejection, and large-scale production of Tregs.

Building on this approach, Klimark et al., constructed an iPSC-derived macrophage line that expressed soluble TNF receptor 1 through Ccl2, a crucial driver of recruitment and proliferation of immune cells, and demonstrated that sTNFR1 can suppress the inflammatory pathway [32]. Additionally, they found that the macrophages could inhibit the inflammatory pathway when stimulated by TNF-α, LPS, and IFN-γ anywhere from 24-72 hours [32]. Since macrophages actively participate in the inflammatory process, this approach requires further study to assess safety and efficacy. Together, these innovative iPSC-based approaches highlight the significant potential of personalized Treg therapies and iPSC-derived macrophage lines in advancing more effective and targeted treatments for rheumatoid arthritis.

1.5 iPSC Therapies in Multiple Sclerosis

Implantation of neural progenitor cells from iPSCs is believed to promote neural function recovery and attenuate disease pathology in animal models of multiple sclerosis. Moreover, leukemia inhibitory factor was observed to secrete from the mouse iPSCs-derived neural progenitor cells and resulted in oligodendrocyte progenitor cell and mature oligodendrocyte survival, differentiation, and remyelination however, not through cell replacement [33]. Xenotransplantation of human iPSCs-derived neural progenitor cells were rejected after intraspinal transplantation in a multiple sclerosis mouse model with no significant recovery observed yet histopathological analysis showed that the transplanted animals displayed reduced demyelination and focal remyelination [34]. While the transplantation of iPSCs was rejected in

the model, these studies demonstrated that oligodendrocyte neural progenitor cells and oligodendrocytes are involved in myelination and can serve as a treatment for multiple sclerosis in animal models in spite of donor health [35].

Wang et al. demonstrated that human iPSC-derived oligodendrocyte progenitor cells can proliferate, migrate, and differentiate into mature oligodendrocytes, as well as produce myelin in immunodeficient mice [31]. Additionally, they fare better in myelination than fetal tissue and can remyelinate denuded axons in progressive multiple sclerosis models. Finally, these cells can also produce compact myelin in shiverer mice which demonstrates potential for autologous cell therapies using iPSCs. However, there is still debate about whether transplanted cells will remain susceptible to triggering factors if defective mutations are not corrected.

To this date, the transplanted sites are limited to the cisterna magna [33], ventricle [36], and corpus callosum [35] which all require an invasive surgical transplantation not including intracranial injection in the thoracic T8-T9 [34]. Though, the intracranial injection is believed to have a worse safety and tolerability for patients. Furthermore, it may be nearly impossible to improve all clinical symptoms of multiple sclerosis by targeting a specific area for iPSC transplantation since lesion locations are diffusive. Further work is required to determine the appropriate selection of transplant sites and disability in addition to comparing the therapeutic effects among different transplant sites.

The effectiveness of marketed drugs to treat MS was validated using iPSCs-based disease models. Multiple sclerosis iPSC-derived neural progenitor cells secrete high-mobility group box-1 which inhibits oligodendrocyte progenitor cell differentiation and reduces their regenerative capacity [37]. The mTOR inhibitor, rapamycin, was found to be able to restore support for oligodendrocyte maturation [37]. Chronic exposure to IFN-γ inhibits iPSCs-derived

oligodendrocyte differentiation primarily in the early stages of reprogramming [38]. Activated PBMCs supernatants impair iPSCs-derived oligodendrocyte differentiation due to IFN-γ secretion [24]. The immunomodulatory drug teriflunomide can partially restore this by reducing immune cell proliferation, particularly in T cells [24]. These findings underscore the utility of iPSC-based models in validating the effectiveness of marketed drugs for treating MS and highlight potential therapeutic interventions by targeting specific pathways.

To summarize, iPSCs have emerged as a powerful tool for advancing our understanding of autoimmune diseases and providing an avenue for modeling immune cell dysfunction at a cellular level. Current autoimmune disease models like the ones described in this chapter have provided valuable insight into the molecular and cellular mechanisms underlying rheumatoid arthritis and multiple sclerosis. By differentiating iPSCs into immune cells, researchers can better understand the interactions between genetic predispositions, disease progression, and enable the development of targeted therapies. However, several challenges remain in the use of iPSCs for autoimmunity modeling such as variation in patient-derived iPSCs, which can result in inconsistent cellular characteristics and limited availability of diverse tissue types. Current protocols remain limited for differentiating into immune cell types restricting the scope of these models. Lastly, genetic modification made to create disease specific iPSCs can introduce unforeseen biases, affecting the reliability of findings. Despite these challenges, iPSC-based models continue to hold great promise for advancing personalized medicine for autoimmunity.

As we deepen our understanding of autoimmunity, it becomes clear that sex differences play a crucial role in disease susceptibility and progression. The next chapter will explore how iPSCs can be harnessed to study these sex-specific immune responses and offer a new perspective on sex-based variations in autoimmune disease mechanisms and treatment strategies.

Chapter 2: Sex-based Differences in Autoimmune Diseases

2.1 Epidemiology and Sex-Based Differences in Autoimmune Diseases

Sexual dimorphism, differences between males and females of the same species, is linked to sex chromosomes because of transcriptional products or from circulating hormones after the development of reproductive tissue. Studies on autoimmune disease all correlate with women being more susceptible to autoimmunity than men and indicate that biological females have more potent immune responses than males [39]. Autoimmune diseases affect women more than men for most pathologies at a rate of 2:1 and develop during extended periods of stress, pregnancy, and large hormonal changes [3]**.** Despite this, there is a lack of reporting differences between biological women and men in presentation, prognosis, and therapeutic treatment of these diseases [40]. Sex differences in autoimmune diseases, particularly in conditions like multiple sclerosis and rheumatoid arthritis, is well-documented, but the mechanisms behind this phenomenon and its potential evolutionary advantages remain unclear [41]. The impact of biological sex on the initiation, progression, and severity of diseases remains poorly understood, despite evidence that health outcomes for both men and women are influenced by sex [42].

It is believed that autoimmunity is linked to hormonal influences contributing to the higher prevalence of autoimmune diseases in females compared to males, with certain diseases like rheumatoid arthritis and multiple sclerosis being much more common in women. While both sexes produce hormones like estrogens, androgens, and progestins, their levels differ between males and females and play a crucial role in reproductive health. Imbalances in these hormones have been linked to inflammation, cell dysfunction, and tissue damage which all contribute to the development of autoimmune conditions. Furthermore, many researchers postulate that there are

ties with the X chromosome explaining why women are more likely to develop autoimmune diseases throughout their lives [39].

The sex chromosome complement is responsible for determining gonadal specification and regulating the concentrations of sex steroids. Past studies using castrated and ovariectomized rodents established that gonadal hormones are the major regulators of sex differences in immune responses [43]. Therefore, the effect of sex steroids on immune function is dependent on the concentration of the specific hormone and cellular expression of the cognate hormone receptor. Sex steroids bind to nuclear hormone receptors in the cytoplasm, then move into the nucleus to influence gene expression. The hormone-receptor complex attaches to DNA hormone response elements, recruiting proteins like activators or co-repressors [44]. These receptors can also inhibit other transcriptional factors like NFκB or AP1 [44], further affecting gene expression. In the absence of a hormone, the receptors can affect gene expression by forming complexes at gene promoter regions. Overall, hormone receptors help to regulate gene expression and cell functions in various ways.

Signaling in the receptors for estrogens, progesterone, and androgens expressed by the innate and adaptive immune cells is responsible for the development and function of immune cell populations. Estrogens are known to enhance and suppress immune responses depending on the concentration of the ligand, receptor, and cell type [45]. Progesterone is a steroid that circulates in higher concentrations in females acting as a ligand for the receptors nPRA and nPRB in addition to the membrane progesterone receptors which are found in immune cells [46]. Progesterone helps to create a tolerogenic environment during periods of infection [47]. In example, progesterone treatment in bone marrow-derived dendritic cells reduced the secretion of TNF and IL-1β and the expression of CD80, which has been shown to lower the ability to

activate T cells [48]. In murine splenic B cells, progesterone reduces mRNA expression of AID, an enzyme critical for antibody development [49]. Androgens which bind to androgen receptors can exert suppressive influence on active immune cell populations and effector immune responses [50]. Androgen receptors are expressed in a variety of immune cells like thymocytes, peripheral T cells, B cells, neutrophils, monocytes, macrophages, type 2 lymphoid cells, and stromal cells. Taken together, androgens, estrogens, and progesterone contribute to sex-specific differences in clinical disease states for autoimmunity.

Building on this understanding of hormonal influence, researchers also believe that the XX sex chromosome complement is strongly associated with susceptibility to autoimmunity, as expression levels of X-linked genes and autosomal genes differ between the sexes [51]. This variation is a significant biological factor that affects all physiological systems in the body including the immune system. Differences in immune cell function and responses to antigens based on sex impact the development and outcomes of various diseases and immune responses. The XX complement provides females with two copies of the X chromosomes, one coming from the male parent and the other from the female parent. One X chromosome is randomly activated in every cell to compensate for gene dosage effects [51]. This occurs through X-chromosome inactivation (XCI) which involves one of the two X chromosomes in female mammals to randomly become inactivated during early development and balancing the dosage of X-linked genes between males and females. Moreover, the X chromosome contains many immune-related genes such as toll-like receptor 7, IRAK1, FOXP3, and CD40L, microRNAs, and histone-modifying enzymes that regulate genes that are responsible for immune response [52]. This XX chromosome complement can lead to allelic diversity that induces immune challenges

[52]. Together, hormone receptors and the XX sex chromosome complement are crucial for understanding genetic sex determination and gene expression in females.

2.2 How Sex-Based Differences Impact Disease Pathogenesis

As described in the previous section, hormonal fluctuations can have significant effects on immune responses. Differences in biological sex refer to differences between males and females caused by the sex chromosome complement, reproductive tissues, and concentrations of sex steroids. Hormonal fluctuations play a pivotal role in regulating immune responses, particularly through the modulation of cytokine production and immune cell function. Estrogen tends to enhance inflammation and promote autoimmune conditions in females, while progesterone has an immunosuppressive effect, balancing immune responses during pregnancy. Testosterone, more dominant in males, tends to suppress inflammation and promotes the activity of regulatory T cells, which may protect against autoimmune diseases development.

Cytokines are signaling proteins that play a central role in immune responses by mediating communication between immune cells. The production and balance of pro-inflammatory and anti-inflammatory cytokines can be influenced by sex and hormonal fluctuations with females having a higher baseline of pro-inflammatory cytokines compared to males. Researchers theorize that this is in part due to estrogen's ability to enhance the activation of immune cells like B-cells and promote inflammation. In contrast, males tend to exhibit lower levels of pro-inflammatory cytokines and a more balanced immune response. The balance of hormones, combined with differences in cytokine production and immune cell responses, can explain gender disparities observed in autoimmune diseases, with women being more susceptible to autoimmune disorders than men.

CD4 and CD8 cells or helper T cells, are crucial components of the immune system and together they ensure a balanced and effective immune response. Sex-dependent differences have been observed in T cell frequencies, with females exhibiting a higher frequency of naïve T cells, CD4 T cells and elevated CD4:CD8 T cell ratios compared to males of the same age [53]. These differences indicate that female immune systems are more robust and likely influenced by sex hormones [53]. Moreover, these differences were replicated in a mouse study where castrated male mice showed thymic enlargement, increased output of naïve CD4 and CD8 T cells, and increased autoimmunity, like female mice. These results suggest that testosterone suppresses lymphopoiesis- the process in which lymphocytes are developed and produced-consistent with androgen effects on T cells during early development [54]. In contrast, estrogens continue to be expressed in later stages of development and their effect on T cells was found to be dose-dependent [55]. A reduction in CD4 and CD8 cells indicates that the body struggles to defend against infections and diseases and would be more susceptible to developing autoimmunity. These ratios illustrate why females are generally more susceptible to autoimmune conditions compared to males.

In females, B cell frequencies tend to be higher and display enhanced B cell survival, maturation, and class-switching [56]. Both androgens and estrogens can suppress B cell lymphopoiesis however, only testosterone reduces numbers of B cells. Estrogen interferes with negative selection and results in increased survival and expansion of high affinity autoreactive B cells [56]. Mice studies using ER-α and ER-β deficient mice demonstrated engagement of either estrogen receptor can alter B cell maturation. However, only ER-α can increase in autoreactive B cells [57]. Compared to T cells, B cells express more ER-β yet ER-α is more highly expressed in

B cells than T cells with higher expression in females than males [58]. This suggests another possibility why females are more prone to autoimmunity.

Testosterone reduces B lymphocyte proliferation and differentiation by lowering B-cell activating factor (BAFF) production and modulating apoptosis regulators like Bcl-2 and NF- κB [59]. In contrast, estrogen boosts B cell proliferation, suppresses apoptosis, and enhances the survival of autoreactive B cells by increasing the expression of BAFF, Bcl-2, CD22, and SHP-2 [56]. Moreover, estrogen can also raise CD80 expression which is crucial for B cell activation [60]. In a mouse study for arthritis [61], male mice exhibited higher frequencies of IL-10 producing regulatory B cells, which are known for immune suppression, in comparison to females. These sex differences in the regulatory B cells in humans needs further investigation.

Innovative models are being developed to better understand sex differences and the roles of the X chromosome in autosomal gene expression [39], [62]. Executing sex-inclusive research studies faces significant challenges, including limited access to female cells and tissues and insufficient resources to expand study scopes. These results indicate that differentiation into B and T cells would not only enhance the modeling of autoimmune disorders but also facilitate the integration of sex as a factor in developing and improving cell therapies given their influence by sex hormones. Overall, these sex-mediated differences result in females typically having a more vigorous immune response, which can be advantageous in fighting infections but also leads to a higher incidence of autoimmune diseases. Incorporating B and T cells into current models of autoimmune disorders, as mentioned in this section, can significantly improve the development of targeted therapies and personalized medicine approaches for immune-related conditions.

2.3 Modeling Sex-Based Differences in Autoimmunity Using iPSCs

Researchers have found that females tend to have stronger innate and adaptive immune responses than males, which is believed to be a survival advantage against infectious diseases, but on average are four times more likely to develop autoimmunity [63]. Despite this, sex differences in autoimmunity are still not widely investigated and often is not acknowledged in immunological research and treatment strategies. Initiatives are moving toward understanding sex-based differences in immune responses, the effect of hormones and genetic factors, as well as differences in the innate and adaptive immune system [63].

The Four Core Genotype Model is a genetic engineering technique that has been established in mice to study the effects of sex chromosomes and gonadal hormones. This model separates the sex chromosome from the gonadal sex, by removing the Sry gene from the Y chromosome and adding it to the chromosome 3, responsible for development to cell signaling and metabolism. This model creates four genotypes: XX females, XY females (without Sry), XY males, and XX males (without Sry) [64]. These studies showed that some X-linked genes were highly expressed in XY females rather than XX females due to lower gene methylation present in the XY female population since they only inherit one X chromosome. Overall, the sex chromosome complements and regulatory elements on sex chromosomes leads to differences in immune gene expression, impacting responses to infections and the development of autoimmune diseases. Moreover, iPSCs offer a unique opportunity to complement the Four Core Genotype Model to enhance the understanding of sex differences in autoimmunity and lead to more personalized and targeted treatments.

The most studied sex-specific aspect of iPSC phenotype is X chromosome inactivation (XCI) which is unique to female somatic cells for dosage compensation. Female iPSCs retain

inactive X chromosomes despite reprogramming [65]. However, reprogramming and long-term culture can induce XCI abnormalities [66]. Early passage iPSCs maintain XCI but higher passage cells tend to reactivate the X chromosome or lose XIST expression, leading to gene expression changes [67]. Male iPSCs can exhibit gene expression patterns like female iPSCs with deranged XCI which indicates broader epigenetic instability [68]. These findings underscore the complexities and epigenetic instability associated with the inactivation of the X chromosome in iPSCs and highlight the need for further research to fully understand and address these challenges in sex-specific cellular models.

2.4 Implications for Personalized Medicine and Gender-Specific Therapies

There is limited research on how these sex-specific differences in iPSC-derived cells impact disease treatments for autoimmunity. While there are little to no sex-specific models for autoimmunity, autoimmune disorders like RA and MS have shown distinct sex-based differences in incidence, severity, and response to treatment as previously reported. Therefore, targeting sex-specific immune mechanisms could lead to more effective and personalized treatment strategies. Models for neurodegenerative disorders such as those for Alzheimer's and Parkinson's have begun to investigate sex differences in cell therapies and drug discovery using iPSCs. One study found sex-specific differences in drug response in iPSC-derived cardiomyocytes [69]. Another discovered that over 12% of genes in iPSC-derived neurons showed sex-specific expression, indicating differences in underlying pathophysiology in mental disorders between the sexes [70]. Therefore, more research is needed to understand the impact of cell sex on iPSC-based treatments in autoimmunity.

Incorporating these sex-based variations into existing iPSC models can provide deeper insights into the pathophysiology of autoimmune diseases. By utilizing iPSCs derived from both

male and female patients, researchers can stimulate the unique cellular and molecular dynamics of autoimmunity as they pertain to each sex. This approach allows for the investigation of how sex hormones, genetic factors, and epigenetic modifications contribute to disease mechanisms and treatment responses. Ultimately, integrating sex bias into existing iPSC models will enhance their relevance and applicability, leading to the development of more personalized and effective therapies for autoimmune disorders.

Furthermore, sex-specific biomarkers can provide valuable insights into the ways in which sex influences health, disease susceptibility, and responses to treatment. By using iPSC models derived from both male and female cells, researchers can uncover important molecular differences between sexes, including gene expression, immune responses, and epigenetic modifications. This approach holds the potential for shedding insight on the underlying biology of sex differences in autoimmune disorders and lead to more accurate diagnostics and personalized therapeutic strategies that account for sex differences. Understanding and targeting these sex-based differences in immune function represents a critical step toward precise and individualized healthcare.

Chapter 3: Comprehensive Review of Literature

3.1 Emerging Patterns of PSC and Sex-Differences

Traditional models have struggled to fully capture the complexity of autoimmune diseases. Advancements in iPSC technology such as the ones covered in the previous chapters, have made it possible to recreate disease-specific phenotypes and investigate the underlying mechanisms of autoimmune diseases. This analysis aims to explore how iPSC technology can be utilized to investigate autoimmunity, integrating these elements to highlight the potential benefits and challenges of this approach.

To model the immune system and develop models of autoimmune disorders, researchers reprogram iPSCs into the cell type of interest by introducing a variety of growth factors during culture [71]. However, differences in reprogramming can arise due to several factors related to biological, genetic, and epigenetic variations. During the reprogramming process, hormones like estrogen and testosterone can influence gene expression and cellular behavior resulting in differences in how these cells are reprogrammed. Additionally, this can affect their subsequent differentiation and functionality in modeling autoimmune disorders.

As mentioned in Chapter 2, X-chromosome inactivation is typically used to equalize dosage compensation of the sex chromosome. It involves the transcriptional silencing of one of the X-chromosomes early in female embryonic development. Xist, a long non-coding RNA, spreads across the X chromosome to maintain the epigenetically silenced state. This silenced state is typically epigenetically inherited during cell division. Researchers theorize that proteins expressed by the inactivated X chromosome can serve as autoantigens if the immune cells have complete tolerance to them which is particularly significant in individuals with skewed XCI.

These factors contribute to the difficulty in developing a reliable test for female predisposition to autoimmunity and observing consistent patterns across all mammals. Additionally, XCI is not observed in all autoimmune disorders which suggests that this theory is not generally applicable. Finally, it is not clear whether the loss of mosaicism in XCI represents skewed initiation of XCI or is due to selective loss of cells carrying a harmful X-linked allele.

An alternative theory proposes that biallelic expression might counteract harmful X-linked mutation and would explain why some autoimmune disorders are more prevalent in males such as type 1 diabetes [72]. Additionally, certain X-linked immunodeficiencies also display similar patterns of incomplete and tissue-specific gene escape from XCI. The escape of specific genes might be influenced by closely linked genes requiring biallelic expression for proper function. New genetic tools are required to understand these gene-specific effects.

Sex differences exist in the epigenetic landscape such as female cells often require more profound DNA demethylation during reprogramming and can impact the differentiation potential of iPSCs. These epigenetic marks influence the efficiency and outcome of reprogramming and are crucial when studying autoimmune disorders. Furthermore, reprogramming somatic cells from patients with autoimmune conditions to iPSCs must account for the unique biological and immunological features of each sex. Finally, differences in cell signaling pathways and cellular dysfunction can influence the experimental conditions required for successful reprogramming and differentiation into iPSCs. Building on these insights, integrating iPSC technology with the study of sex differences presents a unique opportunity to unravel the complexities of autoimmune diseases and develop personalized treatment strategies.

3.2 Literature Gaps and Unexplored Areas

As previously mentioned, significant efforts are required to integrate iPSC technology for modeling and investigating autoimmunity. To properly understand and model sex differences in autoimmunity, researchers must focus on periods of hormonal transitions in females. Autoimmunity is more commonly seen in women predominantly before or during childbearing years. Therefore, it is crucial that future studies focus on these time periods and understand why hormonal fluctuations occur and the influence they have over immune responses. Additionally, careful consideration must be taken to distinguish the effects of chromosomes from hormonal influences. The role of psychosocial factors like stress and social support in influencing sex differences in autoimmune diseases require more attention. Understanding how these factors interact with biological mechanisms can provide new insights.

Longitudinal studies are required for long-term research that tracks the progression of autoimmune diseases in males and females over time. This would help to identify how sex-specific factors influence disease onset, progression, and response to treatment. These studies should include diverse populations with a broader range of demographics that can help researchers provide a more comprehensive understanding of sex differences in autoimmune diseases. Addressing these gaps will help improve understanding in autoimmune disorders and lead to more effective and personalized treatments.

3.3 Clinical Translation and Therapeutic Implications

Improvements in XCI, gene expression stability, and epigenetic stability in human iPSC models can significantly enhance their utility for studying autoimmune disorders and translating

findings into clinical applications [73]. Improvements in XCI can be accomplished through in vitro induction to induce XCI more effectively in iPSCs to help create accurate and stable models of diseases which is crucial for understanding how autoimmune disorders manifest. Furthermore, modifying the epigenetic landscape of iPSCs, such as altering histone modifications and DNA methylation patterns, can improve the efficiency and stability of XCI. Moreover, researchers should investigate the reactivation of the inactive X chromosome in iPSCs, which can provide insights into the mechanisms of XCI and its role in disease modeling. Factoring in XCI, gene expression, and epigenetic stability into existing autoimmune models can help researchers identify and understand the underlying mechanism that contributes to the higher prevalence of autoimmune diseases over others. These advancements can lead to more effective and personalized treatments for autoimmune diseases.

Furthermore, incorporating hormonal influences and genetic factors into clinical approaches for sex-specific models of autoimmune diseases can lead to more personalized and effective treatments. Clinicians can develop strategies to manage symptoms more effectively during periods of hormonal changes. Incorporating genetic information into the treatment plan ensures that patients are receiving the most appropriate and effective therapies corresponding to their genetic makeup. By integrating these factors into clinical practice, providers can offer more comprehensive and individualized care ultimately improving better health outcomes.

Sex-specific models of neurodegenerative diseases can provide valuable insights into autoimmune disorders due to the shared mechanisms of neuroinflammation and immune system involvement. Both neurodegenerative diseases like Alzheimer's and Parkinson’s and autoimmune disorders like multiple sclerosis and rheumatoid arthritis involve the dysregulation of the immune system. Additionally, studying sex-specific differences in already existing models of

neurodegenerative diseases, researchers can better understand how sex hormones and genetic factors influence immune responses.

Further exploration of molecular and epigenetic changes that occur within autoimmune diseases is needed to study gene expression, DNA methylation, and histone modifications. Epigenetic modification can significantly enhance the modeling and drug discovery for autoimmune disorders when applied in conjunction with iPSC technology. Altering DNA methylation patterns can help in reprogramming iPSCs to better mimic the epigenetic state of specific cell types involved in autoimmune diseases and can set the foundation for more accurate disease models in addition to potential drug targets. Techniques that alter chromatin structure can be employed to create more physiologically relevant iPSC derived cell models and improve the study of gene-environment interactions in autoimmune disorders and enhance the discovery of novel therapeutic approaches. By leveraging these epigenetic modifications, researchers can create more accurate and effective models of autoimmune diseases and overall lead to a better understanding and treatment. By combining these approaches, researchers can create more precise and effective models for studying autoimmune disorders and lead to better understanding and treatment options that are tailored to each sex.

Chapter 4: Conclusion

4.1 Summary of Key Findings

While the exact mechanisms underlying the development of autoimmunity remains unknown, researchers theorize that gene expression, hormonal fluctuations, and environmental influences play a vital role in modulating immune responses. The use of human iPSCs has been revolutionary in allowing researchers to create models of autoimmune disease that are more representative of human physiology. iPSC technology allows researchers to work directly with a patient's cells and holds the potential to develop personalized treatments that improve clinical outcomes. However, these models still fall short because they do not represent the entire immune system. It is difficult to properly reprogram and differentiate iPSCs into immune cell types with existing protocols. To reprogram somatic cells into iPSCs requires reactivation of pluripotency genes and resetting the epigenetic landscape and affecting overall efficiency. These differences can arise from biological, genetic, and epigenetic variations. Additionally, there is significant variation between autoimmune disorders that makes it difficult to establish a one-size-fits-all model that is representative of the presentation, initiation, and progression of these disorders.

There is substantial evidence indicating that autoimmunity affects more women than men, suggesting a sex bias in autoimmune diseases. This supports the theory that hormonal fluctuations play a significant role in the development and progression of autoimmune disorders. Yet, the impact of biological sex on the initiation, progression, and severity of these disorders remains poorly studied and understood. Furthermore, this sex disparity is believed to be linked to hormonal and genetic factors that are tied to the X chromosome. Despite this many models of autoimmune disorders do not report a focus on sex differences in their study design or simply exclude sex entirely. Sex differences also appear in the reprogramming process due to hormones

influencing gene expression and cellular behavior such as female cells requiring more profound DNA methylation during reprogramming. Additionally, there is limited report of how differences in sex in iPSC-derived cells affects cell therapies and drug delivery for autoimmune disorder treatments. Utilizing cells from both sexes will allow researchers to stimulate the unique cellular and molecular dynamics of autoimmunity. Incorporating sex-based variations into existing models of autoimmune disorders can provide a better understanding into the pathophysiology of autoimmune disorders.

4.2 Contributions to the Field

The contribution of iPSCs to the field of autoimmune disease research and cell therapies is profound because they offer the opportunity to study immune cell dysfunction in a patient-specific context, improve drug discovery, and contribute to the development of personalized therapies. By reprogramming patient-specific somatic cells into iPSCs, researchers can generate patient-derived models that reflect the hormonal and genetic factors that contribute to autoimmune disorders. Additionally, these models can be differentiated into a variety of immune cells such as T cells and B cells, enabling researchers to directly study immune cell dysfunction in the context of specific autoimmune disorders. Moreover, iPSCs can differentiate into human-specific immune cells and offer insights into immune tolerance breakdown and autoantibody production which are hallmarks of autoimmune disease. While challenges exist, ongoing advancements in iPSC technology and genetic manipulation hold the promise of revolutionizing the treatment of autoimmune disorders and offer a tailored solution to restore immune balance and improve patient outcomes.

By considering sex differences, researchers can develop more comprehensive models that factor sex-specific genetic, epigenetic, and hormonal influences and improve the general understanding of autoimmune diseases. Additionally, these models will be able to reflect the true nature of autoimmune diseases in both females and males, improving the applicability of findings. Recognition of sex differences in autoimmunity through PSC research will strengthen and improve personalized treatment strategies that are more effective for individual patients based on their sex. Moreover, studying sex differences will uncover novel therapeutic targets that are specific to each sex and potentially lead to the development of new drugs and treatments.

4.3 Final Thoughts

Continued PSC research will lead to the refinement and optimization of disease models, enhancing their utility and accuracy with time. Adopting a holistic approach that factors sex differences will drive the field forward and lead to overall comprehensive and effective solutions for autoimmune diseases. Advancements and innovations in technology such as next-generation sequencing can provide a new avenue to study sex differences more effectively. Gaps in current autoimmunity such as developing protocols to differentiate into all immune cell types, modeling a variety of autoimmune disorders, and sex bias require ongoing research. Furthermore, integrating diverse fields such as epigenetics, genetics, and immunology to address sex-based differences can be applied to various populations and healthcare systems globally. By incorporating these considerations, researchers can underscore the pivotal role that integrating pluripotent stem cell (PSC) research with the study of sex differences plays in advancing our overall understanding of autoimmune diseases and improving patient outcomes.

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