IgG4-Related Disease: An Antigen Discovery Project

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Cyrus Washington

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Thesis Advisor: Dr. Shiv Pillai Cyrus G. Washington

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Abstract

IgG4 related disease is a multi-organ disease whose clinical manifestations often mimic infectious, malignant and inflammatory disorders [1]. Prior to 2003, many well-defined eponyms were given to diseases whose causation was unknown. Further clinical investigations of these seemingly idiopathic diseases revealed many histopathological similarities. Originally identified in the pancreas as autoimmune sclerosing pancreatitis, the systemic characterization of the disease lends to its ability to present remarkably similar histopathological findings in distal locations [2]. Clinically significant, and often shared, findings of the disease include storiform fibrosis, increased lymphoplasmacytic infiltrate rich in Igg4-positive plasma cells and tumefective lesions. Elevated Igg4 concentrations in serum have also been identified in some patients with IgG4-related disease, while a >40% IgG to IgG4 ratio is present in tissues [3]. While a vignette of the pathophysiology has been loosely elucidated, the specific causative players are less characterized.

T cell studies of patients with IgG4 related diseases have implicated this subset of cells as a key player in the progression of the disease. Mechanistically, the role of T cells has been hypothesized not been clearly defined. GWAS studies of patients with Chrohn's diseases identified a non-coding polymorphism at which the minor (G) allele is associated with a milder course of Chrohn's disease [4]. The identified SNP in the FOXO3A transcription factor was shown to limit inflammatory responses by the reduction of the pro-inflammatory cytokine TNFa by TGFB1, and the increase in the

production of anti-inflammatory cytokines such as IL-10. Activated T-cells elicit their mechanisms of actions in a very polar like fashion. Therefore, in the context of IgG4 and the role of T-cells, studying the FOXO3 transcription may provide context to the role of these subset of cells in a patients with this disease. This antigen discovery project seeks to define a subset of antigens that are key in eliciting the activation of B cells in this disease. Furthermore, the identification of snps that lead to a milder prognosis may also help clarify the pathogenesis of IgG4 related disease.

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Pillai Lab:

Vinay Mahajan M.D. Ph.D
Cory Perugino DO
Kelly Xing
Xin Kai
Isabella Fraschilla
Grace Yuen Ph.D
Joe Daccache
Imad Awan
Vinay Viswanadham
Faisal Alsufyani M.D.

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Chapter 1: Introduction to IgG4-Related Disease

1.1 The History of IgG4-Related Disease

IgG4-Related Disease (IgG4RD) is a systemic, chronic inflammatory syndrome characterized by storiform fibrosis, tumefective lesions, and obliterative phlebitis. Histopathologically, IgGRD is characterized by the infiltration and accumulation of IgG4-secreting and expressing plasma cells in the tissue. In the past, many specific diseases such as Reidel's thyroiditis, Mikulicz's disease, Küttner tumor, type I autoimmune pancreatis, prostatitis, retroperitoneal fibrosis, and some others were thought to be distinct, idiopathic diseases (1-8). Better diagnostic tools, that provide more information about these diseased sites, have implicated the aforementioned diseases to fall under the IgG4RD umbrella.

At the turn of the 21st century, IgG4RD experienced a massive increase in awareness and knowledge due to its popularity in medical publications. Despite this, gaps in the diseases history have made it difficult to fully characterize the disease in an epidemiological sense. IgG4RD diagnosis necessitates a biopsy of the affected organ, followed by immunostaining and certain diagnostic screenings. Therefore, identifying the disease's true prevalence, presently and historically, proves to be difficult [1]. The idea of extrapolating the prevalence of the disease in the population from the incident rate of its common features is also fruitless, as previous attempts undermine current data. For example, type 1 autoimmune pancreatitis is a common feature of IgG4RD with an estimated prevalence of 2-2 cases per 100,000 populations in Japan [9]. However, the pancreas is only one out of a gamut organs affected by IgG4RD. The incidence rate of

each specific organ's prevalence in the disease is also unclear and therefore provides little context to the behavior of the disease in populations [1].

Present data implicates middle-aged to elderly male as being disproportionately affected by IgG4RD outnumbering their female counterparts three to one. Varied organs express differentially across the sexes for unclear reasons, and currently no data exist that link a genetic component to this disease [1].

1.2 IgG4 Molecule

Heterogeneity of the clinical manifestations of IgG4RD constrains a definitive diagnosis to immunostaining of the biopsied tissue specimens. Basis for diagnosis are contingent upon the IgG4-positive plasma cell population whereby a ratio of IgG to IgG4-positive plasma cells must be at least 40% (health patients 1-4%) [1,3,10]. Despite the large flux of $IgG4^+$ antibody infiltrates, their role in IgG4RD is still a topic of debate. Traditional conditions produce $IgG4^+$ molecules after prolonged antigen exposure and secretion of Interleukin-4 and Interleukin 10, but in IgG4RD the class switching mechanism is still being studied.

Structurally, subclass IgG4 molecules differ from its other IgG counterparts. Amino acid serine replaces proline causing non-covalent associations and inefficient disulfide bridges [11-13]. This change in amino acid results creates a weaker association between the heavy and light chains. A covalent bond between one heavy and one light chain creates hemi-IgG4 molecules that have a predilection to dissociate and reassociate randomly with distinct hemi-IgG4 molecules that differ in binding specificity [10]. This phenomenon, known as 'fab arm exchange' accounts for the antibodies aversion to associating with each other, low affinity for Fc receptors, and their low affinity for

complement. Their low affinity for each other, (which in turn leads to the formation of less conglomerates) and their low affinity for Fc receptors and C1 complement, are all reasons why IgG4 molecules have traditionally been viewed as non inflammatory molecules [10] (Figure 1).

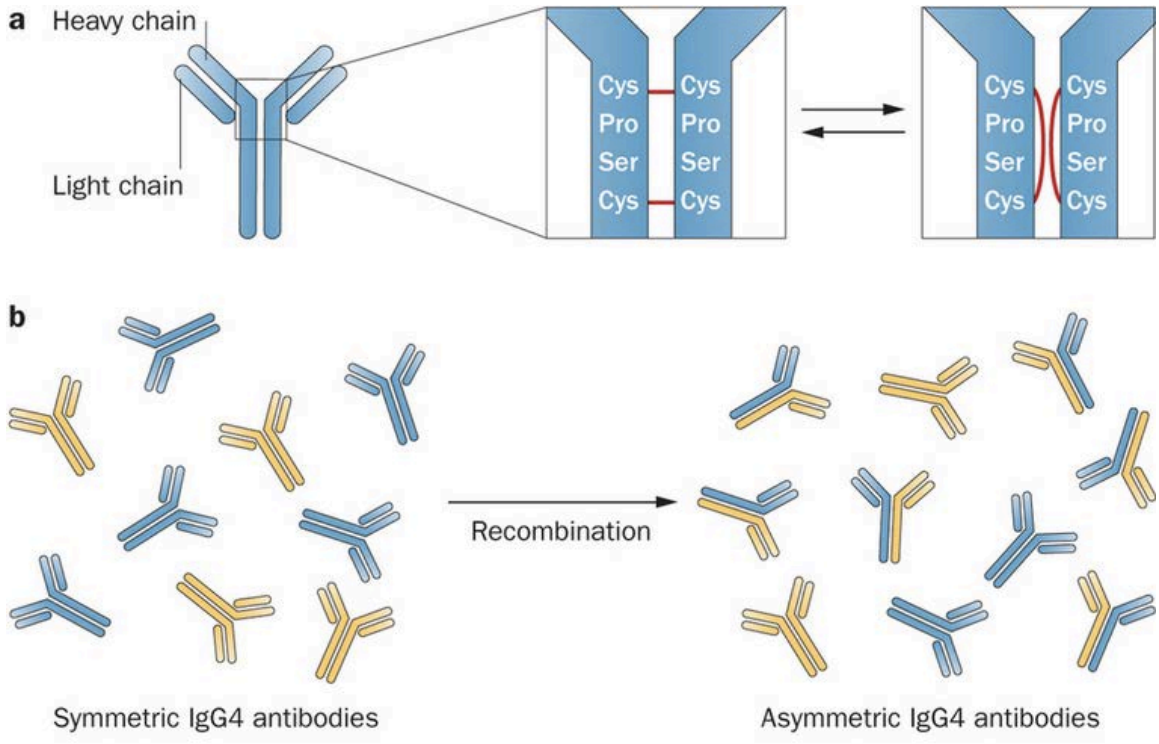

Figure 1: Fab arm exchange (Cortazar and Stone.2015. Nature Reviews Nephrology)

IgG4RD is also characterized by the presence of autoantibodies but their specificity for this disease this poor. Antigens such as carbonic anhydrase II, pancreatic secretory trypsin inhibitor, and lactoferrin have all been described in patients with IgG4RD. These autoantibodies function in pathogenesis is still unclear as there is no direct evidence that suggest that they are of the IgG4 subclass [14].

1.3 Pathological Features of IgG4-Related Disease

Clinical presentation of IgG4RD varies among the affected organ necessitating immunostaining of tissue biopsies for definitive diagnoses. Key pathologies that are specific to IgG4RD include lymphoplasmacytic infiltration, obliterative phlebitis, and storiform fibrosis. Polyclonal lymphocytes and plasma cells, as well as eosinophils are normally present in large populations in affected tissues [1] (Figure 2).

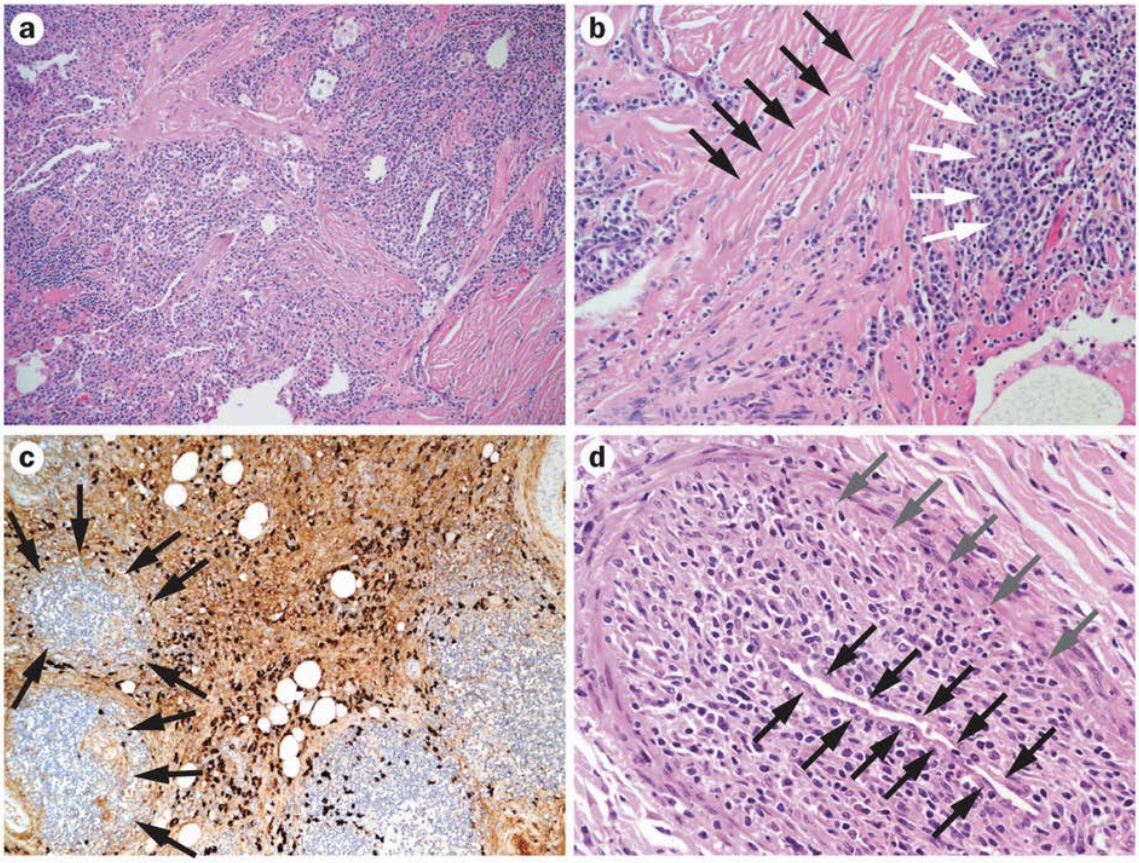

Figure 2: Pathological features of IgG4RD in the lung. A- Lymphoplasmacytic infiltrate and diffuse cellular infiltrates are interwoven with extensive storiform fibrosis (Low magnification). B- Lymphoplasmacytic infiltrates (white arrows), Storiform fibrosis (Black arrows) C- Biopsy of patient with IgG4- related retroperitoneal fibrosis. D- Obliterative phlebitis in a lung biopsy sample. (Cortazar and Stone.2015. Nature Reviews Nephrology)

Histological findings of storiform fibrosis (scarring) are another characteristic of IgG4RD. Storiform fibrosis is characterized by its random, swirly pattern. The progression and formation of storiform fibrosis include the creation of a cellular component of small spindle cells with little collagen formation. Collagen formation increases in production whole the cell component decrease, producing fibrotic foci that consist mostly of collagen [1].

Obliterative phlebitis is the partial or complete obliteration of a vein as result of lymphoplasmacytic infiltrates in the wall of the venous channel and lumen. Degrees of obliteration range from partial to full obliterations, which require elastin stains for identification. Partial obliteration is also consistent with IgG4RD diagnosis [15-17].

1.4 Pathophysiological Mechanisms

IgG4RD's inception is a topic that is still a mystery like many other autoimmune diseases. Studies suggest that a break in immune tolerance occurs as a result of molecular mimicry. Self-reactive T-cells bypass immune checkpoints due to an insult by an exogenous antigen whose sequence is similar to self-peptides. These polarized, self-reactive $CD4^+$ T-cells initiate the onset of disease at specific organs through the induction of the fibrotic pathological process through cytokine secretion [1]. Downstream, the recruitment of other immune cells exacerbates this process.

Mechanistically, the elucidation of IgG4RD's pathophysiology has been a mystery. Stone et al. hypothesized two mechanisms in their IgG4RD review published in 2014. The first hypothesis implicates a subset of undefined CD-4 positive T-cells that activate innate immune cells, including macrophages, myofibroblasts, and fibroblasts.

Scarring is the result of fibroblast activation while the lymphoplasmacytic infiltrates, activated plasmablast and $CD4^+$ T-cells, induce swelling of the damaged tissue. Their second hypothesis, also not well defined, implicates IgG4 secreting plasmablasts, plasma cells, and IgG4 antibodies in feedback negative regulatory process [1]. The former of these two hypotheses has been supported in future studies.

While the role of IgG4 antibodies in the pathogenesis of the disease is still unclear, studies have shown that an increase in an oligoclonal expanded subset of B cell infiltrates that are $CD19^+$ $CD20^-$ $CD27^+CD38^+$, are a strong indicator of IgG4RD disease activity. Serum levels of IgG4+ plasmablast may or may not increase in patients with IgG4RD, but in each case a decrease is seen after treatment with an anti-CD20 mAb (rituximab)[14]. Fab arm exchange renders the IgG4 antibodies unable to bind Fc receptor and activate compliment. Additionally, $IgG4^+$ plasmablast constantly undergo somatic hypermutation over the course of the disease and upon relapse, distinct plasmablast clones emerge. These findings suggest that a self-reactive disease process is being orchestrated via the recruitment of naïve B cells into T-cell dependent responses [14].

Recent work by Pillai et al. has shed light on the role of T-cells. These findings have suggested a more causative role of a poorly defined $CD4^+$ T-cell subset that are $SLAMF7^+$, profibrotic, and secrete both pro-inflammatory and anti-inflammatory cytokines. These $SLAMF7^+$ IL-1β-, IFN-γ-, TGF-β1- secreting expand clonally and are present in affected tissues of patients with active IgG4RD. Secretion of pro-inflammatory such as IL-1β and IFN-γ is speculated to induce inflammation of the diseased tissue and recruit other immune cells that may drive pathogenisis. Although TGF-β1 is known for

its profibrotic properties, its role in this context is still being investigated [18]. Such a unique combination of cytokine secretions implicates this subset of T-cells as a driver in the disease process of IgG4RD.

1.5 FOXO3 Transcription Factor

Transcription factors are adaptor molecules that detect regulatory sequences in the DNA and target the assembly of protein complexes that control gene expression [19]. Their activation can lead to the downstream expression of products that can influence the dynamics of a process or its general surroundings. A good example of this is T-cell lineage commitment where the activation of certain transcription factors in naïve $CD4^+$ T-cells terminally commits the cell, which dictates the type of cytokines the cell secretes [20] (Figure 3).

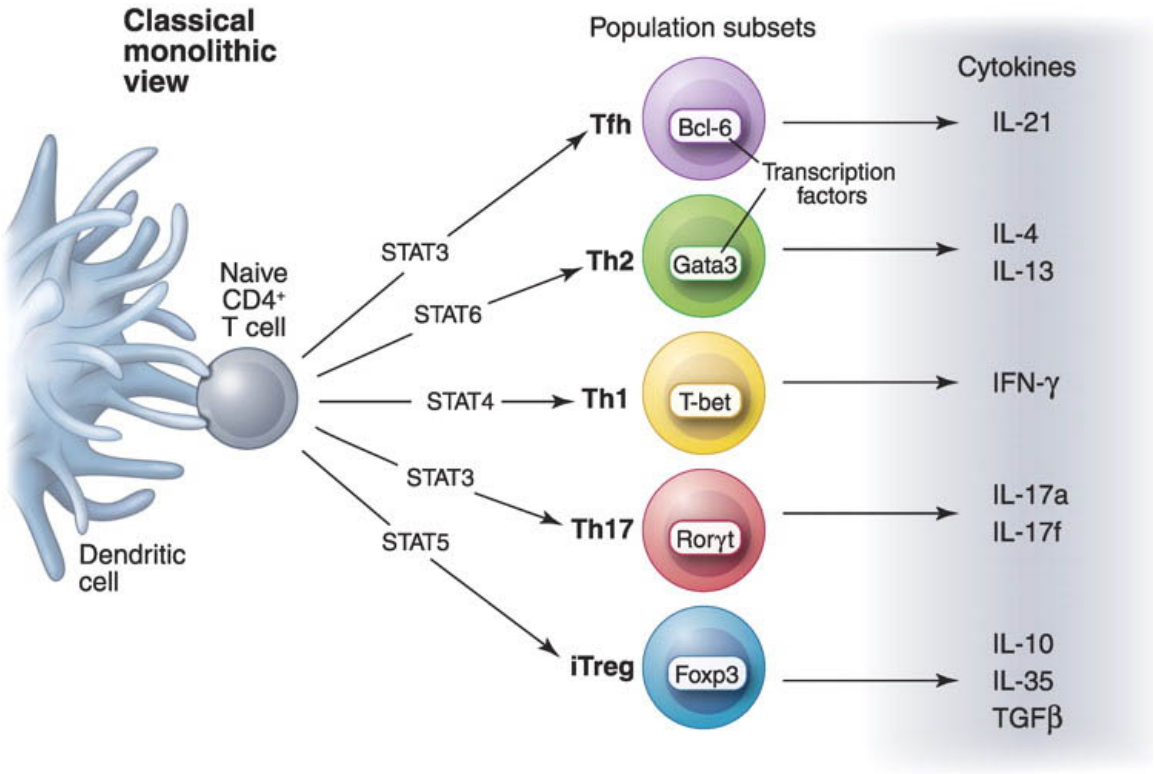

Figure 3: Helper T cell differentiation (O'Shea and Paul. 2010. *Science*)

Forkhead box transcription factors are widely expressed and are named after the Drosophila Melangoster gene fork head (fkh). Its vast expression and presence in many different species has lead to its classification into subfamilies, such as FoxA, FoxP, FoxO, etc. Fox genes functions include control of cell cycle, epithelia cells differentiation, formation of the inner ear, placental development, and many other important biological processes [21].

In 2012, Ken Smith et al. conducted a Genome Wide Association Study (GWAS) on a cohort of patients with Chrohn's Disease. The purpose of this study was to elucidate the mechanisms of cytokine signaling pathways and its affect on the prognosis of the disease. The study discovered a noncoding single-nucleotide polymorphism in the FOXO3A gene [4]. FOXO3A encodes FOXO3, a transcription factor that functions in the suppression of inflammatory cytokine production by dendritic cells and in limiting the inflammatory sequelae of viral infections [22-24].

The snp replaces a G for a T in the in the noncoding region of the gene. Presence of a minor allele (G) has been associated with a milder course of rheumatoid arthritis and Chrohn's disease. The mechanism includes the induction of a TGFb-1 dependent pathway in monocytes, which reduces the production of pro-inflammatory cytokines such as TNFα, and the increase in anti-inflammatory cytokines such as IL-10 in monocytes [4]. With a newly defined role of T cells and TGF-β1 in IgG4RD, it is now of special interest of our group to study our cohort of IgG4RD patients for noncoding snps in FOXO3A gene.

The causality and underlying mechanisms involved in autoimmune diseases is often unclear due to the absence of a single player in the disease, where a specific agent

can be implicated as the catalyst of the disease, and because of the tendency for well defined cell sets to deviate from their characteristic phenotypes. IgG4RD falls into the category of a systemic autoimmune disease that presents with varying clinical manifestations. In this study we have created a system that will allow us to further study the prognosis and development of the disease. The luciferase protein fusion vector will allow us to identify self-antigens that incite and perpetuate the disease. With this, antibodies can be created to target these agents and impede the downstream activation of immune cells.

Ken Smith's GWAS analysis of monocytes in patients with Chrohn's Disease motivated our group to study snps in patients with IgG4RD. While we are still in the process of increasing our cohort, our initial findings suggest that this snp may be influential in the prognosis of patients with IgG4RD. This finding supports the idea that there is a genetic component to the prognosis of the disease and that T-cells are a driver in the pathophysiology of the disease.

Chapter 2: Antigen Discovery Experimental Procedures

2.1 Methods

2.1.1 Overview

Our two-fold project seeks to provide a more comprehensive picture of IgG4RD pathologically and genetically. The antigen discovery project utilizes a luciferase protein fusion vector to identify self-antigens that elicit the activation of the immune response. Our snp study and analysis of IgG4RD patients who carry the minor allele will provide more context to the genetics of the disease and shed light on the drivers of the disease.

2.1.2 Luciferase Protein Fusion Vector

Utilizing a luciferase immunoprecipitation system (LIPS), we created a fusion vector with our own proteins of interest. Our collaborators identified 20 proteins of interest in IgG4RD patients using a platform called PLATO (Parallel Analysis of Translated ORFs), which we are using for our initial studies and analysis with the LIPS system. PLATO is a method that combines *in vitro* display of full-length proteins with analysis by high-throughput DNA sequencing [25].

Drosophila Gateway vector collection was used as a backbone vector. pHFW and pHWF, suitable for n-terminus and c-terminus fusions respectively, served as backbone vectors. Vectors were linearized through polymerase chain reaction (PCR) with primers: fwd 5'-ATGGACTACAAAGACCATGACGGTG-3', and rev 5'-GGTGGCGGAGCTCACCAC-3' (Figure 4).

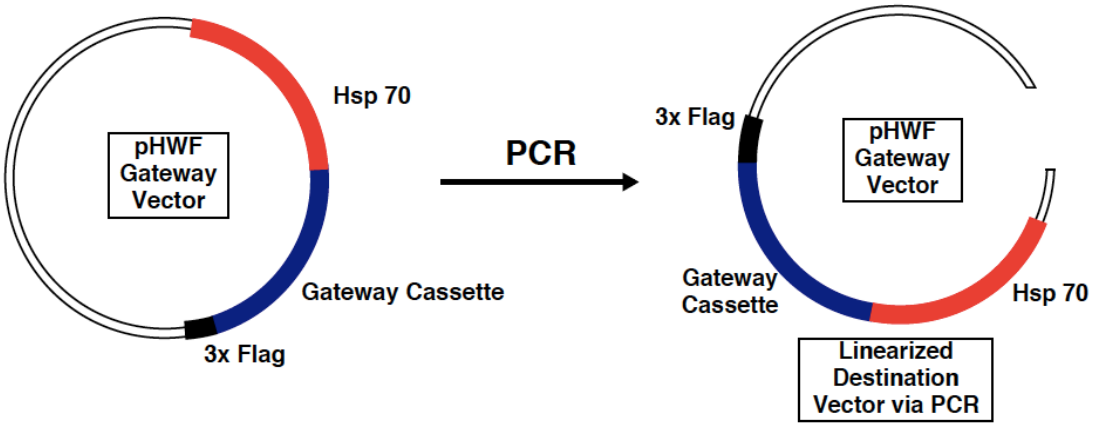

Figure 4: Creation of linearized Gateway Vector after PCR

pNL 1.1 vector was used as a DNA template in a PCR using primers: fwd 5'-ATGGTCTTCACACTCGAAGATTTC-3', and rev 5'- CGCCAGAATGCGTTCGCA-3' (Figure 5).

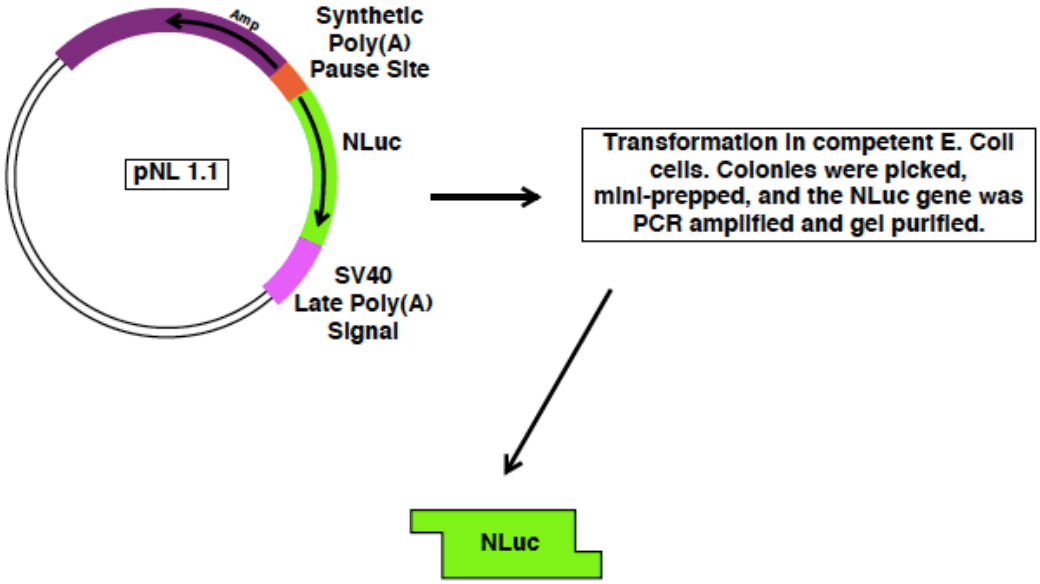

Figure 5: Creation of linearized, isolated NLuc gene segment after PCR

NLuc and vector PCR products were gel purified and DNA fragments were joined in a Gibson assembly reaction (Figure 6).

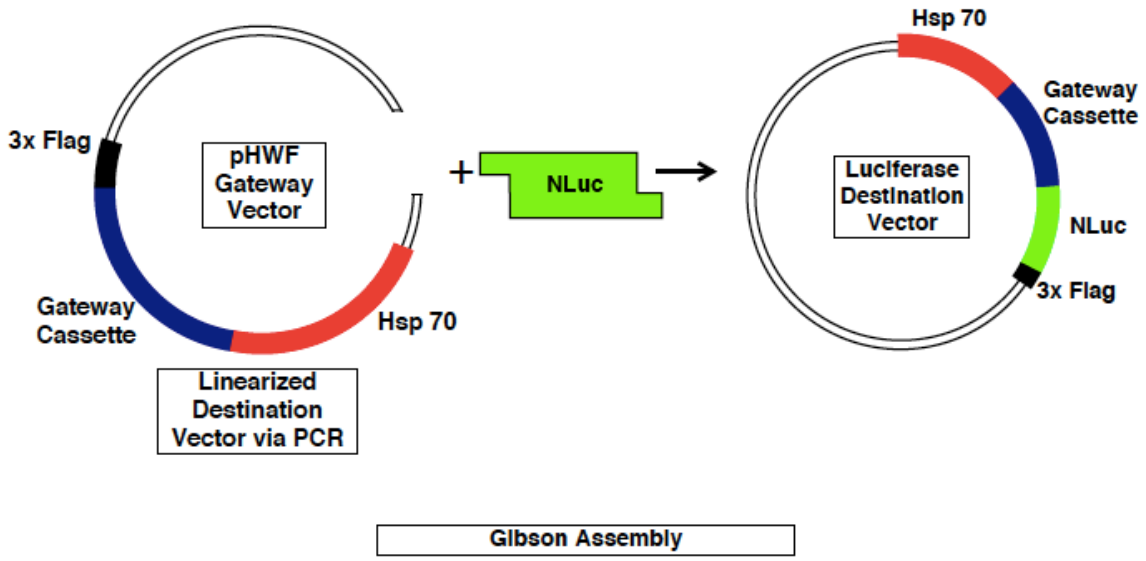

Figure 6: Nluc and Gateway vector fusion by Gibson assembly

Gibson assembly uses a 5' exonuclease to chew back the 5' end sequences and expose the complimentary sequences for annealing. Polymerase activity then fills the gaps in the annealed region followed by DNA ligase that then seals the nicks and covalently links the DNA fragments together [26]. Recombinant plasmids (luciferase destination vector) and entry clones containing ORFs (Open Reading Frames) of interest were transformed in One Shot ccB Survival 2 $T1^R$ chemically competent cells into a gateway cloning reaction. Entry clones' ORFs contain genetic sequences that code for proteins of interest as previously mentioned. Gateway cloning employs clonase activity to excise the gene from entry clones and integrate them into the destination vector. Genes of interest in entry

clones are flanked attL sequences, which are used to locate, and recombine the targeted genes DNA sequence with the attR sequences on the destination vector (Figure 7).

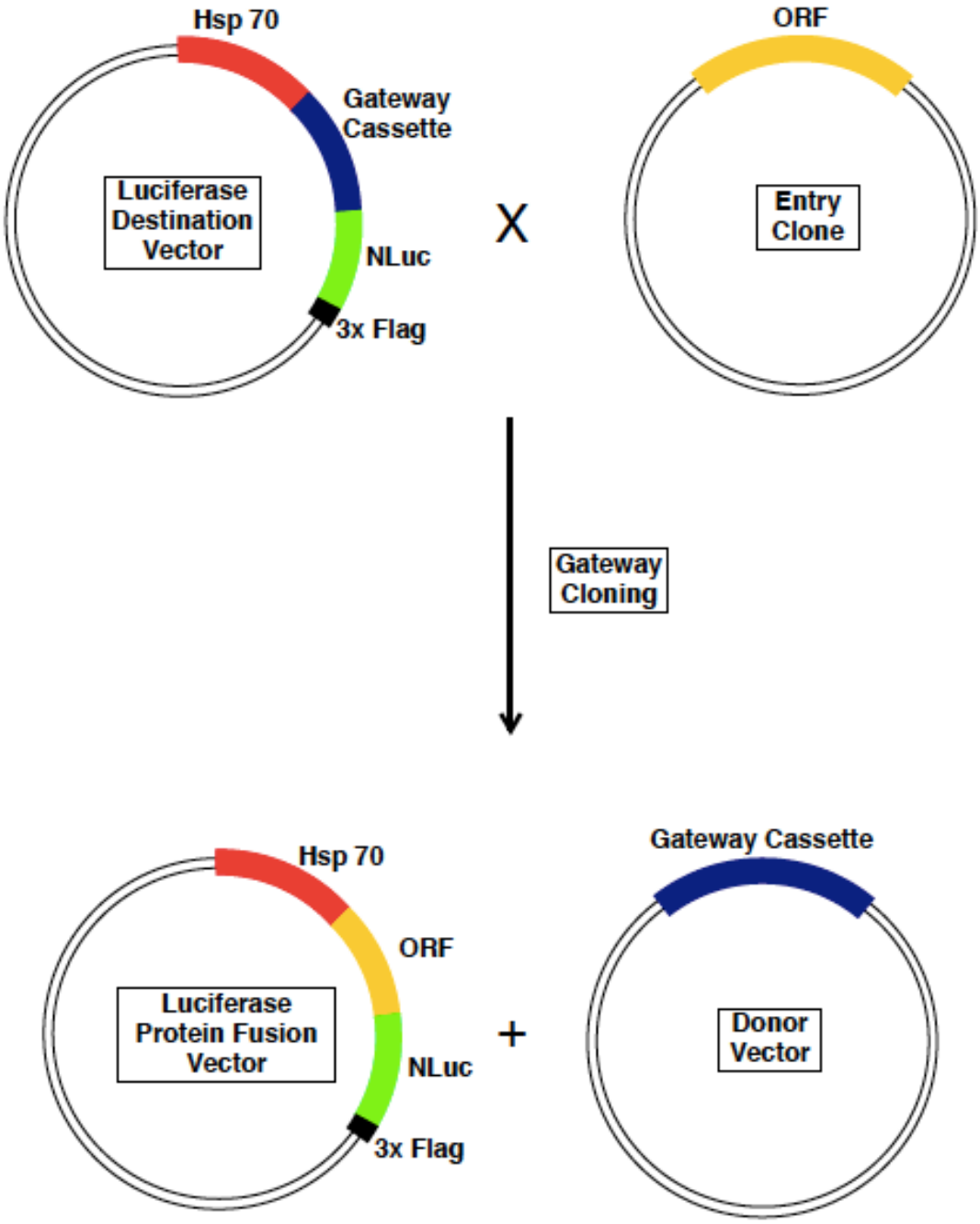

Figure 7: Protein Luciferase fusion vector by Gateway Cloning

This is the final step in the vector creation protocol and results in the creation of the luciferase protein fusion vector (expression clone) needed to perform the LIPS Assay (Figure 8).

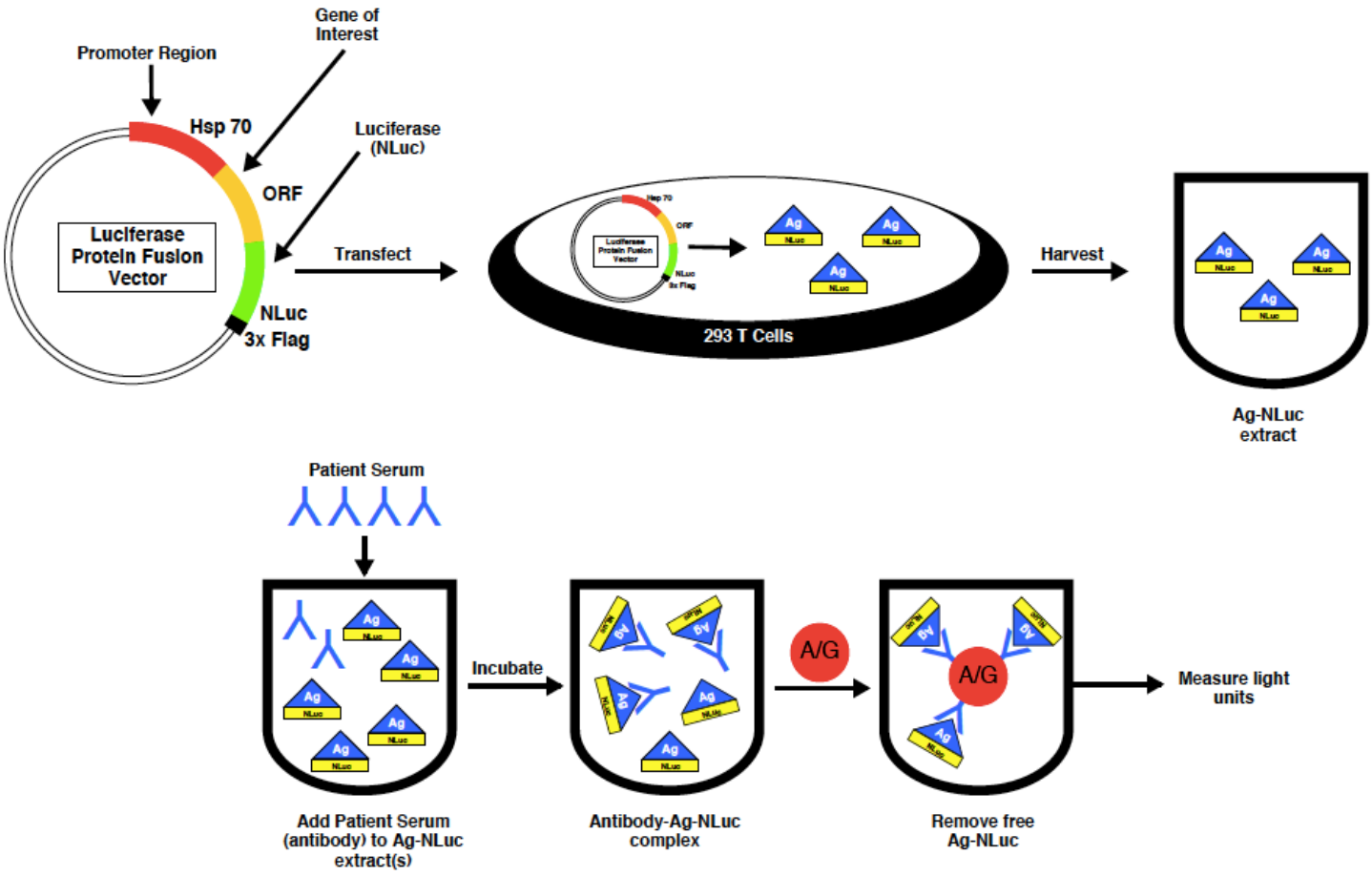

Figure 8: LIPS Assembly Schematic

The LIPS system is a fluid-phase assay used to measure antibodies. It utilizes mammalian cells to produce luciferase-tagged antigens that are then exposed to antibodies [26,27]. The resulting antigen-antibody complexes are then sequestered using a protein A/G beads and a luciferase substrate, furimazine, is added to the system. For our

system we used the Nanoluciferase reporter instead of Renilla Luciferase for its smaller size, brighter luminescence, and greater sensitivity (confirm and find reference) [29]. The relative amount of antibody can then be assessed by the amount of light produced when adding Nanoluciferase's substrate.

Our luciferase fusion vectors include a promoter region –HSP70, an ORF that includes the genetic sequence for our antigen (protein of interest), a fused genetic sequence coding for our reporter probe (Nanoluciferase) and a 3x flag region. Our collaborators on this project have compiled a list 20 proteins of particular interest in patients with IgG4RD. This analysis was conducted using a PLATO system. Luciferase protein fusion vectors were transfected in mammalian 293 T cells. Lysates were harvested with patient's serum and antibody concentrations were assessed from the amount of luminescence was produced when furimazine was added to the system. Autoantibodies can be measured using this system where high luminescence corresponds to a higher number of autoantibodies to self-antigens. Luminescence is measured using a luminometer.

2.1.3 FOXO3 Transcription Factor

Whole blood from 50 patients with IgG4RD was obtained and processed for nucleic acids. FOXO3A gene sequence was amplified via polymerase chain reaction using primers - Fwd 5'- TACGCATACGTTGTTGGAGGTT-3' and Rev 5'-GGTCCCGAGGCTAAATGGAA-3'. Presence and size of sequence was confirmed using gel electrophoresis in which the expected size of the FOXO3a sequence is expected to be 307 bp long. Samples were then sent for Sanger Sequencing at MCB CORE. Sanger

Sequencing results will be analyzed for the snp confirmation at the 251 base pair position.

Chapter 3: Results and Discussion

3.1 Results

3.1.1 Antigen Discovery-LIPs System

Our LIPS system sought to discover self-antigens that may be exacerbatory in patients with IgG4RD. We were successful in creating an N-terminus luciferase protein fusion vectors that included antigens from our list of proteins that are of high interest. Using a luminometer, we have been able to confirm that our fusion vectors do work and have the capacity to report information about the antigen and the antibody in the context of IgG4RD

3.1.2 FOXO3 Transcription Factor

Two patients were removed from our study after set parameters disqualified them from our study. Qualified patients have IgG4RD. IgG1, IgG4, absolute eosinophils, and IgE concentrations were measured. 5 out of the 48 patients were positive for a snp in the FOXO3A gene in the 251 allele position. Out of the 48 patients, 0 out of the 5 patients with a snp fall within the upper quartile of ranges for serum concentrations of IgG1, IgG4, and IgGE. 1 out of the 5 patients fall within the upper quartile of ranges for serum concentrations of absolute eosinophils. Upper quartiles have been associated with disease relapses [30] (figure)

3.2 Discussion

The purpose of this study was to create a system that would permit the detection of autoantigens in patients with IgG4RD. This new information would be advantageous in elucidating the mechanisms behind the diseases pathophysiology and provide insight that could be used in the treatment of this disease. To date, its classification, as an "orphan disease" is no misnomer [31] There have been many attempts to identify the players of the disease but none have been able to definitively pinpoint its cause. Some studies have linked the presence of a snp in the gene sequence that codes for CTLA-4 and Fc receptor-like 3 to IgG4RD [32,33]. Other studies have linked the HLA alleles- DRB1*0405 and DQB*0401- in Japanese men to IgG4RD [34]. While its causes are still unknown, knowing what drives the disease remains a special interest.

The LIPS assay is a very specific approach to identify auto antigens that may be important in patients with IgG4RD. The 20 culprit proteins that we have created fusion vectors were chosen after a PLATO analyst our collaborators conducted with patients with IgG4RD. We expect that this system will provide more context and information about the players that are perpetuating the constant activation of the immune system. Additionally, we expect to gain a better profile of the antibodies that are reacting with the autoantigens. The LIPS assay proves to be more specific and sensitive than ELISAs and may provide more detailed information on the autoantibody profiles that are reactive in our system [35].

For many years, prior to sophisticated scientific instruments, the binary nature of the immune system was widely accepted and provided less fluidity of the cells each branch encompassed. With more knowledge and advanced tools we are seeing that

previous strict constructs of the immune system are becoming less rigid and grayer. A great example of this is the unique phenotype of T cells that have been discovered in patients with IgG4RD. This SLAMF7^{+}, IL-1β, IFN-γ, and TGF-β1- secreting T cell subset exhibits both pro-inflammatory, anti-inflammatory, and pro-fibrotic characteristics. These T-cells have been implicated in driving somatic hypermutation of the CD19^{+} CD20^{-} CD27^{+}CD38 subset of B-cells and driving disease pathology [16]. The new role of this subset of T-cells in the context of disease also proposes the opportunity for more studies to be conducted in the IgG4RD milieu.

We also sought to shed light on a novel mechanism in the diseases pathophysiology that implicates the presence of a genetic component in the diseases pathology and prognosis. Our cohort's serum levels for IgG1, IgG4, IgE, and Absolute Eosinophils provided a representative range that we would expect to see in the IgG4RD patient population. Additionally, the trends that we have noticed in our population, with regards to snp frequency, and disease prognosis, are also consistent with current literature.

Our 48 patient cohort study suggests that there is a genetic component associated with the prognosis of patients with IgG4RD. In concordance with current literature, 10% of our cohort was positive for the presence of a snp in the FOXO3A gene which conferred to milder prognosis. Patients' with a snp serum levels of IgG1, IgG4, IgE, and Absolute eosinophils predominately did not fall within the upper quartile, which is associated with relapse of disease [23,30]. Mechanistically, we hypothesize that this transcription factor is affecting our system in the same fashion, exhibited in Smith's GWAS paper. The presence of a snp increases the rate of transcription, which can

activate certain cytokine dependent pathways resulting in an increase/decrease in the secretion of cytokines. Ultimately these cytokines exhort their functions in the IgG4RD milieu and can lead to change in the concentration of antibodies, plasmablast, and prevalence of class switching.

3.3 Project Limitations

The specificity of our assay is a limiting aspect of our system. Our Luciferase-protein fusion vectors are constructed based on results from PLATO and therefore, in theory, it's possible that our results may not be completely representative of all of the proteins that may be autoantigens in patients with IgG4RD. Our system of identifying autoantigens isn't all-inclusive, but it is a good start to learning about the pathophysiology of the disease.

Our genetic analysis of patients with IgG4RD invites us to conclude that patients who are positive for a snp in FOXO3A gene will have a milder prognosis. While our findings support literature and follow published trends, our cohort is too small to make definitive conclusions. Statistical significance and trends can not be assumed based on our small sample size. We can however demonstrate that our data supports published literature. In the future we will be increasing our cohort size so trends, if any, can be identified.

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