PURIFICATION OF SIMPL ANTIBODY AND IMMUNOFLUORESCENCE OF SIMPL SUB-CELLULAR LOCALIZATION IN RESPONSE TO TNFα- AND IL-1

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Abstract

Steven B. Cogill

PURIFICATION OF SIMPL ANTIBODY AND IMMUNOFLUORESCENCE OF SIMPL SUB-CELLULAR LOCALIZATION IN RESPONSE TO TNF-α AND IL-1

SIMPL is a transcriptional co-activator that alters the activity of transcription factor, NF-κB. In response to pathogens, cytokines such as Interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF) signal through the IL-1 and TNF-α receptors, respectively, which are found on various cell types. Activation of these receptors can result in the nuclear localization of NF-κB where it enables the transcription of several different genes key in the innate immune response. Endogenous co-localization of the SIMPL protein with NF-κB in response to these same cytokine signals has yet to be demonstrated. Polyclonal antibody generated against a truncated version of the SIMPL protein was purified from the sera obtained from immunized rabbits using affinity chromatography. The antibody was found to have a high specificity for both the native and denatured form of the protein as demonstrated by the lack of nonspecific bands observed in immunoprecipitations and Western blotting. The antibody was utilized in immunofluorescence experiments on mouse endothelial cells that were either unstimulated or were stimulated (IL-1 or TNF-α). In the absence of cytokine, SIMPL was localized in both the cytoplasm and the nucleus as opposed to NF-κB which was almost exclusively localized in the cytoplasm. In the presence of IL-1, the concentration of SIMPL in the nucleus was increased, and in the presence of TNF-α, the concentration of SIMPL in the nucleus was even greater. Results of this study identified future routes for SIMPL antibody isolation as well as to

demonstrate that endogenous SIMPL protein nuclear localization may not be solely dependent upon TNF-α signaling.

Maureen A. Harrington PhD, Chair

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List of Abbreviations

BSA	Bovine serum albumin
BME	β-mercaptoethanol
CAK1	Cyclin dependent protein kinase-activating kinase
dd	double distilled
DAPI	4’,6-diamidino-2-phenylindole, dihydrochloride
ELISA	enzyme-linked immunosorbent assay
ES	embryonic stem
EST	expressed sequence tag
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
Ig	immunoglobulin
IL-1	Interleukin-1
IRAK-1	Interleukin-1 receptor associated kinase
KO	knock-out
LPS	Lipopolysaccharide
MEF	Mouse embryonic fibroblast
MIP	maximum-intensity projection
mPLK	mouse pelle-like kinase
NF-κB	Nuclear Factor-κB
PAMPs	pathogen-associated molecular patterns
PAGE	poly-acrylamide gel electrophoresis
PVDF	polyvinylidene fluoride
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween 20
PCR	polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
RHD	Rel Homology Domain
Risc	RNA-induced silencing complex
SIMPL	signaling molecule that interacts with mouse pelle-like kinase

TIGM	Texas Institute for Genomic Medicine
Tlr 4	Toll-like receptor 4
TNFα	Tumor Necrosis Factor α
TNF-R1	Tumor Necrosis Factor Receptor-1
UV	ultraviolet
WT	Wild type

INTRODUCTION

The goal of my thesis was to further the study of a transcriptional co-activator of the nuclear factor that binds the enhancer of the κ chain (NF-κB). This molecule is called signaling molecule that interacts with mouse pelle-like kinase (SIMPL). This introduction will act to give a broad overview of the immune response and inflammation to illustrate the importance of NF-κB and the far reaching effects of its regulation. Also the introduction will focus on some of the biotechnological aspects of the project, including knockout mouse production through the use of gene trap vectors as well as the purification and production of antibodies and their applications. The introduction is concluded by describing the work that has previously been done by the Harrington lab on SIMPL and its interacting protein, interleukin-1 receptor associated kinase (IRAK-1).

A. The immune response

The immune system is an organism's defense against foreign elements such as pathogens or other possibly harmful agents. There are two arms of this system, the innate and adaptive, which must operate in a coordinated fashion to mount an appropriate response. The innate immune system is an evolutionarily conserved system that acts in a non-specific manner towards pathogenic insults. The innate response acts to initiate inflammation, recruit other innate immune cells to the site of infection, and mobilize the adaptive response. It is activated through the recognition of the general signatures of pathogenic agents known as pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway 2002). Innate response cells such as macrophages are localized throughout tissues and organs. The macrophage cells which are distinct to their respective tissues or organs can act as the initial detectors of pathogenic insults through receptors which are constant from cell to cell within a tissue (Glaros et al. 2009; Gordon 2007). PAMP receptors can include the family of toll-like receptors; for example toll-like receptor 4 (Tlr4) recognizes lipopolysaccharides (LPS) found in the cell wall of gram negative bacteria. The LPS binds to Tlr4 and can activate transcription factors including NF-κB which in turn up regulates the transcription of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) (Beutler et al. 1985; Du et al. 1999; Li et al. 2002). These pro-inflammatory cytokines are secreted by the macrophages, and act to initiate the

inflammatory response. The adaptive immune cells or lymphocytes act as secondary responders which are targeted towards specific antigens. The adaptive arm of the immune system also has the capability to mount a memory response which is more rapid and greater in magnitude than the native adaptive response as well as the innate response. Another species of local innate immune cells, the dendritic cells, are one of the first coordinating links between the innate and adaptive response. These cells when activated become antigen presenting cells that transport antigens to the lymph tissues that house the lymphocytes (B and T cells) (Austyn et al. 1983; Qi et al. 2006). Naive helper T cells are activated by antigens binding to T cell receptors specific to the antigen, and this causes a clonal expansion of T cells which contain the targeted receptor. These cells in turn differentiate into cytotoxic T cells as well as memory T cells. The helper T cells remain in the lymph tissue where they secrete cytokines and chemokines to manage the activity of B cells, cytotoxic T cells, and macrophages (Hommel 2004; Andersen et al. 2006; Bonilla and Oettgen 2010) The B cells which also undergo receptor specific clonal expansion upon exposure to antigens secrete antibodies that target antigens which allows for degradation by natural killer cells. The antibodies also work with the complement system to target pathogens. Other roles of antibodies include blocking key receptors due to binding as well as segregation of possibly key proteins both of which may hinder pathogen survival (Carroll 2008; Zola 1985). Activation of NF-κB through T cell receptors and B cell receptors plays a key role in lymphocyte maturation, survival, activation, and clonal expansion (Gerondakis and Siebenlist 2010).

B. Inflammation

Acute inflammation arises in tissues and organs in response to threats to the organism such as pathogenic invasion or tissue damage. The inflammation response is the remodeling of the microenvironment at the site of the specific insult to allow for the effective removal of the threat in question and a return to homeostasis for the organism. The effects of inflammation were observed on a macroscopic scale centuries ago which led to the common indicators of inflammation, redness, swelling, pain, and heat (Libby 2007). These symptoms arise from changes in the blood vessels at the site of infection to allow for the influx of immune cells. Alterations to the vessels include clotting, secretion of proteins to adhere circulating immune cells to the vessel wall, and development of

looser junctions between adjacent cells for greater permeability (Nathan 2002). These changes are initiated by the secretion of the pro-inflammatory cytokines TNFα and interleukin-1 (IL-1) by the local macrophages which act on endothelial cells (Goerdt et al. 1987; Schorer et al. 1986). Activation of NF-κB through cytokine receptors is essential to the inflammatory response. For example tissue factor, a protein secreted by the endothelial cells to aid in the clotting process is transcriptionally controlled by NF-κB (Bierhaus et al. 1995; Zhang et al. 1999). Pro-inflammatory signaling acts to remove any pathogens present, and upon its removal, the response initiates tissue repair through production of anti-inflammatory molecules (Nathan 2002).

Implications of inflammatory response dysregulation are diverse and far reaching. An overly robust inflammation response could result in extensive cellular damage. Elevated levels of pro-inflammatory cytokines including TNFα and IL-1 have been linked with organ sepsis (Cavaillon et al. 2003). In contrast, a weakened initial response can lead to an overwhelming infection (Hotchkiss et al. 1999). Chronic inflammation contributes to the pathogenesis associated with diseases such as arthritis and atherosclerosis (Libby 2007). In breast cancer, the invasion of immune cells to tumors has also been shown to alter the local environment mainly through the secretion of cytokines to promote growth and even metastasis (DeNardo and Coussens 2007). The effect of inflammation on various diseases is currently an intense area of research.

C. Production of knockout mice through gene trapping

In studying the function of a target protein, reverse genetic methods are commonly employed. These involve the removal or "knock out" (KO) of the gene encoding the protein of interest and the studying of the resulting phenotype. Previous studies done by the Harrington lab utilized small hairpin RNA (shRNA) to "knock down" the steady-state level of SIMPL protein. The SIMPL shRNA was effective in knocking down SIMPL RNA and protein levels to further elucidate its role in TNFα induced activation of NF-κB (Benson et al. 2010). To study the phenotypic effects of a complete loss of SIMPL protein and further understand its function, a KO strain of mice was produced using gene trapping technology.

Gene trapping is a high throughput and thus cost effective reverse genetics technique. Currently there are commercially available libraries of mouse embryonic stem (ES) cells

that contain targeted disruptions in a majority of the mouse genes (Zambrowicz et al. 2003). The technique relies on random insertions of a gene trap cassette throughout the genome in mice ES cells using either retroviral transfection or transformation with plasmids. The insertion event is detectable through the insertion of a lacZ gene or by insertion of a gene that confers antibiotic resistance. In modern gene trap vectors, the selectable marker is promoterless, and the cassette contains a splice acceptor site that causes a splice variant where the marker sequence is in frame with upstream exons. Another feature is the poly A tail which produces a functional transcript to express the selectable marker as well as disrupting the transcription of the remaining sequence. The selection process also allows for the trapping of genes that are not actively expressed in ES cells (Lee et al. 2007; Zambrowicz et al. 1998). Due to the mapping of the genome and the extensive databases of expressed sequence tags (EST), reverse transcription-polymerase chain reaction (RT-PCR) can be used to identify trapped genes (Zambrowicz et al. 2003; Lee et al. 2007; Zambrowicz et al. 1998). In this study the targeting vector promoted the transcription of an exon that contained three different stop codons to prevent the translation of the sequence.

D. Antibodies

Antibodies which are also known as immunoglobulins (Ig) are a group of glycoproteins that function in a humoral capacity. They are expressed exclusively by mature B lymphocytes (Carroll 2008; Zola 1985). The antibody structure consists of two respectively identical heavy and light chains consisting of constant and variable regions, and the chains are joined together by disulfide bonds. The heavy chain contains three constant regions that are preceded by a variable region on its amino terminus. The light chain has only one constant region which is also preceded by a variable region. There is a hinge in the heavy chain upstream of the two carboxyl constant regions near the disulfide bond which essentially "juts" out the chains to give the antibody a "Y" shape. The portion of the antibody that lies upstream of the hinge which contains the variable regions is designated either FAb or Fab'^{2} dependent upon whether the division is made above or below the disulfide bond that joins the two heavy chains. The remaining four heavy chain constant components makeup the Fc region (Guddat et al. 1993; Kirkham and Schroeder 1994). Five classes of Ig's exist in humans: IgM, IgA, IgD, IgE, and the most common,

IgG. The different Ig classes differ in their respective Fc regions which target them towards specific cell types expressing complementary Fc receptors (Bournazos et al. 2009). Antigens are bound by the variable regions of an antibody which determine its specificity for an antigen. Lager molecules such as proteins can contain several binding sites for antibodies known as epitopes. Due to an increased mutation rate and recombination of the gene segments coding for the variable regions in both chain types, millions of different combinations can be formed which allows for the expression of individual antibodies specific to nearly every known compound (Kirkham and Schroeder 1994).

1. **Applications**

Antibodies are diverse in their uses in diagnostic assays. They are commonly used to characterize proteins. *In vitro* applications include Western blot analysis, immunoprecipitation, and enzyme linked immunosorbent assays (ELISA). In Western blot analysis and ELISA, antibodies are used to confirm the presence of a target protein usually in a complex mixture such as cell or tissue lysate. Western blots and ELISA's detect the presence of a target protein through the use of either the primary antibody or a secondary antibody that recognizes the primary antibody. The primary or the secondary antibody can be detected by its conjugation to fluorescent molecules, radioactive labels, or peroxidase enzymes which catalyze a reaction to produce a signal (Burnette 1981; Engvall and Perlmann. 1971). Immunoprecipitation reactions are used to isolate a target protein from complex mixtures. Antibodies against the target protein in a lysate are bound by the Fc region to Protein A or G coupled sepharose beads which precipitate out the target protein through the application of centrifugal force. This technique is commonly used to determine the physical interactions of the target protein as any proteins that complex with the target protein will also be precipitated from the solution. Once isolated the protein and additional complexing proteins can be detected by Western blotting or mass spectrometry (Lal et al. 2005). Cellular based assays that utilize antibodies include immunofluorescence microscopy and fluorescence activated cell sorting (FACS). In immunofluorescence microscopy, cells are fixed to allow for the organization of the cell to be maintained and to allow for antibodies to permeate the cell and bind the target protein. Secondary antibodies conjugated with a fluorescent molecule

are commonly used to detect the primary antibody. The technique can be used to determine the sub-cellular localization of proteins within a cell at the time of fixation, and immunofluorescence was used in this study to show the localization of the target protein SIMPL (Catino et al. 1979).

2. **Production**

Antibodies produced by various techniques generate polyclonal or monoclonal antibodies. A broad range of engineering techniques can be performed *in vitro* or *in vivo* to generate the antibodies. Although other techniques may yield a greater specificity, polyclonal antibodies offer a more robust detection. The polyclonal and monoclonal production techniques differ in the number of distinct clonal B cells producing antibody (Köhler and Milstein 1975; Lipman et al. 2005). In this study, polyclonal antibody was used. Polyclonal antibodies are produced by exposing a naive or previously unexposed animal to a target antigen which in turn causes the animal to produce antibodies against the foreign substance. The best choice of animal to inoculate would be a young adult of the species due to the peak effectiveness of its immune response and its lack of exposure to a wide range of antigens. If the animal has been exposed to other antigens, then contamination of the polyclonal antibody can occur as well as cross reactivity. Also, ideally a sufficient phylogenetic distance needs to exist between the species that produced the antigen and the species that is to be inoculated (Leenaars and Hendriksen 2005). An inoculated animal will produce a panel of antibodies recognizing different epitopes of the antigen which in this study is the SIMPL protein. The antibodies against the target protein can then be harvested from the animal's serum (Lipman et al. 2005; Leenaars and Hendriksen 2005).

3. **Purification**

The route of antibody purification from a complex solution is dependent upon the source. In this study, the source was serum from inoculated rabbits which contains a heterogeneous mixture of antibody. Even though the end product was polyclonal not all of the antibodies present in the serum is desired, therefore affinity column chromatography represented the best option to purify antigen specific antibody. Affinity columns use the target antigen, a fragment of the antigen, or a synthesized mimetic of the antigen to isolate the targeted antibodies. The antigen is coupled to what will make up the

column bed and act as the stationary phase and the solution containing the antibody of interest will act as the mobile phase. In most instances as well as in this study, the antigen is coupled to sepharose beads (Frenkel 1974; Roque et al. 2007).

E. Elucidating the role of SIMPL protein

1. NF-κB activation

NF-κB is a family of transcription factors that greatly impacts the fate of the cell. It can affect the fate of cells in many ways including induction of anti-apoptotic signaling, regulation of cell cycling, and it is known to have an impact on cell adhesion and cell migration (Cardoso and Oliveira 2003; Pham et al. 2003; Takeuchi and Baichwal 1995). Aberrations in its activation have been linked to cancer as well as other diseases such as atherosclerosis and diabetes (Bours et al. 1994; Nakajima et al. 1994; Mollah et al. 2008).

NF-κB exists as either a homo or heterodimer. Five separate genes can contribute subunits to the protein. In its most common form it is a heterodimer of one of the three Rel gene products and either p50 or p52 which are derived from larger precursors. All of the NF-κB proteins contain what is referred to as a rel homology domain (RHD) which forms the dimers and binds DNA, but only the three Rel genes (c-Rel, RelA and RelB) contain a transactivation domain. The activation of NF-κB contains many intricacies, but there are two basic schema for its translocation into the nucleus. Essentially, NF-κB is sequestered to the cytoplasm by its binding to inhibitory factor IκB of which there are several forms. IκB can be subsequently phosphorylated by the IκB kinase (IKK) complex which is made up of IKKα, β, and γ. This phosphorylation event causes the dissociation of IκB from NF-κB and targets the IκB protein for ubiquitination and eventual degradation by the proteasome. Once NF-κB is free, it localizes to the nucleus where it binds DNA and increases or decreases the transcription of genes involved in the immune response. This is what is referred to as the canonical pathway (Figure 1). Another possible pathway for the activation of NF-κB is the alternative pathway where a Rel protein precursor contains an inhibitory domain that acts to prevent nuclear localization of the protein. It can later be processed by the proteasome leading to activation of the complex (Vallabhapurapu and Karin 2009).

2. The importance of mPLK/IRAK-1

To further describe the activation of NF-κB in response to innate immune signals, the immune system signaling pathways of *Drosophila* were studied by the Harrington lab. It was already known that *Drosophila* contained a homologue of NF-κB and its inhibitor IκB. Upstream of these two proteins in the same pathway was the serine/threonine kinase pelle. Through comparative sequence analysis, a homologue was isolated from mice that was named mouse pelle-like kinase (mPLK) (Trofimova et al. 1996). The human homologue of this kinase was found through immunoprecipitation studies of the interleukin-1 receptor and was named the interleukin-1 receptor associated kinase (IRAK-1) (Cao et al. 1996). Further studies focused on the role of mPLK/IRAK-1 in the transcription of known NF-κB activated genes in response to certain innate immune response signalers namely IL-1 and TNFα. It was determined that the presence of mPLK protein does indeed impact the level of NF-κB activated transcription in response to IL-1, but it was also found that the same is true for TNFα signaling. This implied that mPLK/IRAK-1 is not only associated with the IL-1 receptor but also the TNFα signaling pathway. Interestingly, mPLK catalytic activity was found to not be needed to induce changes in the activity of NF-κB induced by IL-1, but mPLK catalytic activity was required in the TNFα induced pathway (Vig et al. 1999) (Figure 2).

3. The discovery of SIMPL

In a search for proteins that interacted with mPLK, a protein was identified using a yeast two hybrid system and mass spectrometry. The corresponding cDNA was cloned, and the amino acid sequence was deduced. The gene was designated SIMPL. The identity of the protein and its association with IRAK-1 was confirmed in immunoprecipitation assays. The size of the SIMPL protein in humans is 29.1 kDa and in mice is 28.8 kDa. SIMPL is also known as IRAK binding protein-1 (IRAKBP-1). Other than its association with mPLK/IRAK-1, at the time of its discovery nothing was known about the protein's function. No conserved domains were found within its primary sequence, but early experiments showed that SIMPL impacted NF-κB transactivation activity (Vig et al. 2001).

It was later discovered that SIMPL acted as a TNFα dependent transcriptional co-activator of NF-κB. Utilizing FLAG tagged SIMPL constructs, it was demonstrated

through immunoprecipitation assays that SIMPL could be found in RelA (also known as p65) containing complexes. The association was dependent upon subsequent signaling of the cells with TNFα. When the cells were treated with the cytokine IL-1, the physical association between SIMPL and p65 was not found. This implied that since p65 went into the nucleus when the cell was stimulated with TNFα that the SIMPL protein was more than likely associating with the protein within the nucleus (Figure 3). The SIMPL protein sequence does contain a nuclear localization signal on its carboxyl terminus, and immunofluorescence studies performed on human embyronic kidney epithelial cells using FLAG tagged SIMPL constructs showed that the protein does indeed localize to the nucleus when over-expressed. It was also shown that a removal of the nuclear localization targeting sequence prevented the protein from entering into the nucleus (Kwon et al. 2004).

The goal of my thesis was to purify SIMPL antibody and to use the purified antibody to study the subcellular localization of SIMPL.

METHODS AND MATERIALS

A. Wild type and SIMPL knockout mice

Heterozygous mice containing a single targeted SIMPL gene were generated by the Texas Institute for Genomic Medicine (TIGM) using the VICTR Vector 48 gene trap vector. The integration of the trap was mapped to the first intron of the SIMPL gene on chromosome 9. The targeted ES cells (129Sv) were injected into pseudopregnant C57Bl/6 mice. Germline transmission of the targeted SIMPL gene was confirmed by polymerase chain reaction (PCR). Two sets of breeding pairs were received from TIGM and have been used to generate two independent knockout (KO) and littermate control mouse lines. The mice are currently being backcrossed with wild type (WT) C56Bl/6 mice.

B. Cell lines

The mouse endothelial cell line was kindly provided by Matthias Clauss (IU School of Medicine).

C. Reagents

NF-κB p65 (c-20) rabbit polyclonal IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human (rHu) TNFα was purchased from Sigma (St. Louis, MO), and rHu IL-1 was provided by Hoffman-La Roche. Purified recombinant Δ23SIMPL protein was generated in *E. coli* and kindly provided by Dr. Millie Georgiadis (IU School of Medicine). The purified Δ23SIMPL protein lacks the first 22 amino acids of SIMPL and contains four additional amino terminal residues. The AminoLink® Immobilization Kit was purchased from Thermo Scientific (Rockford, IL). The protein assay kit utilized in the Bradford assays was purchased from Bio-Rad (Hercules, Ca). Complete® Mini Protease Inhibitor Cocktail Tablets were purchased from Roche (Indianapolis, IN).

D. Antibody purification

The Δ23SIMPL protein was sent to Covance Research Products (Denver, PA) for antibody generation in rabbits. Two rabbits [086 and 087] were inoculated with the protein, and serum was isolated for each specimen. The isolated sera were initially stored at -80 ºC and later stored at -40 ºC. The Δ23SIMPL protein (5.6 mg/ml) was stored in 50

mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer at -40 ºC. To the AminoLink column, 2.24 mg of Δ23SIMPL protein was coupled. Antibody purification from the rabbit sera was performed accordingly with the manufacturer's protocol (Pierce). Antibody solutions that were not dialyzed were stored at 4 ºC in elution buffer (0.2 M glycine-HCl) neutralized to pH 8 with 1 M Tris-HCl (pH 9) and 0.05% (vol/vol) sodium azide. For long term storage, antibody solutions were kept at -40 ºC.

1. **Dialysis**

Antibody solutions isolated from the column (8 ml) were dialyzed overnight at 4 ºC. Samples were pipetted into SnakeSkin Pleated Dialysis Tubing from Thermo Scientific (Rockford, IL) with a molecular weight cutoff of 7,000 Da. The dialysis tubing was placed in 4 L of phosphate buffered saline solution (PBS) at pH 7.4 with a spin bar set at 60 rpm.

2. **Stringent column wash**

Using a P100 Pipetteman, two 10 ml volumes of PBS solution were passed through the SIMPL affinity column. Next, five 10 ml volumes of guanidinium (4 M) were passed through the column. To wash the column, five 10 ml volumes of PBS were passed through the column. Then five 10 ml volumes of NaCl (1 M) were passed through the column. Finally, the column was washed again with five additional 10 ml volumes of PBS. Representative 1 ml aliquots were taken for each wash.

E. Protein quantification

Protein concentration was determined by two different methods, Bradford assay and ultraviolet (UV) absorbance at 280 nm. The protein concentrations of lysed tissue samples were measured by Bradford assay. The concentrations of purified protein samples were measured by UV absorbance with the exception of the first two antibody purifications (086B and 087B) which were measured via Bradford assay.

1. **Bradford assay**

Bovine serum albumin (BSA) standards and samples (5 µl respectively) were diluted in 800 µl of 0.25 M Tris-HCl at pH 8 and 200 µl of Bio-Rad Protein Assay Dye. Solutions were mixed by inversion. The absorbance for both the standards and the samples were read at 595 nm in a Beckman DU-62 spectrophotometer with the buffer and dye solution acting as a blank. The absorbance values for the standards were plotted

using the Microsoft Excel program. A linear regression fit was applied to generate a standard curve to determine the protein concentrations of the samples.

2. **UV Absorbance**

The absorbances of purified protein samples (2 µl each) were measured using a NanoDrop (ND-1000) Spectrophotometer at 280 nm. The instrument was blanked with 2 µl of the respective sample solutions. The general reference setting of 1 Abs = 1 mg/ml was applied to determine the sample protein concentrations.

F. SDS-PAGE and Coomassie blue staining

Protein samples (2 µg-5 µg) were diluted 1:1 with Laemmli sample buffer containing 5% β-mercaptoethanol (BME). The diluted samples were then placed in a boiling water bath for 5 min. Samples were next loaded onto 10% polyacrylamide gels with a 5% stacking gel. Polyacrylamide gel electrophoresis (PAGE) was performed in Tris-glycine sodium dodecyl sulfate (SDS) running buffer (0.3% (wt/vol) Tris, 1.44% (wt/vol) glycine, 0.1 % (wt/vol) SDS at pH 8.3). Gels were ran at 80 V until the dye front passed through the stacking gel and at 100 V through the separating gel until the dye front reached the bottom of the gel. Gels were washed briefly with double distilled (dd) H_2O and then incubated in destaining solution (10% (vol/vol) glacial acetic acid, 10% (vol/vol) methanol) for 10 min. Next, gels were washed a second time in dd H_2O and then incubated in Coomassie blue stain for 20 min. The stained gels were left in destain solution with a Kimwipe to act as a wick for the dye until the desired resolution was reached. All destaining and staining steps were performed on a rocking platform.

G. Western blotting

Tissue samples isolated from littermate control and KO mice were frozen in liquid nitrogen and ground with a mortar and pestle into a fine powder. Samples to be stored long term were kept at -40 ºC. The ground tissue samples were lysed in PLC buffer (50 mM HEPES at pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, ½ tablet of protease inhibitor per 10ml of solution) at 4 ºC for 20 min on a rotating Nutator. The lysis solutions were then sonicated with a Branson Sonicator and centrifuged at 14,000 x g for 20 min. Supernatants were collected for Western analysis and pelleted materials were discarded. Immobilon-P polyvinylidene fluoride (PVDF) membranes purchased from Millipore (Billerica, MA) with a pore size of 45 µm were

used to capture transferred proteins. Membranes were activated by submersion for 2 min in ddH_2O, 2 min in methanol, and finally 5 min in transfer buffer (10 mM N-cyclohexyl-3-aminopropanesuolfonic acid (CAPS) containing 15% methanol). Polyacrylamide gels were placed in a liquid transfer apparatus with the activated membrane, Whatmann paper, and transfer buffer. Proteins were transferred at 20 V overnight (~16 h) at 4 ºC. The membranes were then removed from the apparatus and washed 3 times for 5 min in PBS with 0.05% Tween 20 (PBST). Membranes were next incubated for 1 h in blocking solution (5% (wt/vol) non-fat dehydrated milk and PBST). Primary antibody was then added to the blocking solution, and the membranes were incubated for 1.5 h with the primary antibody. Following this the membranes were washed again in PBST 3 times for 5 min each. Membranes were next incubated in blocking solution containing horse radish peroxidase conjugated secondary goat anti-rabbit antibody for 1 h. Membranes were then washed a final time in PBST 3 times for 5 min. All blocking and antibody incubations took place at room temperature on a rocking platform. The ECL or ECL Plus kit, depending on the expected strength of the signal (GE Healthcare) was added to the membranes for 1 min. Membranes were then exposed to Kodak film and processed in a commercial grade film developer.

H. Immunoprecipitation

Tissue samples (250 µg-1 mg) were diluted to 1 ml in PLC buffer containing protease inhibitors. Primary antibody (1 µg) against the protein of interest was added, and the solution was kept for 2 h at room temperature on a Nutator. Upstate Protein A Agarose Fast Flow beads mixed in a 10% slurry with PLC buffer were added (100 µl) to the sample. Samples were incubated for 1 h at room temperature on a Nutator. Next, the samples were spun briefly at 14,000 x g in an Eppendorf microfuge, and the supernates were removed by aspiration. Pelleted materials were washed 3 times by re-suspension in 1 ml PLC buffer and repetition of the centrifugation and supernatant aspiration steps. Final pelleted materials were re-suspended in 35 µl of Laemmli sample buffer containing 5% BME and placed in a boiling water bath for 10 min. Samples were then centrifuged for 5 min at 14,000 x g to remove insoluble materials. Supernates were aspirated and loaded onto polyacrylamide gels.

I. Immunofluorescence

All cells were grown on coverslips submerged in cell culture media in 24-well plates. To prepare for the immunostaining, cells first underwent a methanol fixation. All media was aspirated from each well, and the cell monolayers were next washed with 1 ml of PBS per well. The PBS was quickly aspirated from the wells, and the plates were placed in a -40 ºC freezer for 30 s. Next, 500 µl of ice-cold methanol were added to each well. Plates were then returned to the -40 °C freezer for an additional 7 min. The methanol was removed by aspiration, and the cell monolayers were washed briefly in PBS. The cells were stored overnight in a PBS solution containing 5% BSA at 4 ºC. The coverslips were then incubated for 1 h with the cell monolayer in direct contact with 80 µl of the primary antibody diluted in PBS. The coverslips were washed a total of 3 times for 5 min on a Nutator in 1 ml of PBS. The coverslips were then incubated without exposure to light for 30 min in direct contact with 80 µl of goat anti-rabbit Alexa Fluor 488 fluorescently labeled secondary antibody (Invitrogen; Camarillo, CA). The coverslips were again washed 3 times for 5 min each on a Nutator in 1 ml of PBS while wrapped in aluminum foil to prevent exposure to light. The first wash contained Hoechst stain (1:1000) which stained the nucleus. The coverslips were then briefly washed in ddH_2O which was wicked away before the coverslips were placed with the cell monolayer side down on 15 µl of Geltol on a glass slide. The slides were allowed to dry overnight unexposed to light.

RESULTS

A. Purification of SIMPL antibody from 086 and 087 rabbit sera

1. Recombinant SIMPL protein is effectively bound to the column material

Earlier attempts by the lab to generate SIMPL specific antibody using affinity chromatography were hindered by contamination of the purified antibody solution with SIMPL protein. We hypothesized that recombinant SIMPL, which can form dimers and trimers when stored at a high concentration (data not shown), was not capable of being completely covalently coupled to the column. Specifically only those SIMPL molecules directly binding to the column material through a primary amine group were covalently coupled; those SIMPL molecules binding to other SIMPL molecules were not covalently coupled to the column material. This would likely cause both SIMPL protein that was bound to other SIMPL molecules and antibody to be eluted from the column during the low pH elution. It had been shown previously that the amino termini of both human and mouse SIMPL proteins are disordered using PONDR analysis (Haag Breese et al. 2006). This region was suspected to be the site of formation for multimeric complexes. A truncated version of SIMPL protein lacking the first 22 amino acids (Δ23SIMPL) was generated and purified, and the Δ23SIMPL was covalently coupled to sepharose beads within a column. A Bradford Assay was used to measure the protein concentration of the post binding wash, and there was no detectable protein in the solution (data not shown) indicating 100% binding efficiency of the recombinant protein to the column.

2. Dialysis of purified SIMPL antibody causes the formation of precipitate

In the initial purification of SIMPL antibody (086B), 10 ml of 086 serum were passed through a 7 ml affinity column. Bound antibody was eluted with 8 ml of low pH glycine buffer that were collected in 1 ml aliquots. The protein concentration of each aliquot was measured using a Bradford assay (Figure 4A). The majority of the protein was eluted into the third and fourth aliquots. Next, 6 ml of the 087 serum were passed through the column (Figure 4B). The protein elution profile for the 087 serum purification (087B) was similar to that of 086B with a noticeably reduced signal due to the smaller volume of

serum. The aliquots from the respective purifications were pooled, and the pooled fractions enriched with protein were dialyzed overnight in PBS. After dialysis, a protein precipitate was found in the dialyzate. It was believed that the formation of precipitate was due to the high concentration of the protein, so the protein containing mixtures were diluted by 50% with PBS and stored at 4 ºC. Precipitate was still observed in the solutions. The antibody isolation procedure was repeated with a smaller volume (2 ml) of the 087 serum (087D). The elution profile for this purification showed a broader distribution of protein concentration among the aliquots (Figure 4C). The pooled aliquots were dialyzed, and precipitate was found in the solution. Another purification was performed with 2 ml of the 087 serum (087F). In this purification, the third aliquot contained the highest protein concentration (Figure 4D). To prevent the protein from precipitating out of solution, the pooled fractions were not dialyzed and stored at 4 ºC in neutralized elution buffer. After 48 h the solution did not contain a precipitate. For all further purifications, there was no dialysis step included in the procedure.

3. **Affinity column eluant contains antibody**

To determine if the 087F serum purification contained antibody, 5 µl of the purified material were subjected to SDS-PAGE. As a control, an equal volume of anti-cyclin dependent protein kinase-activating kinase 1 (CAK1) antibody was run in an adjacent lane. The gel was then stained with Coomassie blue dye (Figure 5). Two bands were visible in each lane corresponding to ~55 kDa and ~25 kDa which are respectively the average sizes of the heavy and light chains of an immunoglobulin (Lipman et al. 2005). The banding patterns of the 087F purification and the control antibody also were similar which strongly suggested that the serum purification contained antibody.

B. Western blotting using purified antibody

1. **087F antibody is capable of detecting low concentrations of purified SIMPL**

Northern blot data and previous attempts to detect endogenous SIMPL protein in adult mouse tissue lysates through Western blotting indicated that SIMPL expression is a relatively low abundant protein (Vig et al. 2001). Thus it was deemed necessary to characterize the lower detection limits of the 087F antibody. Three amounts of the purified Δ23SIMPL protein (160 ng, 16 ng, and 1.6 ng) were subjected to Western

blotting with the 087J antibody (Figure 6). This sensitivity test showed extremely dark bands for all three amounts indicating that a strong signal was obtainable with 1.6 ng of the purified target protein.

2. **Immunoprecipitation reactions show SIMPL contamination of the 087F antibody**

Adult mouse testes and kidney tissue had been shown previously to have high SIMPL gene expression, so they were selected to validate the 087F antibody (Vig et al. 2001). To act as negative controls, testes and kidney tissue were also isolated from SIMPL KO mice. The tissues were lysed, and to further concentrate the endogenous SIMPL protein, immunoprecipitation using the 087F antibody was employed. In these experiments, tissue lysate was incubated with 2 µg of the 087F antibody, and the 087F antibody was used to probe the Western blot as well. The blot showed a band corresponding to the approximate size of SIMPL protein in both wild type tissue lanes. A corresponding band of lesser intensity was visible within the SIMPL KO tissue lanes (Figure 7). Due to the intensity of the band at ~29 kDa for the wild type tissue, the 087F antibody appeared to be capable of detecting endogenous SIMPL, but the corresponding band in the KO tissue lane was an indication of contamination within the 087F antibody purification. To confirm the contamination, a Western blot was performed on the 087F antibody and the last 086 serum purification (086H). The two purifications were self-probed with the same antibody to detect if SIMPL protein was present (Figure 8). Both purifications show a band that corresponds to the approximate size of SIMPL protein.

C. Stringent washing of the Amino-Link column

1. **High concentrations of guanidinium are effective in removing excess protein from the affinity column**

It was hypothesized that the truncated SIMPL protein still may have been capable of some degree of multimer formation, and the Δ23SIMPL protein which was not directly linked to the column was being eluted into the antibody solutions. To remove the excess protein, a strong denaturant (4 M guanidinium) was passed through the affinity column followed by a high salt solution (1 M NaCl) to disrupt the multimeric complexes. Then elution buffer was passed though the column to mimic the conditions during an elution of the antibody. All wash steps were preceded and followed by a PBS wash. During this

stringent wash of the column, representative aliquots were taken for the solutions that were passed through the column, and the protein content of each solution was measured by UV absorbance (Figure 9). The guanidinium wash contained the highest protein concentration, and the other washes eluted little to no protein. It should be noted that the elution buffer signal shown on the graph was an average of eight 1 ml aliquots collected from the column wash. Since the profile yielded little to no signal for all of the aliquots (all negative reads were set to 0), it was deemed that a representative average was sufficient.

2. **Stringent wash and aliquot analysis allow for isolation of uncontaminated antibody**

To determine the effectiveness of the stringent wash, 2 ml of 087 serum were purified for anti-SIMPL antibody using the affinity column (87J). The elution profile was measured with UV absorbance (Figure 4F). The protein content appeared to peak in aliquot 2 and and then again in aliquot 5, and it was believed that both polyclonal antibody and SIMPL protein were being eluted off of the column. During previous purifications, all aliquots were pooled, but due to the irregularities of the elution profile for the 087J purification, the aliquots were not pooled. To determine if the contaminating protein was present, a Western blot was performed on 2 µg of protein from aliquots 2-7 (aliquots 1 and 8 did not appear to have a sufficient amount of protein for use based on the elution profile). Since the 087F antibody had been shown capable of detecting the recombinant SIMPL protein regardless of contamination, it was used as the primary antibody to probe the blot (Figure 10). All of the aliquots contained a band at ~29 kDa with the exception of aliquot 2 which appeared to be clear of any detectable contamination. Based on the intensity of the bands, it appeared that the contaminant band subsequently increased in intensity from aliquot 3 to aliquot 6, and then in aliquot 7 the intensity appears to lessen. Also detectable in the blot were the bands corresponding to heavy and light chain, which acted as loading controls. The 087J aliquot 2 purification (referred to as 087J) was used for all further experimentation. All of the purifications that were performed are outlined in Table 1 which acts as a flow chart of the conditions that had been attempted coupled with volumes and concentrations of the resulting purified antibodies.

D. Validation of the 087J antibody

1. 087J antibody binds native SIMPL protein

To determine the capability of the 087J antibody to bind native endogenous protein, immunoprecipitation experiments were performed. Lysed testes tissue from SIMPL KO and littermate mice were used as both a negative and positive control respectively. The 087J antibody was used for both the immunoprecipitation and as the primary antibody for the probing of the Western blot. To control for any signal that may have stemmed from the protein A beads, 100 µl of the protein A beads at a 10% slurry were incubated for 1 hr in PLC lysis buffer, and to control for signal from contamination of the 087J purification, antibody was incubated with beads in PLC lysis buffer for 1 hr. Both negative controls as well as the lysed tissue samples were subjected to SDS PAGE and subsequent Western blotting as per the immunoprecipitation procedure (Figure 11). It can be seen that there is a dark band at 27-29 kDa in the littermate testes tissue lane that is not present in the SIMPL KO testes tissue lane. The two negative control lanes also appeared to be clear of this band. A replicate experiment performed concurrently yielded similar results. The 087J antibody was capable of binding the native form of SIMPL protein in the wild type testes tissue to allow for its precipitation out of solution. The 087J antibody was also capable of detecting the concentrated denatured form of SIMPL protein in a Western blot to produce a strong signal with a high degree of selectivity. Experiments performed under identical conditions three days after the completion of the immunoprecipitation experiments and one week after the purification of the 087J antibody gave results that showed an increase in overall background (data not shown). Adjacent bands to the SIPML protein band in the littermate testes tissue lane were found in the previously clear negative control and SIMPL KO testes tissue lanes. To observe the individual bands in better detail, the experiment was repeated under identical conditions, but during the SDS PAGE, a larger gel was ran to further space out the bands to detect if there still remained a unique band in the littermate testes tissue lane (Figure 12). There was a band present in the littermate testes tissue lane at 27-29 kDa that was not present in the remaining lanes, but it had less intensity than what had been observed in the previous blots. The 087J antibody was still capable of detecting the native form of SIMPL protein, but its effectiveness and specificity had lessened under the storage conditions used.

2. **087J antibody detects SIMPL protein and a splice variant in testes tissue blot**

To further characterize the 087J antibody a Western tissue blot was performed on 0.25 mg of testes tissue isolated from littermate and SIMPL KO mice. The 087J antibody was used as the primary probe (Figure 13). A unique band was observed in the littermate testes tissue lane at 27-29 kDa, and a band of equal intensity was detected at 26 kDa in both the littermate and SIMPL KO lane. Since the insertion of the gene trap vector had been confirmed with PCR (data not shown), it was hypothesized that the band present in both the SIMPL KO and littermate lanes was a SIMPL protein isoform arising from an mRNA splice variant. A search for SIMPL in the Expressed Sequence Tags database through the National Center for Biotechnology Information showed that there exists splice variant RNA that is expressed in the C57BL/6 strain of mice. The splice variant RNA would generate a protein 26.3 kDa in size which was the approximate size of the protein observed in the Western blot. This alternative splicing would result in the loss of exon 2 and the splicing of exon 1 directly to exon 3. The gene trap vector present in intron 1 would be excised in this sequence allowing for expression in SIMPL KO mice.

E. Immunofluorescence

1. **SIMPL protein exhibits passive nuclear localization and is concentrated by NF-κB subunit p65 in the nucleus of endothelial cells**

SIMPL protein has been shown previously to impact TNFα but not IL-1 induced NF-κB activity which indicates that SIMPL may lie in the TNFα signaling pathway (Kwon et al. 2004). Therefore, it was of interest to determine if endogenous SIMPL protein localizes to the nucleus in the presence of pro-inflammatory cytokines IL-1 and TNFα as well as the subcellular localization of SIMPL in the absence of cytokines. Since the 087J antibody had been validated to be free of contaminating SIMPL protein and to be capable of detecting SIMPL in its native structure in immunoprecipitation reactions, it was used in immunofluorescence confocal microscopy experiments to determine SIMPL subcellular localization kinetics. Mouse endothelial cells were chosen for their relatively expansive cytoplasm to allow for clearer imaging of the localization. Additionally, endothelial cells play an important role in the inflammatory response. The cells were grown to 40% confluency on coverslips. To ensure that the cytokine conditions were

capable of inducing an inflammatory response in the endothelial cells, the localization of NF-κB was monitored in control groups of cells. Two groups of endothelial cells were incubated with 50 ng of rHu IL-1 and rHu TNFα respectively for 20 min. The cells underwent methanol fixation and were then stained for the p65 subunit of NF-κB. Acting as the negative controls were two groups of endothelial cells that were not treated with cytokines and stained for SIMPL and p65 respectively. The cells which were stained for the p65 subunit are shown in Figure 14. In terms of nuclear localization of the p65 subunit, the TNFα signaling was the most effective with a strong overlap of the signals for the DAPI stain and the green fluorescence of the secondary antibody corresponding to the location of the p65 subunit. There is also nuclear localization of p65 in response to the IL-1 signaling, but it is less than that of TNFα as can be seen by remaining fluorescence in the cytoplasm and less overlap of the blue and green in the image. The negative control endothelial cells stained for p65 localization showed an area clear of signal in the nucleus for the 488 nm (green) channel. The cells which were stained for SIMPL protein are shown in Figure 15. In the negative control, there was an even distribution of the SIMPL protein throughout the cell with no definitive nuclear localization as was seen in the p65 stained positive control cells or cytoplasmic localization as was seen in the p65 stained negative control cells. With the IL-1 treated cells, SIMPL nuclear localization appears to be intermittent, with some of the cells showing strong nuclear localization for the protein and other cells showing no distinct localization. The strongest nuclear localization of the SIMPL protein can be seen in the TNFα treated cells. Nearly all of the cells in the representative image show an overlap of the DAPI and green fluorescence with limited signal detected within the cytoplasm. For a closer investigation of the overlap of the DAPI and green fluorescence channels, representative maximum image projections (MIP's) were taken of nuclei for each of the immunostained SIMPL cell groups (Figure 16). MIP's map the plane of the signals on the borders of the image in relation to the 2 dimensional image shown in the center. In the IL-1 stimulated cells, the SIMPL MIP shows distinct blue and green signals that appear to be within the same plane of each other, but the representative MIP for the TNFα stimulated cells shows a bright teal corresponding to a much stronger signal overlap of the DAPI and secondary signals.

DISCUSSION

Modulation of NF-κB transactivation activity by a co-activator would aid in explaining the complex behavior of this transcription factor. It would help to explain differences that arise when different activators of NF-κB are examined. It could also serve as a potential drug target to possibly lessen the impact of cytokine signaling from TNFα and IL-1 which would impact diseases such as chronic inflammation, diabetes, or even cancer. It would offer a finer control than currently available drugs which act to target TNFα and its receptors directly. Also, work on SIMPL acts to further describe the complexity of the TNFα signaling pathway. A direct association has been shown between mPLK/IRAK-1 and TNF R1, and subsequently a physical link has been shown between SIMPL and mPLK/IRAK-1 (Vig et al. 1999; Vig et al. 2001). One goal of this thesis was to identify a direct endogenous link between SIMPL co-localization in the nucleus with p65 and TNFα signaling. The primary goal was to learn about the challenges of antibody purification and characterization using a low abundant and not well characterized protein such as SIMPL.

A. Δ23SIMPL's effectiveness in the purification of SIMPL antibody

It was hypothesized that deleting the disordered region near the amino terminus of the protein would help prevent oligomerization of the recombinant SIMPL protein. As to whether this prevented the formation of polymers, no direct evidence is presented in this thesis. It was shown that Δ23SIMPL protein eluted off of the column with the antibody fractions. Since the reaction that acts to bind primary amine groups to the column yields a covalent bond, it seems unlikely that an affinity for an antibody would be sufficient enough to break this bond and allow the protein to elute off of the column bound to the antibody. More likely is that there still remained the formation of polymers within the concentration of the purified Δ23SIMPL that was used to make the column and that the Δ23SIMPL that was eluted was never directly linked to the column. The formation of these multimers has been shown to be concentration dependent (unpublished data). One possible solution to be enacted in further purifications could be to lessen the concentration of the protein that is to be linked to the column. Although the 2 mg that were loaded were within the recommended capacity for the column, less protein could

have been theoretically loaded. Decreasing the amount of Δ23SIMPL could lower the yield of purified Ab, but it may also prevent contamination. Lowering the concentration that the bacterial protein is stored at (see **Methods and Materials**) could also possibly reduce oligomerization.

B. The effectiveness of washing an AminoLink column with various solvents

By the time that the contamination of the antibody purifications had been discovered, almost 20 ml of sera had been passed through the column. Thus it seemed possible that most of the Δ23SIMPL that was not directly bound to the column had been removed with the elution of the previous antibody. However to be certain, the stringent wash described above was proposed to remove all the remaining protein not directly bound. The use of 4 M guanidinium acted as a denaturant to possibly disrupt the Δ23SIMPL interactions with itself. It is possible that the use of such a strong denaturant could have had deleterious effects on the column or prevented the purification of antibodies that detected the native structure of SIMPL. This wash was the most effective in the elution of detectable protein off of the column during the wash. But Δ23SIMPL was later detected in the column fractions. It can be assumed that the wash was not sufficient to remove all of the unwanted protein or that the guanidinium acted to weaken the direct binding of Δ23SIMPL. The latter is an unlikely possibility. The high NaCl concentration solution was used in the hopes that it might be sufficient enough to disrupt any hydrogen bonding between the Δ23SIMPL proteins. The effectiveness of this wash remains dubious as it did not produce any detectable protein in the analyzed flow through. The argument could be made that all of the unwanted protein was removed in the previous wash, but this would fail to explain its presence in the following antibody purification. To further test the effectiveness of the high salt wash, another wash could be performed on a newly made column without the previous guanidinium wash to detect if any detectable protein is eluted. However, this is outside of the scope of this thesis.

C. The differential elution of bound and unbound SIMPL antibody

The problem of contamination was ultimately solved by taking advantage of differences in the elution times of the unbound antibody and the Δ23SIMPL bound antibody. By collecting the sample in 1 ml aliquots and thus separating the contents of the solution based on elution times, we were able to obtain an uncontaminated sample of

polyclonal antibody. It remains to be seen if this would have been effective in the initial purification as all of the previous purifications had been pooled soon after they had been measured for protein content, and this solution was enacted after the stringent wash. Further purification attempts on remade columns could perhaps allow for smaller aliquots to be collected increasing the chances of collecting an uncontaminated fraction.

D. Uncontaminated antibody allowed for the detection of SIMPL

Initially we had been unsuccessful in our attempts to detect the protein in Western blots and immunoprecipitation reactions with the previous purifications of the antibody. The first immunoprecipitation experiment showed that the 087J antibody could not only recognize the SIMPL protein in its native structure but also in its denatured state in a gel as well. The signal was strong, and there was very little background in the initial experiment. The lane for the KO tissue showed no signal which validated previous PCR results (unpublished data) that showed a successful insertion of the gene trap vector into the first intron of the SIMPL gene. The Western blots on the same tissue types showed that a detectable signal could be obtained without concentrating the protein using an immunoprecipitation reaction. The specificity of the antibody is shown by almost a complete lack of background in the blot. There is a strong band in the KO tissue lane that is also seen in the littermate tissue lane with an equal intensity which is interesting because of both its intensity as well as its size relative to that of SIMPL. The band that is not present in the KO lane that is in the littermate lane is only slightly larger in size and is of equal intensity as the other bands. Initially it was hypothesized that this upper band may be a post translational modification of the SIMPL protein, but this would imply that the gene trap vector had been unsuccessful in preventing the expression of the full gene which in light of the PCR evidence as well as observed phenotypic changes between the KO and the littermate mice seemed highly unlikely. The gene trap vector that was used had in place three different stop codons for each possible reading frame to prevent translation of the sequence even if it were transcribed in its entirety. A remaining possibility was that the upper and lower bands seen in the littermate lane of the testes tissue blot were in fact splice variants of the target protein, and the presence of the lower band in the SIMPL KO lane could be explained by a splicing out of the gene trap vector. A database search of expressed sequence tags did show that a transcript that spliced

together the first exon to the third exon for the SIMPL sequence was found in mouse testes tissue. This variant would have removed the gene trap vector found in the first intron. The size shift between the upper band and lower band was measured using the molecular weight ladder as the standard curve, and it was found that the shift corresponded to a loss of the portion of the protein sequence that would have been coded for in the second exon. If the band seen in the SIMPL KO tissue lane were the splice variant, then it can be assumed that the folding of this protein sequence could be different than that of the full SIMPL protein which possibly prevented the 087J antibody from detecting the variant in the immunoprecipitation reactions. One definitive method of confirming the identity of the bands would be to isolate them from the gel and to perform mass spectrometry analysis, but none has been performed as of yet. Alternatively, isolation of the corresponding RNA could be attempted.

E. SIMPL concentrates in the nucleus of endothelial cells in response to TNFα and IL-1

Previous work done by this lab had shown that stimulation of fibroblasts with TNFα caused the formation of a complex containing p65 and SIMPL. Also, using immunofluorescence to detect an over-expressed flag-tagged SIMPL protein showed almost an exclusive concentration of the protein in the nucleus. However, it should be noted that this was seen without TNFα stimulation (Luo et al. 2007). From the first set of immunofluorescence experiments we performed in which the localization of the p65 subunit was detected, it was evident that the concentration of respective cytokines was sufficient to study NF-κB activation. From the representative images, it appears that in the presence of equal concentrations of TNFα and IL-1, the former induces a stronger effect as measured by the p65 dependent signal seen in the nucleus compared to the signal in the cytoplasm. This was to be expected. These results are definitive in terms of cytoplasmic versus nuclear localization. Control, unstimulated cells clearly show almost a complete concentration of a p65 dependent signal in the cytoplasm. For the groups of cells where SIMPL localization was measured, it appears that the SIMPL protein shuttles in and out of the nucleus in the absence of exogenously added cytokine. This is seen in the non-stimulated cells where the signal appears to be equally dispersed throughout the cells. The TNFα stimulated cells do appear to show a stronger concentration of signal in

the nucleus than the non-stimulated cells. To a lesser extent, there is also an increase in the concentration of SIMPL in the nucleus for the IL-1 stimulated cells. This is contradictory to previous models that stated that IL-1 dependent activation of NF-κB is SIMPL independent (Kwon et al. 2004). It should be noted that the IL-1 and TNFα concentrations used for these experiments were five-fold greater than what had been used previously by this group. Based on the strength of the nuclear localization of the p65 subunit being stronger for the TNFα signaling than for the IL-1 signaling and that the same was seen to a lesser degree in SIMPL localization coupled with the fact that the non-stimulated cells showed an even dispersion of SIMPL, one could assume that SIMPL is not actively localized to the nucleus but possibly merely retained there by the p65 subunit with which it interacts. When the p65 subunit is not in the nucleus, the SIMPL protein shuttles in and out of the nucleus with little preference.

F. Possible degradation of the 087J antibody

After the 087J antibody had been purified as stated above, it was stored in the neutralized elution buffer with 0.05% sodium azide to prevent bacterial growth. The pH of the solution had been measured to be at 8-9, and the protein concentration was 0.5 mg/ml. The initial results obtained for the immunoprecipitation reaction in which the 087J antibody was used to both precipitate SIMPL protein and probe for it were not able to be reproduced with the same low background and strong signal for the protein bands. Identical immunoprecipitation experiments that were performed later showed that the signal for the protein and decreased and the background increased. Since the band did not disappear as it was detected in a larger gel, it can be assumed that the antibody itself had been altered rather than the tissues. This was also seen in the form of less intense fluorescence signal in repeats of SIMPL immunofluorescence experiments with 087J antibody that had been stored for greater than one week (data not shown). It is possible that the stability of the antibody was compromised due to its storage conditions. It was kept at a low concentration in a relatively large volume of solution. Future purifications of the antibody would be stored in the same solution with BSA added to lessen the deleterious effects of storage at a dilute protein concentration.

G. Future directions of study with a functional SIMPL antibody

One primary goal would be to determine all SIMPL protein interactions, and this could be accomplished with a good antibody against the target protein. An immunoprecipitation reaction for SIMPL could be performed, and one could simply remove the beads from the solution and apply mass spectrometry to determine what proteins are present. To determine if there is a direct interaction between endogenous p65 and endogenous SIMPL and if the interaction is cytokine dependent, a similar experiment could be carried out on lysates from tissues or cells that had and had not been exposed to TNFα. This experiment could also be performed through Western blotting following the initial immunoprecipitation as the two target proteins are known. The role of IRAK-1 in SIMPL signaling could be further elucidated by attempting to induce phosphorylations or other post-translational modifications in the SIMPL protein through cytokine signaling in WT and IRAK-1 null mutants. Using a Western blot, shifts in the size of the SIMPL protein could be detected with a well characterized SIMPL antibody. It would also be interesting to observe if the null IRAK-1 mutant cells would be capable of inducing SIMPL nuclear localization through immunofluorescence experimentation. Utilizing Western blotting techniques one may also be able to observe detectable differences in the SIMPL protein or its expression during certain disease states such as chronic inflammation or diabetes. These are only some of the experiments which become available with an effective antibody against SIMPL protein. They would act to further clarify the role of this signaling molecule which could drastically impact the study of inflammation.

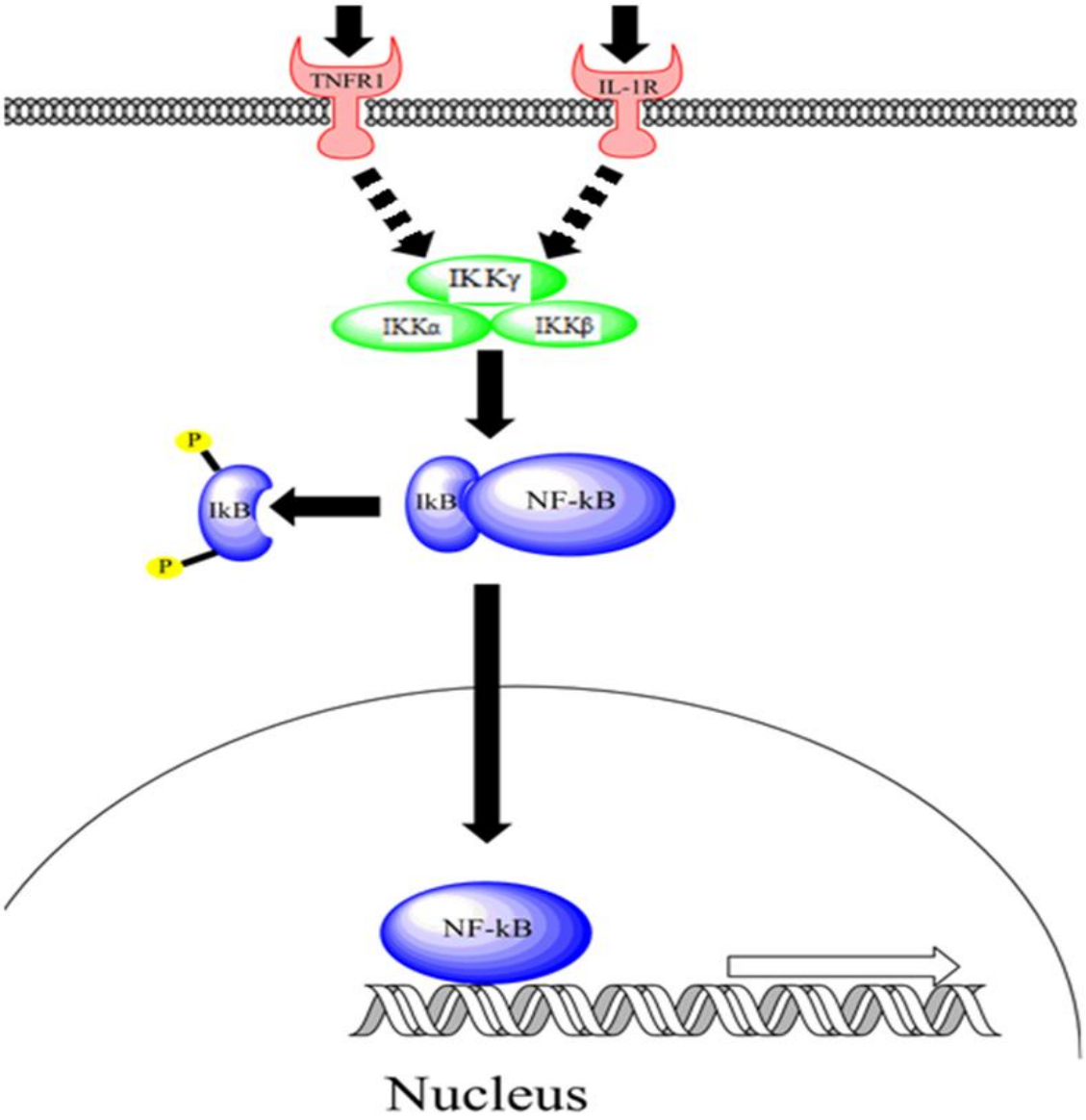

Figure 1. NF-κB activation. Dashed arrows represent the indirect activation of the IKK complex which in turn phosphorylates IκB. This causes IκB to dissociate from the NF-κB complex and to be targeted for ubiquitination and degradation. In the absence of IκB, NF-κB localizes to the nucleus and affects the transcription of inflammatory genes.

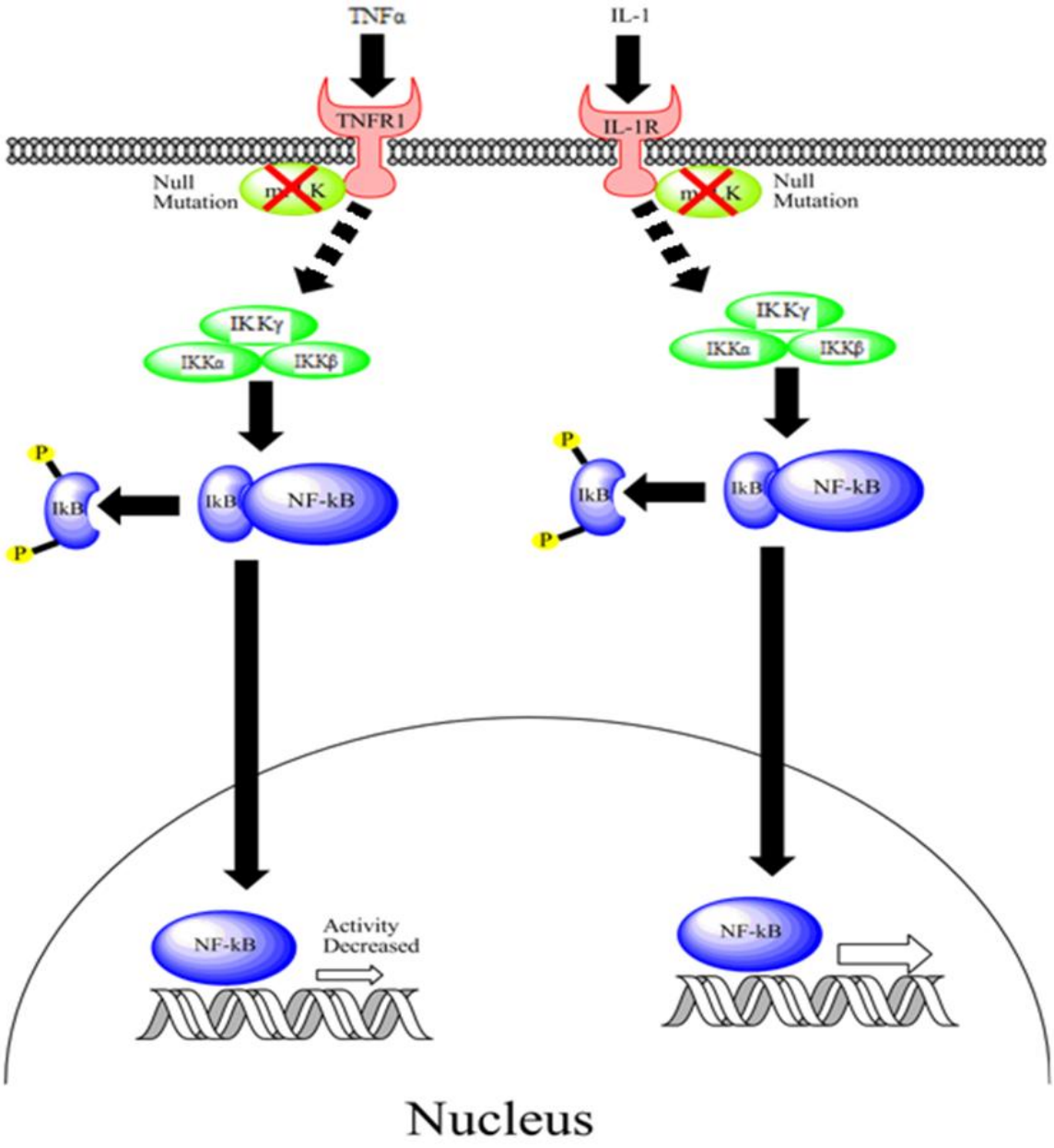

Figure 2. Cytokine activation of NF-κB in the presence of catalytically inactive IRAK-1. The TNFα signaling pathway is inhibited by inactivation of IRAK-1 catalytic activity, and the IL-1 signaling pathway is maintained by the structural presence of the IRAK-1 protein independent of its catalytic activity.

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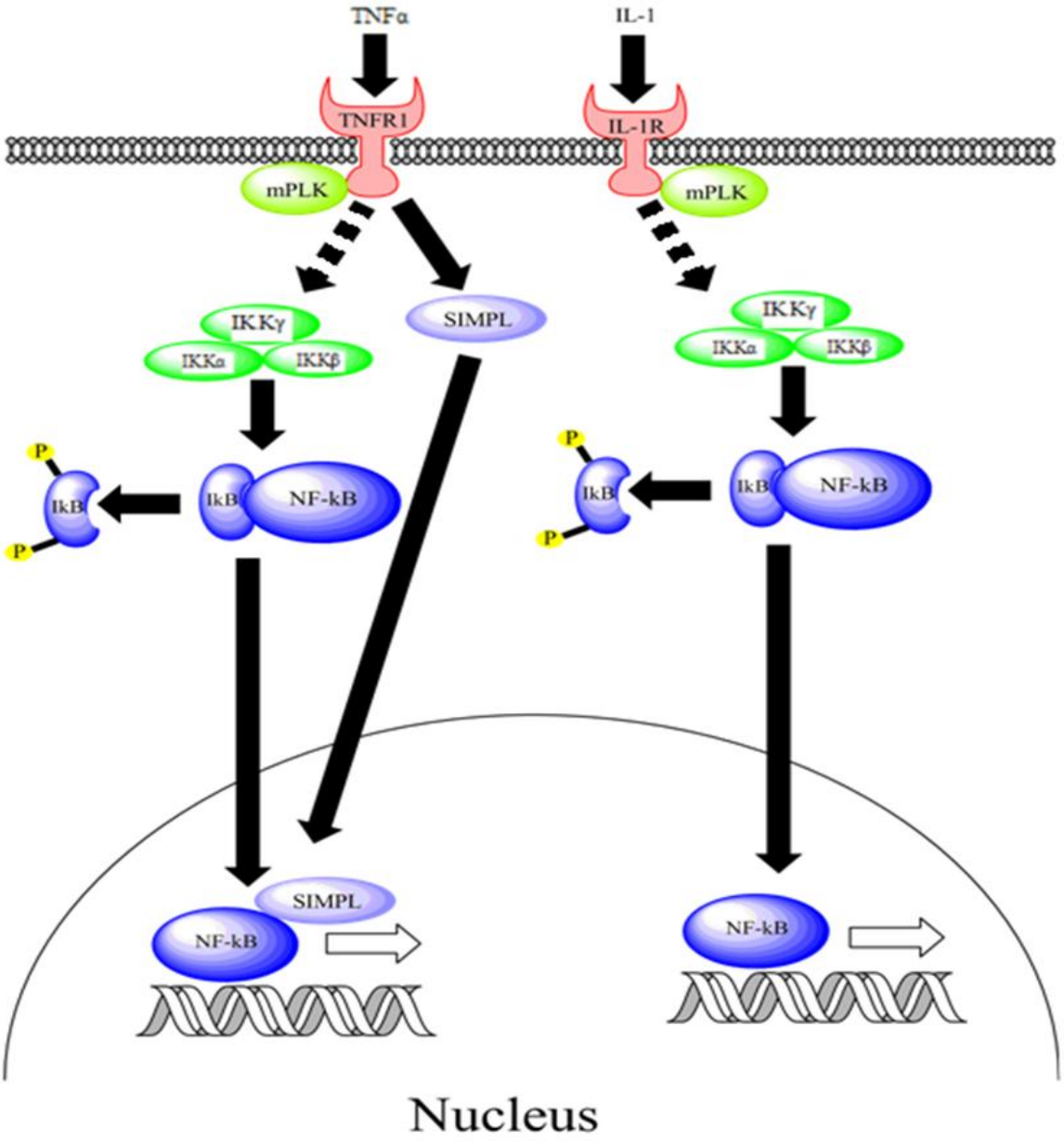

Figure 3. Proposed model of SIMPL activation and interaction with NF-κB.

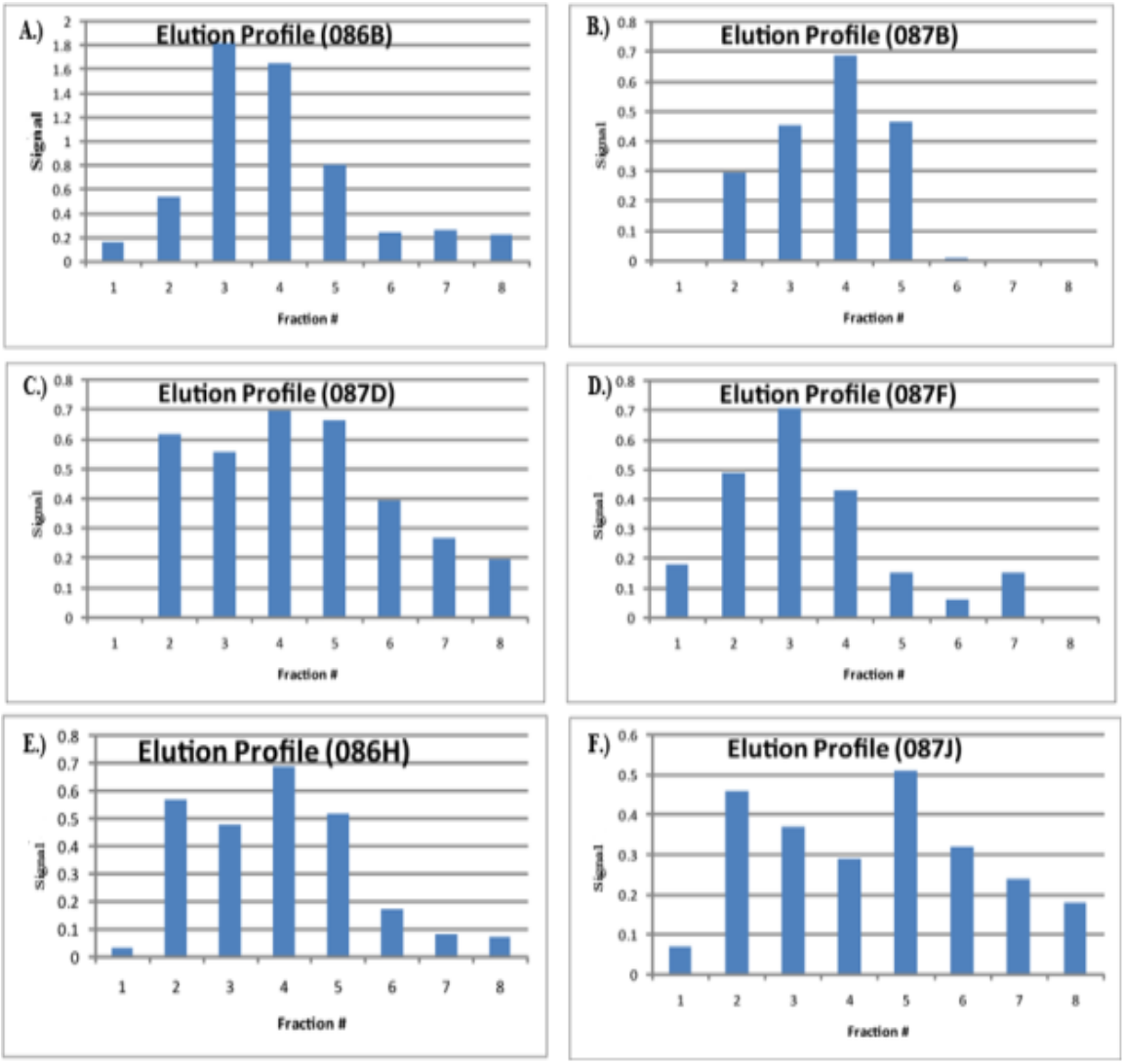

Figure 4. Elution profiles for the purifications of SIMPL antibody. The 1 ml fractions collected during the elution are plotted against the relative protein content.

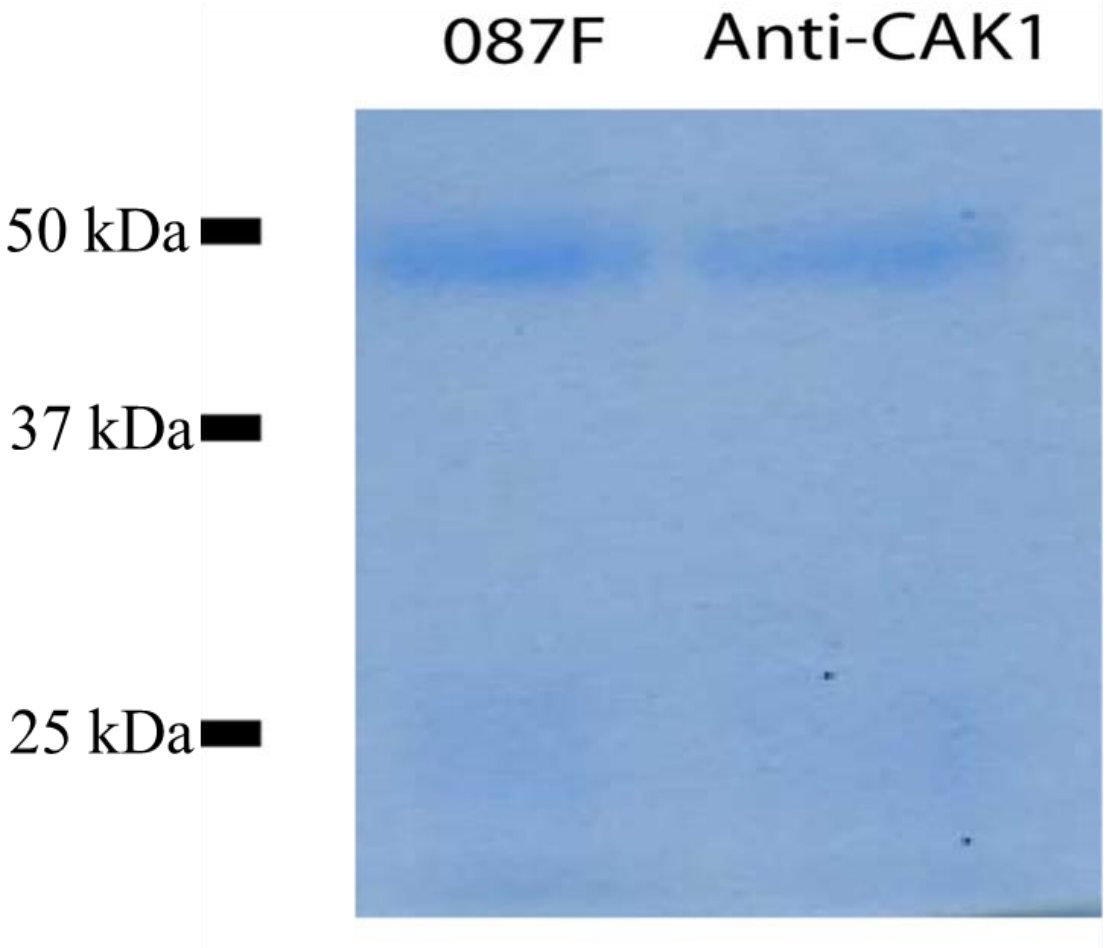

Figure 5. PAGE gel containing isolated protein stained with coomassie blue. The material isolated from the 087F animal was analyzed along with pure CAK1 antibody to compare staining patterns.

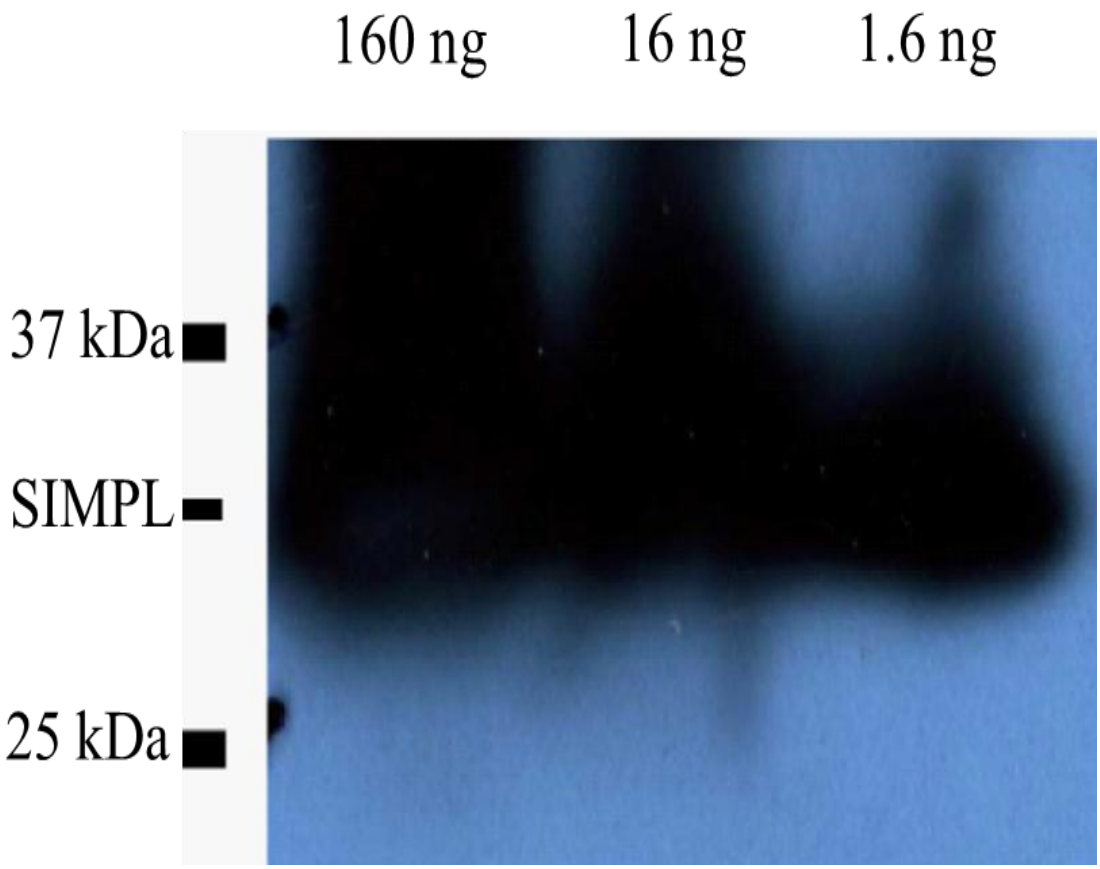

Figure 6. Western blot of known quantities of purified Δ23 SIMPL with the 087F purification acting as the primary antibody. The blot shows the strength of the SIMPL signal in the absence of other proteins.

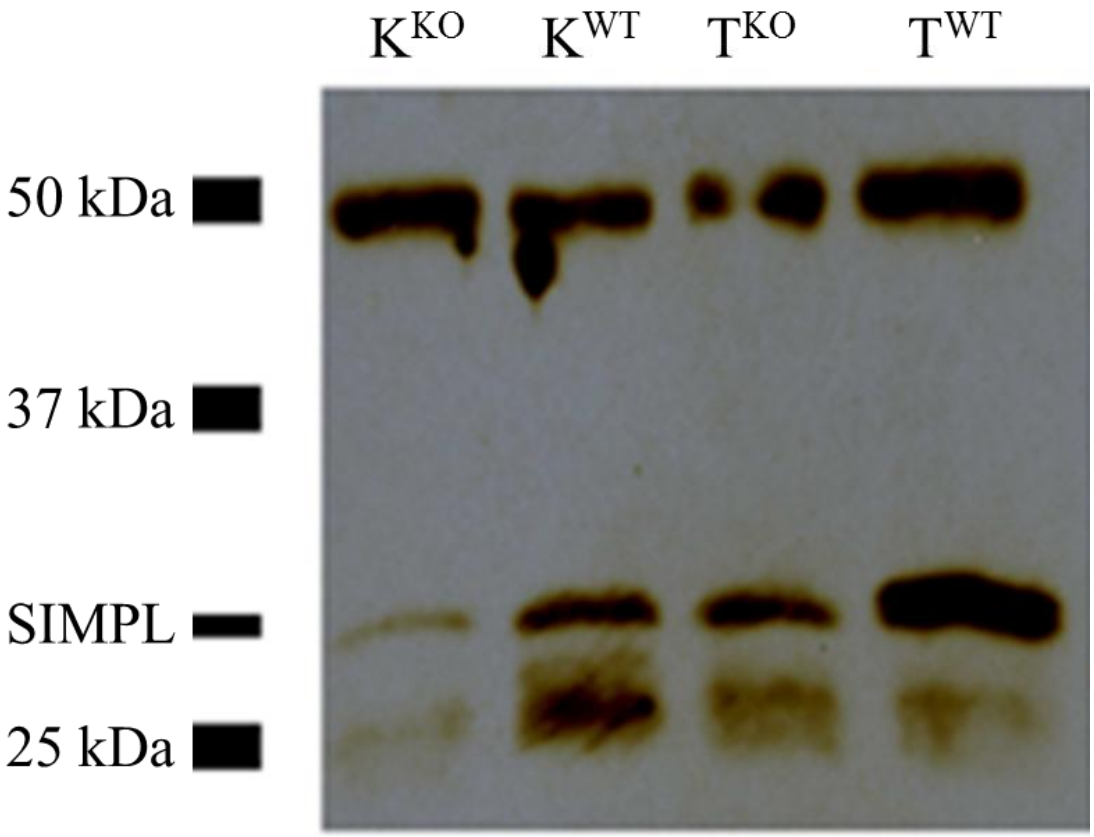

Figure 7. Immunoprecipitation of tissue lysates from SIMPL KO and WT mice. The 087F purification was used for the precipitation as well as the primary antiserum to concentrate the target protein. K=kidney and T=testes.

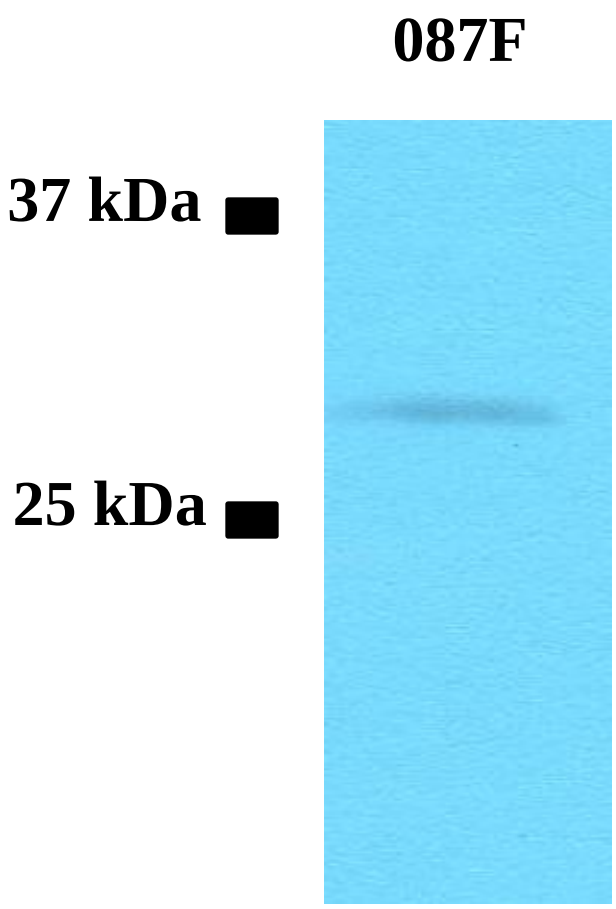

Figure 8. Western blot of the affinity purified 087F antibody. An aliquot of the antibody was probed with the antibody solution. This experiment was designed to determine whether the SIMPL protein was present in the affinity purified 087F antibody.

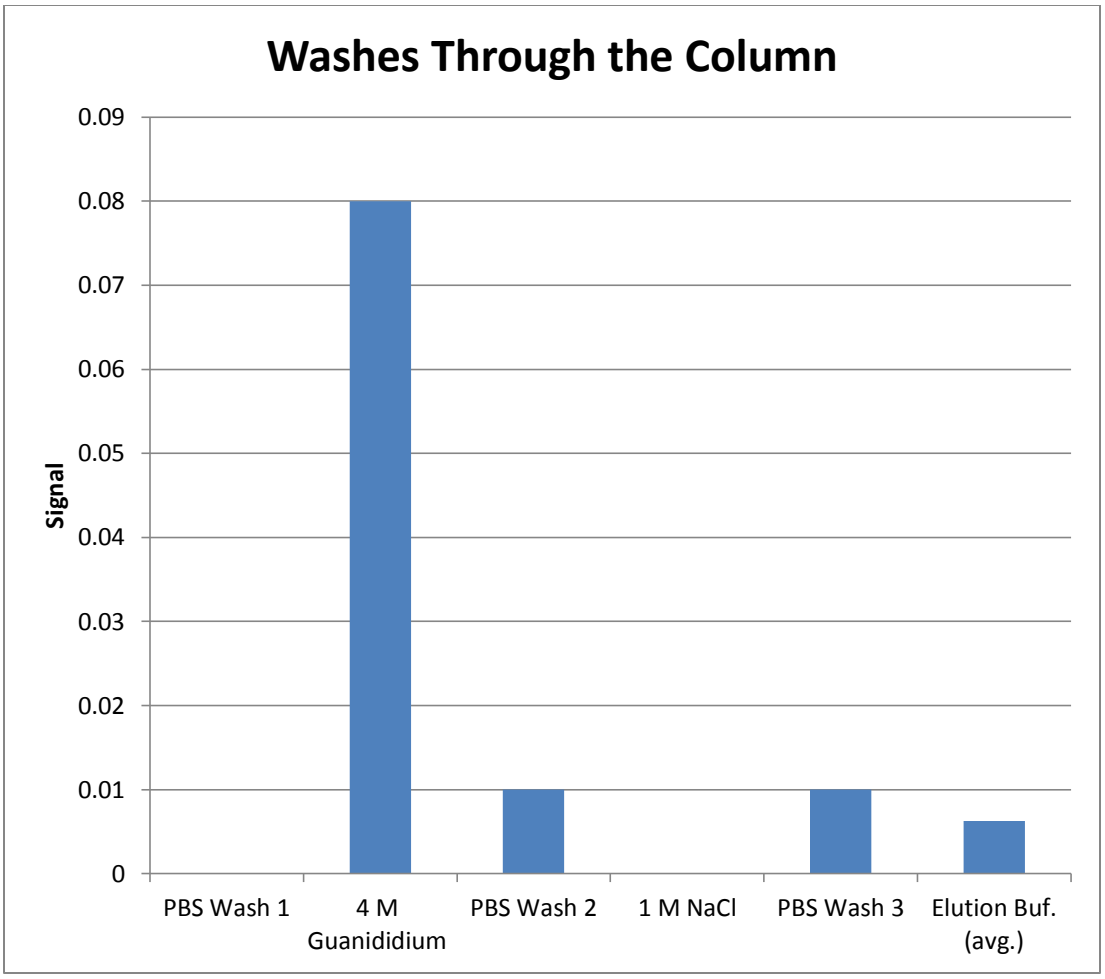

Figure 9. The protein content in aliquots of the column fractions during the stringent washing of the SIMPL affinity column.

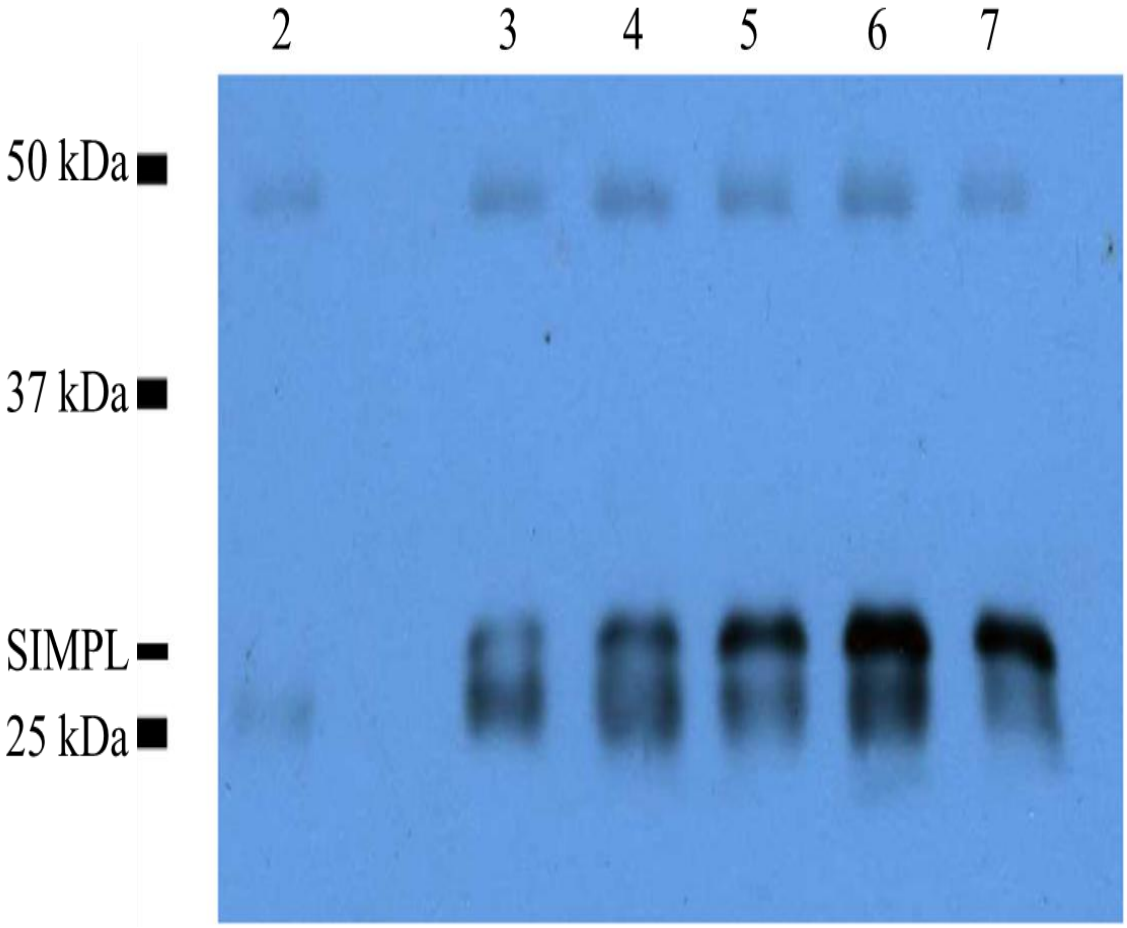

Figure 10. Western blot of column aliquots from the 087J purification. The blot was probed with the 087F antibody.

Table 1. Flow chart that outlines the various antibody purification attempts.

Sample Name	Description	Volume (mL)	Concentration (µg/µl)	Date Run
086A	Serum flow through. Stored at 4 ^{o}C	10	NA	
086B	Elutant that was dialyzed O/N in PBS. Later diluted. Stored at -40 ^{o}C	12	0.687	2/2/2009
087A	Serum flow through. Stored at 4 ^{o}C	5.75	NA	
087B	Elutant that was dialyzed O/N in PBS. Later diluted. Stored at -40 ^{o}C	7	0.349	2/3/2009
087C	Serum flow through. Stored at 4 ^{o}C	3.5	NA	
087D	Elutant that was dialyzed O/N in PBS. Stored at -40 ^{o}C	8	Discarded	2/5/2009
087E	Serum flow through. Stored at 4 ^{o}C	2	NA	
087F	Elutant that was not dialyzed. Stored at -40 ^{o}C	8	0.24 (280 read)	2/21/2009
086G	Serum flow through. Stored at 4 ^{o}C	1.75		
086H	Elutant that was not dialyzed. Stored at -40 ^{o}C	6	0.47 (280 read)	3/6/2009
087I	Serum flow through. Stored at 4 ^{o}C	1.75		
087J Al2	Elutant that was not dialyzed. Only aliquot 2. Stored at 4 ^{o}C	1	0.46 (280 read)	4/15/2009

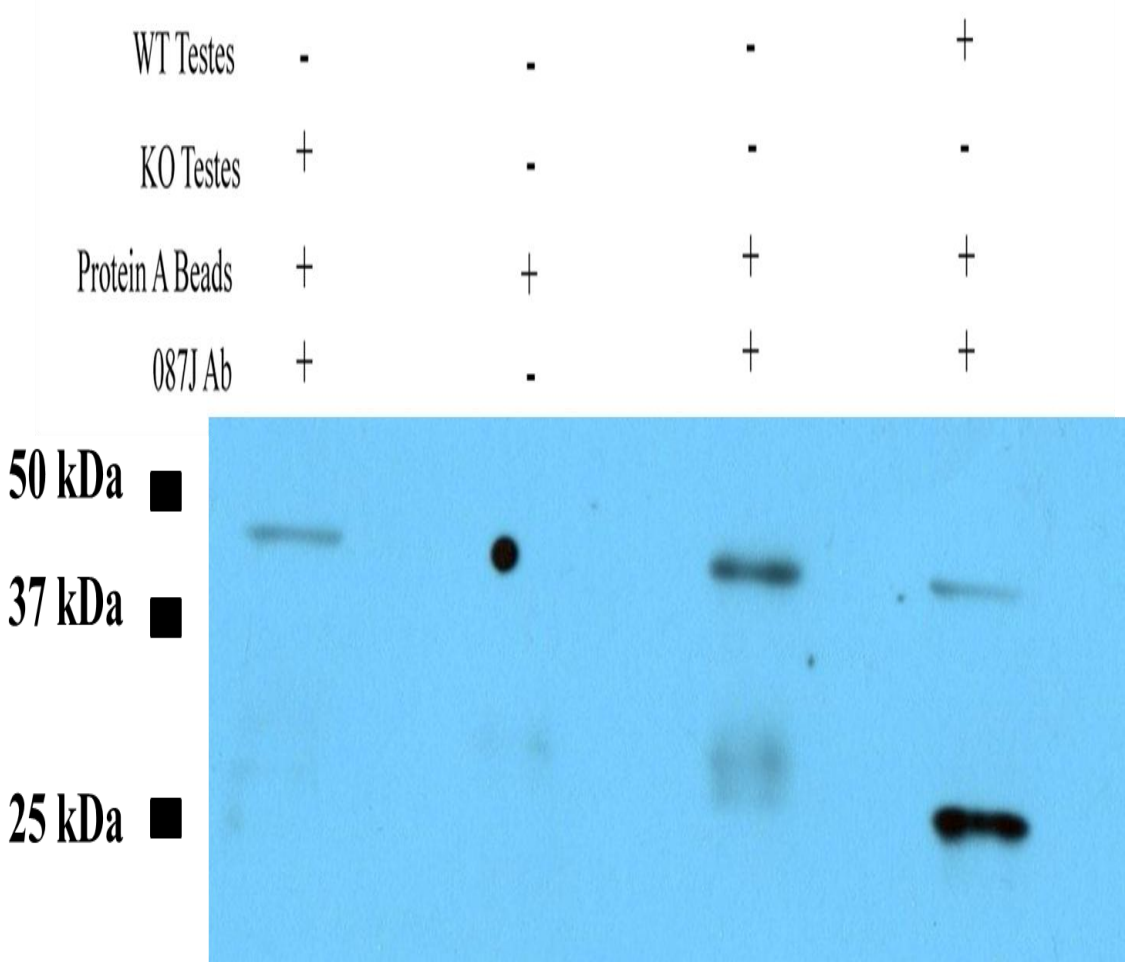

Figure 11. Immunoprecipitation of the WT and SIMPL KO testes tissue lysate. For both the precipitation and the primary probe, the 087J purification was used. For negative controls, two precipitations were performed without tissue lysate, and one contained antibody and one did not. Ab=antibody

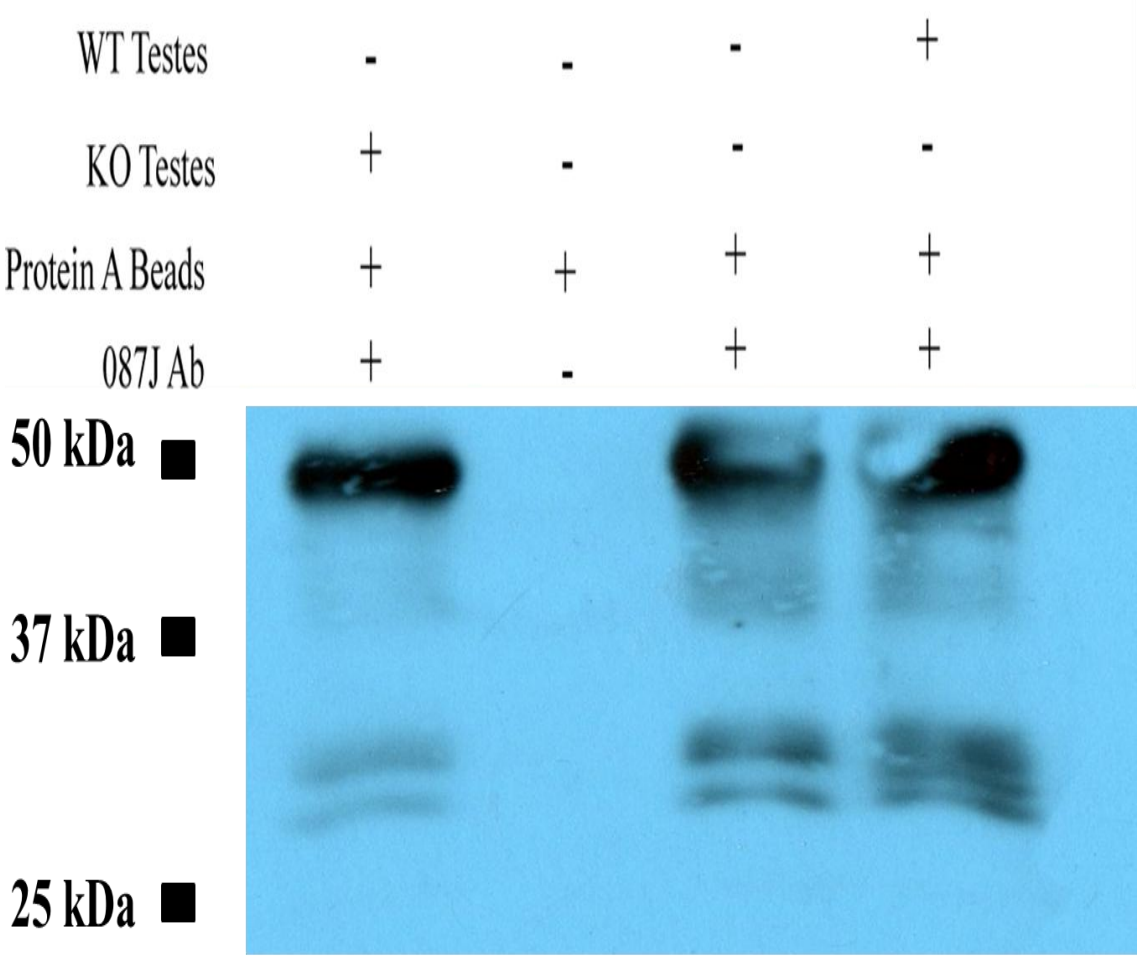

Figure 12. A repeat of the experiment shown in Figure 9 that was performed 1 week later. The gel that was ran was much larger the original that was used in order to obtain a sufficient separation of the bands. Ab=antibody

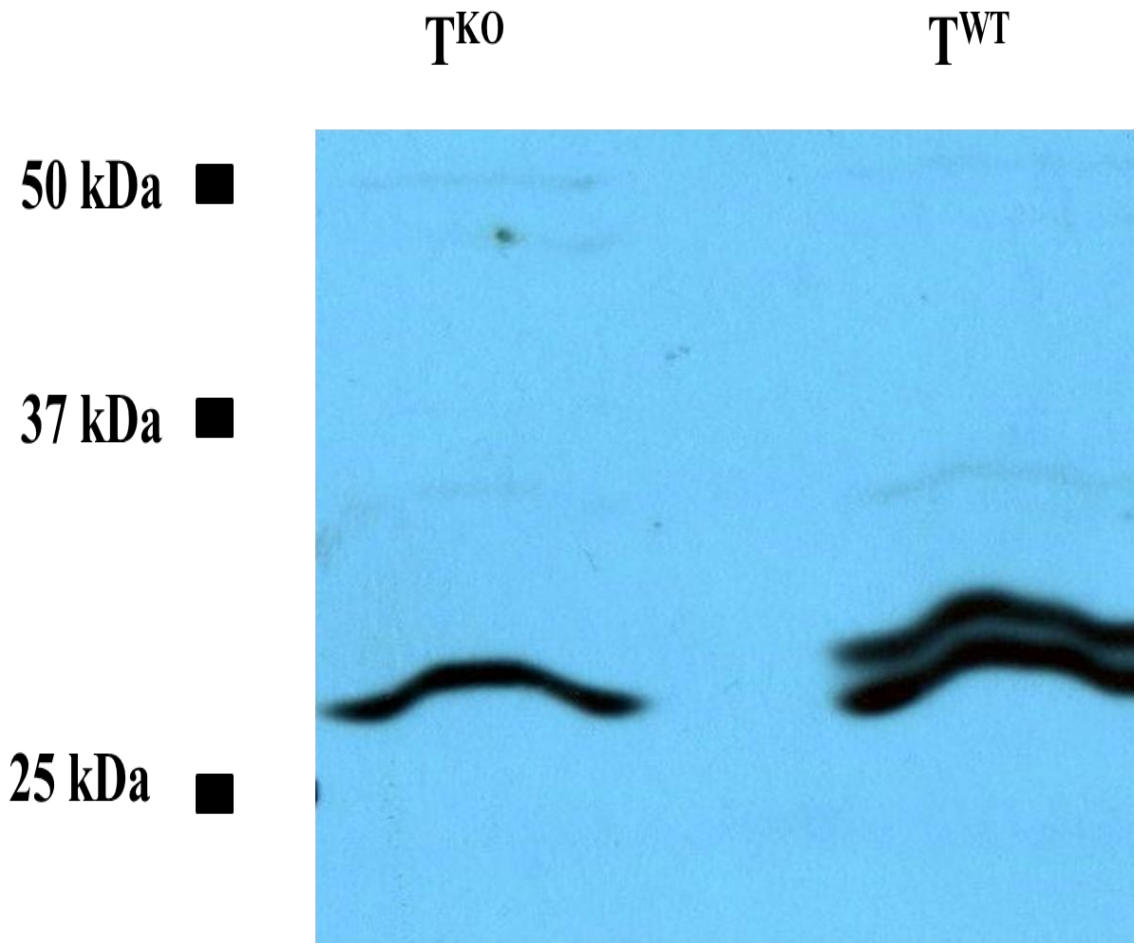

Figure 13. Western blot of WT and SIMPL KO testes tissue lysate.

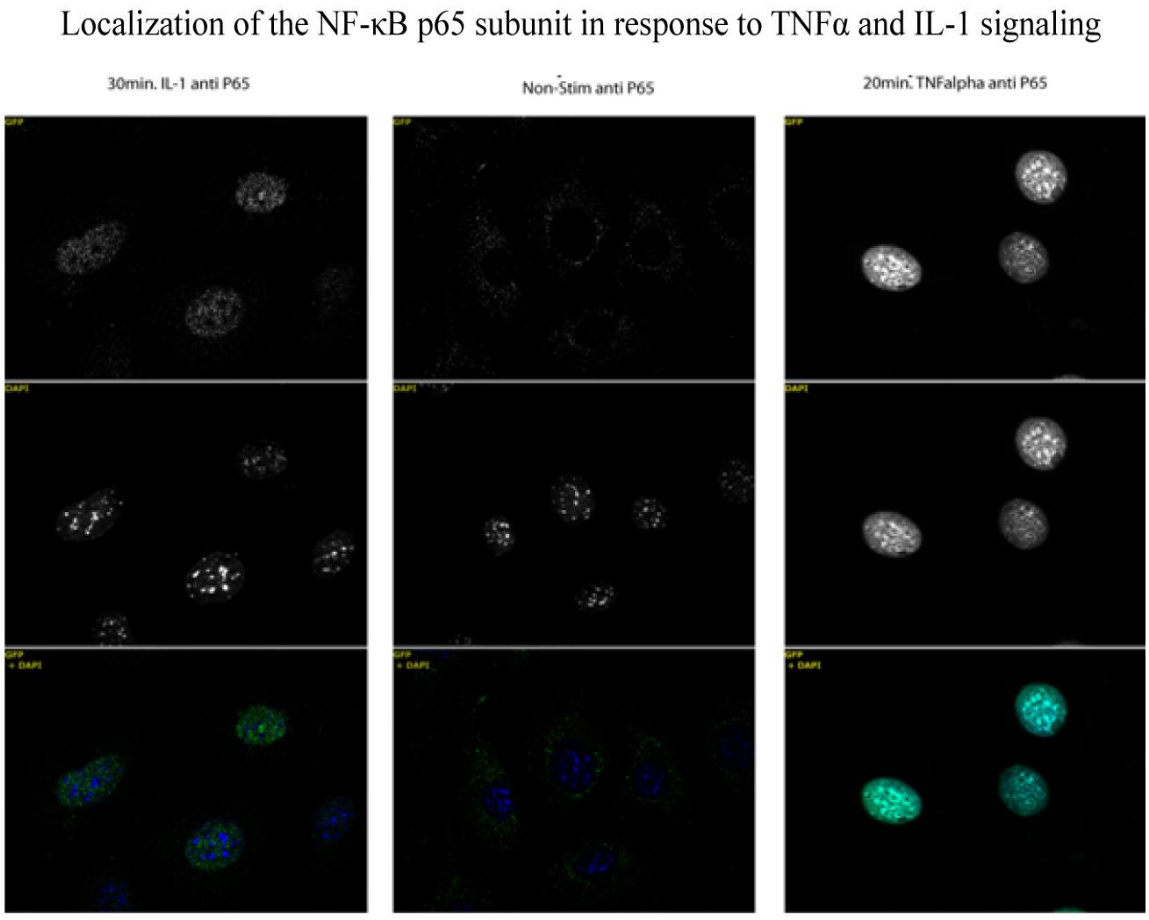

Figure 14. Immunostaining of the p65 subunit in NF-κB for human endothelial cells. The cells were stimulated with IL-1 and TNFα to induce a localization response. The top three image captures are the grayscale of the green 488 nm channel corresponding to the secondary antibody fluorescent signal. The middle three are the grayscale for the blue DAPI channel. The bottom three are the color combined images.

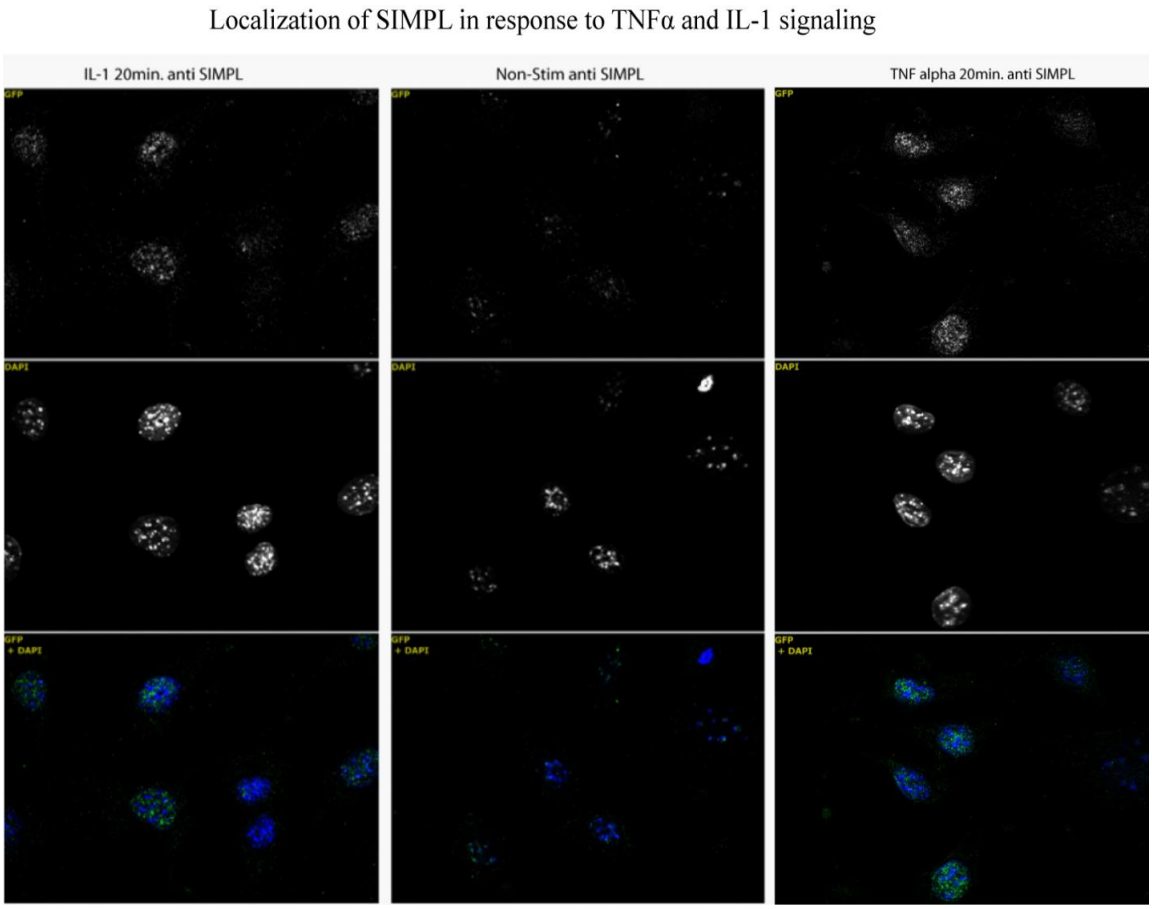

Figure 15. Immunostaining of the SIMPL protein for human endothelial cells. The cells were stimulated with IL-1 and TNFα to induce a localization response. The top three image captures are the grayscale of the green 488 nm channel corresponding to the secondary antibody fluorescent signal. The primary antibody used was the 087J purification. The middle three are the grayscale for the blue DAPI channel. The bottom three are the color combined images.

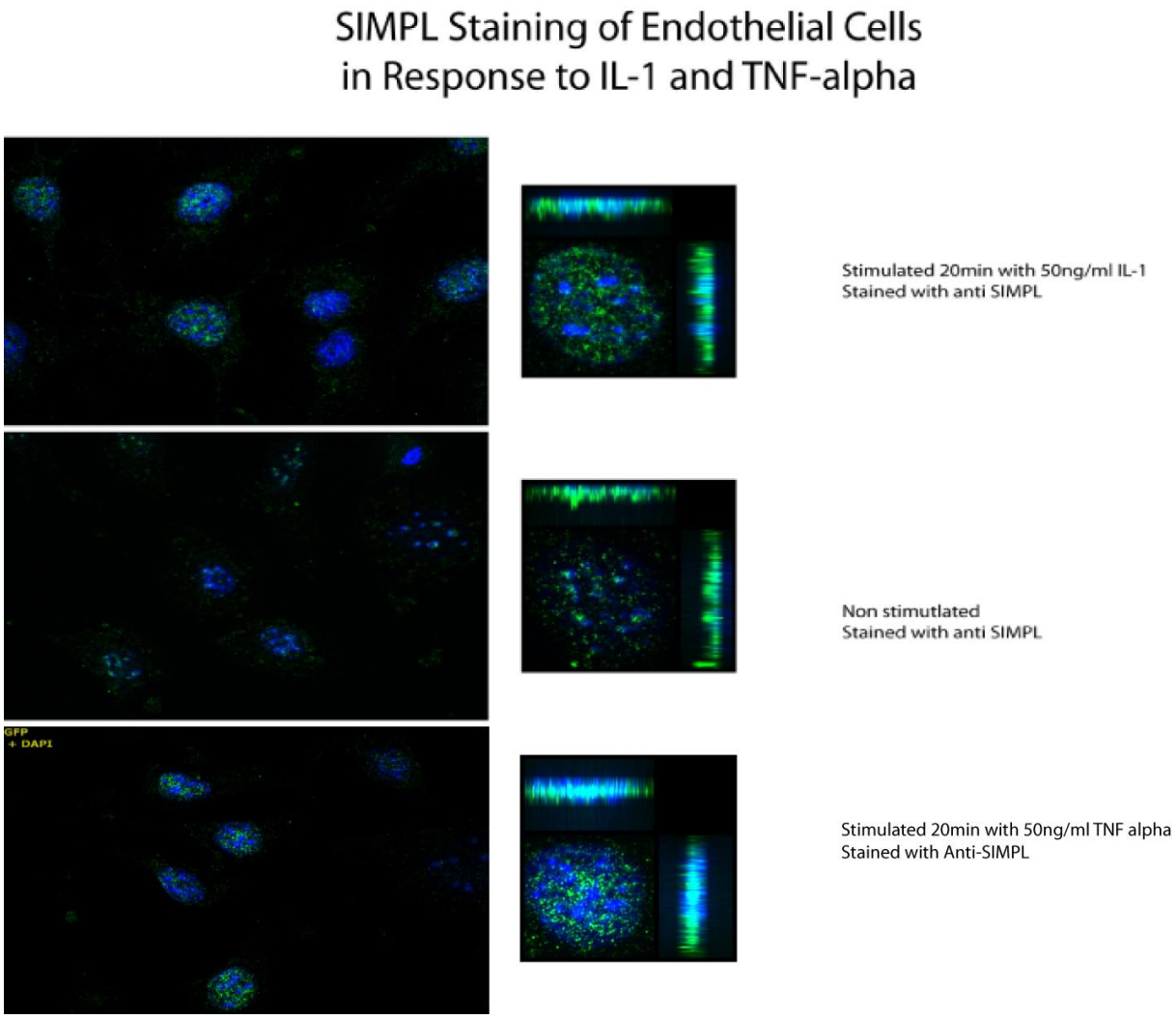

Figure 16. The color combined images from Figure 13 with corresponding MIP's from selected sections of the image captures.

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Curriculum Vitae

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Education

University of Wisconsin-Eau Claire
Bachelor of Science, Biochemistry/Molecular Biology
May 2005
Indiana University
Graduate Certificate in Biotechnology
May 2009
Indiana University
Master of Science, Biochemistry/Biotechnology Track
January 2011

Research

Isolated and validated antibody against a target protein
Elucidated unique cell signaling inflammatory pathway

Related Skills

- Experienced with sub-cloning, plasmid isolation, HPLC, PAGE, Western blots, RTPCR, automated liquid handlers, preparing media and plates, and pH meters
- Also familiar with Microsoft Office, Swiss PDB Viewer, and BLAST
- Proficient in analyzing protein content and activity

Employment History

Dow AgroSciences

Contract Research Associate *January 2010-Present*

- Developed RTPCR and endpoint PCR assays for quality control
- Isolated, quantitated, and normalized DNA
- Assisted in the writing of reports to present data

IU Department of Biochemistry and Molecular Biology

Graduate Student *January 2009-Dember 2010*

- Performed various experiments including protein assays such as Western Blots and immunofluorescence and purifications to elucidate a cellular pathway

Eli Lilly

Biochemist I-Contract Employee *October 2007-March 2008*

- Ran HPLC to determine protein concentration and purity
- Performed PH and SDS PAGE measurements to further characterize samples

Beckman Coulter

Research and Development Temporary Employee *April 2006-October 2007*

- Collected and analyzed data on the performance of various automated liquid handlers and plate readers
- Summarized results, wrote reports, and outlined procedures
- Assisted in the design of testing procedures