Characterization of Autoreactive CD4+ T cell Hybridomas

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by

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ABSTRACT

Autoimmune diseases are diverse disorders arising from abnormal cellular and humoral responses to self-antigens in the immune system. However, the underlying cause of autoimmunity remains unclear. In this study, ready-made autoreactive $CD4^+$ T cell hybridomas (containing autoreactive $CD4^+$ lymphocytes from scurfy TCR^{mini} mice with $BW\alpha^-\beta^-$ thymoma line) were cloned and their antigen receptors (TCRs) were characterized to decipher the specificity of autoreactive TCRs. This approach was used to understand the mechanisms of autoreactivity in the immune system and assist in redirecting abnormal $CD4^+$ T cells present in the peripheral repertoire of healthy individuals for therapeutic advantage. Some hybridoma clones expressed $CD4^+$ and $V\alpha2^+$ TCRs with unique amino acid sequences and they upregulated $Nur77^{GFP}$ expression upon autologous DCs stimulation and a stronger expression of $Nur77^{GFP}$ in the presence of aCD3 stimulation. Our data suggests that these activated hybridoma clones TCRs have an elevated binding affinity for self-peptides/MHCII complex and are autoreactive.

INDEX WORDS: autoreactive $CD4^+$ T cell hybridomas, $V\alpha2^+$ TCR, $Nur77^{GFP}$, autologous DCs, anti-CD3, scurfy TCR^{mini} mice, $BW\alpha^-\beta^-$ thymoma line.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

1.1 Autoimmune Diseases

Autoimmune diseases are diverse disorders arising from abnormal cellular and humoral responses to self-antigens in the immune system (Ermann & Fathman, 2001). The prevalence rate varies by ethnicity and geographical location (Roberts & Erdei, 2020), and it is most prevalent among women, although it affects people of all ages (Pisetsky, 2023). However, the underlying cause of autoimmunity remains unclear. Approximately 5-8% of the population in the United States is affected by autoimmune diseases, the third most common disease in the industrialized world. The disease can be specific to an organ or systemic depending on whether the autoimmune response targets a particular tissue or widespread antigens (Fairweather, 2007), and it is identified by the presence of self-reactive antibodies and inflammation, mononuclear phagocytes, autoreactive T lymphocytes, and plasma cells (autoantibody-producing B cells).

1.2 Disease Mechanism

Lymphopenia accompanies some cases of autoimmunity; lymphopenia is a condition associated with a decline in lymphocytes (Gleeson *et al*., 1996). However, during lymphopenia, homeostatic proliferation governs the body's expansion of T cells specific to an antigen (La Gruta *et al*., 2000). In this case, lymphoid space and costimulatory cytokine IL-21 promotes the expansion of antigen-specific T cells (King *et al*., 2004), and IL-21 is secreted by activated $CD4^+$ T cells, which contributes to stimulating T cell proliferation (Parrish-Novak *et al*., 2000). Given the fact that T cells dividing homeostatically can eventually acquire functional traits of pathogenic T cells after several steps of replication (Surh & Sprent, 2000), in turn, it favors initiation of autoimmunity as it comprises of self-antigen specific responses (King *et al*., 2004). Since not all autoimmune-prone patients are lymphopenic, other factors also contribute to autoimmunity. The main predispositions are genetic susceptibility to single gene/multiple gene mutations (Pisetsky, 2023), as well as HLA (Human Leukocytes Antigens) polymorphic genes (Muñiz-Castrillo *et al*., 2020), environmental influences such as smoking and pesticides also induce oxidative stress linked with autoimmunity (Khan & Wang, 2020), presence of bioagents like neoantigens, unexposed antigens, molecular mimicry, and cross-reactive antigens (Angum *et al*., 2020), and acute or chronic stress, for example, PTSD and traumatic stress (Black, 2003).

1.3 Central and Peripheral Tolerance Mechanisms of T cell

Central and peripheral tolerance are coping mechanisms of the immune system used to eliminate lymphocytes with receptors that attract self-antigens (Xing & Hogquist, 2012). A loss of self-tolerance or breakdown in self-tolerance leads to the generation of autoreactivity in the body (Pisetsky, 2023). Immune tolerance represents a state of immunological non-responsiveness arising from the tolerance mechanism of the central and peripheral systems involving B and T cells (Bluestone & Anderson, 2020).

Central tolerance is induced when T cells develop in the thymus and B cells in the bone marrow (Nemazee, 2017). Moreover, clonal deletion of self-destroying T cells is the primary mechanism of T cell tolerance in the thymus (Benoist & Mathis, 2012). During the T cell repertoire formation, the T cell receptor genes undergo rearrangement to give rise to highly diverse receptor molecules responsive to non-self-antigens but normally unresponsive to autoantigens. Premature T cells expressing moderate MHC molecule/self-peptide avidity undergo positive selection. In contrast, during negative selection when the T cell receptor (TCR) on the Thymocyte has a high affinity for self-MHC molecule/peptide complexes, it leads to apoptosis, which eliminates many pathogenic T cells and prevents their escape into the periphery (Pisetsky, 2023).

Peripheral tolerance, a crucial process in the periphery of lymphoid tissues (ElTanbouly & Noelle, 2021), is primarily regulated by Tregs, the absence of these cells from birth in mice and humans initiates autoreactive immune responses. These cells play a pivotal role in restraining autoreactivity through their suppressive mechanisms, which involve the production of immunosuppressive cytokines (IL-10, IL-35, and TGFβ), competition for growth factors, and cell-cell contact (Sojka *et al*., 2008). The unique characteristic of Tregs is the expression of FOXP3, a transcription factor that serves as a marker (Wing *et al*., 2019). Mutations in FOXP3 genes and the CTLA-4 receptors often lead to severe conditions (Dejaco *et al*., 2006), underscoring the paramount importance of Tregs in maintaining peripheral tolerance.

Tregs secrete IL-10 to hinder the production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α and proliferation of activated self-reactive CD4 T-cells (Moore *et al*., 2001), TGFβ to reduce proliferation of T -cell by stopping the production of IL-2 (Wan & Flavell, 2007), and IL-35 to arrest cellular proliferation in inflammatory environment (Collison *et al*., 2007). Moreover, Tregs compete for growth factors and ultimately deprive the target effector cells of IL-2, leading to death in the target effector cells (Pandiyan *et al*., 2007). So, when Tregs strongly express CD25, there is a reduction in effector T cell proliferation (Rudensky, 2011), eventually maintaining peripheral tolerance. IL-2 enables self-destroying T cells to expand in vitro coculture, while production of interleukin two by endogenous target T-cell remains suppressed (Sojka *et al*., 2005). Interleukin 2 also activates Tregs and enhances the production of IL-10 by Tregs (Barthlott *et al*., 2005).

In a cell-dependent manner, Tregs alter/terminate antigen-presenting cell (APC) function (Shevach, 2009) by downregulating/inhibiting the upregulation of two main costimulatory molecules on APC: CD80 and CD86 (Read *et al*., 2006), through binding of LAG-3 on Tregs to MHCII molecules expressed by immature DCs resulting to the generation of inhibitory signals to suppress DC immunostimulatory capacity of effector lymphocytes (Liang *et al*., 2008), and via the interaction of neuropilin-1 on Tregs with immature DCs to restrict the access of effector cells to the APC (Lourenço & La Cava, 2011).

1.4 Regulatory T cells

Regulatory T cells (Tregs) regulate immune response to pathogens and tolerance (Chen *et al.,* 2019). However, when Tregs are depleted, the self-destructive CD4 T cells react against body antigens (self-peptides) residing in the peripheral repertoire of healthy individuals, thereby precipitating autoimmunity (Cebula *et al.,* 2019). Two subsets of Tregs exist: thymic Tregs and peripherally induced Tregs (Schmitt & Williams, 2013). Thymic CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (tTregs) are generated in the thymus through high avidity MHCII/T cell receptor interactions (Larkin *et al*., 2008). The broad repertoire of tTregs enables the recognition of abundant self and non-self-antigens (Wing & Sakaguchi, 2010). tTregs (CD25^{high}) occupy up to 2% of total CD4$^+$ T cells in humans (Fontenot *et al*., 2003), while 5-10% of tTregs of the CD4$^+$ T cell peripheral subpopulation belong to mice and are characterized by the expression of CD25 (IL-2 receptor α-chain) and CD45RB^{low} (Bluestone & Abbas, 2003), whereas peripherally Induced CD25$^-$CD4$^+$/CD25$^+$CD4$^+$ Tregs (pTreg) arise from CD4$^+$ Foxp3$^−$ conventional T cells in the periphery that are induced to express Foxp3 *in vitro* via T cell activation when TGF-β1 and IL-2 are present (Davidson *et al*., 2007). In healthy mice and humans, the percentage of pTregs falls in the range of 2 to 30% of the total population of circulating Tregs (Thornton *et al*., 2010), and pTregs often recognize non-self-antigens (Hsieh *et al*., 2012).

1.4.1 CD4$^+$ T cell Characteristics

Naive CD4$^+$ T cells originate from hematopoietic-derived stem cells in the bone marrow, where these stem cells give rise to lymphoid or myeloid progenitors. The T cell precursors move to the thymus. Besides, the unique interplay between the Notch-1 signaling pathway and the GATA-3 transcription factor, amongst others, profoundly influences the transitioning of the lymphoid progenitor to a precursor cell (Dontje *et al*., 2006). CD4$^+$ T cells and specific immune cells express CD4 (a transmembrane glycoprotein belonging to the immunoglobulin superfamily) as a surface marker (Ashish *et al*., 2008). In mice and humans, the proportion of naive CD4 T cells for a given epitope generally ranges from 1 to 100 million cells. Still, it varies significantly per epitope and MHCII allele from 100 to 3000 cells per mouse (Geiger *et al*., 2009).

CD4$^+$ T cells use their T cell receptor to perform immune surveillance; the T cell receptor (TCR) interacts with class II major histocompatibility complex (MHC-II), known for presenting peptides from extracellular or phagosome sources. Antigen-presenting cells (APCs), such as dendritic cells, macrophages, and B-cells, express MHC-II. When naive CD4$^+$ T cells are fully activated through three activation signals from APCs (cognate antigen through the TCR, co-stimulation through co-stimulatory receptors, and inflammatory cytokines), they undergo proliferation and differentiate into specialized effector subsets that address different types of immune responses (Annunziato *et al*., 2015). Following the infection clearance, a fraction of the body's effector cells persist as long-lasting memory cells (Künzli & Masopust, 2023). Several CD4$^+$ T cell subsets: T helper 1, T helper 2, T helper 17, T helper 22, regulatory T cells, and T follicular helper cells have been identified and are well-characterized in humans over the last three decades, though they were initially characterized in mice (Zhu *et al*., 2010). The STAT4 molecule and T-bet mediate the Th1 immune response; Th1 produces IL-2, IFN- γ, IFN-α, IFN-β and express CXCR3 and CD161 (Kaiko *et al*., 2008), which allows them to participate in the development of autoimmune diseases and strengthen cell immunity against intracellular pathogens (Cano & Lopera, 2013). Th2 secretes 6 cytokines; IL-4, IL-5, IL-9, IL-13, IL-10, and IL-25, this cell expresses CCR4 and ICOS against extracellular pathogens and allergens (Paul & Zhu, 2010). Th17 is triggered in response to inflammatory diseases like bronchial asthma, rheumatoid arthritis, transplant rejection, and systemic lupus erythematosus (Hemdan *et al*., 2010); this specific cell generates IL-17A, IL-17F, and IL-22 (Kamali *et al*., 2019). Th22 plays a vital role in autoimmune disorders (Kamali *et al*., 2019), infectious diseases, and tumorigenesis (Pan *et al*., 2022); they produce IL-13, IL-22, and TNFα (Kamali *et al*., 2019). Tfh prompts B cells to differentiate into plasma cells and memory B cells and the production of Antibodies (Ab) via expression of CXCR5, BCL-6 transcription factor, IL-21, IL-4, and IL-10 (King, 2009). CD4$^+$ T cells enhance the development of functional, pathogen-specific memory cells by lowering TNF-related apoptosis-inducing ligand (TRAIL) expression on responsive CD8$^+$ T cells (Swain *et al*., 2012). Overall, CD4$^+$ T cells are popular for their pleiotropic functions, from activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as nonimmune cells, and in the suppression of immune reactions (Luckheeram *et al.,* 2012).

1.5 T cell Receptor

T cell receptors (TCRs) are a diverse set of specialized receptors expressed on the surface of T-cells. The adaptive immune system uses these receptors to confer protection against several pathogens, and a collection of TCRs is included in the repertoire (Dupic *et al*., 2019). Humans possess 10^{18} distinct TCR chains (Janeway, 2005), and mice account for 10^{15} (Davis & Bjorkman, 1988). The α chain and β chain account for over 90% of the T-cell receptors in humans (Woodsworth *et al.,* 2013). Besides, accumulating evidence shows that the TCR α-chain predicts the orientation of a receptor on a peptide-MHC complex while being in close contact with it (Stadinski *et al*., 2011); it also changes the mode of interaction of the TCR β-chain with pMHC and the TCR's overall specificity (Yokosuka *et al*., 2002). Amongst the three hypervariable complementary-determining regions (CDRs), CDR1 and CDR2 have the V gene and support the interaction and stabilization of the TCR-MHC complex (Rosati *et al*., 2017). The complementarity determined region 3 (CDR3) of the β chain represents the area where the antigen binds to the T-cell receptor. The CDR3 domain is approximately 45 nucleotides in length; it comprises the VJ (TCR-α) or VDJ (TCR-β) junction and represents a primary source of TCR variability and diversification among sequences (Woodsworth *et al.,* 2013). Most of the binding specificity of the TCRs to the antigen-MHC complex is also determined by the CDR3 region (Yang *et al*., 2018). Therefore, most recent studies focus on the region "CDR3" because of the high variability and short nucleotide sequence, making it easier to capture in a single sequence read (Weinstein *et al*., 2009). The term "T cell receptor diversity" is relevant in assessing the extent of immune convergence, that is, similarities of clonotypes (a population of T cells with an identical TCR) between people (Madi *et al*., 2014).

1.6 Scurfy Mouse Model

The focus and recognition of $CD4^+$ T cells in humans and mice are similar (Javier & Christopher, 2004). Many mouse models of autoimmunity modified by the transgenic expression of self-reactive T cell receptors, vaccination using self-antigens, or adoptive transfer of activated self-reactive T cell clones have established a clear role of CD4 T cells in disease pathogenesis (Totsuka *et al.,* 2007). The Scurfy mouse model: SfC57BL/6 (an autoimmune-prone mouse), which has been used extensively in our group's study on autoimmunity, basically features dysfunctional Tregs and develops lethal autoimmunity that stems from polyclonally activated CD4 cells (Ramsdell & Ziegler, 2014). Scurfy mice suffer from disease dependent on lymphoproliferation with multiorgan inflammation specific to the skin, lungs, liver, and reproductive organs, such as testicles and accessory reproductive structures (Sharma *et al*., 2009). Indeed, they die around 24-28 days old (Chen *et al*., 2005), but they can also have an extended lifespan of 8-20 weeks by introducing the $Fas^{lpr/lpr}$ gene (Zheng *et al*., 2007). The unrestrained activity of autoreactive CD4+ T cells (T_{eff}) is the central mediator of inflammation, infiltrating tissues and recruiting other inflammatory cells, eventually resulting in tissue damage (Suscovich *et al*., 2012). A similar feature of FoxP3 mutation in scurfy mice was identified in humans with immune dysregulation, Poly endocrinopathy, enteropathy, and X-linked syndrome, classified as IPEX syndrome (Wildin *et al*., 2001), with the presence of anti-nuclear antibodies (ANAs), anti-double-stranded DNA (anti-dsDNA), anti-histone, and anti-smith antibodies (Hadaschik *et al*., 2015). So, T cells isolated from this mouse were used to produce T cell hybridomas.

Based on existing knowledge, the scurfy mutation arises from a Foxp3 mutation in hematopoietic lineages. This mutation releases aberrant $CD4^+$ cells in the scurfy model, facilitated by thymic atrophy, and proinflammatory cytokines support the proliferation of autoreactive clones (Chang *et al*., 2005).

1.7 TCR Mini Mouse Model

TCR^{mini} mouse model is a transgenic mouse that carries transgenic T cells with the same TCRβ chain (Vβ14, Dβ2, Jβ2.6) and TCRα chain that restrains the recombination of the Vα2.9 segment to either Jα26 or Jα2 (**fig 1**). $CD4^+$ $CD8^-$ thymocytes in the transgenic model highly express Vα2 and Vβ14 and thymic selection shapes the T cell repertoire in TCR^{mini} mice such that the length of the CDR3α chains on the thymocytes becomes more constrained, demonstrating less diversity of TCRs or low frequency of heterogenous TCRs (Pacholczyk *et al.*, 2006). Thus, the importance of using the mice model with restricted TCR repertoire is to achieve T cell clones bearing identical TCRs, making it easier to comprehend its diversity. In the TCR^{mini} mice, there is an overlap between TCRs of immature or naive T cells and regulatory T cells. Although a significant portion (68%) of TCRs used by the naive T cells were detected on Tregs, a lower percentage (39%) of Tregs TCRs is shared with naive T cells (Pacholczyk *et al.*, 2007).

Additionally, this model shares essential phenotypic and functional characteristics with the wildtype since Tregs in their thymus and peripheral relatively express CD62L and CD103 in a very similar pattern with the wildtype. Transgenic mice comprise regulatory T cells with more diverse TCRs as opposed to naive T cells with less diversity of TCRs (Pacholczyk *et al.*, 2006). It was also observed that the CDR3α region of the immature T cell and regulatory T cell bears the same length in the thymus and peripheral of the constrained model, which is a unique eight amino acid long CDR3α region. Another thing is the presence of the ten most abundant CDR3 TCRα regions in both naive and regulatory T cells' thymic and peripheral subpopulations. Out of these 10, only two are popular to both T cells, which translates to the same TCRs capable of making selection of the immature T cells and regulatory T cells (Pacholczyk *et al.*, 2006).

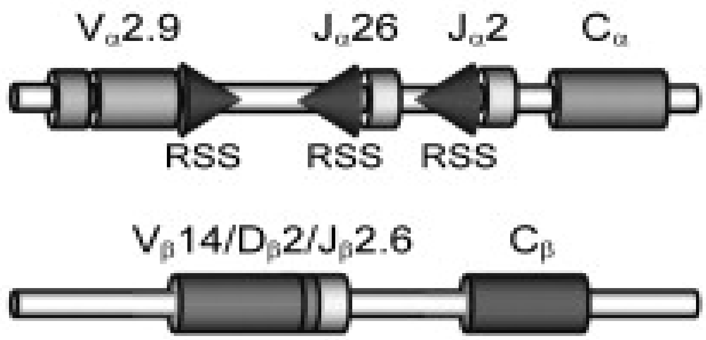

Figure 1: TCRα and TCRβ Constructs in TCR^{mini} Mice with the Vα2.9, Jα26, and Jα2 Segments in an Unrearranged Form (Pacholczyk *et al*., 2006).

1.8 Scurfy TCR Mini Mouse

The two models, the SfTCR^{mini} Foxp3^{GFP} model and the SfTCR^{mini} Nur77^{GFP} model are derivatives of the original SfC57BL/6 mouse; due to the GFP expression, they display TCR functional affinity for self-antigens (Kuczma *et al*., 2009). In the case of the SfTCR^{mini} Foxp3^{GFP} model, abnormal Tregs can be distinguished from readily activated CD4$^+$ Foxp3^{GFP-} cells, while the other reporter strain (SfTCR^{mini} Nur77^{GFP}) only classifies effector T cells according to their T-cell receptor's signaling strength (Cebula *et al*., 2019). SfTCR^{mini} mice suffer from a multi-organ autoimmune disease that resembles the scurfy mice. They display symptoms of numerous infiltrations of CD4$^+$ cells in the lungs, liver, and skin with signs of lymphadenopathy and splenomegaly at six weeks of age, and they eventually succumb to lethal autoimmunity from 7-10 weeks old (Cebula *et al*., 2019).

Cebula *et al*. (2019) discovered that 65-85% of CD4$^+$ Foxp3^{GFP-} T cells from SfTCR^{mini} mice display upregulated levels of CD71 (proliferation marker) and phenotypically express CD44$^+$ CD62L$^-$, hybridomas derived from these scurfy CD4$^+$ Foxp3^{GFP-} lymphocytes usually augment PD-1 expression upon stimulation by C57BL/6 DCs, and less than 1% of SfCD4$^+$ Foxp3^{GFP-} hybridomas are activated by C57BL/6 $H2M^-$ Li^- DCs which express majorly empty class II MHC molecules A^b at normal levels due to impaired antigen processing (Tourne *et al*., 1997), suggesting that A^b autoantigens trigger the activation of $SfCD4^+$ $Foxp3^{GFP-}$ hybridomas, and their TCR do not attract self-peptides presented ex-vivo by SfC57BL/6 or C57BL/6 DCs already loaded with organ-specific lysates (Cebula *et al*., 2019). In addition, the TCRα CDR3 regions examined in the activated $CD4^+$ clones retrieved from the scurfy mice significantly correspond with those present in the clones from the healthy mice with a value of 0.3 to 0.7 based on the mutual information index. Besides, 34% of the autoreactive TCRs of CDR3α regions sequenced from activated $CD4^+$ $Foxp3^-$ cells (in total, 49% of combined TCRs were sequenced from the scurfy and normal mice) implies that potentially pathogenic clones occupy a more significant segment of TCR repertoire in healthy mice. Unlike other TCRs from $CD4^+$ $Foxp3^{GFP-}$ cells in normal mice, the SfTCR uniquely responds ex vivo to C57BL/6 derived DCs, and its T cell repertoire is highly diverse (heterogenous), possibly due to a massive influx of low-abundance clones (Cebula *et al*., 2019).

1.9 Hybridoma Techniques

T-cell hybridomas have been valuable for monitoring antigen-specific cellular, molecular, and functional events of monoclonal cell populations (Kubota & Iwabuchi, 2014). Hybridoma cell lines maintain antigen specificity for an extended period due to their intrinsic feature, which is attributed to their tendency for continuous growth in cultures as opposed to the original T cell clones because they gradually lose their antigen specificity over time when kept unstimulated (Kruisbeek, 2001). In this context, readymade autoreactive polyclonal CD4 T cell hybridomas populations were employed. The T cell-derived hybridomas were formed through the fusion of an antigen-sensitized CD4^{+}Foxp3^{GFP-} cells (from scurfy mice) with BWα^{-}β^{-} thymoma line that has been stably transfected with Nur77^{GFP} reporter (BWNur77^{GFP}).

1.9.1 Nur77

Nuclear receptors (NRs) are structurally characterized into five regions (A or B, C, D, E, and F region) and three main domains: N-terminal domain, conserved DNA-binding domain, and C-terminal ligand-binding domain (Brelivet *et al*., 2012). Generally, the relevance of the NR4A sub-family of orphan NRs (NR4A1/Nur77, NR4A2/Nur1, NR4A3/Nor1) has been recognized as molecular switches in cell survival and inflammation (Mohan *et al*., 2012). Nur77 is localized in the nucleus of normal tissues due to the presence of nuclear localization sequence (NLS) in its protein structure (Wu & Chen, 2018). While the expression of Nur77 is denoted as a specific reporter of TCR signaling strength in murine reporter mice (Zikherman *et al*., 2012), as demonstrated in this study, its role as a modulator of autoreactive T cells during negative selection cannot be ignored (Ohashi, 2003).

1.9.2 BWα^{-}β^{-} cell line

BWα^{-}β^{-} was derived from the BW5147 line, previously extracted from a thymic lymphoma in an AKR/J mouse by Hyman and Stallings (1974). Since 1989, BWα^{-}β^{-} has been recognized as a T cell fusion partner of choice. This cell line and its parent, BW5147, has an inherent expression of CD4 except for CD8; hence, it imposes some constraints on T cell hybridization, resulting in impairment of the functionality of CD8 T cell as its only applicable to CD4^{+} MHCII and unconventional αβ T cells as well as a significant fraction of γδ T cells. Another limitation BW5147 has is the lack of CD3, making it irrelevant to investigations related to transfected or transduced TCR constructs but only practicable for T cell hybridomas and their TCR-dependent responses

(White *et al*., 2020); it was, however, resolved by introducing a multi-cistronic retroviral vector encoding all murine CD3 subunits with GFP into the BW$\alpha^-\beta^-$ cell line (Transduction), and a more stable version of the BW5147 cell line that functionally expresses extraneous TCRs was ultimately generated (Dunst *et al*., 2020).

1.10 Hypothesis and Rationale

Immunization of a suitable mouse model with autoantigens is hypothesized to yield functional autoreactive CD4$^+$ T cells, expressing specific T cell receptors (TCRs), providing a powerful platform to dissect autoimmune T cell responses. Undoubtedly, autoreactive CD4$^+$ T cells are key drivers of autoimmune disease pathogenesis. However, there is limited knowledge about the specific mechanisms that trigger these T cells. While our groups' recent studies identified the prevalence of potentially autoreactive CD4$^+$ T cells in the periphery repertoire of healthy mice with many self-reactive TCRs overlapping with those present in the scurfy mice, it lacked insight into the specificity of their T-cell receptors, limiting our understanding of what makes them potentially pathogenic.

Besides, studying these rare and elusive cells directly from patients presents a significant challenge. Hence, this study is focused on addressing these gaps by utilizing T-cell hybridoma technology, which offers a compelling solution. CD4$^+$Foxp3$^-$ T cells used for hybridomas production originated from sick autoimmune-prone mice, and subsequent fusion of these autoreactive T cells with immortal tumor cells generates hybridomas of autoreactive CD4$^+$ T-cell origin that naturally retain the antigen specificity of the main CD4$^+$ T cell while gaining the ability for continuous growth in culture. So, characterizing clones (monoclonal populations) of self-reactive CD4$^+$ T cell hybridomas could significantly facilitate the global identification and quantification of these cells to advance our understanding of autoimmunity, diagnosis, and inform therapeutic development for the disease.

1.10.1 Significance of Study

The cloning and characterization of autoreactive CD4$^+$ T cell-derived hybridomas and their antigen receptors hold significant implications for advancing our understanding and treatment of autoimmune diseases. This study gives insights into immune tolerance mechanisms and autoimmune pathology. This knowledge is crucial for developing targeted immunotherapies for restoring immune tolerance and assessing the response of these hybridomas to autoantigens to modulate or eliminate autoreactive T cells.

1.10.2 Aims and Objectives

I. To collect CD4$^+$ T cells hybridoma and generate single clones (from 6 cell lines) with a specific antigen receptor.

II. To assess clones that are positive for CD4$^+$ and TCR-Vα2 using a FACS machine (Cytoflex, Beckman).

III. To determine the response of each autoreactive CD4$^+$ hybridoma clone to self-peptides.

IV. To sequence their TCRα chain CDR3 region.

2.0 MATERIALS AND METHOD

2.1 Cell Culture

50x conc HAT was diluted with culture media to 1x working solution. Subsequently, cryopreserved hybridoma T cells were thawed at 37°C. The thawed cells were resuspended in 10 ml of the culture medium in a test tube (15 ml). The supernatant was carefully discarded after centrifugation (1500 rpm, 7 minutes). The pellet was resuspended in 10 ml of fresh medium and transferred to a culture flask. After microscopic observation, the cells were transferred to the incubator at 37°C to culture and were consistently passaged over the culture period.

2.2 Cell Freezing

The contents (10 ml) of culture bottles containing desirable hybridoma T cell clones were transferred to pre-labeled 15 ml test tubes. Cells were centrifuged at 1500 rpm for 7 minutes, and the supernatant was discarded from each test tube. Cell pellets were resuspended in 500 µl of fresh HAT medium and transferred to individual freezing tubes, to which 500 µl of freezing medium (containing DMSO) was added. The freezing tubes were stored in the -80°C freezer.

2.3 Hybridoma Cloning

Hybridoma T cells in the culture bottle were counted and diluted to obtain a cell density of 60 cells per plate. For this purpose, a serial dilution was performed (e.g., 300,000/ml cell concentration was diluted 100x followed by another 10x dilution). Subsequently, 100µl of the diluted cell suspension was distributed to each well of 96-well plates.

Plates were incubated at 37°C for approximately ten days to allow for clonal growth. Only plates with less than 30% growing clones were considered clonal. Single clones were transferred to

0.5ml of pre-warmed (37°C) HAT media in 24-well plates, and expression of CD4 and TCR Vα2 was evaluated by flow cytometry. $CD4^+$ TCR $V\alpha2^+$ hybridomas clones were diluted gradually with an additional 0.5ml of HAT media for cell survival and growth. Clones were further expanded in 3ml culture medium on 6-well plates or 10ml flask before cryopreservation at -80°C.

2.4 Cell Staining and Flow Cytometry

Approximately 200,000 hybridoma T cells were added to each well of a 96-well plate. The plate was centrifuged at 1500 rpm for 7 minutes to pellet the cells. The supernatant was carefully aspirated from the wells. 10 µl of HBSS buffer (containing conjugated anti-CD4-APC (1:1000) and anti-TCR-Vα2-PE (1:1000) antibodies) was added to each well, except for the control well, which received only HBSS buffer (unstained control), and the plate was placed in the refrigerator for 30 minutes. 200 µl of HBSS buffer was added to each well to wash away unbound antibodies. The plate was centrifuged at 1500 rpm for 7 minutes. Cell pellets were resuspended in 150 µl of fresh buffer, and samples were analyzed on a FACS machine (Cytoflex Beckman).

2.5 Hybridoma Stimulation Assay

Live clones expressing $CD4^+$ and TCR $V\alpha2^+$ were selected for the stimulation test. Cell suspension (200,000 cells per test) was transferred from the culture flask to 15 ml test tubes. The test tubes were centrifuged at 1500 rpm for 7 minutes. The supernatant was discarded without disrupting the cell pellets. Activation of T cell hybridomas was tested by incubating the cells with BMDCs (25000 per well) and a final volume of CTM 200 µl per well. The test was set for 3 conditions (hybridoma only, hybridoma DCs, and hybridoma DCs + aCD3) and each test was repeated three times for statistical purposes. The positive control aCD3 (1mg/ml) was diluted 2000x in DCs suspension mixed with hybridomas. The plate was incubated overnight at 37°C to stimulate cells.

After 16 hours, the plate was centrifuged (1500 rpm, 7 minutes), and the supernatant was discarded. Cells were stained with anti-CD4-APC and anti-TCR-Vα2-PE, and $Nur77^{GFP}$ upregulation was evaluated by flow cytometry.

2.6 RNA Extraction and Quantification

2 ml of cell suspension from each clone was transferred to a 12 x 75 mm tube, and the test tubes were centrifuged at 1500 rpm for 7 minutes. After the supernatant was discarded, 3 ml of PBS buffer was added to each test tube, followed by centrifugation at 1500 rpm for 7 minutes. 350 µl of Buffer RLT was added to resuspend the cell pellet in the tubes. The mixture was homogenized approximately five times using a BD insulin syringe, and 350 µl of 70% ethanol was added to the lysate and transferred to a RNeasy mini spin column to spin at 14,800 rpm for 1 minute. Once the supernatant was discarded, 700 µl of Buffer RW1 was added, followed by centrifugation (14,800 rpm, 1 minute) and supernatant removal. Following this, 500 µl of Buffer RPE was added and centrifuged at the same speed and time, the supernatant was discarded, and the step with Buffer RPE was repeated but centrifuged at 14,800 rpm for 2 minutes. RNeasy mini spin columns were transferred to a new 2 ml collection tube and centrifuged again (14,800 rpm, 1 minute) to eliminate residual ethanol. The mini spin columns were moved to the pre-labeled 1.5 ml collection tubes. 30 µl of RNase-free water was added directly to the spin column membrane. It was centrifuged (14,800 rpm, 1 minute) to elute the RNA. 1 µl of each RNA sample was used to measure the concentration using Nanodrop (the absorbance of an RNA sample is measured at 260 and 280 nm, with purity A260/A280 ratio of 1.8 – 2.0). Then, RNA was stored at -80°C.

2.7 First-Strand cDNA Synthesis and RT-PCR

For cDNA synthesis, up to 10 µg of total RNA from single clones of monoclonal populations was retrieved from the -80^{o}C freezer and combined with 7 µl RT Mix (RNase-free water, 50ng/ul of Random Hexamers, 10mM dNTPs), then incubated for 5 mins at 65^{o}C. Following this, 10 µl of RT Mix (10X RT buffer, 25mM $MgCl_2$, 0.1M DTT, 40U/µl RNase out, 200U/µl Superscript RT) was added to the denatured product in a sterile PCR plate and was briefly centrifuged at 1500 rpm. cDNA synthesis was performed in the PCR cycler for 10 minutes at 25^{0}C and 50 minutes at 50^{o}C. The cDNA synthesis reaction spun for a few minutes at 1500 rpm. In a new PCR plate, 2 µl of cDNA was added to 18 µl of PCR1 mix (RNase-free water, 10X buffer AP, 2µM β-actin sense primer: 5-GCTCG TCGTC GACAA CGGCT C-3, 2µM β-actin antisense primer: 5-CAAAC ATGAT CTGGG TCATC TTCTC-3, 5U/µl Accuprime polymerase) excluding the negative control (RT-mix only) and positive control (cDNA from hybridoma with already known TCR CDR3 sequence) and amplified; PCR1 (conditions: 94^{o}C for 2 mins; 32 cycles of 94^{o}C for 10 secs, 56^{o}C for 30 secs, 72^{o}C for 50 secs followed by 72^{o}C for 2mins, and 4^{o}C till finish) and spun at 1500 rpm once the product is ready. Then, 18 µl of PCR2 mix (RNase-free water, 10X Buffer AP, 10µM β-actin sense primer: 5-GCTCG TCGTC GACAA CGGCT C-3, 10µM β-actin antisense primer: 5-CAAAC ATGAT CTGGG TCATC TTCTC-3, 5U/µl Accuprime polymerase) was distributed to another PCR plate containing 2 µl of template (PCR1 product). The reaction was spun down and amplified; PCR2 (conditions: 94^{o}C for 2 mins; 32 cycles of 94^{o}C for 10 secs, 59^{o}C for 30 secs, 72^{o}C for 40 secs followed by 72^{o}C for 2mins, and 4^{o}C till finish) and 4 µl of PCR product was run on 1% agarose gel at 90 volts.

2.8 Agarose Gel Electrophoresis

1% Agarose gel containing 2g of Agarose powder in 200ml of TBE Buffer, and 10µl of Ethidium bromide was prepared in an Agarose tray with carrier and allowed to solidify for 1hr. Visible bands were visualized by BIORAD software.

2.9 PCR Product Purification

The volume of the PCR reaction mixture was adjusted to 50µl using milli-Q water, and samples were directly dispensed onto the membrane of the NucleoFast® 96 PCR plate. Subsequently, the PCR product was suctioned in a vacuum (adjusted to -0.4 to -0.6 bar) for 15 minutes. Then the membrane was washed with 100µl milli-Q water, and vacuum (adjusted to -0.4 to -0.6 bar) was applied again. 25µl of milli-Q water was added to the PCR membrane to elute the DNA. Then the purified DNA was vortexed for 5 minutes using a thermomixer, transferred into a new PCR plate, and the DNA was quantified by a nanodrop machine.

2.10 Sanger Sequencing

Purified PCR products were sent to Sanger sequencing (by Azenta) for further processing to determine the specific sequence and length of the CDR3α region located between the defined sequences of the TCRα V(J)segments.

Table 1: List of Reagents and Kit**.**

REAGENT	REF/ Clone	LOT NO	BASE	COMPOUND NAME	VOL.	CONC.
CTM	10-010-CV	15323003	Minimum Essential Medium (MEM)	CORNING	500 mL	1X
Fetal Bovine Serum (FBS)	N/A	AD18110280	N/A	HyClone™	500mL	N/A
L-Glutamine	25030-081	N/A	N/A	Gibco	200mM	100X
Tumor Coctile	N/A	N/A	N/A	N/A	N/A	N/A
Freezing Media	N/A	N/A	Medium + DMSO	N/A	N/A	N/A
Dimethyl Sulfoxide (DMSO)	25-950-CQC	04121005	N/A	CORNING	250mL	N/A
Hanks' Balanced Salt Solution (HBSS)	21-022-CV	28423003	N/A	CORNING	500mL	1X

Phosphate Buffered Saline (PBS)	21-040-CV	21422010	N/A	CORNING	500mL	1X
Unconjugated anti-CD3	145-2C11	B375765	N/A	BioLegend	1mg 360µL	2.81 mg/mL
APC anti-CD4	GK15	B356962	N/A	N/A	100µg 500µL	0.2 mg/mL
PE anti-TCR vα2	B20.1	B285405	N/A	N/A	100µg 500µL	0.2 mg/mL
Hypoxanthine-Aminopterin-Thymidine Supplement (HAT)	21060-017	N/A	N/A	Gibco	100mL	50X
RNeasy Mini Kit	74106	175026541	N/A	QIAGEN	N/A	N/A
Buffer^{AP}	55190	2408010	N/A	Invitrogen	1.23mL	10X
β-actin Sense Primer	N/A	N/A	N/A	N/A	Up to 10µM	N/A
β-actin Antisense Primer	N/A	N/A	N/A	N/A	Up to 10µM	N/A

Accuprime™ TaqHIFI	12346-094	2452712	N/A	Invitrogen	100rxn	N/A
RT Buffer	53032LT	2794103	N/A	Invitrogen	1mL	10X
Random Hexamers	51709	2383521	N/A	Invitrogen	250µL (50ng/µL)	N/A
10mM dNTP mix	Y02256	2443953	N/A	Invitrogen	250µL	N/A
0.1M DTT	Y00122	2449878	N/A	Invitrogen	250µL	N/A
25mM $MgCl_2$	Y02222	2829403	N/A	Invitrogen	500µL	N/A
RNase OUT	51535	2754145	N/A	Invitrogen	100µL (40U/µL)	N/A
Superscript® III Reverse Transcriptase	N/A	924900	N/A	Invitrogen	10,000U (200U/µL)	N/A
Agarose powder	BP160-500	222777	N/A	Fisher	500g	N/A
Ethidium Bromide	214-984-6	36H3687	N/A	SIGMA	25g	N/A
TBE Buffer	N/A	N/A	N/A	National diagnostics	N/A	10X

3.0 RESULT

3.1 Establishment of Single Clones from Autoreactive CD4+ T cell Hybridomas

A polyclonal population of autoreactive CD4+ T cell hybridomas were cloned to obtain a monoclonal population of CD4+ T cell hybridomas expressing TCRs with a unique specificity. A total of 6 different hybridoma cell lines were cloned simultaneously on 96-well plates after serial dilution. H1009, H1010, H1011, H1014, H1015, and H1063. H1014 line was highly proliferative compared to other cell lines and generated the highest number of single clones which was 95, H1009 (59), and H1063 (44), while H1010, H1011, and H1015 yielded 20, 10, and 22 respectively (**Figure 2 & Table 2**).

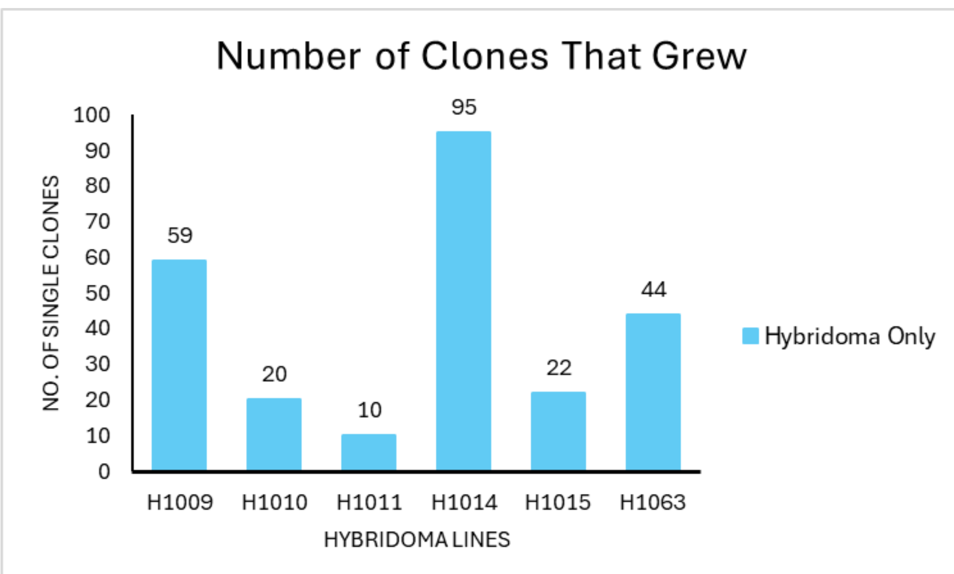

Figure 2: Number of Clones That Grew.

Table 2: Number of Clones That Grew.

HYBRIDOMA	CLONE NAMES	NUMBER OF CLONES OBTAINED
H1009	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59.	59
H1010	1, 2, 3, 4, 5, 6, 7 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.	20
H1011	1, 2, 3, 4, 5, 6, 7, 8, 9, 10.	10
H1014	1, 2, 3, 4, 5, 6, 7 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95.	95

H1015	2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24.	22
H1063	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26,27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44.	44

3.2 To Determine Clones Expressing $CD4^+$ and TCR $V\alpha2^+$.

Antibodies against $CD4^+$ and TCR Vα2 chain were used to screen the hybridomas. Because they originate from $CD4^+$ lymphocytes it was necessary to check if the live clones of the hybridoma cells express $CD4^+$ molecule and TCR. Individual clones were stained with conjugated anti-CD4-APC and anti-TCR Vα2-PE and checked on the FACS machine. (**Figure 3**) The $CD4^+$ $V\alpha2^+$ population was checked for each live clone of H1014 and 39% were $CD4^+$ $V\alpha2^+$. In contrast, many H1011 clones were $CD4^+$ $V\alpha2^+$ (80%). 68% were $CD4^+$ $V\alpha2^+$ for H1015, 54% for H1009 were also $CD4^+$ $V\alpha2^+$, and H1063 accounted for 52%, whereas for H1010 only 9% inherently expressed $CD4^+$ and TCR $V\alpha2^+$ and the proportion was significantly lower than other cell lines (**Figure 4 & Table 3**). Therefore, only hybridoma clones expressing both molecules were used for further testing.

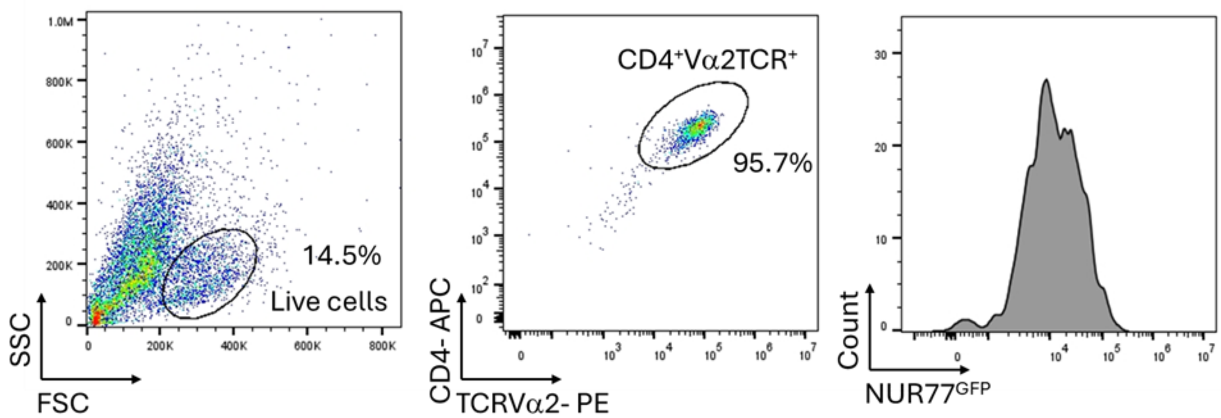

Figure 3: Gating Strategy for CD4⁺ TCR Vα2⁺ Staining.

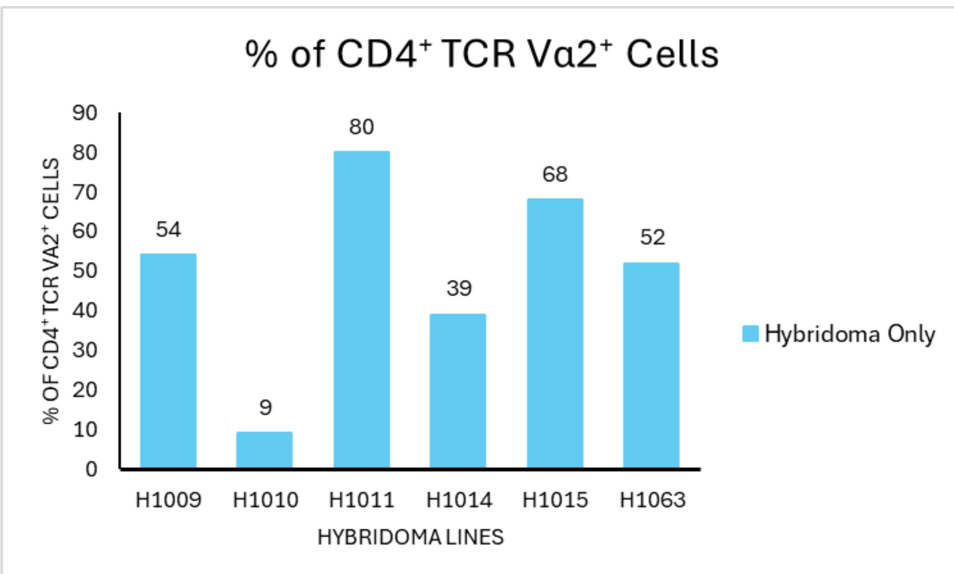

Figure 4: Percentage of Hybridomas Expressing CD4⁺, TCR Vα2⁺.

Table 3: Number of Hybridomas Expressing $CD4^+$, $V\alpha2^+$.

HYBRIDOMA	**CLONE NAMES ($CD4^+$ $V\alpha2^+$)**
H1009	1, 5, 6, 8, 9, 14, 16, 18, 19, 20, 22, 23, 25, 26, 27, 30, 31, 33, 34, 35, 36, 37, 39, 42, 46, 49, 53, 54, 55, 56, 57, 59.
H1010	1, 2, 3, 5, 7, 12, 18, 19, 20.
H1011	1, 4, 5, 6, 7, 8, 9, 10.
H1014	3, 4, 5, 10, 12, 13, 14, 15, 16, 19, 21, 25, 27, 31, 34, 45, 48, 49, 50, 51, 52, 54, 61, 65, 66, 68, 69, 71, 72, 76, 79, 85, 86, 88, 92, 94, 95.
H1015	2, 3, 5, 6, 9, 10, 11, 12, 13, 15, 16, 18, 19, 20, 24.
H1063	2, 3, 4, 5, 7, 8, 9, 10, 12, 13, 14, 15, 18, 20, 27, 28, 33, 34, 35, 36, 39, 40, 42.

3.3 To Check If $CD4^+$ TCR $V\alpha2^+$ Hybridoma Clones Are Stimulated by DCs Presenting Self-Peptides.

The stimulation assay was repeated 3 times for each line for statistical data using autologous DCs and the $CD4^+$ $V\alpha2^+$ hybridoma clones. The stimulation assay was run to find out if the hybridoma clones were responding to self-peptides. The $CD4^+$ $V\alpha2^+$ hybridoma clones were stimulated by autologous APCs (bone-marrow-derived DCs of the A^bWT C57BL6 mouse) to reproduce the specificity of autoreactive $CD4^+$ T cells for self-peptides in vitro culture. Autologous APCs can easily mimic this condition as presented in vivo. Hybridoma only was unstimulated control, whereas anti-CD3 stimulation which caused the strong hybridoma activation served as a positive control.

$Nur77^{GFP}$ upregulation was checked, and an increase in $Nur77^{GFP}$ shows that the hybridoma clones were stimulated by DCs and express TCRs that are autoreactive (**Figure 3 (histogram)**).

59.2% of $CD4^+$ $V\alpha2^+$ clones from H1009 responded to DCs stimulation, and to aCD3 stimulation. 10% of $CD4^+$ $V\alpha2^+$ clones from H1011 responded to DCs stimulation, and to aCD3 stimulation. 88.9% of $CD4^+$ $V\alpha2^+$ clones from H1010 responded to DCs stimulation, and to aCD3 stimulation. 36.5% of $CD4^+$ $V\alpha2^+$ clones from H1063 responded to DCs stimulation and to aCD3 stimulation. 51.3% of $CD4^+$ $V\alpha2^+$ clones from H1014 responded to DCs stimulation and to aCD3 stimulation. 13.2% of $CD4^+$ $V\alpha2^+$ clones from H1015 responded to DCs stimulation and to aCD3 stimulation (**Figure 5**). For each line, there were identical responders for DCs stimulation and aCD3 stimulation because the autoreactive $CD4^+$ T cell hybridoma lines were carefully selected and tested before cloning and they responded to autologous DCs stimulation and aCD3 stimulation.

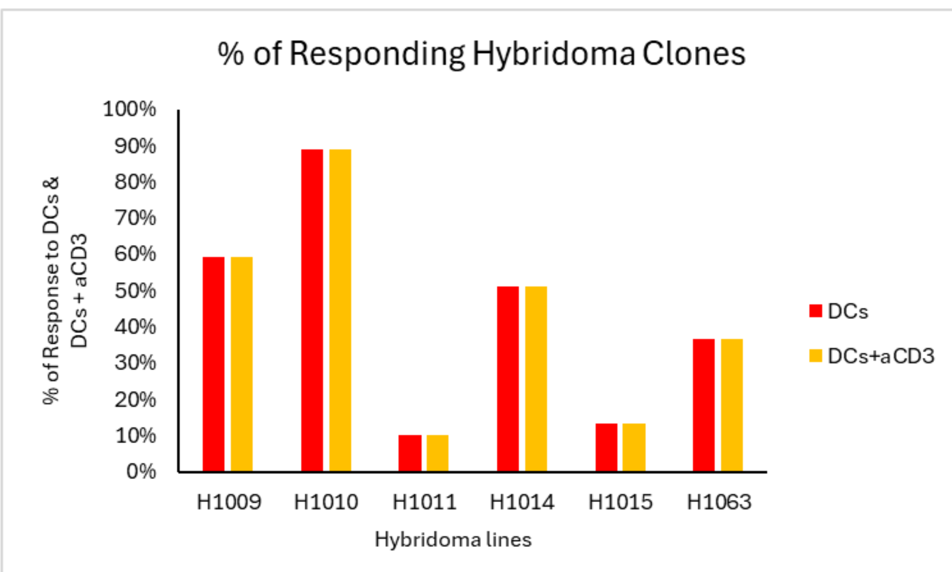

Figure 5: Percentage of Hybridoma Clones Responding to DCs Stimulation and aCD3 Stimulation.

A typical example of CD4^{+} Vα2^{+} hybridoma clones that responded to autologous DCs and aCD3 is displayed in **Figures 6, 7, and 8**. H1010.19 upregulated Nur77^{GFP} with an 8-fold increase over the base level (hybridoma only) after overnight stimulation with A^{b}WT DCs. Nur77^{GFP} increased with a stronger intensity of 166-fold change over the base level ($3.0 \times 10^3 \pm 8.4 \times 10^1$) when we cocultured hybridoma + A^{b}WT DCs with aCD3. In addition, the average Nur77^{GFP} fold change over the background for A^{b}WT DCs and A^{b}WT DCs+aCD3 was greater than that of H1011.9. We calculated the T-tests of Nur77^{GFP} mean fluorescence (average intensity of Nur77^{GFP} expression) between hybridoma only and A^{b}WT DCs, hybridoma only and A^{b}WT DCs+aCD3, A^{b}WT DCs and A^{b}WT DCs+aCD3. The result showed that the difference in Nur77^{GFP} mean expression was statistically significant (p-value ≤ 0.05) between all the 3 comparisons (**Figure 6)**.

Upon stimulation with A^{b}WT DCs, H1011.9 elevated Nur77^{GFP} expression by a 2-fold increase over the background (hybridoma only). When aCD3 was added to hybridoma + A^{b}WT DCs, the expression was stronger with a 37-fold change over the background ($8.2 \times 10^3 \pm 2.1 \times 10^3$). However, the average Nur77^{GFP} fold change increase for A^{b}WT DCs and aCD3 was lower than the other 2 hybridoma clones- H1015.5 and H1010.19. We analyzed the T-tests of the Nur77^{GFP} mean fluorescence between hybridoma only and A^{b}WT DCs, hybridoma only and A^{b}WT DCs+aCD3, A^{b}WT DCs and A^{b}WT DCs+aCD3. We realized that the difference in Nur77^{GFP} mean expression for the 3rd comparison (A^{b}WT DCs and A^{b}WT DCs+aCD3) and the 2nd comparison (hybridoma only and A^{b}WT DCs+aCD3) was highly significant ($p \leq 0.001$), while the difference in Nur77^{GFP} mean expression for the 1st comparison (hybridoma only and A^{b}WT DCs) was statistically significant ($p \leq 0.05$) (**Figure 7**).

We stimulated H1015.5 with A^bWT DCs and it augmented $Nur77^{GFP}$ expression with a 185-fold increase over the background (hybridoma only). Later, we added aCD3 to hybridoma + A^bWT DCs which caused a more intense expression of $Nur77^{GFP}$ with a 652-fold change over the background ($4.5 \times 10^2 \pm 2.9 \times 10^1$). Besides, the average $Nur77^{GFP}$ fold change for A^bWT DCs and aCD3 was more than the average $Nur77^{GFP}$ fold change of H1010.19 and H1011.9. We ran T-tests for the $Nur77^{GFP}$ mean fluorescence between hybridoma only and A^bWT DCs, hybridoma only and A^bWT DCs+aCD3, A^bWT DCs and A^bWT DCs+aCD3. The result displayed a very significant difference ($p \leq 0.01$) in the $Nur77^{GFP}$ mean fluorescence for the 1st comparison (hybridoma only and A^bWT DCs) and the 2nd comparison (hybridoma only and A^bWT DCs+aCD3), whereas there was a significant difference ($p \leq 0.05$) in the $Nur77^{GFP}$ mean fluorescence for the 3rd comparison (A^bWT DCs and A^bWT DCs+aCD3) (**Figure 8**). Overall, all 3 hybridoma clones were stimulated by autologous DCs (A^bWT DCs) and aCD3, and they responded to the self-peptide/MHCll complex presented by the autologous DCs.

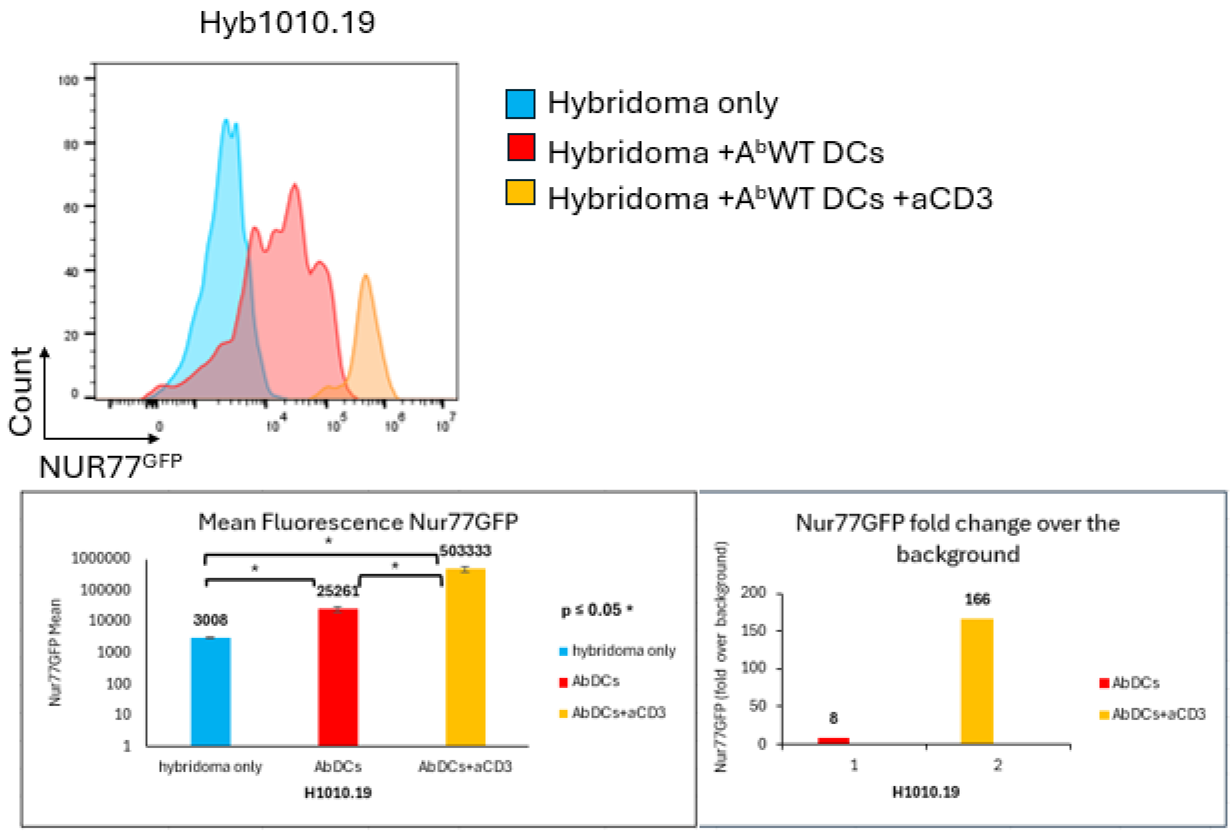

Figure 6: Examples of Responders to Autologous DCs and aCD3 (H1010.19)

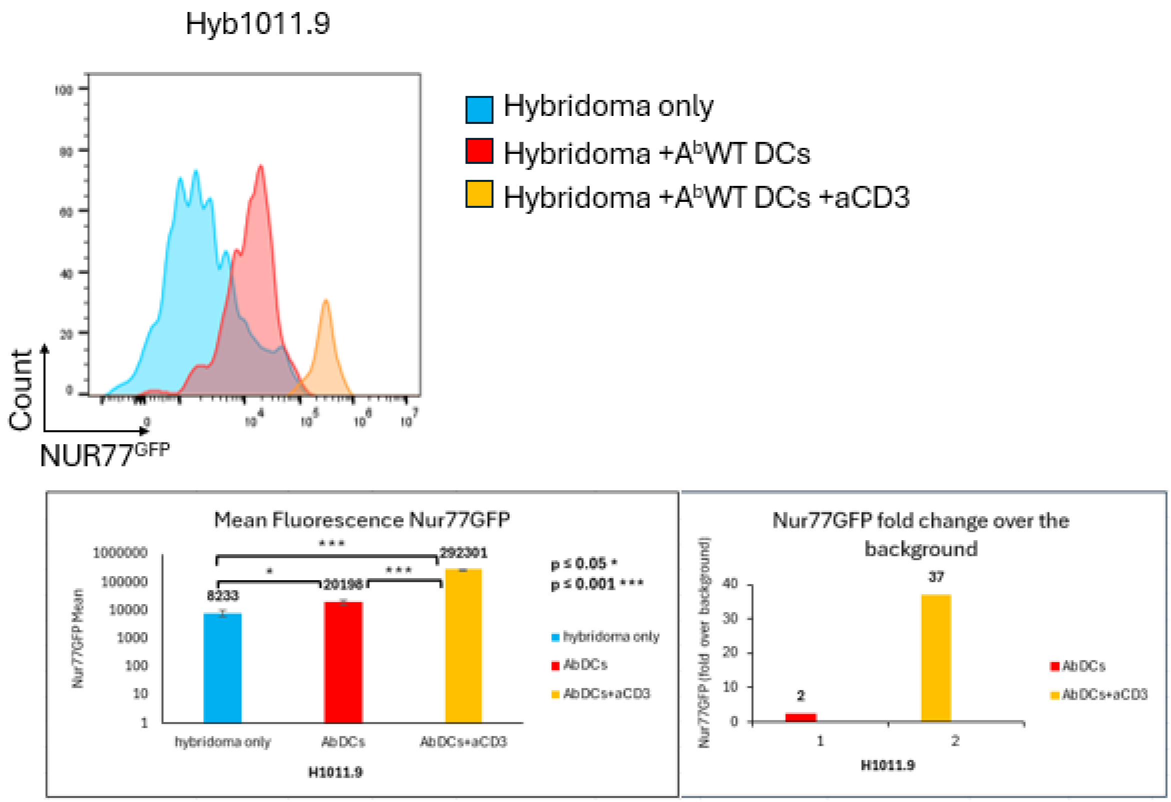

Figure 7: Examples of Responders to Autologous DCs and aCD3 (H1011.9).

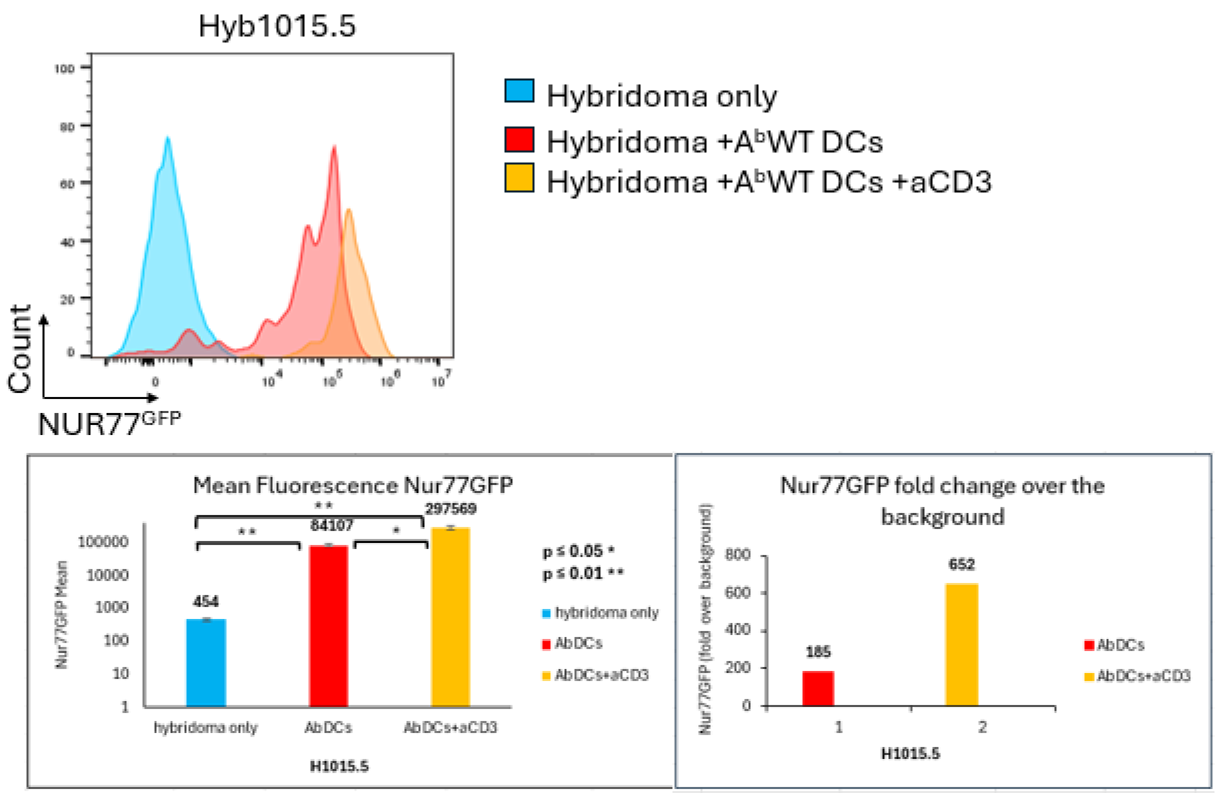

Figure 8: Examples of Responders to Autologous DCs and aCD3 (H1015.5).

3.4 Clones Selected for RNA Extraction.

A total of 12 clones (H1010.18, H1010.20, H1011.5, H1011.9, H1015.5, H1015.6, H1014.3, H1014.12, H1063.5, H1063.15, H1009.54, and H1009.57) that express a high level of Nur77^{GFP} expression were selected. Hence, their RNA was isolated (**Table 4**), and reverse transcribed to cDNA. The TCRα (CDR3 region) gene fragment was amplified via PCR sequence.

Table 4: RNA Concentration (ng/µl) of Hybridoma Clones.

HYBRIDOMA/CLONE NO	**RNA CONC (ng/µl)**
H1009.54	1823.4
H1009.57	1850.3
H1010.18	10.3
H1010.20	7.7
H1011.5	11.4
H1011.9	15.2
H1014.3	344.7
H1014.12	168.8
H1015.5	101.9
H1015.6	237.1
H1063.5	294.7
H1063.15	19.4

The PCR products of the selected clones were separated by agarose gel electrophoresis and were visualized on BIORAD software to verify if the CDR3α gene fragments were amplified. The result displayed a visible 330 bp band of DNA across all clones, confirming that the amplification worked (**Figure 9**).

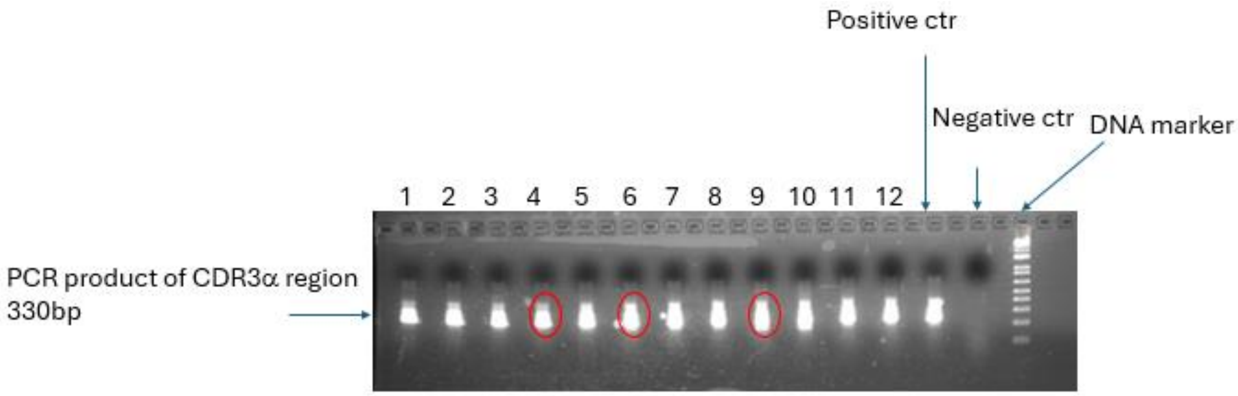

Figure 9: Agarose Gel Electrophoresis of the PCR Amplified TCRα Gene (CDR3 Region).

3.5 TCR Sequences of Selected Clones.

The T cell receptor of the 12 selected clones (H1010.18, H1010.20, H1011.5, H1011.9, H1015.5, H1015.6, H1014.3, H1014.12, H1063.5, H1063.15, H1009.54, and H1009.57) was sequenced to identify the nucleotide sequences present in their variable region. However, there were discrepancies in the sequenced data of 9 clones- H1010.18, H1011.5, H1015.6, H1014.3, H1014.12, H1063.5, H1063.15, H1009.54, and H1009.57 as non-specific sequences were observed in the variable region which is likely due to contamination that might have occurred during any stage of the procedures carried out in the process of the experiment or perhaps the clones were initially mistaken for a monoclonal population. The TCR sequences for H1010.20, H1011.9, and H1015.5 are shown in **Table 5**.

Table 5: TCR Sequence Reading for H1010.20, H1011.9, and H1015.5.

HYBRIDOMA CLONES	NUCLEOTIDE SEQUENCE	AMINO ACID SEQUENCE
H1010.20	ACCTACTTCTGTGCAGCAAGTGGNGGAGGACTAAGT	TYFCAASGGGLS
H1011.9	ACCTACTTCTGTGCAGCAAGTGGCGGTGGCAACTATCAGTTGATCTTGGGC	TYFCAASGGGNYQLIWG
H1015.5	ACCTACTTCTGTGCAGCAGGACTAAGT	TYFCAAGLS

4.0 DISCUSSION

The transgenic mouse model has provided a powerful platform for dissecting the pathogenic mechanisms of autoreactive $CD4^+$ T cells in T-cell-mediated autoimmune diseases. In this report, we cloned 6 ready-made autoreactive $CD4^+$ T cell hybridomas (containing autoreactive $CD4^+$ lymphocytes from scurfy TCR^{mini} mice with $BW\alpha^-\beta^-$ thymoma line) and characterized their antigen receptors (TCRs) to decipher the mechanisms of autoreactivity in the immune system and assist in redirecting abnormal $CD4^+$ T cells present in the peripheral repertoire of healthy individuals for therapeutic advantage. The findings indicate a disparity in clonal expansion among the six hybridoma lines, with H1014 being the most proliferative with 95 single clones. The high clonal output of H1014 may be due to its unique intrinsic property compared to other hybridomas. This observation aligns with the findings of Mitra and Tomar (2021), demonstrating that variations in clonal output among hybridomas are often linked to inherent genetic properties unique to specific cell lines.

Moreover, the FACS analysis revealed significant variation in the hybridoma clones expressing $CD4^+$ and TCR $V\alpha2^+$ across different hybridoma lines, with H1011 exhibiting the highest number of clones (80%) co-expressing both molecules. Such variability in clone numbers may arise from cell viability or extended culture period which contributes to the loss of surface markers and T-cell receptors consistent with the findings of Sharma *et al*. (2011) that iTreg hybridomas lose their ability to express Foxp3-GFP upon induction when cultured for a very long period.

Besides, the stimulation assay demonstrated a varying number of $CD4^+$ and TCR $V\alpha2^+$ hybridoma clones responding to autologous dendritic cells (DCs) stimulation and anti-CD3 stimulation across the 6 hybridoma lines. Furthermore, co-stimulation with anti-CD3 amplified the response of all hybridoma clones. Notably, H1015.5 showed a 652-fold increase in Nur77^{GFP} expression when aCD3 was added to hybridoma + A^{b}WT DCs culture. This drastic increase points to the pivotal role of aCD3 engagement in TCR signaling and subsequent T cell activation, as reinforced by Menon *et al*. (2023) that anti-CD3 provides a potent signal that bypasses the need for strong peptide-MHC interactions. The statistical significance of Nur77^{GFP} mean expression in H1010.19, H1011.9, and H1015.5 supports the notion that TCR engagement in the presence of aCD3 can significantly boost autoreactive T cell activation (Skapenko *et al*., 2005; Matic *et al*., 2013).

From the total clones we generated for all the cell lines, we extracted RNA from 12 clones and sequenced the TCRα region of their T cell receptors because they strongly expressed Nur77^{GFP} after stimulation with autologous APCs. However, there was a significant reduction in the sample size that was sequenced due to contamination from different sources. Therefore, we only obtained results (**Table 5**) for 3 clones (H1010.20, H1011.9, and H1015.5). The identification of specific TCR sequences is vital for investigating T cell interactions with self-peptides as highlighted by Kovaleva *et al*. (2023).

5.0 CONCLUSION

These findings demonstrate the high binding to self-peptides/MHCII complex by H1010.20, H1011.9, and H1015.5, and their unique sequences and suggest that these clones are likely to have autoreactive TCRs. Therefore, the data suggests that once these hybridoma clones TCRs are activated by autologous DCs, they upregulate Nur77^{GFP} expression, affirming their autoreactive nature and capacity to recognize self-peptide/MHCII complexes. These findings validate the potential of T-cell hybridoma technology as a valuable tool for investigating the role of autoreactive T cells in autoimmunity.

5.1 Limitation of the Study

One significant limitation of this study was the occurrence of non-specific sequences in the TCR variable regions of 9 hybridoma clones out of the 12 sequenced samples, likely resulting from contamination during the experimental process or potential misidentification of monoclonal populations. This affects the diversity of T-cell receptor characterization across the hybrid lines hindering the comprehensiveness of how various clones favor autoreactive outcomes. Additionally, the study was confined to $CD4^+$ TCR $V\alpha2^+$ hybridoma clones, which may not fully represent the broader diversity of autoreactive T cell populations. Furthermore, the in vitro nature of the assays may not entirely reflect the complex interplay in vivo, thereby limiting the direct clinical applicability of the findings.

5.2 Recommendation

For future studies, it is highly recommended that the sample size be expanded for a greater diversity of clones and optimize the purification test for contaminant clones that disrupt TCR sequencing to ensure that unintended targets are removed before sequencing. It is also essential to expand the scope of future studies by capturing a more diverse spectrum of auto-reactive T-cell populations, ensuring the comprehensiveness of how different TCRs contribute to autoimmunity.

Moreover, conducting in vivo studies of autoimmunity will be crucial for validating the clinical relevance of these findings. Such studies could explore the impact of modulating TCR signaling on disease progression, thereby bridging the gap between basic research and its real-world applications in autoimmune disease management.

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CURRICULUM VITAE

JUMOKE RAJI-AYOOLA

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PROFILE SUMMARY

Experienced molecular biologist with proficiency in PCR amplification, RT-PCR, DNA/RNA extraction, Cell culture, Flow cytometry, hybridoma cloning, and more. Conducted in-depth research on autoimmunity and T lymphocyte function, showcasing strong problem-solving and analytical abilities. Also skilled in quality assurance/quality control, regulatory compliance, and bioethics, aiming to contribute to innovative biomedical research and ethical initiatives.

EDUCATION

- **MSc. Biomedical Sciences and Enterprise** **2023 - 2024**
  Georgia State University, Atlanta **CGPA:** 4.19 / 4.30
- **BSc. Animal Breeding and Genetics** **2014 - 2021**
  Federal University of Agriculture, Abeokuta **CGPA:** 4.31 / 5.0

WORK EXPERIENCE

Graduate Research Assistant, Institute for Biomedical Sciences, *Georgia State University* **2023 - Present**

- Investigated cellular and molecular mechanisms of autoimmunity using T-cell hybridoma technology, cell culture, RT-PCR, hybridoma cloning, cell staining, cell stimulation, and flow cytometry.
- Analyzed immune tolerance mechanisms and characterized autoreactive CD4⁺ T cell hybridomas and their TCRs.
- Studied ontogeny and function of T lymphocytes and conducted mouse genotyping via PCR amplification.
- Analyzed data collected using Flowjo software and Microsoft Excel.

Regulatory Affairs and Bioethics Intern, *Center for Bioethics and Research* **2021 - 2022**

- Conducted comprehensive reviews of proposed research protocols and presented research on the ethical implications of genome editing technologies in relation to current and emerging regulatory frameworks at an international conference, fostering dialogue on responsible research practices. Organized bioethics seminars to promote ethical awareness and compliance, leveraging communication skills and bioinformatics tools (DNAsp v.5, MEGA 5.2) for educational purposes.
- Developed and implemented a biosafety training program, resulting in a 20% reduction in laboratory incidents within the first year. Collaborated with biosafety professionals to create training materials that integrated bioethics principles into safe laboratory conduct.

Research Assistant, *Federal University of Agriculture Abeokuta* **2021**

- Utilized SNP genotyping and molecular software tools like DNAsp v.5 and MEGA 5.2 to assess genetic diversity in animal populations.
- Collaborated internationally to establish a GWAS pipeline for identifying genetic markers linked to disease-resistance traits in livestock. Presented innovative data visualization techniques at a national symposium, receiving accolades.

RELEVANT RESEARCH AND PROJECTS

- Cloning and characterization of autoreactive CD4 T cell hybridomas and their antigen receptors (TCRs).
- Assessing the genetic diversity and ancestry of Kuri cattle using mitochondrial DNA D-loop in Nigeria.

CERTIFICATIONS/WORKSHOPS

- **Biomedical Responsible Conduct of Research**, *Georgia State University* **2023**
  The training focuses on promoting objectivity and ethical standards in research at Georgia State University, particularly through the Policy on Financial Disclosures in Sponsored Research.

- **Bloodborne Pathogens Training**, *Georgia State University* **2023**
  The training module aims to educate University System of Georgia employees and students on bloodborne pathogens, their transmission modes, and preventive measures to mitigate exposure risks.

- **Hazardous Waste Awareness Training**, *Georgia State University* **2023**
  The online training program complements USG Right-to-Know training, mandated by state law, aiming to educate USG employees on the proper identification, handling, and disposal of hazardous wastes, emphasizing their importance and benefits.

- **Biological Data Science Summer Workshop**, *Drexel University, Rowan University and The University of Chicago* **2022**
  The program focuses on the application of data science techniques in biology, targeting core areas in computational biology and bioinformatics.

- **Vaccine Design Workshop**, *Institute of Bioinformatics and Molecular Therapeutics (IBMT)* **2022**
  Concentrated on vaccine design principles, covering areas such as immunology, computational biology, and molecular modeling techniques.

AWARDS

- **IBMS Graduate Research Assistantship Award 2024** | *Georgia State University*.

PROFESSIONAL MEMBERSHIP/LEADERSHIP/VOLUNTEER SERVICE

- **North Carolina TSA State Conference 2024 |** Judge, Evaluated both High school/ Middle school participants for the competitive event on biotechnology, medical technology, and forensic technology.
  https://drive.google.com/file/d/11P9YNJYi6xng60Pe5e8tENCwmwrKzVxG/view?usp=drivesdk
- **The Ohio Academy of Science State Science Day 2024** | Judge, Evaluated research projects of students in biomedical and health sciences.
  https://drive.google.com/file/d/1_qmnkuFzRGK8ci7xcEdU6LhR1Lay0HQm/view?usp=drivesdk
- **Center For Bioethics and Research |** Team Lead, Bioethics seminars and events for a group of 50 people.
- **Community Development Group |** Team Lead, Public health campaigns, and managed the distribution of Insecticide-treated nets.

PROFESSIONAL SKILLS

Mammalian Cell Culture | Cloning | DNA Extraction & RNA Extraction | DNA Purification | Agarose Gel Electrophoresis | RT-PCR | PCR Amplification | Immunoassay | Mice Handling | Pipetting | Flow Cytometry | Data Collection | Data Analysis & Data Interpretation | Troubleshooting | Quality Control | Research & Laboratory Skills | Laboratory Inventory Management | Presentation | Communication | Attention to Detail | Critical Thinking | Accuracy | Collaborative mindset | Leadership.