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USING TIRF MICROSCOPY TO ANALYZE STIMULATED AND BASAL STATE B-CELL MHC II CLUSTERING IN RESPONSE TO AGEING AND DIETARY FISH OIL

By

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July 2013

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This research focused on developing an efficient TIRF microscopy approach to evaluate membrane protein organization. More specifically, the data demonstrate that TIRF microscopy can detect changes in *ex vivo* B-cell MHC II lateral organization using a monoclonal antibody under differing conditions. MHC II clustering is dependent on the underlying lipid environment and upon LPS stimulation MHC II expression is dramatically increased. Using mice fed a fish oil or control diet for three weeks, or using mice aged for nine months, we imaged splenic B-cell MHC II clustering using TIRF microscopy. We also used LPS to stimulate B-cells from both experimental conditions to determine if either ageing the animals or feeding them fish oil could affect MHC II clustering. We then determined cluster quantity, size, and intensity using the NIH ImageJ software. The data showed that neither a relevant dose of fish oil, nor aging the mice approximately nine months, had an affect on MHC II clustering in the absence of LPS stimulation. However upon LPS stimulation, MHC II clustering dramatically changed in aged mice as well as fish oil fed mice compared to control animals. Taken

together, the data establish the TIRF microscopy protocol as a relevant alternative tomore costly and time consuming approaches to address membrane protein clustering. Moreover, either ageing the animals or feeding them fish oil significantly affects MHC II clustering upon stimulation with LPS.

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by

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TABLE OF CONTENTS

Chapter 1: Literature Review

1.1 *The plasma membrane and membrane microdomains*

The plasma membrane consists of an amphipathic phospholipid bilayer and membrane proteins and each of these components serve several important biological functions necessary for life to exist. In 1925, the findings of Gorter and Grendel demonstrated the first evidence for the existence of phospholipid bilayers using red blood cells [1]. Their results were later bolstered in the 1950's with electron micrographs demonstrating visual confirmation of the lipid bilayer [2]. Since these early investigations into the plasma membrane, the scientific understanding of what the plasma membrane is and how it functions has advanced tremendously. The theoretical underpinning of the most widely taught membrane model is the Singer and Nicolson fluid mosaic model first published in 1972 [3]. The fluid mosaic model hypothesizes unrestricted and random movement of lipids and proteins that make up the plasma membrane [3]. The Singer and Nicolson model predicts proteins are mainly monomeric and dispersed at relatively low concentrations in a "sea of lipids" with little to no preferential association between proteins or proteins and lipids [3, 4]. While this model was the initial paradigm that built the foundational understanding of the molecular components of the plasma membrane, the unrestricted lateral diffusion and lack of lateral heterogeneity described in the Singer and Nicolson model has been challenged over the past forty years [4, 5]. With the advancements in microscopy, cell biophysics, molecular biology, and other technologies and techniques it has become apparent the plasma membrane is not an unrestricted fluid bilayer where membrane lipids and proteins move about freely and interact randomly [3, 4]. Instead, as Donald Engelman states, "membranes are more mosaic than

fluid" containing much more protein than suggested by the fluid-mosaic model and, while Singer and Nicolson described the "standard conceptualization of membrane architecture", the generalizations made in their model are widely ineffective at describing our current understanding of the structure and function of the plasma membrane [4].

Since the development and subsequent publication of the fluid mosaic model a substantial amount of data demonstrate that the membrane exhibits lateral heterogeneity in its structure through preferential association of its molecular components [5]. The membrane is now characterized into the inner and outer leaflets within which particular lipids are found. Four phospholipids characterize more than half of the lipids in the plasma membrane: phosphotidylserine, phosphotidylethanolamine, phosphotidylcholine, and sphingomyelin [4, 6, 7]. Phosphotidylcholine and sphingomyelin are mainly found in the in the outer leaflet of the plasma membrane while the phosphotidylethanolamine and phosphotidylserine are typically found in the inner leaflet [7]. Glycolipids and cholesterol are other lipids that make up the membrane with cholesterol being a major constituent in ratios similar to that of phospholipids [8]. Glycolipids are exclusively found in the outer leaflet of the membrane due to its sugar moieties [7]. Within these lipid constituents of the plasma membrane, proteins and protein complexes interact with the extracellular environment and stimuli in order for the cell to function. Studies investigating the interactions and relationships of lipids and proteins in the membrane, i.e. membrane dynamics, demonstrate that instead of randomness and unrestricted movement of these two components there exists a preferential association of lipids, proteins, and lipid-protein domains of the plasma

membrane [5, 9]. Unique associative properties of particular lipids demonstrate lateral, or lipid, phase separation [5]. For example, in vitro studies demonstrate sphingolipid and cholesterol self associate to form domains thus demonstrating lateral segregation is possible within membranes based on lipid interactions alone [5]. The phase separation demonstrated in these studies was cholesterol dependent. The interactions between saturated fatty acyl chains of membrane lipids and cholesterol are favorable leading to an increase in membrane thickness and segregation into what are known as liquid order phases or microdomains [5]. These microdomains are vital for compartmentalizing particular proteins and lipids that create a functional patchiness that facilitates protein-protein interactions to help propagate protein signaling and cellular function [5, 10, 11]. Some studies argue that most, if not all, plasma membrane proteins are clustered into protein “islands” that are dependent on their surrounding lipid environment to remain intact [12]. However, multiple membrane proteins are known to cluster in response to a ligand binding a specific receptor. This causes multiple proteins to be recruited and signaling complexes to be formed. This process is often dependent on membrane microdomains known as “lipid rafts” [5, 13]. Lipid rafts consist of sphingolipids, glycosphingolipids, and cholesterol [5, 6].

Lipid rafts were proposed to explain how glycosphingolipids are enriched at the apical surface of polarized epithelia cells [14]. Thus, rafts represent a segment of nonrandom membrane organizing functionality within the membrane. The lipid component of lipid rafts are vital, however the protein within the rafts are what carry out the function recognizing ligands and initiating signaling. In a state where a ligand is not present, lipid rafts are transient dynamic nanoscale microdomains that are cholesterol

dependent. These nanoscale rafts can be stabilized and coalesce through interactions of proteins with ligands and signaling complexes to form larger “raft platforms” and, in theory, a “raft phase” that is more dependent on the lipid-protein/protein-protein interactions than lipid-lipid self association [5]. However, not all proteins contain the properties to associate and compartmentalize within rafts. Thus, one characterization of proteins is whether they are raft or non-raft resident proteins. This is demonstrated by antibody crosslinking of proteins that cluster with rafts excluding other proteins that do not show a sterol preference. Thus, many membrane receptors necessary for cellular function are known to localize within lipid rafts upon ligand stimulation [5, 8, 15, 16]. The ability for the membrane to form this raft based heterogeneity to selectively compartmentalize proteins that propagate signaling is a vital function that maintains both cell and whole organism function [8, 15, 16].

1.2 *B-lymphocytes and protein clustering*

Lymphocytes are a class of white blood cells that include B and T-lymphocytes[17]. Both cell types are vital for adaptive immunity. B-lymphocytes are responsible for humoral immunity while T lymphocytes are responsible for cell meditated immunity [17]. The former is the primary cell type discussed in this thesis and will be referred to as B-lymphocytes and B-cells interchangeably. B-lymphocytes originate in the fetal liver and adult bone marrow from hematopoietic multipotent stem cells [18]. The development of B-lymphocytes in the bone marrow are defined by the expression of cell surface markers that are used to characterize what stage of development the B-lymphocytes are in [18]. Immature B-lymphocytes are defined by the expression of membrane bound immunoglobulin mu (IgM) on their surface [17, 18]. When

IgD is concurrently expressed on the surface the B-cell it is considered a mature naïve B-cell. They are considered naïve because the B-cell has not encountered its specific antigen with an epitope capable of activating that particular B-cell [17]. Each membrane bound immunoglobulin recognizes a specific antigenic epitope unique to that B-cell [17, 19]. Following the development of a mature naïve B-lymphocyte, B-cells enter circulation and secondary lymphoid organs (e.g. lymph nodes and the spleen) where they encounter their specific antigen and functionally mature [18]. Membrane bound immunoglobulins, such as IgM, are a critical component of the B-cell receptor (BCR) whose function is responsible both for B-cell development in the bone marrow and functional maturation in the periphery [18]. The BCR also contains Ig-alpha and Ig-beta, which have cytoplasmic domains that contain immunoregulatory tyrosine based activation motifs (ITAMS) [18, 20]. Since the cytoplasmic tails of the membrane bound immunoglobulin component of the BCR are not capable of transducing signaling events, it is Ig-alpha and beta that initiate intracellular signaling events through phosphorylation of their ITAM domains [20, 21]. Thus in a mature naïve B lymphocyte in the periphery, the IgM component of the BCR binds the specific antigen while Ig-alpha and beta facilitate the initiation of the intracellular signaling events leading to B-cell activation [10, 21]. The functional endpoint of B-lymphocytes after encountering immunogenic antigens within secondary lymphoid organs is to produce antibodies through differentiating into plasma cells or forming memory B-cells that are capable of producing antibodies upon a later encounter with that specific antigen [18]. These antibodies are soluble immunoglobulins specific for the same antigen that is initially recognized by the BCR. There are five

isotypes of immunoglobulins, determined by the constant region of their heavy chains, that are found in both the BCR and as antibodies: IgM, IgD, IgA, IgE, and IgG.

Lymphocytes and their membrane bound immunoglobulins have played an integral part in helping to amend and evolve the fluid-mosaic membrane model [4]. In the 1970's murine splenic B-cells showed capping of IgM and IgD to one 'pole' of the cell in response to specific antisera [22]. Moreover as early as 1976, four years after the fluid mosaic model was published, IgM and IgD were shown to co-cap in response to specific antigens [22]. This cocapping demonstrated that the prediction of nonrandom interactions between proteins of the plasma membrane by the fluid mosaic model was incorrect. Lymphocytes were also used to demonstrate that the plasma membranes and its molecular components are very dynamic, constantly changing depending what environment the lymphocyte is responding to. However, the discovery of supramolecular protein complexes using lymphocytes, as well as other cell types, has been critical to both understanding immunological function of organisms as well as understanding the interactions of lipids and proteins within the plasma membrane. For example, Susan Pierce and colleagues research addressing the initiation of B-cell activation demonstrates that once presented with antigen through planar bilayers, B-cells containing specific BCRs capable of recognizing that particular antigen initially form microclusters of immobilized BCRs leading to eventual recruitment and localization of larger quantities of BCRs to immunological synapse between the artificial bilayer and the B-cell [21]. Moreover, they demonstrated that initial BCR microclusters recruit lipid rafts containing the Src family kinase Lyn to proporgate signaling and begin the process of activating the B-cell. Other studies addressing B-cell recognition of T-cell dependent

antigens, antigen processing, and subsequent antigenic peptide presentation to $CD4^+$ T-cells are some of the most eloquent examples of membrane dynamics and protein clustering [23, 24]. In secondary lymphoid organs such as the spleen B-cells encounter T-cell dependent antigens that require not only the recognition of BCR but also the function of another membrane raft resident protein major histocompatibility complex II (MHC II). MHC II is a central protein for antigen presentation to CD4+ T cells via antigen presenting cells (APCs) [25]. In APCs, such as the B-cell, MHC II is loaded intracellularly with specific peptides derived from a processed antigen [26, 27]. It then presents this peptide-MHC II complex to CD4+ T cells for recognition through a cognate T cell receptor (TCR) [28, 29]. How the T cell is able to find the specific peptide-MHC II complex for its TCR is highly debated. However, multiple studies indicate that MHC II will cluster on APC's once loaded with peptide [30]. Clustering of peptide-MHC II complexes depends on the ability of the complex to activate the T cell [30]. This means the more agonistic peptide-MHC II complexes associate with one another while the less agonistic associate together. This allows for efficient recognition of peptide-MHC II complexes that will recognize the T cell receptor of CD4+ T cells and is vital for responding to T-dependent antigens [30]. This process is vital for the development of plasma cells, germinal centers, and memory B-cells that make up humoral immunity and is raft dependent. MHC II expression has been shown to sensitive to activation with lipopolysaccharide using flow cytometry and its spatial distribution is sensitive to changes in the lipid composition of the plasma membrane. However, the majority of the studies that investigate MHC II peptide presentation to $CD4^+$ T-cells, expression, or clustering utilize transfection models or other methods that bring into question the

physiological relevance of the techniques and protocols. This research utilizes murine splenic B-cells MHC II to develop a different approach to study the micron scale MHC II clustering utilizing total internal reflection fluorescent microscopy.

1.3 Fish oil

Fish oil is found in different types of oily fish and is rich in n-3 polyunsaturated fatty acids (n-3 PUFAs), also known as omega-3 fatty acids [31, 32]. This terminology is derived from the position of the double bond that closest to the last methyl group or "omega" carbon. Counting the terminal methyl on the acyl chain as the first carbon, omega-3 fatty acids have the closest double bond on the third carbon. While there are several omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the bioactive omega-3 fatty acids found in fish oil. These two long chain omega-3 fatty acids can be derived from the simplest member of the omega-3 fatty acid family, alpha-linolenic acid [33]. However, this conversion is poor *in vivo* and to obtain significant amounts of DHA and EPA each must be consumed through the diet [33]. Moreover, increased intake of DHA and EPA lowers the amount of arachidonic acid. This is beneficial as arachidonic acid is a substrate for eicosanoids that have well-established roles in inflammatory conditions such as asthma [33].

Fish oil is currently being explored as immunotherapeutic agent to suppress several inflammatory diseases [34, 35], but it cannot be classified solely as an immunosuppressant because other studies demonstrate that it can also enhance specific immune responses [36, 37]. For example, fish oil can attenuate Th1 T-cell function while enhancing Th2 T-cell function [38, 39]. Thus, immuno-suppression depends on the cell and type of immune function studied. Moreover, since the mechanism of fish oil is

not known, it makes it difficult to predict how it will respond in different physiological conditions and limits the ability to use fish oil in a clinical setting. Several mechanisms are suggested to explain how fish oil functions and each of theses are not considered to be mutually exclusive [40]. While fish oil can act via membrane receptors such as GPR120 it has also been postulated to alter the lipid content of the plasma membrane and thereby affect protein clustering and function [33]. More specifically, it has been hypothesized that the ability of fish oil to affect the composition of the lipid component of the membrane could affect membrane order and thus subsequent membrane microdomains such as lipid rafts. This could thereby affect intracellular signaling and gene expression.

1.4 Ageing

Ageing is the progressive and irreversible loss of function accompanied by increasing mortality with advancing age. How the ageing process affects the immune system is a matter of debate. Generally speaking, the classical view was considered that the immune was in a unidirectional decline in function with age [41]. However, more recently the model has shifted towards a more balanced view of immunosenescence in which particular immunological functions and cell populations increase while others decrease. For example, Th1 cytokines are increased while Th2 cytokines are decreased [42]. Thus, determining how ageing affects the immune system cannot be generalized and must be put into context as to what immunological cell type and function one is examining.

Another paradigm of aging is the inability to produce an optimal immune response to vaccines and infectious agents [42]. One explanation for such a decline to

vaccines is that ageing reduces the humoral response in both a qualitative and quantitative manner. High affinity antibodies produced by specific subsets of differentiated B-cells make up the humoral immune response [18]. In aged animals germinal center reactions and that drive B-cell differentiation and somatic hypermutation to produce antibodies with high affinity for their particular antigen are effected greatly [42]. This is potentially from a combination of intrinsic B-cell defects and non-intrinsic B-cell defects such as decreased T-cell and follicular dendritic cell function. Interestingly, the majority of the literature concerning how ageing affects these and other immunological responses focus more on T-cells and dendritic cells and not B-cells [41]. The few studies that have addressed B-cell defects with ageing have focused how ageing affects B-cell subset population and antibody formation and production [42]. While this research is vital in developing an understanding of how ageing affects the immune system much more research is needed to determine exactly what ageing does to differentially affect the immune system. One limiting factor to studying ageing are the models available and the translation of physiological relevance of these models to humans [42]. While some studies have used human immune cells to reach conclusions on how ageing affects immune function and cell populations these findings are often different than what are found in mice. Thus, much more research is needed in all facets of study regarding the immune system in aged animals and humans.

1.5 Total internal reflection fluorescence microscopy

The history of the microscope is unclear regarding its origin. The first attempt to piece together the history of the instrument was Dr. Charles Singer in 1914 [43]. Dr. Singer, along with others, believed the first use of magnification was likely “spheres of

glass filled with water". In addition in the third century B.C. writings clearly discuss the optical properties of curved mirrors [43]. However, the use of microscopy to address the molecular underpinnings of cellular processes begins with the discovery and use of lenses [43]. In 1590, building on the use of lenses for eyesight, Hans and Zaharias Janssen are credited with making the first compound microscope [43]. The expansion of the instruments importance came through Robert Hooke's publication Micrographia in 1665. It was Hooke who coined the term cell when using a microscope to view a piece of cork [44]. Anthony Van Leeuwenhoek drew on Hooke's publication using the microscope and considerably improved the optical quality and use of the instrument. Leeuwenhoek's [45] contributions were so significant some have credited him as the father of microscopy [45].

Since the time of Hooke and Leeuwenhoek, many have contributed to the evolution of microscopes in several fields of science establishing the instrument as vital for scientific research. One major advance in the field of microscopy came with the development of total internal reflection microscopy (TIRF) also known as evanescent wave microscopy. While the concept of total internal reflection had been used in a nonmicroscopic spectroscopy the first form of total internal reflection used in microscopy coupled with fluorescence was done by Dr. Axelrod in 1981 [46]. Total internal reflection combined with fluorescence allows for the ability to confine fluorescence excitation to a thin section (however, just how thin is a matter of debate) close to the cell-substrate interface in an aqueous environment [47]. This technique provides a very good signal to noise ratio by minimizing out of focus background fluorescence. Thus, TIRF has numerous applications in biochemistry and cell biology. Traditionally it has been difficult

with epifluorescense or confocal microscopy to studying events at or near the plasma membrane because these events are obscured by fluorescence that originates within the cytosol [47]. Due to the thin sectioning available through TIRF, biological processes occurring at the plasma membrane are not difficult to image.

Through the use of TIRF many cellular actions and components are able to be studied with extreme clarity such as actions of the cytoskeleton, endo- and exocytosis, and proteins found in the plasma membrane. For example, TIRF revealed that microtubules extend immediately adjacent to the plasma membrane in vesicle trafficking[48]. Another expansive area of focus involving studies that use TIRF are to reveal intracellular processes of clathrin-coated vesicles during endocytosis [49]. However, using TIRF to explore the spatial and temporal dynamics of membrane proteins has been expansive. Dr. Susan Pierces work evaluating the protein molecular organization up the activation of B-cells exemplifies the utility of TIRF [21]. Through using antigen load planar bilayers attached to glass coverslips and live cell imaging Dr. Pierce has challenged the view that the B-cell receptor requires a multivalent antigen to induce crosslinking. Her data collected with TIRF points to an intrinsic ability of the B-cell receptor to cluster and initiate signaling without the requirement of multivalent antigens [21].

Other excellent examples of TIRF contributions to expand on membrane protein molecular organization is the work of Dr. Sam Cushman and Dr. Jonathan Bogan concerning glucose transporter 4 [50, 51]. While differing in their conclusions of the regulation and molecular pathway of GLUT4, both used adipocytes transfected with a fluorophore tagged to GLUT4 proteins. More specifically, Dr. Cushman designed an

eloquent system with primary adipocytes where in which he used a pH sensitive fluorophore attached to GLUT4 to determine its docking and dispersion processes at the plasma membrane [51]. While these studies are impressive and utilize TIRF as the primary instrument for study, these techniques consume both time and budget. The data here present an alternative method to more cumbersome approaches.

Chapter 2: Methods

2.1 Mice and cells

Splenic B220$^+$ B-cells were isolated from male C57BL/6 mice as previously described [52], regardless of the treatments and/or conditions used to evaluate B-cell MHC II clustering. Purity of the B-cells was verified with flow cytometry as previously described [37]. For experiments utilizing dietary fish oil, mice were fed either a control or fish oil diet for three weeks. The content of the fish oil diet is 5% of the total fat by weight, which represents approximately four grams of fish oil consumed per day for a human, which models current recommended dosage for treating hypertriglyceridemia. Experiments using methylbetacyclodextrin (MBCD), cytochalasin D, and lipopolysaccharide were conducted using splenic B220$^+$ B-cells from mice approximately the same age as those fed the control and fish oil diets. Splenic B-cells used in the ageing experiments were from mice that were nine months old. Select studies from the fish oil and aged mice were conducted as blinded experiments. All experiments followed the guidelines set in place by East Carolina University for the euthanasia and humane treatment of animals.

2.2 Reagents, equipment, and antibodies

B-cells were stained with 2 ng/μl of M5/114 MHC II antibody conjugated to Cy3. MHC II expression and clustering was measured using an Olympus IX-71 microscope modified with Olympus's cell TIRF system as described previously [37]. Experiments treating B-cells with lipopolysaccharide (Sigma) and methylbetacyclodextrin (Sigma) were done at the same dosage and time frame as previously published [37]. For some

experiments, B-cells were treated with cytochalasin D (Sigma) at 2 μg/mL for 15 minutes to prevent actin polymerization. In all experiments, cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes (Electron Microscopy Sciences) before staining with MHC II.

2.3 TIRF Protocol

During the isolation of the splenic $B220^+$ B-cells, autoclaved sterile glass cover slips (Corning) placed in 60 mm culture dishes were coated with poly-D-lysine (Sigma) for 30 minutes in a sterile environment. The number of cover slips to be coated was determined by the number of samples needed with one cover slip placed in one culture dish. Each sample was done in duplicate. Coating the slides for a longer than 30 minutes did not increase the number of cells adhered nor did it improve TIRF image resolution. When covering the slides with sample, it was imperative that enough poly-D-lysine was added to completely cover the glass cover slips and that the cover slips were not floating on top of the poly-D-lysine solution but submerged within the solution. During the 30-minute coating process, isolated splenic $B220^+$ B-cells were counted and suspended in fresh RPMI media containing 5% fetal bovine serum, 2 mM L-glutamine, and 2% penn/strep (RPMI media) at a concentration of 1 million per mL and placed in the incubator at 37^0 C.

Following the 30-minute incubation period, the poly-D-lysine was removed. The poly-D-lysine solution covering the cover slips could be reused up to two times. Thus, if the poly-D-lysine had not been used more than twice the excess was placed into an appropriate container of used poly-D-lysine solution. After the removal of the poly-D-lysine, each 60 mm culture dish containing a coated glass cover slip was washed three

times with a 1X phosphate buffer solution (PBS) at two minutes per wash. Following the third wash, the 1XPBS was aspirated off and the glass cover slip was suctioned to the bottom of the 60 mm culture dish so that the cover slip did not move around in the culture dish during the experiment. To achieve this, a 200 µL pipette tip was placed over the glass Pasteur pipet that was inserted into the tubing of the aspirator. The tip of the pipette tip was cut to increase the flow rate and then all remaining 1X PBS solution was removed, including the 1X PBS between the glass cover slip and the culture dish, effectively 'adhering' the glass cover slip to the 60 mm culture dish.

The next step was to prepare 625,000 B-cells in 125 uL of RPMI media to be adhered to the cover slips. Thus, 1 FACS tube per sample was loaded with 1 mL of isolated B-cells that were previously suspended at 1 million per mL after being isolated from the spleen. Then each sample was spun in a centrifuge at 23^0C for 5 minutes at 300 G. Following centrifugation, the RPMI media was fully decanted onto a paper towel. Then 200 µL of 37^0C RPMI media was added to each FACS tube containing the 1 million pelleted B-cells. Using a pipette set to 125 µL, the pelleted B-cells were gently resuspended into the 200 µL of RPMI media. Then 125 µL of resuspended B-cells were carefully added to the center of the corresponding cover slip within each culture dish for each sample. Then the samples were delicately placed in the incubator at 37^0C for 30 minutes to allow the B-cells to adhere.

After adhering the cells, the samples were removed from the incubator and washed once for no more than 30 seconds with 1X PBS. Following the quick wash, 2 mL's of 4% PFA fixative was added to each sample and they were placed in the dark. The cells were then fixed for 30 minutes. During this incubation a small section of a lab

bench was cleaned with 70% ethanol and then water drops were added to the cleaned area and a 5 inch by 5 inch piece of parafilm was placed on top. All air bubbles were smoothed out and this effectively 'adhered' the parafilm to the benchtop. This was used to place the MHC II antibody working solution for the adhered/fixed cells to be stained.

After the samples had been fixed, they were washed three times with 1X PBS containing 2% bovine serum albumin (BSA). The 2% BSA allows for blocking to decrease non-specific binding of the antibody. Each wash with the 1X PBS solution containing 2% BSA lasted for five minutes. During the last wash, 100 µL of the MHC II staining solution (3 ng/µL) was added for each sample onto the parafilm. (i.e. if there are five samples add five 100 µL droplets onto the parafilm.) After setting up the staining solutions and conducting the final wash out of the fixative, each sample was added to its respective 100 µL drop of staining solution. This was accomplished by taking the 60 mm culture dish, pinching the sides to create a gap between the cover slip and the culture dish large enough for a pair of tweezers to pick up the sample. Then, being sure not to scrap off the adhered cells with the tweezers, the sample was taken out of the culture dish and placed onto the 100 µL of staining solution for that sample. This was done for each sample and then the samples were covered, in order to prevent photobleaching the fluorophore. Samples were stained for MHC II at 23^0C (room temperature) for one hour.

After the samples were stained, the tweezers are again used to pick the glass cover slip off the staining solution and then placed back into a 60 mm culture dish. The cover slip was placed into the culture dish so that the side of the cover slip that the cells are adhered to was facing up away from the bottom of the dish to prevent the B-cells

from being scrapped off by the rubbing against the bottom. After placing the samples back into their respective culture dishes, each was washed three times with ice cold 1X PBS for four minutes per wash. Following the last wash, each sample was mounted onto a microscope slide (Fischer Scientific) with 100 µL of 1X PBS in-between the glass cover slip with the cells adhered and the microscope slide. The edges of the glass cover slip were painted over with finger nail polish so to seal the adhered cells between the glass cover slip and microscope slide. The 100 µL of 1X PBS prevents the cells from drying out and keeps the samples viable for longer. The samples can then be imaged or stored at 4^0C.

2.3 Pharmaceutical treatment protocol

For experiments using MβCD and cytochalasin D, treatment was conducted after adhering the splenic B-cells to the glass coverslip. Briefly, parafilm was added to a separate 60 mm culture dish where sterile water was placed between the parafilm and dish to 'adhere' the parafilm to the bottom of the dish. Then, 500 µL of MβCD or cytochalasin D was added on top of the parafilm and the sample was placed on top of the solution for treatment. Both treatments were conducted at 37^0C for 15 minutes. The remainder of the protocol followed the same steps as outlined in the previous section.

2.4 Total internal reflection fluorescence (TIRF) microscopy instrument and analysis

TIRF instrumentation relied on an Olympus IX-71 microscope and motorized cell^{TIRF} illuminator [11]. TIRF images were acquired over a range of penetration depths from 100-150 nanometers to demonstrate total internal reflection and to determine what penetration depth had the best resolution and lowest background. All images in this study are at a penetration depth of 100 nanometers with manual adjustments to the

angle offset to reduce the background fluorescence. TIRF images were compared to images acquired in the wide-field setting to ensure total internal reflection was reached. Images from B-cells stained for MHC II were analyzed using NIH ImageJ (http://rsbweb.nih.gov/ij/). The average cluster number, intensity, and relative size was determined by creating a region of interest for each cell, as confirmed by DIC, and subtracting out the background fluorescence. Then a threshold was set to analyze cluster parameters. Figure A below represents an example of the image analysis by demonstrating how a threshold is set.

For LPS studies comparing non-treated control cells to LPS treated cells, cells were normalized by determining the average Feret's diameter of all cells analyzed for each particular treatment (i.e. control or LPS treated). Then all three cluster parameters (i.e. quantity, size, and intensity) were divided by the average Feret's diameter for that treatment.

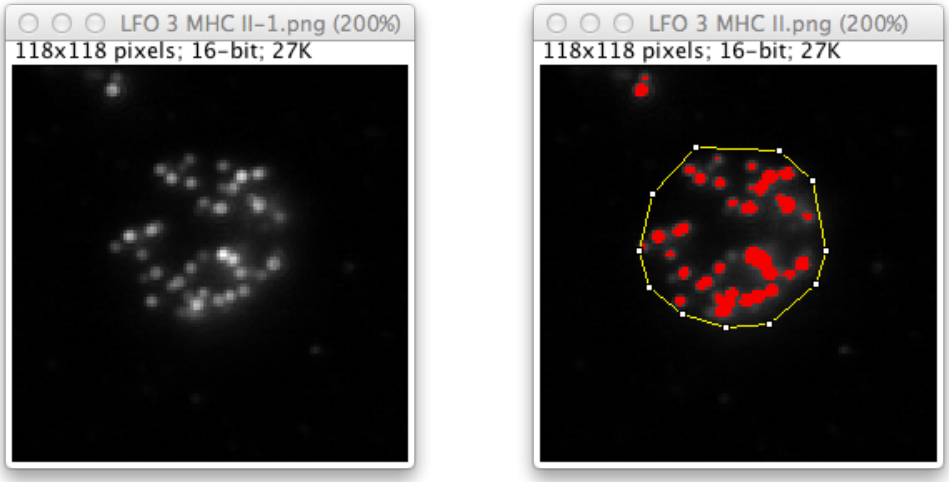

Figure A: Representative images from Image J analysis. The B-cell was magnified considerably and the background was subtracted. Then a region of interest was drawn around a cell and the threshold was set.

Chapter 3: Results

3.1 Pharmacological agents MβCD and Cytochalasin D disrupt B-cell MHC II lateral organization.

We first tested if it was possible to measure changes in MHC II lateral organization using TIRF microscopy by treating splenic B-cells with pharmacological agents capable of disrupting protein organization. Figure 1A shows representative images of B-cell MHC II clustering from control cells and those treated with MβCD and cytochalasin D. MβCD treatment resulted in a 42% decrease in number of MHC II clusters compared to the control (Figure 1B). In contrast, cytochalasin D treatment had no effect on the average number of clusters (Figure 1C). The average intensity per cell was not affected by either pharmacological treatment compared to control cells (Figure 1D & 1E). Moreover, treatment of B cells with MβCD or cytochalasin D was not associated with any change in the surface expression of MHC class II molecules as measured by flow cytometry correlating with the intensity data analysis from the TIRF images (data not shown). Finally, the average cluster size as, as measured with Image J using a defined threshold, was not affected by MβCD or cytochalasin D (Figure 1F and 1G). Taken together, the micron scale images acquired using TIRF microscopy allowed for changes to be detected in B-cell plasma membrane MHC II lateral organization.

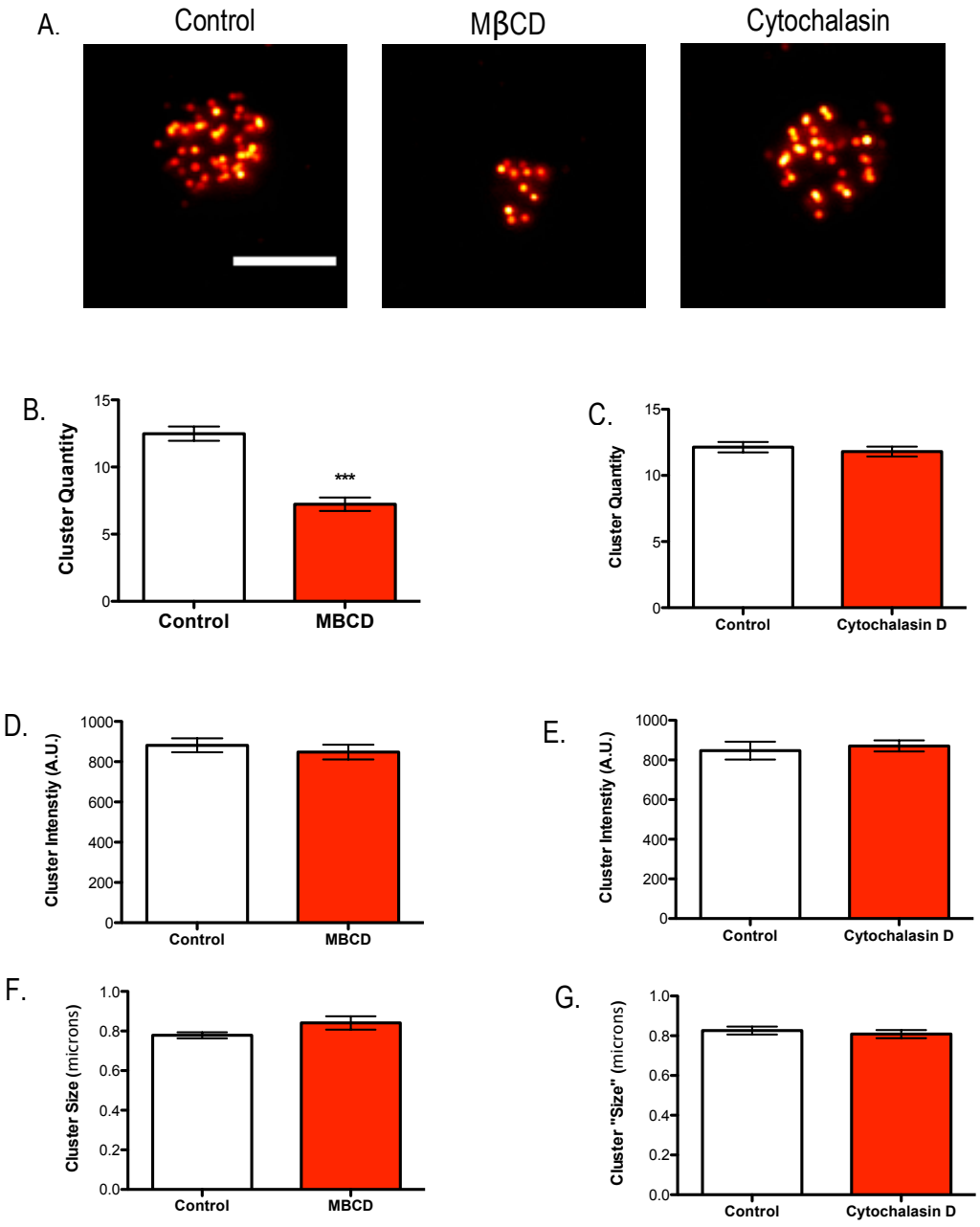

Figure 1: Cholesterol depletion and actin disruption have differential effects on MHC II clustering. (A) Representative TIRF images of MHC II staining from control, MβCD, and cytochalasin D treated B-cells. (B) Quantification of the average number of MHC II clusters expressed B-cells treated with MβCD. (C) Quantification of the average number of MHC II clusters expressed in the region of interest adhered for B-cells treated with cytochalasing D. (D) Average intensity of MHC II clusters of B-cells treated with MβCD. (E) Average intensity of MHC II clusters of B-cells treated with cytochalasin D. (F and D) Average cluster size of MHC II clusters on the micron scale for MβCD and cytochalasin D treatment respectively. Scale bar represents 5 microns. Asterisks represent different from control ***P < 0.001.

3.2 Lipopolysaccharide stimulation effects MHC II plasma membrane clustering.

Lipopolysaccharide (LPS) binds a complex containing Toll Like Receptor-4 subsequently initiating a signaling cascade that leads to downstream activation [(53)]. One consequence of LPS stimulation in immune cells expressing MHC II is substantially increased expression of MHC II protein on the plasma membrane [(54)]. Typically, measuring this increase in protein expression is done using flow cytometry and indeed we verified that *ex vivo* LPS stimulation of splenic B-cells caused a stark increase in total surface levels of MHC II compared to unstimulated controls (data not shown). Therefore, we next determined how LPS stimulation affected MHC II membrane lateral organization using TIRF microscopy.

Figure 2A are representative images of B-cells with no LPS stimulation and twenty-four hour *ex vivo* LPS stimulation (1µg/ml). Due to the dramatic increase in cell size, the data were normalized to the amount of the surface area of the B-cell adhered. Correlating with the flow cytometry data, TIRF microscopy demonstrated a dramatic increase in MHC cluster number (Figure 2B). Interestingly, both cluster size and intensity were significantly decreased (Figure 2C & 2D) compared to control cells. Taken together, the results demonstrate that LPS not only increases MHC expression but that LPS activation affects MHC II clustering.

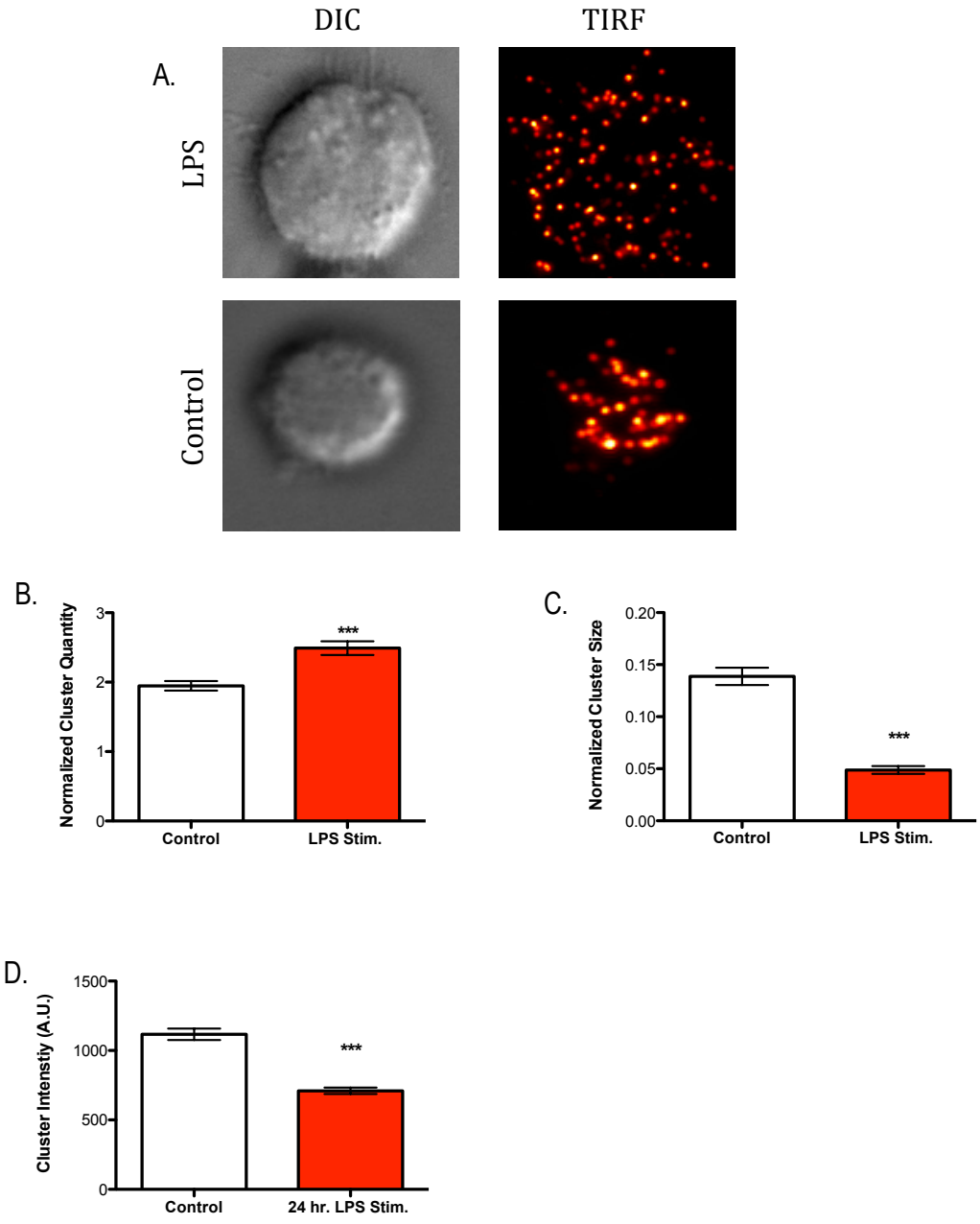

Figure 2: TIRF is able to detect changes in B-cell MHC II expression with LPS stimulation. (A) Representative differential interference contrast (DIC) and TIRF images of MHC II staining on B-cells stimulated with LPS for 24 hours. (B) Quantification of the average number of MHC II clusters expressed in the region of interest adhered. (C) Average MHC II cluster size on the micron scale. (D) Average cluster intensity of MHC II clusters. Asterisks represent different from control ***P < 0.001.

3.3 Fish oil does not affect MHC II lateral organization.

MHC II molecules partially cluster on the plasma membrane within lipid rafts prior to any stimulus such as LPS or formation of the immunological synapse [55]. We previously demonstrated that dietary fish oil is capable of disrupting lipid raft clustering upon stimulation (i.e. crosslinking GM1 molecules modeling a ligand) without determining how that affected raft resident protein clustering [37]. Thus, we determined if mice fed a fish oil diet for three weeks could disrupt basal state MHC II clustering compared to mice fed a control diet. Figure 3A shows representative TIRF images of MHC II clustering on the plasma membrane of B-cells from mice fed the control and fish oil diets. We measured the average B-cell cluster quantity (Figure 3B) and size (Figure 3C) and observed no differences between the diets on the micron scale. Similarly, fish oil had no effect on MHC II cluster intensity (Figure 3D) on a micron scale.

We then determined if splenic B-cells from fish oil fed mice differed from mice fed the control diet in MHC II lateral organization after LPS stimulation for 24 hours. Figure 3E are representative images of B-cells from each diet stimulated with LPS. Cluster quantity was elevated compared to non-stimulated cells, which corresponds with increased surface intensity as measured with flow cytometry (data not shown). This agrees with other studies that have indicated increased MHC II expression and increased cell size with LPS stimulation [54].

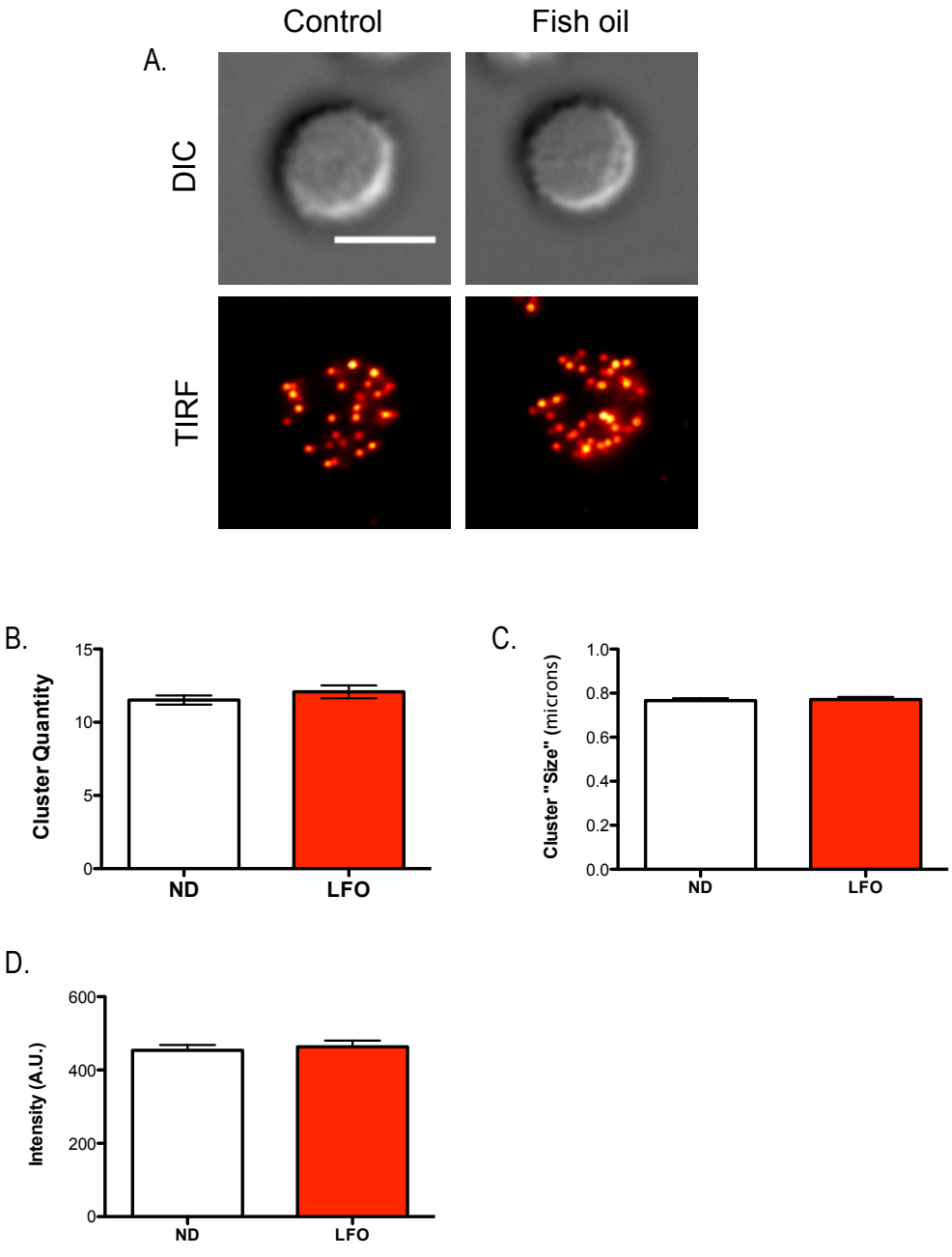

Figure 3: Fish oil has no effect on B-cell MHC II lateral organization.
(A) Representative differential interference contrast (DIC) and TIRF images of MHC II staining on B-cells isolated from mice fed either the control or fish oil diet. (B) Quantification of the average number of MHC II clusters expressed in the region of interest adhered. (C) Average MHC II cluster size on the micron scale. (D) Average cluster intensity of MHC II clusters. Scale bar represents 5 microns.

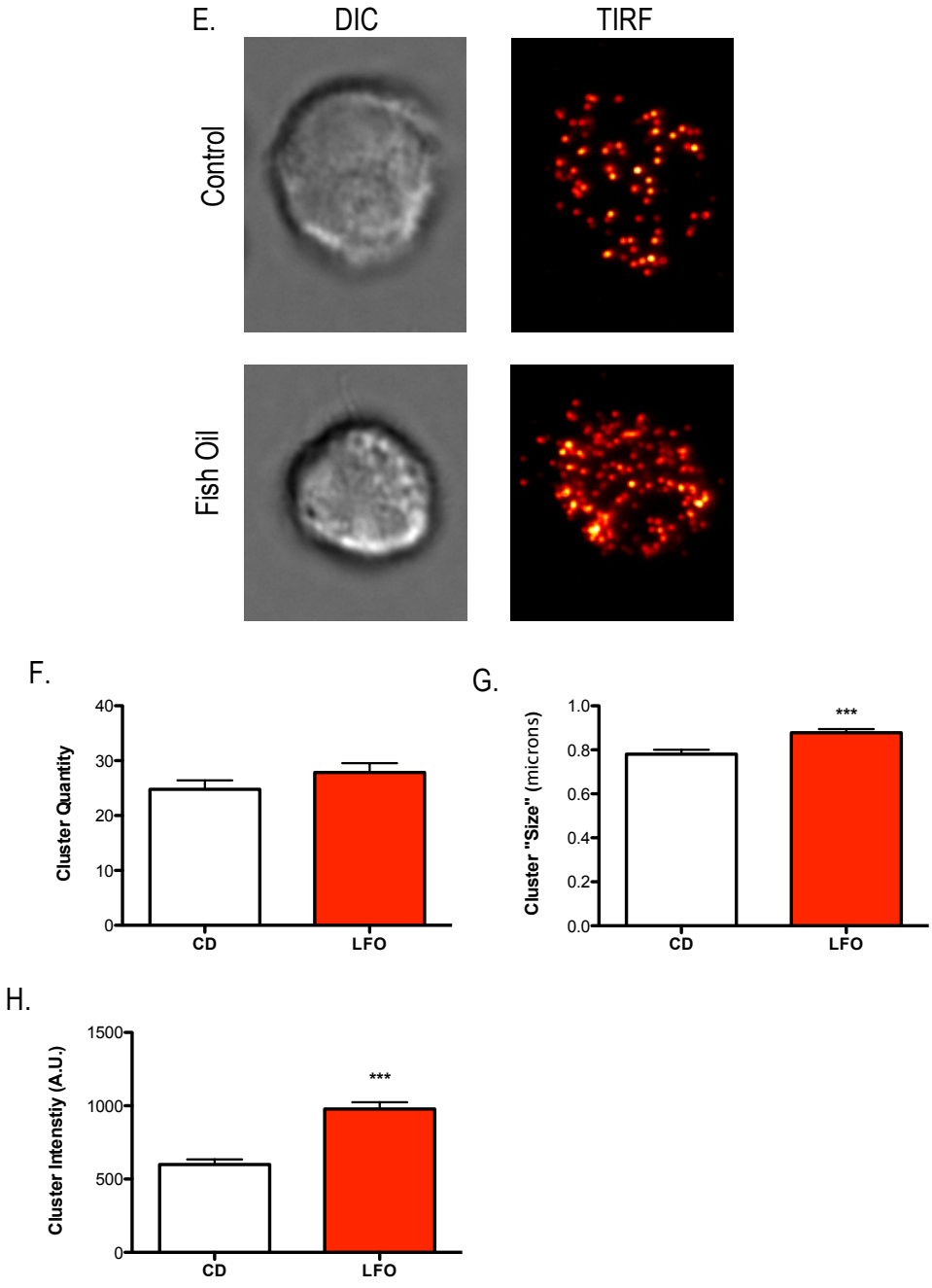

Figure 3: Fish oil effects B-cell MHC II lateral organization upon LPS stimulation.
(E) Representative differential interference contrast (DIC) and TIRF images of MHC II staining on B-cells isolated from mice fed either the control or fish oil diet and stimulated with LPS for 24 hours. (F) Quantification of the average number of MHC II clusters expressed in the region of interest adhered. (G) Average MHC II cluster size on the micron scale. (H) Average cluster intensity of MHC II clusters. Asterisks represent different from control ***P < 0.001.

While slightly elevated in fish oil fed mice, cluster quantity showed no statistical difference between the two diets when stimulated with LPS (figure 3F). However, both cluster size and intensity was significantly increased in aged mice fed fish oil in comparison to mice fed the CD (figure 3G and 3H). To ensure these differences were not due to changes in the surface area adhered we quantified the area adhered as done in the non-diet stimulated cells as no differences were discovered (data not shown).

3.4 B-cells from aged mice

Finally, we addressed how MHC II clustering is affected in an aged phenotype. Aging has been demonstrated to decrease B-cell numbers, physiological function, as well as diminish the amount of omega-3 fatty acids that are present in the plasma membrane [41, 42]. Therefore, we analyzed clustering quantity, intensity, and number using TIRF as done in each of the previous experiments. Figure 4A are representative images of B-cells from young and aged mice stained for MHC II. Cluster quantity showed no difference between B-cells from young mice and B-cells from aged mice (Figure 4B). Similarly, cluster size and intensity did not differ between either phenotype (Figure 4C & D).

Then, as was done in the fish oil experiments, the aged splenic B-cells were isolated and stimulated with LPS for 24 hours. Figure 4E are representative images of between young and aged B-cells stimulated with LPS. When examining cluster parameters cluster quantity and size were significant (Figure 4F and 4G). However, cluster intensity did not change (Figure 4H).

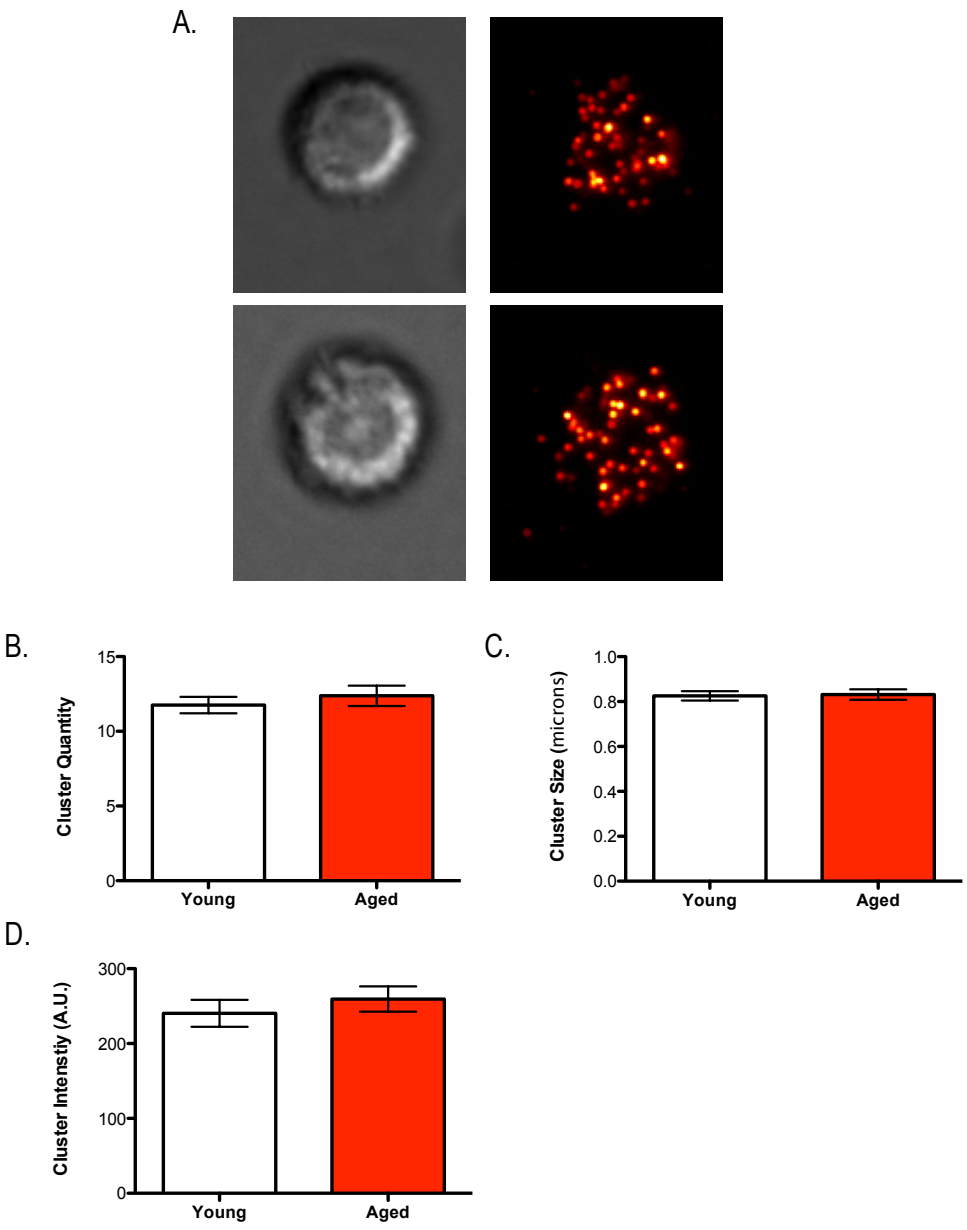

Figure 4: Aging has no effect on B-cell MHC II lateral organization.
(A) Representative differential interference contrast (DIC) and TIRF images of MHC II staining on B-cells isolated from mice young and aged mice. (B) Quantification of the average number of MHC II clusters expressed in the region of interest adhered. (C) Average MHC II cluster size on the micron scale. (D) Average cluster intensity of MHC II clusters.

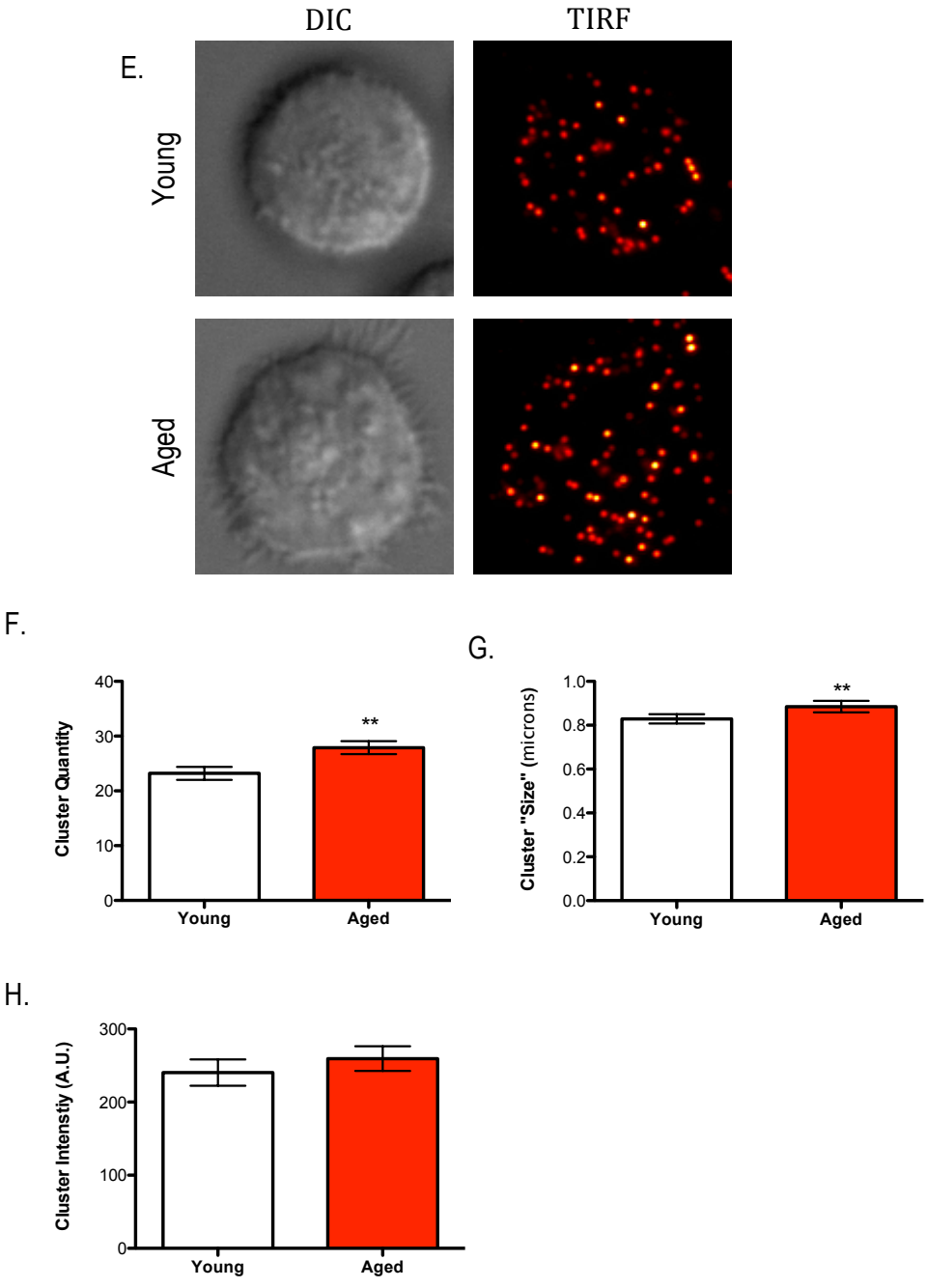

Figure 4: Aging effects B-cell MHC II lateral organization upon LPS stimulation.
(E) Representative differential interference contrast (DIC) and TIRF images of MHC II staining on B-cells isolated from mice that were either young or aged and stimulated LPS for 24 hours. (F) Quantification of the average number of MHC II clusters expressed in the region of interest adhered. (G) Average MHC II cluster size on the micron scale. (H) Average cluster intensity of MHC II clusters. Asterisks represent different from control **P < 0.005.

Chapter 4: Discussion

This research focused on developing an efficient TIRF approach to evaluate membrane protein organization. More specifically, these data demonstrate that TIRF microscopy can detect changes in *ex vivo* B-cell MHC II lateral organization using a monoclonal antibody under differing conditions. First, it was demonstrated that the TIRF protocol is viable and capable of detecting changes in MHC II membrane organization using pharmacological agents capable of disrupting protein clustering. Next, the data showed that neither a relevant dose of fish oil, nor aging the mice approximately nine months, had an affect on MHC II clustering in the absence of LPS stimulation. Taken together, the data establish the TIRF protocol as a relevant alternative to more costly and time consuming approaches to address membrane protein clustering.

4.1 MβCD disrupts ex vivo B-cell MHC II clustering

The rationale for studying *ex vivo* B-cell MHC II organization was based on past studies conducted in our laboratory evaluating lipid raft size using differing forms of microscopy [(56)]. Multiple studies have shown that MHC II localizes within lipid rafts in both the absence and presence of a stimulus [(55)]. Some have even proposed that newly synthesized MHC class II molecules are trafficked directly to lipid raft domains [(57)]. Thus, it was a logical protein candidate to evaluate if the TIRF protocol was efficacious.

MHC II clustering is dependent on the underlying lipid environment and the protein resides in lipid raft-like domains to enhance antigen presentation to T-cells [(58)]. We first used pharmacological agents capable of disrupting either the lipid environment (MβCD) or the underlying cytoskeleton that influences protein organization in the plasma membrane (cytochalasin D). Previously, Vrljic et al. showed that removal of

cholesterol with MβCD decreased MHC class II diffusion [59]. The TIRF data appear to be in agreement with this study as MHC II cluster quantity was diminished. Removing cholesterol from the plasma membrane also generates solid-like domains [60], i.e. containing lipids with a high melting temperature. This process slows protein diffusion and limits the number of protein clusters dispersed around plasma membrane as can be seen in Figure 1A.

The actin cytoskeleton also serves a role in clustering membrane proteins on the plasma membrane surface [61]. It was previously demonstrated that B cells treated with cytochalasin D did not change the diffusion or clustering of membrane bound MHC II proteins [55, 62]. The data presented here correlate with this finding, as TIRF images revealed no difference in any of the MHC II clustering parameters upon quantification. While other studies have demonstrated that cytochalasin D does affect MHC II protein clustering this may be a result of scale. Meaning, the cytoskeleton may impact nanoscale spatial distribution of MHC class II molecules but may not remodel the surface organization to the extent that it can be observed at the micron scale. Taken together, the data show that the approach used to evaluate MHC II on the micron scale with TIRF is capable of detecting differences in MHC II clustering as removing cholesterol from the membrane with MBCD caused a substantial decrease in MHC II cluster number compared to the control.

4.2 No changes in MHC II lateral organization in the basal state

Studies with immortal B-cells suggest that MHC II proteins are partially clustered in the basal state (i.e. in the absence of a stimulus) [12, 55]. However, what role these clusters play in cellular function is unclear. Using the experimental conditions of dietary

fish oil and aging we investigated the same cluster parameters as with the pharmacological treatment using our TIRF protocol. The merits of choosing dietary fish oil have already been discussed however the rationale for studying MHC II clustering in the context of aging is multifaceted. Aging in humans and animal models results in the inability to make an optimal immune response to vaccines and infectious agents [41, 42]. A potential contributing factor to the age-adapted immune response is the affect aging has on B-cell subsets and function [41, 42]. One hallmark or aging is a decreased humoral response and molecular immunological mechanisms in B-cells [41, 42]. Thus B-cell MHC II was again a logical choice to study since it is a vital protein in propagating the humoral immune response.

Thus, we determined the cluster quantity, size, and intensity in the context of dietary fish oil and aging. In all three categories the data remained unchanged in either experimental condition. Its important to note that while on the micron scale there is no change in MHC II lateral organization it does not mean any change is not present or possible. Clustering in the absence of stimulus is extremely dynamic and on a much smaller scale than when protein is engaged with a ligand or, as with MHC II, the immunological synapse [12, 55, 63]. Thus, one explanation for our TIRF protocol not being able to detect changes is due to the nanoscale heterogeneity displayed by membrane microdomains containing MHC II [5]. These proteins have an intrinsic ability to cluster however the clustering becomes more efficient upon the loading of derived antigenic peptide [23, 25]. Therefore, moving forward it would be of interest determine if our TIRF approach could detect changes in MHC II clustering at the B-T cell immunological synapse *ex vivo* in the context of aging and dietary fish oil.

4.3 TIRF microscopy revealed changes in MHC II clustering upon ex vivo LPS stimulation.

LPS is a component of the cell wall of gram-negative bacteria capable of activating B-cells and leads to the production and secretion of small proteins that can induce inflammation known as proinflammatory cytokines [64]. The other, more relevant, aspect to LPS stimulation is it dramatically increases the expression of MHC II and cell size [54, 65]. LPS is also known as a PAMP (pathogen associated molecular patterns) because of its repetitive epitope that stimulates TLR4 [66]. Recognition of LPS by TLR4 involves four proteins: the LPS binding protein, CD14, lymphocyte antigen 96 (commonly referred to as MD-2), and TLR4. CD14/MD-2/TLR4 must cluster together and form a trimolecular complex in order to activate the cell [66]. Recent data published in Nature Immunology demonstrates that, outside of its classical function in antigen presentation, MHC II can act as a signal transducer to promote TLR4 signaling [67]. Moreover, in the context of increased MHC II expression in response to LPS, to our knowledge no other study address how the *ex vivo* B-cell MHC II clustering changes using TIRF upon LPS stimulation.

Previous data from our laboratory show that mice fed the fish oil diet have an enhancement of B-cell activation in the presence of LPS [37]. We previously hypothesized that one possible explanation for this data is the fish oil increased lipid rafts size and therefore could affect proteins involved in B-cell activation. While a correlation between lipid raft size and enhanced cellular function is hypothetical, other studies suggest this conclusion as well [68, 69]. Meaning, the “larger” the size of the lipid raft the stronger the signaling response. However, understanding what “increased lipid

raft size" means for proteins that localize within rafts is unclear. Since MHC II localize in rafts it is an ideal target of study.

We first wanted to determine how B-cell MHC II clustering was affected by a 24 hour *ex vivo* LPS stimulation in the absence of any other condition such as fish oil or age. As expected, the surface adhered increased when compared to unstimulated cells because the cell size was increased due to LPS stimulation as reported by multiple studies and our own flow cytometry data (data not shown). However, our data reveal that upon normalizing the clustering parameters for area of the surface of the cell that adhered to the glass slides, MHC II cluster quantity was increased while cluster size and intensity was decreased. Increased cluster number can be rationalized with increased expression of MHC II. However, when coupled with the cluster size and intensity data it becomes more significant. The data suggest that upon LPS stimulation, not only does MHC II increase in content, the way in which MHC II proteins cluster is changed. The clusters are smaller and less intense, which would not be revealed in studies that use excessive staining or over expression of MHC II proteins tagged with a fluorophore. In those instances, fluorophore saturation and background fluorescence become issues in resolving those particular cluster parameters. Meaning, as more MHC II proteins are expressed on the plasma membrane they cluster in smaller quantities, which individually take up less fluorophore than larger clusters would. Of course, this is assuming that the amount of fluorescently labeled antibody is kept at a relatively low concentration (i.e. not saturating) which is one aspect utilized in our protocol. Thus, the data reveal, for the first time, that *ex vivo* B-cell MHC II lateral organization changing up LPS stimulation.

4.4 Fish oil effects ex vivo B-cell MHC II clustering when stimulated LPS

After discovering LPS changes in MHC II clustering could be distinguished using our TIRF protocol we addressed how animals fed the control diet and fish oil diet for three weeks affected MHC II clustering after LPS stimulation for 24 hours. While there was no change between the diets in regards to cluster quantity the data revealed that dietary fish oil increased cluster quantity and size. This data is intriguing as it has been hypothesized by many that fish oil makes the membrane more fluid allowing for easier association between membrane receptors [70, 71]. Indeed, our own data regarding increased lipid raft size upon fish oil treatment would seem to correlate well with larger LPS stimulated MHC II clusters that are more intense. However, more data must be collected before attempting to correlate lipid rafts and MHC II clustering. Future dual labeling studies involving lipid raft staining with MHC II staining would shed more light on this potential correlation. One would expect if MHC II expression was substantially increased to the point of having physiological relevance our data would show a dramatic increase in all three clustering parameters.

4.5 Aged splenic B-cells display different MHC II clustering with LPS stimulation

Stimulating splenic B-cells from aged mice resulted in an increase in cluster quantity and size without a corresponding increase in intensity. Moreover, the standard intensity units of measure were significantly lower with aged B-cells compared to untreated and fish oil treated B-cells. However, the intensity was elevated compared to B-cells young animals but again not significant. These data are difficult to interpret as there is very little literature on expression in splenic B-cells from aged animals. It has been shown that omega-3 polyunsaturated fatty acids are decreased in the plasma

membrane with aging so perhaps the fluidity of the membrane is limited causing the size of the clusters to increase. However, this is speculative as a corresponding increase in cluster intensity and decrease in cluster quantity would be expected. Much more investigation is needed to truly determine the significance of these data. It would be of interest to determine how aging [41, 42] affects lipid raft clustering as we have done in our previous publications to provide more insight into the capability of the aged plasma membrane to organize in response to a stimulus. Moreover, determining how signaling is affected in response to LPS stimulation and the formation of the immunological synapse using splenic B-cells from aged mice would help address the underlying gaps of knowledge.

4.6 Conclusion

Collectively these data demonstrate the validity of our TIRF protocol. It appears the major differences regarding MHC II lateral organization are not in the basal state but upon activation as our most recent publication demonstrates [63]. This correlates with our data that demonstrate lipid raft clustering is diminished up dietary fish oil intervention where we cross-linked GM1 molecules using cholera toxin B to mimic a protein binding its ligand. More data is needed to determine what the physiological relevance of LPS stimulated MHC II clustering is and if there is any interaction between MHC II and TLR-4. Moreover, it should be noted that the cluster parameters here are in the context of the TIRF protocol and ImageJ analysis. For example, the cluster size parameter should not be taken as the actual size of an MHC cluster *in vivo* but rather an approximation to detect differences in treatment. Moreover, the size measurement can be easily influenced by clusters that are in close proximity to one another with the region

of interest. The closer the proximity of the clusters the higher the likely hood the threshold will recognize only one large cluster as opposed to multiple smaller clusters. Thus, there are much more eloquent systems such as Dr. Pierces research in which planar lipid bilayers were attached to glass cover slips to study “live” B-cell receptor clustering [21]. By no means is this a comparison to such innovative and complex systems. Each TIRF protocol and approach have their benefits and drawbacks. Our approach does not allow for the benefits of live cell imaging however it is extremely difficult to transfect primary cell lines for live cell imaging. Even upon transfection the physiological relevance of such systems is questionable. The approach taken with our protocol attempts to bridge the gap by studying *ex vivo* protein clustering with a monoclonal antibody at a low concentration. This approach to TIRF is to be coupled with other forms of microscopy, as well as other experimental approaches, to address membrane organization and protein clustering.

Bibliography

1. Gorter E, Grendel F. On bimolecular layers of lipoids on the chromocytes of the blood. J Exp Med 1925; 41:439-43.
2. Robertson JD. The molecular structure and contact relationships of cell membranes. Prog Biophys Mol Biol 1960; 10:343-418.
3. Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. Science 1972; 175:720-31.
4. Engelman DM. Membranes are more mosaic than fluid. Nature 2005; 438:578-80.
5. Simons DLaK. Lipid rafts as a membrane-organizing principle. Science 2009; 327:46-50.
6. Jacobson K, Mouritsen OG, Anderson RG. Lipid rafts: At a crossroad between cell biology and physics. Nat Cell Biol 2007; 9:7-14.
7. Calder PC. The relationship between the fatty acid composition of immune cells and their function. Prostaglandins Leukot Essent Fatty Acids 2008; 79:101-8.
8. Simons K, Ehehalt R. Cholesterol, lipid rafts, and disease. J Clin Invest 2002; 110:597-603.
9. Lindner R, Naim HY. Domains in biological membranes. Exp Cell Res 2009; 315:2871-8.
10. Tolar P, Sohn HW, Liu W, Pierce SK. The molecular assembly and organization of signaling active b-cell receptor oligomers. Immunol Rev 2009; 232:34-41.
11. Kurosaki T. Molecular dissection of b cell antigen receptor signaling (review). Int J Mol Med 1998; 1:515-27.
12. Björn F Lillemeier JRP, Zurab Surviladze, Bridget S Wilson, and Mark M Davis. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. Proc Natl Acad Sci U S A 2006; 103:18992-7.
13. Yaqoob P. The nutritional significance of lipid rafts. Annu Rev Nutr 2009; 29:257-82.
14. Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997; 387:569-72.
15. toomre KSaD. Lipid rafts and signal transduction. Nature Reviews Molecular Cell Biology 2000; 1:31-41.
16. Gupta N, DeFranco AL. Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated b-lymphocyte activation. Mol Biol Cell 2003; 14:432-44.
17. Tedder TWLaTF. B lymphocytes: How they develop and function. Blood 2008; 112:1570-80.
18. Hayakawa RRHaK. B cell development pathways. Annu Rev Immunol 2001; 19:595-621.
19. DeFranco AL. Molecular aspects of b-lymphocyte activation. Annu Rev Cell Biol 1987; 3:143-78.
20. DeFranco AL. The complexity of signaling pathways activated by the bcr. Curr Opin Immunol 1997; 9:296-308.

21. Tolar P, Sohn HW, Pierce SK. Viewing the antigen-induced initiation of b-cell activation in living cells. Immunol Rev 2008; 221:64-76.
22. Goding JW, Layton JE. Antigen-induced co-capping of igm and igd-like receptors on murine b cells. J Exp Med 1976; 144:852-7.
23. Hiltbold EM, Poloso NJ, Roche PA. Mhc class ii-peptide complexes and apc lipid rafts accumulate at the immunological synapse. J Immunol 2003; 170:1329-38.
24. Sumen C, Dustin ML, Davis MM. T cell receptor antagonism interferes with mhc clustering and integrin patterning during immunological synapse formation. J Cell Biol 2004; 166:579-90.
25. Al-Daccak R, Mooney N, Charron D. Mhc class ii signaling in antigen-presenting cells. Curr Opin Immunol 2004; 16:108-13.
26. Neefjes JJ, Ploegh HL. Intracellular transport of mhc class ii molecules. Immunol Today 1992; 13:179-84.
27. Wubbolts R, Neefjes J. Intracellular transport and peptide loading of mhc class ii molecules: Regulation by chaperones and motors. Immunol Rev 1999; 172:189-208.
28. Zech T, Ejsing CS, Gaus K, de Wet B, Shevchenko A, Simons K, Harder T. Accumulation of raft lipids in t-cell plasma membrane domains engaged in tcr signalling. Embo J 2009; 28:466-76.
29. Lang P, Stolpa JC, Freiberg BA, Crawford F, Kappler J, Kupfer A, Cambier JC. Tcr-induced transmembrane signaling by peptide/mhc class ii via associated ig-alpha/beta dimers. Science 2001; 291:1537-40.
30. Vogt AB, Spindeldreher S, Kropshofer H. Clustering of mhc-peptide complexes prior to their engagement in the immunological synapse: Lipid raft and tetraspan microdomains. Immunol Rev 2002; 189:136-51.
31. Gebauer SK, Psota TL, Harris WS, Kris-Etherton PM. N-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. Am J Clin Nutr 2006; 83:1526S-35S.
32. Bailey N. Current choices in omega 3 supplementation. Nutrition Bulletin Wiley Online Library 2009.
33. Calder PC. Mechanisms of action of (n-3) fatty acids. J Nutr 2012; 142:592S-9S.
34. Calder PC. N-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006; 83:1505S-19S.
35. Siriwardhana N, Kalupahana NS, Moustaid-Moussa N. Health benefits of n-3 polyunsaturated fatty acids: Eicosapentaenoic acid and docosahexaenoic acid. Adv Food Nutr Res 2012; 65:211-22.
36. Chang HR, Arsenijevic D, Pechere JC, Piguet PF, Mensi N, Girardier L, Dulloo AG. Dietary supplementation with fish oil enhances in vivo synthesis of tumor necrosis factor. Immunol Lett 1992; 34:13-7.
37. Rockett BD, Teague H, Harris M, Melton M, Williams J, Wassall SR, Shaikh SR. Fish oil increases raft size and membrane order of b cells accompanied by differential effects on function. J Lipid Res 2012; 53:674-85.
38. Arrington JL, Chapkin RS, Switzer KC, Morris JS, McMurray DN. Dietary n-3 polyunsaturated fatty acids modulate purified murine t-cell subset activation. Clin Exp Immunol 2001; 125:499-507.

39. Khan NA, Yessoufou A, Kim M, Hichami A. N-3 fatty acids modulate th1 and th2 dichotomy in diabetic pregnancy and macrosomia. J Autoimmun 2006; 26:268-77.
40. Shaikh S. Biophysical and biochemical mechanisms by which dietary n-3 polyunsaturated fatty acids from fish oil disrupt membrane lipid rafts. Journal of Nutrional Biochemistry 2012; 23:101-5.
41. Globerson A, Effros RB. Ageing of lymphocytes and lymphocytes in the aged. Immunol Today 2000; 21:515-21.
42. Frasca D, Blomberg BB. Effects of aging on b cell function. Curr Opin Immunol 2009; 21:425-30.
43. Singer C. Notes on the early history of microscopy. Proc R Soc Med 1914; 7:247-79.
44. Mazzarello P. A unifying concept: The history of cell theory. Nat Cell Biol 1999; 1:E13-5.
45. Toledo-Pereyra LH. The strange little animals of antony van leeuwenhoek surgical revolution. J Invest Surg 2009; 22:4-8.
46. Axelrod D. Cell-substrate contacts illuminated by total internal reflection fluorescence. J Cell Biol 1981; 89:141-5.
47. Mattheyses AL, Simon SM, Rappoport JZ. Imaging with total internal reflection fluorescence microscopy for the cell biologist. J Cell Sci 2010; 123:3621-8.
48. Rappoport JZ, Simon SM. Real-time analysis of clathrin-mediated endocytosis during cell migration. J Cell Sci 2003; 116:847-55.
49. Rappoport JZ, Taha BW, Lemeer S, Benmerah A, Simon SM. The ap-2 complex is excluded from the dynamic population of plasma membrane-associated clathrin. J Biol Chem 2003; 278:47357-60.
50. Xu Y, Rubin BR, Orme CM, Karpikov A, Yu C, Bogan JS, Toomre DK. Dual-mode of insulin action controls glut4 vesicle exocytosis. J Cell Biol 2011; 193:643-53.
51. Lizunov VA, Matsumoto H, Zimmerberg J, Cushman SW, Frolov VA. Insulin stimulates the halting, tethering, and fusion of mobile glut4 vesicles in rat adipose cells. J Cell Biol 2005; 169:481-9.
52. Rockett BD, Harris M, Raza Shaikh S. High dose of an n-3 polyunsaturated fatty acid diet lowers activity of c57bl/6 mice. Prostaglandins Leukot Essent Fatty Acids 2012; 86:137-40.
53. Lanzavecchia A, Sallusto F. Toll-like receptors and innate immunity in b-cell activation and antibody responses. Curr Opin Immunol 2007; 19:268-74.
54. Barrachina M, Gonalons E, Celada A. Lps upregulates mhc class ii i-a expression in b lymphocytes at transcriptional and at translational levels. Tissue Antigens 1999; 54:461-70.
55. Gombos I, Detre C, Vamosi G, Matko J. Rafting mhc-ii domains in the apc (presynaptic) plasma membrane and the thresholds for t-cell activation and immunological synapse formation. Immunol Lett 2004; 92:117-24.
56. Shaikh SR, Rockett BD, Salameh M, Carraway K. Docosahexaenoic acid modifies the clustering and size of lipid rafts and the lateral organization and surface expression of mhc class i of el4 cells. J Nutr 2009; 139:1632-9.

57. Poloso NJ, Muntasell A, Roche PA. Mhc class ii molecules traffic into lipid rafts during intracellular transport. J Immunol 2004; 173:4539-46.
58. Anderson HA, Hiltbold EM, Roche PA. Concentration of mhc class ii molecules in lipid rafts facilitates antigen presentation. Nat Immunol 2000; 1:156-62.
59. Vrljic M, Nishimura SY, Moerner WE, McConnell HM. Cholesterol depletion suppresses the translational diffusion of class ii major histocompatibility complex proteins in the plasma membrane. Biophys J 2005; 88:334-47.
60. Nishimura SY, Vrljic M, Klein LO, McConnell HM, Moerner WE. Cholesterol depletion induces solid-like regions in the plasma membrane. Biophys J 2006; 90:927-38.
61. Chichili GR, Rodgers W. Clustering of membrane raft proteins by the actin cytoskeleton. J Biol Chem 2007; 282:36682-91.
62. Umemura YM, Vrljic M, Nishimura SY, Fujiwara TK, Suzuki KG, Kusumi A. Both mhc class ii and its gpi-anchored form undergo hop diffusion as observed by single-molecule tracking. Biophys J 2008; 95:435-50.
63. Rockett BD, Melton M, Harris M, Bridges LC, Shaikh SR. Fish oil disrupts mhc class ii lateral organization on the b-cell side of the immunological synapse independent of b-t cell adhesion. J Nutr Biochem 2013.
64. Loppnow H, Libby P, Freudenberg M, Krauss JH, Weckesser J, Mayer H. Cytokine induction by lipopolysaccharide (lps) corresponds to lethal toxicity and is inhibited by nontoxic rhodobacter capsulatus lps. Infect Immun 1990; 58:3743-50.
65. Ozcan E, Garibyan L, Lee JJ, Bram RJ, Lam KP, Geha RS. Transmembrane activator, calcium modulator, and cyclophilin ligand interactor drives plasma cell differentiation in lps-activated b cells. J Allergy Clin Immunol 2009; 123:1277-86 e5.
66. Lu YC, Yeh WC, Ohashi PS. Lps/tlr4 signal transduction pathway. Cytokine 2008; 42:145-51.
67. Hassan GS, Mourad W. An unexpected role for mhc class ii. Nat Immunol 2011; 12:375-6.
68. Fessler MB, Parks JS. Intracellular lipid flux and membrane microdomains as organizing principles in inflammatory cell signaling. J Immunol 2011; 187:1529-35.
69. Janes PW, Ley SC, Magee AI. Aggregation of lipid rafts accompanies signaling via the t cell antigen receptor. J Cell Biol 1999; 147:447-61.
70. Alexander JW. Immunonutrition: The role of omega-3 fatty acids. Nutrition 1998; 14:627-33.
71. Vognild E, Elvevoll EO, Brox J, Olsen RL, Barstad H, Aursand M, Osterud B. Effects of dietary marine oils and olive oil on fatty acid composition, platelet membrane fluidity, platelet responses, and serum lipids in healthy humans. Lipids 1998; 33:427-36.