Quantification and Molecular Characterization of Anti-citrulline Antibodies in Rheumatoid Arthritis

A Thesis

Presented to

the Faculty of the Department of Chemical Engineering

University of Houston

In Partial Fulfilment

of the Requirements for the Degree

Master of Science

in Chemical Engineering

by

Anthony Lie

August 2012

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ABSTRACT

Autoimmune diseases are believed to result from the inability of the immune system to distinguish self and non-self. Rheumatoid Arthritis (RA) is a systemic autoimmune disease that leads to joint destruction and affects more than 1.5 million adults (Centre for Disease Control). Auto-antibodies against citrullinated proteins (ACPA) are present in approximately 60-75% of RA patients with 96% specificity. Although the importance of ACPA, both as a causative agent and diagnostic marker, has been established, the reactivities of single ACPA and the eptiopes are unknown. Isolation and characterization of protein targets of ACPA would shed light on the underlying mechanism of RA and would offer (i) earlier diagnosis and (ii) routes for therapeutic intervention. Here, we employ a novel high-throughput methodology, *microengraving*, based on fabricated nanowells arrays, to isolate antibodies from stimulated memory B cells of RA patients. In conjunction with single-cell RT-PCR amplification this technique is employed to perform molecular characterization of ACPA antibodies.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

ACPA: Anti-citrullinated Protein Antibodies, present in RA patient sera

Antibody: Y-shaped protein produced by plasma cell capable of specific binding to antigen via paratope

Antigen: Protein expressed in pathogen surface

Anti-perinuclear: other RA autoantibodies specifically bind to perinuclear factor

AF: Alexa Fluor, commercial fluorophore dye molecules by Invitrogen

Autoimmune: the state when immune system attack own tissues

Bone marrow: flexible tissue found in the interior of bones responsible for producing new blood cells

Calcein Violet: optimal dye for distinguishing live cells through the presence of ubiquitous intracellular esterase activity

CCP: Cyclic Citrullinated Peptide, a synthetic peptide used in the current commercial RA diagnostic test

cDNA: complementary DNA synthesized from reverse transcription of mRNA

CD19: protein expressed in surface of Memory B cells

Citrulline: deimination of L-arginine with help of Peptidyl Arginine Deiminase enzyme

Cytokines: cell-signaling protein molecules that are secreted and used extensively intercellular communication

DNA: double stranded base pairs that encode genetic information

Effector: An organ or cell that acts in response to a stimulus

ELISA: biology assay capable of detecting antigen or antibody using immunosandwich principle

Enzyme: catalyst of biological reaction

Epitope: part of antigen that is recognized by immune system (paratope)

Etiology: origination of disease

Fillagrin: filament-associated protein that binds to keratin fibers

Gene: molecular unit of heredity of a living organism that includes DNA and RNA sequence

Hapten: small molecule that can elicit an immune response if it attached to large carrier such as a protein

HEK293: suspension cell line originally derived from human embryonic kidney cells grown in tissue culture capable of rapid production of protein or antibody

Humoral: immune system that mediated by antibody

Immunoglobulin: see *antibody*

Immunoprecipitation: technique of precipitating a protein antigen from solution using an antibody that specifically binds to target antigen

Isotype: genetic variations in the constant regions of the heavy and light chains

Lymphocytes: white blood cells responsible in human immune system

Memory B cell: B cell subtype that formed following primary infection

Microengraving: high throughput screening using nanowell arrays up to single cell resolution

mRNA: messenger RNA used in all living organism as genetic information template for protein synthesis

Oligos: short nucleic acid polymer synthesized as template for RT-PCR reaction

Paratope: part of antibody which recognize epitope of antigen

Pathogenesis: see *etiology*

Pathogen: microorganism such as a virus, bacteria, fungus that causes disease in human, animal or plant host

PBMCs: peripheral blood mononuclear cells, Memory B cell is a sub-population of PBMCs

PDMS: widely used silicon-based organic polymer in microfluidic biology application

Phagocytosis: cellular process of engulfing solid particles by the cell membrane as the major mechanism to remove pathogens

Plasma cell: B cell subtype that secretes large volume of antibodies

Polymerase Chain Reaction: molecular biology technique capable of amplifying from single piece of DNA to generate exponential numbers of DNA copies

Polypeptide: short polymers of amino acid monomers linked by peptide bonds

Poly-L-Lysine: small natural homopolymer of the essential amino acid L-lysine capable of improving cell or protein adherency by charge

Primers: see *oligos*

Protease: any enzyme that hydrolyse peptide bonds that link amino acids together in the polypeptide chain or protein

Reverse Transcription: process of making single-stranded DNA from a RNA template

Ribosome: component of cells that translate mRNA genetic code into protein synthesis via translation process

RT-PCR: amplification of cDNA copies from mRNA by utilizing reverse transcription reaction initially

Sera: blood components excluding blood cells and clotting factor

Somatic Hypermutation: extremely high rate of mutation differentiation of B cell receptor genetic locus to provide efficient genetic rearrangement

Synovial fluid: a viscous, non-Newtonian fluid found in synovial membrane to reduce friction between cartilages of synovial joints during movement

Titer: the lowest dilution possible that still yield positive result

I. INTRODUCTION

Background

Rheumatoid arthritis (RA) is a systemic inflammatory auto-immune disease that affects multiple joints in the human body and is estimated that greater than 120,000 adults in the U.S. are diagnosed with RA each year (CDC).[1,2,3] Although RA is rarely fatal, it contributes to prolonged disability with the need for lifelong pain management.[4] The inflammatory process primarily affects the lining of the joints (synovial membrane), and the inflamed synovial membrane in turn leads to erosion of the cartilage and bone (Figure 1).[5] The symptoms of RA include swelling, pain, redness, and joint structure deformity.[4]

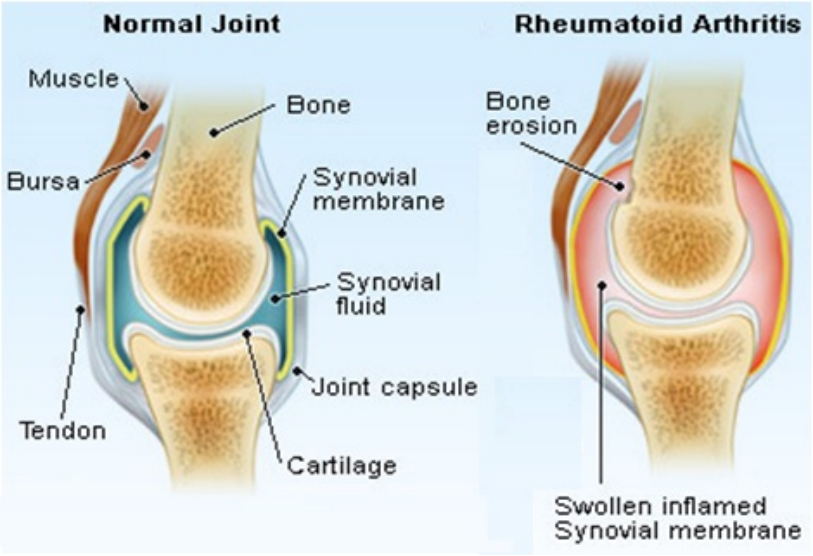

Figure 1: Normal and Rheumatoid Arthritis Joints.[6]

The origin of RA remains unclear, however it has been established that both humoral and cellular immunity play an important role in disease progression.[7,8,9] The etiology of RA may include a combination of: genetics (*Protein tyrosine phosphatase,*

non-receptor type 22 gene which linked to several autoimmune diseases), reproductive hormonal responses, prior infections, diet, and tobacco use.[10,11]

Human Adaptive Immune System

The human immune system consists of innate and adaptive components that serve complementary roles in combating foreign pathogens. Although they are classified into different categories, the interaction between them is dynamic and provides synergistic protection. Innate immunity provides the first line of defense against pathogen exposure and the response is generic with regards to the nature of the pathogen.[12] The response time is typically within hours of the first infection and the major components of the innate immune system are phagocytic cells (cells able to ingest foreign pathogen), complement systems, and anatomical barriers.[12] In the event of a second infection of the same pathogen, the innate immune system will respond no differently than the first response. On the other hand, the adaptive immune system is a second line of defense against pathogen and is highly pathogen-specific. This response has slower kinetics (typically days after primary infection) and the major component of the system is lymphocytes that include cytotoxic T (T_C) cells, T helper (T_H) cells, plasma cells, and memory B cells.[12] The adaptive immune system itself can be divided into two types: cellular and humoral immunity.[12] The cellular immunity involves effector T lymphocyte cells such as activated T_H cells and T_C cells. For instance, cytokines, cell-signalling molecules which are secreted by T_H cells, could activate phagocytic cells to ingest and kill the target pathogens more effectively. In contrast, the humoral immune system utilizes the antibody in recognizing and binding

specifically to the target antigen.[12] The humoral adaptive immune system mediated by B cells is highly diverse because of somatic hypermutation (V(D)J recombination to rearrange the antigen receptor gene).[12] This mechanism allows the arrangement of genes to generate a library of different antigen receptors that will be expressed in the adaptive immune cells.[12] As a result of the gene rearrangement, the change of gene (DNA) in each cell is irreversible. Therefore, all of the progeny (offspring) of that cell will inherit the same genes encoding the same receptor specificity.

Humoral Immunity

In the humoral immune system, antibodies (proteins secreted by B cells) function by binding to the antigen and promote its elimination by several mechanisms: the antibody-antigen can cross-link forming clusters that are more easily ingested by phagocytic cells binding the antibody-antigen may also activate the complement system that promotes ingestion by phagocytic cells. The origin of antibody production begins with an activation phase of the B cells that involves the process of presenting antigen by macrophages.[12] Macrophages digest microscopic pathogen which have antigen on their surface and signals to T_H cells.[12] Upon co-stimulation with T_H cells, naïve B cells differentiate into plasma cells that secrete antibodies in a large volume against the antigen that macrophages displayed.[12] The irreversible differentiation also produces memory B cells that are useful in recall responses against the same antigen.[12]

Antibody

Humoral response is mediated by antibodies which are glycoproteins belonging to the immunoglobulin superfamily, therefore the terms *antibody* and

immunoglobulin are used interchangeably.[12] The generic structure of antibody consists of two heavy chains and two light chains with the antigen binding site (paratope) located at the tip of Y (Figure 2). These chains comprise the variable and constant regions in which the variable region has a different peptide sequence that varies with the antigen being recognized, while the constant region as the name suggests is largely invariant with the antigen being recognized. The antibodies can be classified based on the constant regions of the heavy chains into different isotypes (IgG, IgM, IgA, and IgE) that each has different functions within the context of the immune system.[12] The antigen binding site in variable regions of both heavy and light chains is the only site where antibody-antigen binding occurs (Figure 2).

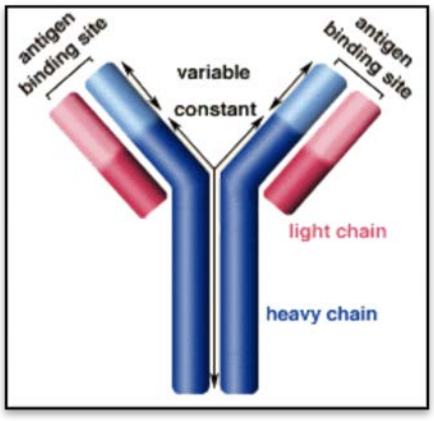

Figure 2: Antibody, a Y-shaped protein consists of two chains, the light and the heavy chains. Heavy chain (blue) with one variable (VH) domain followed by a constant domain (C_H1), a hinge region, and two more constant (C_H2 and C_H3) domains. Light chain (red) with one variable (VL) and one constant (C_L) domain. Antigen binding sites (paratope) are located in the variable region of both chains (VH and VL). Heavy and light chains are connected by disulfide bonds. [13]

B Cells

The B cell lineage is originated in the bone marrow and differentiated into plasma cells and memory B cells upon costimulation with T_H cells.[12] Plasma cells actively secrete antibodies into circulation in large volume, ranging from hundreds to

thousands of antibody molecules per second per cell while memory B cells are present in circulation to prepare for secondary response to the same antigen.[12]

Autoimmunity

In the event of autoimmune disease, both humoral and cellular immunity have different roles in disease origination and progression of the disease. Examples of autoimmune diseases include diabetes mellitus type 1, systemic lupus erythematosus, psoriasis, and rheumatoid arthritis.[14] In rheumatoid arthritis (RA) patients, autoantibodies (antibodies that react to the patient's own tissues) have been identified. These autoantibodies are collectively called *Anti-Citrullinated Protein Antibody (ACPA)* because they bind specifically with 96% specificity to citrullinated proteins with prevalence of 60-75% among RA patients.[15] A review of the research efforts over the last decade reveals several important findings with regards to the development of arthritis: (i) the appearance of ACPA precedes the onset of the disease and can be used as an early diagnostic marker and (ii) immunity towards citrulline-modified proteins underlies arthritis (at least in rodents).[16,17] Therefore, it is imperative to determine the sequence of ACPA from RA patients in an effort to understand the pathogenesis of RA.[18]

Citrullination

Citrullination is a deimination reaction that occurs when L-arginine is post-translationally modified to L-citrulline in the presence of Peptidyl Arginine Deiminase (PAD) enzyme (Figure 3).[19] This enzymatic conversion results in the loss of a positive

charge from arginine to citrulline which could lead to improper protein folding and enhanced degradation by proteases.[20]

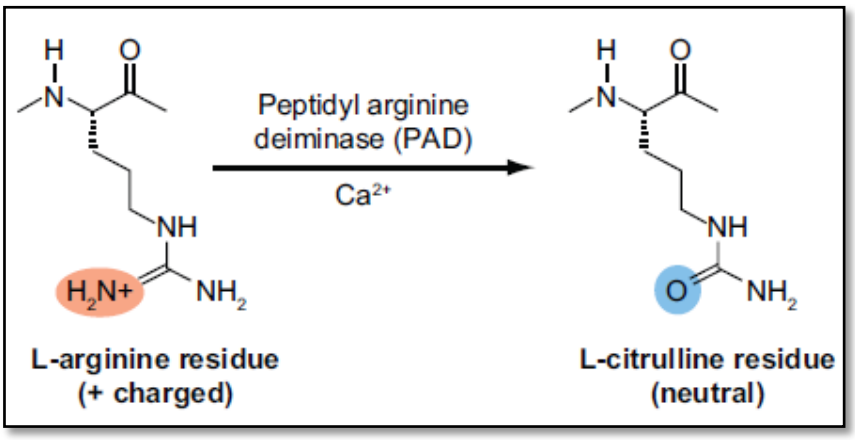

Figure 3: Post-translational modification of positively charged amino acid arginine to neutral citrulline in a deimination (citrullination) reaction. Deimination is catalyzed by a group of calcium dependent Peptidyl arginine deiminase (PAD) enzymes. The terminal nitrogen atom of the arginine sidechain is replaced by oxygen in the reaction. The reaction uses one water molecule and yields one ammonia molecule as a side product. [21]

Although studies have shown that ACPA is prevalent in RA patients with high specificity, the sequence of ACPA is still unknown. The precise molecular targets that serve as the target for ACPA is also unknown, however ACPA bind specifically to a synthetic molecule named *Cyclic Citrullinated Peptide (CCP).*[22]

Cyclic Citrullinated Peptide

Cyclic Citrullinated Peptide (CCP) is a synthetic antigen used in the diagnostic test for RA by utilizing the binding of ACPA in the RA patient sera to CCP.[23] The molecule consists of a 21 amino acid peptide with citrulline at the 9th position (Figure 4).[24] The discovery of using CCP as a molecular target antigen of ACPA originated from a study that showed fillagrin as the common target of the antibodies that are used in anti-keratin and anti-perinuclear RA diagnostic tests.[25] Subsequently, it was demonstrated that the fillagrin-specific reactivity of RA sera is dependent on

deimination (citrullination) of arginine residues in filaggrin-derived peptides.[26] Different variations of the citrullinated peptide were then systematically explored and the arrangement of CCP proved to have the highest specificity among other candidates.[26]

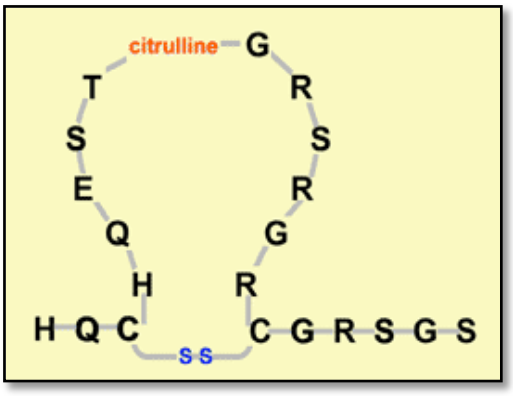

Figure 4: Cyclic citrullinated peptide (CCP), 21 amino acid length synthetic peptide with citrulline at the 9th position from N-termini. CCP is the target peptide for CCP-based RA diagnostic assay (high specificity assay, 96%). ACPA antibodies present in the RA patient sera bind to CCP coated on the plate. [27]

High throughput single-cell assay for isolation of antibody from memory B cells

Successful isolation and molecular characterization of ACPA and protein targets of ACPA would shed light on the underlying mechanism of RA. In order to screen for ACPA, memory B cells available in the human blood will be stimulated to enable ACPA secretion *in vitro*. Therefore it is important for a methodology to be able to screen and retrieve genetic information from the single cell level. The acquired genetic information is essential to produce and characterize ACPA antibodies.

Flow cytometry is a powerful technique that permits the quantification and sorting of cells. Flow cytometry has previously been employed to successfully isolate antibodies from memory B cells.[28,29] One example is the isolation of Tetanus Toxin (TT)-specific from human memory B cells using flow cytometry.[28] TT is produced by

the gram-positive bacteria *Clostridium tetani* and infants are routinely vaccinated against tetanus.[28] Consequently, most adults have memory B cells that are directed specifically against TT. By utilizing flow cytometry, Phycoerythrin-conjugated recombinant TT C-Fragment (rTT.C) was used to detect the TT positive memory B cells. [28]

Phycoerythrin (PE) is a fluorescent protein molecule harvested from cyanobacteria and red algae.[12] Cell sorting in flow cytometry was performed for rTT.C^+ $CD27^+$ memory B cells from healthy donor peripheral blood and cultured on CD40L-L cells with presence of IL-21 for 36 hr. [28] The cell cultures were then transduced with STAT5bER-IRES-GFP and cultured for seven more days in order to immortalize B cells. [28] An inducible active mutant of the transcription factor Signal Transducer and Activator of Transcription 5 (STAT5) inhibits the differentiation of B cells while increasing their replicative life span.[28] Also, the use of an Internal Ribosome Entry Site (IRES) allows simultaneous expression of a protein of interest and a fluorescence marker (GFP).[28] The cells were then sorted for rTT.C^+ $CD27^+$ and transferred to microcultures for 2 more weeks. At the end of 2 weeks, rTT.C^+ STAT5bER^+ memory B cells were sorted and expanded further in the culture. The majority of clones expressing IgM and IgG were specific for TT which determined by IgM and IgG specific TT ELISA. [28] This study demonstrated that the STAT5bER methodology combined with flow cytometry can be used to obtain B cell lines with the capacity to secrete antigen-specific antibody in this case it was TT specific. [28]

Another independent study utilized flow cytometry to isolate the antibody against conserved epitopes of Human immunodeficiency virus (HIV), surface protein gp140, which are recognized by a set of antibodies expressed by memory B cells of HIV-infected individuals.[29] To isolate these memory B cells, fluorescence-activated cell staining (FACS) was performed on the patient PBMCs utilizing biotinylated gp140 to stain and successfully identify a distinct population of gp140 binding memory B cells.[29] Single memory B cells were then sorted to the 96-well plate PCR plate and cDNA synthesis was performed followed by PCR reaction to amplify the DNA sequences of heavy and light chains of the antibodies.[29] To produce the recombinant antibodies, human embryonic kidney cell line (HEK-293T) was used and the antibodies present in the supernatant of cell cultures were tested on the ELISA plate coated with gp140 antigens.[29] Finally, 502 antibodies were able to be cloned and among these were composed of 50 independent clones from memory B cells of six HIV-infected patients.[29]

There are however several disadvantages to using flow cytometry for screening antibodies such as: (i) sensitivity, (ii) numbers of B cells, and (iii) screening of plasma cells. The first disadvantage is that flow cytometry can only detect the antibodies that are surface-bound to the memory B cells, not the secreted antibodies. The second is that flow cytometry typically utilizes million of cells which can be problematic especially when interrogating memory B cells from the synovium.[30] Third, plasma cells from bone marrow of RA patients cannot be screened since plasma cells do not have a membrane bound form of the antibody (plasma cells only secrete antibodies).

By comparison, our assay could overcome these disadvantages by screening ACPA both from memory B cells and plasma cells using nanowells as a container for each cell in the experiment.

Nanowell Arrays

The design of nanowell arrays comprises of 84,672 nanowells equally spaced.[31] The production of the microarray can be accomplished by molding Polydimethylsiloxane (PDMS), a biocompatible polymer widely used in microfluidic chips, with silicon oxide wafer which has 50 μm cube posts arranged as microarrays (Figure 5).[31] The wafer is then mounted in a custom stainless steel template and serves as the template for making replicas in PDMS (Figure 5).[31] The PDMS is cast onto the wafer and cured thermally. The PDMS can then be detached from the wafer and rendered hydrophilic by plasma oxidation.[31]

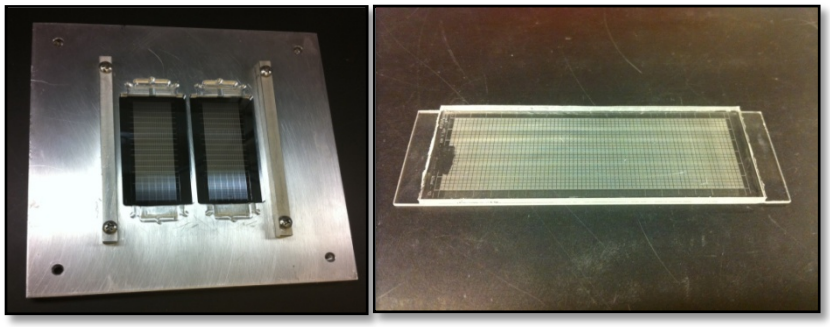

Figure 5: Nanowell arrays are fabricated using a soft lithography method employing polydimethylsiloxane (PDMS) and molding cast. A patterned silicon oxide wafer with a metal molding cast is used as a template for soft lithography (left). The fabricated arrays consist of 84,672 nanowells (50 μm x 50 μm x 50 μm) with a 1 mm thickness. The nanowell array is bonded to a standard glass slide (3 inch x 1 inch) upon curing (right).

Microengraving

Microengraving is a method to detect the secretion of antibodies or cytokines from these immune cells through the formation of immunofluorescent sandwiches

assembled on functionalized glass placed in contact with the Nanowell arrays (Figure 6). The glass slide is functionalized with poly-L-lysine and coated with desired antibodies (capture antibodies) capable of capturing target molecules (target antibodies or cytokines). The final step of the sandwich is to add detection antibodies which are capable of only detecting target molecules which bind to the glass slide via the capture antibodies. The detection antibodies are conjugated with fluorophore and upon excitation with a laser, positive signals signify the site where the target molecules are binding.

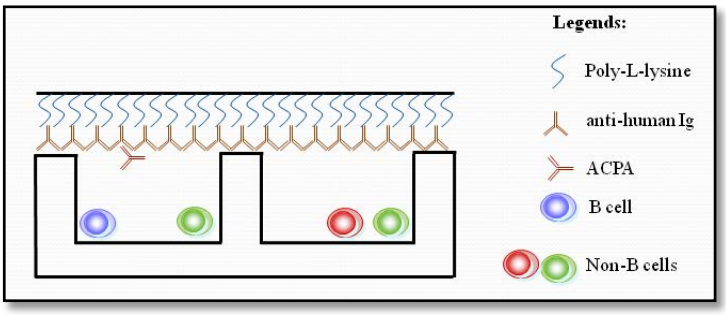

Figure 6: Schematic representation of the hybridization step between the nanowell arrays and the glass slide, creating a closed system environment. Antibodies secreted from B cells are capture locally where the nanowell is positioned. Antibodies captured from B cells are detected by the addition of detection antibodies.

In the RA project, the target antibodies secreted from human B cells (ACPA) will be captured by the anti-human Ig antibody within an incubation period of 2 hr. The subsequent step after the incubation period is to detect the target antibodies that bind to the capture antibodies on the glass slide. Detection can be accomplished as a two-step process. First, the glass slide is incubated with biotinylated CCP to enable the capture of this molecule by the autoantibodies secreted from the B cells (Figure 7).

Second, after adequate washing the glass slide is incubated with streptavidin-AF 647 (fluorophore) and also with labeled anti-human Ig (Figure 7).

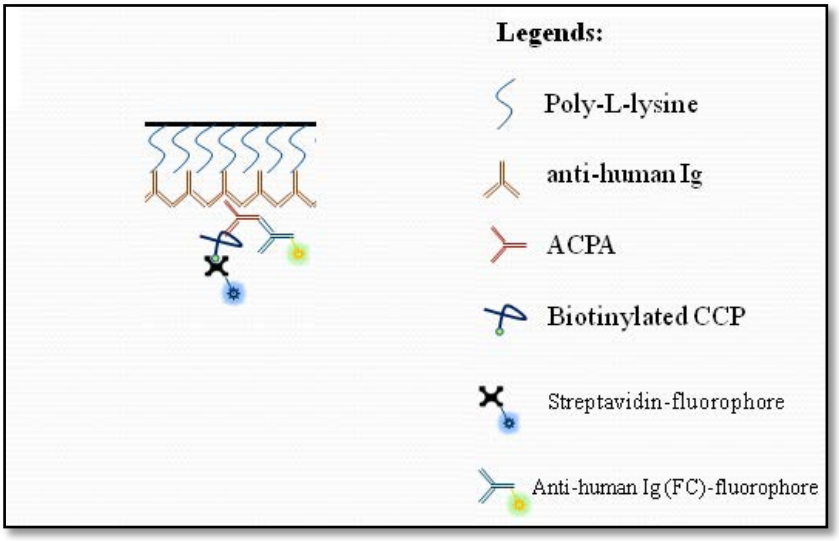

Figure 7: Schematic representation of antibody sandwich for ACPA. All antibodies (including ACPA) secreted from the RA patient B cells bind to capture antibodies (anti-human Ig) on the glass slide. Biotinylated CCP is added to the screen specifically for ACPA among all antibodies bound on the glass slide. Streptavidin-AF 647 is added subsequently to detect ACPA (streptavidin binds to biotin) while the anti-human IgG-AF 488, anti-human IgA-AF 532 are added to determine the antibody isotype.

In parallel, the cells in the nanowell arrays are incubated and stained with anti-CD19 (B cell marker) and Calcein violet (live cell marker) and imaged using an epifluorescent inverted microscope.

Enzyme-Linked ImmunoSorbent Assay

ELISA (Enzyme-Linked ImmunoSorbent Assay) is a biochemical assay for detecting antibodies or antigens of interest with high sensitivity.[32] A capture antibody or antigen is immobilized on the surface of a 96-well plate via passive adsorption.[33] The passive adsorption occurs through hydrophobic interactions between polystyrene and non-polar protein residues.[33] In indirect ELISA setup, the antigen is coated onto the polystyrene well and incubated for a time period. Washing procedures are typically

performed to remove residual antigen or antibody that is not coated on the well. The next step is to provide a specific antibody that will bind to the antigen previously coated on the wells. The detection antibody conjugated with the enzyme is then provided and will bind to the specific antibody available in the wells. The final step is to add substrate which only turns into the product (color solution) upon the presence of the enzyme. The reaction will form a color in the solution and the intensity is recorded using the ELISA plate reader/luminometer. In the RA project, the CCP peptide is used as an antigen and the ACPA present in the patient sera is the specific antibody that binds to CCP. Next, the anti-human IgG/IgA with the horseradish peroxide enzyme will be added to catalyze the reaction upon substrate addition in the final step.

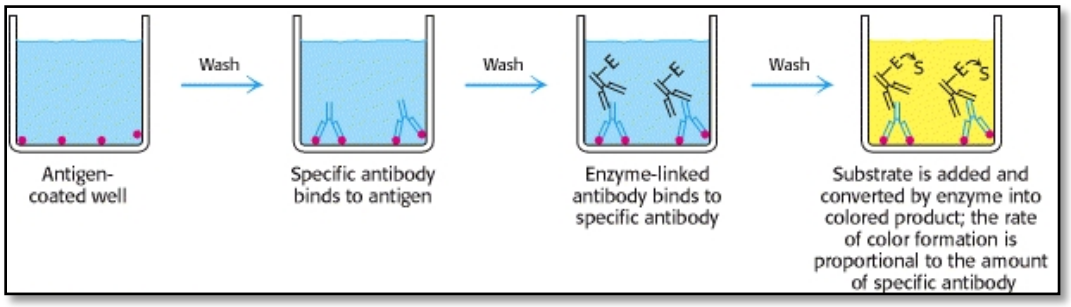

Figure 8: Schematic representation for Enzyme Linked Immunosorbent Assay (ELISA) for the detection of antibody/antigen.[34]

Spotting Assay

The spotting assay is an immunosandwich assay that utilizes the same principle as the ELISA test. In the spotting assay, CCP is coated on the glass slide instead of polystyrene plates in ELISA and the reactivity of the RA patient sera is determined via the formation of immunofluorescent sandwiches coated on the glass slide. The

intensity of the fluorophore emission is proportional to the concentration of specific antibodies present in the patient sera.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a method to exponentially amplify the DNA of interest from mRNA available in the sample. The genetic code encoded in mRNA was located inside the memory B cells and needed for the translation process.[35] The translation process in the ribosome utilized mRNA genetic code to produce a specific polypeptide chain that folds to protein or the antibody downstream.[36]

After reverse transcription was finished, the next step was to amplify the cDNA molecules exponentially by the polymerase chain reaction. In the subsequent PCR the cDNA was exponentially amplified, making the copies of cDNA by the addition of a single nucleotide base available in the solution with the presence of the polymerase enzyme.[36] The final product was a double-stranded DNA encoding the gene of interest. In the RA project, mRNA from ACPA-secreting B cells is reverse-transcribed to produce its complementary DNA. The PCR cycles amplify the DNA copies that encode the ACPA genes.

Agarose gel electrophoresis was performed to characterize the DNA products by separating different DNA product according to their sizes.[37] It worked by applying an electric field to move the negatively charged molecules through an agarose matrix.[37] Shorter molecules moved faster and migrated further than longer molecules

because the shorter molecules migrated more easily through the gel pores compared to longer molecules.[37]

II. OBJECTIVES

The general objective of the project is to determine the target molecule that ACPA binds in the synovial fluid of RA patients, while the preliminary objective is to obtain the gene sequence of ACPA. Although the diagnostic test for RA has been established[23], the role of ACPA in disease mechanism is largely unknown and the hypothesis is by identifying the molecules (citrullinated protein) that bind to ACPA, the progression of RA can be better studied and the etiology of RA disease can be fully understood.

Our first objective is to maximize the frequency of B cells secreting antibodies (IgG, IgA, IgE, IgM) from the healthy patient. After the frequency is maximized, the subsequent step is to retrieve B cells of interest by the CellCelector robot from each well. RT-PCR is then performed to amplify the variable heavy (VH) and variable light (VL) gene from a single B cell. This set of preliminary experiments is to ensure that we have: (i) an adequate number of B cells actively secreting antibody, (ii) the ability to pick a single B cell from a specific well, and (iii) the ability to amplify the gene from the B cell of interest.

III. RESULTS & DISCUSSION

Frequency of immunoglobulin secretion in healthy patient B cells

To increase the probability in screening ACPA from the RA patient, we have optimized the overall immunoglobulin secretion frequency in PBMCs (Peripheral

Blood Mononuclear Cells) from the healthy patient (B cells are a subpopulation of PBMCs). Fresh PBMCs were isolated from healthy patient blood by using a density gradient Ficoll-paque protocol.[38] Subsequently, an aliquot of ~1 million cells in 300 µL was stimulated for four days (5% CO_2/37^oC) in R-10 medium with soluble CD40 ligand (sCD40L), Interleukin 21 (IL-21), and anti-APO1 to promote antibody secretion.[39] Soluble CD40L engaged with the surface B cell expressing CD40 to mimic T cells-mediated activation.[37] Activated B cells undergone division, antibody isotype switching, and differentiation to secrete antibodies.[40] The same principle applied with IL-21 as it bound to IL-21 receptors on B cells and promoted further differentiation of antibody isotype.[41] Anti-APO1 prevented programmed cell death (apoptosis) during the stimulation process.[42] After four days stimulation period, ~300,000 PBMCs in 300 µL were loaded on the nanowell arrays thus distributing 8,000-120,000 PBMCs in all of the nanowells. The data confirmed that we could detect antibody secretion from single B cells in the nanowells with the frequency of antibody secreting B cells ranged 5-13% from all live B cells. The frequency of each isotype was 3-10% for IgG, 0.5-1% for IgM, and 0.5-1% for IgA from all live B cells population. Stimulating PBMCs from three healthy patients for different stimulation periods showed that the four days stimulations yielded higher immunoglobulin secretion than the seven days stimulation period (Figure 9). Based on these preliminary results we decided to stimulate the PBMCs of the RA patients for four days.

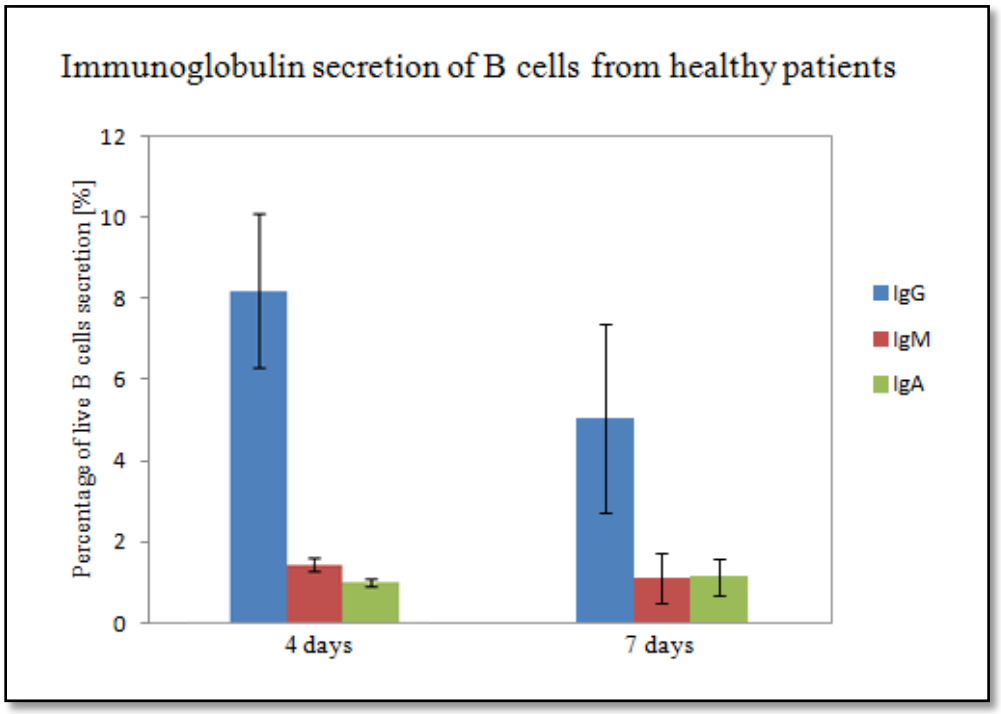

Figure 9: Frequency of immunoglobulin secretion in stimulated PBMCs (n=3). Stimulation was performed for four and seven day periods with sCD40L, IL-21, anti-APO1. Immunoglobulin secretion assayed by microengraving. The error bars represent the standard error of the mean (standard deviation divided by the square root of the sample size).

Cells retrieval process

Memory B cells screened by microengraving were retrieved by the CellCelector robot in the next step. In order to determine which B cells satisfied all the conditions that we have set, systematic analysis must be performed. First, we observed CD19 expression (B cell marker) and the viability by staining the B cells with immunofluorescence: anti-CD19-AF 568 for B cell marker and calcein violet for the live cell marker (Figure 10 A). The microscopy images of the nanowell array were analyzed using MabAnalyze (custom script) generating a spreadsheet of each identifiable cell with anti-CD19-AF intensity and calcein violet intensity. From this information, the intensity gating for both channels were performed in Microsoft

Access thus producing a subpopulation of live and CD19 positive cells. Second, the microengraved response of the immunoglobulin secretion on the glass slide was revealed by immunofluorescent staining and read with a microarray scanner. The readings from the microarray scanner gave immunofluorescent spots with different intensities at each fluorescent channel. Custom blocks of array were utilized to correctly determine the location of spots which we set as positive spots by manual observation (high fluorophore intensity compared to the background). Then we analyzed the observed spots by flagging positive spots in each channel: IgM in 594 nm channel, IgG in 488 nm channel, IgA in 532 nm channel, and IgE in 635 nm channel thus a list of flagged spots with each channel intensity was created (Figure 10 B). The result was exported to Microsoft Access to be matched with the microscopy data retrieved previously thus creating a list of cells to be retrieved. Third, the CellCelector camera provided the images of before and after picking therefore we could ensure that the B cell of interest was transferred correctly from the nanowells to the target tubes (Figure 10 C). An example for the process integration of single live B cells secreting IgM-isotype antibody was observed (Figure 10 A-C).

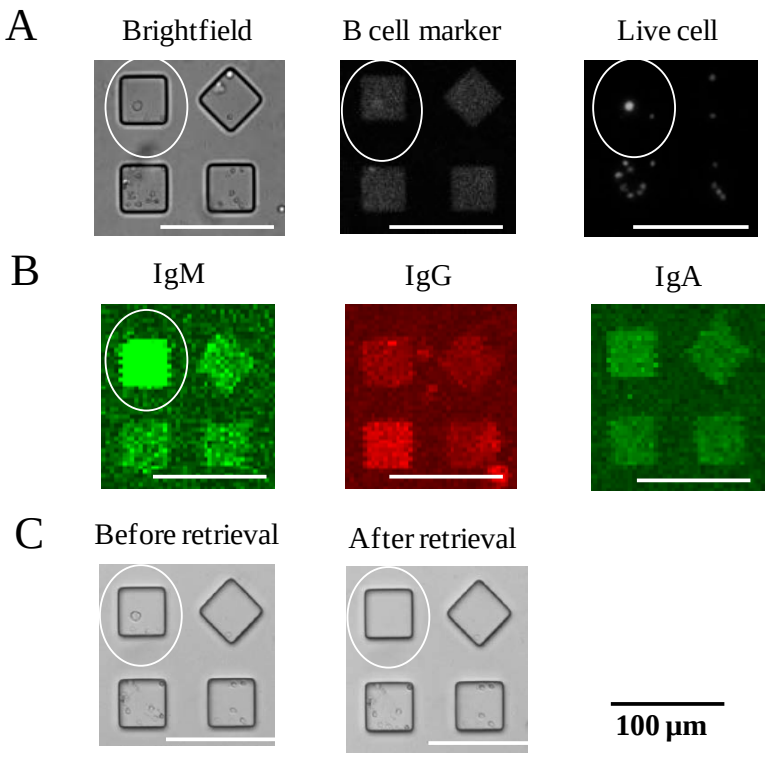

Figure 10: Integrated single-cell analysis of immunoglobulin secretion from B cells. (A) Representative composite micrograph showing a live B cell. Live B cells were identified as CD19 positive and Calcein Violet positive. (B) IgM secretion was observed on a microengraved glass slide matched to the live B cell shown in Figure 10A (white circle). Immunoglobulin isotypes were identified for IgM, IgG, and IgA. (C) Images of the nanowell before and after the retrieval by the CellCelector robot containing live B cell secreting the IgM isotype.

Amplification of variable heavy and variable light chain genes from single B cells

After single B cells were retrieved independently (no cells pooling), the mRNAs from the single B cell of interest was obtained by lysing the cell in a PCR tube. This process served as a starting template for the RT reaction in which the mRNAs of each B cell were reverse-transcribed to cDNA and subjected further to two rounds of PCR amplifications. Amplified PCR products for VH and VL were run on a 0.8% agarose gel electrophoresis accompanied by negative control (no B cell, only the PBS solution has undergone the whole RT-PCR procedure). We successfully amplified the heavy chain from 4/9 single cells and light chains from 3/9 single cells

(Figure 11). In order to produce a full-length antibody, both heavy and light chain genes from the same B cell must be successfully amplified. From the successful amplifications, there were only three corresponding sets of heavy and light chain (Figure 11). This preliminary experiment indicated that we were able to amplify variable heavy and variable light chain genes set from the single memory B cell with a 33% success rate.

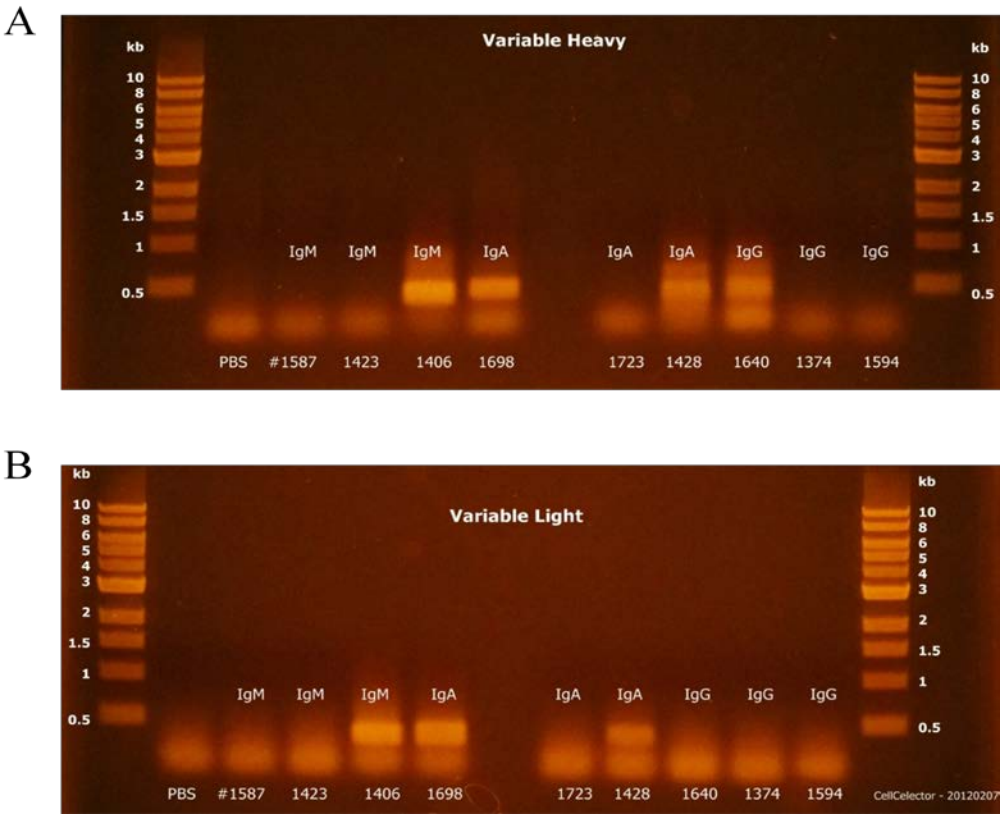

Figure 11: Combined RT and nested PCR on a single cell. mRNAs isolated from the single B cells were used as the template for cDNA synthesis and two rounds of PCR amplifications. Immunoglobulin genes (VH & VL) were amplified independently. (A) Specific VH PCR products were observed in lane 1406, 1698, 1428, and 1640 (size approximately 400 bp). (B) Specific VL PCR products were observed in lane 1406, 1698, and 1428 (size approximately 400 bp). PBS lanes were run as negative control. Immunoglobulin isotypes were determined from microengraving.

RA patient sera reactivity in ELISA

In order to pre-screen patient samples for CCP reactivity, we designed an in house ELISA test using CCP peptide, similar to the commercial CCP ELISA. The ELISA was performed on the sera of two different RA patients (RA01 and RA02) and a healthy patient (control). Sera reactivity was determined by performing serial dilutions (1:100, 1:500, 1:1000, 1:5000 dilutions) on each patient sera (Figure 12). The result showed that the patient RA02 exhibited higher titers compared with patient RA01 while the healthy patient exhibited nearly negligible ACPA presence in sera (Figure 12). Therefore we decided to screen the ACPA-secreting-B cells from RA02 as the patient sera exhibited high titer of the ACPA antibody.

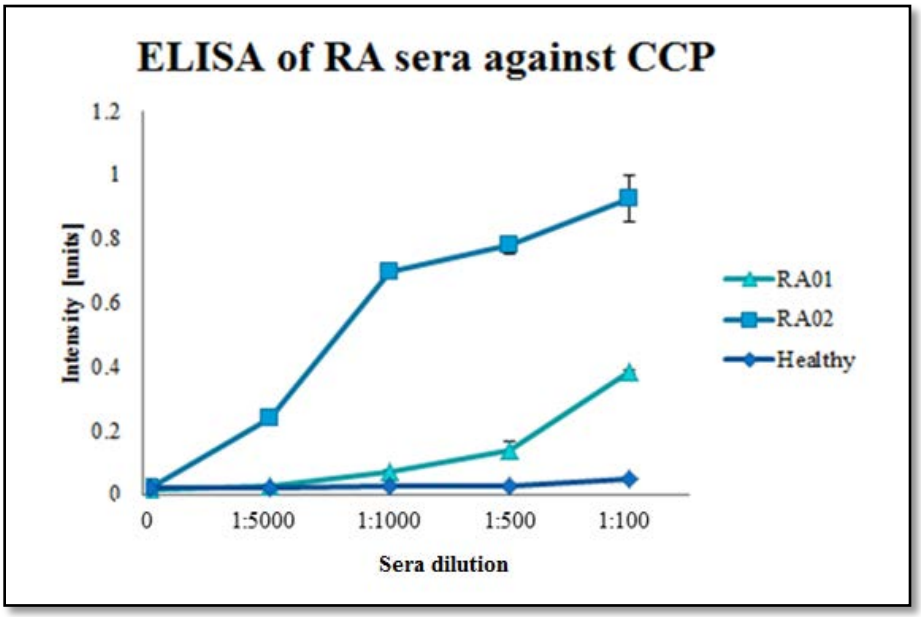

Figure 12: CCP reactivity sera from RA patients. Serial dilutions of patient sera were compared. CCP peptide (25 µg/ml) was coated on the ELISA plate. The ACPA captured from the patient sera were detected by the rabbit anti-human IgG conjugated with horseradish peroxide (HRP). The error bars represent the standard error of the mean (standard deviation divided by the square root of the sample size).

RA patient sera reactivity in Spotting Assay

We further performed a spotting assay experiment to determine qualitatively the ACPA level in sera from three patients, to confirm that the immunosandwich could be performed on a glass slide, and to verify the ELISA result. The result confirmed the immunosandwich worked on the glass slide where CCP, ACPA, and anti-human IgG/IgA were incorporated (Figure 13). The assay also confirmed that the RA02 patient has higher ACPA level in serum in comparison with RA01 and the healthy patient (1:100, 1:500, 1:1000 sera dilution) which in accordance with the ELISA result (Figure 13). We have observed no signal from the healthy patient even in the highest sera dilution (1:100) which suggested that the only ACPA specifically bound to CCP and not other antibodies that were present in patient sera.

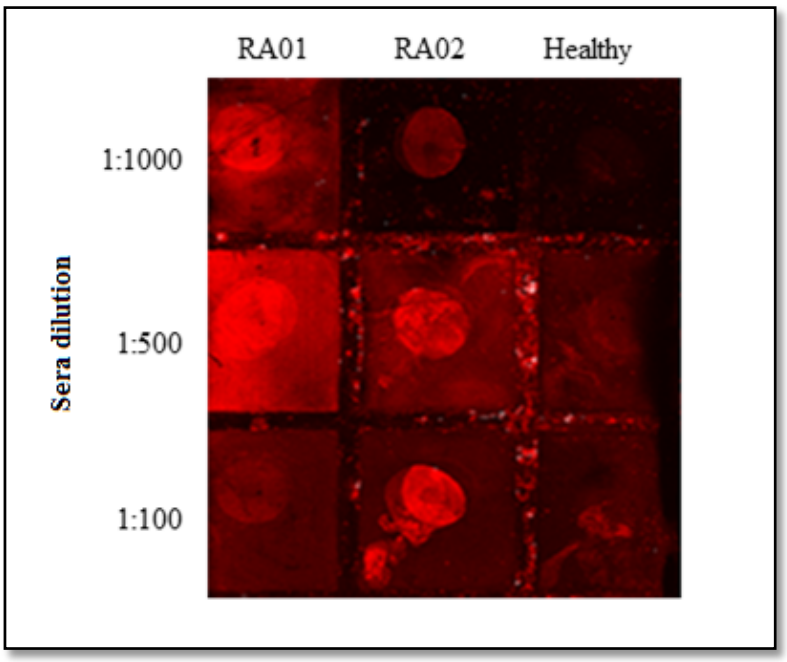

Figure 13: Spotting Assay experiment of RA patient sera reactivity at various concentrations. CCP peptide (25 µg/ml) was coated on glass slide. The ACPA captured from the patient sera were detected by goat anti-human IgG-AF 647. These results confirmed with ELISA results.

Microengraving with RA patient

Microengraving with the RA patient was performed with the sandwich described previously (Figure 7). An aliquot of ~1 million PBMCs in 300 µL from RA02 patient was stimulated for four days (5% CO_2/37^oC) with CD40L, IL-21, and anti-APO1. After the four day stimulation period, the PBMCs were loaded on the nanowell arrays with ~300,000 cells in 300 µL and microengraved to screen for ACPA. We detected ACPA and determined specifically their isotype (IgA and IgG isotype), which was in accordance with the previous report.[43] For the microengraving experiment performed on the RA02 patient there were 96 ACPA spots (69 spots had IgG isotype, 27 spots had IgA isotype) with 10,846 PBMC cells (1,014 cells were CD19 positive/Calcein violet positive) were present in the nanowell arrays (Figure 14). These 96 ACPA spots were analyzed and matched with 1,014 live B cells from the microscopy, and out of these 96 spots, 37 live B cells were retrieved by the CellCelector and stored at -80^oC. The amplification of the variable heavy and light chain of ACPA genes is currently being performed by RT-PCR.

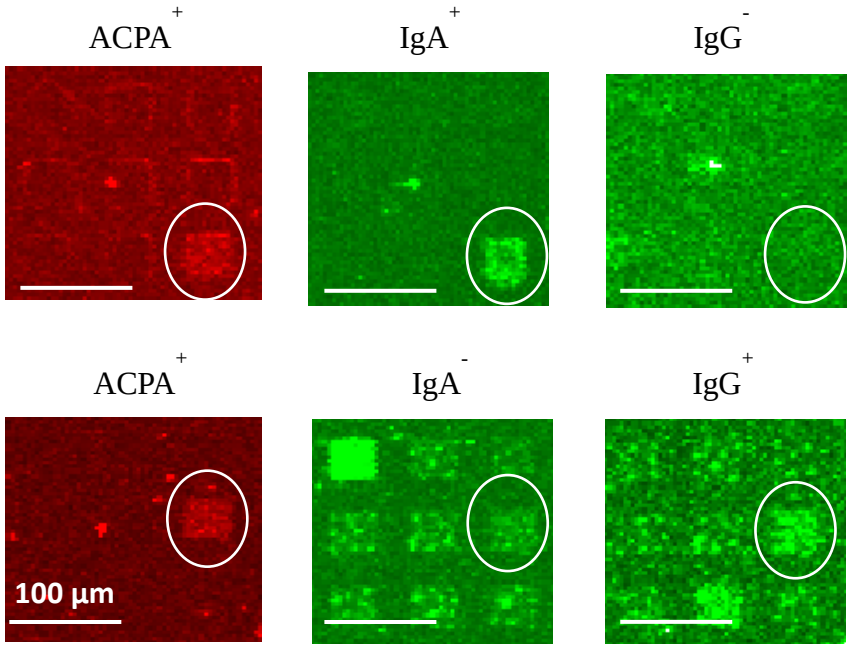

Figure 14: ACPA-secreting B cells from RA02 patient were screened by microengraving. Both IgA and IgG isotypes of ACPA were observed (white circle).

IV. SUMMARY & CONCLUSIONS

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease which occurs in multiple joints of the body.[3] The disease compromised the quality of life and was the major contributor to disability in the US.[4] The symptoms of RA include swelling, pain, redness, and joint structure deformity.[4] The etiology of RA is largely unknown however studies find that there is set of autoantibodies that react specifically to citrullinated protein and they are known as ACPA.[17] By determining the sequence of ACPA and the molecular target in the synovial fluid of RA patients, the etiology of RA will be better understood.[17]

With the in purpose of determining the sequence of ACPA, a high throughput single cell assay, microengraving, is utilized to screen stimulated memory B cells that

secrete ACPA in RA patients using stimulation parameters that previously determined by experiment with healthy patients (Figure 9). We have shown that gene amplification using RT-PCR can be performed in the single cell level with a 33% success rate. We have also shown that microengraving, cell retrieval, and RT-PCR can be performed as an integrated process. The microengraving result of ACPA is promising as we were able to detect both isotypes IgG and IgA of ACPA which confirms with the literature (Figure 14).[47] We are currently amplifying the variable heavy and variable light of ACPA. With successful amplification performed previously in the healthy patient case, we are confident that we could determine the sequence of ACPA in the near future.

V. METHODOLOGY

Fabrication of Nanowell Arrays

Nanowell arrays of 1-mm-thick polydimethylsiloxane (PDMS) were fabricated by the soft lithography technique using silicon oxide as arrays template.[31] The arrays template consisted of fabricated 50 µm cubic posts of equal distance and was silanized with *(tridecafluoro*-1,1,2,2-*tetrahydrooctyl)-1-trichlorosilane* (UCT, Bristol, PA) to ensure easy detachment of PDMS.[31] Sylgard 184 silicon (Dow Corning, Midland, MI) was used as PDMS elastomer material in soft lithography process. [44] PDMS was cured for 2 hr at 80^{o}C and 84,672 nanowells (50 µm x 50 µm x 50 µm) were present in PDMS which bonded to a standard 3 inch x 1 inch glass slide.

Poly-L-lysine glass slide

The poly-L-lysine glass slide provided the binding capability for the capture ntibody on the glass slide. Glass cleaning solution was made by dissolving 50 g of

NaOH (Sigma, St. Louis, MO) in 110 ml of DI water, and 390 ml of 73% EtOH (BDH, Dubai, UAE). The poly-L-lysine solution was made by combining 814 ml DI water, 100 ml of PBS (Cellgro, Manassas, VA), 86 ml of poly-L-lysine 0.01% solution (Sigma, St. Louis, MO). Standard glass micro slides of 3 inch x 1 inch (Corning, Corning, NY) were placed in a stainless steel rack and the whole assembly was placed inside a glass container with a working volume of 600 ml. The glass cleaning solution was added onto the glass container until all the micro slides were submerged. The glass container was rocked for 2 h and then carefully rinsed with DI water. To remove residual NaOH, five washes were performed with DI water. The micro slides were allowed to sit overnight in the last DI water washing solution. On the next day, the DI water was replaced with poly-L-lysine solution and the glass container was rocked for 2 hr. To remove residual poly-L-lysine solution, three washes were performed with DI water. The micro slides were dried with microarray centrifuge (Labnet, Edison, NJ) and transferred to the oven for further drying (50^{o}C/20 min). The dried micro slides were stored in a vacuum for at least two weeks before coating capture antibodies.

Cell media

The cell media used in the experiments were RPMI-PLGH, R-10, and freezing media. RPMI-PLGH was prepared by combining RPMI-1640 of 500 ml (Cellgro, Manassas, VA), Penicillin/Streptomycin of 50,000 U/50 mg (Cellgro, Manassas, VA), L-Glutamine at 2 mM (Cellgro, Manassas, VA), and Hepes solution at 10 mM (Sigma, St. Louis, MO). R-10 was prepared by mixing the Fetal Bovine Serum/FBS (Atlanta Biologicals, Lawrenceville, GA) in RPMI-PLGH at 10% by volume. The freezing

media consisted of 90% FBS (Atlanta Biologicals, Lawrenceville, GA) and 10% DMSO (Sigma, St. Louis, MO).

PBMCs harvesting

PBMCs were harvested using density gradient Ficoll-Paque protocol.[43] 14 ml of Ficoll-Paque™ (GE Health) and 20 ml of RPMI-PLGH media were brought to room temperature prior of starting density gradient Ficoll-Paque protocol. Healthy patient blood was drawn from an anonymous healthy donor and the Buffy coat (blood without plasma) was prepared on the same day (Gulfcoast Regional Blood Center in Houston, Texas). 10 ml of Buffy coat was transferred in a 50-ml conical tube and the volume was brought to 30 ml using RPMI-PLGH. 14 ml of Ficoll-Paque was added underneath the mixture by lowering the pipet to the bottom of the conical. All conical tubes were centrifuged at 700 xg for 30 min at room temperature with a minimum acceleration and no brake. Aspiration was performed slowly from the top until at least 10 ml of media in each tube. The mononuclear layer was harvested using 10 ml pipet and collected in one 50-ml conical tube. Media was added to the conical tube up to 45 ml and the tubes were centrifuged at 700 xg for 10 min. The washing process was repeated two more times and the washed pellets were resuspended with 10 ml of R-10 media. The washed pellets were frozen in freezing media at ~10 million cells/ml aliquots. RA patient PBMCs were purchased commercially (Astarte Biologics, Redmond, WA).

Memory B cells stimulation

PBMCs were prepared at a concentration of ~3 million cells/ml in 300 µL R-10 in a 15-ml conical tube. The stimulation reagents were added into the solution: CD40L (R&D System) at concentration of 2.5 µg/ml, IL-21 (BD Bioscience) at concentration of 50 ng/ml, and anti-APO1 (Bender MedSystems) at concentration of 5 µg/ml.[44] The PBMCs were incubated (5% CO_2/37^oC) for a period of four days.

B cells enrichment

B cell enrichment was performed by using the EasySep™ protocol.[45] PBMCs were prepared at a concentration of 5 million cells/ml in PBS + 2% FBS with 1 mM EDTA. PBMCs were placed in a round bottom, non-tissue culture-treated 96 well plate (Brand, Wertheim, Germany). EasySep™ Human B Cell Enrichment Cocktail (Stemcell Technologies) was added at 50 µL/mL concentration per well. The solution was mixed and incubated at room temperature for 10 min. EasySep™ D Magnetic Particles (Stemcell Technologies) were vortexed for 30 sec to ensure that the particles are in a uniform suspension with no visible aggregates. EasySep™ D Magnetic Particles were added at 100 µL/mL concentration per well. The solution was mixed and incubated at room temperature for 5 min. The cells suspension was gently mixed by pipetting up and down 2 - 3 times. The 96 well plate was placed onto the EasyPlate Magnet (Stemcell Technologies), ensuring that the plate sat securely on the magnet. The plate was incubated for 10 min. After the incubation time was done, the enriched cell suspension was carefully pipetted transferred to a new well. The negatively selected, enriched B cells in the new well were now ready for use.

Cells loading

Prior to cell loading, the chip was first cleaned by plasma-oxidation for 1 min at high RF setting (PDC-32G, Harrick Plasma). To maintain hydrophilicity, 15 mL of PBS was prepared on a petridish and the chip was placed facedown. Density of cells was determined by using a hemacytometer. The cells were resuspended to 500,000 cells/ml in 300 µL of R-10 media. Nanowell arrays were inverted to face up and the PBS was aspirated until a thin layer of PBS remaining on the chip. Nanowell arrays were then rinsed with 5 ml of R-10 to fill all wells with media and R-10 was aspirated until a thin layer of R-10 was remaining. The cells were then loaded onto the chip by dispensing dropwise and incubated for 10 min to ensure the cells in the bottom of wells. Observation under a standard tissue-culture microscope was performed to ensure high occupancy and distribution of cells in the wells. In the event of insufficient cell loading, additional loadings were performed until the desired occupancy and distribution of cells were met. Final rinsing of the chip was performed with 5 ml of RPMI-PLGH and the chip was ready for microengraving.

Microengraving: glass slide

To perform microengraving, a poly-L-lysine glass slide was coated with a capture antibody of donkey anti-human Ig (Jackson ImmunoResearch, West Grove, PA) at 25 µg/ml in borate buffer (sodium borate 50 mM, sucrose/trehalose 50 mM, NaCl 50 mM, pH 9). 80 µL of capture antibody solution was loaded on the glass slide and immediately covered with a cover slip carefully. After 1 hr of incubation at room temperature, the glass slide was washed with 3% milk/PBST for 10 min on a rocking

platform. The glass slide was washed subsequently with PBST 0.05% (0.05% Tween in PBS) for 5 min. The final washing was with PBS for 5 min and rinsed briefly with DI water. The slide was then dried by a microarray centrifuge, sandwiched with cells-loaded nanowell arrays chip using the hybridization chamber (Agilent Technologies, Santa Clara, CA) and incubated for 2 hr (5% CO_2/37^oC). Post-hybridization, the glass slide was detached from the chip while ensuring that the chip was immersed in PBS. The glass slide was washed in 1% milk/PBST for 10 min, PBST 0.05% for 5 min, and PBS for 5 min. The glass slide was rinsed briefly with DI water and dried using a microarray centrifuge. The glass slide was then incubated with CCP peptide (Anaspec, Fremont, CA) at 25 µg/ml in 80 µL of PBS. After 1 hr of incubation at room temperature, the glass slide was washed with PBST 0.05% for 5 min and PBS for 5 min. The glass slide was rinsed briefly with DI water and dried in a microarray centrifuge. The final incubation of the glass slide was with the detection antibodies: anti-human IgG-AF 488 (Jackson ImmunoResearch, West Grove, PA), anti-human IgA-AF 532 (BD, Franklin Lakes, NJ), anti-human IgM-AF 594 (BD, Franklin Lakes, NJ), and streptavidin-AF 647 (Sigma, St. Louis, MO) was performed in 80 µL of PBS, carefully covered with the cover slip for 1 hr at room temperature. The glass slide was washed with PBST 0.05% for 5 min and then PBS for 5 min. The glass slide was rinsed briefly with DI water and dried with a microarray centrifuge.

Microengraving: nanowell arrays

After the detachment of the nanowell arrays from the glass slide sandwich, the microscopy of cells was performed using an Axio Observer Z1 Epifluorescence

inverted microscope (Zeiss, Jena, Germany). The chip was incubated with an aliquot of 300 µL PBS with anti-CD19-AF 532 (BD, Franklin Lakes, NJ) at 4 µg/ml and Calcein Violet (Invitrogen, Grand Island, NY) at 1 µg/ml using a coverslip for 15 min before microscopy. Post-microscopy, the chip was transferred to 4-well plate (NUNC, Rochester, NY), immobilized with 0.8% noble agar (Gibco, Grand Island, NY), and immersed in 10 ml of PBS solution until the coverslip detached. The chip was then stored at 4^{o}C until the B cell retrieval process.

Cells retrieval

The target tubes, 0.1 mL PCR tubes (Invitrogen, Grand Island, NY), were prepared for the retrieval process using the CellCelector robot (ALS, Jena, Germany). An aliquot of 2 µL RT buffer 5x (Invitrogen, Grand Island, NY) and 2 µL sterile DI water was added to the 0.1 ml PCR tubes. The 4-well plate containing the chip was transferred from 4^{o}C and placed on the motorized stage of CellCelector. The CellCelector glass microtip was connected to a motorized syringe filled with mineral oil and utilized capillary pressure to perform cell retrieval process. The data exported to the CellCelector software (explained in the analysis section) determined the exact location of B cells on the chip. The retrieved B cells were transferred one cell at a time to target PCR tubes and stored in -80^{o}C for RT-PCR experiment.

RT-PCR

The RT-PCR experiment consisted of (i) Reverse Transcription, (ii) PCR 1, (iii) Nested PCR (PCR 2) using single cell RT-PCR established protocol.[58] The reverse transcription reaction started with lysing the cells with 3 µl of 5% NP-40

(Sigma, St. Louis, MO) and 1 µl of 5 pmol/µl of constant region RT primers (Integrated DNA Technologies, Coralville, IA) (Table 1) in a 0.2 ml PCR tube (Axygen, Union City, CA). The PCR tubes were placed in a Veriti thermocycler (Applied Biosystems, Carlsbad, CA) with heating at 65^{o}C for 3 min and cooling at 25^{o}C for 3 min. An aliquot of 2 µl of RT buffer 5x (Invitrogen, Grand Island, NY), 2 µl of DTT (Invitrogen, Grand Island, NY), 1 µl of 10mM dNTP (Takara, Shiga, Japan), and 0.5 µl Superscript III RT at 200 U/µl (Invitrogen, Grand Island, NY) was added to each PCR tube. The tubes were kept at 37^{o}C for 1 hr and heated to 70^{o}C for 10 min to produce cDNA products. Two aliquots of 8 µl cDNA products were used as a template for first PCR cycles of variable heavy and variable light. For variable heavy, an aliquot of 8 µl cDNA product, 6 µl of PCR buffer 10x (Takara, Shiga, Japan), 1.6 µl of 10 mM dNTP (Takara, Shiga, Japan), 0.5 µl of leader sequence primer mixture at 20 pmol/µl for each primer (Integrated DNA Technologies, Coralville, IA), 0.5 µl of constant region primer mixture at 20 pmol/µl for each primer (Integrated DNA Technologies, Coralville, IA), 1 µl of 2.5 U/µl Taq Polymerase (Takara, Shiga, Japan), and 48 µl of sterile-filtered H_2O.

Three cycles of pre-amplification were employed in the first PCR (heating to 94^{o}C for 45 s, 45^{o}C for 45 s, 72^{o}C for 1 min 45 s) followed by thirty cycles of amplification (94^{o}C for 45 s, 50^{o}C for 45 s, 72^{o}C for 1 min 45 s) and final 10 min incubation at 72^{o}C. An aliquot of 3 µl PCR 1 product, 5 µl of PCR buffer 10x (Takara, Shiga, Japan), 1.25 µl of 10 mM dNTP, 1 µl of nested PCR primer mixture at 20 pmol/µl for each primer (Integrated DNA Technologies, Coralville, IA), 1 µl of

constant region III primer at 20 pmol/µl for each primer (Integrated DNA Technologies, Coralville, IA), 1 µl of 2.5 U/µl Taq Polymerase, and 38 µl of sterile-filtered H_2O was added in new PCR tubes. Thirty cycles of amplification were employed in the second PCR by heating to 94°C for 45 s, 50°C for 45 s, 72°C for 1 min 45 s and final 10 min incubation at 72°C. The PCR 2 products were run using agarose gel electrophoresis.

Oligonucleotides used as primers in single-cell PCR

Description	Name	Sequence[a]
cDNA synthesis		
Constant regions	CµI	5′-GCAGGAGACGAGGGGGA
	CγI	5′-AGGG(C/T)GCCAGGGGGAA
	CκI	5′-AACAGAGGCAGTTCCAGA
	CλI	5′-TGTGGCCTTGTTGGCTTG
First PCR		
VH leader sequences	VHL-1	5′-TCACCATGGACTG(C/G)ACCTGGA
	VHL-2	5′-CCATGGACACACTTTG(C/T)TCCAC
	VHL-3	5′-TCACCATGGAGTTTGGGCTGAGC
	VHL-4	5′-AGAACATGAAACA(C/T)CTGTGGTTCTT
	VHL-5	5′-ATGGGGTCAACCGCCATCCT
	VHL-6	5′-ACAATGTCTGTCTCCTTCCTCAT
Vκ leader sequences	VκL-1	5′-GCTCAGCTCCTGGGGCTCCTG
	VκL-2	5′-CTGGGGCTGCTAATGCTCTGG
	VκL-3	5′-TTCCTCCTGCTACTCTGGCTC
	VκL-4	5′-CAGACCCAGGTCTTCATTTCT
Vλ leader sequences	VλL-1	5′-CCTCTCCTCCTCACCCTCCT
	VλL-2	5′-CTCCTCACTCAGGGCACA
	VλL-3	5′-ATGGCCTGGA(T/C)C(C/G)CTCTCC
Constant regions	CµII	5′-CAGGAGACGAGGGGGAAAAG
	CγII	5′-GCCAGGGGGAAGAC(C/G)GATG
	CκII	5′-TTTCAACTGCTCATCAGATGGCGG
	CλII	5′-AGCTCCTCAGAGGAGGG(C/T)GG
Second PCR		
V_H framework-1 regions	VH-1	5′-TTGCGGCCGCCAGGT(G/C)CAGCTGGT(G/A)CAGTC
	VH-2	5′-TTGCGGCCGCCAG(A/G)TCACCTTGAAGGAGTC
	VH-3	5′-TTGCGGCCGC(G/C)AGGTGCAGCTGGTGGAGTC
	VH-4	5′-TTGCGGCCGCCAGGTGCAGCTGCAGGAGTC
	VH-5	5′-TTGCGGCCGCGA(G/A)GTGCAGCTGGTGCAGTC
	VH-6	5′-TTGCGGCCGCCAGGTACAGCTGCAGCAGTC
Vκ framework-1 regions	Vκ-1	5′-CATAAGATCTCG(A/C)CATCC(A/G)G(A/T)TGACCCAGT
	Vκ-2	5′-CACCAGATCTCGAT(A/G)TTGTGATGAC(C/T)CAG
	Vκ-3	5′-CACCAGATCTCGAAAT(T/A)GTG(T/A)TGAC(G/A)CAGTCT
	Vκ-4	5′-CACCAGATCTCGACATCGTGATGACCCAGT
Vλ framework-1 regions	Vλ-1	5′-TATTAGATCTCCAGTCTGTGCTGACTCAGC
	Vλ-2	5′-TATTAGATCTCCAGTCTGCCCTGACTCAGC
	Vλ-3	5′-CACCAGATCTCTCCTATGAGCTGAC(T/A)CAGC
Nested constant regions	CµIII	5′-AGGTCTAGAGAAAAGGGTTGGGGCGGATGC
	CγIII	5′-AGGTCTAGAGAC(C/G)GATGGGCCCTTGGTGGA
	CκIII	5′-TATTCCATGGAAGATGAAGACAGATGGTGC
	CλIII	5′-CATTCCATGGGGGAACAGAGTGACCG

[a] Degenerate positions are shown in parentheses. Restriction enzyme sites are underlined.

Table 1: List of primers (oligos) for RT-PCR experiment.[46]

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed with 0.8% agarose gel (Gibco, Grand Island, NY) in TAE 1x buffer (Fisher, Waltham, MA) by using an established protocol.[47] An aliquot of 3 µl of each PCR 2 product was combined with 2 µl of 6x loading dye (NEB, Ipswich, MA), and 7.5 µl of TAE 1x buffer. The mixtures were loaded to each lane on the gel and an aliquot of 0.5 µg of 1 kb DNA ladder (NEB, Ipswich, MA) with 2 µl of 6x loading dye and 7.5 µl of TAE 1x buffer was loaded as a standard. The parameters for electrophoresis were 110 V for 40 min running time. After the electrophoresis was performed, the gel was transferred into container with 50 ml of TAE 1x buffer and ethidium bromide (NEB, Ipswich, MA) at 0.5 µg/ml. The agarose gel was rocked for 15 min in a rocking platform for DNA staining. After the staining, the agarose gel was exposed to UV light and DNA bands of different length were observed.

ELISA

ELISA was performed based on the established protocol.[48] An ELISA 96-well polystyrene plate (NUNC, Rochester, NY) was coated with streptavidin at 50 µg/ml (Sigma, St. Louis, MO). The plate was sealed and incubated at 4^{o}C overnight. Each well was aspirated and washed 5 times with 300 µL of washing buffer (PBS 1x, 0.05% Tween-20) per well. The plate was inverted and blotted on absorbent paper to remove any residual buffer. Each well was blocked with 200 µL of 10% BSA (Sigma, St. Louis, MO) in washing buffer and incubated at room temperature for 2 hr. The wells were aspirated and washed 5 times with 300 µL of washing buffer. The plate was

inverted and blotted on absorbent paper to remove any residual buffer. The plate was then coated with CCP peptide at 25 µg/ml. The plate was sealed and incubated at room temperature for 1 hr. After incubation, the wells were aspirated and washed 5 times with 300 µL of washing buffer. The plate was inverted and blotted on absorbent paper to remove any residual buffer. 100 µL aliquots of diluted human plasma (1:100, 1:500, 1:1000, 1:5000 dilutions) were added to respective wells. The plate was sealed and incubated at room temperature for 45 min. The wells were aspirated and washed 5 times with 300 µL of washing buffer. The plate was inverted and blotted on absorbent paper to remove any residual buffer. An aliquot of 100 µL HRP CCP 3.1 IgG/IgA Conjugate (Inova Diagnostics, San Diego, CA) was added to each well. The plate was sealed and incubated at room temperature for 1 hr. The wells were aspirated and washed 5 times with 300 µL of washing buffer. The plate was inverted and blotted on absorbent paper to remove any residual buffer. 100 µL of TMB substrate (Thermo, Waltham, MA) was added to each well and the plate was incubated at room temperature for 10 min. After the incubation, the absorption (OD 450 nm) was detected by using Infinite M300 Microplate Reader (Tecan, Männedorf, Switzerland).

Spotting Assay

The poly-L-lysine glass slide was coated with 2 µL of streptavidin (50 µg/ml) by pipetting and incubated for 1 hr at room temperature in a humidified container to avoid drying. The streptavidin spots were aspirated and the glass slide was blocked with 30 mL of blocking buffer (10% BSA in PBS 1x, 0.05% Tween-20) for 2 hr at room temperature. The blocking buffer was removed and the slide was washed for 10

min in 30 mL of washing buffer (1X PBS, 0.05% Tween-20). The washing buffer was decanted and the slide was washed for 5 min in 30 mL of PBS. The PBS solution was removed and the glass slide was immersed briefly in 30 mL of DI water. The slide was then dried in the microarray centrifuge. An aliquot of 2 µL of CCP peptide (25 µg/ml) was coated by pipetting in the exact location where streptavidin was previously coated. The glass slide was incubated at room temperature for 1 hr in humidified container to avoid drying. The CCP spots were aspirated and the glass slide was blocked with 30 mL of 3% BSA in washing buffer for 10 min at room temperature. The blocking buffer was decanted and the slide was washed for 10 min in washing buffer followed by 5 min washing in PBS. PBS solution was decanted and the glass slide was immersed once in 30 mL of DI water followed by drying in microarray centrifuge. The glass slide was assembled in Microarray Microplate 96 (Arrayit, Sunnyvale, CA) and aliquots of 150 µL diluted human plasma (1:100, 1:500, 1:1000 dilutions) were added to each well in the microplate. The microplate was sealed and incubated at room temperature for 45 min. After incubation, The diluted human plasma were aspirated and blocked with 30 mL of 1% BSA in washing buffer for 10 min at room temperature. The blocking buffer was decanted and the slide was washed for 10 min in washing buffer followed by 5 min washing in PBS. The PBS solution was decanted and the glass slide was immersed once in 30 mL of DI water followed by drying in the microarray centrifuge. An aliquot of 80 µl goat anti-human IgG-AF 647 (Jackson ImmunoResearch, West Grove, PA) at 1 µg/ml was used as detection antibody, evenly coated the glass slide with coverslip. The slide was incubated at room

temperature for 1 hr under no light exposure. The washing buffer was used to washed the slide for 5 min followed by PBS washing for 5 min. The PBS solution was decanted and the glass slide was immersed once in 30 mL of DI water followed by drying the glass slide in a microarray centrifuge. The glass slide was scanned for a fluorescence signals at 647 nm using GenePix 4200A Microarray Scanner (Molecular Devices, Sunnyvale, CA).

Data Analysis

The analysis could be divided into: (i) microscopy, (ii) antibody secretion, (iii) cell retrieval. First, microscopy was performed on the chip loaded with PBMCs stained anti-CD19 (B cell marker) and Calcein violet (live cell marker). Each microscopy image consisted of a block of 7 x 7 wells and the imaging was performed for each channel (brightfield, anti-CD19, and Calcein violet channel). The resulting images (.tiff format) were analyzed by MabAnalyze (custom script) and produced a list of unique well code with intensity values of each channel. The resulting data from MabAnalyze were exported to Microsoft Excel where the histograms of the log intensity for CD19 positive and live cells were produced. The histograms provided the minimum cut-off values of intensity for both channels. These cut-off values were used as initial iteration intensity values by manually observing the microscopy images. The manual observation validated the MabAnalyze analysis and the population of live CD19 positive cells was determined after manual observation (Figure 15).

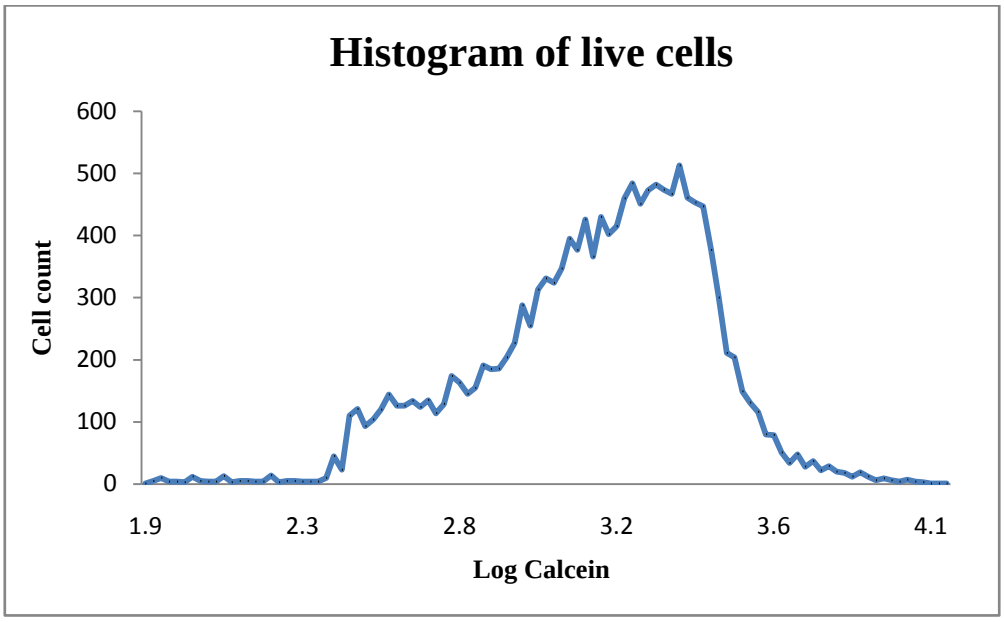

Figure 15: Histogram of live cells based on Calcein violet channel intensity.

The microscopy data from Microsoft Excel was exported to Microsoft Access to provide a platform for mapping the data from the antibody secretion by microarray scanner. The microarray scanner provided the data of the location of B cells that secreted immunoglobulins with their isotypes. Built-in arrays of block from the microarray scanner software were used as a template to flag positive spots for different channels. The resulting data were in the format of a block number with corresponding intensity values for each channel and the resulting data was exported to Microsoft Excel for further manipulation. The block number from each spot was transformed to a unique identification number which corresponded to the mapped location in the microscopy. The microarray scanner data was exported to Microsoft Access and the process of matching the unique identification number from both sets of data was performed. The process gave the population of live CD19 positive cells that secreted immunoglobulin with the isotype information. In the case of multiple B cells that

existed in the same well, Microsoft Access was able to differentiate them and only the case for the single B cell in one well included in the final analysis. An example of the analysis result was the distribution of the immunoglobulin isotype secreted by live CD19 positive cells/live B cells (Figure 16).

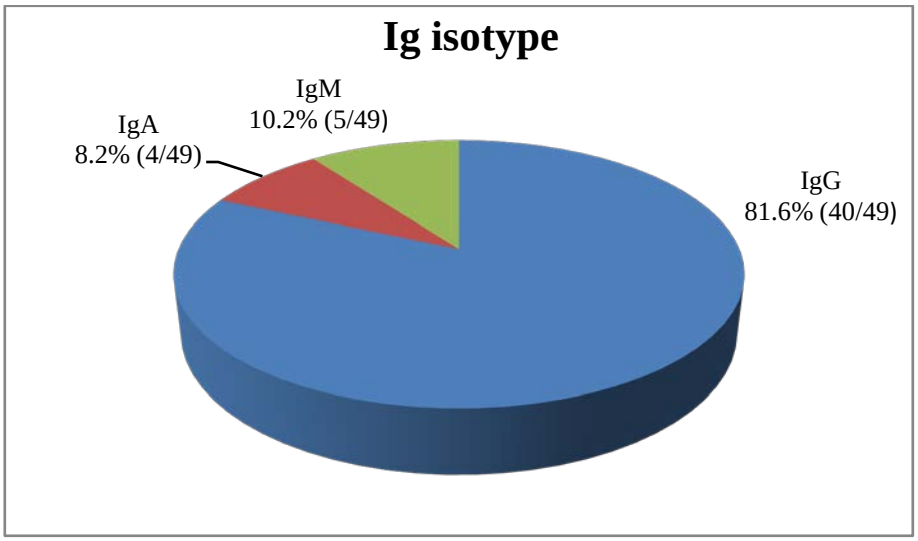

Figure 16: Immunoglobulin isotype distribution from live B cells. Pie chart indicating the relative percentages of live B cells secreting an isotype of antibody following the four day stimulation.

Lastly, based on the combined microscopy and antibody secretion matching data, the B cells of interest were retrieved by the CellCelector robot. The unique identification numbers each B cell had in the Microsoft Access analysis were transformed to sets of block, row, and column (.csv file) that the CellCelector recognized. An automated retrieval process was performed after the file was exported to CellCelector.

VI. FUTURE DIRECTIONS

The short term goal is to amplify the retrieved B cells from the RA patient experiment (comprising of a total of 49 B cells retrieved from 3 microengraving experiments) and to express and produce the antibodies using HEK293 cell line. The

antibody production from the time of DNA transfection to HEK293 cell line will take 3-4 weeks.[49] After that time period, the supernatant of the culture will also be retrieved and the reactivity of the produced antibody could be assessed using ELISA and the spotting assay. From the produced antibodies, screening will be performed to determine which antibodies possess high reactivity against CCP and the genes of those antibodies will be sequenced commercially. If out of those 49 B cells retrieved, there is no antibody reactive to CCP, then we will repeat the microengraving experiment with other RA patients. Our collaborator, Dr. Sandeep Agarwal (UT-Health Science, Houston) will provide blood from the RA patients and the downstream strategy in screening ACPA antibodies will be the same as described above.

The long term goal is to utilize these ACPA antibodies to perform an immunoprecipitation experiment with synovial fluid and bone marrow with the purpose to acquire the specific citrullinated protein(s) that ACPA bind to in the synovial fluid and also to assess whether the protein(s) are also present in bone marrow. Another appealing idea is to test the reactivity of ACPA antibodies against protein in patient bone as we know that bone erosion is one of the symptoms of late stage RA disease.

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