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AN INVESTIGATION OF THE ROLE OF BASIGIN-VARIANT-1 IN THE IMMUNE RESPONSE WITHIN THE RETINA

by

Abigail Delanie Vera Tompa

A thesis submitted to the Department of Biology in partial fulfillment of the requirements for the degree of Master of Science in Biology

UNIVERSITY OF NORTH FLORIDA

COLLEGE OF ARTS AND SCIENCES

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Abstract

The neural retina is considered an immune privileged area, in that the eye attempts to suppress the inflammatory response to preserve vision. If there is damage to the blood-retina barrier, such as in diabetes or macular degeneration, it is possible for monocytes to travel out of the blood vessels and into the retina. While studying retinoblastoma, a research group determined that the protein, Basigin, through its extracellular domain, elicits an immune response by enhancing transcription of the pro-inflammatory cytokine interleukin-6 (IL-6). Basigin is cell adhesion molecule that belongs to the immunoglobulin superfamily. The Basigin gene has two main transcripts, that produce the proteins Basigin-variant-1 and Basigin-variant-2, that differ in the extracellular region. The expression of Basigin variants differ, in that variant-1 is specifically expressed by photoreceptor cells in the neural retina, and variant-2 is expressed throughout the body, including monocytes of immune cells and Müller glial cells of the retina. Because a previous study by this laboratory indicates that the two Basigin gene products interact via their immunoglobulin (Ig) extracellular domains in the retina, the aim of the present study was to determine if the binding region in the Ig0 domain of Basigin-variant-1 is responsible for eliciting the immune response observed in monocytes. In addition, specific amino acids within the binding region in the Ig0 domain were tested for their ability to bind to Basigin-variant-2, and their ability to induce an immune response in monocytes. The data indicate that recombinant versions of the Basigin-variant-1 Ig0 domain do not stimulate production of IL-6 in mouse RAW 264.7 monocytes. The results from the binding assay show the mutated proteins had reduced binding to Basigin-variant-2 when compared to the Ig0 protein, however, no significant difference in binding was observed. These data suggest that the Ig0 domain of Basigin-variant-1 does not stimulate a proinflammatory response in monocytes. The results of the current study

contradict the data of a previous study, which suggests that the true receptor for Basigin-variant-1 Ig0 domain is not expressed in the mouse cell line used in this current study. It remains to be determined if the ability of Basigin-variant-1 to induce an immune response is species-specific.

Chapter 1- Introduction

The neural retina is the tissue within the eye that allows one to see. It is organized into layers of different types of neuron and supportive glial cells and, as part of the brain, is protected by the retinal pigmented epithelium which creates a barrier between the neural retina and the periphery (reviewed by Kaji et al., 2018). Unfortunately, as humans age, one is prone to diseases of the neural retina, which contribute to vision problems, including blindness (Wong et al., 2014; Yau et al., 2012). A frequent type of retinal disease in advanced age is macular degeneration, estimated to affect 196 million people globally (Wong et al., 2014). Macular degeneration is characterized by the accumulation of membranous debris on both sides of the retinal pigmented epithelium, ultimately leading to neurodegeneration of the center of the retina (reviewed by Al-Zamil and Yassin, 2017). Diabetic retinopathy is also a retinal disease significantly impacting a large population, affecting 93 million people worldwide (Yau et al., 2012). In diabetes, the retina is vulnerable to microvascular damage due to high metabolic and oxygen demands (reviewed by Shah, 2008). The capillaries in the back of the eye can deteriorate and leak fluid into and under the retina causing blurring of vision (reviewed by Shah, 2008). The treatment of retinal diseases, such as macular degeneration and diabetic retinopathy, is challenging due to the structure of the retina, and the ocular barriers that protect the eye from pathogens (reviewed by Hosoya and Tachikawa, 2009).

Structure and integrity of the retina

The retina is a thin layer of tissue that is located at the posterior portion of the eye, and functions by converting light into neural signals (reviewed by Masland, 2012). There are several different cell types that are arranged in layers to make up the composition of the retina. The

outermost region of the retina consists of the retinal pigmented epithelium, which is responsible for the maintenance and survival of photoreceptor cells, as well as regulating the transport of nutrients and waste to and from the retina (reviewed by Boulton and Dayhaw-Barker, 2001). Next, photoreceptors form a single layer and consist of two specialized cells known as rods and cones (reviewed by Masland, 2012). Rods are specialized for vision in dim light and contain a light-sensitive pigment, rhodopsin. Cones are specialized for vision in bright light, and contain a light-sensitive pigment, opsin. Bipolar cells exist in a layer between the photoreceptors and retinal ganglion cells. The function of bipolar cells is to transfer information from the rods and cones to ganglion cells. The retinal ganglion cell layer is located near the inner surface of the retina. Ganglion cells possess axons that project to the visual cortex of the brain (reviewed by Masland, 2012). Horizontal cells modulate information from photoreceptors to bipolar cells while amacrine cells modulate information from bipolar cells to retinal ganglion cells (Chaya et al., 2016). Lastly, Müller glial cells extend throughout the various layers of the neural retina. They interact with all the neurons in the tissue and have a supportive role for photoreceptor cells (Philip et al., 2003).

The neural retina is considered an immune privileged area, in that the eye attempts to suppress the inflammatory response to preserve vision (reviewed by Zhou and Caspi, 2010). The blood retinal barrier is composed of an inner and outer barrier to regulate fluid and molecular movement between the ocular vascular beds and the retinal tissues (Cunha-Vaz et al., 2011; Figure 1, Tomi and Hosoya, 2008). The inner blood retinal barrier consists of tight junctions between blood vessel endothelial cells within the retina, which protects the retina from the inner retinal vasculature (Cunha-Vaz et al., 2011). The continuous retinal blood vessel endothelial cell layer rests on a basal lamina that is surrounded by end feet of astrocytes and processes of Müller

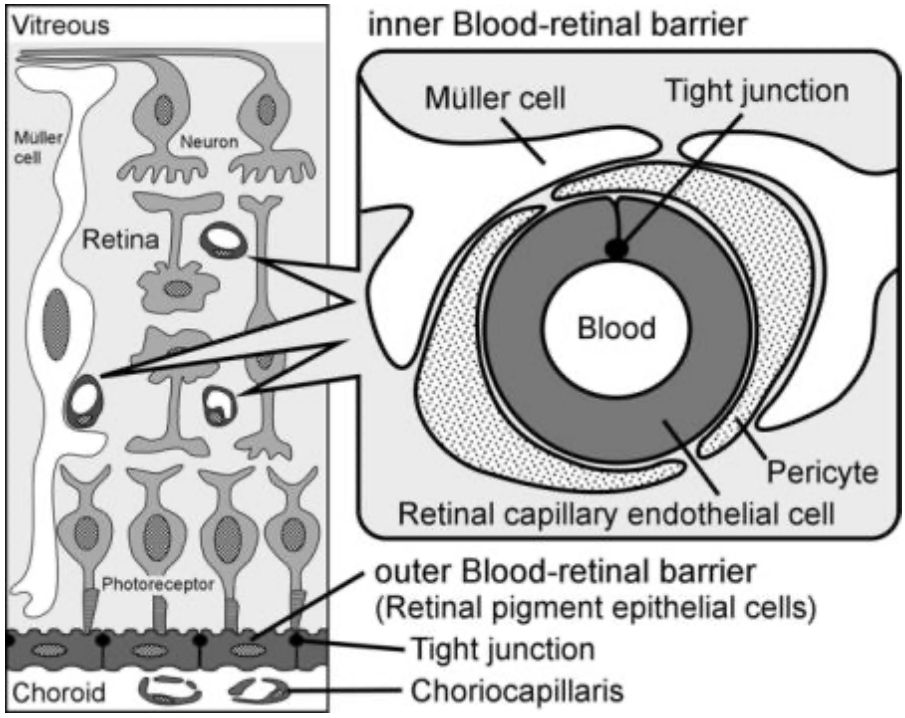

Figure 1. Blood Retinal Barrier. The diagram illustrates the inner blood retinal barrier composed of retinal capillary endothelial cells, as well as the outer blood retina barrier composed of retinal pigmented epithelial cells (Tomi and Hosoya, 2008).

glial cells. Pericytes are also enclosed in the basal lamina, however, do not form a continuous layer. Astrocytes, Müller glial cells, and pericytes maintain integrity of the blood retinal barrier by transmitting regulatory signals to the endothelial cells to indicate changes in the microenvironment (Cunha-Vaz et al., 2011). The tight junctions of the retinal pigment epithelium form the outer blood-retina barrier to protect the retina from direct contact with systemic blood circulation of the choroid (Lau and Taylor, 2009). The basolateral side of the retinal pigmented epithelium interacts with the fenestrated choroid capillaries, thereby playing a vital role in the regulation of nutrients from the blood to the photoreceptors, as well as elimination of waste products (Cunha-Vaz et al., 2011). The retinal pigmented epithelium produces several immunosuppressive growth factors and neuropeptides to suppress T cell activation (Lau and Taylor, 2009). Therefore, the blood-retina barrier keeps leukocytes and other immune cells out of this neural tissue (Lau and Taylor, 2009). If there is damage to the blood-retina barrier, such as in diabetes or macular degeneration, it is possible for monocytes to travel out of blood vessels and into the retina.

Transport of nutrients through the blood retinal barrier

Neuronal cells of the retina, including photoreceptors, require high amounts of oxygen and glucose to function (reviewed by Hosoya and Tachikawa, 2009). Since the blood retinal barrier limits metabolic substrates from passive diffusion, carrier-mediated transporters are required to supply hydrophilic nutrients, such as glucose, lactate, and amino acids to the retina (reviewed by Hosoya and Tachikawa, 2009). Transport of glucose across the blood retinal barrier is facilitated by glucose transporters that permit ATP and Na+ independent passive movement. Glucose transporter-1 (GLUT-1), the most ubiquitously expressed member of the GLUT family,

is localized to the apical and basolateral membranes of the retinal pigmented epithelium (Swarup et al., 2019; Gospe et al., 2010). GLUT-1 is also expressed on the membranes of endothelial cells, inner segments of photoreceptors, and Müller glial cells (Swarup et al., 2019; Gospe et al., 2010; Kumagai et al., 1994). Transport of monocarboxylates, such as lactate, across the blood retinal barrier is mediated by a family of proton-coupled monocarboxylate transporters (MCTs) (Halestrap and Price, 1999). Both monocarboxylate transporter-1 (MCT-1) and monocarboxylate transporter-3 (MCT-3) are expressed on the retinal pigmented epithelium (Philp et al., 2001). Additionally, MCT-1 is expressed by photoreceptors, endothelial cells, and Müller glial cells (Philp et al., 2003).

In the retina, glucose is transported from photoreceptor cells to Müller glial cells via GLUT-1 (Philp et al., 2001). Due to Müller glial cells possessing few mitochondria, the glucose is metabolized to lactate for use in anerobic glycolysis. Then, lactate is shuttled to photoreceptors and converted to pyruvate to fuel oxidative phosphorylation. Excess lactate is shuttled out of the retina via MCTs into the choroidal venules (Philp et al., 2001).

Immunological aspects of cell adhesion molecules

Cell adhesion molecules (CAMs) are known to play important roles in the immune system by initiating and sustaining a state of inflammation against pathogens (reviewed by Ren et al., 2011). The immune system is responsible for initiating this line of defense. There are two branches within the immune system, known as the innate and adaptive immune system (reviewed by Medzhitov and Janeway Jr., 2000). The innate immune response is a non-specific defense mechanism that is activated by chemical properties of antigens (Parham, 2015). The innate system is the first line of defense that is ready from birth, in which the primary goal is to

establish a state of inflammation in response to a foreign body. If innate immunity fails to eliminate a pathogen due to its limited specificity, then the adaptive system will be activated (Parham, 2015). The adaptive immune system is a highly specific system that includes an antigen-specific response with two specialized classes of lymphocytes, T cells and B cells (reviewed by Medzhitov and Janeway Jr., 2000). Lymphocyte receptors recognize pathogens by using cell-cell surface receptors of just one molecular type (Parham, 2015). The lymphocyte receptors are encoded by genes that are cut, spliced, and modified during lymphocyte development. Thus, the adaptive system requires more time to activate, however, the response is stronger and there is a memory of infection (Parham, 2015).

Specifically, in innate immunity, adhesion molecules are known to play a role in the recognition of foreign entities (Garver et al., 2008). These transmembrane glycoproteins are characterized by their structural and functional differences (reviewed by Ren et al., 2011). The four families of adhesion molecules consist of immunoglobulin-like molecules, integrins, cadherins, and selectins (reviewed by Homrich et al., 2016). Members of integrins, selectins, and cadherins are all calcium dependent. Members of the immunoglobulin superfamily mediate calcium-independent cell adhesion and contain at least one Immunoglobulin (Ig)-like domain in their extracellular region (reviewed by Homrich et al., 2016). The Ig-like domains are composed of two opposing antiparallel β-pleated sheets, stabilized by a disulfide bridge (reviewed by Wong et al., 2012). The immunoglobulin superfamily is a diverse family of proteins, including major histocompatibility complex class I and II molecules, proteins of T cell receptors, virus receptors, and cell surface glycoproteins (reviewed by Wong et al., 2012). The structure of the immunoglobulin (Ig) domain is ideal for ligand binding, in that it accommodates a broad amino acid range (Garver et al., 2008). Thus, the Ig superfamily possess a prominent role in cell surface

reception and pathogen recognition (Garver et al., 2008). Intercellular adhesion molecules (ICAMs), vascular adhesion molecules (VCAMs), and activated leukocyte adhesion molecules (ALCAMs) also belong to the Ig superfamily and are vital in leukocyte trafficking (reviewed by Harjunpaa et al., 2019). Many molecules within the Ig superfamily also contain a cytosolic domain, which transmits signals into the cell therefore activating signaling pathways within the cell (reviewed by Homrich et al., 2016).

Toll-like Receptors

The discovery of Toll-like receptors (TLRs) revealed that pathogen recognition within the innate immune system was specific, in that pattern recognition receptors (PRRs) located on effector cells detect invading pathogens via pathogen associated molecular patterns (PAMPs) (reviewed by Kawai and Akira, 2010). The structure of TLRs consist of a single spanning transmembrane domain with ectodomains containing leucine-rich repeats that determine the recognition of PAMPs, referred to as the leucine-rich repeat region (Figure 2; reviewed by Kawai and Akira, 2010). The variation in the number of leucine repeats provides Toll-like receptors with their variation of microbial ligands. There is an additional signaling domain on the cytoplasmic side of the membrane that is referred to as Toll Interleukin-1 Receptor (TIR) domain (Parham, 2015). The TIR domain is a motif in the cytoplasmic tail that recruits cytoplasmic adaptor proteins when activated and is required for the initiation of downstream signaling pathways (reviewed by Kawai and Akira, 2009).

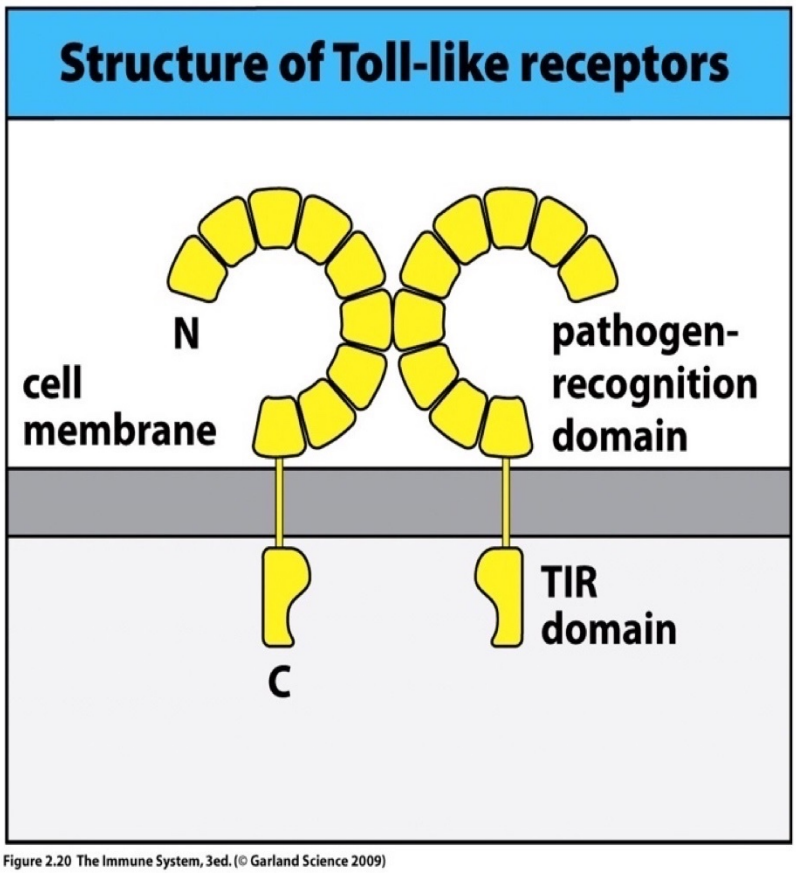

Figure 2. Structure of Toll-like receptors. A Toll-like receptor protein is a transmembrane protein consisting of a Toll-Interleukin-1 Receptor (TIR) domain on the cytoplasmic side of the membrane. The TIR domain is utilized for signal transduction. There is a pathogen-recognition domain located on the extracellular side that is rich in the hydrophobic amino acid leucine. The leucine-rich repeat region (LRR) is key for the recognition of pathogens (Parham, 2009).

Phosphorylation Cascade of Toll-like Receptor 4

Toll-Like Receptor 4 (TLR4) is essential for the recognition of lipopolysaccharide (LPS), which is a molecule located in the outer membrane of Gram-negative bacteria (reviewed by Kawai and Akira, 2010). After LPS is released from bacterial surfaces, it is bound on the surface of macrophages by a protein called CD14 (Figure 3, reviewed by Kuzmich et al., 2017; Parham, 2015). However, LPS can also be picked up by a soluble LPS-binding protein (LBP) in the plasma, followed by deliverance to CD14 on the macrophage surface. The TLR4 dimer associates with a leucine-rich protein, MD2, and together form a complex with CD14 and LPS. The extracellular recognition of the pathogen causes the TIR domain of TLR4 to associate with a particular TIR domain of an adaptor protein, either MyD88 or TRIF (Parham, 2015). Thus, TLR4 is known to activate two signaling pathways, the myeloid differentiation primary response gene 88 (MyD88) pathway and the TIR-contains adaptor inducing IFNβ (TRIF) pathway (reviewed by Kawai and Akira, 2009).

TLR4 binds to MyD88 when it is embedded in the plasma membrane and will bind to TRIF if it is internalized within an endosome (Figure 3, reviewed by Kuzmich et al., 2017; reviewed by Kawasaki and Kawai, 2014). Sorting adaptors, TIR-domain containing adaptor protein (TRAM) and TRIF-related adaptor molecule (TIRAP), are utilized to recruit TRIF and MyD88 to TLR4, respectively. The activation of TRIF induces pathways that lead to the activation of transcription factor interferon regulatory transcription factor 3 (IRF3), and NF-κB (reviewed by Kawai and Akira, 2010). Though both pathways elicit transcription of cytokines, the focus will remain on the MyD88 pathway. TLR4 recruits TIRAP at the plasma membrane, followed by the recruitment of MyD88 to initiate a phosphorylation cascade that activates the transcription factors nuclear factor κB (NF-κB), and activator protein 1 (AP-1) (reviewed by

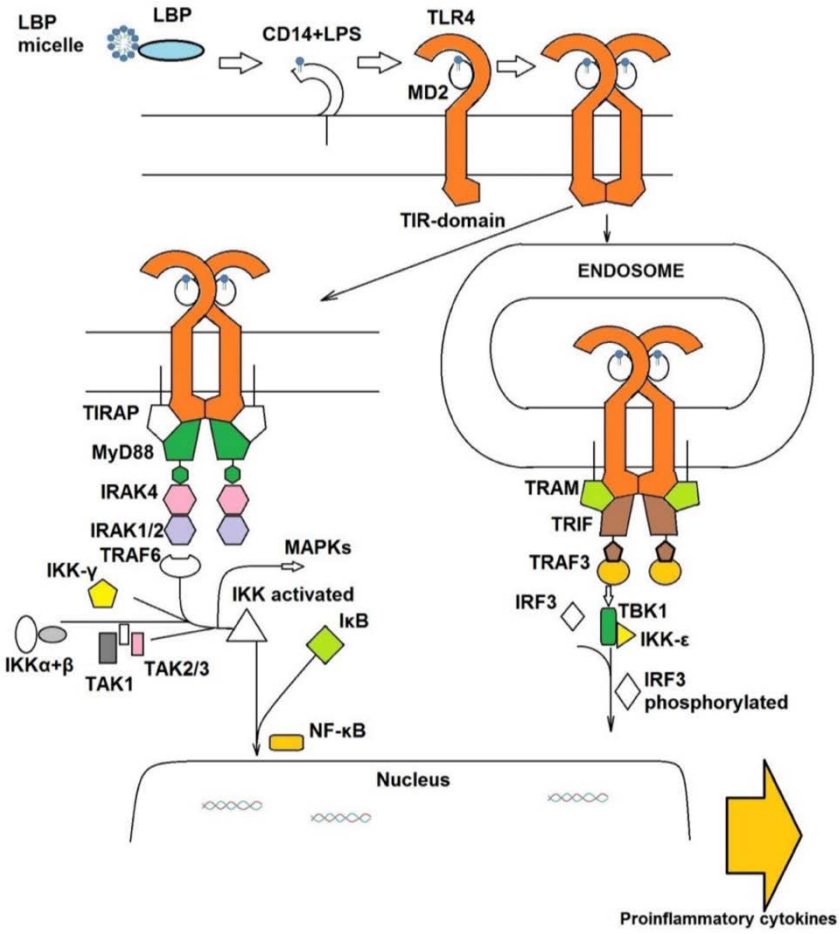

Figure 3. Toll-like Receptor 4 signaling pathway. TLR4 identifies lipopolysaccharide as its pathogen-associated molecular pattern (PAMP). With the help of accessory proteins, cluster of differentiation 14 (CD14) and LPS binding protein (LBP), LPS is transferred to the TLR4-MD2 heterodimer. The TLR4-MD2 complex dimerizes, causing the recruitment of TRAM and TIRAP to the intracellular domain of TLR4. TLR4s located in the endosome possess the adaptor protein, TRAM, which recruits TRIF. Activation of TRIF induces alternative pathways that lead to the activation of transcription factor interferon regulatory transcription factor 3 (IRF3). IRF3 promotes the transcription of inflammatory cytokines. TLR4s located at the plasma membrane possess TIRAP, which recruits MyD88. Upon MyD88 activation, a phosphorylation cascade results in the activation of transcription factor, NF-κB. NF-κB translocation inside the nucleus allows the transcription of proinflammatory cytokines (reviewed by Kuzmich et al., 2017).

Kawasaki and Kawai, 2014). In the resting state of a macrophage, NF-κB is sequestered in the cytoplasm by the Inhibitor of NF-κB (IκB) (Ernst et al., 2018). While NF-κB is bound by IκB in the cytoplasm, the translocation signal of NF-κB is masked. However, upon infection, IκB is phosphorylated by IκB kinase complex (IKK), allowing NF-κB to be released from the cytoplasm. NF-κB undergoes post-translational modifications and translocates into the nucleus (Ernst et al., 2018). The activation of AP-1 is achieved through the mitogen-activated protein kinase (MAPK) pathway. Thus, activation of MyD88-dependent pathway is necessary for the induction of inflammatory cytokines (reviewed by Kawai and Akira, 2010).

Basigin gene products

The protein Basigin, also known as EMMPRIN and CD147, is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily, and functions as a cell adhesion molecule (reviewed by Muramatsu, 2015). The Basigin gene is located on chromosome 10 in mice and differential splicing generates two main transcripts. Both isoforms of Basigin possess the same transmembrane domain and short cytoplasmic tail, however, differ in the extracellular region (reviewed by Muramatsu, 2015). Basigin-variant-2 possesses two extracellular Ig-like domains, encoded by exons 1-7 (Figure 4; Ochrietor et al., 2003). Basigin-variant-1 possesses three extracellular Ig-like domains and includes exon 1A, which is located between exon 1 and 2 (Figure 4; Ochrietor et al., 2003). The expression of the Basigin variants is different as well, in that Basigin-variant-1 is specifically expressed by the photoreceptor cells in the neural retina, and Basigin-variant-2 is expressed in most, if not all, tissues of the body (Ochrietor et al., 2003). This includes monocytes (immune cells), Müller glial cells of the neural retina, the retinal

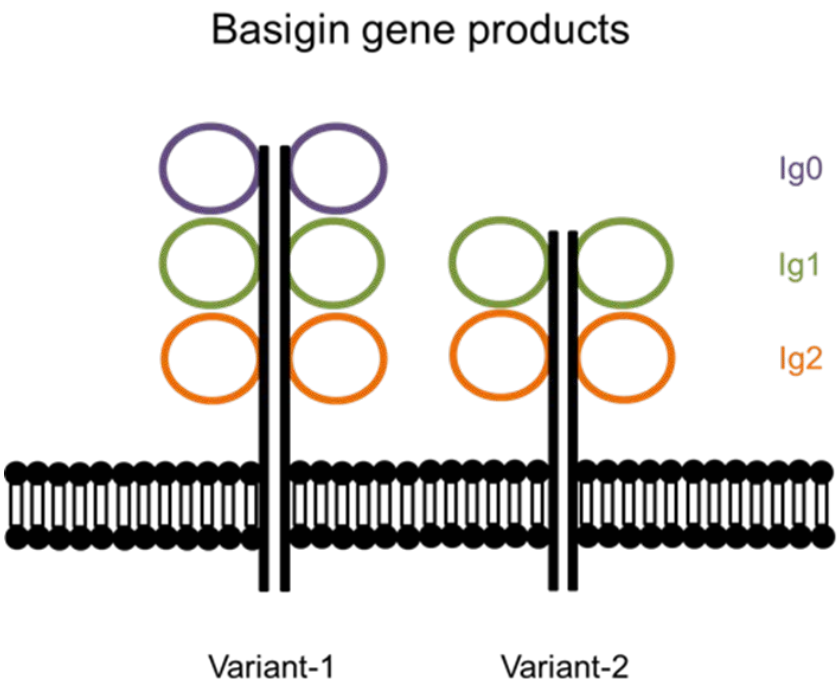

Figure 4. Basigin gene products. Basigin gene products are members of the Immunoglobulin (Ig) superfamily with extracellular Ig domains, a single-pass transmembrane domain, and a short cytoplasmic tail. Basigin-variant-1 possesses three extracellular Ig-like domains while Basigin variant-2 possesses two extracellular Ig-like domains (Ochrietor et al., 2003).

pigmented epithelium of the eye, and blood vessel endothelial cells (reviewed by Muramatsu, 2015).

Basigin gene products are thought to associate with each other in the neural retina to form an association between photoreceptor neurons and Müller glial cells. It is thought that the two cell adhesion molecules interact to allow for efficient transfer of nutrients from the Müller cells to the photoreceptor cells via monocarboxylate transporters (Philip et al., 2003). The absence of Basigin gene products in the neural retina, as in the Basigin null mouse, results in misexpression of MCT-1, limited electrical signaling by the photoreceptors, and blindness (Philp et al., 2003). Thus, failure of the interaction to occur does not result in structural deficits in the neural retina, but rather metabolic defects due to the misalignment of nutrient transporters between two cell types (Ochrietor et al., 2001; Philip et al., 2003).

Previous studies by this laboratory, using recombinantly expressed proteins representing the variant-1 Ig0 domain, sought to determine if the Ig0 domain of Basigin-variant-1 binds to Basigin-variant-2. The amino acid sequence of the Ig0 domain is highly conserved among species, suggesting a conserved function, specifically to bind Basigin-variant-2 (Ochrietor et al., 2003). Binding assays using recombinantly expressed proteins containing Ig0 domain amino acids and endogenous Basigin-variant-2 suggest that the two proteins interact (Figure 5; J. Ochrietor, personal observation). Specifically, the variant-2 binding region within the Ig0 domain was localized to the amino half of the Ig0 domain (Figure 5; J. Ochrietor, personal observation). Within the amino half of the Ig0 domain is a sequence of amino acids with significant sequence identity to L1cam, which is known to interact with Basigin-variant-2 in the brain (Figure 6A, J. Ochrietor, personal observation; Heller et al., 2003). A recombinant protein representing the amino acids with significant homology to L1cam also binds to endogenous

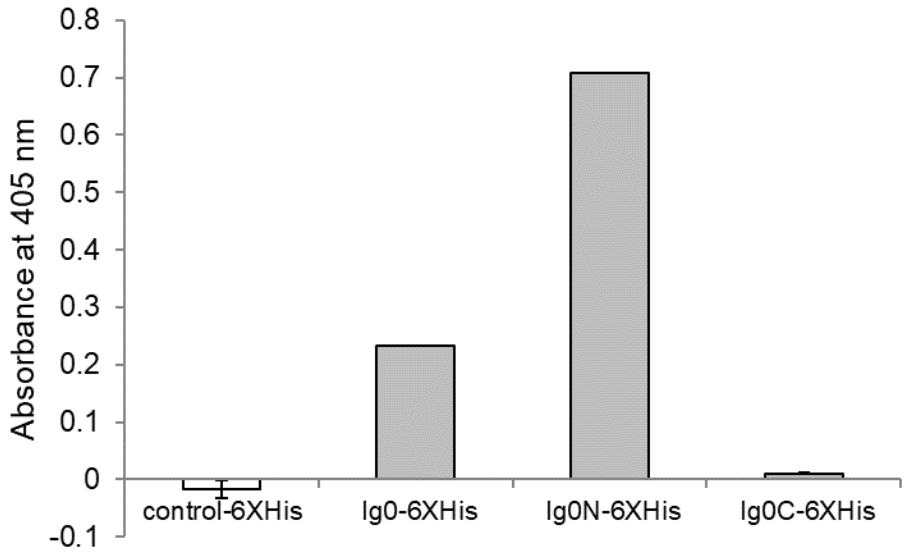

Figure 5. The amino half of the Ig0 domain of Basigin-variant-1 binds to Basigin-variant-2. Sandwich ELISA analyses were performed by capturing endogenous mouse Basigin-variant-2, followed by incubation with recombinant protein probes consisting of the vector-encoded amino acids (control-6XHis), the Basigin-variant-1 Ig0 domain (Ig0-6XHis), the amino-half of the Basigin-variant-1 Ig0 domain (Ig0N-6XHis), or the carboxy-half of the Basigin-variant-1 Ig0 domain (Ig0C-6XHis). The absorbance reflects the ability of a recombinant protein probe to interact with endogenous Basigin. Error bars represent the standard deviations.

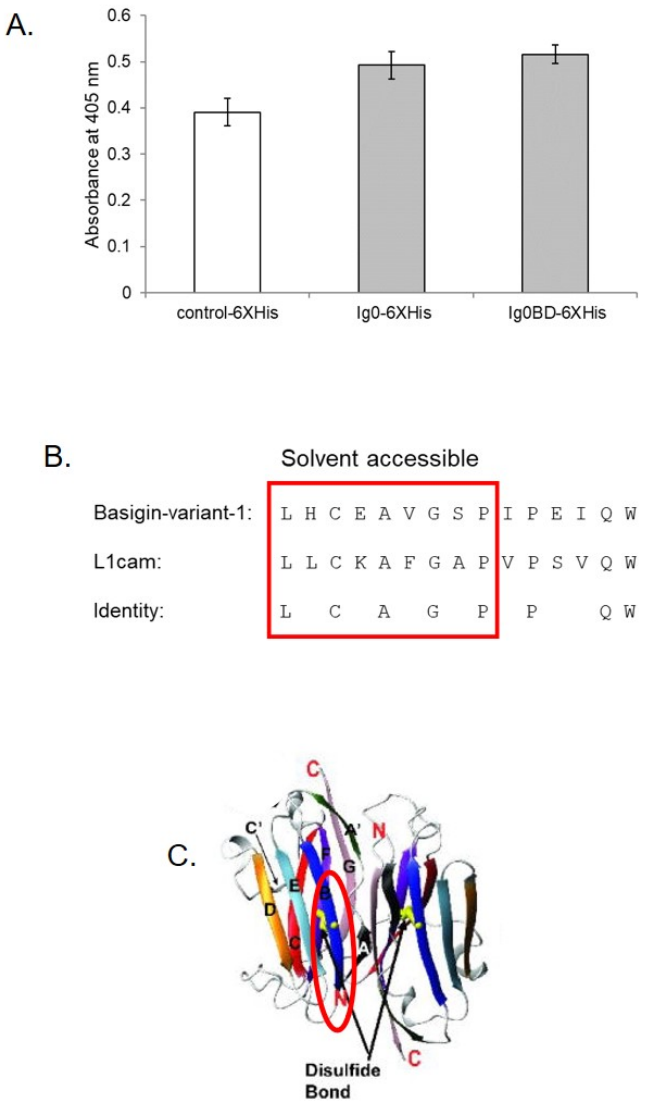

Figure 6. The variant-2 binding motif within the Ig0 domain of Basigin-variant-1. (A) Sandwich ELISA analyses were performed by capturing endogenous mouse Basigin-variant-2, followed by incubation with recombinant protein probes consisting of the vector-encoded amino acids (control-6XHis), the Basigin-variant-1 Ig0 domain (Ig0-6XHis), or the amino acids with significant homology to L1cam (Ig0BD-6XHis). The absorbance reflects the ability of a recombinant protein probe to interact with endogenous Basigin-variant-2. Error bars represent the standard deviations. (B) Amino acid sequences of the extracellular domains of Basigin-variant-1 and L1cam were aligned via BLAST software. Significant amino acid identity (50%) was observed between the two sequences in the region shown. Amino acids that are identical in both Basigin-variant-1 and L1cam are indicated. The red box indicates solvent accessible amino acids. (C) The crystal structure of Basigin-vairant-1 Ig0 domain adopts an Immunoglobulin-like domain fold, comprising of 9 anti-parallel β-strands and 2 α-helices (adapted from Redzic et al., 2011). The red oval indicates the location of the amino acids sequence homology to L1cam.

Basigin-variant-2 (Figure 6B; J. Ochrietor, personal observation). A study in which the crystal structure of the Basigin-variant-1 Ig0 domain was resolved revealed that a portion of the sequence with homology to L1cam is solvent accessible and therefore an appropriate candidate for the Basigin-variant-2 binding domain (Figure 6C; Redzic et al., 2011).

Role of Basigin gene products in the immune response

There is previous research indicating the role of Basigin in immune response signaling. Basigin functions as a signaling receptor for cyclophilin A and B (Yurchenko et al., 2010). Extracellular cyclophilins induce chemotactic responses of peripheral immune cells. Addition of cyclophilin A or B to Basigin-variant-2 expressing cells resulted in an influx of Ca2+ and activation of ERK1/2 kinases. ERK1/2 kinases activate the transcription factor nuclear factor κB (NF- κB), followed by the synthesis of proinflammatory cytokines. The amino acid residues proline 180 and glycine 181 in the extracellular domain of Basigin-variant-2 are vital for this signaling to occur (reviewed by Yurchenko et al., 2010). The interaction between Basigin-variant-2 and cyclophilin most likely leads to activation of integrins because cell migration is enhanced following treatment (reviewed by Muramatsu, 2015).

Additionally, it was determined that the extracellular Ig0 domain of Basigin-variant-1 elicits an immune response (Redzic et al., 2011). This observation was uncovered while exposing a human monocyte cell line (THP-1) and a human kidney cell line (HKC) to a recombinant version of the Basigin-variant-1 extracellular domain and observing an increase in expression of the pro-inflammatory cytokine IL-6 (Redzic et al., 2011). Although this is an intriguing finding, the biological relevance of this observation is yet to be determined. Basigin-variant-1 is only

expressed in the neural retina, which is an immune privileged tissue and carefully restricts immune cell migration into the retina.

One hypothesis that seeks to explain the biological relevance of the ability of Basigin-variant-1 Ig0 to induce and immune response in monocytes proposes that a signal transduction cascade utilizing Basigin-variant-2 is used. In this hypothesis, Basigin gene products interact in a healthy retina to hold photoreceptors and Müller glial cells together. Each interacts with MCT-1 on their respective cell to aid in the efficient transfer of metabolites from the Müller cells to the photoreceptors (Figure 7A). However, upon damage to the blood-retina barrier, as a result of retinal damage like that seen in macular degermation and diabetic retinopathy, it is thought that Basigin-variant-1 on photoreceptor cells acts as a ligand for Basigin-variant-2 on monocytes that may have infiltrated the neural retina through a breach in the blood-retina barrier to stimulate the production of IL-6 (Figure 7B). Because there are no consensus sequences for signal transduction molecules to bind within the short cytoplasmic domain of the Basigin gene products, it is hypothesized that another membrane-associated protein serves to transduce the signal leading to IL-6 expression. A study suggested that the transmembrane domain of Basigin has a glutamate residue that may interact with two positively charged histidine residues within the transmembrane domain TLR4 (Brown, 2016). Basigin protein hydrophobic residues within the transmembrane segment also stabilize the interaction *in vitro* (Brown, 2016).

The aim of the present study is to determine if the region of the Basigin-variant-1 Ig0 thought to bind to Basigin-variant-2 is the same region that elicits an immune response in monocytes. It is hypothesized that the region does induce an immune response and that signaling cascades used by TLR4 are responsible for the subsequent expression of IL-6. The results of these studies may establish a potential mechanism for the immune aspects of diseases like

macular degeneration and diabetic retinopathy and provide a better understanding of the role of Basigin gene products in the immune response.

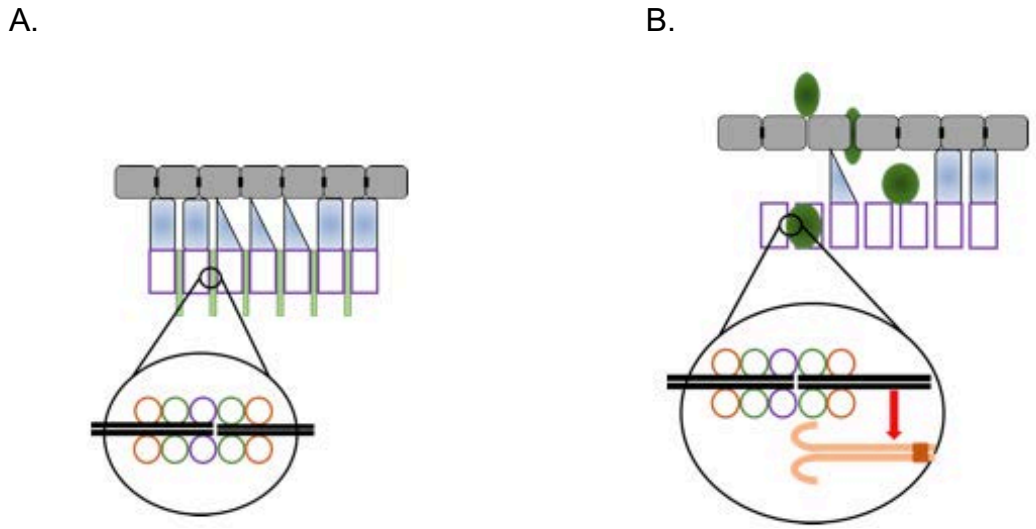

Figure 7. Interactions of Basigin gene products in the neural retina. (A) In a healthy retina, the Ig0 domain of Basigin-variant-1 on photoreceptor cells (outlined in purple) binds to the extracellular domain of Basigin-variant-2 on Müller cells (green). The retinal pigmented epithelium (gray) is the blood-retina barrier. (B) In a damaged retina, the retinal pigmented epithelium becomes breached, and monocytes (dark green) can enter the neural retina. The Ig0 domain of Basigin-variant-1 on photoreceptor cells (outlined in purple) binds to the extracellular domain of Basigin-variant-2 on monocytes (dark green). Basigin-variant-2 interacts with TLR4 (orange) on the surface of the monocyte to initiate intracellular signaling.

Chapter 2- Methods

Recombinant protein expression

The pET102 vector (Invitrogen Corporation, Carlsbad, CA) is a bacterial expression vector designed to produce recombinant proteins containing a tag of six histidines at the carboxy-terminus that allows for efficient purification and identification (Figure 8). The vector codes for a total of 157 amino acids, including the histidines. The pET102 vector was used to generate expression plasmids containing the cDNA coding for the entire Ig0 domain of Basigin-variant-1 (pET102-Ig0-6XHis), the amino-half of the Ig0 domain of Basigin-variant-1 (pET102-Ig0N-6XHis), and the region of the Ig0 domain of Basigin-variant-1 thought to interact with Basigin-variant-2 (pET102-Ig0BD-6XHis; found within the amino-half of the domain) by Dr. Ochrietor during her post-doctoral studies (Figure 9). The pET102 plasmid was also ligated without a cDNA insert and verified by sequencing (pET102-control-6XHis; Finch et al., 2009). In addition, the plasmid containing the cDNA coding for the entire Ig0 domain in which the coding sequences for individual amino acids are each mutated to glycine (pET102-Ig0E/G-6XHis, pET102-Ig0C/G-6XHis, pET102-Ig0L/G-6XHis, pET102-Ig0P1/G-6XHis) were previously generated by Dr. Ochrietor using the pET102-Ig0 plasmid and the QuikChange II XL protocol (Agilent Technologies, Santa Clara, California; Figure 10).

To transform the competent BL21 *E. coli* cells, 50 μL of cells were combined with 1 μL of the appropriate plasmid (Invitrogen Corporation). The cells were incubated on ice for 15 minutes, and then at 42°C for 30 seconds. The cells were combined with 250 μL of Super Optimal broth (SOC) and incubated for 1 hour at 37°C with shaking at 225 rpm. After an hour, the SOC solution was transferred to 10 mL of Lysogeny broth (LB) containing carbenicillin (50 μg/mL) and incubated at 37°C overnight with shaking at 225 rpm.

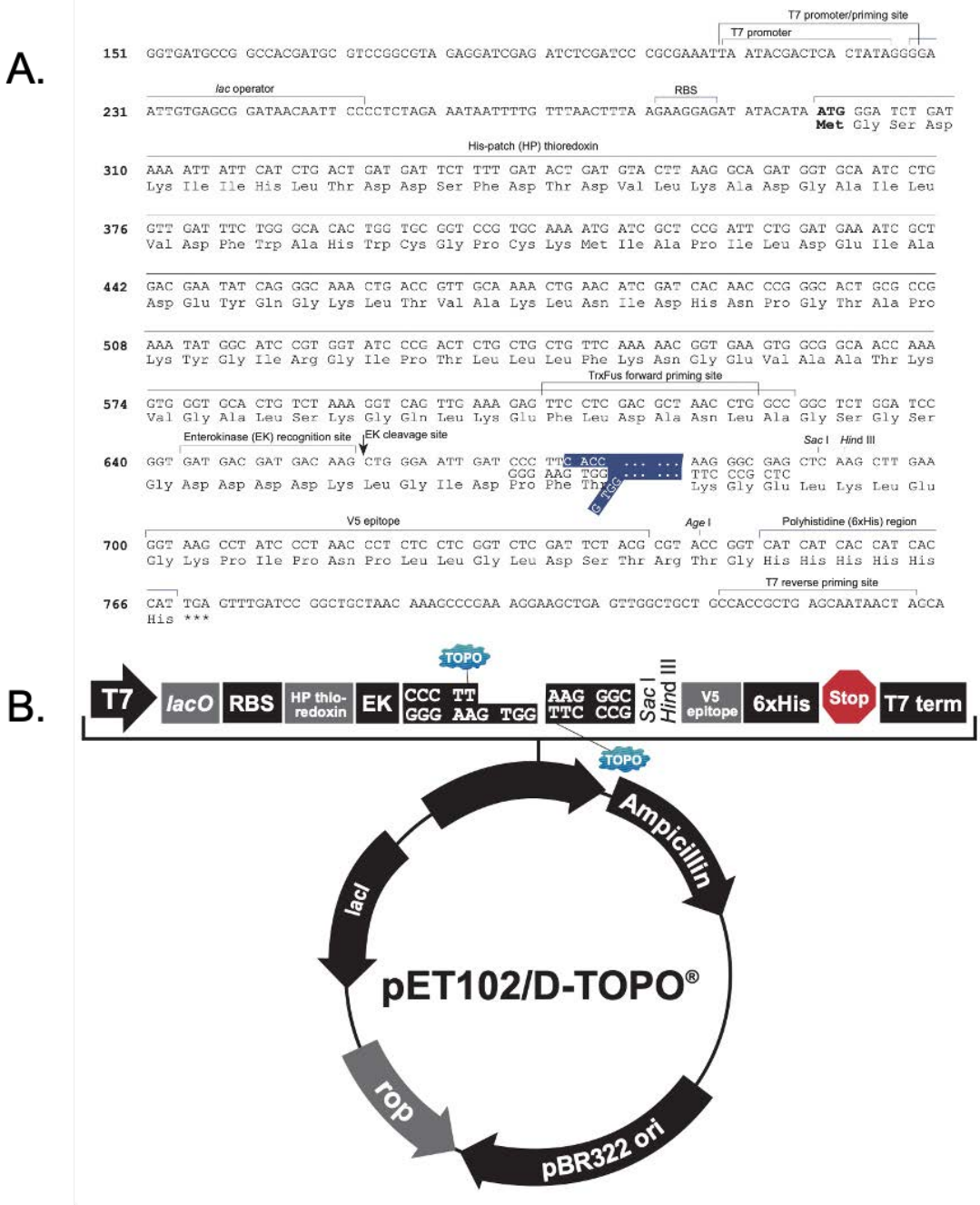

Figure 8. pET102/D-TOPO plasmid. (A) The pET102 vector codes for 157 amino acids and utilizes a T7 promoter system. There is a polyhistidine (6XHis) region at the carboxy-terminus for identification and purification. (B) This figure illustrates the pET102/D-TOPO plasmid map with the selectable marker ampicillin.

Figure 9. The amino acid constructs of the Basigin-variant-1 Ig0 domain. cDNA for the entire Ig0 domain of Basigin-variant-1 (Ig0), the amino-half of the Ig0 domain of Basigin-variant-1 (Ig0-N), and the region of the Ig0 domain of Basigin-variant-1 thought to interact with Basigin-variant-2 (Ig0-BD; found within the amino-half of the domain) were cloned into the bacterial expression plasmid pET102 (Invitrogen Corporation, Carlsbad, CA). This plasmid allows for recombinant protein expression using BL21 *E. coli* cells (Invitrogen Corporation).

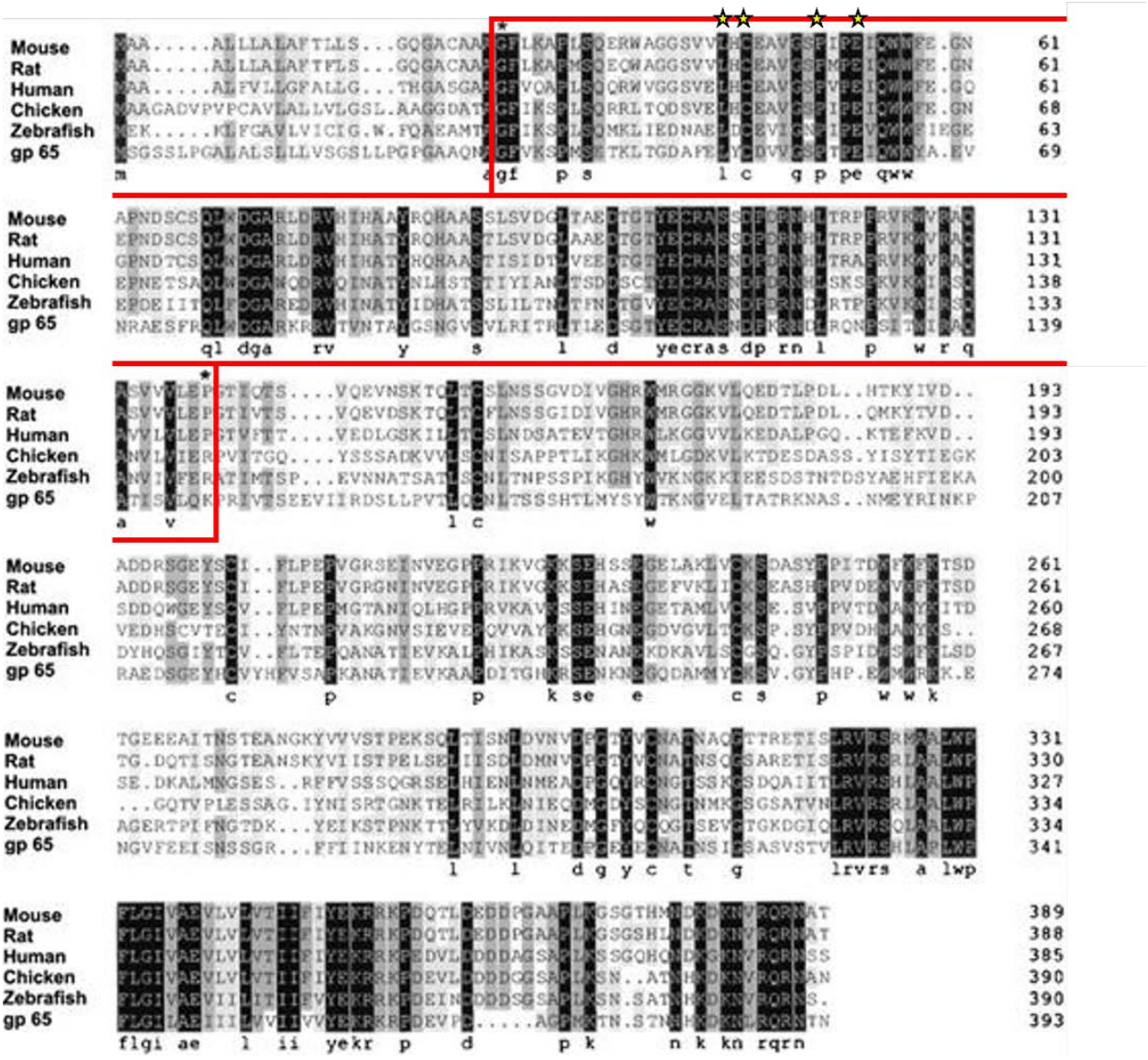

Figure 10. Basigin-variant-1 amino acid sequence alignment. The amino acid sequences for mouse, rat, human, chicken, and zebrafish Basigin-vairant-1, as well as rat neuroplastin gp65 were aligned. There is significant homology (80% sequence identity) within the amino-terminal Ig0 loop, as indicated by the red box. The stars illustrate the individual amino acids that were each mutated to glycine (adapted from Ochrietor et al., 2003).

The optimal conditions for expressing each recombinant protein varied dependent upon pH, duration of growth, oxygen, and temperature (Table 1). For pET102-Ig0BD-6XHis, pET102-Ig0N-6XHis, and pET102-control-6XHis, the competent *E. coli* cells (10 mL) were combined with 250 mL of LB broth containing carbenicillin (50 μg/mL) and incubated for 4 hours at 37ºC with shaking at 225 rpm. Then, 1mM final concentration of Isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to the cells and incubated for 18 hours at 37°C with shaking at 225 rpm (Trial 5, Trial 6, Table 1). For the entire Ig0 domain of Basigin-variant-1, as well as the Ig0 domain mutations, the competent *E. coli* cells (10 mL) were combined with 250 mL of LB broth containing carbenicillin (50 μg/mL) and incubated for 4 hours at 30ºC with shaking at 225 rpm. Then, 1mM final concentration of IPTG was added to the cells and incubated for 3 hours at 30°C with shaking at 225 rpm (Trial 11, Table 1).

Recombinant protein purification

For protein purification, several variables were optimized for each of the recombinant proteins produced (Table 2). In general, the bacterial culture was pelleted by centrifugation at 3,000 rpm for 15 minutes. The mass of the cell pellet was determined, and the cell pellet was resuspended in 10 mL xTractor Cell Lysis Buffer (Clontech Laboratories, Inc., Mountain View, CA) per 1 g of cell pellet. Then 100 μL of lysozyme (10 mg/mL) and 20 μL of DNAse I (5 U/mL) per 10 mL of xTractor Buffer was added to the lysate. The mixture incubated for 20 minutes at room temperature, then was centrifuged at 12,000 rpm for 20 minutes, and the protein lysate was retained. Two milliliters of the TALON metal affinity resin (Clontech Laboratories, Inc.) were prepared by washing with equilibrium buffer (50 mM of NaH_2PO_4, 300 mM of NaCl) and centrifuged at 700 ×g for 2 minutes. The cell lysate was added to the resin and incubated for

Table 1. Variables tested for optimal recombinant protein expression in BL21 *E. coli* cells

	pH of LB Broth	Flask Size	Volume of LB Broth	Temperature of Starter Culture	Temperature of LB Broth	Time to induction	Time of growth for starter culture	Incubation time after induction
Trial 1	6.5	250 mL	100 mL	37°C	37°C	3 hours	20 hours	20 hours
Trial 2	7.2	250 mL	100 mL	37°C	37°C	3 hours	20 hours	20 hours
Trial 3	7.2	500 mL	100 mL	37°C	37°C	3 hours	18 hours	18 hours
Trial 4	7.2	500 mL	250 mL	37°C	37°C	3 hours	18 hours	18 hours
Trial 5	7.2	500 mL	100 mL	37°C	37°C	4 hours	18 hours	18 hours
Trial 6	7.2	1 L	250 mL	37°C	37°C	4 hours	18 hours	18 hours
Trial 7	7.2	1 L	250 mL	37°C	20°C	No induction	18 hours	96 hours
Trial 8	7.2	1 L	250 mL	37°C for 3 hours, 23°C O/N	23°C	No induction	18 hours	96 hours
Trial 9	7.2	1 L	250 mL	37°C	37°C	No induction	18 hours	96 hours
Trial 10	7.2	1 L	250 mL	29°C	23°C	No induction	18 hours	96 hours
Trial 11	7.2	1 L	250 mL	30°C	30°C	4 hours	18 hours	3 hours

Table 2. Troubleshooting for protein purification

Variables tested
Centrifuge speed
Addition of protease inhibitor
Increased number of washes with equilibrium buffer
Increased time of wash with equilibrium buffer
Addition of imidazole to equilibrium buffer
Addition of Triton X-100 to equilibrium buffer
Equilibrium buffer composition (0.5 M NaCl)
Lower pH of equilibrium buffer
Decreased volume of TALON metal affinity resin (Clontech Laboratories)

20 minutes at room temperature, followed by overnight incubation at 4°C. The mixture was centrifuged at 700 ×g for 5 minutes, and the supernatant was removed. The resin was washed twice with 10 mL of equilibrium buffer and subsequent centrifugation at 700 ×g for 5 minutes. The resin was resuspended in 1 mL of equilibrium buffer, and then transferred to a gravity column. The resin was washed with 5 mL of equilibrium buffer by gravity flow. The recombinant protein was eluted by the addition of 5 mL of elution buffer and 0.5 mL fractions were collected.

The protein concentration was determined using the Bradford-Coomassie assay (Pierce™ Thermo Fisher Scientific, Waltham, MA) following the instructions of the manufacturer. A standard curve was generated by serial dilutions of bovine serum albumin (BSA) from a concentration of 2 mg/mL to 0.2 mg/mL. Five microliters of each BSA dilution, as well as recombinant protein fractions were transferred to individual wells of a 96-well plate. Each of the wells received 250 μL of Bradford-Coomassie binding reagent, and the samples were analyzed using a Bio-Tek plate reader at an absorbance of 595 nm (Biotek Instruments, Winooski, VT). A logarithmic regression line was generated to determine the concentration (mg/mL) of the recombinant protein fractions.

SDS-PAGE and Immunoblot

To ensure the presence of the recombinant protein, the retained protein fractions were separated using a 4% to 12% Bis-Tris polyacrylamide gel (Invitrogen Corporation, Carlsbad, CA) in NuPAGE buffer (Invitrogen Corporation) at 200 V. The proteins were transferred to a nitrocellulose membrane (Millipore Corporation, Burlington, Massachusetts) at 30 V for 1 hour.

Proteins were visualized by staining with Fast Green for two minutes, followed by three washes with a detaining solution consisting of water/acetic acid/methanol (5:1:5).

To determine if the co-eluted proteins in the Ig0-6XHis fractions were bacterial or proteolytically digested, the blot was incubated overnight in Blotto (tris buffered saline [TBS] containing 2.5% dry milk and 0.2% Tween-20). The Blotto was replaced with another 10 mL of Blotto containing anti-6XHis mouse antibody (diluted 1:500 in Blotto). The blot was incubated at 37°C for 1 hour with shaking, and then the primary antibody solution was removed, and the blot was washed with 10 changes of TBS. Another 10 mL of Blotto containing alkaline phosphatase-conjugated goat-anti-mouse secondary antibody (AP-GAM; diluted 1:1000 in Blotto) was added to the blot. The blot was incubated at 37°C for 30 minutes with shaking, and then the secondary antibody solution was removed. The blot was washed with 10 changes of TBS, and the AP substrate was added to the blot. The blot incubated at room temperature until a purple signal had developed then the substrate was removed with water.

Treatment of RAW 264.7 cells

Mouse monocytic RAW 264.7 cells, a generous gift from Dr. Terri Ellis, University of North Florida, were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Thermo Fisher Scientific, Waltham, MA) supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS; HyClone, Cytiva, Marlborough, Massachusetts) at 37°C with 5% CO_2. The day before treatment, approximately 215,983 RAW 264.7 cells were plated in serum-free RPMI 1640 medium in a 24-well plate and incubated overnight at 37°C (Corning Incorporated, Corning, NY). The RAW 264.7 cells were treated with 5 μM of recombinant versions of the Basigin-variant-1 Ig0 domain and incubated for 24 hours at 37°C. Controls include cells treated with a control protein generated from the expression vector used to make the

Basigin-variant-1 recombinant proteins, cells treated with D-PBS (Gibco Thermo Fisher Scientific), and cells treated with LPS (1 μg/mL; InvivoGen, San Diego, California). Each treatment was performed in quadruplicate. The cell culture medium was stored at -20°C.

An additional experiment was performed by plating 1,000,000 RAW 264.7 cells in serum-free RPMI 1640 medium in 35 mm Petri dishes. The RAW 264.7 cells were treated with 10 μM of the recombinant version of the entire Ig0 domain and incubated for 24 hours at 37°C. The control included cells treated with D-PBS (Gibco Thermo Fisher Scientific). Each treatment was performed in quadruplicate. The cell culture medium was stored at -20°C.

RAW 264.7 cell protein lysates

RAW 264.7 cells were harvested and homogenized with 400 μL of D-PBS. The cells incubated on ice for 10 minutes, followed by centrifugation at 15,000 rpm for 10 minutes. The protein concentration was determined via the Bradford Coomassie protein assay as previously described.

Immunocytochemistry

Immunocytochemistry was performed using RAW 264.7 cells to verify the expression of Basigin-variant-2. RAW 264.7 cells were plated on Lab-Tek chamber slides (Nunc – Thermo Fisher Scientific) in serum-free RPMI 1640 medium (HyClone, GE Healthcare- BioSciences) and allowed to attach. The cells were fixed by incubation in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes at room temperature and washed with several changes of PBS. The cells were incubated overnight in 500 μL of blocking solution (TBS containing 0.1% Tween 20 and 2% normal goat serum; Pierce/Thermo Scientific) overnight at 4°C. The cells

were incubated with an antibody specific for Basigin gene products (Ochrietor et al., 2003; diluted to 1 μg/mL in blocking solution) for 1 hour at 37°C and then at 4°C overnight. After washing with 10 changes of TBS, the cells were incubated with 250 μL of Alexa 488-conjugated goat-anti-rabbit secondary antibody (diluted 1:1000 in blocking solution; Invitrogen Corporation) for 1 hour at 37°C. DAPI (Invitrogen Corporation) was added to the first of several changes of TBS. Coverslips were mounted with 30% glycerol containing p-phenylenediamine (Sigma Chemical Company) and the cells were viewed with an Olympus Fluoview F1000 confocal microscope (Tokyo, Japan).

IL-6 expression in RAW 264.7 cells in response to recombinant Ig0 domain

A mouse IL-6 ELISA (Thermo Fisher Scientific) was utilized to quantify IL-6 expression in the cell culture medium. The IL6 standard was reconstituted to 10,000 pg/mL with Standard Diluent Buffer. Serial dilutions of the standard were performed to generate dilutions ranging from 500 pg/mL to 7.8 pg/mL. One hundred microliters of the standards and the cell culture medium were each plated into separate wells. The standards and culture media samples were plated in duplicate. The chromogen blank wells remained empty. The samples were incubated at room temperature for two hours, followed by washing the wells four times with wash buffer. One hundred microliters of Mouse IL-6 Biotin Conjugate solution was added to each well, with the exception of the chromogen blanks, and was incubated at room temperature for 30 minutes. The wash step was performed again. One hundred microliters of Streptavidin-HRP Solution was added to each well, with the exception of the chromogen blanks, and the plate was incubated at room temperature for 30 minutes. The wells were washed. One hundred microliters of Stabilized Chromogen was added to each well and the reaction developed in the dark at room temperature

for 30 minutes. The reaction was stopped with the addition of 100 μL of stop solution to each well. The samples were analyzed using a Bio-Tek plate reader at an absorbance of 450 nm. A linear regression line was generated to determine the concentration (pg/mL) of mouse IL-6 in each sample. A one-way ANOVA was utilized to analyze the data generated from the ELISA.

Enzyme-linked immunosorbent assay (ELISA) binding assays

Sandwich ELISA analyses served as binding assays. One hundred microliters of the antibody specific for Basigin gene products (0.5 μg/ml in PBS) was coated onto wells of a 96 well plate. Wells designated as "blank" received 100 microliters of PBS and no antibody. The plate was incubated overnight at 4°C. Unbound capture antibody was removed by three washes with PBS-Tween (PBS-T; PBS containing 0.25% Tween-20). The wells were blocked by incubation in a solution of bovine serum albumin (BSA; diluted 1:10 in PBS) for 30 minutes at 37°C. The BSA was removed, and the wells were washed with PBS-T. One hundred microliters of RAW 264.7 protein lysates (diluted 100 ug/mL in PBS) were added to appropriate wells and incubated for 30 minutes at 37°C. Unbound RAW 264.7 proteins were removed, and the wells were washed three times with PBS-T. One hundred microliters of the appropriate recombinant protein were added to the wells of the plate: Ig0-6XHis, Ig0E/G-6XHis, Ig0C/G-6XHis, Ig0L/G-6XHis, Ig0P1/G-6XHis (10 μM in PBS). The plate was incubated at 37°C for 30 minutes. Unbound recombinant proteins were removed, and the wells were washed three times with PBS-T. One hundred microliters of anti-6X-His mouse primary antibody (Clontech Laboratories; diluted 1:500 in PBS) was added. The plate was incubated for 30 minutes at 37°C. The primary antibody was removed, and the wells were washed three times with PBS-T. One hundred microliters of alkaline phosphatase (AP)-conjugated secondary antibody (goat-anti-mouse;

Pierce/ThermoScientific; diluted 1:1000 in PBS) was added to the wells for 30 minutes at 37°C. The secondary antibody was removed, and the wells were washed three times with PBS-T. A reaction was initiated by adding 100 µl of AP substrate (PNPP, Pierce/ThermoScientific) to the wells. The solution was incubated until a color change from clear to yellow was seen, and the reactions were stopped by the addition of 50 µl of 2N NaOH. The absorbance at 405 nm in each well was recorded using a Bio-Tek plate reader (Winooski, VT). The raw data were analyzed, and bar graphs were constructed using Microsoft Excel software (Redmond, Washington).

Chapter 3- Results

Recombinant protein expression and purification

The aim of the present study was to investigate if the region of the Basigin-variant-1 Ig0 thought to bind to Basigin-variant-2 is the same region that elicits an immune response in monocytes. To achieve this aim, expression plasmids containing the cDNA coding for the entire Ig0 domain of Basigin-variant-1 (pET102-Ig0-6XHis), the amino-half of the Ig0 domain of Basigin-variant-1 (pET102-Ig0N-6XHis), and the region of the Ig0 domain of Basigin-variant-1 thought to interact with Basigin-variant-2 (pET102-Ig0BD-6XHis) that were previously generated (post-doctoral studies, Dr. Ochrietor) were used in the present study for recombinant protein expression. The plasmid containing the cDNA coding for the entire Ig0 domain in which the coding sequences for individual amino acids were each mutated to glycine (pET102-Ig0E/G-6XHis, pET102-Ig0C/G-6XHis, pET102-Ig0L/G-6XHis, pET102-Ig0P1/G-6XHis) that were previously generated (post-doctoral studies, Dr. Ochrietor) were also used in the present study for recombinant protein expression. In addition, the pET102 plasmid was also ligated without a cDNA insert, verified by sequencing, and used to generate an appropriate control recombinant protein for the study (pET102-control-6XHis; Finch et al., 2009). Although each of the recombinant proteins had been expressed, purified, and used in ELISA binding assays in the past, expression of these proteins proved to be challenging for the present study, which led to a series of experiments to optimize the conditions for each protein.

The expression of each protein construct varied dependent upon pH, duration of growth, oxygenation, and temperature (Table 3). For optimal protein expression of all constructs generated, it was required that flask size be at least four times the total volume and the LB broth was pH 7.2. The ability to attain mid-log phase of bacterial growth was also important for protein

Table 3. Conditions for optimal recombinant protein expression in BL21 *E. coli* cells

	pH of LB broth	Flask size	Volume of LB broth	Temperature of starter culture	Culture incubation temperature	Time to induction	Time of growth for starter culture	Incubation time after induction	Protein expression
Trial 1	6.5	250 mL	100 mL	37°C	37°C	3 hours	20 hours	20 hours	Not optimal
Trial 2	7.2	250 mL	100 mL	37°C	37°C	3 hours	20 hours	20 hours	Not optimal
Trial 3	7.2	500 mL	100 mL	37°C	37°C	3 hours	18 hours	18 hours	Not optimal
Trial 4	7.2	500 mL	250 mL	37°C	37°C	3 hours	18 hours	18 hours	Not optimal
Trial 5	7.2	500 mL	100 mL	37°C	37°C	4 hours	18 hours	18 hours	Optimal for Ig0BD-6XHis, control-6XHis, Ig0N-6XHis
Trial 6	7.2	1 L	250 mL	37°C	37°C	4 hours	18 hours	18 hours	Optimal for Ig0BD-6XHis, control-6XHis, Ig0N-6XHis
Trial 7	7.2	1 L	250 mL	37°C	20°C	No induction	18 hours	96 hours	Not optimal for Ig0-6XHis, Ig0E/G-6XHis, Ig0C/G-6XHis, Ig0L/G-6XHis, Ig0P1/G-6XHis
Trial 8	7.2	1 L	250 mL	37°C for 3 hours, 23°C O/N	23°C	No induction	18 hours	96 hours	Not optimal for Ig0-6XHis, Ig0E/G-6XHis, Ig0C/G-6XHis, Ig0L/G-6XHis, Ig0P1/G-6XHis
Trial 9	7.2	1 L	250 mL	37°C	37°C	No induction	18 hours	96 hours	Not optimal for Ig0-6XHis, Ig0E/G-6XHis, Ig0C/G-6XHis, Ig0L/G-6XHis, Ig0P1/G-6XHis
Trial 10	7.2	1 L	250 mL	29°C	23°C	No induction	18 hours	96 hours	Not optimal for Ig0-6XHis, Ig0E/G-6XHis, Ig0C/G-6XHis, Ig0L/G-6XHis, Ig0P1/G-6XHis
Trial 11	7.2	1 L	250 mL	30°C	30°C	4 hours	18 hours	3 hours	Optimal for Ig0-6XHis, Ig0E/G-6XHis, Ig0C/G-6XHis, Ig0L/G-6XHis, Ig0P1/G-6XHis

expression, with a minimum of 4 hours needed to reach an optical density at 600 nm (OD_{600}) of 0.5. These adaptations resulted in optimal protein expression for Ig0BD-6XHis, Ig0N-6XHis, and control-6XHis, but not Ig0-6XHis (Table 3, Figure 11). The temperature of incubation for both the starter culture and the protein expression culture was critical for the longer Ig0-6XHis proteins, with 30°C, rather than 37°C showing to be the optimum conditions for expression (Table 3). Finally, the time of IPTG induction was also critical, with expression of the shorter proteins after 24 hours, but the longer proteins after 4 hours (Table 3).

Protein purification was achieved through the utilization of cobalt resin with affinity for the six histidine carboxy-terminal epitope tag on the recombinant proteins. Initially, the purification of recombinant protein was impeded during the generation of the protein lysate. Increasing the size of the cell pellet yielded increased protein isolation. In addition, the centrifugation speed was critical in the formation of the protein lysate (Table 4). The centrifugation speed of 12,000 rpm was the optimal speed for all the recombinant proteins except for Ig0BD-6XHis and Ig0N-6XHis, which required 10,000 rpm.

The result of affinity chromatography should be the independent isolation of the recombinant protein of interest (Figure 12). While this was true for most of the proteins (Figure 12), the entire Ig0 domain (Ig0-6XHis) co-eluted with various other proteins (Figure 13). Therefore, adjustments were made to the protocol to absolve the protein eluting with contaminating proteins (Table 2). Unfortunately, none of these variables improved the purity of Ig0-6XHis (Figure 13). An immunoblot using an antibody specific for 6XHis suggested that the additional proteins are bacterial proteins, and not proteolytically digested Ig0-6XHis protein (Figure 14).

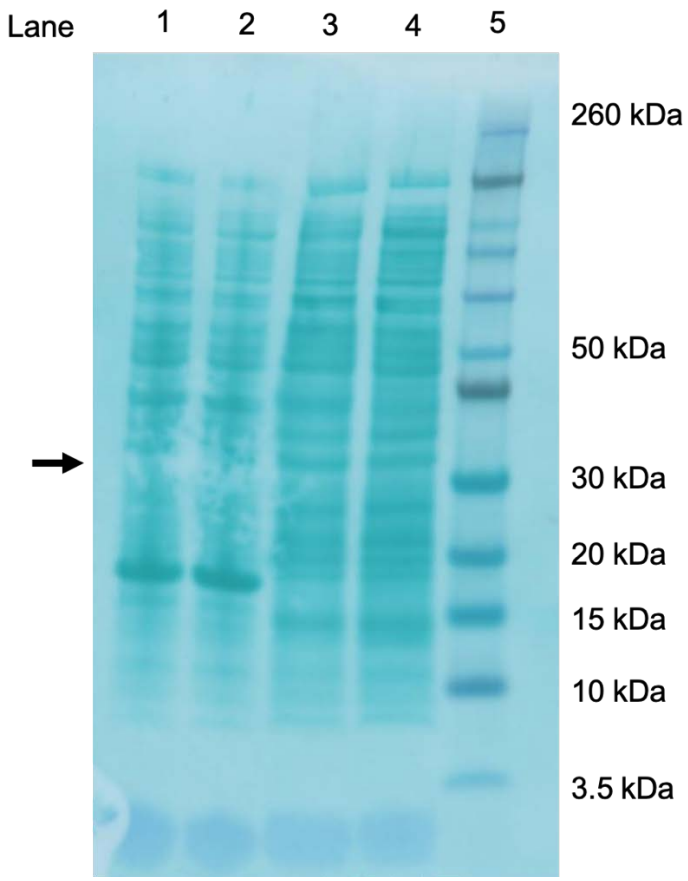

Figure 11. Verification of recombinant protein expression. The expression cultures of were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Lanes 1 and 2 display the culture of control-6XHis while lanes 3 and 4 display the culture of Ig0-6XHis. Lane 5 contains the Novex Sharp Pre-Stained Protein Standard. The control recombinant protein can be seen in lanes 1 and 2 at 17 kDa. No recombinant protein expression of Ig0-6XHis is observed in lanes 3 and 4. The arrow indicates the molecular weight (~30 kDa) of Ig0-6XHis.

Table 4. Optimal centrifugation speed for formation of protein lysate

	10,000 rpm	11,000 rpm	12,000 rpm	10,000 ×g	11,000 ×g	12,000 ×g
Ig0-6XHis	−	−	+	−	−	−
Ig0BD-6XHis	+	−	−	−	−	−
Ig0N-6XHis	+	−	−	−	−	−
Ig0E/G-6XHis	−	−	+	−	−	−
Ig0C/G-6XHis	−	−	+	−	−	−
Ig0L/G-6XHis	−	−	+	−	−	−
Control-6XHis	−	−	+	−	−	−

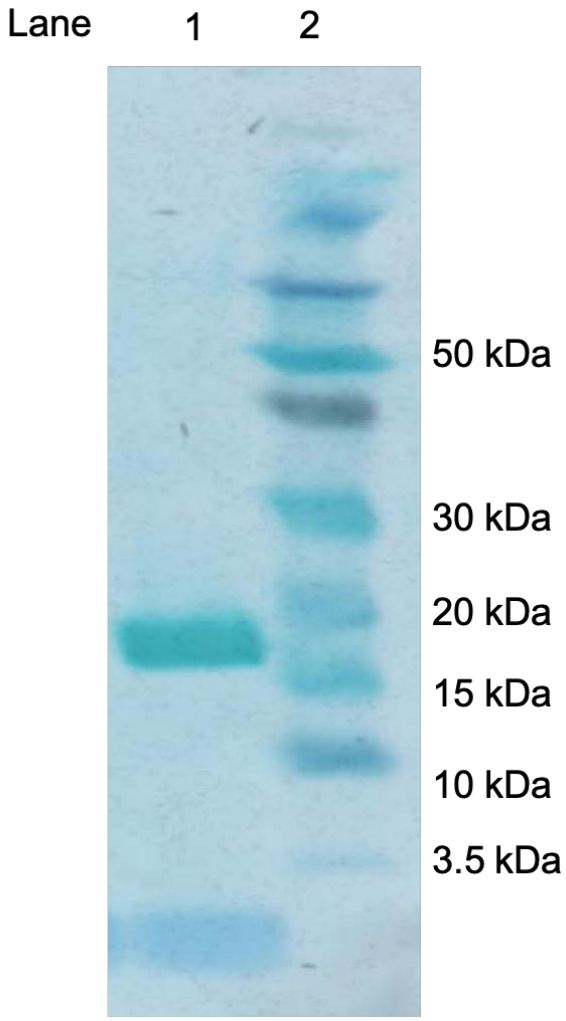

Figure 12. Analysis of control-6XHis protein fraction. Affinity chromatography was performed to purify the control recombinant protein. Lane 1 indicates a signal at 17 kDa, which signifies the molecular mass of control-6XHis. Lane 2 contains the Novex Sharp Pre-Stained Protein Standard.

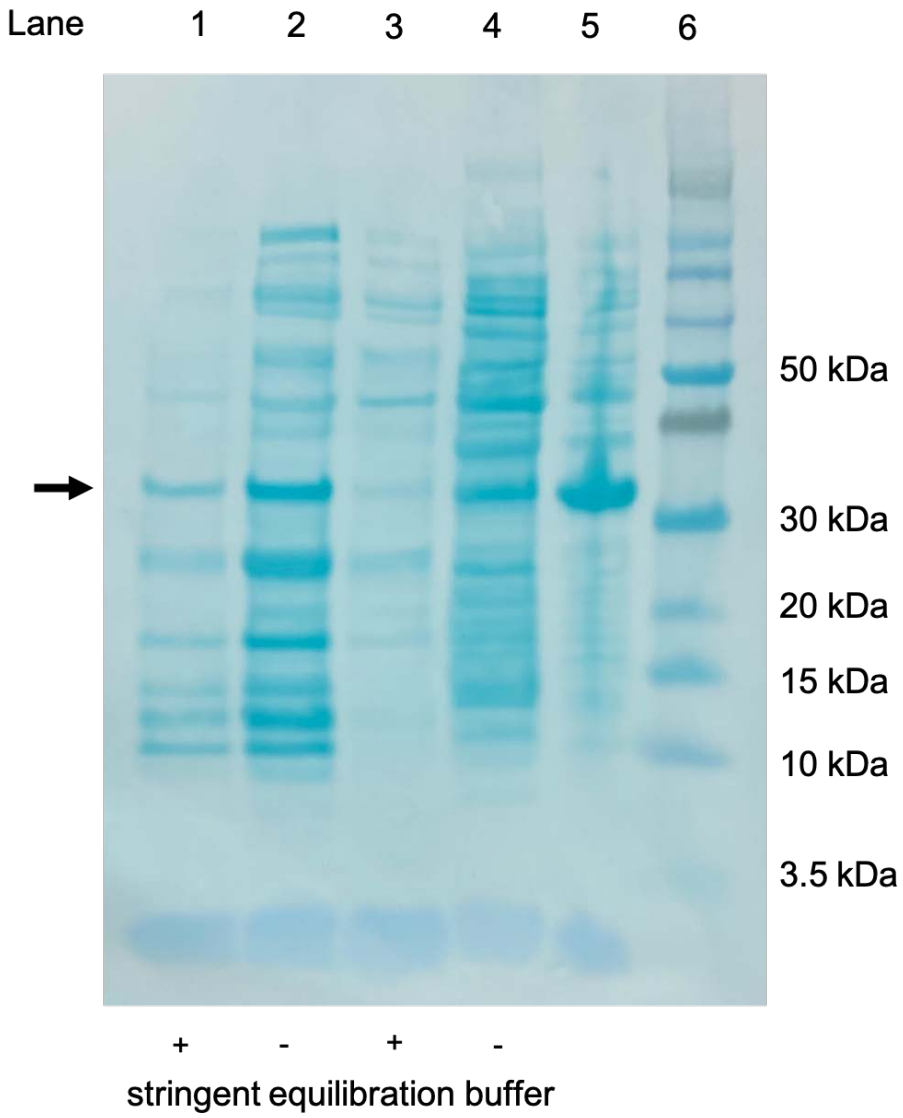

Figure 13. Analysis of Ig0-6XHis protein fraction and culture. Ig0-6XHis recombinant protein and culture samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Lanes 1 and 3 indicate Ig0-6XHis fractions that were purified with a stringent equilibration buffer while lanes 2 and 4 indicate Ig0-6XHis fractions that were purified with a less stringent equilibration buffer. Lane 5 shows the Ig0-6XHis culture. Lane 6 contains the Novex Sharp Pre-Stained Protein Standard. The arrow indicates the molecular weight (~30 kDa) of Ig0-6XHis.

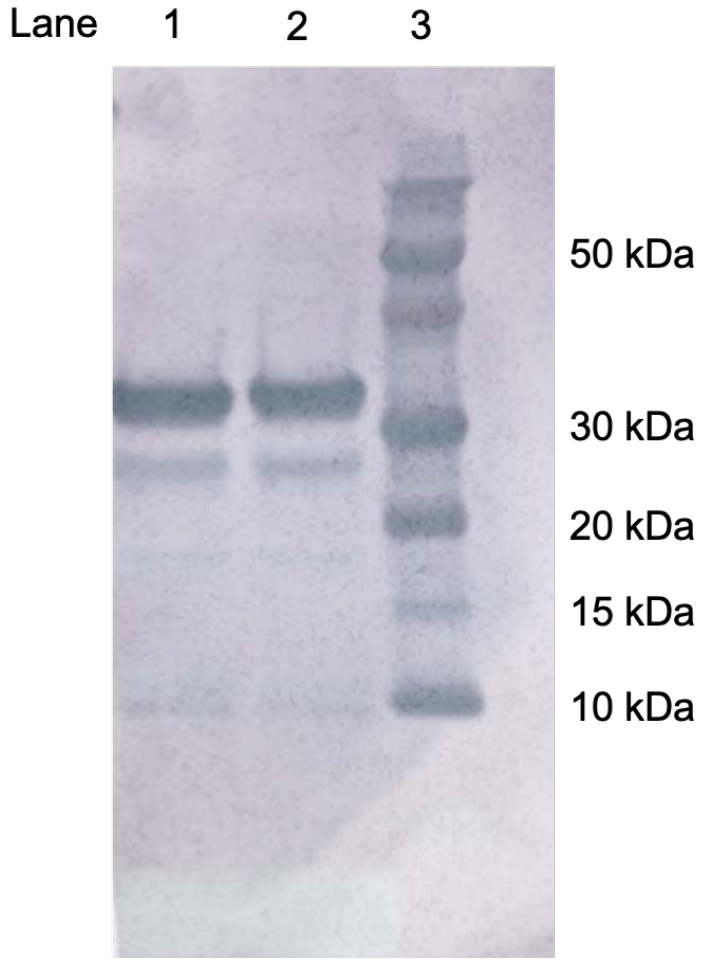

Figure 14. Immunoblot of six histidine carboxy-terminal epitope tag. Lanes 1 and 2 contain Ig0-6XHis proteins and contaminants. The main signal ~30 kDa is the Ig0-6XHis protein. The other co-eluted proteins seen on the electroblot (as shown in Figure 13) are not positive for the 6XHis tag. Lane 3 contains the Novex Sharp Pre-Stained Protein Standard.

Identification of Ig0 domain amino acids used to bind Basigin-variant-2

Because of the experienced protein expression issues, the region of the Ig0 domain thought to bind to Basigin-variant-2 was identified, but the amino acids within the region were not directly tested for their ability to bind Basigin-variant-2. Therefore, an ELISA binding assay was used to determine which amino acids within that region may contribute to the interaction with Basigin-variant-2. Endogenous Basigin was captured and probed with the various Ig0-6XHis recombinant proteins (Figure 15). Although each of the mutated proteins showed reduced binding to Basigin variant-2 (as measured by absorbance) when compared to the Ig0-6XHis protein, no significant difference was observed ($p=0.88$ via one-way ANOVA; Figure 15).

Induction of IL-6 expression in RAW 264.7 monocytes

The RAW 264.7 cell line, derived from mouse monocytes, was selected for the IL-6 expression analyses. The cell line expresses Basigin-variant-2 at the cell surface, as verified by immunocytochemical analyses (Figure 16). RAW 264.7 cells were incubated with LPS for 24 hours to establish that RAW cells can express IL-6. Analysis of the results indicate treatment of LPS led to significant IL-6 expression, in comparison to treatment of D-PBS ($p=0.001$; Figure 17).

To determine if the region of the Ig0 domain thought to bind to Basigin-variant-2 is also responsible for eliciting IL-6 expression, RAW 264.7 cells were treated with 5 μM of the entire Ig0 domain (Ig0-6XHis), the amino-half of the Ig0 domain of Basigin-variant-1 (Ig0N-6XHis), the region of the Ig0 domain of Basigin-variant-1 thought to interact with Basigin-variant-2 (Ig0BD-6XHis), or a control protein generated from the expression plasmid (control-6XHis).

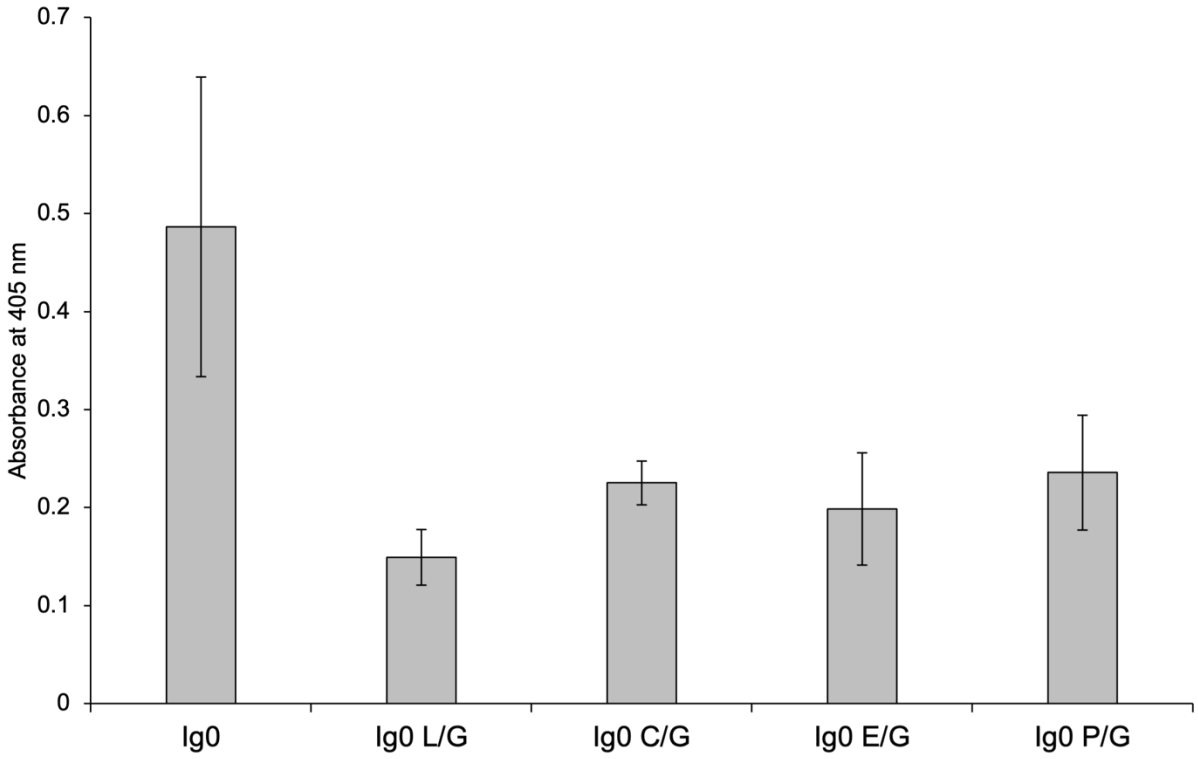

Figure 15. ELISA binding assay investigating amino acid(s) responsible within Ig0 domain for binding to Basigin-variant-2. Endogenous Basigin-variant-2 was captured with recombinant Ig0 protein, as well as recombinant variant-1 Ig0 domain in which amino acids were mutated to glycine. Binding between recombinant protein and endogenous Basigin-variant-2 was measured using an alkaline phosphatase detection system and spectrophotometric assay at 405 nm. The binding assay illustrates that interaction of Basigin-variant-2 and the Ig0 domain of variant-1 is no greater than binding of the mutated Ig0 recombinant proteins to Basigin-variant-2. All runs were performed in triplicate. Error bars represent the standard error.

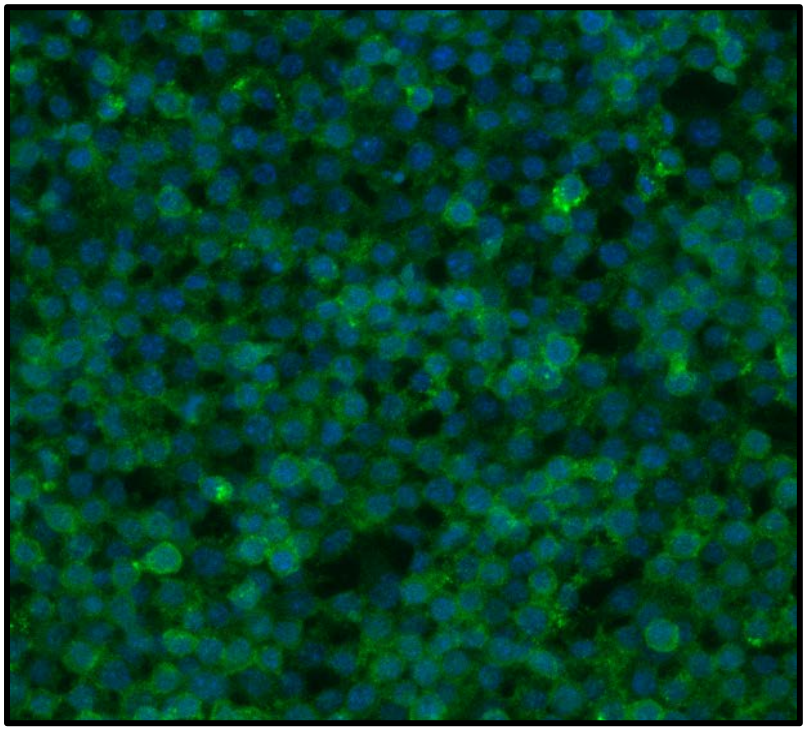

Figure 16. Basigin-variant-2 is expressed on the surface of RAW 264.7 cells. The green fluorescence indicates Basigin-variant-2 expression. The blue fluorescence indicates DNA.

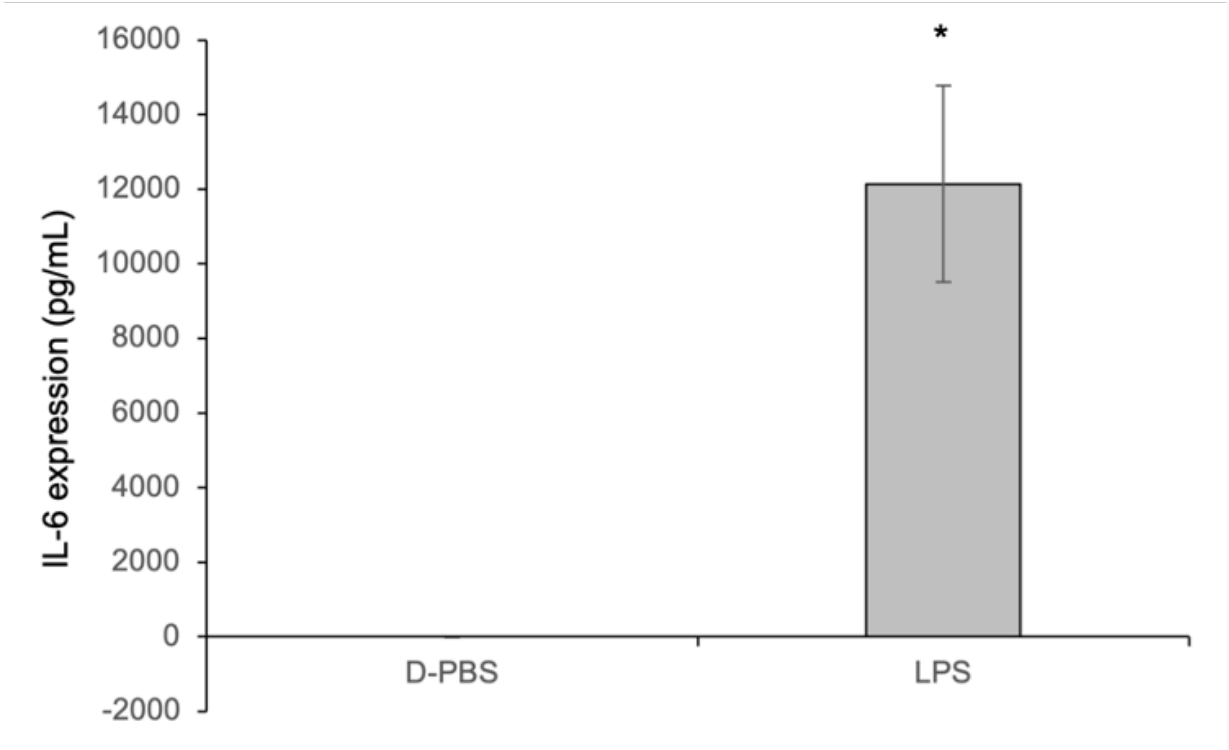

Figure 17. RAW 264.7 cells express Il-6 in response to treatment with LPS. The average pg/mL of IL-6 in culture medium exposed to D-PBS or LPS is shown. The experiment was performed in triplicate. The error bars represent the standard error. * indicates a p-value of less than 0.05 in comparison to D-PBS

The supernatant was assayed for the presence of IL-6 expression via a quantitative IL-6 ELISA. The data indicate neither that the Ig0 domain, the amino-half of the Ig0 domain, nor the binding domain for variant-2 of the Ig0 domain stimulate IL-6 expression in RAW 264.7 cells (Figure 18A). A similar effect was observed when the concentration of the Basigin-variant-1 Ig0 protein was increased to 10 μM. Treatment of the recombinant control protein significantly increased IL-6 expression, in comparison to D-PBS treatment ($p=2.06x10^{-6}$; Figure 18A).

To investigate whether changes in amino acids within the binding region of the Ig0 domain of Basigin-variant-1 were able to induce expression of IL-6, RAW 264.7 cells were treated with 5 μM of the recombinant versions of the mutated Ig0 domain. Similar levels of IL-6 expression were observed when RAW 264.7 cells were treated with the entire Ig0 domain (Ig0-6XHis), the L/G mutation, the C/G mutation, or the P/G mutation (Figure 19). IL-6 production was significantly greater for treatment of Ig0-E/G, in comparison to Ig0-6XHis treatment ($p=0.0001$; Figure 19).

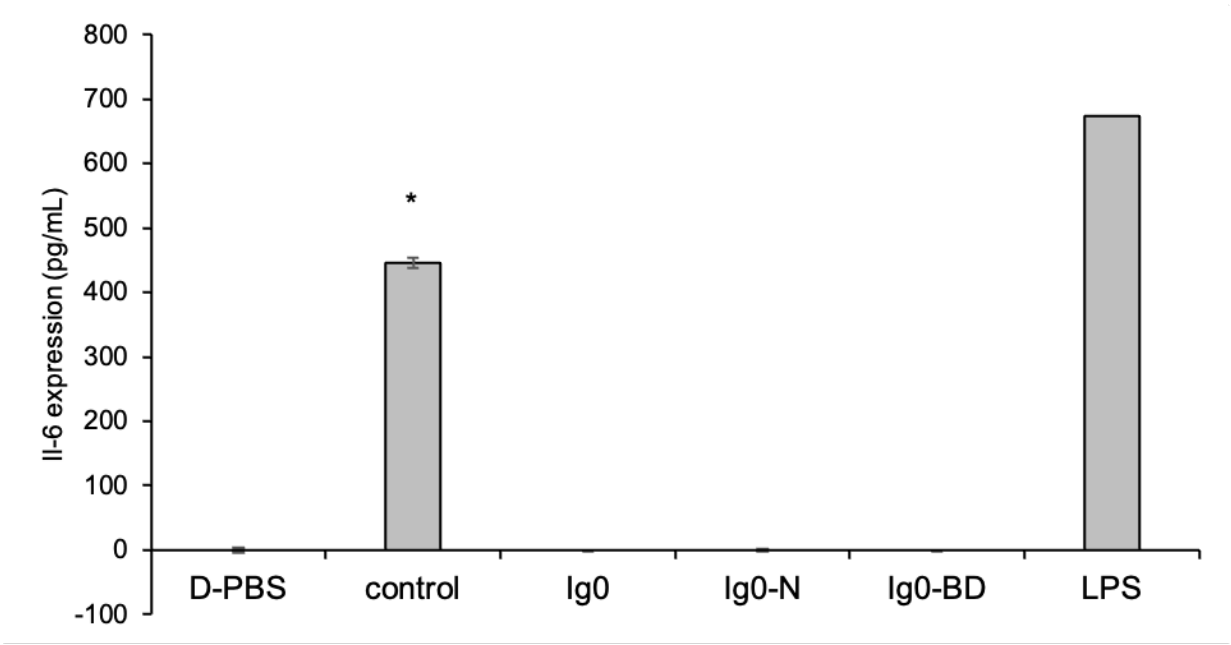

Figure 18. Induction of IL-6 in response to treatment of recombinant Ig0 variants. Five μM of recombinant versions of the entire Ig0 domain (Ig0), the portion of the domain determined to bind to Basigin-variant-2 (Ig0-BD), the amino half of the domain (Ig0-N) were incubated with RAW 264.7 cells. Controls include cells treated with control protein generated from the expression vector used to make the Basigin-variant-1 recombinant proteins, cells treated with D-PBS, and cells treated with LPS. The supernatant was assayed for the presence of IL-6 expression via a quantitative IL-6 ELISA. All runs were performed in quadruplicate. The error bars represent the standard error. * indicates a p-value of less than 0.05 in comparison to D-PBS

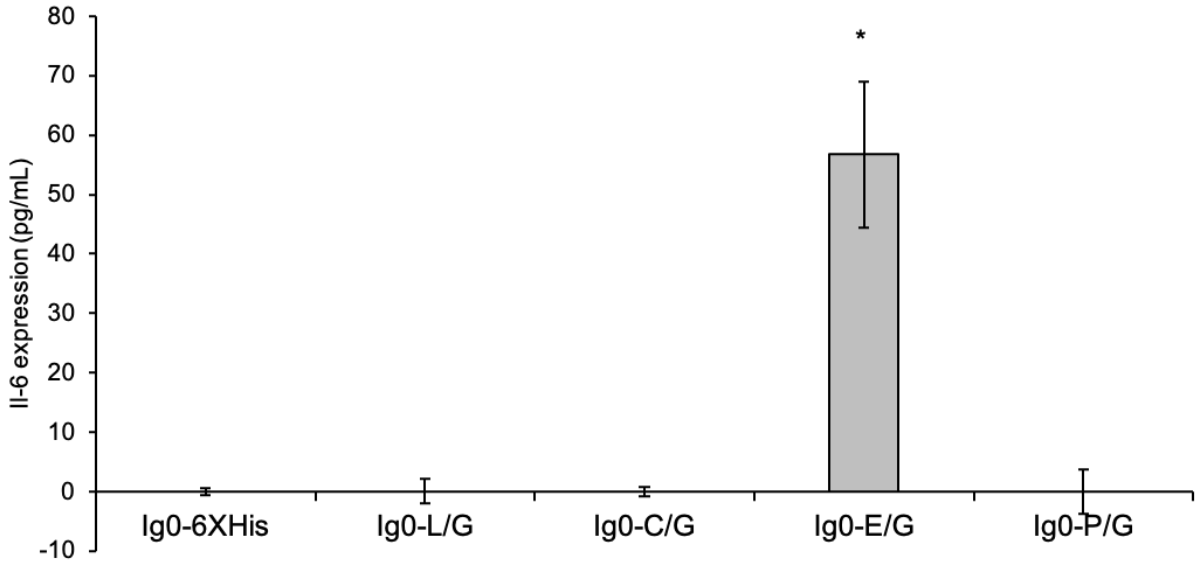

Figure 19. **Induction of IL-6 in response to treatment of recombinant Ig0 mutations.** A recombinant version of the entire Ig0 domain (Ig0-6XHis) and the mutated variants of the Ig0 domain (Ig0-L/G-6XHis, Ig-0-C/G-6XHis, Ig0-E/G-6XHis, Ig0-P/G-6XHis) were incubated with RAW 264.7 cells. The supernatant was assayed for the presence of IL-6 expression via a quantitative IL-6 ELISA. All runs were performed in quadruplicate. The error bars represent the standard error. * indicates a p-value of less than 0.05 in comparison to D-PBS

Chapter 4- Discussion

The Basigin gene codes for two main protein products, including one that is expressed throughout the body and one that is neural retina-specific in expression. The Ig0 domain of Basigin-variant-1, which is the retina-specific form of the protein, is highly conserved throughout evolution, which suggests a conserved function (Ochrietor et al., 2003). Because Basigin gene products are members of the immunoglobulin superfamily, the Ig0 domain of Basigin-variant-1 was hypothesized to participate in cell-cell interactions within the retina (Ochrietor et al., 2003). More recent studies have implicated this protein in metabolism and immune responses (Ait-Ali et al., 2015; Redzic et al., 2011). Specifically, reports demonstrate that the Ig0 domain of Basigin-variant-1 induces expression of IL-6 in a human monocyte cell line (THP-1) and a human kidney cell line (HKC; Redzic et al., 2011). A region of the Ig0 domain of Basigin-variant-1 has been hypothesized to bind to Basigin-variant-2 to form an association between photoreceptor neurons and Müller glial cells in the neural retina (Ochrietor et al., 2003). Basigin-variant-2 is expressed on white blood cells, including monocytes (reviewed by Muramatsu, 2015), and therefore may provide a signaling mechanism through which IL-6 expression could be induced in the THP-1 cells. Therefore, the purpose of the present study was to determine if the region thought to be the Basigin binding domain of the Ig0 domain is the same region of amino acids used to stimulate IL-6 expression in monocytes. Many difficulties were experienced when expressing the recombinant proteins for this study. However, significant amounts of protein were eventually obtained so that the aim of the study could be carried out. The results indicate that the Ig0 domain of Basigin variant-1 does not stimulate the expression of IL-6 in mouse RAW 264.7 monocytes.

During the course of the study, it was exceedingly difficult to express the recombinant proteins necessary for the experiments. Critical factors for soluble expression of recombinant proteins in BL21(DE3) *E. coli* cells include cell density, as well as time of induction (reviewed by Sahdev et al., 2008; reviewed by Ashayeri-Panah et al., 2017). To increase cell density, expression conditions, such as oxygenation, pH, temperature, and nutrients can be modified to enhance recombinant protein production (reviewed by Sivashanmugam et al., 2009). Reports indicate that optimization of bacterial expression conditions is protein dependent (reviewed by Sivashanmugam et al., 2009). In general, the highest yield of protein is obtained through induction at mid-log phase (reviewed by Ashayeri-Panah et al., 2017). At higher cell densities, the metabolic activity of the cells declines due to depleted nutrient, oxygenation, as well as high levels of carbon dioxide (reviewed by Kaur et al., 2018). These conditions decrease the recombinant gene expression, thereby decreasing protein expression (reviewed by Kaur et al., 2018). At lower cell densities, there is a metabolic drag on the cells that contain the plasmid, thus they are selected against when the cells rapidly divide. Therefore, inducing protein expression at low cell densities often causes plasmid loss. In the present study, it was determined that modifying the expression conditions to increase the cell density positively impacted the protein expression of the shorter Ig0 proteins, but this effect was not retained on the longer Ig0 proteins.

In past studies in the Ochrietor laboratory, instead of generating the entire Ig0 domain protein via IPTG induction and overnight incubation at 37^{o}C, expression via the "leaky" T7 promoter occurred over four days at room temperature. This methodology was deemed appropriate because rapid expression induced by IPTG resulted in little to no protein expression. The entire Ig0 domain interferes with the proliferation and homeostasis of BL21(DE3) *E. coli* cells, thereby induction causes a slower growth rate, low final cell density, and cell death

(reviewed by Rosano and Ceccarelli, 2014). However, because the entire Ig0 domain could not be expressed via the T7 "leaky" promoter, the protein expression protocol was adapted by lowering the incubation temperature. Studies indicate growing *E. coli* at low temperature improves the solubility of difficult proteins by increasing the stability and correct folding patterns (Sahdev et al., 2008). Low temperature has this effect due to the fact that hydrophobic interactions influencing inclusion body formation are temperature dependent. In addition, lower incubation temperatures suppress any expression associated toxic phenotype that is typically observed at 37°C (Sahdev et al., 2008).

The purification of the entire Ig0 domain was also problematic, in that the protein co-eluted with various other proteins. There were multiple variables tested to decrease non-specific binding, however, none proved advantageous. It was initially suspected that the eluted proteins contained a six-histidine tag, believing they were proteolytically digested Ig0-6XHis protein. However, an immunoblot suggested the additional proteins are bacterial proteins. Previous research shows the utilization of immobilized metal affinity chromatography (IMAC) in the presence of native *E. coli* proteins that have a high affinity for divalent cations is problematic (reviewed by Bolanos-Garcia and Davies, 2006). According to previous research, the majority of eluted bacterial proteins are stress responsive proteins, which are induced in stress conditions such as nutrient starvation, heat shock, and oxidative damage. Therefore, the relative level of contaminant *E. coli* protein is dependent upon culture conditions (reviewed by Bolanos-Garcia and Davies, 2006). This suggests that although the conditions for expression of the entire Ig0 domain allowed for recombinant protein production, they were not optimal for the bacterial cells. The native proteins from *E. coli* that are commonly co-purified, signifying stressful conditions, possess the same molecular weight as the proteins co-eluted with the entire Ig0 domain

(reviewed by Bolanos-Garcia and Davies, 2006). Thus, confirming the conditions for expression of Ig0 were not favorable.

For the cell based studies, it was determined that the RAW 264.7 cell line was appropriate based on its expression of Basigin-variant-2 and ability to response to LPS. Despite this, the entire Ig0 domain did not stimulate the expression of IL-6 in the mouse monocyte cell lines. This was surprising, as it was expected that a false positive would have been observed because of the contaminating bacterial proteins in the recombinant protein sample. This lack of IL-6 expression in RAW 264.7 cells in response to the Ig0 domain contradicts previous data observed in THP-1 monocytes (Redzic et al., 2011). This difference could be a result from quantification of IL-6 expression, as Redzic et al. (2011) quantified fold increase of IL-6 while the current study quantified the concentration of IL-6 expressed in pg/mL. Thus, the degree of difference in IL-6 expression observed could be due to absolute quantification versus relative quantification. In addition, Redzic et al. (2011) utilized a human monocytic cell line while the current study utilized a mouse monocytic cell line, so the differences observed could be attributed to the variation of cell line. Lastly, the experimental control for Redzic et al. (2011) included cells that were not treated, and cells treated with buffer. The lack of control protein generated by the expression plasmid through which the recombinant proteins were made Redzic et al.'s (2011) study, suggests that the difference in observation could be attributed to a lack of a control. Normalizing the data to unstimulated cells, or cells treated with buffer could lead to overestimation of the expression of IL-6.

The quantitative ELISA IL-6 data also indicated that the recombinant control protein significantly increased IL-6 expression, in comparison to D-PBS treatment. The response observed by the control protein suggests the plasmid ligated without a cDNA insert has the

ability to stimulate IL-6 expression. When ligated in the presence of water, rather than cDNA product, the "insert" consists of the four amino acids Aspartate, Isoleucine, Valine, Glutamine. The data suggest that this sequence, which is not found within the Ig0 recombinant proteins, is proinflammatory. Future studies should investigate the utilization of the pET102/TOPO-D plasmid as an effective control in the induction of IL-expression.

An additional quantitative IL-6 ELISA assay was performed to understand if specific amino acid residue(s) changed the ability of the Ig0 domain protein to stimulate IL-6 expression. Similar levels of IL-6 expression were observed for the entire Ig0 domain and the Ig0 mutations, except for Ig0-E/G. Ig0-E/G was the only treatment that stimulated IL-6 expression, suggesting that mutating the glutamate to glycine removes the inhibition of IL-6 expression. The significance of this observation has yet to be determined.

The amino acid residues vital in the interaction of Basigin-variant-1 and variant-2 were also investigated. Although each of the mutated proteins showed reduced binding to Basigin-variant-2 when compared to the Ig0 protein, no significant differences were observed. It is believed that the small sample size and the lack of the ability to perform replicate experiments was the chief explanation for the lack of significance. Due to the trend towards significance, it is expected that there will be a significant difference in binding by increasing the statistical power.

In conclusion, the results of this study indicate that higher cell densities are needed for optimal protein expression, and expression conditions, such as oxygenation, pH, temperature, and nutrients can be modified to enhance cell growth. In addition, the Ig0 domain of Basigin-vairant-1 does not induce IL-6 expression in RAW 264.7 monocytes. The results of the current study contradict previous data that suggests the extracellular domain of Basigin-variant-1 domain induces an immune response via IL-6 production in THP-1 monocytes (Redzic et al., 2011). The

questions remains whether a retina-specific protein can truly induce and innate immune response.

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Abigail D. Tompa and Judith D. Ochrietor
The immune response in the neural retina is not
mediated by the Ig0 domain of Basigin-variant-1
American Society of Cellular Biology
Virtual Event, December 2020

Abigail D. Tompa and Judith D. Ochrietor
The Ig0 domain of Basigin variant-1 stimulates
IL-6 expression in RAW 264.7 monocytes
American Society of Cellular Biology
Washington, D.C., December 2019

Abigail D. Tompa and Judith D. Ochrietor
The Basigin-variant-2 binding domain in the Ig0 domain of Basigin-variant-1
stimulates an immune response in the mouse monocyte RAW 264.7 cell line
Experimental Biology Conference
Orlando, Florida, April 2019

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Showcase of Ospreys Advancements in Research and Scholarship Jacksonville,
Florida, April 2019

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