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Comparative Characteristics of Integrin αDβ2 Binding to Native Fibrinogen and Fibrinogen Modified by DHA Oxidation During Inflammation

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by

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ABSTRACT

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2-ω-carboxyethylpyrrole (CEP) is a product of docosahexaenoic acid (DHA) oxidation, which forms covalent adducts with different proteins. CEP-modified proteins can interact with macrophage receptor, integrin αDβ2. This study aims to compare αDβ2 binding to its physiological ligand, fibrinogen, and CEP-modified fibrinogen, which is formed during inflammation. We hypothesize that modification of fibrinogen changes its ligand-binding properties to integrin αDβ2 which can affect macrophage migration and retention. Recombinant αD I-domain and αDβ2-transfected HEK293 cells were used for the experiments. Using biolayer interferometry, we found that the affinity of αD I-domain binding to fibrinogen-CEP was higher than fibrinogen and inhibited by the anti-CEP antibody. In agreement, αDβ2-transfected cells demonstrated stronger adhesion to fibrinogen-CEP and this adhesion was significantly inhibited by polyglutamic acid that mimics CEP-mediated binding. These findings suggest that αDβ2's interaction with DHA-modified extracellular matrix (ECM) proteins significantly increases macrophage adhesion and may serve for macrophage retention during chronic inflammation.

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CHAPTER 1. INTRODUCTION

The immune system reacts and plays its part in defending the body when it is subjected to harsh and stressful conditions, external or internal foreign stimuli, or both. Inflammation is one of these responses. Specialized cells enable the immune system to recognize and eliminate harmful and foreign stimuli while also starting the healing process. Time is a crucial element in the process of inflammation since it determines and categorizes the stage of inflammation, which can be acute—rapid response and brief symptomatic manifestation—or chronic—considerably longer duration of inflammation. However, the injury's origin and the immune cells' ability to treat and resolve the issue also affect the type of inflammation, the intensity, and the effects. As evidenced by research, inflammation is the root cause of the great majority of chronic diseases such as arthritis, diabetes, obesity, and cardiovascular diseases[1-4].

The fact that the immune system and inflammatory responses are linked to a wide range of physical and mental health problems that predominate current morbidity globally and have been acknowledged as the leading cause of death in the world today—with more than half of all fatalities being linked to inflammation-related diseases like chronic kidney disease, ischemic heart disease, and neurodegenerative disorders—has been one of the most important clinical discoveries of the past 20 years.[5,6]

In general, the immune system's main goal is to get rid of harmful or alien cells including bacteria, viruses, fungi, parasites, and damaged cells from the body. The innate and adaptive components make up the immune system. Even though each sub-system's duties for maintaining the body's integrity are distinct, they cooperate to eliminate dangerous antigens—non-self-cellular matter—from the body as quickly and

effectively as possible [7]. However, when human disease situations arise that cause any of these immune systems to become alert, inflammation invariably sets in and plays a major role, giving rise to 2 main forms: acute and chronic inflammation[8]. A combination of innate and adaptive immune cells, stromal fibroblast matrix proteins, blood and lymphatics, and soluble molecular mediators including plasma proteins, cytokines, and chemokines are all involved in an inflammatory milieu[9,10]. Although distinguishing between the regulated (acute) and dysregulated (chronic) forms of inflammation has been difficult, modern molecular biology methods have made this significantly more achievable and attainable[11].

Acute and Chronic Inflammation

As a general rule, inflammation is produced whenever the body detects "risk" in the form of infections, trauma, or ischemia events, or physical, chemical, or mechanical damage. The acute form of inflammation is the response to abrupt or severe bodily injury that attracts leukocytes, plasma proteins, and much later antibodies to the location of the lesion or infection. It lasts for a couple of days[12]. Chronic inflammation develops when the activity of the different immune cells that have been recruited is protracted and persistent [13]. This would continue to happen whether there was an outside stimulus or not for months or years. Therefore, both acute inflammation and time are major contributors to chronic inflammation.

A direct inflammatory route made up of a series of actions including inducers, sensors, mediators, and effectors constitutes the acute inflammatory response[14], which is an immediate and adaptive reaction. The procedure initiates with the presence of one or more inducers, including pathogenic microorganisms, foreign substances, or signals

from necrotic cells or damaged tissues, whether they are pathogenic or not. These inducers activate specialized molecules, known as sensors, which then trigger effectors, such as tissues and cells, as well as mediators, which are endogenous compounds. The mediators can either activate or inhibit inflammation, induce pain, and trigger tissue healing[15]. As was previously mentioned, a variety of mediators, including toll-like receptors (TLRs) on macrophages and dendritic cells, arachidonic acid mediators on mast cells, complements C3a and C5a, and Hageman factor, start this cascade of acute inflammatory pathways in tissues. Ultimately, the specific types of immune cells and mediators involved are highly varied and depend on a range of factors, such as the type of inducing agent, the duration of the injury, and other genetic factors[16].

Contrarily, chronic inflammation shares many traits with acute inflammation but often manifests as a low-grade, long-lasting condition that leads to reactions that deteriorate tissue. There are several mechanisms that could be the cause of persistent inflammation. The synthesis and release of cytokines, which can accelerate or control the inflammatory response and change the morphology of adjacent cells, could be the result of damaged non-immune cells and activated immune cells, which is frequently detrimental to proper tissue function[17]. Another possibility is that pathogen-killing leukocytes that have invaded tissues chronically synthesize and release reactive chemicals, eventually harming the structural and cellular components of tissues[18].

Inflammatory Response

In order to defend against insults brought on by a variety of substances and to maintain tissue homeostasis, various cell types are activated during the inflammatory response [19]. This multifaceted response to tissue stress, injury, and infection is crucially

regulated at the level of gene transcription and is made up of a number of coordinated regulatory sets of genes that encode functionally essential aspects regulated by transcription factors, as well as a number of transcriptional co-regulators and chromatin modifications[20].

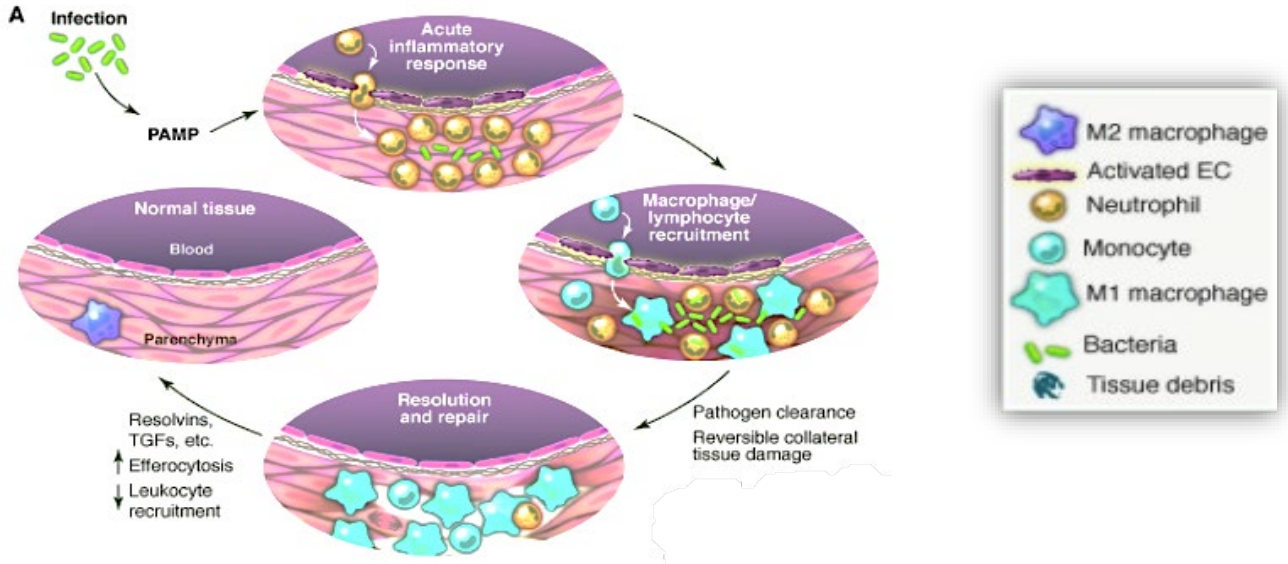


Figure 1: Acute inflammatory response. Reprinted with permission from AAAS [20]. Acute inflammation response to bacterial infection. Neutrophils first migrate to the site of inflammation to infiltrate the tissue, then monocyte-derived macrophages clear the pathogen and restore parenchyma.

The inflammatory response follows a carefully planned series of actions so that following the initial stimulus, there is a huge influx of inflammatory cells at the site of harm. The signals from these events changes the local profile of adhesion molecules and produces a chemotactic gradient that draws in cells from the circulation. An inflammatory response is initiated by the production of soluble mediators and chemokines from locally dwelling cells such as vascular endothelial cells, dendritic cells, macrophages, and interstitial fibroblasts. The first inflammatory cells to reach the site of

injury are polymorphonuclear neutrophils (PMNs), which extravasate and migrate to the affected area. Downstream signals then recruit specialized mononuclear cells, including lymphocytes and monocytes/macrophages. Macrophages take a role in inflammatory/immune effector cell generation, mobilization, activation, and control[22]. Studies have also demonstrated the significance of metabolism in the inflammatory response and how the various metabolic pathways affect the inflammatory response and the fate of immune cells.

However, the inflammatory response can be divided into first and second waves based on the type of immune cell that responds. In the first wave neutrophils are the first immune cells to go to the site of inflammation during the initial inflammatory response. Neutrophils that are circulating in the blood can enter the affected tissue through leakage, which is guided by a chemokine gradient created by local cells and other chemoattractant molecules. This process is facilitated by changes in blood flow, vascular permeability, and alterations in the molecular surface expression of cells lining the channel wall[23]. Macrophages are key players in the second wave of inflammation after neutrophil infiltration. As was previously stated, circulating monocytes exit the bloodstream and extravasate into the tissue, where they then undergo macrophage differentiation[24]. The extensive variety of immune system functions performed by monocyte-derived macrophages, from phagocytic clearance of insult and cell recruitment to later aiding healing and tissue regeneration during the resolution phase of inflammation, are enhanced by their highly malleable nature and dynamic character.

Chronic Inflammation

Chronic inflammation is characterized histologically by lymphocyte and macrophage presence, vascular proliferation, fibrosis, and tissue necrosis. Chronic inflammation is a key factor in the development of cardiovascular and metabolic diseases. The resolution phase, which comes after a successful inflammatory response occurs once the danger signal or the harm has been resolved, is defined as the time when the inflammatory reaction gradually shuts down. To restore homeostasis, the resolution phase is essential[25]. In some situations, if the immune system's ability to switch between pro-inflammatory and anti-inflammatory states is compromised, the resulting effect is chronic inflammation[19].

Resolution for Chronic Inflammation

A progressive change in the inflammatory profile of macrophages towards anti-inflammatory or recovery macrophages characterizes the resolution of inflammation. Failure of inflammation resolution is the primary cause of chronic inflammatory diseases including arthritis, colitis, or asthma, which are associated with irreversible tissue damage and a higher risk of developing cardiovascular disease, cancer, and osteoporosis[26-28]. Therefore, it follows that the resolution process is strictly regulated. Numerous molecular effectors regulate this process, such as the IGF1 pathway[29], the p38 MAPK regulator MKP1/DUSP1, and the transcription factors C/EBP (CEBPB) and NFIX[30]. These anti-inflammatory macrophages have a variety of effects on neighboring cells, including stimulating myogenesis's final stages of differentiation and fusion, remaking the extracellular matrix (ECM), and promoting angiogenesis, which occurs

concurrently with myogenesis[31]. Phagocytosis of debris also aids in the resolution of inflammation, which is required to begin tissue repair and regeneration[31].

Roles of Macrophages in Resolution of Chronic Inflammation

The inflammatory process is typically very closely controlled, involving both signals that start and keep the inflammation going, as well as signals that stop it. Unchecked inflammation leads to cellular and tissue damage and notifies the immune cells when there is an imbalance between the two signals[32]. Macrophages were first referred to as phagocytes since they were discovered to be engaged in the phagocytotic process that helps maintain homeostatic processes of tissue resorption and the acquisition of nutrients[33]. Years of further study also revealed that macrophages played a role in cellular immune responses[34]. They play a number of roles in the body, including the metabolism of iron, bilirubin, calcium, lipids, and amino acids among numerous other functions as reviewed by Mosser et al (2021)[34]. This helps keep the amounts of these chemicals in the body relatively constant[32-34]. They are also referred to as transducers since they gather information from various tissues and then transmit it to regulate crucial chemical reactions for the particular operation of each organ[34].

The mononuclear phagocyte system is made up of cells with a similar lineage that specializes in phagocytosis. However, they perform a minimum of three main tasks, including immunomodulation, phagocytosis, and antigen presentation[35]. In sequential processes of active cell recruitment to the site of infection, detection of microorganisms, phagocytosis, and killing of ingested bacteria, they demonstrate a general functional response role in host defense. Furthermore, they generate biologically active chemicals that play a variety of crucial roles in both innate and adaptive immune responses.

Although macrophages can have a wide range of different phenotypes, they can be generically categorized into M1-like or M2-like states based on their activation mechanisms, molecular expression, and behavioral patterns. The classically activated M1-like state is typically thought to be pro-inflammatory, whereas the alternatively activated M2-like state is typically thought to be anti-inflammatory[36].

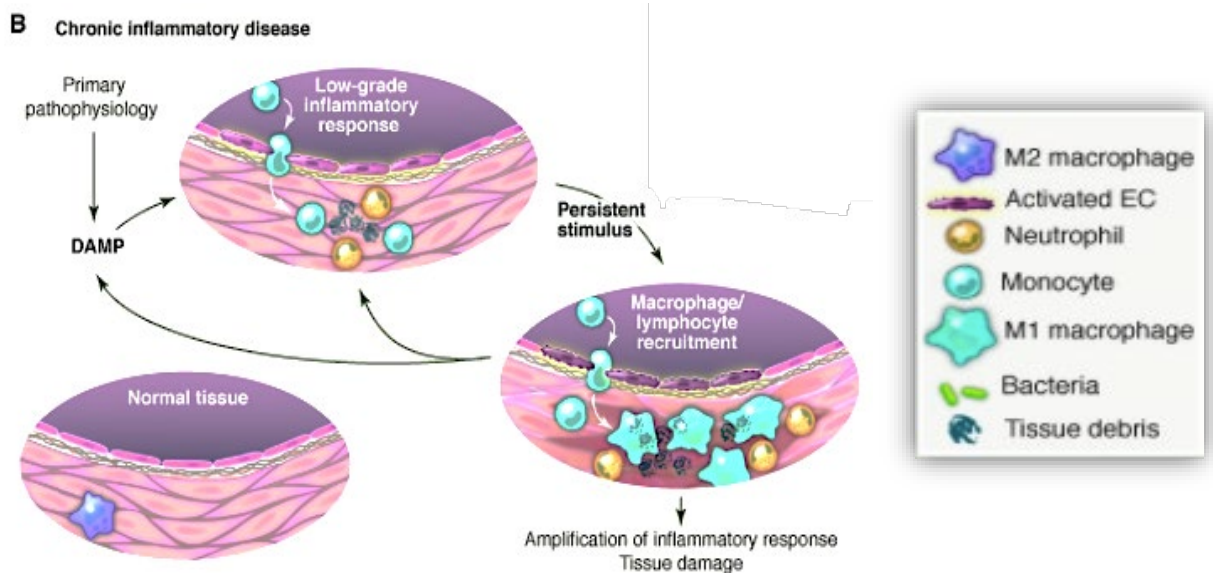


Figure 2: Chronic Inflammation Reprinted with permission from AAAS [20] Chronic inflammatory reactions arise when immune cells fail to eliminate the stimuli that provoke an inflammatory response. The persistence of these stimuli amplifies the inflammatory response, preventing the resolution of inflammation and causing damage to host tissue.

Usually, M1-type macrophages exhibit significant phagocytic activity at the site of inflammation to eliminate stimuli that can influence the activity of neighboring cells and clear any debris. Additionally, these macrophages promote inflammation by stimulating the recruitment of additional immune cells to the site and generating a range of pro-inflammatory cytokines, chemokines, and other inflammatory mediators[37]. When the inflammatory stimulation is resolved, the macrophage population is thought to change

from expressing an M1-like phenotype to an M2-like phwenotype[38]. By releasing anti-inflammatory molecules, M2-like macrophages decrease the inflammatory response. These cells also regulate the actions of other cells, assist in tissue regeneration, and promote healing by guiding the repair process. As the damaged tissue is repaired, immune cells evacuate the area, hence promoting recovery[39].

Pro-inflammatory macrophages

Pro-inflammatory damage-associated macrophages, which predominantly develop from circulating monocytes, are the first to invade the wounded area. Macrophages linked to inflammatory damage produce pro-inflammatory mediators[40]. Preventing or reducing the migration and adhesion of pro-inflammatory macrophages to the inflammatory site is an attractive approach for managing and preventing chronic inflammatory reactions, as these macrophages are the primary cellular component in these lesions and are also responsible for a significant amount of the damage caused by chronic inflammation. The migration and adherence of monocytes and macrophages during inflammation are primarily mediated by a critical subfamily of cell adhesion receptors known as the β2 integrins, which are a part of the larger integrin family.

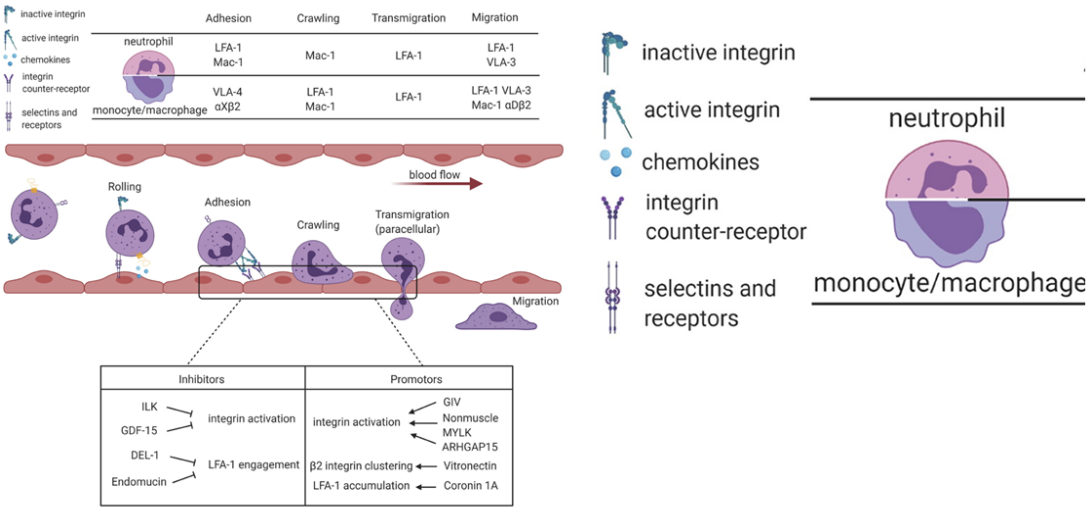


Figure 3: Leukocyte Adhesion Cascade. The image is from reference [41]. The process begins with the rolling of leukocytes along the endothelium facilitated by the interaction between selectins on the endothelial cells and their ligands on the leukocytes. This is followed by firm adhesion, as the leukocytes bind tightly to the endothelial cells through the integrins on their surface. The leukocytes then undergo transmigration, across the endothelium and into the site of infection or injury. Finally, the leukocytes migrate toward the source of chemoattractants, such as bacterial or viral antigens, cytokines, or chemokines, to initiate the immune response.

The Integrin Family

The transmembrane cell adhesion molecules known as integrins are heterodimeric and composed of an alpha (α) and a beta (β) subunit organized in various dimeric combinations. Diverse extracellular ligands are more or less attractive to these complexes. Integrins are vital for various biological processes, including cell

proliferation, migration, apoptosis, tissue healing, inflammation, infection, and angiogenesis. These receptors are widely distributed and exist in various forms that can bind to numerous ligands. Integrins regulate cellular development, proliferation, migration, signaling, cytokine activation, and release. They play a critical role in maintaining homeostasis and signaling by facilitating interactions between cells and the (ECM).

In terms of ligand specificity, integrins can be divided into four groups: those that bind collagen (integrins α1β1, α2β1, α10β1, and α11β1), those that recognize the RGD (Arg-Gly-Asp) peptide motif (integrins α5β1, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, and αIIbβ3), those that bind laminin (α3β1, α6β1, α7β1, and α6β4), and those that are expressed exclusively on leukocytes (αLβ2, αMβ2, αXβ2, and αDβ2). Leukocyte function-associated antigen-1, Mac1/CR3 (macrophage-1 antigen, complement receptor 3), 150.95/CR4 (complement receptor 4), and CD18/CD11d, respectively, are formed when the β2 integrin subunit (CD18) pairs with one of the four subunits (αL, αM, αX, and αD). Most leukocytes express αL, while myeloid cells primarily express αM, αX, and αD [42].

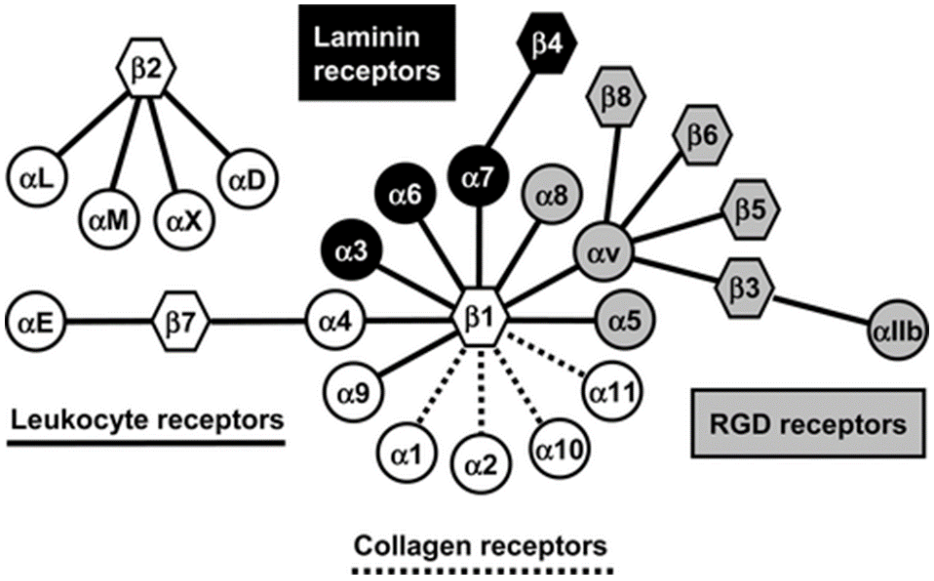


Figure 4: The Integrin Family. Integrins, a family of transmembrane proteins, are categorized into four groups based on their ligand specificity. The first group includes integrins α1β1, α2β1, α10β1, and α11β1, which recognize collagen. The second group includes integrins α5β1, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, and αIIbβ3, which recognize the RGD motif. The third group includes integrins α3β1, α6β1, α7β1, and α6β4, which bind laminin. The fourth group includes integrins αLβ2, αMβ2, αXβ2, and αDβ2, which are expressed exclusively on leukocytes.

Integrin Structure

The structures of integrins are critical for their proper function in normal physiology, including the maintenance of tissue and organ structure, developmental processes, and wound healing. Additionally, integrin-dependent adhesion complexes play a role in the development of diseases such as cancer. Evidence suggests that certain integrins interact with receptor tyrosine kinases (RTKs), leading to the activation

of signaling pathways necessary for tumor invasion and metastasis. Specifically, integrin signals facilitate cancer cell detachment from neighboring cells, reorientation of polarity during migration, and survival and proliferation in unfamiliar microenvironments[43]. Integrins are made up of numerous proteins that can be thought of as serving three different purposes: structure, signaling, and control of the integrin adhesion complexes. For example, Arf GTPase-activating proteins (Arf GAPs), members of the GTPase family, were discovered in this complex and carry out all three of these tasks, demonstrating the impact of their various roles in regulating the formation, maturation, and dissolution of integrin adhesion complexes[44].

The integrin family comprises 24 heterodimeric transmembrane adhesion receptors and cell-surface proteins, consisting of αβ pairs that form the structure. These non-covalent connections involve α and β subunits, and it is known that these pairs involve 18 α and 8 β subunits[45]. The integrin subunit αβ pairings control intracellular adhesion complex formation, control downstream signaling, and determine the specificity of the integrin to a given ligand. It is noteworthy that the first two integrins to be recognized, namely lymphocyte function-associated antigen 1 (integrin αLβ2) and macrophage antigen 1 (integrin αMβ2), obtain their specificity from specific α subunits, but they both share the same β subunit[46].

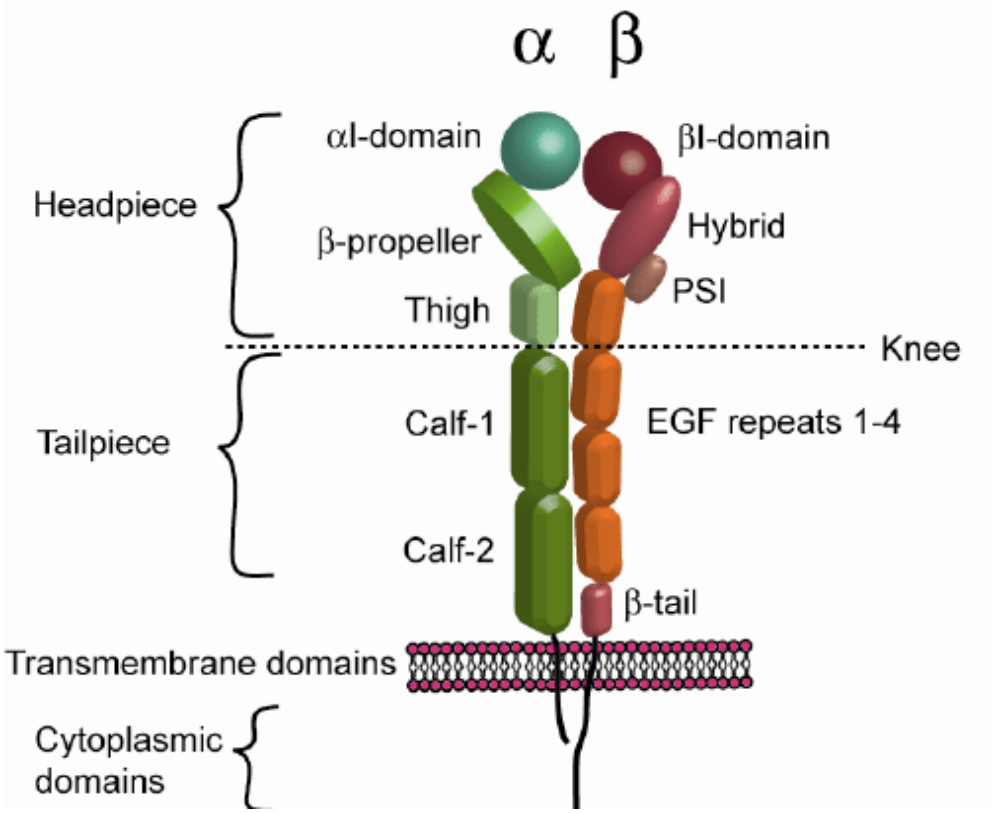


Figure 5: Integrin Structure. The integrin molecule consists of two subunits, α and β, each with a large extracellular domain that contains ligand-binding sites, a transmembrane domain, and a short cytoplasmic tail. The α and β subunits interact through noncovalent bonds to form a heterodimeric complex. The extracellular domain contains a head region composed of two subdomains, the β-propeller and the α/β I domains, and a leg region that connects the head to the transmembrane domain.

α Subunit. The α subunit of integrin consists of several domains that form the leg structure supporting the integrin head. This includes a seven-bladed propeller, a thigh, a calf-1 domain, and a calf-2 domain. The final few propeller blades at the lower side away from the ligand-binding surface have EF-hand domains that bind to Ca^{2+}. Notably, nine of the integrin α chains possess an I-domain, which is a 200 amino acid domain present in the integrin α subunits[47]. When present on an integrin, the I-domain is an

exclusive ligand-binding site. The αI-domain, found on integrins, serves as a unique ligand-binding site. Integrins containing the αI-domain interact with various ligands due to their 13 extracellular domains distributed across the two subunits.

β Subunit. The β subunit of integrin consists of several domains, including four cysteine-rich epidermal growth factor (EGF) repeats, a hybrid domain, a plexin-semaphorin-integrin (PSI) domain, and a βI-domain[48]. Apart from some variation found in the β4 tail, which has roughly 1000 amino acids, it generally contains a cytoplasmic tail that is 75 amino acids long[49]. Although the α subunit of each integrin determines ligand specificity, the β chain plays a crucial role in binding to acidic residues in intercellular adhesion molecules (ICAMs) and cytoplasmic adapters which promote cellular adhesion with the ECM. Integrins interact with the actin cytoskeleton through talin- and kindlin-binding motifs in the cytoplasmic regions of their subunits via these adapters[50].

However, there is a lengthy list of integrin ligands, and these include several of the main components of the ECM[51]. Integrins are expressed on cell surfaces, often with a bending, closed headpiece in a low-affinity conformation. Both extracellular and intracellular signaling can activate high-affinity binding and intracellular signal transduction. They are expressed in immune cells and participate in the molecular mechanisms of inflammatory reactions and diseases as a result of their wide range of functions[52].

Beta2 (β2) integrins

Leukocytes are the sole cells that express β2 integrins. The corresponding β2 integrin's specific functional characteristics are defined by the

corresponding α subunit, but functional redundancy is present in all β2 integrins. They coordinate the uptake of external substances including complement-opsonized pathogens, facilitate adherence to neighboring cells and to elements of the ECM, regulate cytoskeletal architecture, and regulate cell signaling[53]. They are also necessary for the movement of leukocytes and other immunological activities such as neutrophil phagocytosis, the formation of ROS, and the activation of T cells[54].

These integrins are activated by inflammatory cytokines during inflammation and encourage cellular adhesion to antagonistic receptors like ICAMs, which facilitate phagocytosis and cytotoxic death. Inflammation causes platelet antigens like glycoprotein Ibα (GPIbα) and integrin receptors on leukocytes like the macrophage-1 antigen (αMβ2) to interact with each other. Transforming growth factor (TGF)-β1's pro-domain is where integrins bind to activate and encourage the release of the protein. The transcription of modulatory family member MRFT-A (myocardin-related transcription factor-A) has been reported to be crucial in cardiac inflammation. Although its absence does not manifest any obvious cardiac problems in the null mice, they were protected from a variety of cardiovascular illnesses[55,56]. Studies by Weng et al (2015) and Liu et al (2018) also demonstrate that it controls inflammation and leads to ROS generation in macrophages[57,58], potentially by enlisting the KDM3A epigenetic machinery and stimulating the transcription of the integrin β2 to mediate macrophage adherence to the vascular endothelia, therefore boosting cardiac inflammation[59]. Furthermore, when a blocking antibody was employed, this effect was diminished. It was also shown that activated neutrophils release ROS that alter the ECM by forming CEP adducts, which act as migratory and sticky ligands for macrophage integrins, notably integrin β2.

Integrin αDβ2. The most recent member of the β2 subfamily to be identified is integrin αDβ2 (CD11d/CD18), which is notably ubiquitously expressed on inflammatory macrophages[60]. It, like the other β2 integrins, has a common β2 subunit and distinct, though homologous, α subunits. In comparison to the other members of the subfamily, it demonstrates multiligand binding properties, has superior recognition specificity, and enables cell adhesion to a variety of ECM proteins, including fibronectin, vitronectin, fibrinogen and CCN1 (Cyr61)[60,61]. One very important role of αDβ2 (CD11d/CD18) that was recently revealed in atherosclerosis and diabetes is its ability to cause pro-inflammatory macrophages to adhere strongly to the oxidatively damaged ECM, allowing them to remain at the site of persistent inflammation[62]. Although little is known about its role in monocyte and macrophage migration, investigations have revealed that it has a role in infection and inflammatory responses in a variety of clinical diseases[63].

DHA Oxidation during inflammation (CEP formation)

Docosahexaenoic acid (DHA) is an omega-3 (n-3) fatty acid with a lengthy acyl chain and a high level of unsaturation. Its acyl chain contains 22 carbons and six double bonds giving its unique physical and functional properties[64]. It is crucial for infant brain growth and functional development. For adult brains to continue functioning normally, DHA is also necessary. DHA deficiency is linked to learning difficulties, whereas consuming plenty of DHA in the diet boosts learning capacity[65]. It is found in cold water and fatty fish, such as salmon and tuna. Along with eicosapentaenoic acid (EPA), it is also present in fish oil supplements. They also play significant roles in human health and disease by acting as ligands for a variety of receptors, lipid mediator precursors, and membrane glycerophospholipid components (GPLs)[66]. Polyunsaturated fatty acids

(PUFA) may be oxidized by reactive oxygen species produced at the site of inflammation, which may result in ECM protein modifications that are macrophage-specific ligands that lead to enhanced inflammation. They are widely available as oxidation substrates and are found in both food sources and cellular membranes. Studies have demonstrated that the oxidation of PUFAs results in a variety of biological activities through its released end products[66,67].

Recently, it was shown that 2-(ω-carboxyethyl) pyrrole (CEP), the by-product of DHA oxidation, is a specific ligand for integrins αDβ2 and αMβ2 at the site of chronic inflammation. This oxidation leads to the generation of CEP adducts with neighboring (ECM) proteins[68]. These CEP-modified proteins aid in the migration of macrophages mediated by the αMβ2- and αDβ2 receptors to the location of the inflammation. CEP is predominantly produced during inflammation, and in individuals with diabetes, it is widely present in adipose tissue and atherosclerotic plaques[69]. Therefore, it was anticipated that CEP could serve as a vital ligand for the retention of αDβ2-mediated macrophages at the site of inflammation, given the αDβ2-specific expression pattern on M1 macrophages. According to a recent theory put forth by Yakubenko et al., during the first wave of inflammation, the oxidative damage produced by neutrophils greatly contributes to the synthesis of CEP and its addition to nearby extracellular matrix components.

How therefore can these contacts be broken down in order to provide a therapeutic effect and accelerate resolution for inflammation, given how much of this αDβ2-CEP-mediated macrophage interaction contributes to inflammation? Research was initiated to find particular inhibitors of the αDβ2-CEP interaction that can reduce the

retention of macrophages during inflammation. Using a specially created peptide library, a sequence (known as P5 peptide) was found. This P5 peptide effectively and precisely inhibited αD–CEP binding. P5 peptide was administered to WT mice on a high-fat diet. It blocked the build-up of macrophages in adipose tissue in a way that was αDβ2-dependent[70].

The group of 2-(ω-carboxyalkyl) pyrrole adducts known as CAP protein adducts are generated via the oxidation of polyunsaturated fatty acids (PUFA)[71]. As such, linoleic acid or arachidonic acid undergoes oxidative fragmentation, producing 9-hydroxy-12-oxododec-10-enoic acid (HODA) or 5-hydroxy-8-oxooct-6-enoic acid (HOOA), respectively. These acids subsequently react with protein to form 2-(ω-carboxyheptyl) pyrrole (CHP) or 2-(ω-carboxypropyl) pyrrole (CPP) adducts. Since these protein adducts can also form from other commonly occurring PUFAs, the presence of CPP or CHP does not exclusively indicate the oxidative damage caused by linoleic, arachidonic, or their esters. However, only DHA generates 2-(ω-carboxyethyl) pyrrole (CEP) adducts by the oxidative cleavage of 4-hydroxy-7-oxohept-5-enoic acid (HOHA), followed by the interaction of this acid with protein. HOHA-PC is a minor oxidative product of DHA-PC that can create peptide-bound CEP adducts, as previously demonstrated[72].

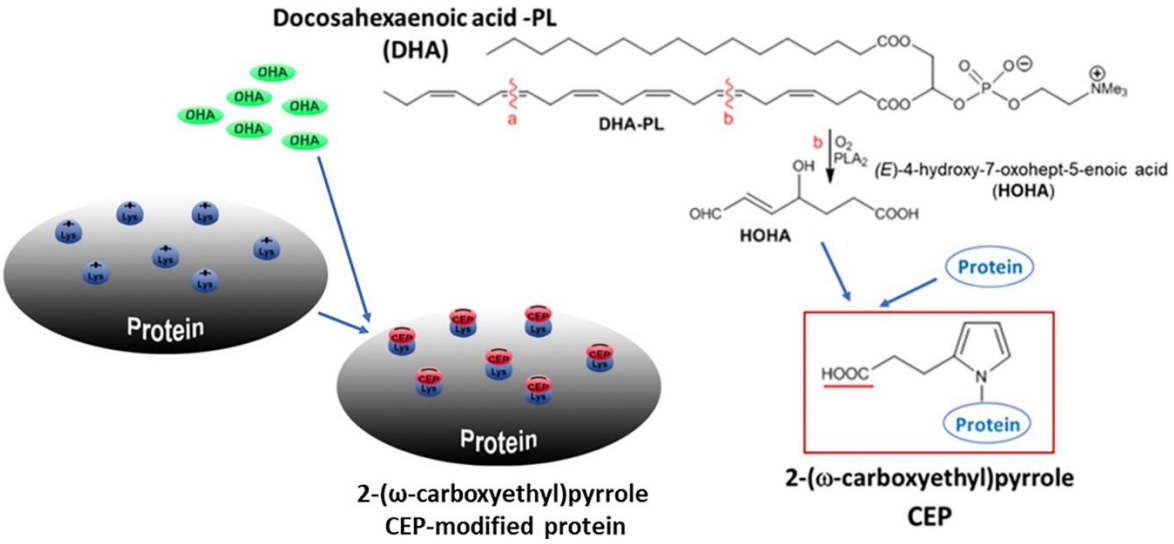


Figure 6: Schematic representation of CEP formation. Reprinted with permission from Casteel et al. The figure depicts the formation of CEP (2-(ω-carboxyethyl) pyrrole) from 4-hydroxy-2-hexenal (HOHA) through a series of reactions. HOHA is generated from the oxidation of omega-6 polyunsaturated fatty acids (PUFAs) and reacts with primary amines to form Michael adducts. These adducts undergo further oxidation and cyclization reactions to form CEP.

Role of CEP-$\alpha_D\beta_2$ Interaction in inflammation

These small by-product CEP molecules have been linked to the stimulation of angiogenesis under pathological circumstances such as during tissue ischemia. Although they are diversely associated with inflammation, developing proangiogenic treatments or medications may be possible using these protein adducts[73].

As was previously mentioned, CEP production is significantly increased in inflamed tissue, and CEP-modified albumin and fibrinogen were found to bind to the αDβ2 and αMβ2 integrins. Can these CEP-modified ECM proteins have a significant impact on the adhesion abilities of M1-polarized macrophages during chronic inflammation? Casteel et al. (2022) used in vitro oxidation assays and digested atherosclerotic lesions in a

study. They showed that CEP created by DHA oxidation formed adducts with ECM proteins such as collagen IV, fibrinogen, and laminin. Collagen I was the one exception. But what functional distinctions exist between fibrinogen altered by the CEP ligand from DHA oxidation and the normal unmodified fibrinogen that would alter the interaction with the integrin αDβ2? The same study discovered that CEP-modified proteins facilitated higher cell adhesion and spreading when compared to native ECM ligands like collagen IV, laminin, and fibrinogen. Additionally, compared to the native fibrinogen ligand, the isolated αI-domain had a greater binding affinity to CEP. By switching a positively charged lysine to a negatively charged CEP, CEP modification transforms ECM proteins into αD-recognition ligands. This boosts M1 macrophage adherence to ECM and encourages macrophage retention during harmful inflammation, autoimmunity, and chronic inflammation[74].

Inflammation-related changes in the ECM frequently involve fibrinogen, a natural integrin αDβ2 ligand. It has been demonstrated that CEP alteration dramatically enhances fibrinogen's ability to adhere to αDβ2 -HEK293 transfected cells. Different proteins that have undergone CEP alteration have enhanced cell adhesion capabilities. However, prior research has indicated that for fibrinogen, cell adhesion can only be accomplished at a maximum concentration of 2-3 g/ml and that it is greatly diminished at greater concentrations[75]. The difference in the binding of integrin αD to CEP-modified and unaltered fibrinogen was assessed using biolayer interferometry. It was discovered that the αD interaction with unmodified fibrinogen was weaker than the αD binding to CEP-modified fibrinogen by a factor of two. Due to a larger affinity caused by a negative charge on a carboxyl group in the structure of CEP, αDβ2 produces a stronger adhesion

to proteins that have been modified by CEP. According to this finding, CEP-modified ECM proteins play an important role in macrophage adherence by producing robust αDβ2 -dependent adhesion via CEP, which may be essential for macrophage retention[75].

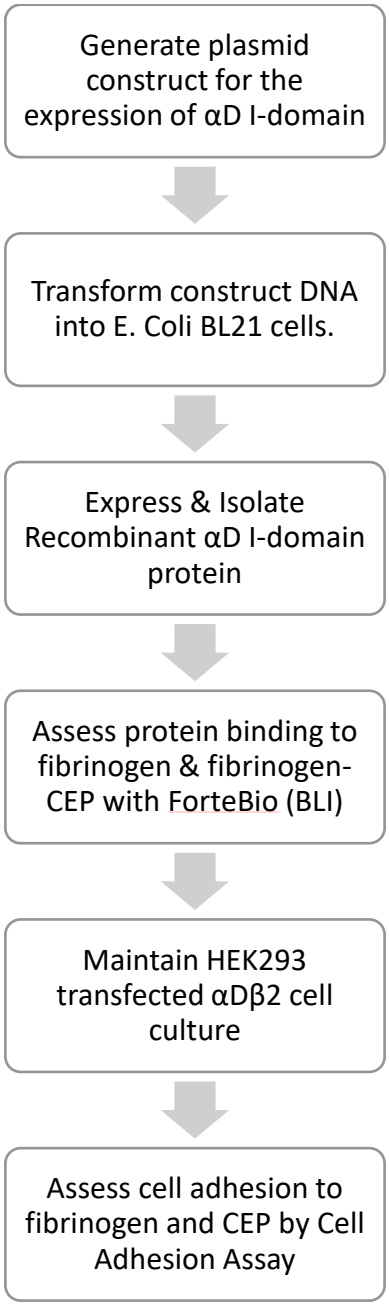
Aims and Hypothesis

The goal of this project is to examine and compare the binding properties of αDβ2 integrin to fibrinogen, a native ECM protein, as well as fibrinogen that was CEP-modified. This comparison will assist in further clarifying the role of the CEP product from DHA oxidation in chronic inflammation and inflammatory diseases.

We hypothesize that the interaction between integrin αDβ2 and fibrinogen-CEP, fibrinogen modified by the DHA oxidation product, will be stronger than its interaction with unmodified fibrinogen and that this increased binding may contribute to macrophage retention at sites of inflammation.

CHAPTER 2. EXPERIMENTAL PROCEDURES

Experimental Design Workflow



Protein Expression

Plasmid Construct

The Pet-28a expression vector was utilized to enable the insertion of the DNA sequence for the active form of the I-domain of recombinant human integrin αDβ2 at the NdeI and XhoI restriction sites. Transcription was carried out by the T7 polymerase binding the T7 promoter present in the expression vector. The expression of the recombinant αD I-domain sequence was controlled by the lac repressor (IPTG-inducible), and resistance to the antibiotic kanamycin was provided by a gene in the vector. The desired protein was isolated and purified by growing the *E. coli* cells that had taken up the vector and expressed the recombinant protein in the presence of kanamycin.

DNA Isolation

Qiagen's QIAprep Spin Miniprep Kit (Catalog # 27104) was utilized to isolate the DNA. Firstly, an overnight culture of *E. coli* was grown in a 37^0C shaker, after which the culture was subjected to centrifugation at room temperature for 3 minutes at 8,000 rpm to spin out the bacterial pellet. The pellet was resuspended in 250 µL of buffer P1 and mixed with 250 µL of buffer P2. Thereafter, 350 µL of buffer N3 was added to the suspension, and it was resuspended. The suspension was then centrifuged for 10 minutes at 13,000 rpm. The supernatant was collected and transferred to a QIAprep 2.0 spin column, and it was then spun for 1 minute. The column was washed with 750 µL of buffer PE, spun again for 1 minute, and then spun for another 1 minute to remove any remaining wash buffer. The spin column was then moved to a new microcentrifuge tube,

and 50 µL of buffer EB was added to elute the DNA. The column was left to stand for one minute and then centrifuged for another 1 minute. Finally, the concentration of the DNA was measured using a Synergy H1 hybrid multi-mode microplate reader.

BL21 Cell Transformation

The transformation of BL21(DE3) competent cells was carried out following the instructions provided by Agilent Technologies (Catalog #200133). Initially, the BL21 competent cells were thawed on ice for 30 minutes, and then 50 ng of ligated αDβ2 DNA was added to the cells. The reaction was left on ice for 30 minutes. While waiting, the SOC medium was heated in a 37 ^{0}C water bath. The transformation reaction was briefly heated in a 42 ^{0}C water bath for 45 seconds and then cooled on ice for 2 minutes. The preheated SOC medium was added to the reaction and incubated at 37 ^{0}C for 1 hour with shaking at 225 rpm. The cells were then plated onto an LB agar containing 100 µg/mL kanamycin using a sterile spreader. The plate was incubated overnight at 37 ^{0}C to allow for colony formation.

Bacteria Cell Culture

From an LB agar plate that had been incubated overnight, a single colony was selected. The colony was transferred to a centrifuge tube containing 3 mL of TB media with 100 µg/mL kanamycin (0.1%). The cells were then grown for 7-9 hours at 37 ^{0}C with intensive shaking at 225 rpm. Following this, 100 µL of the culture was used to inoculate 100 mL of TB media with 100 µg/mL kanamycin, and the cells were cultured overnight at 37 ^{0}C with shaking at 225 rpm. The 100 mL culture grown overnight was then scaled up to 2 liters by adding 1.9 L of autoclaved TB media with 100 µg/mL kanamycin. The 2-liter culture was then equally divided into 4 flasks using sterile

techniques. Finally, the 4 flasks were cultured at 37 ^{0}C with shaking for 1.5 – 2 hours until the optical density reached 0.9 – 1.0, indicating the optimal growth condition of the cells.

IPTG-Induced Protein Expression

The initiation of recombinant protein αDβ2 expression was carried out by adding isopropyl β-d-thiogalactopyranoside (IPTG) to the bacterial cell culture, with a final concentration of 1 mM. This was achieved by adding 0.5 mL of IPTG to each of the 4 flasks, each containing 500 mL of cell culture. IPTG was added to bind to the lac operon's repressor protein, causing the release of control from the T7 promoter, and allowing for the expression of the recombinant protein. The culture was then grown for an additional 4 hours at 25 ^{0}C with shaking at 225 rpm. The culture was then transferred to 4 different 500 mL tubes for the next step of harvesting.

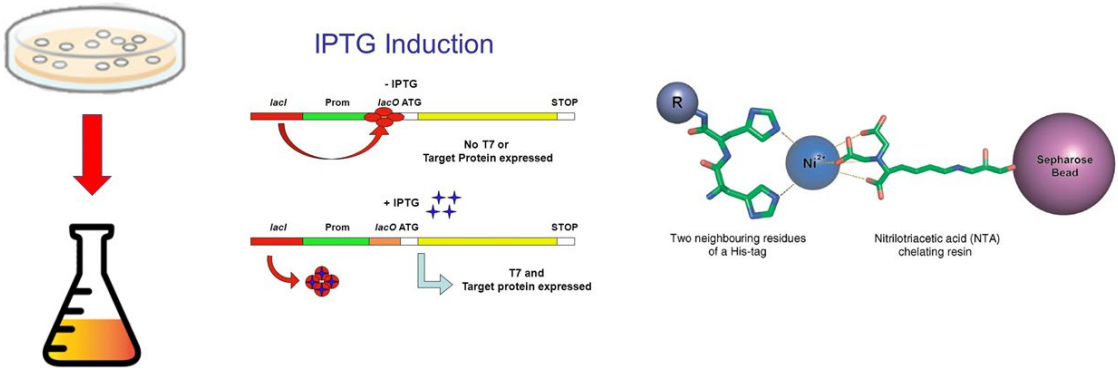


Figure 7: Protein expression from bacterial culture. This figure shows the process of protein expression from bacterial culture. The bacterial cells were initially grown in

culture, and the expression of the recombinant protein was initiated by adding IPTG to the culture at a final concentration of 1 mM. The lysed cell was then purified using Ni-NTA column affinity chromatography to isolate the recombinant protein of interest. The purity and quantity of the purified protein were then analyzed using SDS-PAGE and BCA assay, respectively.

αD I-domain Protein Isolation

Bacterial Cell Lysis by Sonication

The harvesting of cells was carried out by centrifugation for 20 minutes at a temperature of 42 ^{0}C and a speed of 6,000 rpm. The centrifugation process resulted in the cells being collected in the form of a pellet, and the supernatant was discarded. The bacterial cell pellet was then resuspended in PBS buffer containing 1% Triton X-100 to break down the cell membrane and release the intracellular components. The bacterial cell pellet lysate was then frozen overnight at -80 ^{0}C. The following day, the cell was thawed using a water bath, and the cell lysate was placed on ice and sonicated three times to disrupt the chromatin. The tube was allowed to rest for 2 minutes in between each sonication. The sonicated lysate was then subjected to centrifugation at 15000 rpm at 42 ^{0}C for 30 minutes. The supernatant, which contained the αD I-domain protein, was filtered through a 0.8 µm syringe, and the pellet was discarded. This step was necessary to remove any remaining cell debris or unbroken cells and to purify the recombinant protein of interest.

Protein Isolation by Ni-NTA Column Chromatography

The isolation of the αD I-domain protein was accomplished by utilizing Ni-NTA Column Affinity Chromatography. This technique utilizes a column filled with Ni-NTA

agarose beads specifically designed to bind to histidine tags, commonly used as protein purification tags. The first step involved stabilizing the protein and blocking non-specific binding by adding imidazole and NaCl to the supernatant, reaching a final concentration of 10 mM imidazole and 300 mM NaCl. After that, the protein-containing supernatant was mixed with HisPur Ni-NTA Superflow Agarose (Thermo Scientific) at 4 °C for 1 hour while being gently shaken. The mixture was then centrifuged at 4°C and 1200 rpm for 10 minutes, and the bottom layer containing the protein bound to Ni-NTA agarose was collected and transferred to a column (Bio-Rad Laboratories) for isolation. The column was washed with increasing concentrations of imidazole 20mM, 60mM, and 80mM until the eluent reached an optical density of less than 0.1, 0.05, and 0.01, respectively. The protein was then eluted with 200mM imidazole and collected into 5 separate 1 mL samples. The protein concentration in each sample was measured using a spectrometer (BioTek Instruments). The samples with the highest protein concentration were collected for further purification by dialysis. This method efficiently purified the target protein while effectively removing any impurities or unwanted proteins.

Protein Dialysis

The purified protein samples were combined and placed into Fisherbrand dialysis tubing (# 21-152-10). Dialysis is a process used to remove small molecules and ions to refine the protein sample. The proteins were dialyzed in three stages. Initially, they were dialyzed in 500 mL PBS buffer with 1M NaCl at 4 °C for 30 minutes with mild shaking. This step is known as buffer exchange and helps to remove any remaining impurities from the previous buffer while also bringing the protein solution to the best physiological condition. Next, the proteins were moved to 500 mL PBS buffer with 500 mM NaCl at 4

°C and left overnight with mild agitation. The following morning, the proteins were dialyzed in 500 mL of 20mM Hepes buffer with 150mM NaCl at 4 °C for 4 hours with mild agitation. The final buffer was chosen based on the protein's biological activity and stability. In this case, the 20 mM Hepes buffer with 150 mM NaCl is a commonly used buffer for protein storage and downstream applications. The protein was collected and aliquoted in 200 µL, and the protein concentration was measured using a spectrophotometer (Synergy H1 hybrid multi-mode microplate reader, BioTek Instruments).

Protein Analysis

Protein Quantification by SDS-PAGE

The protein samples of interest were prepared by mixing them with SDS sample buffer (24 µL sample, 6 µL SDS sample buffer) and heating the mixture at 95 °C for 5 minutes to denature the proteins. The protein standard was loaded onto a separate lane of the SDS-PAGE gel, and the gel was run using the SDS-PAGE running buffer according to standard protocols. The gel was stained with Coomassie blue staining solution for 30 minutes to visualize the separated protein bands. The gel was then destained with a destaining solution to remove excess Coomassie blue. Finally, the gel was imaged using a gel imaging system (G Box). The intensity of the protein bands from the samples of interest was compared to the protein standard curve to determine the concentration of each protein in the samples. The accuracy of protein quantification using SDS-PAGE was affected by various factors, including the efficiency of protein extraction, the quality of the sample preparation, and the variability in protein binding to the Coomassie blue dye.

Protein Quantification by Bradford Assay

The protein quantification method using the Bradford assay was carried out. Protein samples were used to quantify protein concentrations by comparing the absorbance values to a standard curve generated from known concentrations of BSA (bovine serum albumin). The Bradford reagent was used to bind to the protein in the samples and produce a color change that was measured using a spectrophotometer. The protein samples were prepared by diluting them to a suitable concentration, and aliquots of the diluted samples were transferred to a 96-well microplate. Standards of known BSA concentrations were also prepared in the same way. The Bradford reagent was added to each well of the microplate, and the plate was incubated at room temperature for 10 minutes.

The absorbance values of the protein samples and standards were measured using a spectrophotometer at a wavelength of 595 nm. The absorbance values were then compared to the standard curve to determine the protein concentration in each sample. The accuracy of protein quantification using the Bradford assay was affected by various factors, including the quality of the Bradford reagent, the accuracy of the spectrophotometer measurements, and the interference from other substances in the samples.

Kinetic Characterization of Protein Interactions by Biolayer Interferometry

The ForteBio K2 Octet System was used to analyze the binding interactions of wild-type αD I-domain protein to several ligands through surface plasmon resonance assays.

Biolayer Interferometry Assay

The interactions between proteins and various ligands were examined using The Amine Reactive (ARG2) ForteBio biosensor. The biosensors were prepared by removing the protective coating through immersion in a buffer solution containing 150 mM Nacl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.02% Tween. Activation of the biosensors was achieved by utilizing N-hydroxysulfosuccinimide (NHS) and (EDC) 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide. The optimal pH for each ligand was determined, and the ligands were then immobilized on the activated biosensor surface. Any remaining unreacted sites on the biosensor were quenched with 1 M ethanolamine. The experiment was performed according to the manufacturer's guidelines, where the biosensor was first immersed in water for 10 minutes and then in a buffer solution containing HEPES, NaCl, $MgCl_2$, and $CaCl_2$. The tested proteins were diluted to appropriate levels in a buffer solution with HEPES, NaCl, $CaCl_2$, $MgCl_2$, and Tween 20 detergent. The data were recorded and analyzed.

Cell Adhesion

Cell Culture

The αDβ2-transfected human embryonic kidney 293 cells were cultured in DMEM-F12 medium, supplemented with 10% FBS, 2 mM glutamine, 15 mM HEPES, 0.1 mg/mL streptomycin, and 0.1 unit/mL penicillin. The cells were maintained in a humidified incubator at 37 °C with 5% CO_2. The culture medium was changed second day. The cells were passaged once they reached 80% confluency. To passage the cells, the culture medium was removed, and the cells were washed with PBS. The cells

were then detached using cell dissociation buffer (Gibco) and resuspended in a fresh medium.

Cell Adhesion Assay

100 ul of fibrinogen-CEP and fibrinogen were coated onto 96-well plates (Immulon, 4HBX, Cambridge, MA) and incubated for 3 hours at 37 °C. Subsequently, the wells were blocked for non-specific binding by post-coating with 200 µL of 0.5% Polyvinylpyrrolidone (PVP) for 1 hour at 37 °C. HEK293 cells that had been transfected and were in DMEM/F-12 were collected, counted, and stained using 10 mM calcein and incubated for 30 minutes at 37 °C. The cells were then washed with PBS and centrifuged at 12,000 rpm for 8 minutes. The supernatant was discarded while the pellet containing the cells was resuspended and diluted to $1x10^6$/mL in HBSS. The plate was washed three times using PBS and then 50 µL of the cell mixture was added to each well and subsequently incubated for 30 minutes at 37 °C. An additional step was added for the polyglutamic acid inhibition of αDβ2 adhesion to fibrinogen and fibrinogen-CEP. Varying concentrations of the polyglutamic acid were added to the cells and incubated for 15 minutes at 37 °C. The fluorescence was measured using a Synergy H1 microplate reader The cells were then washed again by adding 200 µL of PBS to each well. The fluorescence was measured again and the data were used to determine the number of adherent cells to each ligand.

CHAPTER 3. RESULTS

Protein Isolation and Purification

To obtain a high yield of the protein, it is important to find the optimal conditions for its expression. To achieve this, an experiment was conducted to optimize the expression of αD I-domain protein using different concentrations of IPTG, temperature, and incubation time. The E. coli BL21(DE3) strain containing the plasmid for αD I-domain protein was used for this experiment. Eight different conditions were tested, including 0.5 mM IPTG at 25 ^{0}C with 4 hours of shaking, 0.5 mM IPTG at 25 ^{0}C with overnight shaking, 1 mM IPTG at 25 ^{0}C with 4 hours of shaking, 1 mM IPTG at 25 ^{0}C with overnight shaking, 0.5 mM IPTG at 37 °C with 4 hours shaking, 0.5 mM IPTG at 37 °C with overnight shaking, 1 mM IPTG at 37 °C with 4 hours shaking, and 1 mM IPTG at 37°C with overnight shaking.

For each condition, a 5 mL culture of *E. coli* was grown in LB media supplemented with the appropriate concentration of IPTG. The cultures were incubated at the designated temperature with either 4 hours or overnight shaking. After incubation, the cells were harvested and lysed using a lysis buffer. The lysate was then centrifuged to remove cell debris, and the supernatant was collected for SDS-PAGE analysis.

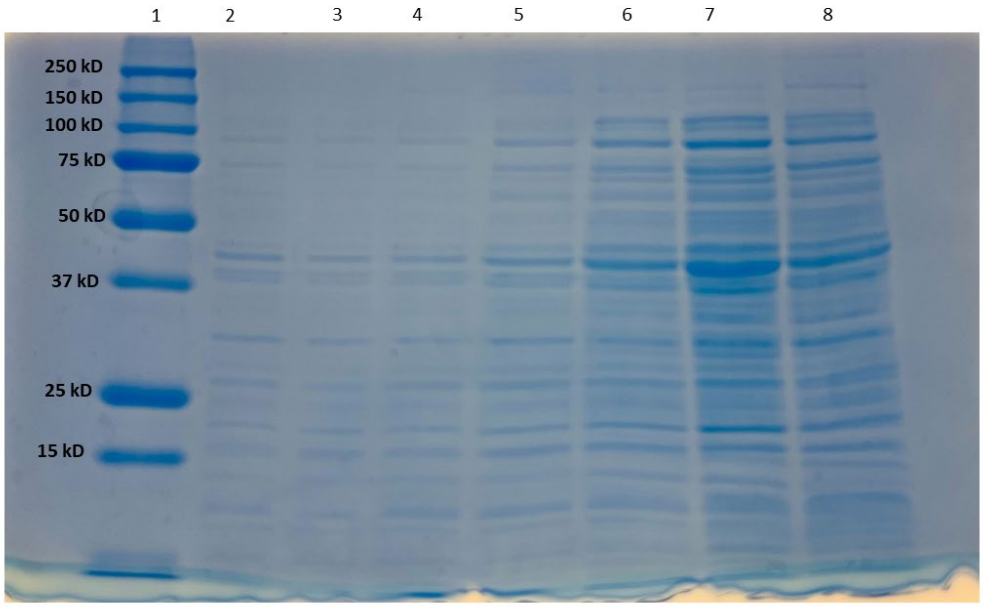


Figure 8: SDS-PAGE: αDβ2 I-domain Protein Expression under different conditions. Lane 1: molecular weight marker, Lane 2: 0.5 mM IPTG at 37 °C with 4 hours shaking, Lane 3: 0.5 mM IPTG at 37°C with overnight shaking, Lane 4: 1 mM IPTG at 37 °C with 4 hours shaking, Lane 5: 1 mM IPTG at 37 °C with overnight shaking, Lane 6: 0.5 mM IPTG at 25 °C with overnight shaking, Lane 7: 0.5 mM IPTG at 25 °C with 4 hours shaking, Lane 8: 1 mM IPTG at 25 °C with 4 hours shaking.

All eight conditions were tested, and the results showed that 1 mM IPTG at 25°C with 4 hours of shaking gave the best expression of the αD I-domain protein. The protein was observed at approximately 21 kDa, which is the expected size of the protein.

A plasmid construct containing the active form of integrin αD I-domain was created by inserting it into the Pet-28a vector. This construct was then used to transform BL21 *E.coli* cells, which were grown and scaled up to 2 liters before protein expression was

induced using IPTG. Ni-NTA affinity column chromatography (Ni-chelating agarose resin) was used to isolate and purify the expressed protein. The protein was further purified using 3-phase buffer dialysis. The final phase ensured the protein was in the appropriate buffer for protein-protein binding assays to test its interactions with fibrinogen and CEP-modified fibrinogen. The purity and quality of the purified protein were evaluated using 15% acrylamide electrophoresis gel, as depicted in Figure 1.

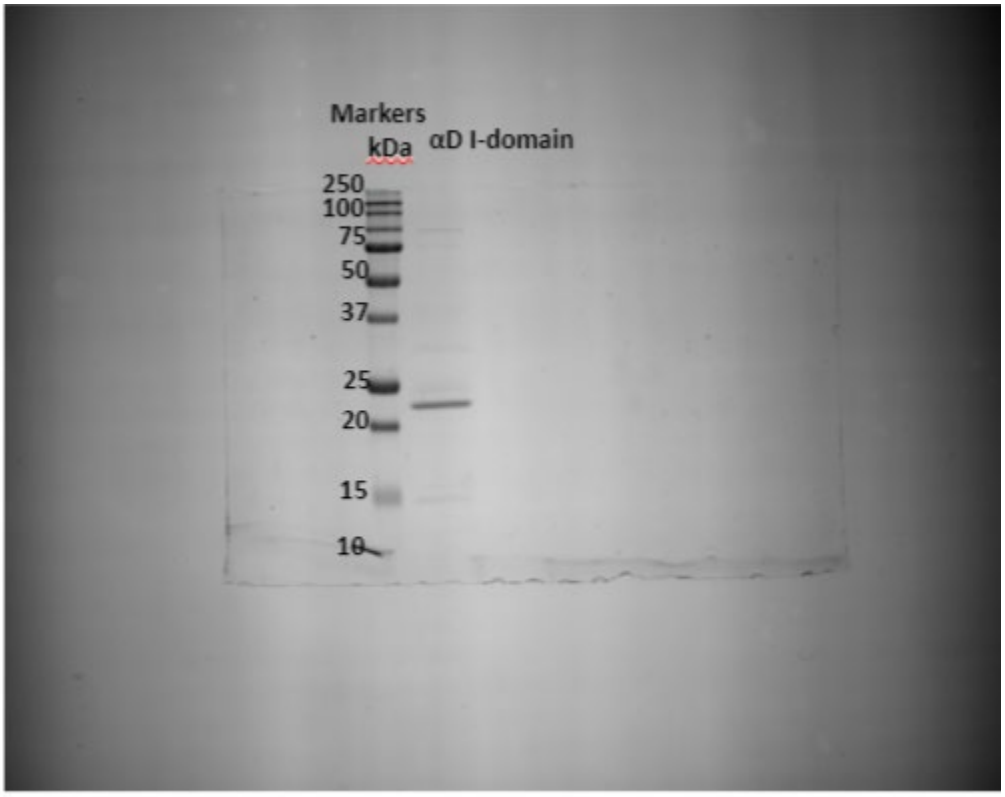


Figure 9: SDS-PAGE analysis of purified wild type αD I-domain protein.

The concentration of the purified protein was determined to be 135.2 µg/mL with Bradford assay, as shown below.

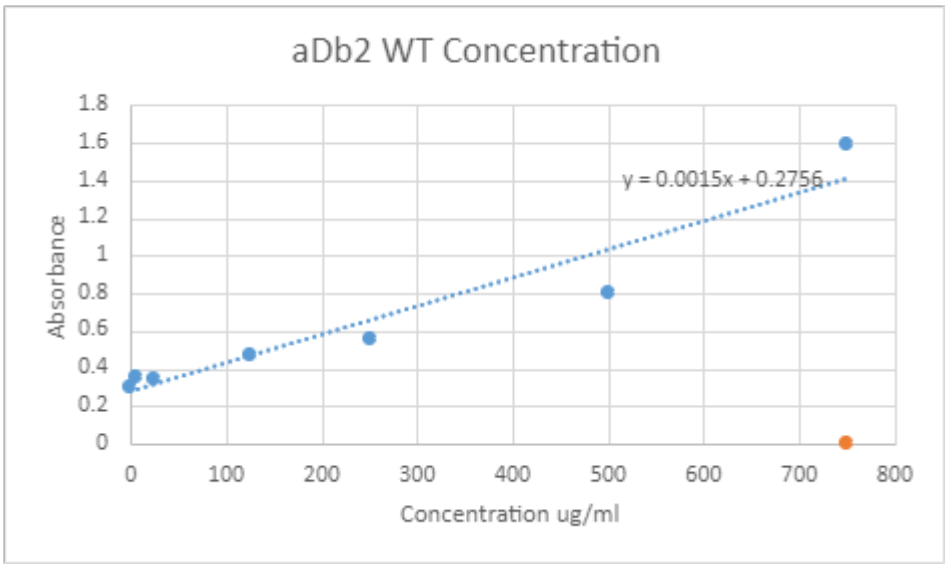


Figure 10: Bradford Assay (BCA) graph for determining αD I-domain protein concentration.

The absorbance of the dye-protein complex is measured, and the concentration of protein in the sample was determined by comparing the absorbance to a standard curve of known protein concentrations from the BCA kit.

αDβ2 Purified I-Domain Demonstrated Stronger Binding to Fibrinogen-CEP than Fibrinogen

The binding interaction between the expressed I-domain protein to natural ligand fibrinogen and CEP-modified fibrinogen was assayed at four different concentrations, 17.5 nM, 64.4 nM, 161 nM, and 320 nM, using the ForteBio Octet K2 bio-layer interferometry (BLI) system. Biotinylated fibrinogen and fibrinogen-CEP were immobilized at pH 5.0 and pH 3.5 respectively. The kinetic protein-protein interaction between αD I-domain and the two ligands was measured in real-time.

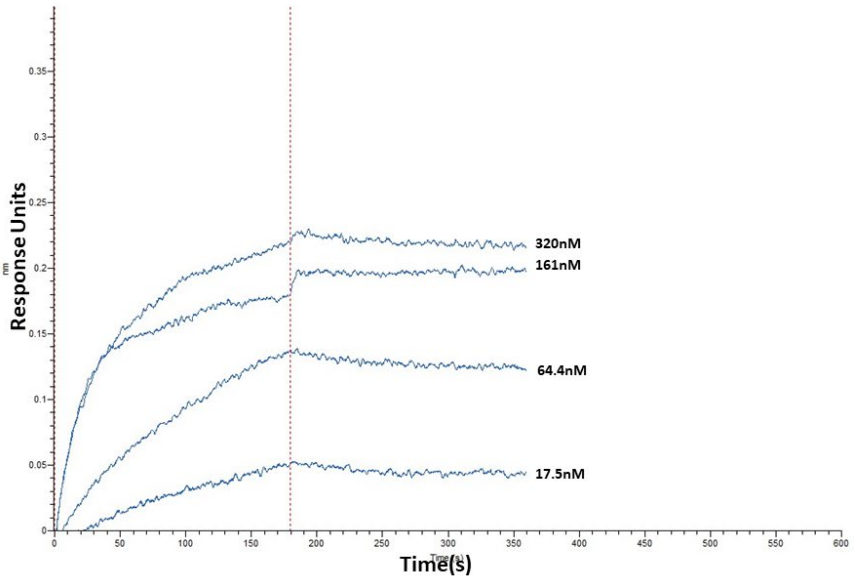


Figure 11: αD I-Domain Binding to Fibrinogen. A representative ForteBio Octet protein-protein assay sensorgram was generated to analyze the interaction between αDβ2 I-domain and fibrinogen, which served as the immobilized ligand on a streptavidin biosensor. The sensorgram showed that the association of the I-domain with fibrinogen occurred from Time 0 to 180 seconds, followed by dissociation of the complex until 360 seconds.

As shown in figure above, the ForteBio Octet protein-protein interaction experiment for αDβ2 and fibrinogen indicate that the purified αD I-domain exhibited concentration-dependent binding to immobilized fibrinogen at pH 5.0. Specifically, the response units increased with increasing concentrations of αD I-domain, with values of 0.04, 0.13, 0.18, and 0.23 observed for concentrations of 17.5 nM, 64.4 nM, 161 nM, and 320 nM, respectively.

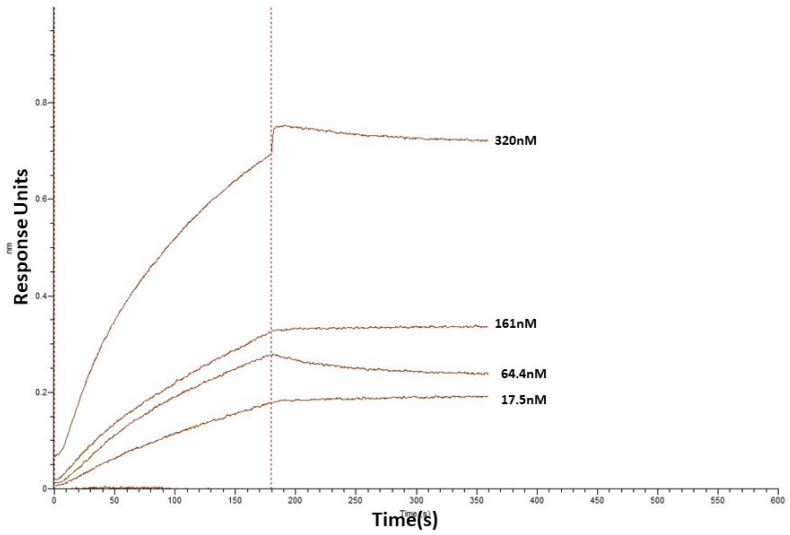


Figure 12: αDβ2 I-domain binding to Fibrinogen-CEP. A representative ForteBio Octet protein-protein assay sensorgram was generated to analyze the interaction between αD I-domain and fibrinogen-CEP, which served as the immobilized ligand on a streptavidin biosensor. The sensorgram showed that the association of the I-domain with fibrinogen-CEP occurred from Time 0 to 180 seconds, followed by dissociation of the complex until 360 seconds.

As shown above, the purified αD I-domain exhibited concentration-dependent binding to immobilized fibrinogen-CEP at pH 3.5. The response units increased with increasing concentrations of αD I-domain, with values of 0.16, 0.26, 0.33, and 0.69 observed for concentrations of 17.5 nM, 64.4 nM, 161 nM, and 320 nM, respectively.

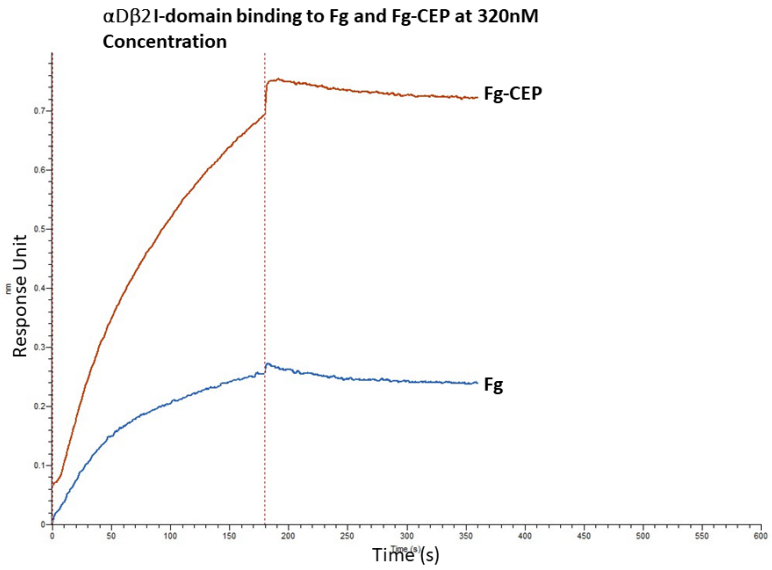


Figure 13: αD I-domain binding to fibrinogen and fibrinogen-CEP at 320 nM concentration. The sensorgram showed that the association of the I-domain with fibrinogen and fibrinogen-CEP occurred from Time 0 to 180 seconds, followed by dissociation of the complex until 360 seconds.

The results of the graph comparing αD I-domain binding to fibrinogen and fibrinogen-CEP at the highest concentration tested (320 nM) demonstrate that αDb2 I-domain exhibits stronger binding to fibrinogen-CEP than to fibrinogen. Specifically, the response unit value for fibrinogen was 0.23, while the response unit value for fibrinogen-CEP was 0.69.

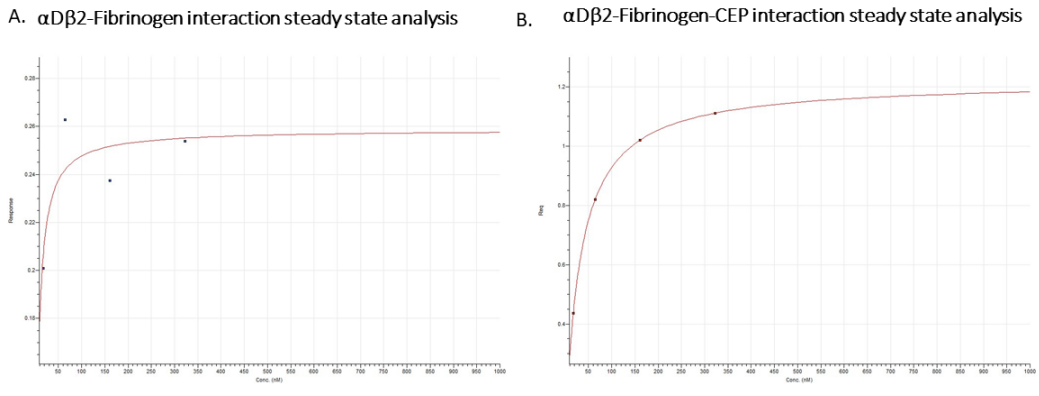


Figure 14: Steady state analysis of the interaction between αDβ2 and (A) fibrinogen or (B) fibrinogen-CEP.

Based on the steady state analysis data provided, the interaction of αD I-domain with fibrinogen and fibrinogen-CEP was compared. A global fitting 1:1 model was used to obtain the results. For the interaction of αD I-domain with fibrinogen, the chi-square value was 0.0023, the R square value was 0.8536, the R max value was 0.2326, and the dissociation constant (Kd(M)) value was 4.6×10^{-8} M while the interaction of αD I-domain with fibrinogen-CEP, the chi-square value was 0.0065, the R square value was 0.6716, the R max value was 0.3022, and the dissociation constant (Kd(M)) value was 1.7×10^{-8} M. This suggest that the interaction of αDβ2 I-domain with fibrinogen-CEP is stronger than with fibrinogen. This is indicated by the lower Kd value (1.7×10^{-8} M) for fibrinogen-CEP compared to fibrinogen (4.6×10^{-8} M). Additionally, the R max value for fibrinogen-CEP (0.3022) was higher than for fibrinogen (0.2326), indicating a higher binding capacity.

Anti-CEP antibody dramatically reduced the binding of αDβ2 I-domain to fibrinogen-CEP

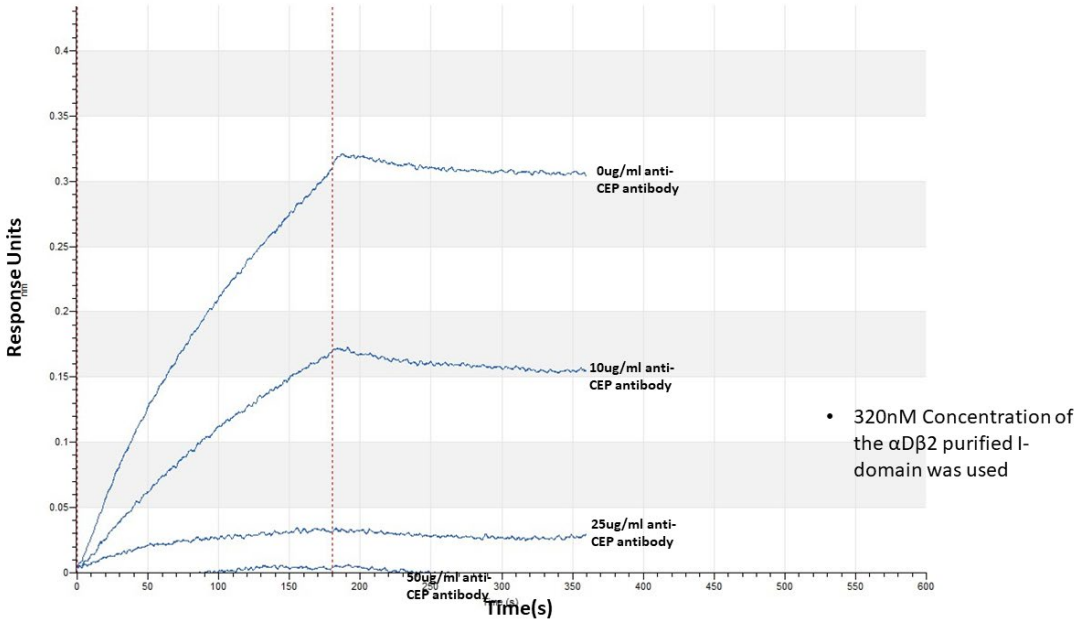


Figure 15. Inhibition of αD I-domain binding to fibrinogen-CEP by anti-CEP antibody. The figure depicts the inhibitory effect of anti-CEP antibody on the binding of αDβ2 I-domain to fibrinogen-CEP. The experiment was performed by pre-incubating different concentrations of anti-CEP antibody (0 µg/mL, 10 µg/mL, 25 µg/mL, and 50 µg/mL) with fibrinogen-CEP coated biosensors before exposing them to αDβ2 I-domain at a concentration of 320 nM. The binding response was measured using BLI and compared to the binding response of αD I-domain to fibrinogen-CEP without pre-incubation with the antibody. The results show that the binding of αD I-domain to fibrinogen-CEP is significantly inhibited by the presence of anti-CEP antibody in a concentration-dependent manner. The maximum inhibition was observed at the highest concentration of the antibody (50 µg/mL) which completely inhibited the association.

αDβ2-Transfected HEK293 cells demonstrated higher adhesion to fibrinogen-CEP than fibrinogen

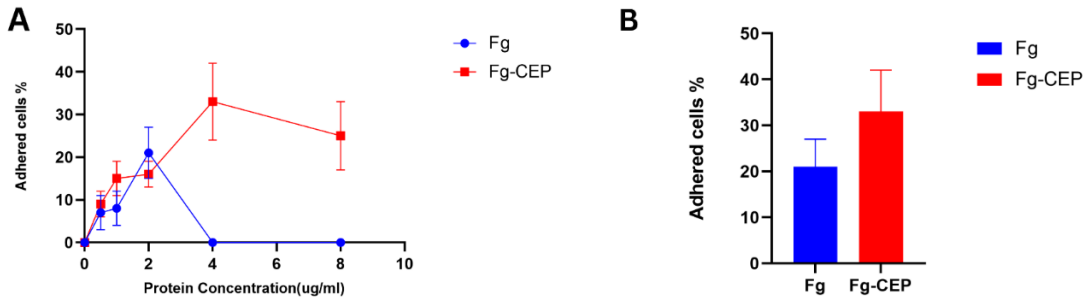


Figure 16:Adhesion of αDβ2-transfected HEK293 cells to fibrinogen and fibrinogen-CEP(A) at optimal concentrations (B). The adhesion assay was performed three times and the results were plotted as mean values ± standard deviation.

The concentrations of ligands used were 1 μg/mL, 2 μg/mL, 4 μg/mL, and 8 μg/mL. The adhesion of αDβ2 to fibrinogen-CEP was found to be stronger than fibrinogen in a dosage-dependent manner, with adhesion to fibrinogen-CEP reaching saturation at 4 μg/mL. In contrast, αDβ2 adhesion to fibrinogen returned to 0% at 4 μg/mL.

Polyglutamic acid markedly decreased the adhesion of αDβ2 transfected cells to fibrinogen-CEP

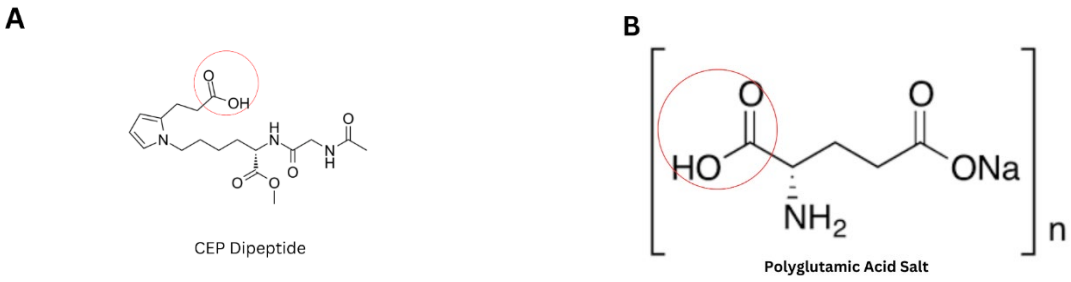


Figure 17: Chemical structures of (A) 2-(ω-carboxyethyl) pyrrole (CEP) and (B) polyglutamic acid.

Polyglutamic acid salt was used to inhibit fibrinogen-CEP binding because it contains multiple negatively charged carboxyl groups which can interact with positively charged amino acids on the integrin αDβ2 receptor. This interaction creates a charge-charge repulsion that reduces or prevents the binding of fibrinogen-CEP to the αDβ2 receptor. This mechanism of action is like that of other negatively charged molecules, such as heparin or sulfated polysaccharides, which have been shown to inhibit integrin-mediated cell adhesion by electrostatic repulsion. In addition to its electrostatic properties, polyglutamic acid has been shown to mimic the structure of CEP in some cases, potentially competing with CEP for binding to the integrin αDβ2 receptor. This suggests that polyglutamic acid may also interfere with integrin binding to CEP by steric hindrance or other mechanisms.

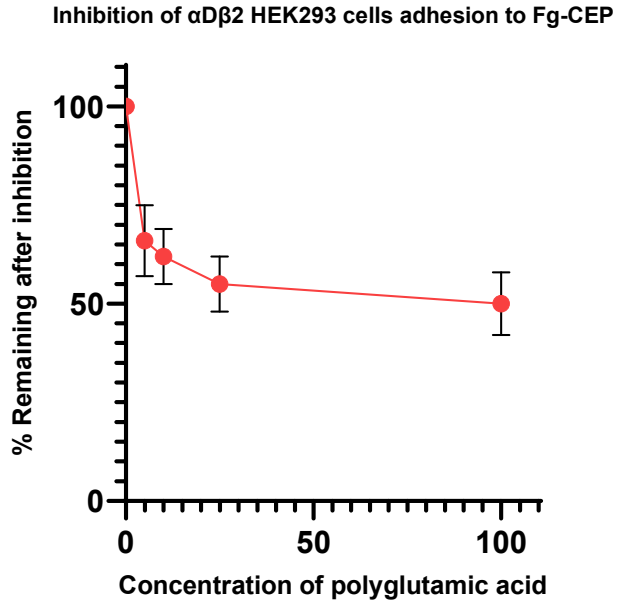


Figure 18: Inhibition of αDβ2-transfected HEK293 cells adhesion to fibrinogen-CEP by Polyglutamic acid.

The data show the inhibitory effect of polyglutamic acid on the adhesion of αDβ2-transfected HEK293 cells to fibrinogen-CEP. Different concentrations of polyglutamic acid (1 µg/mL, 5 µg/mL, 25 µg/mL, and 100 µg/mL) were added to the cells that were incubated with the coated wells for 30 minutes. The cells that adhered to the wells were then quantified using a microplate reader.

The results showed that polyglutamic acid inhibits the adhesion of αDβ2-transfected HEK293 cells to Fg-CEP in a concentration-dependent manner. At the lowest concentration of polyglutamic acid (1µg/ml), there was a slight decrease in cell adhesion compared to the control group. However, at higher concentrations (25µg/ml, and 100µg/ml), there was a significant decrease in cell adhesion, with the highest concentration of polyglutamic acid resulting in about 50% inhibition of cell adhesion to Fg-CEP.

CHAPTER 4. DISCUSSION

Integrin αDβ2 is a crucial molecule involved in the adhesion and retention of macrophages within inflamed tissue. Inflammation causes circulating monocytes to migrate toward the site of inflammation and differentiate into tissue macrophages. Once macrophages reach the inflamed tissue, integrin αDβ2 present on their surface binds to extracellular matrix proteins like fibrinogen, which are abundant in the inflammatory microenvironment. This binding allows macrophages to firmly adhere to the site of inflammation, leading to their subsequent accumulation in the tissue.

The retention and accumulation of macrophages in the inflamed tissue are crucial for effective immune responses to pathogens and tissue repair. Integrin αDβ2-mediated adhesion of macrophages not only enhances their retention at the site of inflammation but also promotes their polarization into a pro-inflammatory phenotype. However, sustained or aberrant activation of integrin αDβ2-mediated signaling can lead to chronic inflammation and tissue damage in various diseases such as atherosclerosis, arthritis, and cancer.

2-(ω-carboxyethyl) pyrrole (CEP) is a byproduct of oxidative stress that is produced in inflamed tissue. The interaction between CEP and integrin αDβ2 plays a vital role in macrophage retention and accumulation in inflamed tissue. Integrin αDβ2 is expressed on the surface of macrophages and binds to CEP present on extracellular matrix proteins like fibrinogen. This interaction activates intracellular signaling pathways, resulting in the increased adhesion, migration, and retention of macrophages within inflamed tissue. Consequently, this leads to chronic inflammation, tissue damage, and disease progressions such as atherosclerosis and age-related macular degeneration.

Inhibiting the binding of CEP to integrin αDβ2 on macrophages is a promising approach to treating chronic inflammation. Preventing macrophage accumulation within inflamed tissue by inhibiting this interaction could help reduce inflammation. Therefore, the development of therapeutics targeting this interaction has become an area of interest. This study's findings highlight the importance of αDβ2-CEP interactions in macrophage retention at sites of inflammation. Therefore, this interaction could be a potential therapeutic target in treating chronic inflammatory diseases.

The binding assay results indicate that the αDβ2 I-domain preferentially binds to fibrinogen-CEP compared to fibrinogen. This indicates a higher affinity of the I-domain for the ligands formed by the oxidation of DHA during inflammation. In addition, adhesion assays showed that αDβ2 exhibits stronger adhesion to fibrinogen-CEP than fibrinogen, further supporting the role of CEP in αDβ2-mediated macrophage adhesion.

The specificity of αDβ2-CEP interactions was also examined in this study. The adhesion inhibition assay showed that polyglutamic acid salt inhibits αDβ2 adhesion fibrinogen-CEP, indicating that αDβ2 binding to these ligands is at least partially mediated by CEP. Furthermore, the results of the antibody-blocking assay suggest that αDβ2 binding to fibrinogen-CEP is specifically dependent on CEP, as the binding activity was decreased in a concentration-dependent manner with increasing concentrations of anti-CEP antibody.

Macrophage retention at sites of inflammation is critical for the resolution of inflammatory responses, as macrophages play key roles in phagocytosis, cytokine production, and tissue remodeling. The ability of αDβ2 to selectively bind to fibrinogen-CEP suggests that this interaction may play a role in the recruitment and retention of

macrophages at sites of CEP deposition, such as in atherosclerotic plaques or in the lung during chronic obstructive pulmonary disease (COPD). Indeed, previous studies have implicated CEP in the pathogenesis of several inflammation diseases[69]. The findings of this study suggest that αDβ2-CEP interactions may contribute to these inflammatory responses by promoting macrophage retention at sites of CEP deposition.

The ability of αDβ2 to selectively bind to fibrinogen-CEP may also have therapeutic implications. Targeting αDβ2-CEP interactions may represent a novel approach to modulating inflammatory responses. For example, the development of small molecules that specifically disrupt αDβ2-CEP interactions may be a promising approach for the treatment of inflammatory diseases. The P5 peptide (a small molecule composed of five amino acids (cysteine, arginine, tryptophan, leucine, and aspartic acid), developed in Yakubenko's lab, is a promising new approach for inhibiting the interaction between integrin αDß2 and CEP, thereby preventing macrophage retention and accumulation in inflamed tissue[70]. This study further supports the potential of the P5 peptide as a therapeutic agent for the treatment of chronic inflammatory diseases by providing additional evidence of the specificity of integrin αDß2-CEP interactions and the importance of CEP in macrophage retention at sites of inflammation. The P5 peptide has shown promising results in inhibiting integrin αDß2-CEP interactions, but further optimization is necessary to improve its pharmacokinetic properties and efficacy. Small molecules with higher affinity and specificity for αDß2-CEP interactions may offer a more effective treatment option for chronic inflammatory diseases. Further research in this area will be important for developing effective therapies and improving outcomes for patients with chronic inflammatory diseases.

However, it should be noted that this study has some limitations. The experiments were conducted in vitro, and the findings may not necessarily translate to in vivo settings. Future studies are needed to examine the role of αDβ2-CEP interactions in animal models of inflammatory diseases. In addition, while this study provides insight into the specificity of αDβ2-CEP interactions, further studies are needed to fully understand the molecular mechanisms underlying these interactions. The potential clinical applications of targeting integrin αDß2-CEP interactions extend beyond the treatment of chronic inflammatory diseases, as the selective binding of αDß2 to fibrinogen-CEP may also have implications for the development of biomaterials for tissue engineering and regenerative medicine.

In conclusion, this study provides evidence that αDβ2-CEP interactions play a crucial role in macrophage retention at sites of inflammation.

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