Anti-Galectin-3 and Anti-Cytokine Antibodies in IgG4-Related Disease

ANTI-GALECTIN-3 AND ANTI-CYTOKINE ANTIBODIES IN IgG4-RELATED DISEASE

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Abstract

IgG4-Related Disease (IgG4-RD) is a chronic, immune-mediated disease that typically manifests as tumor-like enlargement of various organs. Prior work suggests a role for plasmablasts and CD4+ cytotoxic T lymphocytes (CD4+ CTLs), both of which are found in the tissue at high numbers, decline in response to therapy and demonstrate clonally-restricted antigen receptors. However, the antigens that drive the clonal proliferation of these B and T cell types remain largely unknown.

Studies employing Next Generation Sequencing, single cell cloning and quantitative mass spectrometry have identified galectin-3 as a dominant auto-antigen in IgG4-RD. Here, we have expanded this work to examine the frequency of anti-galectin-3 auto-antibodies and quantify plasma galectin-3 levels in a large, clinically diverse cohort of IgG4-RD patients. Anti-galectin-3 auto-antibodies were detected in 6/121 (5%) of the cohort. Five of these 6 subjects were also found to have anti-galectin-3 antibodies of the IgG4 subclass. 15.5% of the subjects were found to have elevated galectin-3 levels in the plasma. Of those subjects with increased plasma galectin-3, 14.3% had concurrent anti-galectin-3 auto-antibodies compared with only 4.7% of those subjects with normal galectin-3 levels. We have also studied the presence of anti-galectin-3 auto-antibodies in 3 of the subjects longitudinally to correlate with disease activity and treatment status. Finally, we have analyzed laboratory parameters and organ manifestations for correlation with elevated galectin-3 levels.

As an alternative antigen discovery approach to that mentioned above, a cytokine microarray has been used to test the plasma from a limited cohort of 13 IgG4-RD subjects. Of the culprit "hits," anti-IL-6, anti-CXCL1 and anti-CCL23 antibodies were confirmed by ELISA in the same cohort. Additionally, a monoclonal antibody from one of these subjects was found to bind CCL23. The current study aims to validate the presence of anti-IL-6 antibodies, anti-CXCL1 antibodies and anti-CCL23 antibodies in our much larger cohort of 130 IgG4-RD subjects.

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Chapter 1

Background

IgG4-Related Disease

IgG4-Related Disease is an immune mediated condition that involves multiple organs. The most common features of this disease are autoimmune pancreatitis, sialadenitis (particularly of the submandibular gland), dacryoadenitis, and IgG4-related retroperitoneal fibrosis.[1]. IgG4-Related Disease is commonly seen in middle-aged to elderly men.[1]

Diagnosis of this disease is done using histopathology, and the common histopathologic features in this disease are lymphoplasmacytic infiltration, obliterative phlebitis, and storiform fibrosis. Fibrosis to some extent is seen in all cases.[1] Another characteristic feature of this disease is the increased numbers of IgG4 plasma cells in the tissue sites.[1] Tissue biopsy and imaging such as PET (to study the extent of organ involvement) are the tools used to diagnose this disease.[1]

Many organs are involved in this disease including the orbits, salivary glands, ear, nose and throat, thyroid glands, lymph nodes, aorta, retroperitoneum, lungs, kidneys, pancreas etc.[1] A common ophthalmic presentation in this disease is dacryoadenitis which is inflammation of the lacrimal gland.[1] Enlargement of the submandibular and other salivary glands as wells as sialadenitis (inflammation of the salivary glands) is also a presentation of this disease. Other clinical features of this disease include lymphadenopathy, retroperitoneal fibrosis, tubulointerstitial nephritis, and autoimmune pancreatitis.[1]

IgG4-RD patients are treated using glucocorticoids, conventional steroid sparing agents and B-cell depletion.[1] Prednisolone therapy (glucocorticoid) shows rapid improvement in clinical manifestations and this therapy is a common first line treatment in IgG4-RD patients.[1]

Azathioprine, mycophenolate mofetil, and methotrexate are conventional steroid sparing agents used in the treatment of IgG4-RD. Rituximab works by depleting CD20 positive cells (B cells). This in turn depletes the plasma cells that produce IgG4 in IgG4-RD.[1]

An expansion of CD20+ pre-plasmablasts and CD19+CD20- plasmablasts were observed in IgG4-RD lesions.[3] CD19+CD27+CD38hi plasmablasts were expanded in a group of IgG4-RD subjects and major clones make IgG4. Rituximab treatment depletes CD19+ B cells as well since CD20+ B cells are their precursors. Some B cells, especially long-lived plasma cells, survive rituximab therapy, but relapse is associated with new VDJ recombination followed by the fresh generation of plasmablasts. Monoclonal immunoglobulins from IgG4 secreting plasmablasts in IgG4-RD subjects have been shown to be self-reactive.[3]

The pathogenesis of IgG4-RD is not well understood. Fibrosis is a common feature of IgG4-RD, but IgG4 antibodies are non-inflammatory since they do not engage activating Fc receptors or complement.[4] CD4+SLAMF7+ cytotoxic T lymphocytes (CTLs) were clonally expanded in IgG4-RD subjects. These cells were found in the blood as well as in the fibrotic lesions in IgG4-RD.[4] Some functional features of the CD4+SLAMF7+ CTLs are the secretion of IFNγ, IL-1β and TGF-β1. TGF-β1 and IL-1β have an established role in fibrosis.[4] The effect of rituximab therapy on CD4+SLAMF7+CTLs was also studied. Rituximab led to the depletion of the CD4+SLAMF7+CTLs. This observation may be due to B cell mediated maintenance of CD4+T cells or dependence of T cells on B cell derived growth factors.[4]

The presence of CD4+CTLs were confirmed in IgG4-RD lesions in patients with dacryoadenitis and sialadenitis.[2] Also, there was a higher number of CD4+GZMA+ CTLs infiltrating into these tissues which correlated with more number of organs involved as well as increased serum IgG4

concentration.[2] CD4+GZMA+TGF-β1+ CTLs are present in IgG4-Related Dacryoadenitis and sialadenitis. CD4+CTLs may induce fibrosis in IgG4-RD, but the pathogenesis remains unclear.[2]

Hypothesis

Activation of the CD4+T cells by B cells may cause the inflammation and fibrosis seen in IgG4-RD. (*Figure 1)*. The antigens involved in the disease remain unknown. This work aims to explore some antigens that may be involved in IgG4-RD.

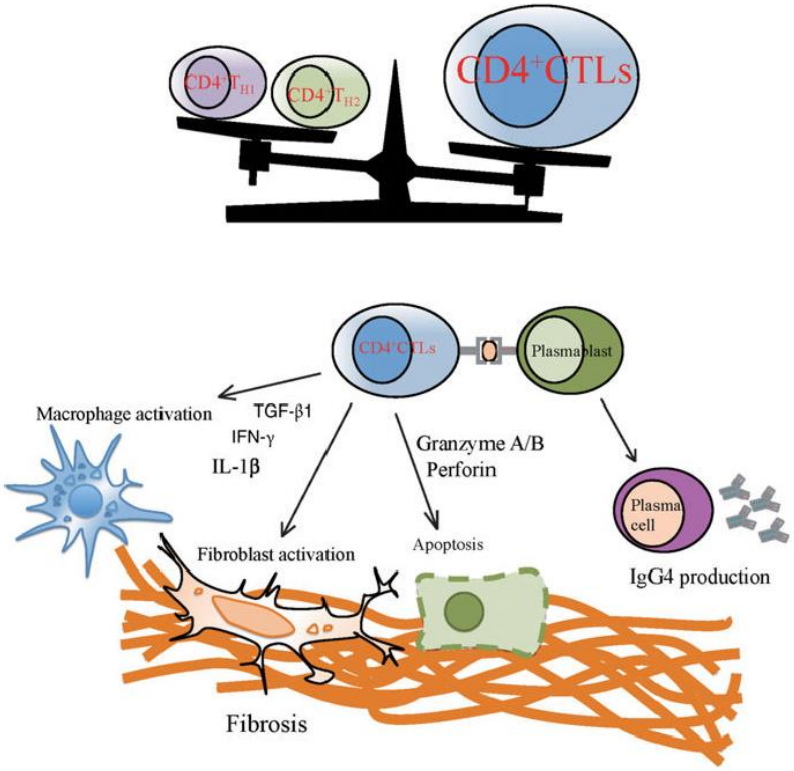


Figure 1: IgG4-Related Disease Model[4]

Identification of Galectin-3 as an auto-antigen in IgG4-RD

Circulating plasmablasts that were expanded in IgG4-RD patients compared to the healthy controls were sorted and their immunoglobulin heavy chain VDJ segments were sequenced by next-generation sequencing.[5] This Next Gen sequencing data suggested that this could be an antigen driven process. This was due to the heavy chain sequencing reads being oligoclonally restricted – mainly 3-5 clones comprising the majority of the plasmablasts. Clones are classified and numbered from 1-5 based on the identical IGH CDR3 amino acid sequences. *(Figure 2).*[5] The same pool of plasmablast was simultaneously cloned into 96 well plates as single cells and both Ig heavy chain and light chains were sequenced from individual cells using PCR and Sanger sequencing. The most frequent clones corresponded to those seen by Next Gen sequencing. The two clones with the highest frequency on Next Gen sequencing were selected. Separate expression vectors including one containing IgG1 constant domains backbone and another the kappa light chain constant domain were used to clone the paired heavy and light chain sequences from these two clones.[5]

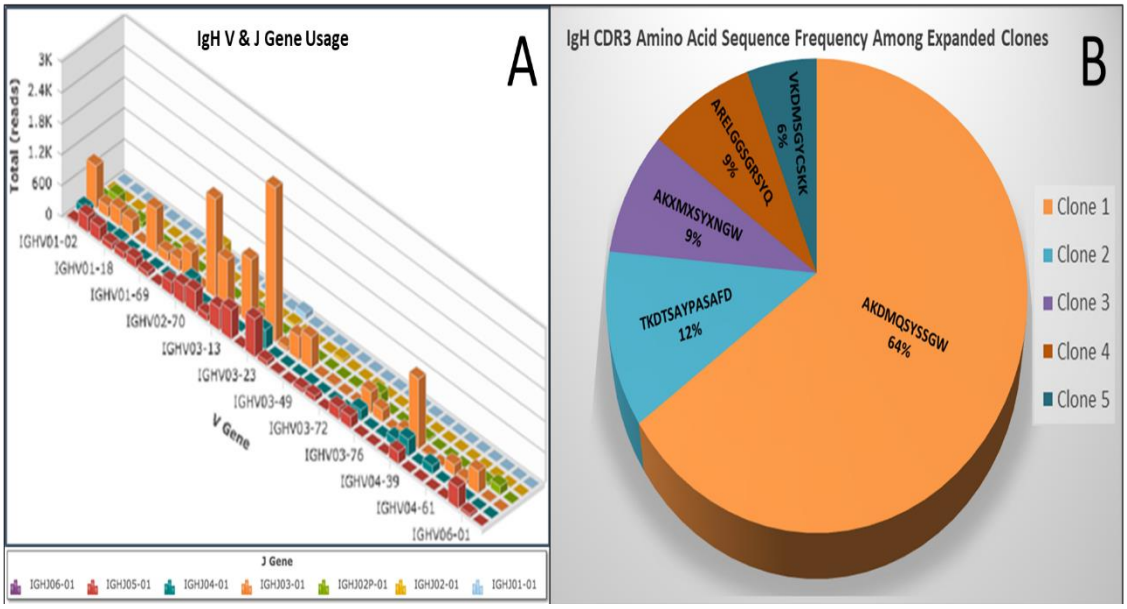


Figure 2: A) Bulk repertoire of sorted plasmablasts IGH V gene usage on x-axis, IGH J gene usage on the z axis and number of sequencing reads on the y-axis. Each bar represents a clonal family. B) Pie-chart showing the frequency of dominantly expanded clones from single cell sequencing. The specific CDR3 amino acid sequence and percentage of wells from the single cell plate are displayed. The dominant clone, labeled "Clone 1" represented 22 of 34 sequenced individual cells.[5]

These monoclonal antibodies were used to identify the location where their cognate antigen was expressed based on immunofluorescence. The patient from whose blood these antibodies were derived had autoimmune pancreatitis and therefore pancreatic tissue was selected for immunofluorescence. The results showed reactivity in the tissues incubated with monoclonal antibodies.[5] *(Figure 3)*

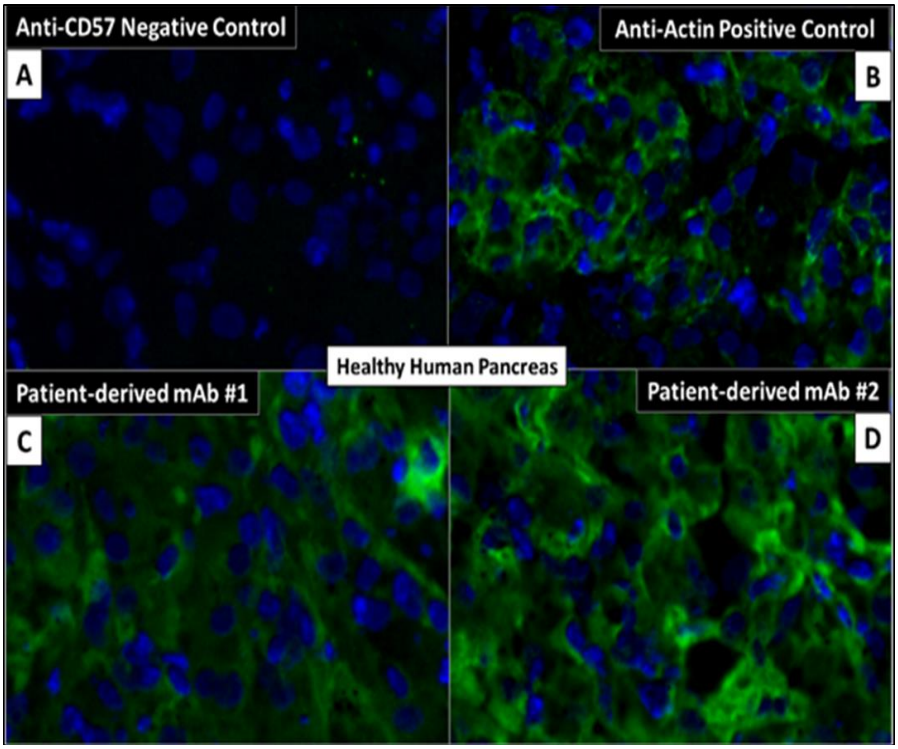
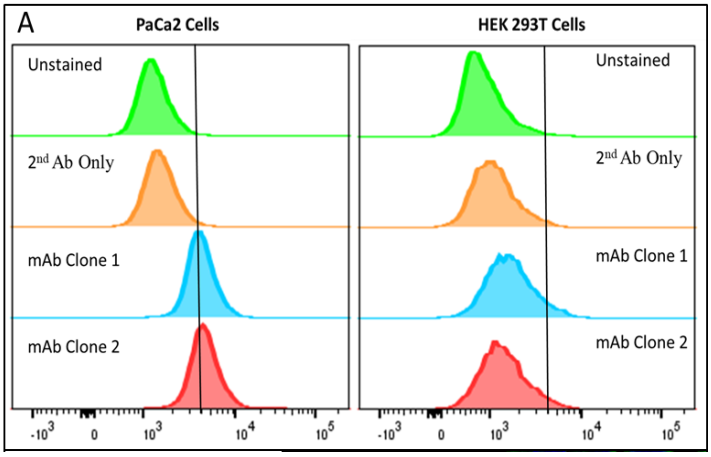


Figure 3: Immunohistochemistry of pancreatic tissue. A-B) Negative(Anti-CD57) and positive(anti-actin) control antibodies. C-D) Dominantly expanded, patient-derived monoclonal antibodies demonstrating intense reactivity.[5]

The pancreatic cancer derived PaCa2 cell line was selected for further experiments. Of the tumor lines tested, the PaCa2 cell line showed greatest reactivity to the patient derived monoclonal antibodies in intracellular flow cytometry data. Immunofluorescent staining of the PaCa2 cell line showed similar reactivity to the monoclonal antibody staining of the tissues.[5] *(Figure 4).*



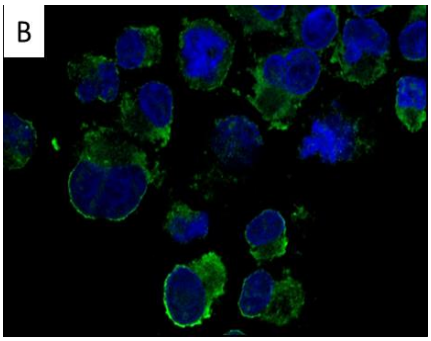


Figure 4: A) Intracellular Flow cytometry showing reactivity of mAbs with PaCa2 cells. Vertical cutoff lines drawn to exclude unstained cells. No difference was observed in HEK293T cells. B) Confocal microscopy of PaCa2 cells stained with biotinylated mAb clone 1 demonstrating a cytosolic staining pattern.[5]

PaCa2 cell lysates were used as source for immunoprecipitation to determine the antigen. After separately covalently attaching the antibodies to protein -A sepharose, the immobilized antibodies were used to affinity- purify their cognate antigens. Following binding and washing off unbound proteins, bound proteins were eluted at low pH, neutralized and the eluate was analyzed by mass spectrometry. Mass spectrometry results showed **Galectin-3** as the protein

which has the highest differential binding for both monoclonal antibodies compared to isotype antibody binding.[5] *(Figure 5)*

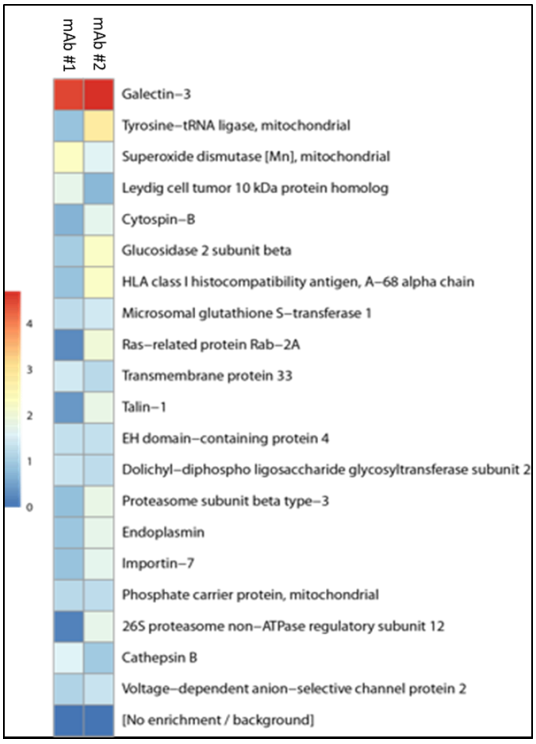


Figure 5: Heat Map showing mass spectrometric results of the protein binding of PaCa2 cell lysate by mAbs. Background was determined using isotype control. Galectin-3 was identified as the antigen with the highest binding affinity for both mAb clones.[5]

Galectin-3

Galectins are β-galactoside binding lectins. Their primary structure is conserved and consists of a carbohydrate recognition domain (CRD) of about 130 amino acids, that binds different self and non-self-glycoconjugates.[6] Galectins preferentially bind glycans which contain N-acetyllactosamine and polylactosamine chains. Due to structural differences in CRDs, the binding specificity of galectins can differ for complex glycoconjugates.[6] Galectins bind to only a

limited number of the large number of galactose-containing glycoproteins. Galectin-binding is not always glycosylation dependent. This suggests a carbohydrate independent function.[6] There are 15 members in the mammalian galectin family that have been identified. The binding specificities and the carbohydrate independent activities for each of the galectins is not understood completely.[6] Galectin-3 lacks a signal sequence for translocation into the endoplasmic reticulum (ER) and it is synthesized on free ribosomes in the cytosol.[9] Galectin-3 has been shown to be secreted from cells by an incompletely understood mechanism called ectocytosis, which is independent of the classical secretory pathway through the ER and Golgi system.[9] A hamster galectin-3 CRD fragment lacking the N-terminal domain is not secreted. Therefore, The N-terminus of galectin-3 has been proposed to contain targeting information for secretion.[9]

Galectin-3 has been studied in various biological processes to understand its role in inflammation. Galectin-3 promotes cell migration by modulating cell-cell adhesion and cell-matrix adhesion. Galectin-3 knockout mice, examined in the context of different disease models show architectural abnormalities in granulomas, tumor stroma and in the intestinal epithelium.[6] The role of galectin-3 has been studied in several autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, primary Sjogren syndrome, systemic sclerosis, polymyositis, dermatomyositis, atopic dermatitis, multiple sclerosis, experimental autoimmune encephalomyelitis, Type 1 diabetes, autoimmune hepatitis, psoriasis and Crohn's disease.[7] A correlation between increased serum levels of galectin-3 and fibrosis of the kidneys has been reported.[8]

In vivo, data shows that in acute inflammation galectin-3 has a pro-inflammatory, protective role whereas in chronic inflammation, galectin-3 plays more of a wound healing, fibrogenic role with

resultant disruption of tissue architecture and organ scarring.[10] In systemic sclerosis, galectin-3 is shown to be an independent predictor of all-cause and cardiovascular mortality.[12] A possible model for how Gal-3 secreted by macrophages and fibroblasts may contribute to tissue fibrosis is shown in figure 6.[11] *(Figure 6).*

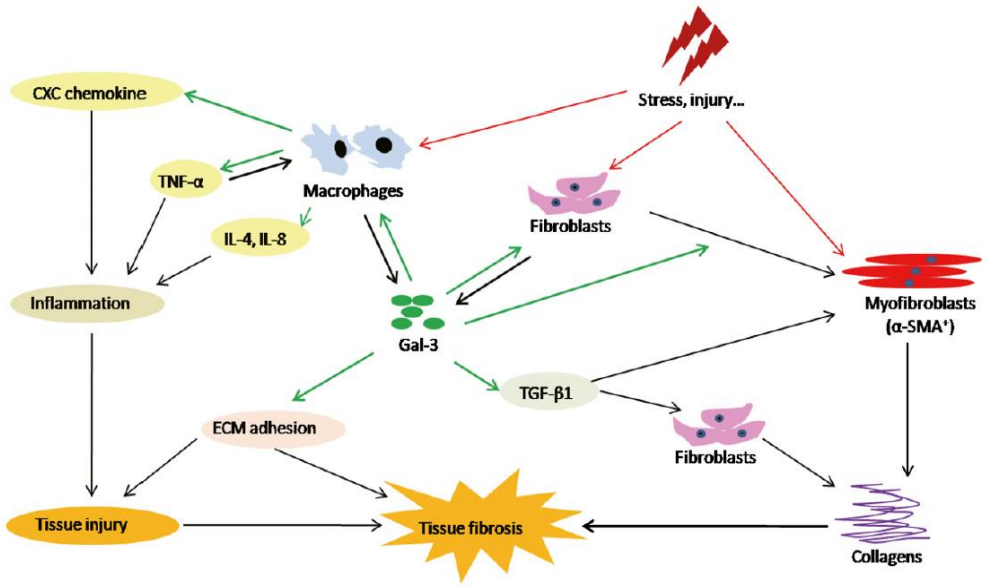


Figure 6: Galectin-3 and its possible contribution to Tissue Fibrosis.[11]

Chapter 2

Materials and Methods

Indirect ELISA

Anti-Galectin-3, Anti-IL-6, Anti-CXCL1 and Anti-CCL23, Anti-Prohibitin Antibodies in IgG4-RD patients were detected using indirect ELISA (Enzyme Linked immunosorbent assay). 96-well Plates were coated with 500 ng/well Galectin-3 (Biolegend Recombinant Human Galectin-3) or 178ng/well IL-6 (Peprotech 200-06, Recombinant Human IL-6), CXCL1 (Peprotech 300-11 Recombinant Human GRO-α/MGSA (CXCL1)) , CCL23 (Peprotech 300-29 Recombinant Human MIP-3 (CCL23)) or Prohibitin (Novus Biological NBP2-23364 Recombinant Human Prohibitin Protein).Volume of protein antigen coated was 100 µl per well. The plates were incubated overnight at 4 C. The plates were washed 3 times with 300 µl PBS (Phosphate Buffered Saline)-Tween-20 (Wash Buffer). 300 µl of Blocking Buffer was added to each well. The plates were incubated overnight at 4 C. The plates were washed 3 times with 300 µl wash buffer. 100 µl of Primary antibody was added in each well. The plates were incubated at 4 C overnight. The plates were washed 3 times with 300 µl wash buffer. The secondary antibody was diluted to different concentrations such as 1:2000 for IgG, 1:1000 for IgG1, IgG2, IgG3, IgG4. 100 µl of Secondary Antibody conjugated to HRP (Anti-Human IgG (Invitrogen Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP), (IgG1 (Invitrogen Mouse anti-Human IgG1 Fc, HRP), IgG2 (Invitrogen Mouse anti-Human IgG2 Secondary Antibody, HRP), IgG3 (Invitrogen Mouse anti-Human IgG3 (Heavy chain) Secondary Antibody, HRP), IgG4 (Invitrogen Mouse anti-Human IgG4 Fc Secondary Antibody, HRP) (For subclass ELISA)) was added. The plates were incubated for 2 hours at room temperature. The plates were washed 3

times with 300 µl wash buffer. 100 µl of OPD substrate was added to all the wells and incubated for 30 to 45 minutes. 100 µl of Stop solution (2M Sulphuric Acid) was added to the wells. The plates were read within 15 minutes of adding stop solution. Microplate reader was used to read the plates at OD 492nm.

Sandwich ELISA

Galectin-3 was quantified using Galectin-3 sandwich ELISA kit (R&D systems DGAL30 Quantikine ELISA Human Galectin-3 Immunoassay). Assay procedure was followed as per the kit instructions.

Statistical Analysis

Different statistical tests were used for the analyzing clinical correlations of galectin-3 levels in patients. Fischer Exact Test was used to compare Organ manifestation data with Galectin-3 levels. Mann-Whitney Test was used to compare laboratory parameters with Galectin-3 levels. The ELISA plate readouts were analyzed using Microsoft Excel. The Graphs from ELISA data were plotted using Graph Pad Prism 7.

Chapter 3

Results

Anti-Galectin-3 Antibodies

Anti-Galectin-3 IgG responses in 121 IgG4-RD patients and 50 Healthy donors were studied using indirect ELISA and shown in Figure 7. Positive responses are 2 Standard deviations above the Healthy Donor mean OD 492 nm.

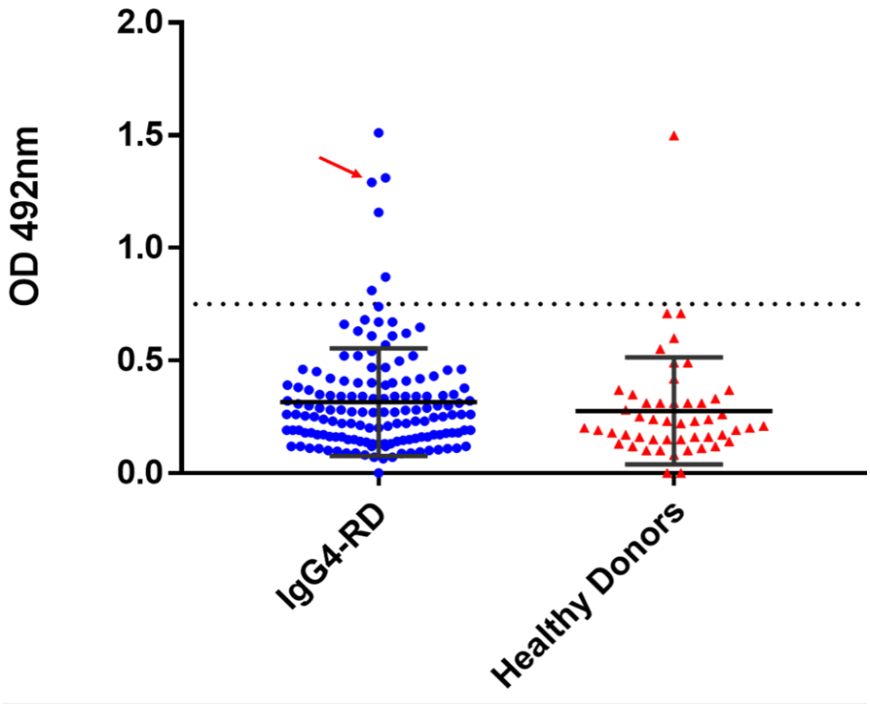


Figure 7: Anti-Galectin-3 Antibodies in IgG4-RD patients. Anti-Galectin-3 antibodies were confirmed with ELISA with 6 out of 121 IgG4-RD patients demonstrating responses including the index subject used to generate mAbs (indicated by small red arrow). Dashed line represents 2 Standard deviations above the Healthy donor mean.[5]

IgG1 specific Anti-Galectin-3 responses in patients with positive Anti-Galectin-3 IgG responses compared with 10 Healthy controls, using indirect ELISA. The results are shown in Figure 8.

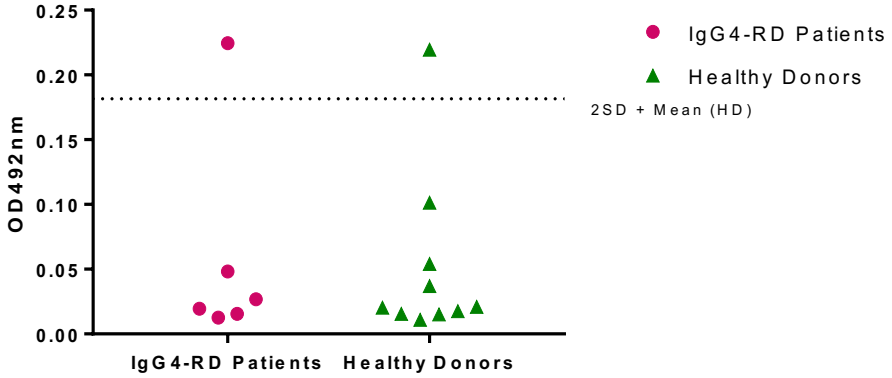


Figure 8: Anti-Galectin-3 IgG1 subclass in IgG4-RD patients. Response to IgG1 specific Anti-Galectin-3 antibody was determined using indirect ELISA. 1 out of 6 IgG4-RD patients have shown response to IgG1 specific Anti-Galetin-3 antibody. Dashed line represents 2 Standard deviations above the Healthy donor mean.[5]

IgG2 specific Anti-Galectin-3 responses in patients with positive Anti-Galectin-3 IgG responses compared with 10 Healthy controls, using indirect ELISA. The results are shown in Figure 9.

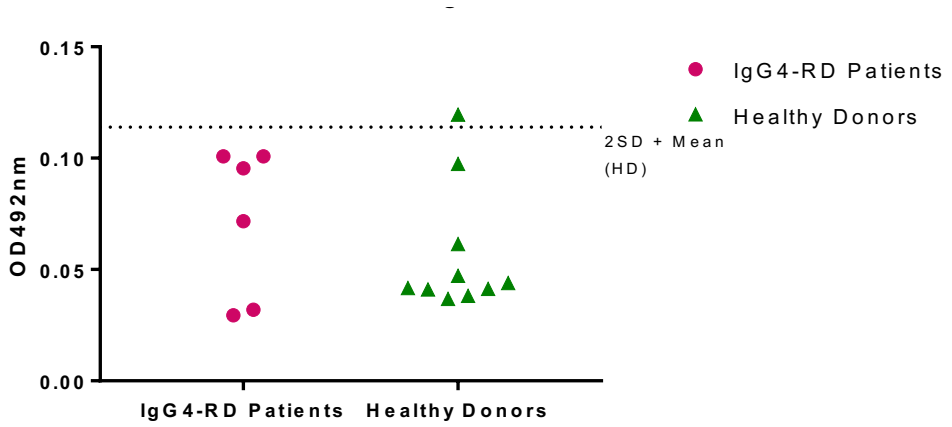


Figure 9: Anti-Galectin-3 IgG2 subclass in IgG4-RD patients. Response to IgG2 specific Anti-Galectin-3 antibody was determined using indirect ELISA. No IgG4-RD patient has shown response to IgG2 specific Anti-Galetin-3 antibody. Dashed line represents 2 Standard deviations above the Healthy donor mean.[5]

IgG3 specific Anti-Galectin-3 responses in patients with positive Anti-Galectin-3 IgG responses compared with 10 Healthy controls, using indirect ELISA. The results are shown in Figure 10.

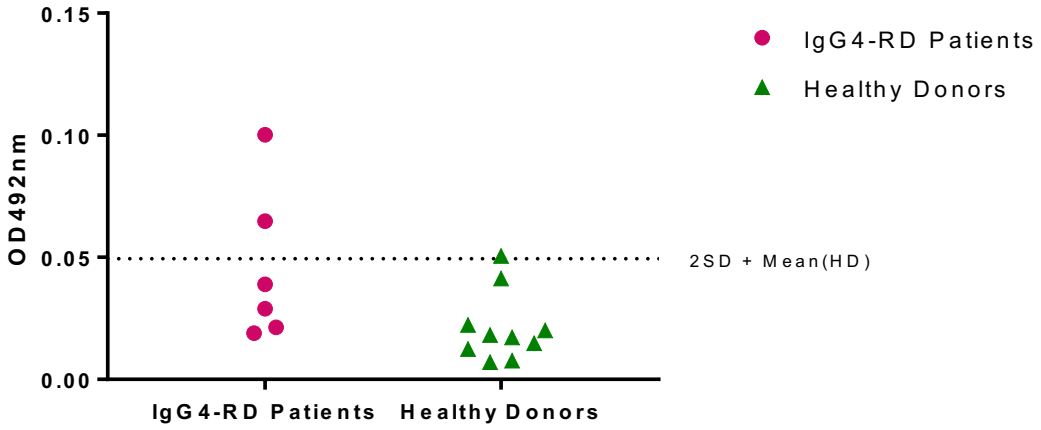


Figure 10: Anti-Galectin-3 IgG3 subclass in IgG4-RD patients. Response to IgG3 specific Anti-Galectin-3 antibody was determined using indirect ELISA. 2 out of 6 IgG4-RD patients have shown response to IgG3 specific Anti-Galetin-3 antibody. Dashed line represents 2 Standard deviations above the Healthy donor mean.[5]

IgG4 specific Anti-Galectin-3 responses in patients with positive Anti-Galectin-3 IgG responses compared with 10 Healthy controls, using indirect ELISA. The results are shown in Figure 11.

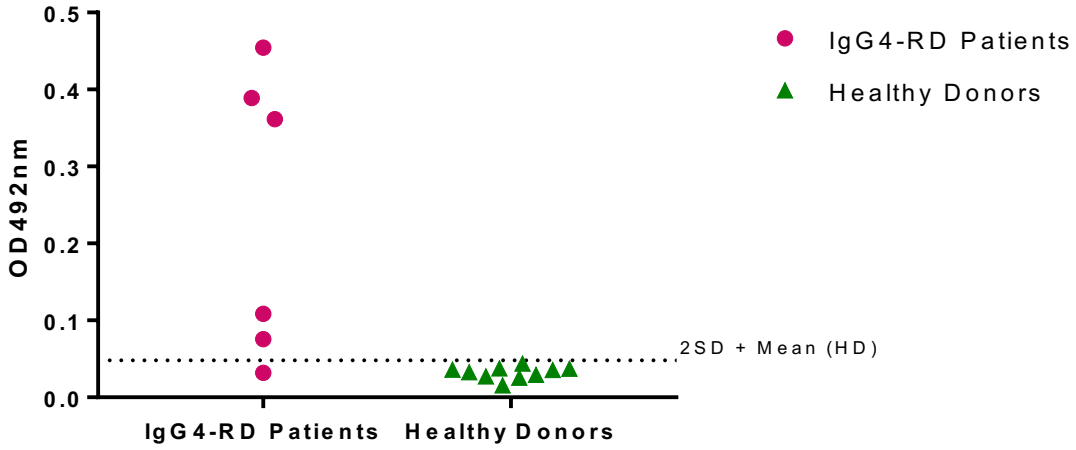


Figure 11: Anti-Galectin-3 IgG4 subclass in IgG4-RD patients. Response to IgG4 specific Anti-Galectin-3 antibody was determined using indirect ELISA. 5 out of 6 IgG4-RD patients have shown response to IgG3 specific Anti-Galetin-3 antibody. Dashed line represents 2 Standard deviations above the Healthy donor mean.[5]

Figure 12 shows Galectin-3 plasma levels in IgG4-RD patients. Galectin-3 was quantified 103 IgG4-RD patients and 50 Healthy Donors using a Galectin-3 sandwich ELISA quantification kit. Galectin-3 levels higher than 2 Standard deviations above the Healthy controls mean Galectin-3 level are high.

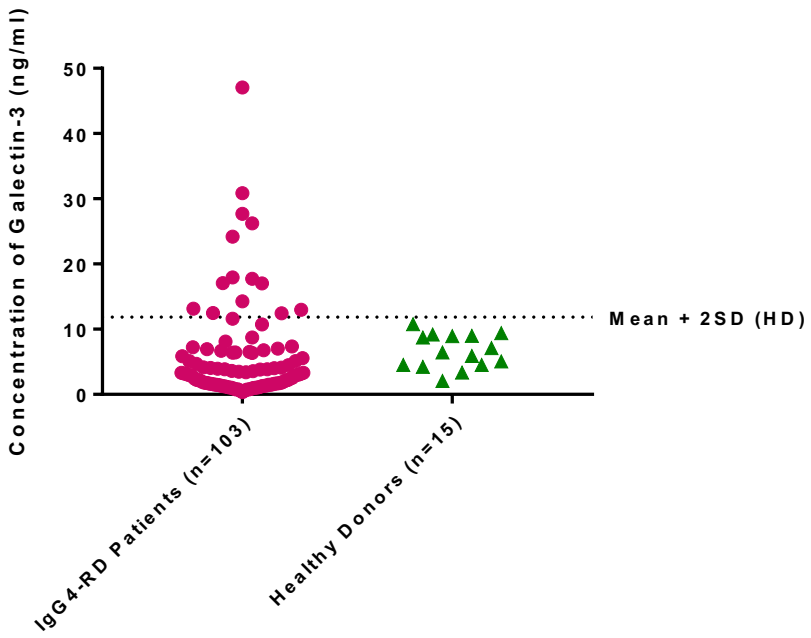


Figure 12: Galectin-3 levels in IgG4-RD patients. Circulating Galectin-3 levels were quantified in 103 IgG4-RD and 15 Healthy Donors using Sandwich ELISA kit. 15.5 % of the IgG4-RD cohort had elevated levels of Galectin-3 compared to healthy donors.[5]

IgG4-RD patients are classified in Figure 13. One group of IgG4-RD patients have Low C3/C4 levels (33 patients) and the other group has normal C3/C4 levels (70 patients). Galectin-3 levels in these two groups of IgG4-RD patients and Healthy Donors are presented in Figure 13 to understand if there is any correlation between C3/C4 levels and Galectin-3 levels.

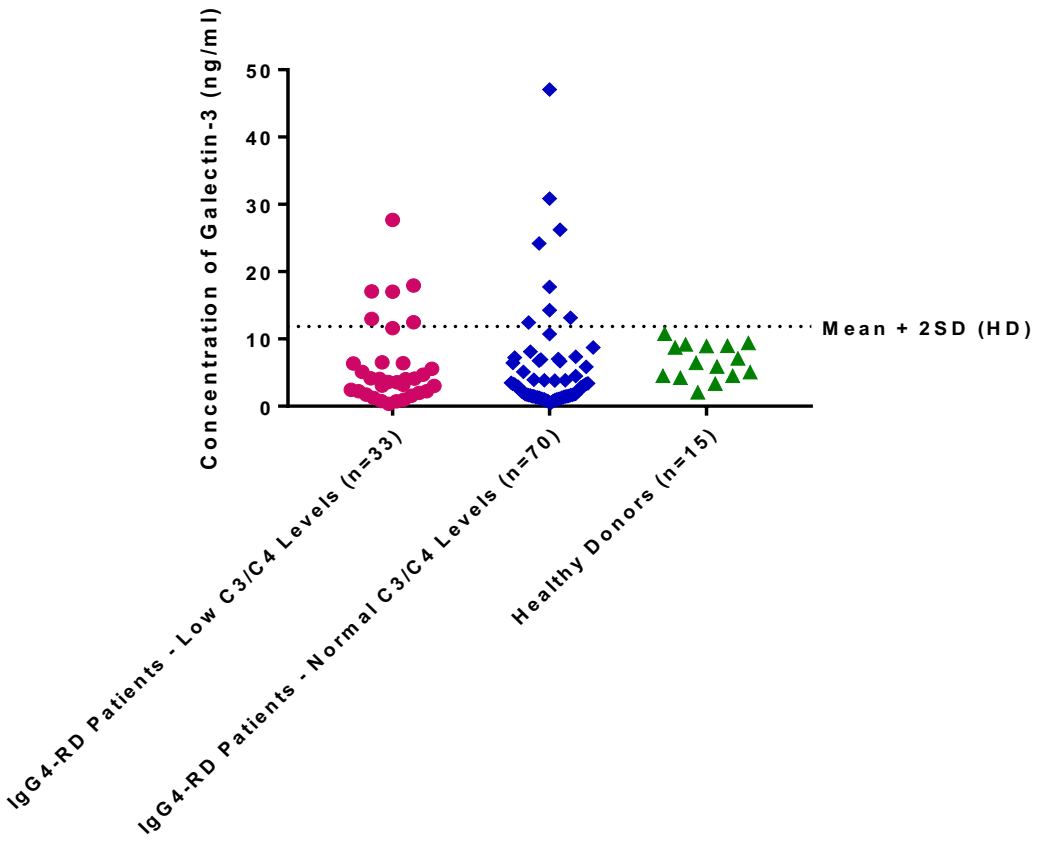


Figure 13: Galectin-3 levels in IgG4-RD patients – separated based on C3/C4 levels.

Circulating Galectin-3 levels were quantified in 33 IgG4-RD patients with low C3/C4 levels, 70 patients with normal C3/C4 levels and 15 age-matched healthy controls. Dashed line represents 2 Standard deviations above the healthy donor mean. There was no correlation between C3/C4 levels and Galectin-3 levels.

Results in Figure 14 show Anti-Galectin-3 IgG antibodies in subjects 365, 336 and 876 at different time points of the plasma collection studied using indirect ELISA. OD 492 nm values (Antibody responses) are being compared to Responder index which has been calculated based on number of organs involved and other laboratory parameters.

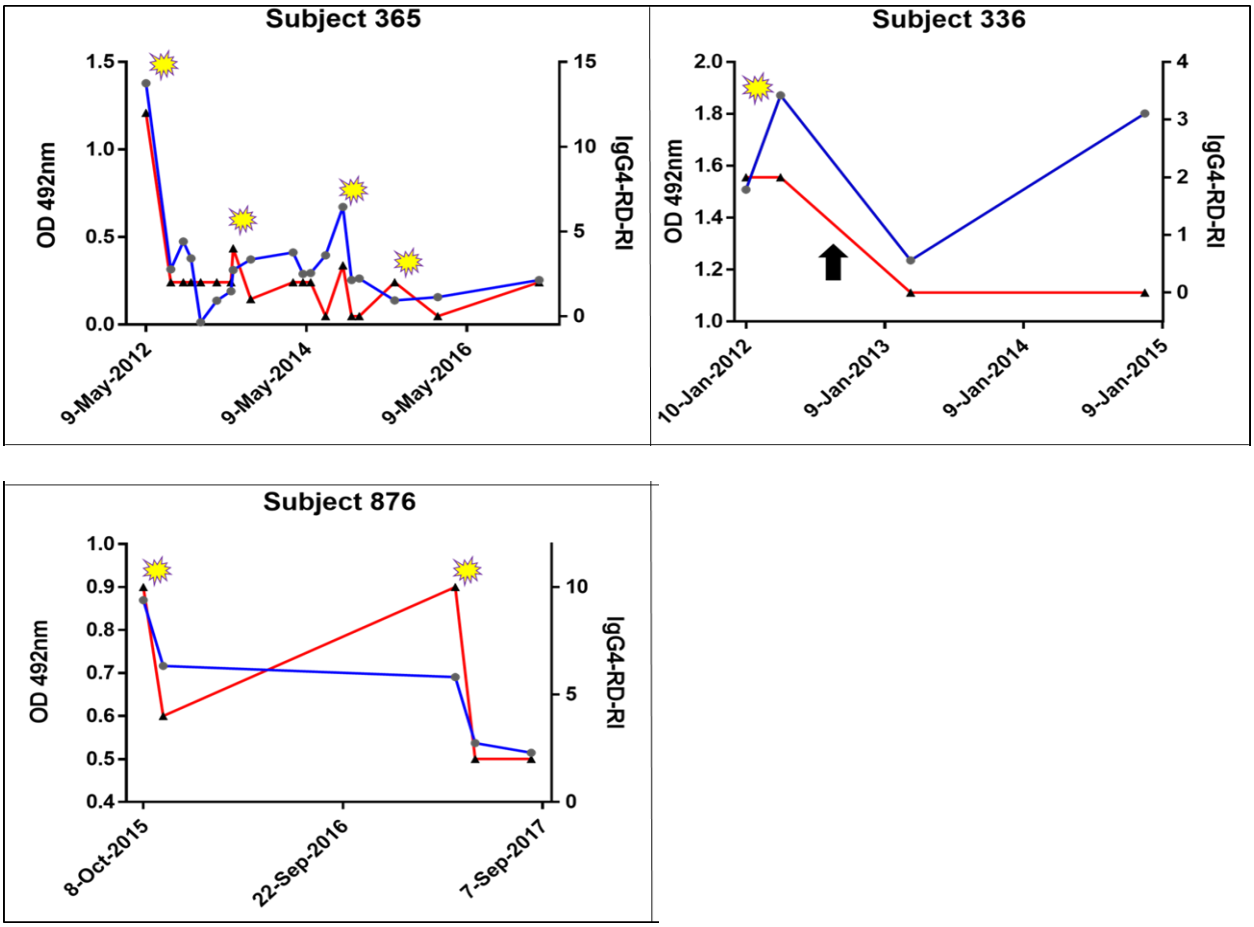


Figure 14: Anti-Galectin-3 Antibodies at Different Time Points. 3 subjects with positive IgG Anti-Galectin-3 antibody responses studied longitudinally by ELISA and plotted against IgG4-RD responder index (RI) on the right y-axis. Yellow symbols indicate rituximab treatment, after which the anti-Galectin-3 antibody titers declined. Black arrow indicates the initiation of low dose prednisone.[5]

Patients were grouped into Galectin-3 normal and high based on the galectin-3 quantification results. Percentage of IgG4-RD patients with different organ involvements are shown in Table 1. Using Fischer Exact test, organ manifestations are compared between IgG4-RD patients with normal and high Galectin-3 levels in Table 1.

Table 1: Clinical Correlations of Organ Manifestations in IgG4-RD patients with high levels of Galectin-3.[5]

Organ Manifestations	Galectin-3 Normal Patients (n=89)	Galectin-3 High (>10ng/ml)(n=16)	p-value (Fisher Exact Test)
Lacrimal glands	22%	38%	0.217285
Salivary glands	58%	44%	0.160526
Biliary tract	17%	13%	1
Pancreas	36%	19%	0.25238
Kidney	21%	25%	0.74766
Lung	28%	0%	0.011034
Retroperitoneum	16%	6%	0.458052

Using Mann-Whitney test, different laboratory parameters are compared between IgG4-RD patients with normal and high Galectin-3 levels in Table 2.

Table 2: Clinical Correlations of Laboratory Parameters in IgG4-RD patients with high levels of Galectin-3.[5]

IQR - Inter quartile range (Q1-Q3)

Laboratory Parameters (Median, IQR)	Galectin-3 Normal Patients	Galectin-3 High (>10ng/ml)	p-value (Mann-Whitney Test)
Total IgG	1448.5(1117.5-1962.5)	1590(1230-4621)	0.17384
IgG1	827(555.5-1058)	872.5(707.15-2067.5)	0.09102
IgG2	513(356.5-690.05)	605(437-1082)	0.101
IgG3	76.4(41.75-160.5)	61(51-128.05)	0.76418
IgG4	267.95(105-547)	299.5(165.1-834.5)	0.29834
C3	105(85.5-142.5)	130.5(59-155.5)	0.92034
C4	23(15-28.5)	20(0-32.5)	0.63122

CRP	4.7(1.5-13.75)	5.4(2.05-8.6)	0.71884
ESR	24(8-55.5)	29(18.5-66.5)	0.16758
IgE	94(28-404)	165(47.5-616)	0.5287
IgM	80(44-140)	64(51.5-91)	0.6818
Anti-Galectin-3 Antibodies	4(4/89)	2(2/16)	

Anti-Cytokine Antibodies

Figure 15 is heatmap from a cytokine microarray showing the Anti-cytokine antibody responses in 13 IgG4-RD patients and 10 Healthy controls.

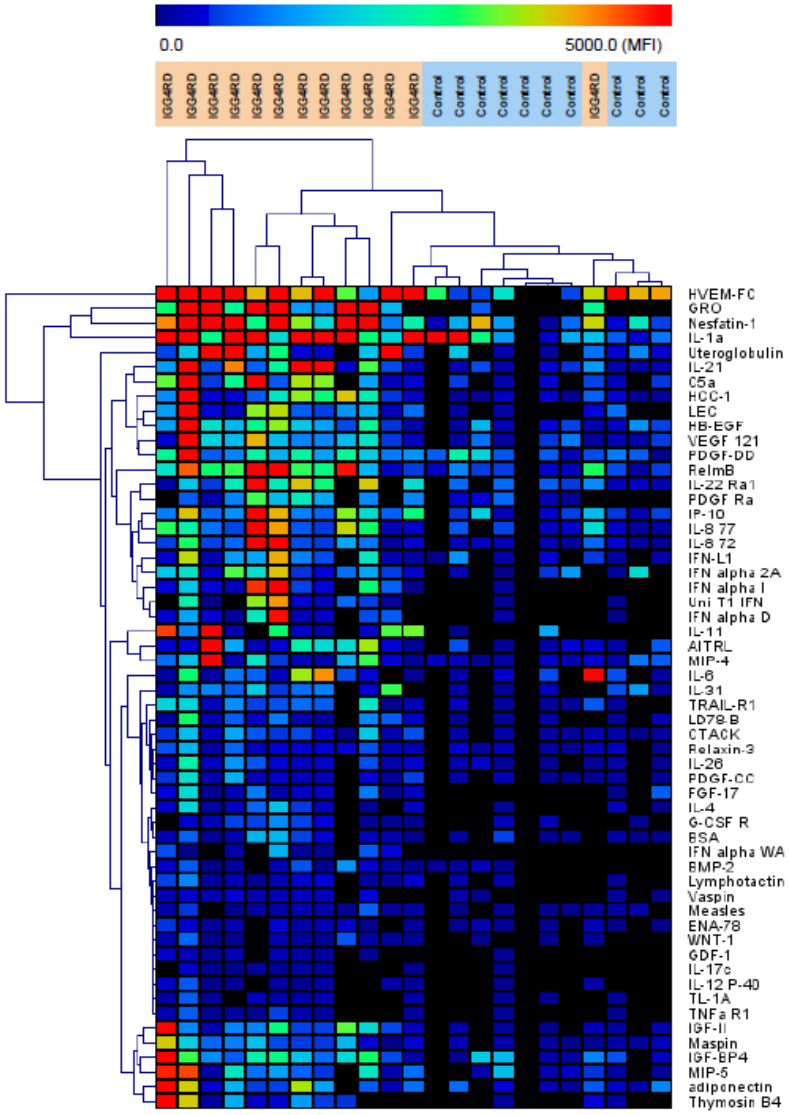


Figure 15: Cytokine microarray showing Anti-Cytokine Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls. Each column corresponds to one patient whereas every row

shows the response to one cytokine/chemokine. Red represents positive response whereas blue represents Black represents no response.

From the heatmap in figure 15, Anti-IL-1a, Anti-IL-4, Anti-IL-17C, Anti-IFNa 2A Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls calculated based on mean fluorescence intensity are shown in figure 16 graphs.

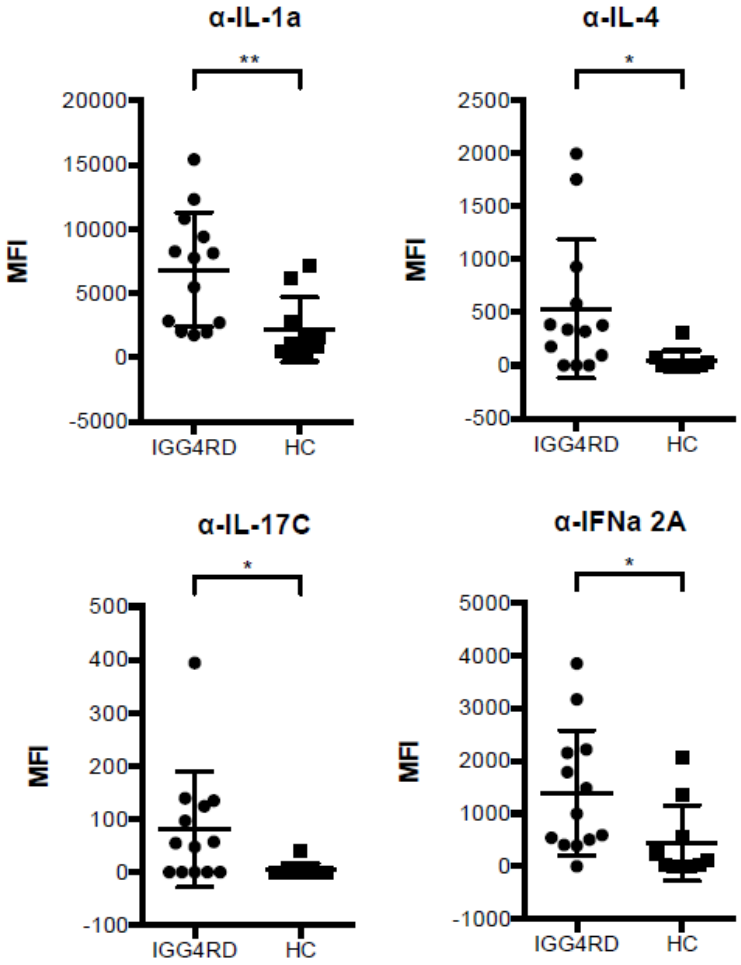


Figure 16: Anti-IL-1α, Anti-IL-4, Anti-IL-17C, Anti-IFNα 2A Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls. Anti-IL-1α, Anti-IL-4, Anti-IL-17C, Anti-IFNα

2A Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls was determined using ELISA and plotted as MFI – Mean Fluorescence Intensity on the y-axis.

From the heatmap in figure 15, Anti-IL-6, Anti-IL-21 Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls calculated based on mean fluorescence intensity are shown in figure 17 graphs.

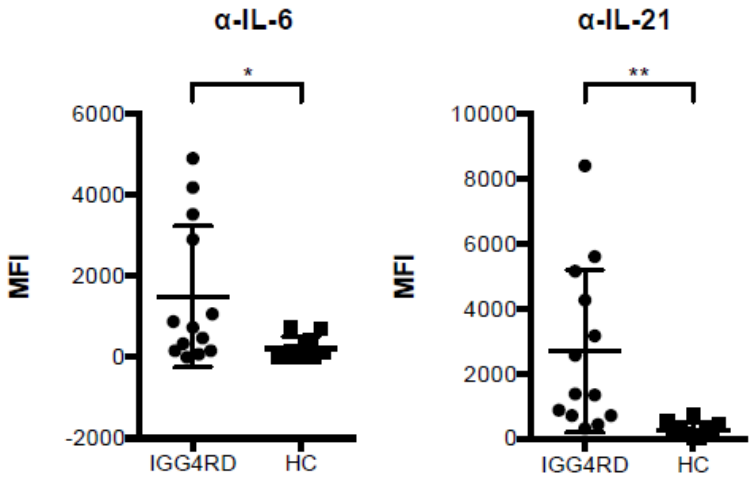


Figure 17: Anti-IL-6, Anti-IL-21 Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls. Anti-IL-6, Anti-IL-21 Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls was determined using ELISA and plotted as MFI – Mean Fluorescence Intensity on the y-axis.

From the cytokine microarray in figure 15, Anti-Gro, Anti-IP-10, Anti-IL-8 77aa, Anti-IGF-II, Anti-HVEM Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls calculated based on mean fluorescence intensity are shown in figure 18 graphs.

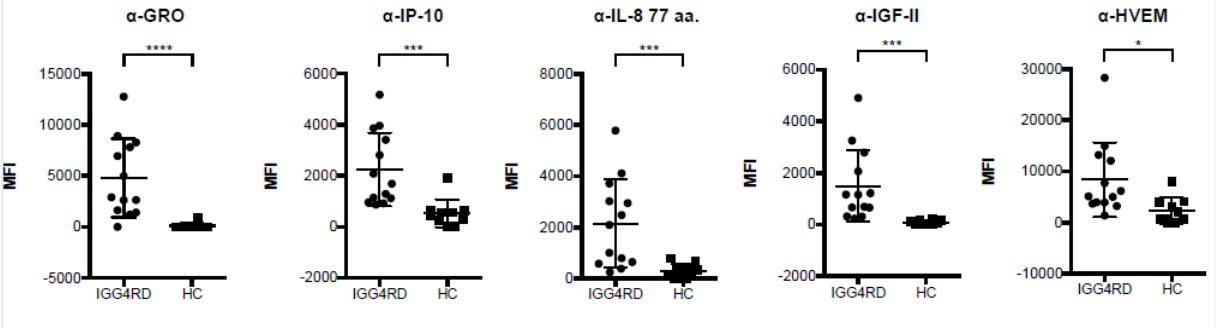


Figure 18: Anti-Gro, Anti-IP-10, Anti-IL-8 77aa, Anti-IGF-II, Anti-HVEM Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls. Anti-Gro, Anti-IP-10, Anti-IL-8 77aa, Anti-IGF-II, Anti-HVEM Antibody responses in 13 IgG4-RD patients and 10 Healthy Controls was determined using ELISA and plotted as MFI – Mean Fluorescence Intensity on the y-axis.

Table 3 shows the anti-cytokine antibody targets grouped based on the p values calculated from the cytokine microarray data.

Table 3: Autoantibody Targets identified in IgG4-RD

* p < 0.05	** p < 0.01	*** p < 0.005	**** p < 0.001
AITRL	C5a	CTACK	GRO
BMP-2	ENA-78	IGF-II	RelmB
G-CSF R	GDF-1	IL-8 77 aa	
HB-EGF	HCC-1	IP-10	
HVEM-FC	IGF-BP4	Nesfatin-1	
IFN alpha 2A	IL-1a	TRAIL R1	
IFN-L1	IL-21	Vaspin	
IL-11	IL-22 Ra 1		
IL-12 p40	Maspin		
IL-17C	Relaxin-3		
IL-26			
IL-4			
IL-6			
IL-8 72 aa			
LD78-B			
LEC			
Lymphotactin			
MIP-5			
PDGF CC			
PDGF DD			
PDGF Ra			
Prokineticin-2			
UNI T1 IFN			
Uteroglobulin			
VEGF 121 aa			
WNT-1			

P-values of unpaired, student's t-test comparing IGG4-RD and healthy controls.

Anti-IL-6 Antibodies

Figure 19 shows Anti-IL6 antibody responses in 13 IgG4-RD patients, 9 Sjogren's syndrome patients, and 10 Healthy controls studied using ELISA.

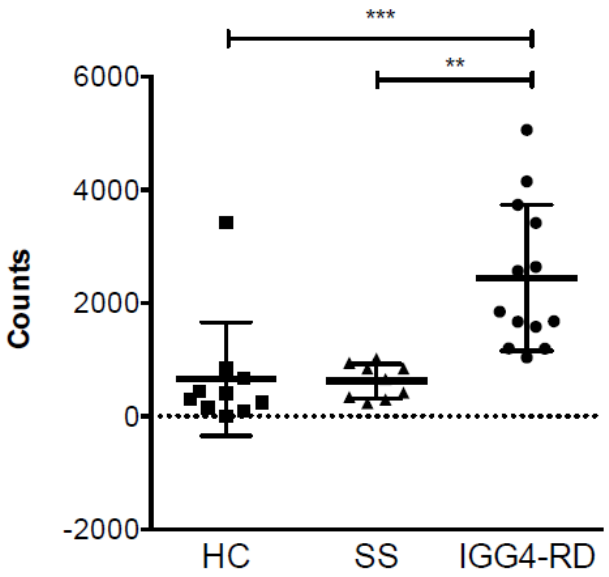


Figure 19: Anti-IL-6 Antibodies detected in a small cohort of IgG4-RD patients. Anti-IL-6 antibody responses studied in 10 Healthy controls, 13 IgG4-RD patients and Sjogren's syndrome patients using ELISA. IgG4-RD patients have demonstrated Anti-IL-6 antibody responses compared to healthy controls and Sjogren's syndrome patients.

Presence of Anti-IL6 antibodies were studied in a large cohort of 131 IgG4-RD patients and 50 Healthy donors using indirect ELISA. Figure 20 shows the result of this study.

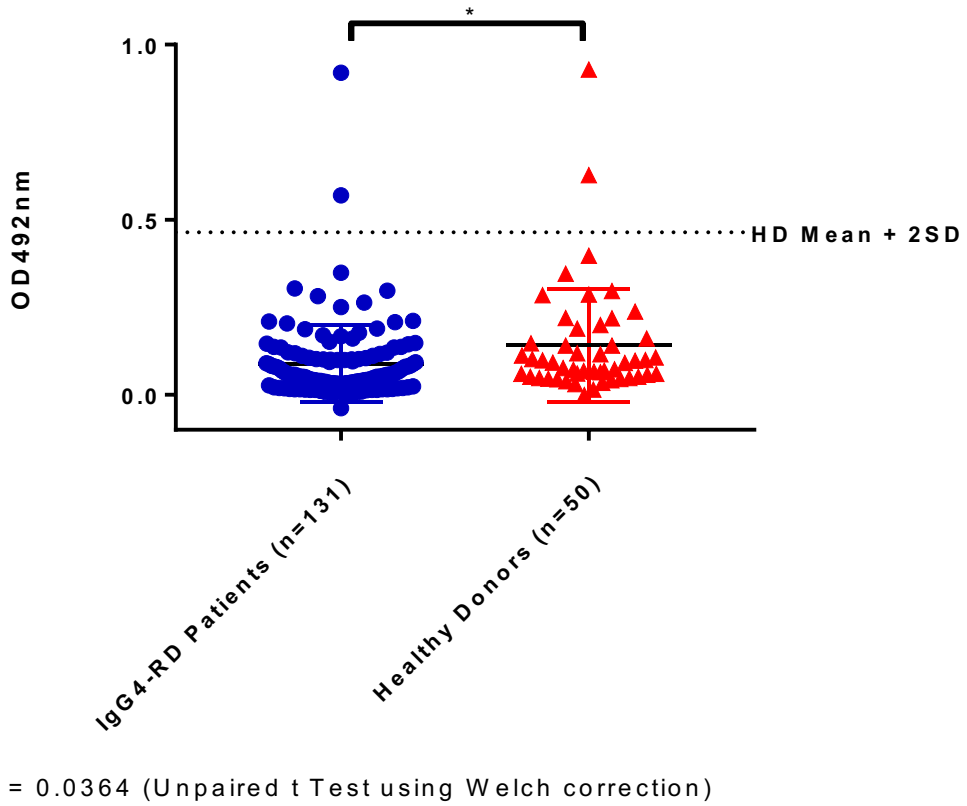
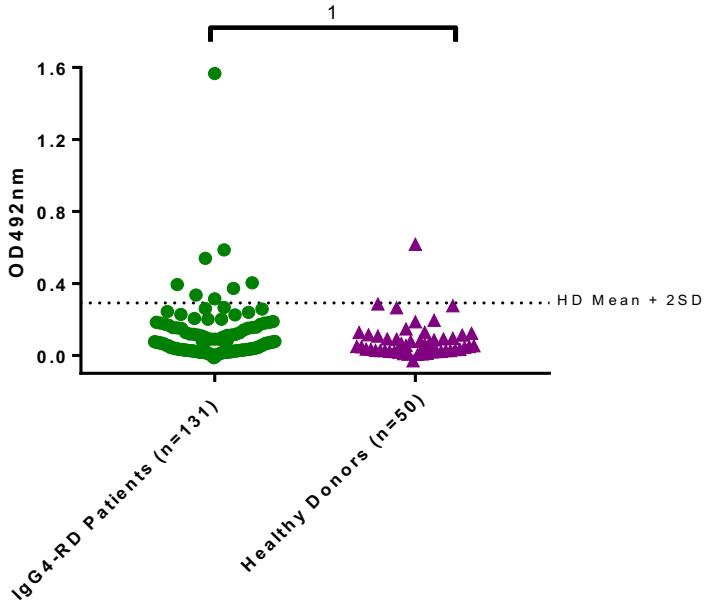


Figure 20: Anti-IL-6 antibody responses in 131 IgG4-RD patients and 50 Healthy Donors. Anti-IL-6 antibody responses were studied using indirect ELISA in 131 IgG4-RD patients and 50 Healthy Donors. 2 out of 131 IgG4-RD patients had shown Anti-IL-6 antibody response. Dashed line represents 2 Standard deviations above the Healthy donor mean.

Anti-CXCL1 Antibodies

Figure 21 shows the presence of Anti-CXCL1 (Gro-Alpha) antibodies studied in a large cohort of 131 IgG4-RD patients and 50 Healthy donors using indirect ELISA.



1 p value = 0.2589 (Unpaired t using Welch correction)

Figure 21: Anti-Groα antibody responses in 131 IgG4-RD patients and 50 Healthy Donors. Anti-Groα antibody responses in 131 IgG4-RD patients and 50 Healthy Donors was studied using Indirect ELISA. 8 out of 131 IgG4-RD patients had shown Anti-Groα antibody response. Dashed line represents 2 Standard deviations above the Healthy donor mean.

Anti-CCL23(MIP-3) Antibodies

Figure 22 shows Anti-CCL23 antibody responses in 13 IgG4-RD patients, 9 Sjogren's syndrome patients, and 10 Healthy controls studied using ELISA.

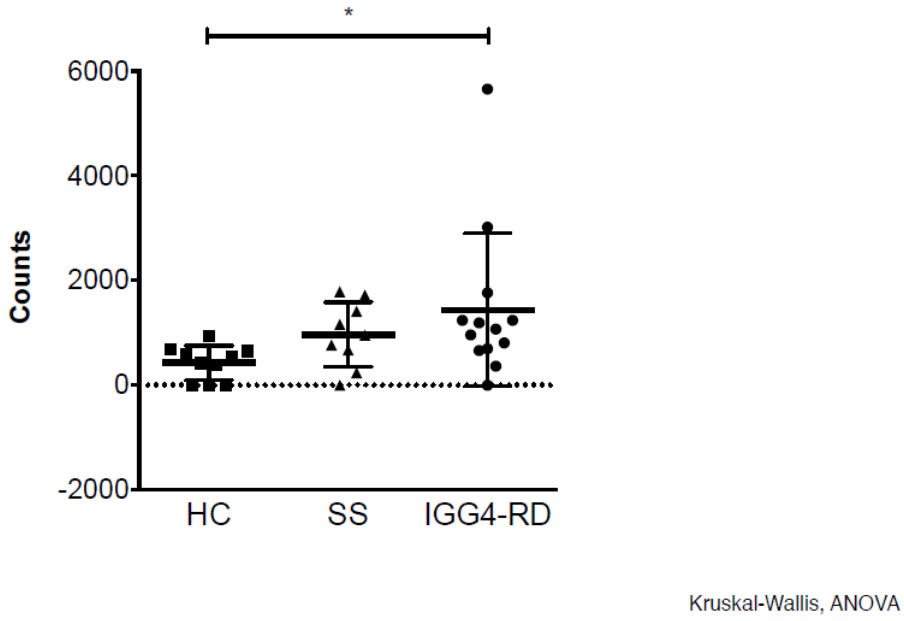


Figure 22: Anti-CCL23 Antibodies detected in a small cohort of IgG4-RD patients. Anti-CCL23 antibody responses studied in 10 Healthy controls, 13 IgG4-RD patients and Sjogren's syndrome patients using ELISA. IgG4-RD patients have demonstrated Anti-CCL23 antibody responses compared to healthy controls and Sjogren's syndrome patients.

Figure 23 shows the presence of Anti-CCL23 antibodies studied in a large cohort of 131 IgG4-RD patients and 50 Healthy donors using indirect ELISA.

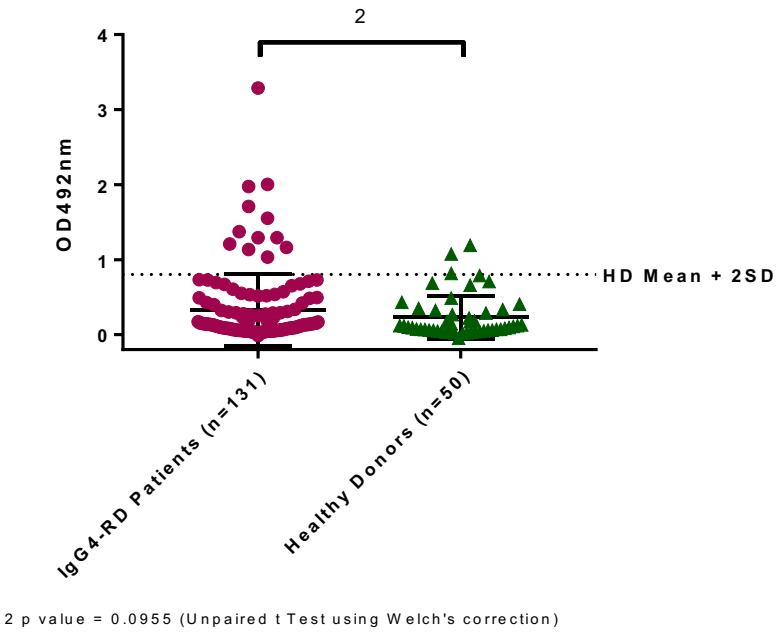


Figure 23: Anti-CCL23 antibody responses in 131 IgG4-RD patients and 50 Healthy Donors. Anti-CCL23 antibody responses in 131 IgG4-RD patients and 50 Healthy Donors was studied using Indirect ELISA. 12 out of 131 IgG4-RD patients have demonstrated Anti-CCL23 antibody response. Dashed line represents 2 Standard deviations above the Healthy donor mean.

Figure 24 shows the Anti-MIP-3 response of an IgG4-RD patient derived monoclonal antibody via ELISA

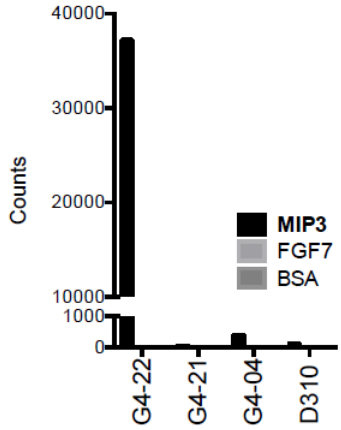


Figure 24: Anti-MIP-3 specific patient-derived monoclonal antibody response. Out of 3 IgG4-RD patients (G4-22, G4-21, G4-04) and a control (D310). Monoclonal antibody from an IgG4-RD patient (G4-22) shows Anti-MIP-3(CCL23) antibody response using ELISA. The monoclonal antibody does not show any anti-FGF7 or anti-BSA antibody response.

Figure 25 shows the Anti-MIP-3 responses of 3 IgG4-RD patient derived monoclonal antibodies from cytokine microarray.

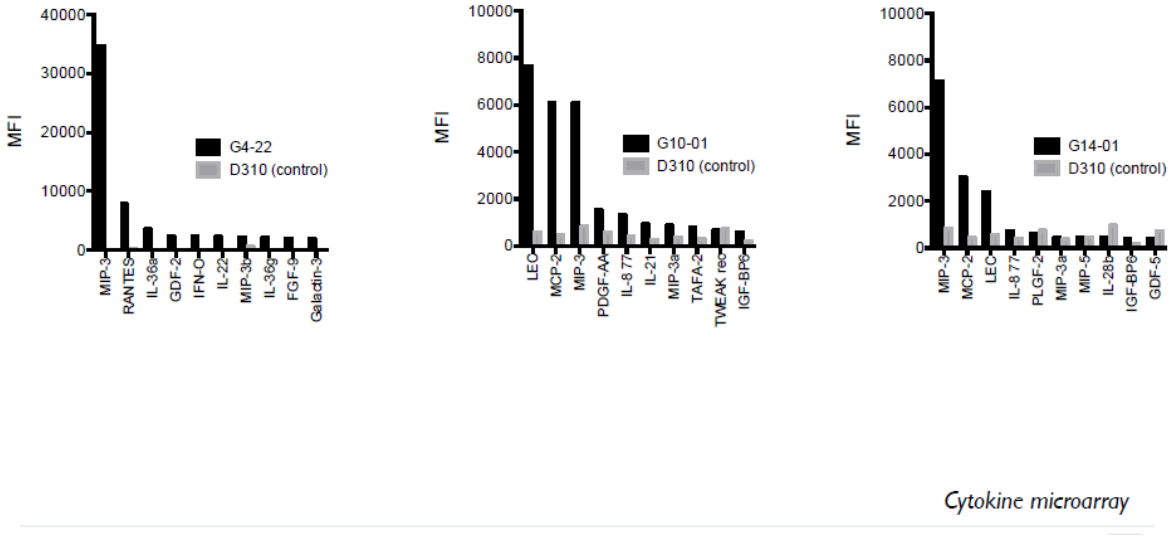


Figure 25: Anti-Cytokine antibody responses of 3 IgG4-RD patients (G4-22, G4-21, G4-04) and a control (D310). All the 3 IgG4-RD patients (G4-22, G4-21, G4-04) show Anti-MIP-3 antibody response compared to the control (D310) which does not have an Anti-MIP-3 antibody response. This graph was plotted based on the cytokine microarray from figure 15.

Figure 26 shows the Anti-MIP-3 responses of 3 IgG4-RD patient derived monoclonal antibodies via ELISA.

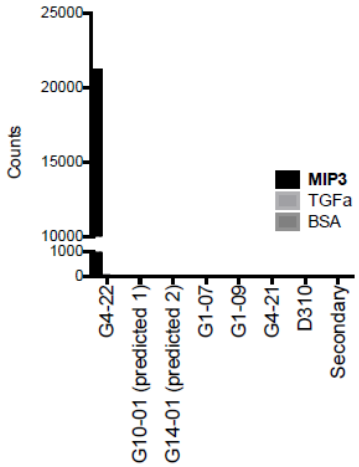
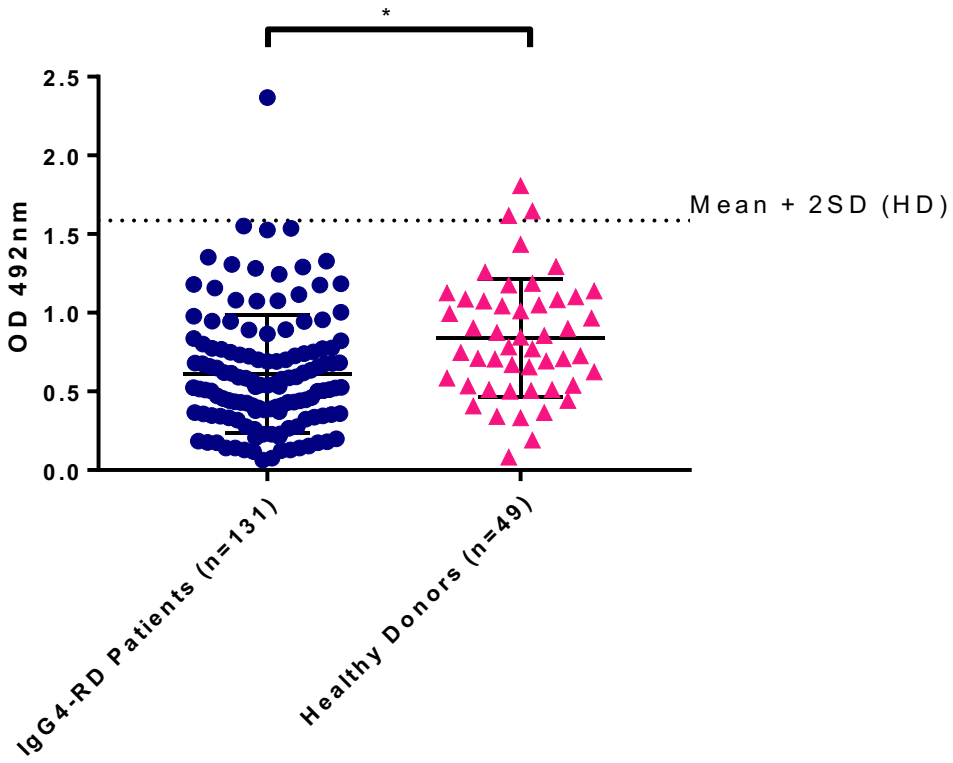


Figure 26: Anti-MIP-3 response shown by 1 out of 3 IgG4-RD patient-derived monoclonal antibody. Anti-MIP-3, Anti-TGFα and Anti-BSA(controls) antibody responses of 5 IgG4-RD patients and 1 healthy control was studied using ELISA. 1 out 5 IgG4-RD patients (G4-22) shows Anti-MIP-3 antibody response.

Anti-Prohibitin Antibodies

Anti-Prohibitin IgG responses in 131 IgG4-RD patients and 49 Healthy donors were studied using indirect ELISA and shown in Figure 27. Positive responses are 2 Standard deviations above the Healthy Donor mean OD 492 nm.



* p value = 0.0005 (Unpaired t Test using Welch correction)

Figure 27: Anti-Prohibitin antibody responses in 130 IgG4-RD patients and 49 Healthy Donors. Anti-Prohibitin antibody responses were studied using indirect ELISA in 130 IgG4-RD patients and 50 Healthy Donors. 1 out of 130 IgG4-RD patients had shown Anti-Prohibitin antibody response. Dashed line represents 2 Standard deviations above the Healthy donor mean.

IgG4 and IgE Anti-Galectin-3 Antibodies

IgG4 Anti-Galectin-3 antibody responses were studied in 121 IgG4-RD patients, 45 IPF (Idiopathic pulmonary fibrosis) Patients and 50 Healthy donors using Indirect ELISA. Figure 28 shows the presence of IgG4 specific Anti-Galectin-3 antibodies in IgG4-RD patients.

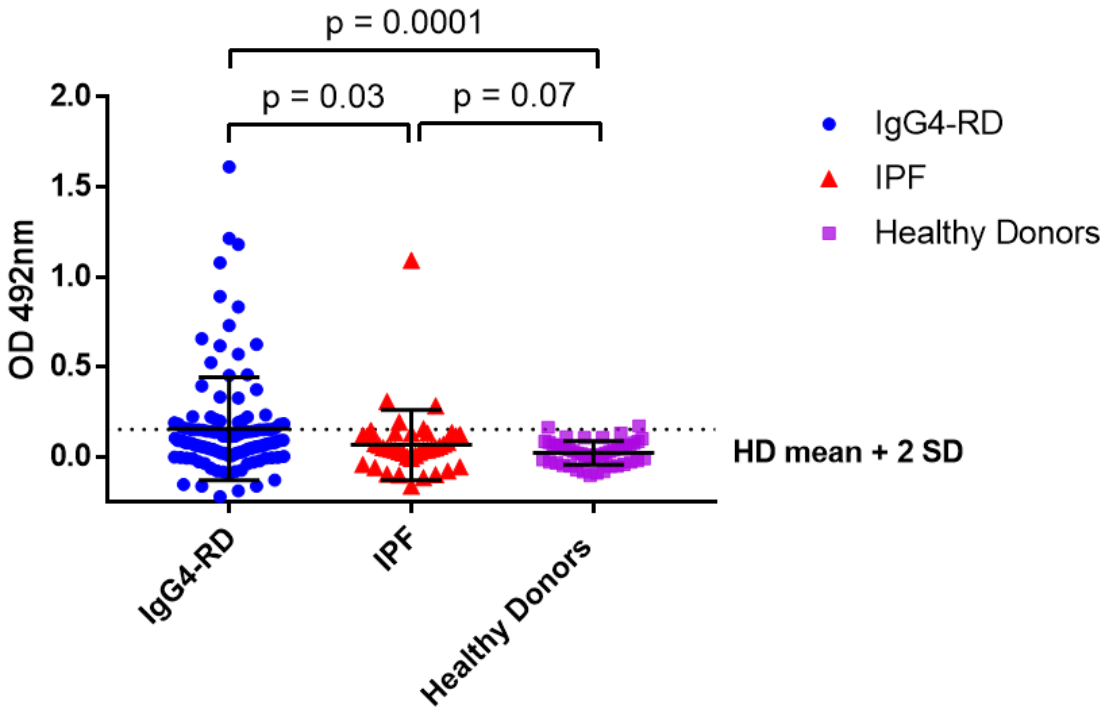


Figure 28: IgG4 specific Anti-Galectin-3 antibody responses in 121 IgG4-RD patients, 45 IPF patients and 50 Healthy Donors. IgG4 specific Anti-Galectin-3 antibody responses in 121 IgG4-RD patients, 45 IPF patients and 50 Healthy Donors was studied using indirect ELISA. 28% of IgG4-RD patients in this cohort are positive for IgG4 specific Anti-Galectin-3 antibodies. Dashed line represents 2 Standard deviations above the Healthy donor mean.[5]

IgE Anti-Galectin-3 antibody responses were studied in 121 IgG4-RD patients, 45 IPF (Idiopathic pulmonary fibrosis) Patients and 50 Healthy donors using Indirect ELISA. Figure 28 shows the presence of IgE specific Anti-Galectin-3 antibodies in IgG4-RD patients.

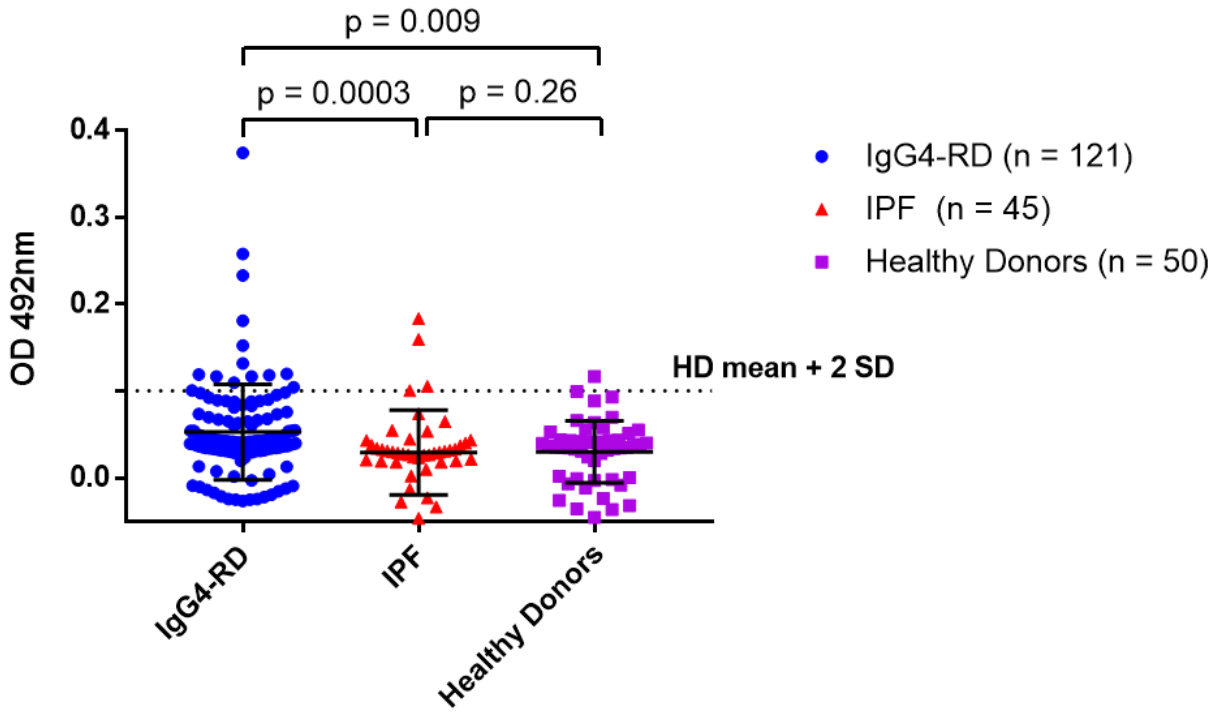


Figure 29: IgE specific Anti-Galectin-3 antibody responses in 121 IgG4-RD patients, 45 IPF patients and 50 Healthy Donors. IgE specific Anti-Galectin-3 antibody responses in 121 IgG4-RD patients, 45 IPF patients and 50 Healthy Donors was studied using indirect ELISA. 10.7% of IgG4-RD patients in this cohort are positive for IgE specific Anti-Galectin-3 antibodies. Dashed line represents 2 Standard deviations above the Healthy donor mean.[5]

3.1 Brief Discussion

Previous work in the lab identified Galectin-3 as a novel autoantigen in IgG4-RD.[5] The presence of Anti-Galectin-3 Antibodies in a large cohort of IgG4-RD patients compared to healthy controls was determined. Anti-Galectin-3 antibody subclass in the positive Anti-Galectin-3 patients was studied. Galectin-3 levels in IgG4-RD patient cohort were quantified and compared to healthy controls. Organ Manifestations and Laboratory parameters were compared to Galectin-3 levels in patients and shown in Table 1 and Table 2.

A Cytokine microarray was used to study Anti-cytokine antibody responses in 13 IgG4-RD patients. Based on this, 3 cytokine/chemokines were chosen for further study. The 3 cytokine/chemokines that were chosen are IL-6, CXCL1(Gro-Alpha) and CCL23(MIP-3). Anti cytokine/chemokines Antibodies were studied in 131 IgG4-RD and compared to 50 Healthy donors.

Additionally, Presence of Anti-Prohibitin antibodies in IgG4-RD patients was also studied since Prohibitin was identified as an antigen in IgG4-RD.[23]

Chapter 4

Discussion

Anti-galectin-3 auto-antibodies were detected in 6/121 (5%) of the cohort. *(Figure 7)* Five of these 6 subjects were also found to have anti-galectin-3 antibodies of the IgG4 subclass. *(Figure 11).* The number is higher when IgG4 specific Anti-Galectin-3 antibodies were studied in 121 IgG4-RD patients. 28% of the cohort was found to have IgG4 specific Anti-Galectin-3 antibodies. *(Figure 28).* High levels of galectin-3 were seen in IgG4 related pancreatitis.[13]. 15.5% of the subjects were found to have elevated galectin-3 levels in the IgG4-RD patient plasma. *(Figure 12).* Of those subjects with increased plasma galectin-3, 14.3% had concurrent anti-galectin-3 auto-antibodies compared with only 4.7% of those subjects with normal galectin-3 levels. *(Figure 12).* There was no correlation between the C3/C4 levels of the patients and elevated galectin-3 levels. *(Figure 13).* Anti-Galectin-3 antibodies of the IgG1, IgG2 and IgG3 subclasses were also studied. *(Figure 8, Figure 9, Figure 10).* 1 patient out of 6 patients who had Anti-Galectin-3 antibodies had IgG1 subclass Anti-Galectin-3 Antibody. *(Figure 8).* No patient had IgG2 Anti-Galectin-3 antibodies. *(Figure 9)*, while 3 out of 6 patients have IgG3 Anti-Galectin-3 Antibody. *(Figure 10).* In addition to IgG and its subclasses , IgE specific Anti-Galectin-3 antibodies were studied in 121 IgG4-RD patients. 10.7 % of the IgG4-RD cohort is positive for IgE specific Anti-Galectin-3 antibodies. *(Figure 29).* Anti-Galectin-3 Antibodies were studied at different time points using ELISA and the antibody levels were compared to the Anti-CD-20 time point and IgG4-RD Responder index. Anti-Galectin-3 antibody levels declined after the Anti-CD20 treatment, which also corresponds to the responder index. *(Figure 14).* Correlation between organ involvement and galectin-3 levels were studied using Fischer exact test, and only a correlation between lung involvement and galectin-3 level was significant.

(Table 1). Clinical correlation between different laboratory parameters and galectin-3 levels were studied using Mann Whitney test, and there was no correlation between laboratory parameters and galectin-3 level. *(Table 2).*

Another approach to study antibodies in IgG4-RD is the cytokine microarray. The heat map from the cytokine microarray shows the Anti-cytokine responses of 13 IgG4-RD patients and 10 Healthy Controls. *(Figure 15).* The different autoantibody targets identified in the cytokine microarray are shown in Table 3. *(Table 3)*

Interleukin-6 (IL-6) is a pro-inflammatory cytokine. The pathogenesis of several autoimmune and chronic inflammatory diseases involves IL-6. Over-production of IL-6 is seen in several autoimmune diseases.[14] An anti-IL-6R monoclonal antibody - tocilizumab is a humanized anti-IL-6R monoclonal Ab of the IgG1 class. Tocilizumab is an approved treatment for Rheumatoid arthritis.[15] Tocilizumab is also in clinical trials for treatment of various other autoimmune diseases.[16] Anti-IL-6 Antibodies were detected in a small cohort of IgG4-RD patients. *(Figure 19)*. Based on this initial data, Anti-IL-6 antibody ELISA was performed in 131 IgG4-RD patients and 50 Healthy Controls. 2 out of 131 IgG4-RD and 2 out of 50 Healthy Controls have Anti-IL-6 antibodies. *(Figure 20).*

CXCL1 is also known as MGSA and Gro-α in humans, is a chemokine that signals via CXCR2. CXCR2 is present on neutrophils. CXCL1 also binds to glycosaminoglycans (GAG) on endothelial and epithelial cells and Extracellular matrix (ECM). CXCL1 plays a role in host-immune response. CXCL1 recruits and activates neutrophils to combat infection.[17] CXCL1 levels in the plasma were increased in Interstitial Pneumonia with Autoimmune Features IPAF compared to idiopathic interstitial pneumonia (IIP), chronic obstructive pulmonary disease (COPD), and healthy controls.[18] CXCL1 induces growth, chemotaxis, and metastasis of several

cancer cell lines.[19] Anti-Groa antibody responses was studied in 131 IgG4-RD patients and 50 Healthy Donors. 7 out of 131 IgG4-RD patients have Anti-Groa Antibodies. *(Figure 21).*

CCL23 stimulate chemotaxis of human THP-1 monocytes and enhances the expression of the marker CD11c (adhesion molecule).[20] Eosinophils are the major CCL23 producing cells.[21] CCL23 upregulates MMP-2 expression in vascular endothelial cells and may play a direct role in angiogenesis.[22] Anti-CCL23 Antibodies were detected in a small cohort of IgG4-RD patients. *(Figure 22).* Anti-CCL23 antibody responses was studied in 131 IgG4-RD patients and 50 Healthy Donors. *(Figure 23).* This showed the presence of Anti-CCL23 antibodies in 12 out of 131 IgG4-RD patients. A patient-derived monoclonal antibody was found to bind to MIP-3 *(Figure 24).* Antibodies from 3 IgG4-RD patients were found to bind to CCL23 based on the cytokine microarray *(Figure 25).* However only 1 monoclonal antibody out of the 3 IgG4-RD patients bound specifically to CCL23 via ELISA. *(Figure 26)*

A recent study published that Anti-prohibitin antibodies were present in 73.5% IgG4-RD patients.[23] However, when performed an ELISA on 131 IgG4-RD and 49 Healthy controls to study Anti-Prohibitin Antibodies, there was no difference between IgG4-RD and Healthy controls. *(Figure 27)*

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