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Complement Receptor-1 Binding to Erythrocytes Under Normal and Pathological Membrane Deforming Conditions

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Complement Receptor-1 Binding to Erythrocytes Under Normal and Pathological Membrane Deforming Conditions

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ABSTRACT

In primates, complement-coated invading microbes and immune complexes are efficiently removed from circulation by binding complement receptor-1 (CR1) on erythrocytes, a process known as immune-adherence clearance. This project examines the nature and functionality of complement binding to erythrocytes under normal and pathological conditions. The data identifies key factors associated with the complement-CR1 ligation pathway promoting membrane deformability, helping elucidate a greater understanding of this important, unique erythrocyte function.

TABLE OF CONTENTS

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BACKGROUND

Erythrocytes: Introduction

Red blood cells (RBCs) are the most common type of blood cell in vertebrates and an essential component to an organism's survival. In the process known as erythropoiesis, as many as two million RBCs per second are produced in the bone marrow. With a lifespan of 120 days, RBCs constantly circulate throughout the body, keeping all other cells alive through oxygenation and removal of immune particles in primates.

Erythrocyte Structure

Erythrocytes are unique among cell types in that they completely lack organelles, including a nucleus. Packed within the cell is the metalloprotein hemoglobin, a dense, iron complex that binds molecules for transport such as oxygen and carbon dioxide. Spanning roughly 8 micrometers in diameter, RBCs are biconcave, with a slight depression in the middle. Given its simple structure and lack of organelles, RBCs have been used for decades as a model system in studying cell membranes, and how biological and chemical signals within the cell affect cell response (Dao et al., 2003).

Scattered throughout RBC membranes are several integral and membrane spanning proteins that interact with the surrounding tissues and circulating immune complexes. The membrane is constructed to allow two types of interactions. The first is horizontal interactions between the networks of skeletal proteins located underneath the membrane. These connections support the structural integrity of erythrocytes, and play a major role in the elasticity and deformability of the membrane. The second plane of

structure involves vertical interactions between the lipid skeletal network and the integral proteins in the membrane, which are important in stabilizing the lipid bilayer (Corrons, 2011).

Membrane Deformability

A key characteristic of the RBC membrane is its ability to bend and fold onto itself. This uniaxial folding affects the size and shape of the cell. The biconcavity of the cell contributes to a greater average surface area than average cell volume (135 μm^2 to 94 μm^3, respectively). Thus, there is a considerable excess area allowing for RBCs to deform (Chien, 1987). Erythrocyte deformability is extremely important for an effective microcirculatory function, given that the diameter of capillaries is 3-4 μm. Blood flow and viscosity are influenced significantly by this ability. Membrane deformability is possible due to two coupled components surrounding the cytoplasm, the lipid bilayer and its underlying scaffolding (Van Dort et al., 2001). The lipid bilayer is composed of a variety of lipids including phospholipids, glycolipids, cholesterol, and sphingolipids. Embedded within, or transecting, the bilayer are several structural proteins, primarily glycophorins and band-3 protein. Underneath the lipid bilayer is a 2D scaffolding system, composed of interconnecting skeletal proteins, predominantly spectrin and actin. The bilayer and scaffolding are anchored together at several locations by bridging proteins, such as ankyrin, in the lipid bilayer, as well as by amphiphilic surfactant molecules. These proteins play a dominant role in membrane structure and its mechanical ability to bend and deform.

The ability of RBCs to fit through capillaries to exchange gases and remove immune complexes is heavily dependent on the deformability of the membrane, its ability to bend and fold without disrupting its function or causing cell fragmentation. Skeletal proteins embedded in the membrane play a crucial role in deformability. The mechanical work attributed by the spectrin network has been shown to depend on the phosphorylation of β-spectrin, adducin, and protein 4.1R (Manno et al., 1995; Manno et al., 2005).

Decreased erythrocyte deformability can have significant consequences on surrounding proteins and tissues. It has been shown that aged cells do not deform as efficiently as younger cells (Wen et al., 1998). Diseases such as sepsis and lupus are also associated with an increase in membrane rigidity. High shear stress, turbulence, and hypothermia are other conditions that can negatively influence overall RBC functionality (Kameneva et al., 1999). In particular, oxidative stress caused by free radicals is especially detrimental to erythrocyte deformability. These reactive oxygen species (ROS) can destroy fatty acids in the membrane and fragment membrane proteins.

Spectrin

Spectrins are the most abundant RBC membrane skeletal proteins, constituting approximately 75% of all membrane associated proteins (Yawata, 2003). They represent the major portion of the scaffold network that interacts with the integral proteins of the membrane. RBC spectrins are composed of a heterodimer, the α-spectrin and the β-spectrin subunits which are structurally aligned in an antiparallel arrangement. It has been shown through electron microscopy that the entire molecule appears as a twisted, rod-

like structure (Yawata, 2003). Spectrin molecules are tightly coiled in their native state, and are able to extend to a more relaxed state when the RBC membrane is stretched. Due to this high level of flexibility, spectrins have been classified as one of the major determinants of cell shape.

β-spectrin in particular has been linked to membrane deformability due to a sequence on the polypeptide that contains at least four phosphorylation sites for casein kinase 1 (CK1). It has been shown that the phosphorylation of β-spectrin at these sites is directly related to membrane deformability (Manno et al., 1995): the phosphorylated state increases deformability. The β-spectrin subunit also contains sites along its sequence that bind to other structural proteins, such as actin, protein 4.1, and ankyrin.

The formation of the mesh structure is predominately determined by the self-association of spectrin heterodimers. The head-head and side-side interactions of the spectrins allow the formation of higher level oligomers, thus creating the cytoskeletal meshwork. In addition, the linkage of spectrins to other RBC membrane proteins is essential for changes in deformability.

Erythrocyte Immune Adherence Clearance

The most well-known function of the RBC is gas exchange, delivering oxygen to the tissues, and taking carbon dioxide away. In primates, however, this is not their sole function. RBCs in primates are also responsible for mediating the process known as immune-adherence clearance, an extremely important, yet relatively less known task of removing both microbes and immune particles from the body **(Figure-1)**. To avoid the buildup of antibody-bound infectious agents and inflammatory particles, primates employ

erythrocytes to remove these complexes from the intravascular space. These particles are coated with complement fragments and bind to a receptor on RBCs known as complement receptor 1 (CR1, CD35) (Nielsen, et al., 2002). This is a very important function, as it prevents deposition of circulating immune complexes inside fragile organs such as the kidney, as well as indirectly activating circulating leukocytes. By recognizing these complement-tagged particles through CR1, RBCs immobilize and transfer them to nearby macrophages for phagocytosis, or to the liver and spleen where complement is removed without damaging the RBC.

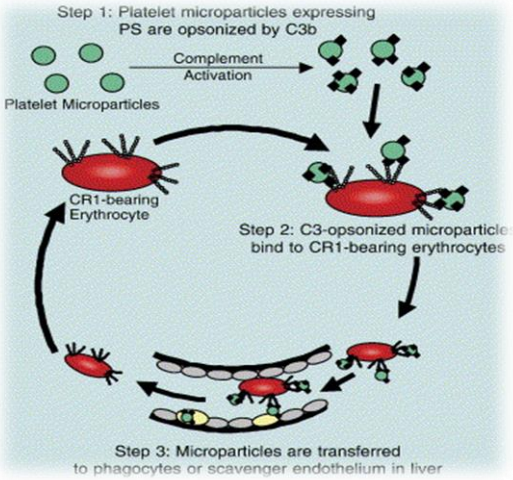

Figure-1: Diagram of Human Immune-Adherence Clearance

The Complement System

The complement system is part of the innate immune system, and an additional tool used to destroy pathogens. This collection of small proteins combines to opsonize (tag) pathogens and immune complexes to facilitate their removal from the body. The process also induces chemotaxis, bringing in macrophages to ingest the complexes by phagocytosis. The final component of complement-mediated lysis is the formation of a membrane attack complex (MAC), which consists of several complement fragments (**Figure-2**). The arrangement of these fragments induces a rush of extracellular fluids into the cell, compromising the integrity of the RBC membrane, and eventually lysing the cell. However, mammalian cells, unlike simple bacteria, possess a range of complement-

regulatory factors that control the pathway before a MAC is formed (Hourcade et al., 1989).

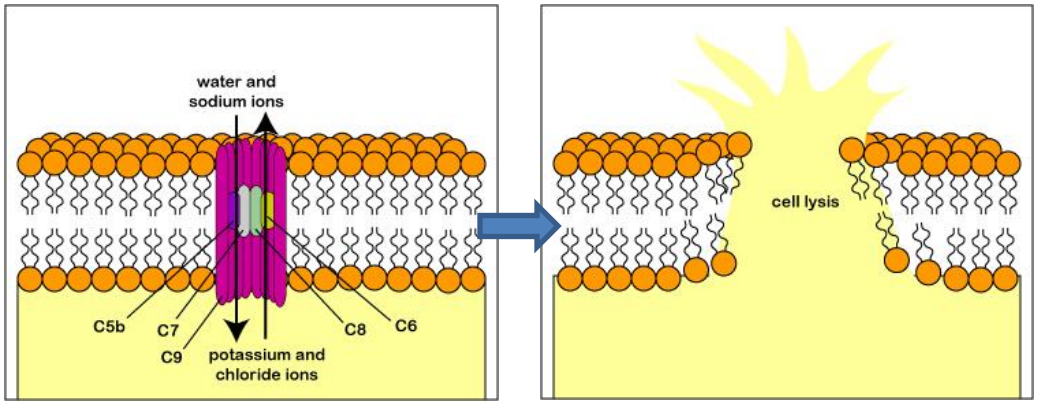

Figure-2: Formation of the MAC Complex. (Kaiser, 2011)

Complement Pathway

The complement system can be activated by three different pathways: the classical, alternative, and lectin pathways (**Figure-3**). All three converge with the formation of the C5 convertases, which leads to the initiation of the MAC (Nielsen et al., 2002). Erythrocytes engage with complement fragments through the classical and alternative pathway, where regulatory complement factors CR1, DAF, and MCP bind to newly formed C3-convertase, a precursor to C5 convertase. This binding disrupts the continuation of the complement pathway, halting the formation of the MAC complex.

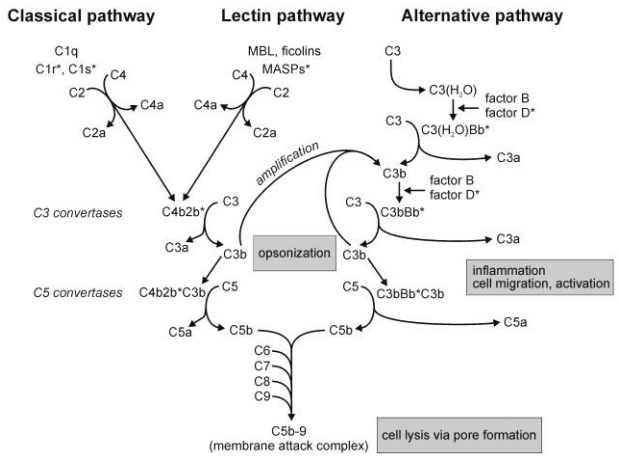

Figure-3: Diagram of the Complement Pathways (Mihaly, 2011)

Complement Activation

The complement fragments specifically related to erythrocyte binding are the C3 and C4 fragments, both of which are found in their precursor form circulating in the plasma. Both fulfill analogous functions in the formation of the C3 convertase of the classical and alternative pathways (Isenman, 1983). In the classical pathway, C4 is converted to its active form when it is cleaved by C1s, a molecule generated from the interaction of C1 and an immune complex. The resulting major fragment, C4b, is involved in the assembly of the C3 convertase (Muller-Eberhard et al., 1966). Additionally, C4b becomes a ligand for the type I complement receptor 1 (CR1) present on blood cells (Bianco & Nussenzweig, 1977). In a similar fashion, C3 is cleaved by a C3 convertase enzyme, producing a major fragment C3b, which can interact with other proteins, as well as ligating to the CR1 receptor (Reid & Porter, 1981).

Complement Receptor 1

RBCs circulate throughout the body nearly half a million times in their lifespan, constantly binding with molecules to ensure a homeostatic environment. The binding is mediated through extracellular peripheral and membrane-spanning proteins, especially complement receptors. Complement receptor 1 (CR1, CD35) is a polymorphic glycoprotein found on most human blood cells, notably erythrocytes, leukocytes, and dendritic cells. It is the central component for binding activated fragments of the complement pathway (Fearon, 1980). This Type 1 transmembrane protein mediates the binding of complement-activated immune complexes to RBCs (Nelson, 1953). It is

through this receptor that erythrocytes are able to bind and remove immune particles from the blood, so it is referred to as the immune adherence receptor.

Complement receptor 1 is anchored to the lipid membrane with an extracellular N-terminal portion composed of 30 repeats of 59-72 amino acids (Klickstein et al., 1987). These repeats fold into long homologous modules (LHR), a conserved feature among the family of complement activation regulators (RCA) (Liszewsi & Atkinson, 1998). There is significant diversity among these modules apart from 4 Cys residues and a conserved Trp (Smith et al., 2002). It is through these structural motifs that CR1 can bind to the activated on C3b and C4b fragments via a thioester bond.

It has previously been shown that CR1 is dispersed across the plasma membrane of circulating human RBCs, and clusters together following ligation by immune particles through interactions with Fas-associated phosphatase-1 (FAP-1) (Ghiran et al., 2008). The receptor undergoes this lateral movement largely because of the spectrin network beneath the membrane (Brown, 2003). It is also important to note that the mean number of CR1 receptors per erythrocyte is genetically determined, with RBCs expressing about 100 CR1/cell for low CR1 expressers, to 500/cell on intermediate CR1 expressers, and 1,000/cell on high CR1 expressers (Wilson et al., 1982). A decrease in receptor number due to disease can affect the transport of these immune particles by erythrocytes. In addition, a low number of CR1 has been linked to a reduced immune complex binding efficiency (Madi et al., 1990).

The activation of CR1 leads to the deposition of C3b and C4b fragments onto a target. These complexes are reversibly bound and are transported by CR1 on

erythrocytes from the peripheral blood vessels to the liver. CR1 is also expressed on other blood cells with various functions.

Glycophorin A

In addition to CR1, the predominant receptor responsible for complement ligation, erythrocytes have another membrane protein that has been shown to bind complement. Glycophorin A (GPA) is a Type I transmembrane protein and a major sialoglycoprotein found on the membrane of erythrocytes (Winzler, 1969). It is characterized as such due to the glycosylated regions found on the extracellular NH_2-end of the protein. GPA is the most abundant protein of the glycophorin family, present at 5-9 x 10^5 copies per cell, amounting to approximately 1.5% of the total RBC membrane protein (Chasis and Mohandas, 1992). It has been linked with complement ligation on RBCs in pathological cases where there is an excessive of C3b/C4b fragments in the intravascular space. This is mediated through the hydroxyl residues on the sialic acid expressed on GPA, which bind covalently to the free thioester groups on the complement fragments. The pathway triggered from this ligation is not fully understood.

Complement Pathology

Activated complement has a beneficial impact on the body's ability to identify and clear microbes. This mechanism is employed by the innate immune system, and has an additional effect in promoting erythrocyte deformability, a vital characteristic of RBCs. However, excess circulating complement has a negative impact on RBCs.

Sepsis (Acute Complement Conditions)

Systemic inflammatory response syndrome (SIRS), also known as sepsis, is an acute illness resulting from a severe host response towards infectious diseases, predominantly bacteria. SIRS is viewed as a complication to an infection when chemicals released throughout the body trigger a chain of events mediated by inflammatory particles that disrupt the function of multiple organ systems. These particles are naturally found in the bloodstream, and as complications arise, blood flow and blood pressure drop, resulting in organ failure. Sepsis can have a range of severity, but is most lethal in the elderly, or patients with a weakened immune system. Despite the advances of modern medicine and pharmaceutical industries to curb these infections, the fight against sepsis and indirect bacterial infections appears to be an endless one, due to microorganisms' ability to evolve and modify their resistance to these drugs. Although complement deposition is a key player in the immune system's arsenal against infectious diseases, it mediates the worsening conditions and complications in septic patients.

Systemic Lupus Erythematosus (Chronic Complement Conditions)

Systemic Lupus Erythematosus (SLE) is a long-term, auto-immune disease that is difficult to diagnose due to its wide range of symptoms. It can affect many tissues and organs of the body, causing inflammation and pain. Increased levels of plasma complement have been identified in connection with many pathological disorders, especially in patients with SLE (Walport, 2002). It is, however, difficult to use these levels in determining the activity of a disease in a clinical setting, because normal

complement levels vary between individuals, and the degree of complement in serum may not accurately reflect its activity in tissues.

Ghiran Lab's Main Interests

The long term interest of the Ghiran lab is to investigate the interactions between complement fragments and peripheral circulatory erythrocytes during normal and pathological conditions. The specific focus is on two signaling pathways triggered by different receptors, the major recipients for complement fragments on RBCs. The lab's approach is driven by the hypothesis that under inflammatory conditions the complement activated CR1 and GPA signaling pathways have opposing effects on mediating the RBC's responsibility of maintaining a homeostatic intravascular environment: ligation of CR1 promotes a non-inflammatory response, while GPA ligation signals a proinflammatory shift. The location of complement deposition can have a critical impact on the overall RBC via changes in membrane deformability, widely considered one of the most important factors controlling microcirculation (Vaya et al., 2002; Koksal et. al., 2002). The impact of these experiments can contribute to ways to prevent and regulate a wide range of pathologies, hereditary and acquired. In particular, sepsis alone in the United States is responsible for over 210,000 deaths each year, with associated costs estimated at over $16.7 billion (Angus et al., 2001).

Innovative Methods

The following methods were developed by the Ghiran Lab to quantify critical RBC functions affected in pathological settings:

- RBC membrane flickering is quantified using time-lapse, positive-low phase contrast microscopy. Membrane flickering is a unique marker for RBC functions that correlates with membrane deformability and oxygen transport across membranes. This is a unique technique we developed to monitor high resolution RBC functions (Costa et al., 2008).

- Unique, 2-D microfluidic channels have been specifically developed to study RBC membrane deformability *in vitro*. These devices allow for precise visualization of RBC deformability in order to observe the subtle differences in physiological and pathological settings faster than any other existing methods.

- Using luminometric methods to quantify the levels of ATP release by RBCs under various conditions. ATP, acting on its cognate (purinergic) receptors, is a significant pro-inflammatory mediator; therefore its extracellular presence is a key indicator on RBC function.

Specific Aims of the Ghiran Lab

A: Define the functional consequences of complement engagement with RBC CR1 and GPA receptors.

Our lab has shown that, under normal conditions, complement fragments deposited on CR1 are transferred more efficiently from RBCs in the microvasculature to macrophages in the liver and spleen when compared to free-floating particles (Nelson, 1953; Pilsczek et al., 2005). It has also been determined that CR1 plays an important role in the immune-adherence clearance of bacteria (Li et al., 2010). In pathological settings, previous reports from other labs have elucidated that the nascent complement fragments excessively generated do not bind to CR1, but rather engage covalently and irreversibly to GPA via sialic acid (Parker et al., 1984). We have shown that this complement-GPA binding results in a decrease in RBC membrane deformability (Karnchanaphanurach et al., 2009), providing the first mechanistic explanation for the alterations in RBC microcirculatory flow in pathologies associated with excessive complement activation: the RBCs are too stiff to efficiently move. This loss of RBC membrane deformability has

functional consequences, decreasing the diffusion of soluble molecules inside RBCs as well as oxygen across the membrane (Sakr et al., 2004; De Backer, 2006).

ATP is a major inflammatory marker actively released by RBCs (Wan et al., 2008; Sprague et al., 2009). Preliminary data has shown that complement-GPA binding significantly increases the overall production and release of ATP from RBCs, with no sign of cell lysis. This is a critical concept, as this increase in ATP production would constantly activate purinergic receptors on higher order immune complexes such as neutrophils, platelets, and endothelial cells (Junger, 2008; Gachet, 2006; Ferrero, 2009), promoting a proinflammatory environment. This study identifies a significant, unregulated source of ATP that could explain the purinergic overload described in septic patients.

B: Delineate the two signal transduction pathways triggered by the engagement of complement on RBCs.

Our hypothesis postulates that complement deposition on RBCs triggers alternate pathways depending on the receptor ligated, with one promoting membrane deformability and the other impairing it. Thus this second aim investigates the mechanisms contributing to the alterations in RBC membrane deformability.

We have shown that both CR1 and GPA promote a Ca^{++} influx. We hypothesized that a stretch-activated cation channel was responsible for the CR1-mediated Ca^{++} influx, with a member of the TRPC (transient receptor potential cation channel) family potentially linked to this pathway. We have also shown that GPA ligation, not CR1, enhances tyrosine phosphorylation of band 3, fully supporting other studies correlating

increased band 3 phosphorylation with decreased membrane deformability (Condon et al., 2007; Saldanha et al., 2007). Further studies on β-spectrin phosphorylation due to CR1/GPA-complement engagement yielded opposite results, where ligated CR1 promoted an increase in phosphorylation, and GPA stimulated a decrease in phosphorylation. The phosphorylation status of skeletal proteins adducin, and protein 4.1 is dependent on protein kinases A (PKA) and C (PKC) (Matsuoka et al., 1996; Manno et al., 2005), while β-spectrin is dependent on casein kinases I (CKI) and II (CKII) (Manno et al., 1995). We therefore postulated that these kinases are key players in the complement signaling pathways.

In order to determine the correct sequence of events, our lab intends to determine the nature of the Ca^{++} influx, and identify the stretch activating cation channel responsible, using Western blotting techniques and flow cytometry readouts. The involvement of specific kinases will further be tested using specific inhibitors and measuring the phosphorylation levels of skeletal proteins, using similar methodologies.

Our lab is also interested in defining the purinergic signaling events that occur as part of GPA-mediated loss of RBC function. It is postulated that converging signaling pathways from GPA ligation and autocrine purinergic receptors promote loss of RBC membrane deformability. ATP release, regardless of the stimulus, is dependent critically on the signaling cascade that includes adenylyl cyclase, PKA, pannexin-1 and P2X/P2Y receptors. Additionally, the involvement of CFTR (cystic fibrosis transmembrane conductance receptor) in this cascade is not fully understood. The role of each part of the cascade will be addressed through inhibitors and agonists for the enzymes responsible for

ATP release and the P2 receptors responsible for the autocrine purinergic signaling in RBCs, and measured by luminometric methods.

C: *In vivo* assessments of complement-altered RBCs in septic and lupus patients

The previous two lab aims are designed for *in vitro* studies on the functional consequences of CR1 and GPA ligation with complement. It is necessary to also measure these loss of function effects *in vivo*. Due to critical differences between the rodent and human immune clearance system, it is impossible to use mice for these studies. Rodent immune adherence is based on the platelet, they lack CR1 as an independent molecule, and their GPA is significantly different from that of humans. Thus, we will use RBCs drawn from septic (acute conditions) and lupus (chronic conditions) patients, two pathologies with known severe complement activation. These will be compared to RBCs under physiological conditions.

Physiological Relevance

The study of these objectives in our lab will have a significant impact on the current understanding of the mechanisms responsible for the mortality and morbidity surrounding all diseases associated with excessive complement activation. By investigating the RBC interactions through CR1 in a normal setting, as well as the GPA and purinergic mechanisms, this research may lead to the development of novel therapeutic approaches to interrupt the signaling cascades responsible for the loss of RBC function and proinflammatory reprogramming.

PROJECT PURPOSE

The purpose of this MQP project was to pursue a portion of our lab's objectives to help determine the functional effects of complement engagement with RBC receptors. This project will help confirm the main hypothesis of our lab that under inflammatory conditions, complement-activated CR1 and GPA signaling pathways have *opposing* effects on mediating the RBC's responsibility of maintaining a homeostatic intravascular environment: ligation of CR1 promotes a non-inflammatory response, while GPA ligation signals a proinflammatory shift.

CR1-mediated Ca^{++} influx will be investigated, as will the stretch-activated cation channel responsible this Ca^{++} influx, using Western blotting and flow cytometry techniques. The functional effects of extracellular ATP released by RBCs upon CR1 ligation will be demonstrated through microscopy and flow cytometry. Additional experiments regarding membrane deformability of RBCs will be conducted using unique 2D microfluidic devices. Complement-opsonized beads will be prepared to mimic fragments circulating the intravascular system, instead of using antibody-mediated binding.

METHODS

Antibodies and Reagents

The following antibodies were used during this project: anti-CR1 monoclonal Ab YZ-1, non-immune IgG1 (BD Biosciences, San Jose, CA); anti-TRPC1 rabbit polyclonal (Santa Cruz, Santa Cruz, CA); T1E3 inhibitor (gift of Yao Xiaoqiang, University of Hong Kong). Secondary Abs included: Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Grand Island, NY); horseradish peroxidase (HRP)-goat anti-mouse IgG, HRP-donkey anti-goat IgG, and HRP-donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Reagents were obtained as follows: Hank's Balanced Salt Solution with Ca^{++} and Mg^{++} ($HBSS^{++}$), Fluo-4-AM, 0.2 μm non-fluorescent sulfate latex microspheres (microbeads) (Life Technologies), IgG-free BSA (Jackson ImmunoResearch), citrate (4% w/v), dextran T500, cold water fish gelatin, glycine (Sigma-Aldrich, St. Louis, MO), GsMTx-4 (Peptide Institute, Osaka, Japan), 4,5,6,7-tetrabromobenzotriazole (TBB) (R & D Systems, Minneapolis, MN), apyrase (adenosine diphosphatase) (Tocris, Bristol, United Kingdom).

Fresh RBC Isolation

Blood was obtained from healthy adult volunteers in accordance with the guidelines of the Institutional Review Board (IRB) of Beth Israel Deaconess Medical Center, and after informed consent was obtained, in accordance with the Declaration of Helsinki. RBCs were obtained either by fingerprick (10-50 μL) or by venipuncture (40 mL) if more was needed. Blood acquired through venipuncture was centrifuged at 300 x

g, and plasma, buffy coat and top layer of RBCs were aspirated. RBCs were washed at least 3 times in $HBSS^{++}$ containing 0.05% IgG-free BSA.

Preparation of Complement-Opsonized Microbeads for Microscopy

Latex beads (0.2 μm) (Life Technologies) were sonicated for separation, and then incubated for 3 minutes with BSA Fluo 647 (stored at -20°C) in $HBSS^{++}$. Beads were washed 5 times to remove excess BSA, and then incubated with anti-BSA IgG for 10 minutes. Beads were washed again, and then incubated with human serum for 8 minutes to prevent the degradation of newly formed C3b/C4b fragments. Beads were spun down and washed 3 times and resuspended in 100 μL $HBSS^{++}$, and then sonicated. The quality of the beads (lack of aggregation) was checked by fluorescence microscopy using a 647 nm filter cube.

Analysis of RBC Calcium Influx

RBCs were preloaded with Fluo-4 AM for 15 minutes at room temperature, washed, and resuspended in $HBSS^{++}$. Due to the ATP-depleting effect of the acetoxymethyl group, all experiments were performed within one hour from Fluo-4 AM loading. Fluorescence levels of RBCs were acquired for 20 seconds using a LSRII flow cytometer (Becton Dickinson, Mountain View, CA) to establish a baseline for intracellular RBC Ca^{++} concentration. Non-fluorescent control or complement-opsonized beads were then added to the RBCs, vortexed briefly, and RBC fluorescence intensity was recorded for 2 minutes. Data was exported as FSC 3.0 files and analyzed using the kinetic module of FlowJo 9.0.1 (Treestar, Inc., Ashland, OR).

Western Blotting Analysis

RBCs (2 μL packed cells) were washed and lysed in 100 μL of 1 x NUPAGE LDS Sample Buffer (Life Technologies) and kept at 37°C. Samples were run on 10% Tris gels (Life Technologies), transferred to nitrocellulose paper (Pierce, Rockford, IL), and blocked with blotting-grade blocker (Bio-Rad, Hercules, CA). Membranes were then incubated with proper primary and secondary antibodies, as indicated in the figure legends and according to the manufacturer's instructions. Blots were developed using the LAS 4000 imaging system (FujiFilm, Edison, NJ).

Flow Cytometry Analysis

Isolated RBCs were incubated for 15-30 minutes with appropriate Abs or inhibitors as noted in the figure legends in 0.5% BSA in $HBSS^{++}$ buffer at 4°C. The cells were then washed and incubated with Alexa Fluor 488-labeled secondary Ab specific for each primary Ab at a dilution following the manufacturer's instructions. For each experiment, at least 10,000 events were recorded using either a FACScan or LRSII flow cytometer (Becton Dickinson), and analyzed using either CellQuest Pro version 4.0.1 (BD Biosciences) or FlowJo 9.0.1 software (Treestar, Inc., Ashland, OR).

RBC Membrane Deformability Assay Using 2D Microfluidic Devices

To test the efficiency of RBC membrane deformability, 2D filters were developed comprising an array of posts with 5x5 μm channel openings using polydimethylsiloxane (PDMS). The design of this experimental setup has been previously described in detail (Shevkoplyas et al., 2006). In order for the sample to flow through the 2D device, the

outlet was connected to a waste reservoir (60 mL syringe) with a 60 cm long piece of PE-60 tubing filled with $HBSS^{++}$. The differences in the level of liquid in the reservoir of the 2D filter device and the level of liquid in the waste reservoir provided the driving pressure pushing the samples through the channels. Zero pressure difference corresponded to the absence of movement of RBCs in the device.

Control and CR1 ligated RBCs were loaded into the inlet reservoir and 'pushed' towards the channels by moving the waste reservoir tubing to a fixed point below the zero pressure, driving RBCs towards the outlet. The passage of RBCs through the 2D filter was recorded using a 40 x 0.75 Ph2 Plan Fluorite objective on a TE300 Nikon inverted microsope, using a Retiga Exi camera (QImaging, Canada) controlled by iVision 4.01 (BioVision, Exon, PA) at a rate of 10 frames/s. The images were analyzed frame by frame to measure the time from initial contact with the channel to egress. Differences in passage time between the control and CR1-ligated RBCs were compared using the Mann-Whitney test, with a p-value of 0.05 or less considered statistically significant.

Determining the Role of ATP in Complement Promoted CR1 Clustering

Alexa Fluor 647 fluorescently-labeled complement-opsonized microbeads were prepared as previously described. RBCs were incubated with microbeads for 20 minutes in the absence or presence of apyrase. RBCs were then washed and fixed in a 0.05% acrolein solution for 5 minutes, and then blocked in a solution containing 0.2% cold water fish gelatin, 5% human serum and 0.2 M glycine for 60 minutes. RBCs were then incubated with 1 μg/mL of directly-labeled Alexa Fluor 488 anti-CR1 (YZ1) mAb for 15 minutes. Cells were washed and imaged using a QImaging Rolera EM-C^2 fitted on an

Olympus BX62 microscope controlled by Slidebook 5.0 (Intelligent Imaging Innovations, Denver, CO).

Statistical Analysis

The statistical analysis tests were performed using Prism Version 4.0 (GraphPad Software).

RESULTS

RBCs are classically described as passive carriers of immune complexes from the intravascular environment to resident macrophages in the liver and spleen. The focus of this project was to investigate the functional consequences that occur following the engagement of RBC complement receptor-1 (CR1) by circulating immune complexes during the immune-adherence clearance process. The results show that the ligation of RBC CR1 increases membrane deformability, triggers a significant Ca^{++} influx, and promotes ATP release that is critical for CR1 clustering and binding efficiency. The most important results leading to these conclusions are presented below.

Ligation of RBC CR1 by Complement Induces a Ca^{++} Influx

Our lab previously reported that RBC CR1 ligation by complement-opsonized particles shifts the receptor into large clusters (Ghiran et al., 2008). We postulated that the ligation of RBC CR1 also triggers an influx of Ca^{++} into RBCs. Importantly, the expression pattern of CR1 on RBCs is genetically determined, with low donors expressing 100 copies of CR1/cell, intermediate donors expressing 500 copies of CR1/cell, and high donors expressing 1000 copies of CR1/cell. Therefore, we next asked whether the different levels of CR1 expression are associated with the magnitude of CR1-mediated Ca^{++} influx.

RBCs isolated from donors with known high or low CR1 levels were loaded with Fluo-4 in the presence of $HBSS^{++}$. RBCs were incubated with non-fluorescent control or complement-opsonized beads to mimic the physiological CR1 ligands. The results

(**Figure-4**) show that ligation of RBC CR1 with complement-opsonized beads promoted a sustained Ca^{++} influx, with RBCs from high CR1 donors displaying a more pronounced influx (15 nM →120 nM) than low CR1 donors (15 nM →60 nM) (**Figure 4A**). In addition, the RBCs from high CR1 donors (1000 copies/cell) bound to fluorescently-labeled complement-opsonized beads at a greater efficiency than RBCs from low CR1 donors (100 copies/cell) (Mean Fluorescence Intensity of 440 to 110) (**Figure 4B**). Taken together, these results show that CR1 ligation triggers a Ca^{++} influx, which is dependent on the genetically determined levels of CR1. It is possible that immune-complexes and complement-opsonized particles could augment the Ca^{++} influx simply by the shear stress experienced by circulating RBCs, so the next step was to search for an RBC Ca^{++} channel that is also a mechanoreceptor.

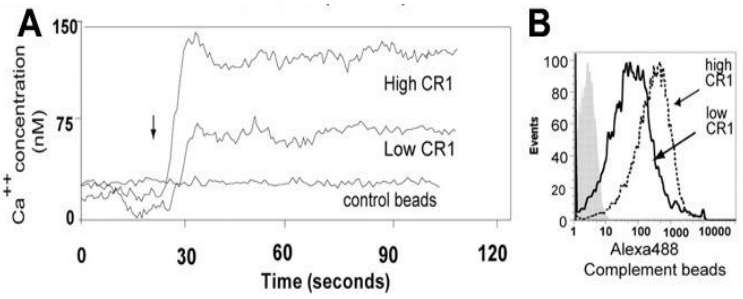

Figure 4: Ligation of RBC CR1 promotes Ca^{++} influx that depends on the genetically determined CR1 levels. A) Ligation of CR1 by complement-opsonized particles promotes RBC Ca^{++} influx. Intra-RBC Ca^{++} concentration of Fluo-4 loaded RBCs from known high and low CR1 expressing donors was measured by flow cytometry for 20 seconds before RBC CR1 was ligated by control or complement-opsonized beads. B) Functional characterization of RBC CR1 using complement-opsonized beads. RBCs from known low and high CR1 expressing donors were incubated with fluorescently-labeled complement-opsonized beads for 30 minutes, washed, and analyzed by flow cytometry.

TRPC1 is Involved in the Ca^{++} Influx Induced by CR1 Ligation

Transient Receptor Potential Channels (TRPC) are a group of stretch-activated ion channels located on the plasma membrane of numerous human cell types. They are typically triggered through mechanical deformations or receptor mediated ligation (Johnson, 1994). To investigate the involvement of a stretch-activated cation channel (SAC) in CR1-mediated Ca^{++} influx, we used GsMTx-4, a peptide isolated from tarantula

venom that acts as a non-specific peptide blocker of cationic SAC. Our data (**Figure 5A**) shows that pre-incubation of RBCs with 1 and 5 μM concentrations of GsMTx-4 progressively inhibited CR1-mediated Ca^{++} influx, measured by flow cytometry. Recently, TRPC1 was shown to promote Ca^{++} influx following stretch activation (Maroto et al., 2005). First, we investigated its presence in RBCs by using immunofluorescence microscopy (data not shown), and immunoblotting methods (**Figure 5B**). RBC lysates were run on a 10% Tris HCl gel, transferred onto nitrocellulose paper, and probed with rabbit anti-TRPC1 mAb. The developed blot shows that the antibody detected a protein running at a MW around 85 kDa (right lane), corresponding with the MW of TRPC1 identified in a testes tissue lysate used as a positive control (middle lane).

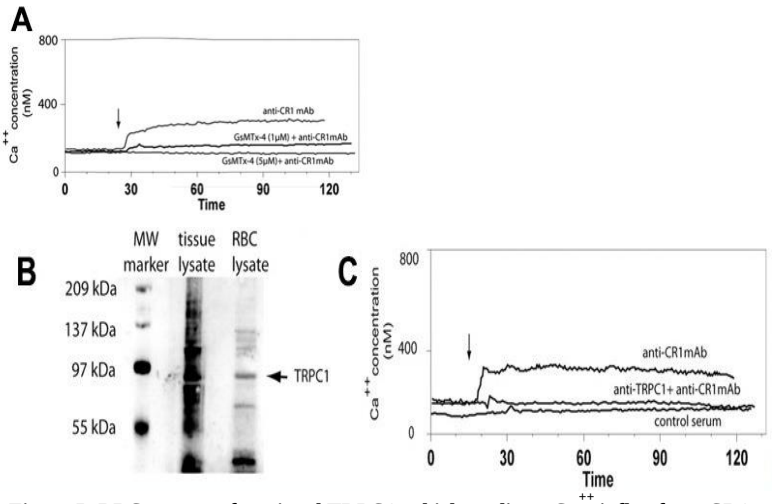

Figure 5: RBCs express functional TRPC1, which mediates Ca^{++} influx from CR1 ligation. A) RBC CR1 ligation promotes a SAC-dependent Ca^{++} influx. Fluo-4 loaded RBCs were incubated with buffer, 1, or 5 μM GsMTX-4 for 30 minutes and then analyzed by flow cytometry for CR1-mediated Ca^{++} influx. B) Immunoblotting detection of TRPC1 in RBCs. Testes tissue lysate (positive control) and RBC lysate were separated by gel electrophoresis using TRIS-HCl gels, transferred onto nitrocellulose paper, and probed rabbit anti-TRPC1 Ab. TRPC1 is seen as a band around 85 kDa (arrows) in both positive control and RBC lysate. C) CR1 ligation triggers a Ca^{++} influx dependent on TRPC1. Fluo-4 loaded RBCs were pre-incubated with control serum or T1E3 (TRPC1 inhibitor) for 20 minutes, washed, and analyzed by flow cytometry for CR1-mediated Ca^{++} influx.

These results (Figure 4, 5A, and 5B) demonstrate that a SAC was important for CR1-mediated Ca^{++} influx, and TRPC1 was identified as a cation channel on RBC membranes. Thus, the next step was to identify TRPC1's functional involvement in the CR1-mediated Ca^{++} influx. In order to do this, a rabbit anti-serum (T1E3) raised against the extracellular portion of TRPC1 was identified as a successful inhibitor of TRPC1 and used for this

experiment. Following methods used previously in Figure 5A, a time dependent assay found that T1E3 successfully prevented CR1-mediated Ca^{++} influx (**Figure 5C**). It is important to note that because the inhibitor came from rabbit serum, RBCs were washed 3 times before CR1 ligation to prevent Ca^{++} influx through a newly formed C5b-C9 complex (MAC), an ungated ion channel. Taken together, these results strongly suggest that TRPC1 is implicated in CR1-mediated Ca^{++} influx.

CR1 Ligation Increases RBC Membrane Deformability

The immune-adherence clearance process occurs in capillaries in the liver and spleen where RBCs (7-8 μm diameter) are forced to deform through 3-4 μm capillaries and through 2-3 μm slits of the wall of the reticulo-endothelial sinusoids to reach resident macrophages. Therefore, we hypothesized that CR1 ligation would increase the RBC membrane deformability. In addition, we questioned whether the levels of CR1 expression on RBCs correlate with the magnitude of RBC membrane deformability, as mediated by CR1 ligation. This question was addressed using a microfluidic filtration device consisting of an array of 320 2D microchannels with a diameter of 4.5 μm and a length of 25 μm. The transit time for RBCs through the narrow channel should inversely correlate with the magnitude of deformability. RBCs from known high and low CR1 expressing donors were incubated with primary anti-CR1 Ab and corresponding secondary Ab, washed, and added to the microfluidic device (**Figure-6**). The time required for RBCs to enter (Frame 3), pass through, and exit the 25 μm channels (Frame 6), were measured and expressed as one value per RBC (**Figure 6A**). Quantifying this data (**Figure 6B**) showed that the number of CR1 molecules per RBC did correlate with

increasing membrane deformability. High CR1 expressing RBCs required about half the time (0.78±.18 s) to pass through the microchannels compared to low CR1 expressing RBCs (1.42±.22 s), and about a third the time required compared to control RBCs (2.21±.18 s). This, in addition to experiments using laser-optical tweezers conducted by our collaborators at Harvard Medical School (Glodek et al., 2010) strongly suggests that CR1 ligation increases RBC membrane deformability directly, a process that likely would increase the efficacy of the immune-adherence transfer.

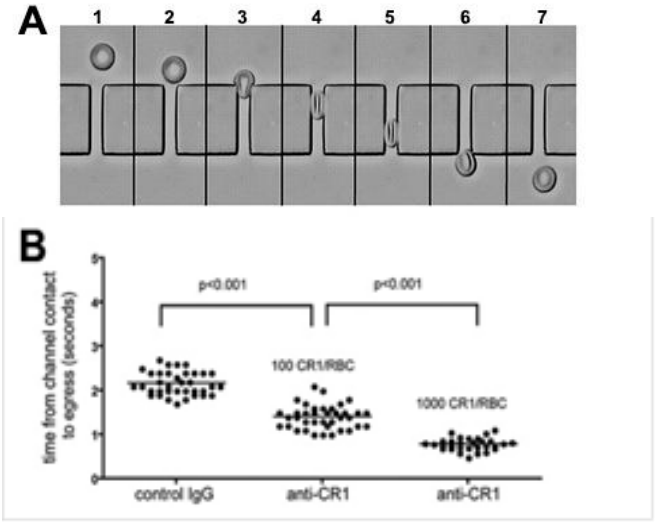

Figure 6: Ligation of CR1 increases RBC membrane deformability. A) Sequential imaging (3 frames/s) of RBC folding upon entering 25 µm long microfluidic device, passing through and exiting, regaining its original shape. Every third frame is shown. B) Ligation of RBC CR1 decreases the time required for RBC to pass through microchannels. The extent of the decrease in the time required for RBCs to pass through the microchannels depends directly on the number of CR1 molecules on the surface of RBCs. Each dot represents one RBC. The results are representative of five independent experiments using different anti-CR1 Ab and different high or low CR1 donors.

CR1-Mediated ATP Release is Critical for the Interaction Between CR1 and Complement-Opsonized Particles

Recently, extracellular ATP was shown to be essential for effective removal of apoptotic particles by professional phagocytic cells. Therefore, we next asked whether CR1 ligation would elicit an ATP release that would be relevant in the immune complex binding and transfer. We showed that ligation of CR1 by complement opsonized beads produced a sustained and robust ATP release (Brodsky, data submitted for publication).

We next asked whether the CR1 promoted ATP release plays an autocrine role in modulating membrane deformability as well as the binding efficacy of CR1 to complement-opsonized particles. To investigate this hypothesis, we incubated RBCs with complement-opsonized beads in the presence or absence of apyrase (ATP adenosine diphosphatase), a molecule that hydrolyzes extracellular ATP. Our results (**Figure-7**) show that the removal of extracellular ATP significantly decreases the binding efficiency from 22.1% positive complement-opsonized beads to RBC to .34% binding, respectively (**Figure 7A**). In addition, our lab also showed that the effect of complement ligation to CR1 depends critically on casein kinase II (CKII) activity (Glodek et al., 2010). Therefore, in a separate experiment, RBCs were pre-incubated with specific CKII inhibitor TBB, and then incubated with complement-opsonized beads (**Figure 7B**). The results show that prevention of RBC ATP release by inhibition of RBC CKII activity decreased the percentage of beads bound to RBC from 31.1% to 17.4%. Importantly, the addition of 20 mM ATP back to the inhibited-RBC restored the binding efficacy to the initial levels (**Figure 7B**, right panel).

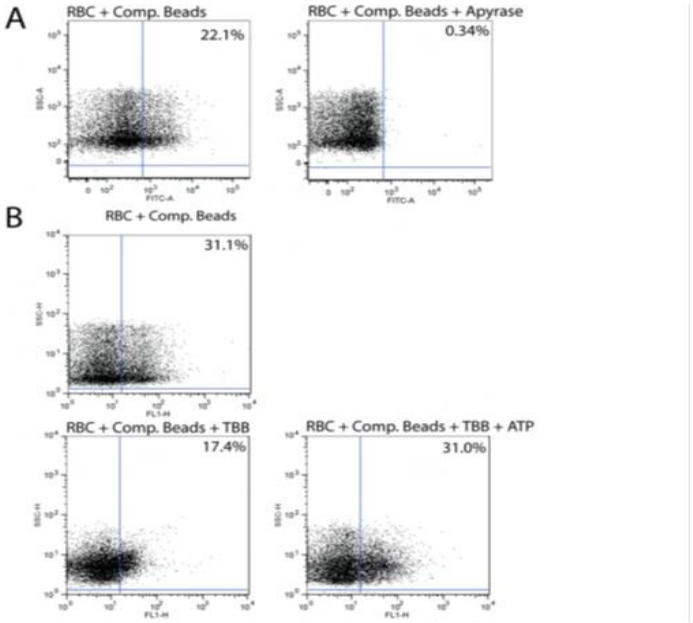

Figure 7: Ligation of RBC CR1 is dependent on the presence of extracellular ATP. A) Hydrolysis of extracellular ATP by apyrase decreases the binding efficiency of complement-opsonized microbeads to RBC CR1. RBCs were pretreated with 5 U/mL apyrase before incubation with either control or complement-opsonized microbeads. Results were measured by flow cytometry. B) Inhibition of ATP release via CKII inhibition decreases binding efficiency of complement-opsonized microbeads to RBC CR1. RBCs were pretreated with 40 μM TBB before incubation with complement-opsonized beads in the absence or presence of 20 nM ATP. Results were analyzed by flow cytometry.

Taken together, these results indicate that extracellular ATP is critical in mediating the binding efficiency of complement-opsonized particles to RBC CR1.

Extracellular ATP is an Essential Component to CR1 Clustering

The results in Figure 7 indicate that extracellular ATP is required in modulating the binding avidity of complement-opsonized particles to CR1 as RBCs circulate from the binding site to the liver and spleen. One possible explanation for these results is that ATP is essential for CR1 to cluster upon complement ligation. To test this, RBCs were incubated with Alexa Fluor 647-labeled complement-opsonized beads in the presence or absence of apyrase (**Figure-8**). The cells were then fixed in 0.5% acrolein, blocked, and incubated with CR1 using Alexa Fluor 488 directly labeled YZ1 anti-CR1 mAB. The fluorescent intensity profiles of CR1 in buffer and apyrase treated RBCs were analyzed through immunofluorescence microscopy against the intensity profile of complement-opsonized particles. The results (**Figure 8**) show that in the presence of apyrase, CR1 (green) was scattered throughout the RBC, compared to the cells in buffer, which appear to cluster around the microbead (red). This suggests that apyrase, or the lack of extracellular ATP, prevented the clustering of CR1, therefore lowering the binding avidity of CR1 to complement particles.

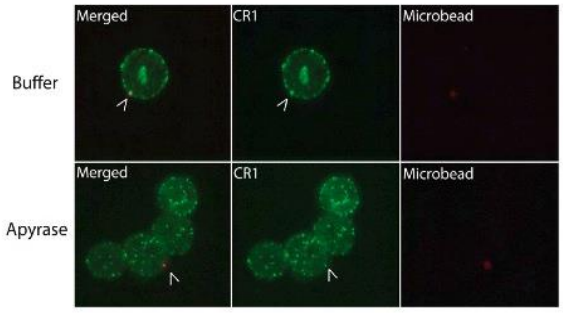

Figure 8: Extracellular ATP is critical in maintaining CR1 clustering. A) Apyrase prevents clustering of CR1 bound complement-opsonized microbeads. RBC in the absence (upper row) or presence of 5 U/mL apyrase (lower row) were incubated with Alexa Fluor 647 complement-opsonized microbeads for 30 minutes, washed, and incubated with Alexa Fluor 488 labeled anti-CR1 Ab.

DISCUSSION

The most important function of RBCs, delivering oxygen to tissues and removing carbon dioxide, is largely dependent on the deformability of the membrane. The dynamic link between the cell membrane and the cytoskeleton underneath is essential for RBCs to deform efficiently and pass rapidly through narrow capillaries. The data presented in this project investigates a lesser known function of RBCs regarding complement binding, and identifies a novel functional consequence of RBC complement receptor-1 (CR1) engagement that is relevant to the process of immune adherence clearance. By binding and removing circulating immune complexes, RBCs help ensure a non-inflammatory environment, by preventing the direct interaction between circulating neutrophils and free proinflammatory immune complexes. We and others have previously shown that CR1 plays a key role in erythrocyte immune adherence clearance. We thus investigated the effect of CR1 ligation on RBC membrane deformability through microchannel networks. The data from this project show that ligation of CR1 significantly decreases RBCs passage time through the channels, which directly depends on the genetically inherited number of CR1 molecules present on RBC membranes.

Previous studies have demonstrated that mechanical stimulation of RBCs increased membrane tension and augmented the permeability for Ca^{++} influx. Here, we showed that ligation of CR1 promotes a Ca^{++} influx, with an amplitude directly dependent on the expression levels of CR1 on RBC membranes. This project identified the presence of TRPC1 on human RBC membranes and its involvement in CR1 mediated Ca^{++} influx, by using a custom made inhibitory antibody against TRPC1.

We previously showed that CR1 is constitutively dispersed on the surface of circulating RBCs and clusters upon ligation with complement-opsonized particles due to its interactions with the scaffolding protein, FAP-1. Others have shown that this distinctive clustering on RBC helps prevent erythro-phagocytosis by inhibiting the zipper mechanism of phagocytosis, a process that requires a continuous, uniform deposition of complement fragments on the target cells (Swanson & Baer, 1995). In addition, ATP release by apoptotic cells was shown to be critical in recruiting macrophages and enhancing phagocytosis of apoptotic cells *in vitro*. We have shown that ligation of RBC CR1 promotes ATP release. This project continued this work by investigating the role of extracellular ATP following CR1 ligation. The loss of complement engagement with CR1 following enzymatic removal of extracellular ATP implicated that ATP maintains a critical role in the process of regulating CR1 clustering and binding capabilities, both crucial processes are necessary for sustaining the shear stress encountered by circulating RBCs and avoiding erythro-phagocytosis by macrophages.

In conclusion, this project demonstrated that ligation of RBC CR1 triggers a significant Ca^{++} influx, and activates a signaling pathway that depends on CKII and PKA activity, leading to a significant increase in plasma membrane deformability through phosphorylation of β-spectrin. Furthermore, our data showed that ligation and clustering of CR1 is critically dependent on the extracellular ATP released as part of this signaling cascade. Recognition of the signaling pathway triggered by CR1 ligation will allow for targeted modulation regarding the signaling events of immune adhesion, and the immune transfer process relevant in pathologies associated with complement deficiencies, such as sepsis, lupus, rheumatoid arthritis, etc. Given the increased functional advantage

(increased membrane deformability, increased efficiency in binding, transporting, and transferring of complement-opsonized particles) of high CR1 expression levels, one can also speculate on the possibility that transfusion of stored blood from high RBC CR1 donor to patients with excessive complement activation could be potentially advantageous in restoring a non-inflammatory environment.

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