INVESTIGATION OF SELENOPROTEIN K FUNCTION AND ASSOCIATED PROTEINS

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULLFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

CELLULAR AND MOLECULAR BIOLOGY

MAY 2012

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ACKNOWLEDGEMENTS

I would like to say mahalo to all the faculty, staff, and students at the Cell and Molecular Biology department for making me feel so welcome, and like I belong. I would like to say thank you to all my family and friends for their support down this difficult road toward today, and encouragement for tomorrow. I must also thank MirusBio for the research grant received to continue my work I would also like to thank everyone at the Berry lab, the Bellinger lab, the LeSaux lab and the Kuwada lab. I would also like to say mahalo nui loa to everyone at the Hoffmann lab for their time and patience in guiding me and always lending a helping hand, and my mentor Dr. Hoffmann for continuous optimism despite the adversity.

Abstract

Selenoprotein K (SelK) is a small (16kD) single pass transmembrane protein localized to the ER. An Src Homology 3 (SH3)-binding domain within the amino acid sequence was exploited to identify potential protein-protein interactions. The effects of SelK deficiency in immune cells and effector calcium flux served as a model system to elucidate a function for SelK.

Immunoprecipitation and mass spectrophotometry identification of potential binding partners returned Arf-GAP2 and pyruvate kinase as possible binding partners. Plasmid DNA vectors were designed for use in the Two-Hybrid system to confirm preliminary data of suspected interactions with the creation of fusion proteins coding for Vav1 or Vav2 SH3 domain and the SelK SH3 binding domain. Protein-protein interaction could not be confirmed in this system however SelK seems to play a role in receptor mediated calcium flux, and subsequent low- level production of nitric oxide by way of neuronal nitric oxide synthase.

The form and content of this abstract are approved, and I recommend its publication.

Signed _____

Chairperson

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Acronyms and Abbreviations

°C	degrees Celsius
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
bp	base pair
BSA	Bovine Serum Albumin
BMDM	Bone marrow derived macrophage
Ca^{2+}	calcium ion
CcL20	Chemokine (c-c motif) Ligand 20
CcL22	Chemokine (c-c motif) Ligand 22
Ccr6	Chemokine receptor 6
cDNA	complementary DNA
CMV	Cytomegalovirus
CO_2	Carbon dioxide
co-IP	co-Immunoprecipitation
CpG	cysteine-phosphate-guanine sequence
CRACM1	Calcium release activated calcium channel
CxCL10	Chemokine (c-x-c) Ligand 10
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagles Media
DNA	Deoxyribonucleic acid
dSelK	Drosophila SelK
EDTA	Ethylenediaminetetraacetic acid
EFSec	Selenocysteyl-$tRNA^{(Ser)Sec}$ Elongation Factor
ELISA	Enzyme-linked Immunosorbent Assay

eNOS	endothelial Nitric Oxide Synthase
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERK	Extracellular-signal regulated kinase
ES	Embryonic Stem Cell
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GDP	Guanine diphosphate
GFP	Green fluorescent protein
GPx	Glutathione peroxidase
GSH	Glutathione
GTP	Guanine triphosphate
IARC	International Agency for Research of Cancer
IFN-γ	Interferon gamma
IgG	Immunoglobulin class G
IL-1a	Interleukin-1 alpha
IP	Immunoprecipitation
IP3	Inositol triphosphate
pI	Isoelectric point
kb	kilobases
kDa	kilodalton
KO	Knockout
LC/MS	Low complexity/Mass spectrophotometry
LPS	Lipopolysaccharide

mM	millimolar
ng	nanogram
NIH	National Institute of Health
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	Phosphoenolpyruvate
PLC	Phospholipase C
ppm	Part per million
PSTK	Phosphoseryl $tRNA^{(Ser)Sec}$ Kinase
RDA	Recommended daily allowance
RLU	Relative light unit
ROS	Reactive Oxygen Species
RT-PCR	Real-Time PCR
SBP2	SECIS Binding Protein 2
SDS	Sodium dodecyl sulfate
Se	Selenium
Sec	Selenocysteine
SECIS	Selenocysteine Insertion Sequence
SecS	Selenocysteine Synthase
SELECT	Selenium & Vitamin E Cancer prevention Trial
SelK	Selenoprotein K
SFK	Src family kinase
SH2	SRC Homology 2 domain

SH3	SRC Homology 3 domain
SOCE	Store operated Calcium entry
SPS2	Selenophosphate Synthetase
STIM1	Stromal interaction molecule 1
TCR	T cell receptor
TLR	Toll-like receptor
tRNA	transfer Ribonucleic Acid
trsp	Selenocysteine tRNA gene
UK	United Kingdom
UTR	untranslated region
WCL	Whole cell lysate
WT	Wild Type
µg	microgram

HYPOTHESIS

The biological role of selenoprotein K (SelK) in immune cells can be revealed by studying the effects of SelK-deficiency on immune cell function and by identifying protein-binding partners.

Due to the increased investment of cellular energy for selenoprotein synthesis and the lethal consequences of its mechanisms deletion, we can infer the biological importance of a selenoproteins. While the biological functions for several selenoproteins have been studied extensively, others remain poorly understood. SelK was first described in 2003, and subsequent research has revealed some important insight into its functional role. The focus of this thesis project was to determine mechanisms by which SelK affects immune cell function. This involved employment of a mammalian two-hybrid system to confirm preliminary data of an interaction with Guanine exchange factors (GEF) Vav1 and Vav2. In other experiments, a non-biased approach was taken using co-immunoprecipitation with V5 tagged SelK to find potential binding partners of SelK.

The biological role of SelK was examined by RT-PCR in wild-type (WT) and SelK knockout (KO) macrophages to assess cellular function and effects of SelK deficiency. WT and KO macrophage effector functions were assessed by evaluating Fcγ receptor-induced mediators to determine the biological functions of SelK in immune cells.

INTRODUCTION

Selenium (Se) is a nutritionally essential trace element found in the soil at varying concentrations around the world. The soil in the United States contains relatively high levels of Se compared to other countries, although Se levels are subject to regional differences. Other nations, most notably parts of China, New Zealand, and the UK, are considered to have Se poor soil. As a result of low Se concentrations, plant uptake is reduced and lower levels enter the food chain and water supply. Correlating with the lower Se found in some of these regions, there is often found a higher prevalence of Se deficiency-related disorders. For example, Keshan disease is a congestive cardiomyopathy found in parts of China, and Kashin-Beck disease is a severe Se deficiency involving reduced iodine and thyroid function resulting in a progressive osteoarticular degeneration (2, 3). Low Se concentrations are also the underlying cause of White muscle disease in livestock, along with deficiencies in reproduction (4). Most of these disease states can be rectified with adequate Se supplementation. The Recommend Daily Allowance (RDA) of Se, according to the NIH, is 20μg for ages 1-3 years, 30μg for ages 4-8, 40μg for ages 9-13, and 55μg for ages 14 and up (5).

Similar to other required nutrients, a proper balance of Se intake and biological utilization must be achieved. The toxicity of Se, selenosis, has long been documented and can lead to cirrhosis of the liver, hair skin and nail maladies, and neurological disorders (6). Debates remain as research continues on the mechanisms of how excess Se is detrimental to cells and tissue.

The Selenium and Vitamin E Cancer Prevention Trials (SELECT) recently halted supplementation in 2009 to over 35,000 male participants in part due to a slightly increased risk that was statistically non-significant in type II diabetes prevalence in the Se supplementation arm. Excess Se has been proposed to initiate

DNA damage and/or oxidative stress accompanied by double stranded breaks, however experimental models have proven inconclusive (7). Although the International Agency for Research on Cancer (IARC) states that there is no significant data to consider Se as carcinogenic to humans (8), other states of disease and pathophysiology may arise from above adequate intake.

Selenoproteins incorporate the Se atom in the form of the 21st amino acid selenocysteine (Sec). Currently 25 selenoprotein genes have been identified in humans and a variety of functions have been elucidated from the repertoire of selenoproteins. Many selenoproteins function as enzymes with most exhibiting antioxidant activity due to the high reduction potential of the Se atom (9). The earliest discovered and most notable selenoproteins are what is now classified as the family of glutathione peroxidases (GPx). This family of enzymes mediates the reduction of reactive oxygen species (ROS) such as hydrogen peroxides or phospholipid peroxides utilizing glutathione (GSH)(10). However, the function of several selenoproteins still remains unclear.

The incorporation of the Sec amino acid into a peptide sequence is achieved through a complex series of events unlike any other amino acid. This all starts with the aminoacylation of Sec tRNA (containing the ACU anticodon) with a serine residue, which is then subsequently phosphorylated by phosphoseryl-tRNA$^{(Ser)Sec}$ kinase (PSTK). Occurring in parallel to this are the actions of selenophosphate synthetase (SPS2), which is a selenoprotein itself and converts dietary Se with ATP to AMP, P_i and a monoselenophosphate/SPS2 complex. Continuing from this point the SPS2 enzyme complex can bind the phosphoseryl tRNA$^{(Ser)Sec}$ along with Sec synthase (SecS) to dephosphorylate the *O*-phosphoseryl tRNA$^{(Ser)Sec}$ and allocate the monoselenophosphate to the tRNA to form the selenocysteyl-tRNA $^{(Ser)Sec}$ (11).

We know these early players and steps to be essential for life as demonstrated by Michael Bösl et al in their demonstration of the Sec tRNA gene transcript (*Trsp*) in mice to be embryonic lethal (12).

Once the Sec tRNA is synthesized, several other *trans*-acting binding proteins collaborate during translation in a unique manner to complete incorporation of the Sec residue at the ribosome. This assortment of binding proteins and mRNA features are commonly referred to as Selenocysteine insertion elements.

The most unique and notable feature of Sec incorporation is the secondary stem loop structure of the mRNA in the 3' untranslated region (UTR). This hairpin loop of the mRNA is a *cis*-acting binding site of the key proteins that enables the reprograming of a conventional UGA stop codon into the codon of Sec incorporation. This mRNA sequence is referred to as Selenocysteine Insertion Sequence (SECIS). All known selenoproteins have at least one SECIS structure, and notably Selenoprotein P has been shown to have 2 SECIS, and can recode up to 10 UGA codons (13). The SECIS and bound proteins form a complex to override the stop codon machinery of the ribosome and continue with the synthesis of the peptide.

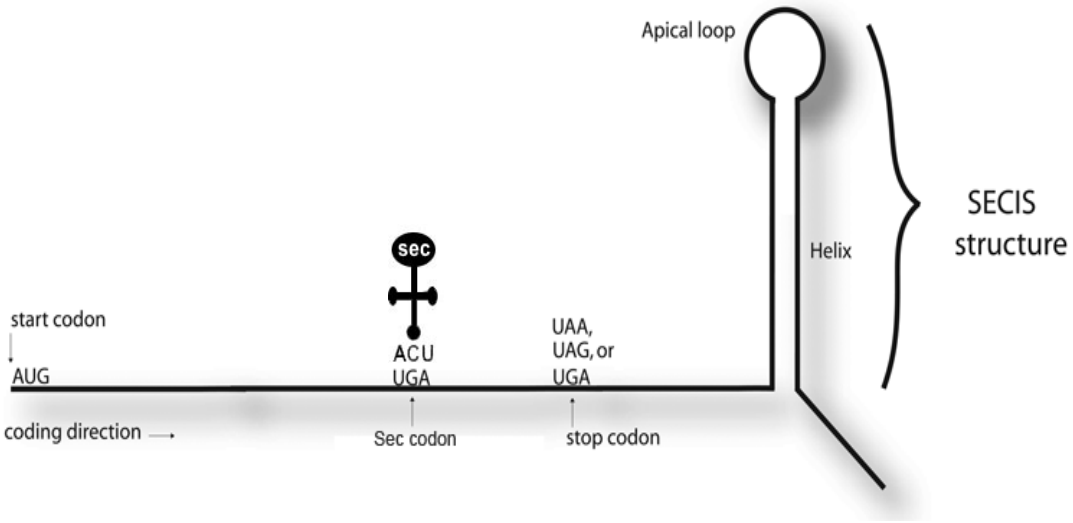

Figure 1. Typical structure of mRNA SECIS. The apical loop is contained in the 3' UTR. At least two stop codons must be included as the first UGA codes for Sec (1)

Among the Sec tRNA binding proteins of these Sec insertion elements is SecP43, which has been shown by Xu et al (14) to be required for methylation of uracil in the wobble position. It is also suspected that SecP43 may be involved in the transporting of the SecS/Sec tRNA complex from the nucleus to the cytoplasm (11).

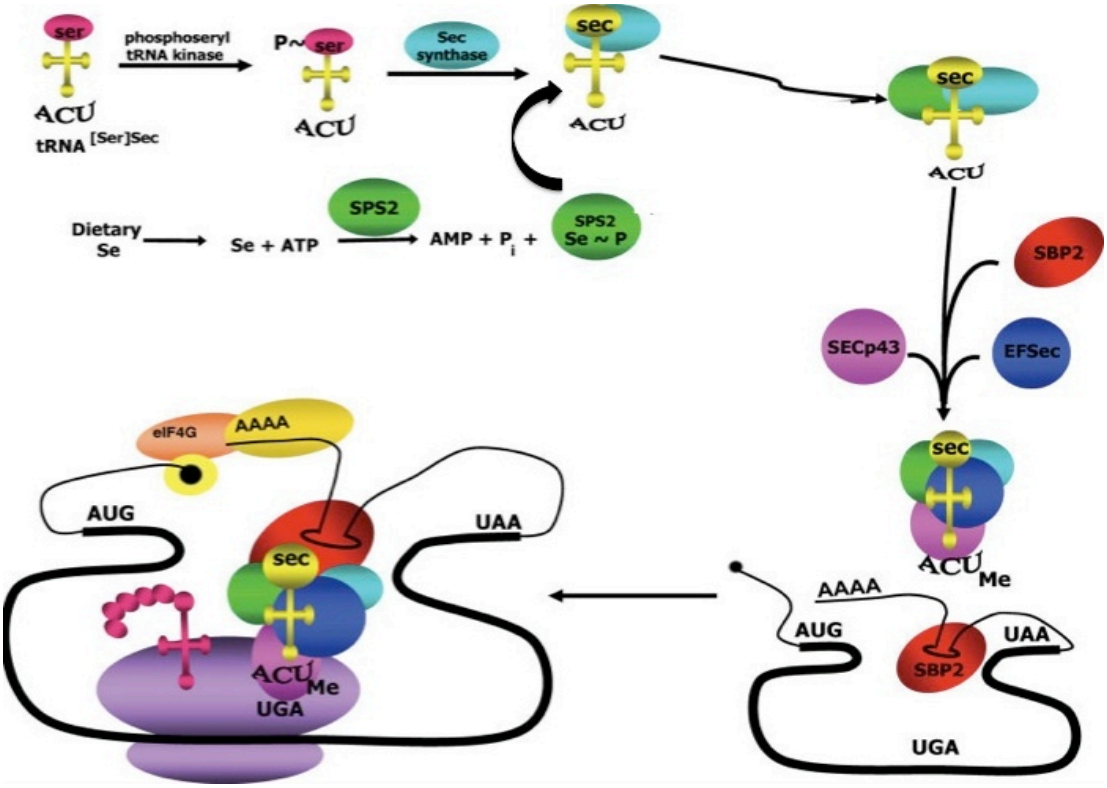

Figure 2. A proposed model mechanism of Sec synthesis and incorporation into protein, enabled by SECIS elements (11). A multiprotein complex must form to bypass normal cell machinery in order to recode the UGA sequence for Sec.

As figure 2 shows, the synthesis of selenocysteyl-tRNA$^{(Ser)Sec}$ and formation of complexes with subsequent binding partners is a complex and highly regulated system. This multistep bypass of conventional cellular machinery for Sec incorporation is therefore a final large investment of energy by the cell.

The series of Sec incorporating proteins are SECIS Binding Protein 2 (SBP2) and Sec Elongation factor (EFSec). SECIS Binding Protein 2 acts as a linker protein with a characteristic internal loop domain to bind within the internal motifs of the SECIS, and also to the large ribosomal subunit during translation (11). Studies have shown co-localization of SBP2 forming complexes with SECIS elements within the nucleus allowing the mRNA to evade nonsense-mediated decay and shuttle to the cytoplasm (15).

The selenocysteyl-tRNA$^{(Ser)Sec}$ Elongation Factor (EFSec) is a final piece to the puzzle of Sec incorporation. With SBP2 associated with the SECIS, EFSec can bind to SBP2 and deliver the tRNA-protein complex to the ribosomal A site for Sec incorporation into the peptide. Once the tRNA has reached the ribosome, EFSec can dissociate from SBP2 elements and reconvene with a subsequent selenocysteyl-tRNA$^{(Ser)\ Sec}$ molecule for Sec incorporation.

As stated before, the evolutionary conserved system of selenoprotein synthesis further indicates the importance of these proteins and emphasizes the impact that a better understanding of their function may have on human health. With this in mind, we have focused our studies on one selenoprotein found at relatively high levels in immune cells, selenoprotein K (SelK).

Selenoprotein K

Gladyshev et al first described SelK in 2003 by computational genomic analyses in experiments including expression of a Se^{75} SelK-GFP fusion protein (16). In that study SelK and SelS were described as novel membrane bound selenoproteins. SelK is a small 94 amino acid peptide that is localized to the endoplasmic reticulum with a predicted a single pass transmembrane region and a minimal N-terminal region in the ER lumen (17). Mouse and human SelK share a

91% amino acid sequence homology, with both containing one Sec residue in proximity to the C-terminus of the protein (17). It has been shown that the Sec residue resides on the cytosolic region of the Golgi apparatus in Drosophila SelK (dSelK) (18), but the Sec orientation has yet to be proven in mammals. Features of SelK include a rigid proline rich structure in the cytoplasm as shown in figure 3, which includes a Src Homology 3 (SH3) binding domain, identified by the consensus sequence R/KxxPxxP. This specialized domain is a notable feature of SelK that may provide insight into its function.

SH3 domains have been implicated, although not exclusively, in scaffolding functions to promote protein-protein interactions for Src Family Kinases (SFK) such as Fyn. Proteins rich in proline tend to be binding partners for SH3 domains, but the diversity of this domain continues to be evaluated with the emergence of new information (19).

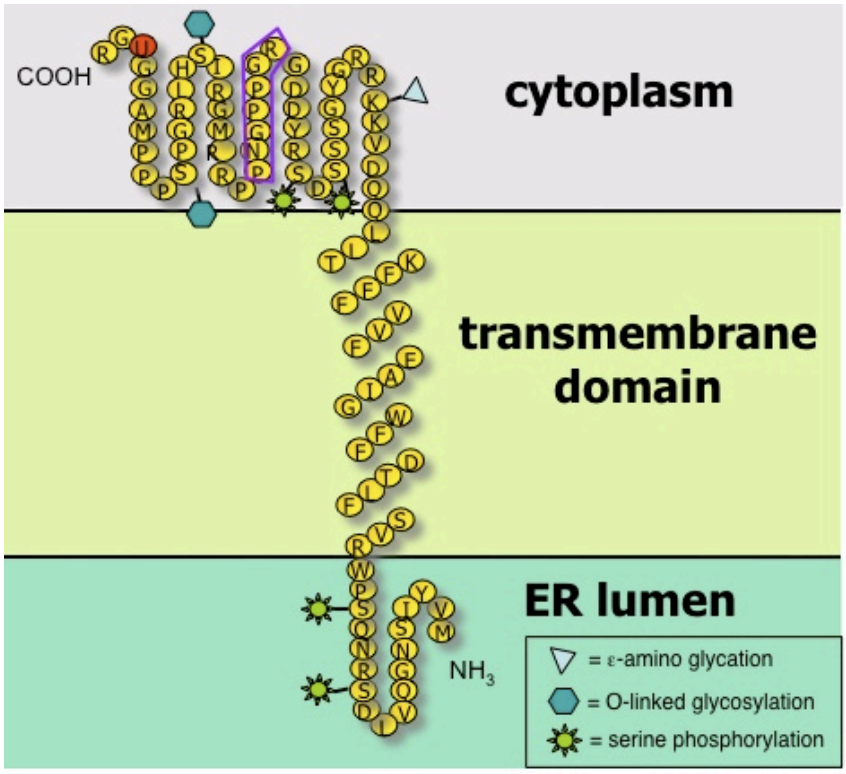

Figure 3. The predicted structure of SelK. Sec residue is shown in red. The SH3 binding domain is outlined in purple. Possible posttranslational modifications of SelK are also suggested (17)

Since SelK seems to show no enzymatic or conserved redox motif found with other selenoproteins (20), the SH3 binding domain has been a key feature in guiding the research of this protein and could prove as a vital piece to the puzzle of elucidating a function for SelK.

Another notable feature of SelK is that it is predicted to run at 10kDa on an SDS (sodium dodecyl sulfate) gel, but is more often observed to migrate characteristically of a 15kDa protein. This is likely a result of the isoelectric point of 10.8, which results from a high abundance of positively charged amino acids that are able to overcome the charge eliminating effect of SDS with SDS-PAGE (17). In correlation to this is the other notable feature of SelK; the predicted transmembrane region contains two acidic residues that reside in the hydrophobic region of the lipid bilayer. The presence of a charged amino acid within the hydrophobic region of the lipid bilayer is energetically unfavorable, and could be involved in a protein-protein interaction with other transmembrane proteins through non-covalent bonds. SelK itself could also be an adapter protein involved in a multiprotein complex formation.

Recently published data has shown that SelK may interact with members of the Derlin protein family, and SelK may associate in a complex involved in ER-associated degradation (ERAD) of misfolded proteins (21). This study also shows an interaction with SelS, which is suggested to share some structural similarities and is also localized to the ER membrane (21). Protein-protein interactions between SelK and other proteins is likely to reveal functional information about SelK, this was the basis of our research project. The N-terminal region located within the ER lumen has no known identifiable features, thus the proposed cytoplasmic C-terminal region was our research focus.

The relative abundance of SelK in different tissues has been established in mice and revealed higher levels of SelK in the spleen and immune cells, and heart tissue containing the least (17). This appears to be the first instance of increased

selenoprotein expression in immune cells. A SelK$^{-/-}$ mouse model was developed (see materials and methods) in order to further understand the functions of this protein and its role in the process of developing immune responses. It has been shown that SelK is required for sufficient receptor-mediated calcium flux in order to elicit a proper immune response involving the activation of neutrophils, T cells, and macrophages (22).

The SelK$^{-/-}$ (KO) mouse was initially developed using a Cre/lox approach in the event that SelK KO was embryonic lethal and tissue-specific or conditional KO could be utilized. Phenotypically, KO mice were found to be healthy and fertile with no abnormal development from that of wild-type (WT)(22). The immune system of KO mice also appears to develop normally despite relatively high expression of SelK in immune cells. This led our research to focus on the effects of SelK KO in the immune system under conditions in which immune cells could be challenged.

Recently published data have shown disregulated calcium (Ca^{2+}) flux as a probable mechanism for the multiplicities of an impaired immune response in KO mice and subsequent KO macrophage, neutrophil, and T cell responses (22). Upon activation of these cells through the T cell receptor (TCR), chemokine receptors, and Fcγ receptors, the binding of a ligand activates phosphoinositide-specific phospholipase C (PLC) which in turn mediates the cleavage of phosphatidylinositol-4,5 bisphosphate to yield inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 then binds to its receptor (IP3R) on the ER membrane resulting in Ca^{2+} release into the cytosol. It is likely that at this step of IP3 binding to its receptor, SelK may convey its function. As Ca^{2+} is released into the cytosol from within the ER, stromal interaction molecule 1 (STIM1) relocates to regions of the ER membrane proximal to the plasma membrane at appositions called puncta. Here STIM1 interacts with Ca^{2+} release activated Ca^{2+} channels (CRACM1) to promote an influx of extracellular Ca^{2+} into the cytosol. This example of store operated calcium entry (SOCE) is a critical

step towards T cell proliferation, chemotaxis, and other activation processes. Hematopoietic STIM1$^{-/-}$ mice have been shown to be defective in SOCE, and subsequently have impaired downstream effects such as phagocytosis in macrophages (23).

SOCE is also important for neutrophil chemotaxis and is also receptor mediated. Hoffmann et al (22) showed decreased migration of KO neutrophils after chemokine receptor stimulation. This was also shown using an in vivo model upon induced peritonitis with a viral mimetic poly (i:c). Several research studies have also shown that proper SOCE signaling cascades through Fcγ receptor stimulation is required for optimal phagocytosis in macrophages (23, 24).

As an integral ER membrane protein SelK has been implicated as playing a role in ER stress response. Although cell lines induced with ER stress reagents such as tunicamycin have linked SelK expression to ER stress elements (21, 25), SelK KO tissues do not show similar results (22). However KO tissues could have likely developed an alternative compensatory mechanism. The rationalization could be made that the impaired Ca^{2+} flux in KO immune cells is merely induced by ER stress. However data using thapsagargin and ionomycin to induce Ca^{2+} flux showed no difference between WT and KO immune cells, suggesting that ER homeostasis and ER stress are not the mechanisms by which SelK deficiency impairs immune cell function (22). In addition, stimulation of SelK deficient macrophages through Fcγ receptors produced lower levels of mediators and signaling compared to WT in a manner that did not involve ER stress (26). Overall, there appears to be a role for SelK in ERAD-mediated shuttling of misfolded proteins from the ER lumen as well as a second independent function related to Ca^{2+} flux in immune cells.

Other research has emerged providing clues as to the biological roles of SelK. Huang et al. found that SelK was a novel target of the proteolytic enzyme calpain. In particular m-calpain was shown to be more active in macrophages than μ-calpain

and m-calpain was found to cleave SelK between Arg_{81} and Gly_{82} (27). The cleavage of SelK by calpain was inhibited by calpastatin upon macrophage activation through Toll like Receptors (TLR). The characteristic Sec residue was cleaved from the nascent peptide soon after synthesis in resting macrophages. Truncated SelK remained in the ER membrane, and this was found to be unresponsive to chemokine induced migration. Full length SelK that predominates in activated macrophages functionally promoted migration. Figure 4 shows the proposed mechanism of m-calpain regulation of SelK in resting and activated macrophages.

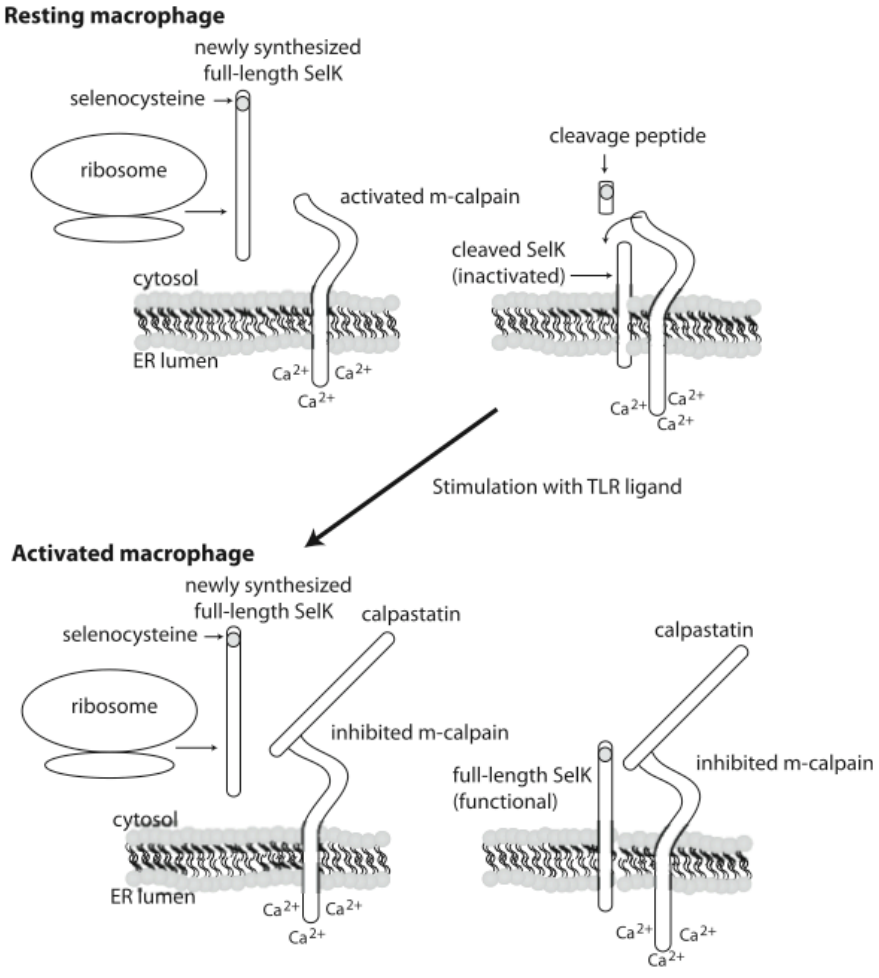

Figure 4. Proposed regulatory mechanism of SelK by m-calpain in macrophages. Newly synthesized SelK is cleaved by m-calpain. Calpain inhibition occurs after TLR stimulation and activation of macrophages and leads to full length SelK (28).

Recent publications have also functionally and structurally compared SelK with Selenoprotein S. SelS was also revealed by Gladyshev et al. at the time of the SelK discovery by the same genomic computational analysis (21). As figure 5 shows SelK and SelS have very similar structures and a may also share similar functions. SelS has also been shown to be a possible component of ERAD complexes along with SelK and has also been suggested to play a role in shuttling misfolded proteins from

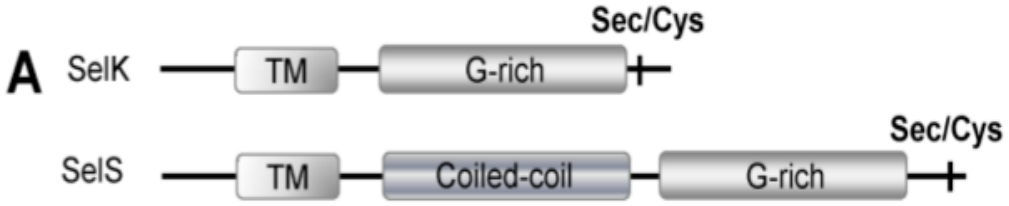

Figure 5. Illustration of domain organization of SelK and SelS. SelK and SelS share a transmembrane domain and a G-rich region. SelK does not contain the coiled-coil domain that SelS does (21)

the ER to reduce ER stress (21, 28). Both proteins are predicted to be transmembrane localized to the ER, but SelK is classified as a type III transmembrane while it remains to be determined if SelS is a type II or type III. Both SelK and SelS encode for the Sec residue within 3 and 2 amino acids, respectively, of the C-terminus. SelK and SelS also both contain regions rich in glycine, proline and arginine. SelS contains a coiled-coil domain for which a proposed homodimer or protein binding site was suggested (17), while SelK contains no such domain. SelS and SelK are both relatively rich in proline (10.9% and 11.4% respectively), however SelS does not have an SH3 binding domain identified in SelK.

Even though debate continues about the biological role of SelK, the predominating theories may not be mutually exclusive. It has been clearly shown that SelK is required for optimal immune response in mice, SelK expression has also been shown to be increased during ER stress in HepG2 cells (28), and under similar experiments SelK has been linked to ERAD complexes. Insights into the structure and functions of SelK have been provided by recent studies, however much more remains to be revealed about the biological role of SelK, and researchers have many avenues to explore.

MATERIALS AND METHODS

Mice: Selenoprotein K knockout (KO) mice were generated by transplantation of embryonic stem cells (ES) into C57BL/6 blastocysts with one wild type (WT) and one floxed SelK allele, $SelK^{wt/fl}$ to generate chimera offspring, to be mated to produce $SelK^{fl/fl}$ mice on a C57BL/6 background. FLP1 transgenic mice purchased from The Jackson Laboratory were bred with $SelK^{fl/fl}$ to generate offspring with Neo cassette removed; these mice could be bred to regenerate $SelK^{fl/fl}$ offspring. These mice were subsequently mated with CMV-Cre transgenic mice, also purchased from The Jackson Laboratory, to produce offspring in which SelK is deleted in all tissue ($SelK^{-/-}$). Polymerase chain reaction was utilized to confirm offspring being SelK KO. Targeted amplification of a 408 base pair product present in WT allele was carried out with forward primer (5' TTC CTG CCC TAG TTG AGT TCT TCT 3'), reverse (5' TGT ATG CCA TTC TTA GTC CAG TTT 3'); and a 1.6kb product in the excised allele with forward primer (5' CGC CTC CGA GAA TTA CAT ACT GA 3') and reverse primer (5' GCT GGG GCC ACG AAG GT 3'). Mice were kept on standard chow with 0.25ppm (part per million) selenium content. Littermate C57BL/6 wild-type controls were generated from mice originally purchased from Jackson Laboratories. All animal experiments were done following approval of University of Hawaii Institutional Animal Care and Use Committee.

Mammalian two-hybrid system: CheckMate™ Mammalian Two-Hybrid system was purchased from Promega containing 5 high copy plasmids pACT, pBIND, PG5luc, pBIND-Id, and pACT-MyoD. All vectors were ampicillin resistant and were cloned in Invitrogen One Shot® TOP 10 chemically competent *E. coli*. Plasmid Maxi-preps were performed by inoculation of ampicillin LB broth incubated overnight at 37^{o} and 250 rpm orbital shake. QIAfilter by Qiagen Maxi-prep was used to purify all plasmids.

Transfection of HEK-293 (ATCC# CRL-1573), NIH 3T3 (ATCC# CRL-1658), and CHO-K1 (ATCC# CCL-61) cell lines were carried out in a Corning Inc. sterile 12 well tissue culture dish, using Lipofectamine 2000 from Invitrogen in a 1:1:1 molar ratio for all experiments. Cell lines were plated at 5 X 10^5 cells per well. Transfected cells were harvested after 72 hours or 90% confluence with Dual-Glo® luciferase assay reagent from Promega containing lysis buffer and required substrate. Firefly Luminescence was measured on SpectraMax M3 plate reader after 10-minute cell lysis incubation. *Renilla* luminescence is constitutively expressed and coded in the pBIND vector and acts as a transfection control, and therefore must be corrected for in final calculations. Stop & Glo® reagent was then added to halt firefly luminescence and provide substrate for *Renilla* luminescence. *Renilla* luminescence was then measured as background for each transfection and firefly luminescence calculated as a relative ratio.

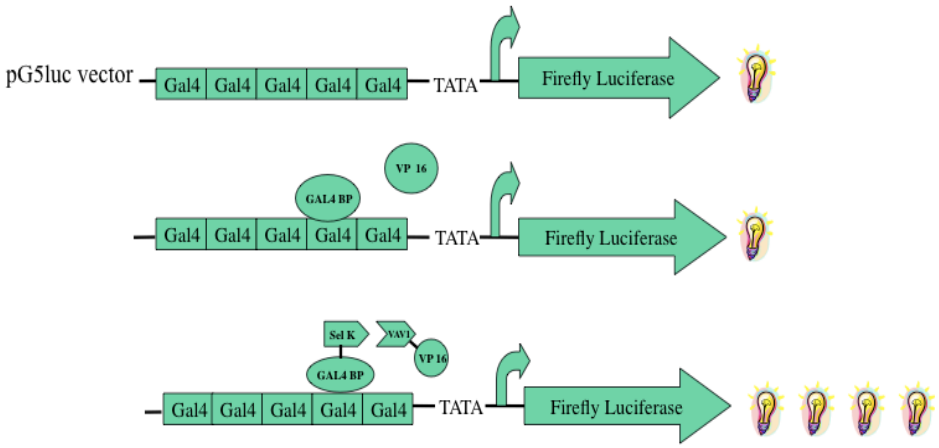

Figure 6. Schematic representation of protein interactions in Mammalian two-hybrid system. Fusion protein GAL4BP-SelK is co-transfected with fusion protein VP16-Vav1, and pG5luc plasmid. Should SelK and Vav1 interact, they would do so upstream of a promoter TATA box and promote transcription of firefly luciferase.

Fusion protein design: Functional domain fragments of SelK, Vav1 and Vav2 were designed using Vector NTI (Invitrogen) as integral components to be inserted into pACT and pBIND vectors. These plasmids encoding fusion proteins for SelK, Vav1 & Vav2 were then constructed by Genscript according to specific design restrictions. The cytoplasmic region of SelK (465bp) containing the SH3 binding domain was cloned and inserted into both pACT and pBIND plasmid vectors at the 3' end of both VP16 and GAL4 sequences keeping the coding sequences in frame. The Sec residue for the SelK sequence was designed to code for cysteine by mutation to TGC. A 722bp fragment encoding Vav1 containing the SH3, SH2, and a second SH3 domain was cloned in the same manner into each vector based on the NCBI sequence. A 905bp fragment of Vav2 containing two SH3 domains was also cloned in the same manner. Each subcloned fusion protein sequence was then confirmed by GenScript.

Antibodies and reagents: Antibodies were purchased from Sigma Aldrich for rabbit polyclonal anti-SelK (HPA008196) for Western blot. Monoclonal anti-GAL4 and anti-VP16 antibodies were purchased from Santa Cruz for Western blots. Monoclonal anti-V5 from GenScript (A01724) for Western blot and Immunoprecipitation. Monoclonal rabbit anti-β actin from Li-Cor technologies (926-42210) was used for control in Western blot. Flourochrome conjugated secondary antibodies were also purchased from Li-Cor. All cell cultures were grown in DMEM with 10% fetal Bovine Serum, and cell counts were measured by use of Millipore Scepter handheld automated cell counter with 60μm tips.

Preparation of Bone Marrow Derived Macrophages: DMEM was forced through resected femur and tibiae bones of CO_2 sacrificed SelK KO mice to harvest bone marrow with a 25 gauge needle into culture dish with DMEM + 10% FBS, maintaining

sterile conditions. Cell clumps were released by shear force through an 18 gauge needle and cell suspensions passed through a 40µm pore cell strainer from Becton Dickinson Falcon. Cells were then collected and plated in DMEM + 10% FBS and allowed to differentiate for 6 days before use.

Immunoprecipitation: RAW cells purchased from ATCC (#TIB-71), and bone marrow derived macrophages (BMDM) were transfected with Mirus Bioscience TransIT 2020 cationic lipid/polymer based reagent and RAW cells were harvested at 48hrs post transfection and BMDM's harvested at 72hrs for Western blot and IP. Cells were lysed in a low salt lysis buffer containing 10 mM Tris pH 7.5, 1% Trition X-100, 5-mM EDTA, proteinase and phosphatase inhibitors, and 5mM NaCl for 10 minutes on ice and centrifuged at 20,000 x g for 10 minutes. Anti-V5 (10µg) antibody was cross-linked to Protein G Dynabeads from Invitrogen using 5mM Bis (sulfosuccinimidyl) suberate from Thermo Fischer. The cross-linking reaction was then quenched with 20mM Tris-HCl before magnetic bead separation from the reaction. Dynabeads were then incubated with whole cell lysates for 1 hour at 37°C with inverted rotation. Protein complexes with V5 tagged SelK were eluted and reduced from magnetically pulled out Dynabeads, and separated by gel electrophoresis on BioRad Criterion TGX gel. Gel portions were then either stained with Coomassie stain for protein by GelCode™ BlueSafe from Thermo scientific, or SilverXpress (Invitrogen) silver staining. Protein bands suspected of being SelK binding partners were then excised from the gel and sent to Applied Biomics for protein identification via LC-MS.

V5 Tagged Selenoprotein K: The sequence for SelK was cloned into pcDNA 3.1 from Invitrogen through restriction enzyme digestion and ligation into pcDNA 3.1 vector by colleague Jeffrey Squires. The V5 tag obtained from Invitrogen was

amplified by polymerase chain reaction with primers designed to incorporate restriction sites Nhe1 and Kpn1. The V5 PCR product was then digested with Nhe1 and Kpn1 restriction enzymes from New England Biolabs, as was the SelK pcDNA3.1 vector. The V5 PCR product was then ligated into SelK pcDNA3.1 by T4 ligase also from New England Biolabs. Figure 7 shows vector map and cloning sites. PCR screens of transformed One Shot Omnimax chemically competent cell colonies were then conducted to confirm proper ligation, as well as Sanger sequencing.

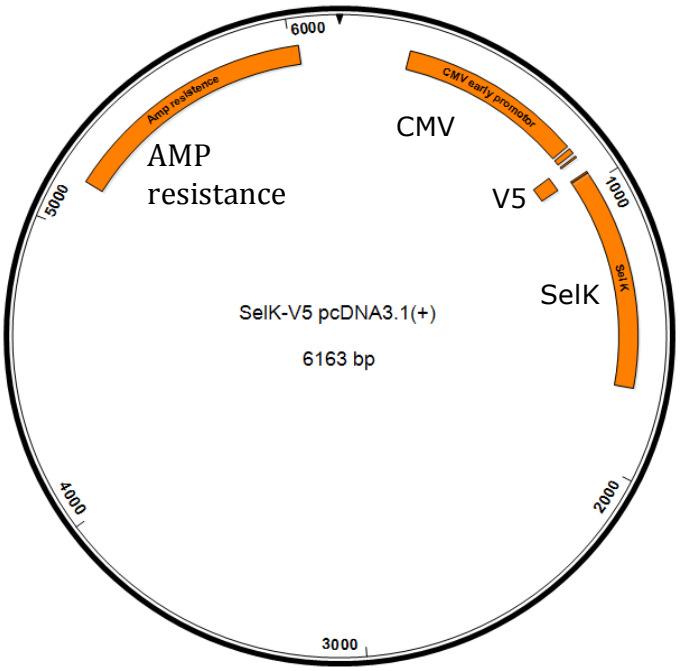

Figure 7. Vector map for SelK with the V5 tag to generate an N terminal fusion protein. The V5 sequence was ligated on the 5' end of SelK using restriction enzymes Nhe1 and Kpn1.

Real-time PCR: Bone marrow derived macrophages were stimulated with BSA-coated beads opsonized with anti-BSA IgG as described by (26). Wild Type BMDM were plated at a concentration of 2 x 10^6 cells/well in a 6 well plate. Cell pellets were harvested at 0, 12, and 24 hours post stimulation and total RNA extracted using RNeasy kit from Qiagen. Complementary DNA (cDNA) was

synthesized using a High Capacity cDNA synthesis kit from ABI containing reverse transcriptase. Real time PCR was carried out with iQ SYBR-Green Supermix from Bio-Rad. PCR reactions included primers for the following; nNOS forward: cat cag gca ccc caa gtt, nNOS reverse: cag cag cat gtt gga cac a, eNOS forward; cca gtg ccc tgc ttc atc, eNOS reverse; gca ggg caa gtt agg at cag, hprt forward; tcc tcc tca gac cgc ttt t, hprt reverse; cct ggt tca tca tcg cta atc. Roche LightCycler 480II conditions consisted of 45 cycles of hybridization at 55°C.

Mouse inflammatory qPCR array: RT-PCR Array plates obtained from SABiosciences contained proprietary lyophilized primers in a 96 well plate format. The plate was designed as a multi-target RT-PCR primer array for 83 common inflammatory mediators, receptors, subunits, signaling ligands, and effectors (Table 1). Five "housekeeping" primers for genes beta Glucuronidase, Hypoxanthine guanine phosphoribosyl transferase 1, Heat shock protein 90 alpha, Glyceraldehyde-3-phosphate dehydrogenase, and beta actin were also included in order to normalize data. Wild Type and KO BMDM were harvested from mice as previously described and plated for 6 days before either stimulation through Fcγ receptors with IgG opsonized BSA for 6hrs, or left unstimulated. Cell pellets were collected by use of QuiaShreder kit mRNA levels were then detected by RT-PCR. Quality control was maintained by use of 3 reverse transcription control wells, 3 PCR control wells, and 1 genomic DNA contamination well. The lyophilized primer was reconstituted with a cDNA master mix and aliquot into a 384-reaction plate in quadruplicates for 45 cycles of PCR hybridization at 55°C.

Table 1. RT-PCR Primer list for inflammatory cytokines and receptors of RT^2 PCR array.

Symbol	Description	Symbol	Description
Abcf1	ATP-binding cassette, sub-family F	**Il13**	Interleukin 13
Bcl6	B-cell leukemia/lymphoma 6	**Il13ra1**	Interleukin 13 receptor, alpha 1
Cxcr5	Chemokine (C-X-C motif) receptor 5	**Il15**	Interleukin 15
C3	Complement component 3	**Il16**	Interleukin 16
Casp1	Caspase 1	**Il17b**	Interleukin 17B
Ccl1	Chemokine (C-C motif) ligand 1	**Il18**	Interleukin 18
Ccl11	Chemokine (C-C motif) ligand 11	**Il1a**	Interleukin 1 alpha
Ccl12	Chemokine (C-C motif) ligand 12	**Il1b**	Interleukin 1 beta
Ccl17	Chemokine (C-C motif) ligand 17	**Il1f6**	Interleukin 1 family, member 6
Ccl19	Chemokine (C-C motif) ligand 19	**Il1f8**	Interleukin 1 family, member 8
Ccl2	Chemokine (C-C motif) ligand 2	**Il1r1**	Interleukin 1 receptor, type I
Ccl20	Chemokine (C-C motif) ligand 20	**Il1r2**	Interleukin 1 receptor, type II
Ccl22	Chemokine (C-C motif) ligand 22	**Il20**	Interleukin 20
Ccl24	Chemokine (C-C motif) ligand 24	**Il2rb**	Interleukin 2 receptor, beta chain
Ccl25	Chemokine (C-C motif) ligand 25	**Il2rg**	Interleukin 2 receptor, gamma chain
Ccl3	Chemokine (C-C motif) ligand 3	**Il3**	Interleukin 3
Ccl4	Chemokine (C-C motif) ligand 4	**Il4**	Interleukin 4
Ccl5	Chemokine (C-C motif) ligand 5	**Il5ra**	Interleukin 5 receptor, alpha
Ccl6	Chemokine (C-C motif) ligand 6	**Il6ra**	Interleukin 6 receptor, alpha
Ccl7	Chemokine (C-C motif) ligand 7	**Il6st**	Interleukin 6 signal transducer
Ccl8	Chemokine (C-C motif) ligand 8	**Cxcr2**	Chemokine (C-X-C motif) receptor 2
Ccl9	Chemokine (C-C motif) ligand 9	**Itgam**	Integrin alpha M
Ccr1	Chemokine (C-C motif) receptor 1	**Itgb2**	Integrin beta 2
Ccr2	Chemokine (C-C motif) receptor 2	**Lta**	Lymphotoxin A
Ccr3	Chemokine (C-C motif) receptor 3	**Ltb**	Lymphotoxin B
Ccr4	Chemokine (C-C motif) receptor 4	**Mif**	Macrophage migration inhibitory factor
Ccr5	Chemokine (C-C motif) receptor 5	**Aimp1**	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1
Ccr6	Chemokine (C-C motif) receptor 6	**Spp1**	Secreted phosphoprotein 1
Ccr7	Chemokine (C-C motif) receptor 7	**Tgfb1**	Transforming growth factor, beta 1
Ccr8	Chemokine (C-C motif) receptor 8	**Tnf**	Tumor necrosis factor
Ccr9	Chemokine (C-C motif) receptor 9	**Tnfrsf1a**	Tumor necrosis factor receptor superfamily, member 1a
Crp	C-reactive protein, pentraxin-related	**Tnfrsf1b**	Tumor necrosis factor receptor superfamily, member 1b
Cx3cl1	Chemokine (C-X3-C motif) ligand 1	**Cd40lg**	CD40 ligand
Cxcl1	Chemokine (C-X-C motif) ligand 1	**Tollip**	Toll interacting protein

Cxcl10	Chemokine (C-X-C motif) ligand 10	**Xcr1**	Chemokine (C motif) receptor 1
Cxcl11	Chemokine (C-X-C motif) ligand 11	**Gusb**	Glucuronidase, beta
Cxcl12	Chemokine (C-X-C motif) ligand 12	**Hprt**	Hypoxanthine guanine phosphoribosyl transferase
Cxcl13	Chemokine (C-X-C motif) ligand 13	**Hsp90ab1**	Heat shock protein 90 alpha (cytosolic), class B member 1
Cxcl15	Chemokine (C-X-C motif) ligand 15	**Gapdh**	Glyceraldehyde-3-phosphate dehydro
Pf4	Platelet factor 4	**Actb**	Actin, beta
Cxcl5	Chemokine (C-X-C motif) ligand 5	**MGDC**	Mouse Genomic DNA Contamination
Cxcl9	Chemokine (C-X-C motif) ligand 9	**RTC**	Reverse Transcription Control
Cxcr3	Chemokine (C-X-C motif) receptor 3	**RTC**	Reverse Transcription Control
Ccr10	Chemokine (C-C motif) receptor 10	**RTC**	Reverse Transcription Control
Ifng	Interferon gamma	**PPC**	Positive PCR Control
Il10	Interleukin 10	**PPC**	Positive PCR Control
Il10ra	Interleukin 10 receptor, alpha	**PPC**	Positive PCR Control
Il10rb	Interleukin 10 receptor, beta	**Il11**	Interleukin 11

RESULTS

Levels of inflammatory cytokine and signaling molecule mRNAs differ between KO and WT macrophages after Fcγ receptor stimulation

Relatively high levels of SelK mRNA have been detected in immune cells (29) and data have also demonstrated that SelK protein expression is increased in the spleen compared to other tissues (22). The development of a SelK KO mouse model allowed us to study the effects of SelK deficiency in the immune system. BMDM cells were cultured from KO and WT bone marrow and were stimulated with IgG opsonized beads to stimulate the Fcγ receptors for 6hrs, or left unstimulated. Levels of mRNA for common inflammatory cytokines and cytokine receptors were measured by way of RT-PCR array plates. Data were normalized to the stable housekeeping mRNA, hprt, and as expected most cytokine or cytokine receptor mRNAs were increased in WT BMDM after Fcγ receptor stimulation (Figure 9). Interestingly, several mRNAs that were increased at higher levels in the KO BMDM compared to WT controls included Chemokine ligand 20 (ccl20), Chemokine ligand 22 (ccl22), Chemokine receptor 6 (ccr6), Chemokine ligand 10 (cxcl10), and Interleukin-1 alpha (Il-1a). These transcripts were among those mRNAs that differed most between WT and KO. Ccl20 and Ccl22 are important chemokines released by epithelial cells and others that recruit neutrophils, monocytes, T cells, and macrophages to the site of infection or trauma (30).

When protein levels for many of these chemokines and cytokines were evaluated, we found no differences between stimulated WT and KO BMDM, and some important cytokines were actually decreased in SelK-deficient BMDM (26). Thus, the increased mRNA levels for several targets in SelK KO BMDM found with this screening assay may indicate a compensatory mechanism in the KO BMDM that

involves increased mRNA levels required for maintaining equivalent levels of secreted cytokines. As part of these analyses, co-author Zhi Huang found that unprimed BMDM secreted low levels of nitric oxide (NO) and KO BMDM exhibited 50% output compared to WT controls (Figure 8). This was in contrast to IFNγ-primed BMDM, which resulted in high output NO that was equivalent between WT and KO BMDM. This suggested to us that SelK played a particular role in unprimed macrophages stimulated through the Fcγ receptors for low output NO.

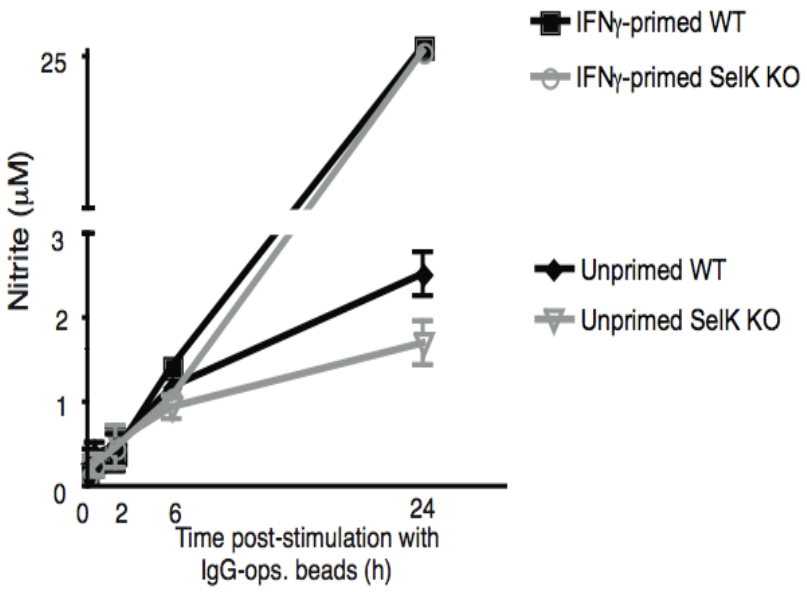

Figure 8. Nitric Oxide output of WT and KO BMDM, primed or unprimed with IFN-γ. SelK KO BMDM secreted less NO than WT controls after 24hrs stimulation through Fcγ receptors (27).

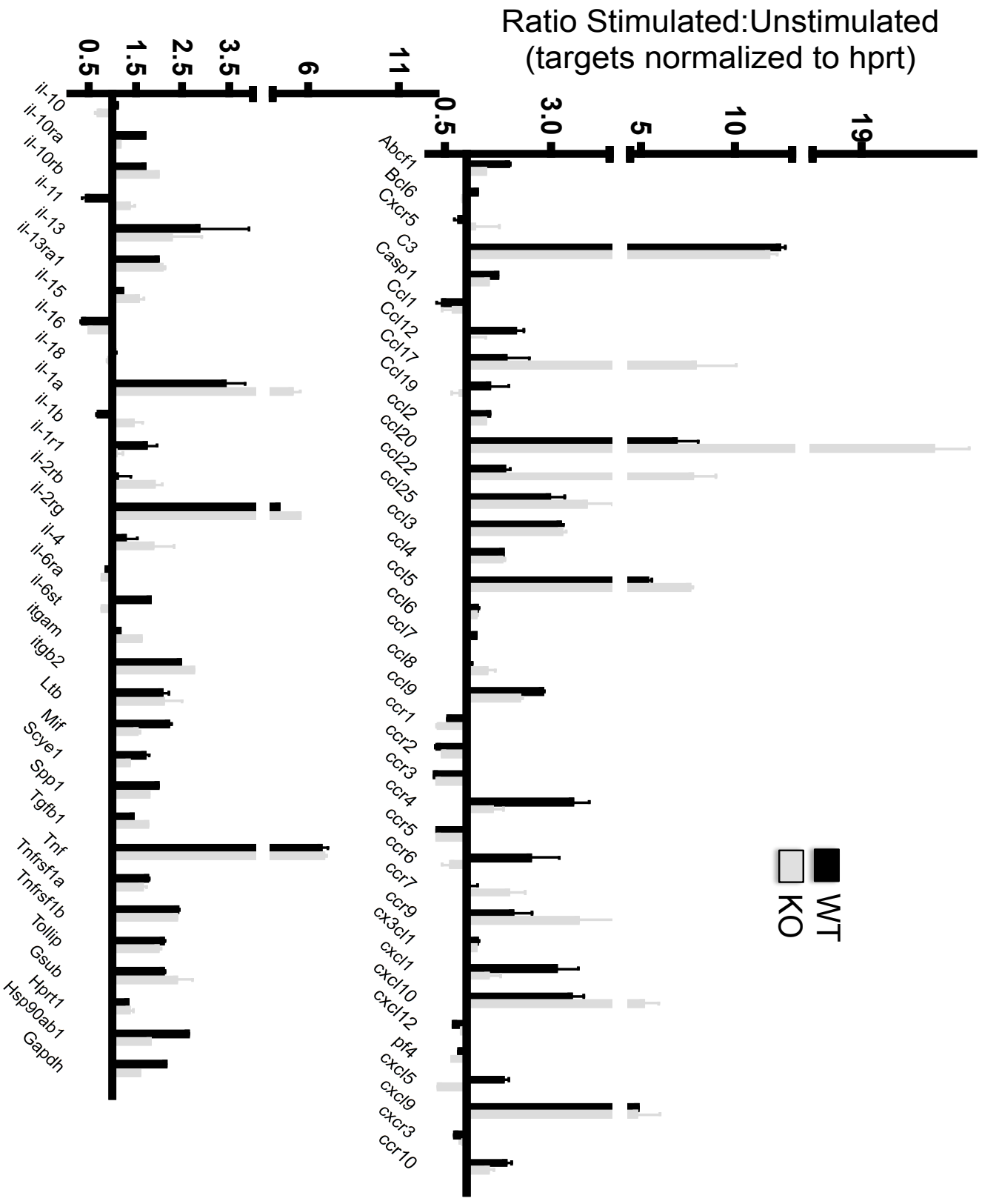

Figure 9. mRNA levels of common inflammatory cytokines and receptors in WT and KO macrophages after 6hr stimulation with IgG opsonized BSA. mRNA levels were detected as a screening tool for possible effects of SelK deficiency in immune cells.

Subsequent experiments demonstrated that SelK was crucial for the calcium flux required for increased activity of neuronal nitric oxide synthase (nNOS). The question remained as to whether this involved increased levels of nNOS mRNA or whether the SelK-dependent calcium flux regulated nNOS at the level of protein synthesis or enzymatic activity. Thus, we next examined the mRNA levels of the nitric oxide synthases (NOS) endothelial NOS (eNOS) and neuronal NOS (nNOS). RT-PCR along with Western blot data of these enzymes demonstrated a particularly significant increase in mRNA (Figure 10) and protein levels for nNOS upon Fcγ receptor stimulation of WT BMDM that did not occur in SelK KO BMDM (26). These results lead to the notion that SelK is required for optimal low levels of NO as a signaling molecule for the macrophage after Fcγ receptor stimulation.

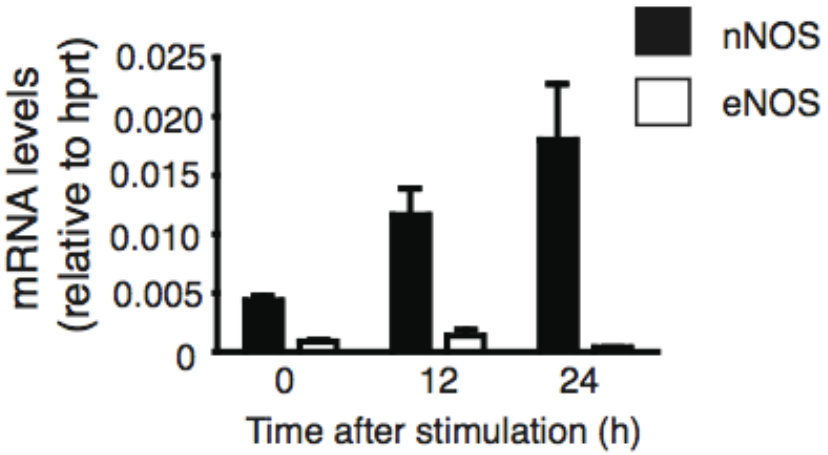

Figure 10. mRNA levels of nNOS and eNOS after Fcγ stimulation in unprimed macrophages Western blot data also confirms increased amounts of protein for nNOS and eNOS after stimulation through Fcγ receptors of macrophages (27).

Employment of a Mammalian two-hybrid system to confirm preliminary binding partners of SelK

We examined the structure of SelK in order to find a possible functional domain in the amino acid sequence. We found within the cytoplasmic region an SH3 binding domain, the consensus sequence being R/KxxPxxP where x is any amino acid. This binding domain is one of the few features of SelK and may provide as a key component to its function. It is also of interest since there is no SH3 binding domain found in the other selenoprotein found in the ER membrane, SelS, thought by some to be functionally similar to SelK, suggesting a unique functional significance in SelK. We analyzed the SelK amino acid sequence using an online computer algorithm called "SH3 hunter" (31), which compares amino acid sequences of queried proteins to known binding partners of SH3 domains. Figure 11 shows the results generated using this computer algorithm, and findings indicated that proteins Vav1 and Vav2 may be possible proteins that bind to SelK, as reflected by relative scores for each.

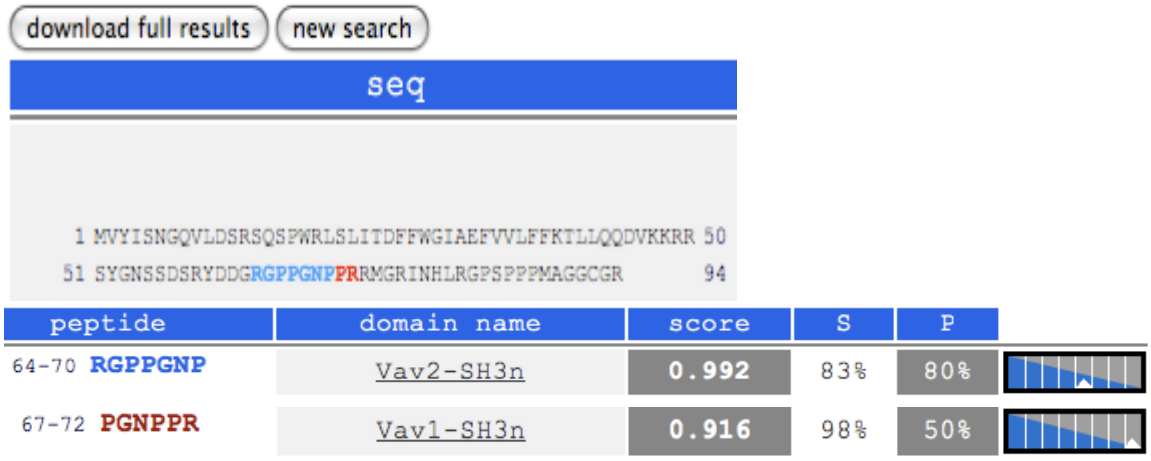

Figure 11. SH3 hunter algorithm results yielded potential binding partners Vav1 and Vav2. These GEF proteins are involved in cytoskeletal rearrangement, and migration

These results led colleague Zhi Huang to perform co-immunoprecipitation experiments using WT BMDM in which Vav1 or Vav2 were immunoprecipitated and the presence of associated SelK protein was detected using anti-SelK immunoblotting. Results indicated SelK was interacting with both Vav1 and Vav2, particularly after stimulation with proinflammatory, TLR ligands including LPS, CpG, and zymosan (Figure 12).

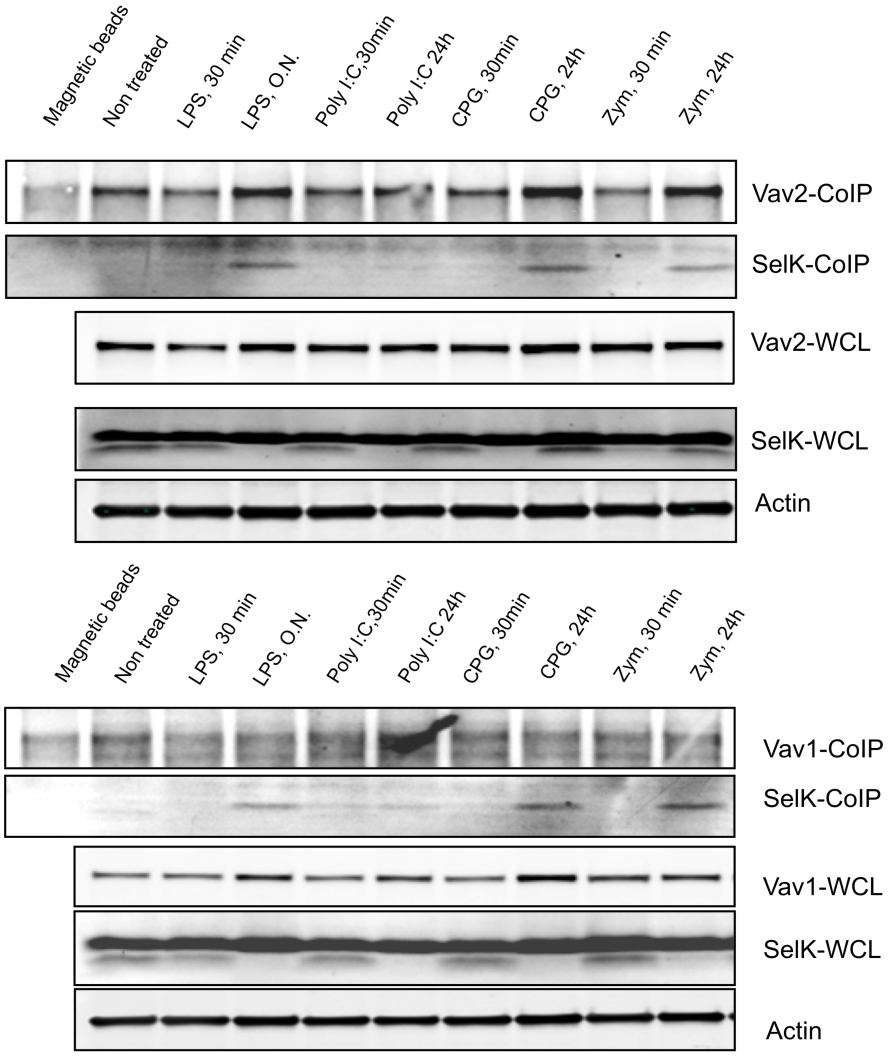

Figure 12. Co-IPs of SelK with Vav1 and Vav2. Increased amounts of SelK were pulled down after overnight stimulation of WT macrophages with LPS, CpG, and Zymozan. Unpublished data from Zhi Huang et al.

Vav proteins have an important role in the regulation of Rho activation, and therefore cytoskeletal rearrangement. This remodeling to the cytoskeleton is critical for a variety of cellular functions such as cell division, growth, adhesion and chemotaxis (32). With Vav proteins having a strong influence on these powerful cellular functions, regulation of Vav machinery can be subverted and transformation of tumors can occur. Vav proteins are classified as an oncogene for this reason, and research has shown the tumorgenicity of disregulated Vav proteins (33).

Further experimentation of protein-protein interactions with SelK led us to attempt confirmation of an interaction with Vav1 by a Mammalian two-hybrid. The two-hybrid system is best known as a screening tool developed in yeast in order to examine protein-protein interactions or protein-DNA binding. The concept of this system was then translated into mammalian cells in order to more accurately model an intracellular in vitro association that may not occur in yeast. To confirm a protein-protein interaction, fusion proteins must be designed as the so-called "bait" and "prey", that are allowed to interact upstream of a reporter gene and promote transcription.

Fusion proteins were designed as such that the bait protein (SelK) is tagged with a portion of a known DNA binding protein, GAL4 binding protein. The prey protein (Vav1) was designed to be a partial structure with the nuclear translocation factor protein VP16. Once the sequence of these fusion proteins is designed and cloned into their respective vectors they are co-transfected along with a reporter gene plasmid that provides as a binding site for the bait/DNA binding protein. Upon binding of the GAL4 DNA sequence, the prey portion of the prey/nuclear translocation fusion protein can interact with its partner and promote transcription of a reporter gene, luciferase, which may then be quantified. Although SelK is described as the bait, and Vav as the prey, we followed the manufacturers recommendation

and also performed each experiment visa versa with Vav as the bait and SelK as the prey.

Due to the complex nature of Selenocysteine insertion into a peptide and complications that may arise with a fusion protein, we decided to design a truncated form of SelK that consisted of the cytoplasmic region and included the SH3 binding domain, and the mutation U92C. After thorough design of the SelK fusion protein we outsourced its construction to GenScript for single nucleotide polymerization and subcloning into vectors pACT and pBIND as to code for VP16 and GAL4 at the amino terminus of SelK.

Following failed attempts of molecular cloning of full length Vav1 and Vav2 into pACT and pBIND, we concluded that the next best course of action was to design truncated forms of Vav and outsource their construction to GenScript in the same matter. We designed Vav proteins to contain the two functional SH3 domains and SH2 domain located at the C-terminus of the full-length peptide. These proteins were also subcloned into pACT and pBIND for and N-terminal tagging of VP16 and GAL4 respectively.

Attempts to confirm the expression of SelK fusion protein constructs in HEK-293 cells by way of Western blot analysis proceeded without success. Figure 13 shows that we were able to successfully detect expression of endogenous SelK and over expression of GFP tagged SelK, but not fusion protein expression for either construct. This effect could be the result of a multitude of factors including antibody specificity, partial or complete burying of the epitope in to the interior of the fusion protein, or steric hindrance. After consulting with Promega of this concern, they informed us that routine confirmation of fusion protein expression by Western blot is not performed. Sequence data of each fusion protein from GenScript confirmed accurate design and construction of all fusion protein constructs.

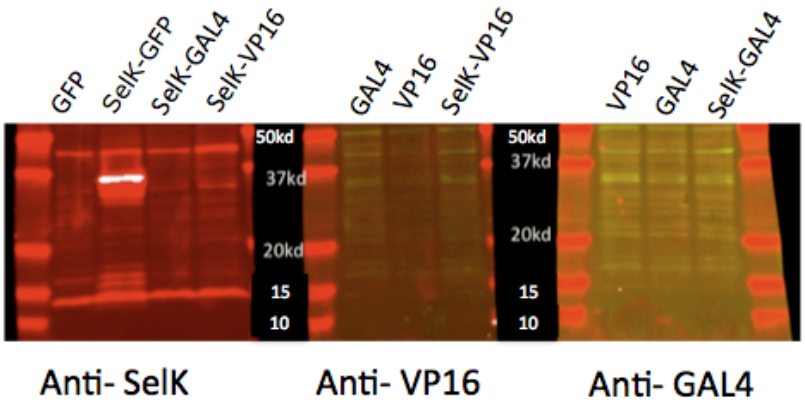

Figure 13. Western Blot analysis reveals fusion protein expression could not be conventionally detected for SelK-VP16 or SelK-GAL4. Predicted sizes for SelK-VP16 are ~20kda, and SelK-GAL4 at ~40kDa.

Considering the fact that after the fusion proteins take on their tertiary folding conformation, their epitope for their respective antibodies may have altered, but due to the proline rich structures of SelK and Vav, and vested interest we continued with experimentation.

Attention next turned to running the luciferase reporter assay. Transfected HEK 293 cells with each construct, appropriate controls, and reporter plasmid in a 1:1:1 molar ratio as recommended in a 12-well plate. Figure 14 is a simplified map of the transfection controls and experimental wells.

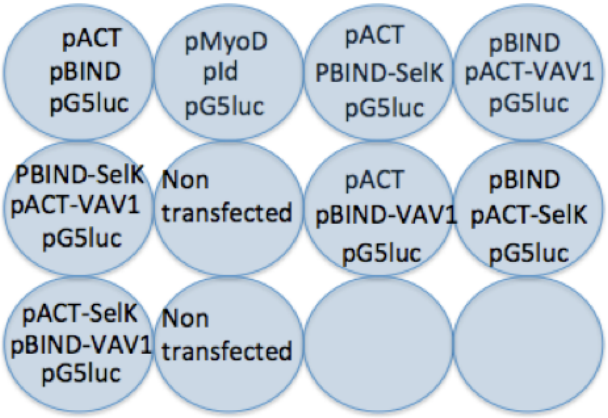

Figure 14. For each experimental luciferase reaction a series of 4 negative controls and one positive control must be also be analyzed for luminescence.

Firefly luminescence was quantified as light forming units and background *renilla* luminescence subtracted. Firefly luminescence has been reported to be stable for up to 2 hours after addition of luciferin substrate (34); therefore initial measurements were taken 10 minutes and 1 hour after addition of luciferin substrate. Addition of Stop and Glo® reagent quenches firefly luciferase and provides substrate for *renilla* luciferase. Figure 15 represents results from one of three independent experiments and relative ratios of luminescence are shown.

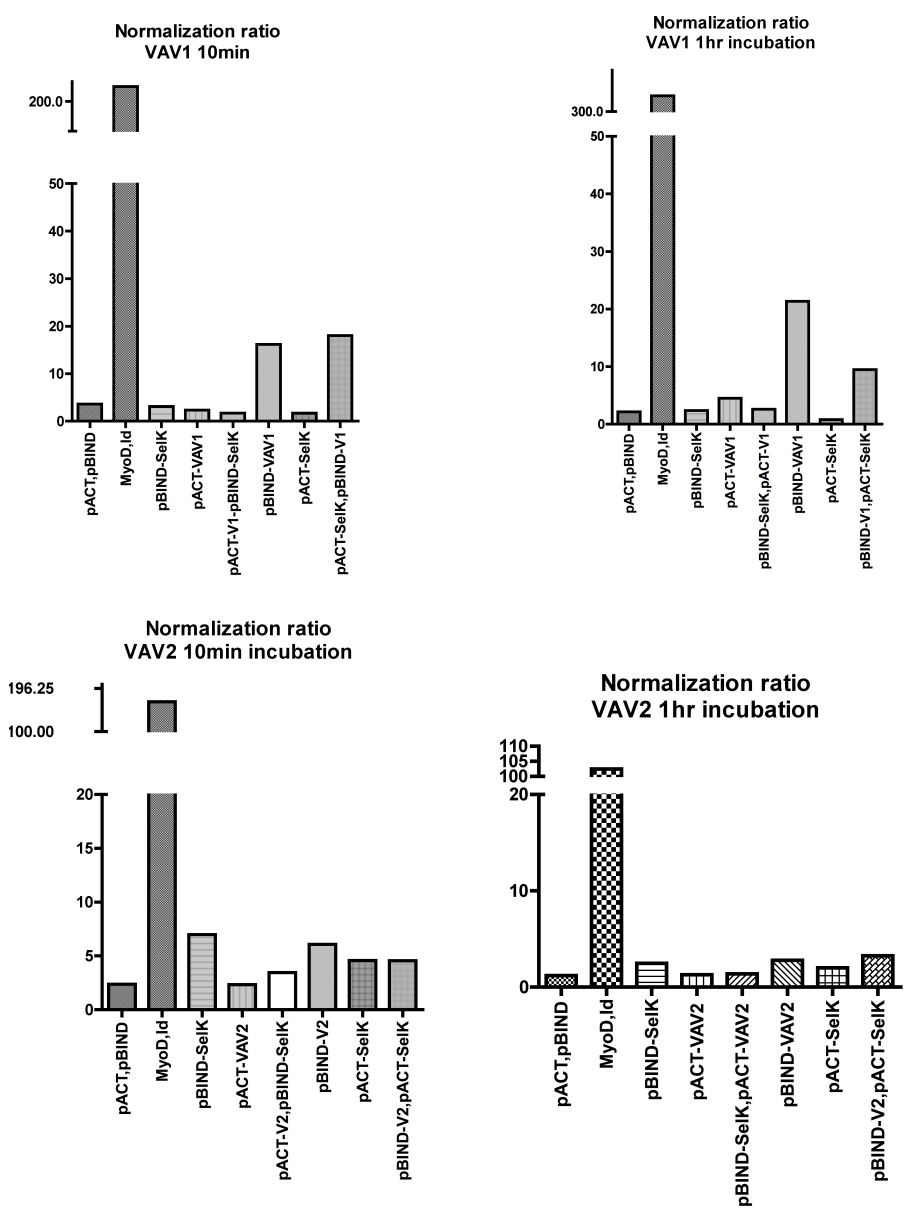

Figure 15. Relative ratios of experimental reactions (pACT-SelK, pBIND-Vav) and visa versa, with positive control MyoD-Id and negative controls. Luciferase light signal is stable for up to 2hrs. Measurements were taken at 10min and 1hr after addition of substrate for Vav1 and Vav2.

The positive control MyoD, Id is known to associate after binding to the multiple GAL4 binding sequence of the pG5luc vector, and initiating transcription of luciferase in upwards of 50,000 RLUs (relative light unit) in certain cell types. Controls are the key to interpretation of these assays, particularly expression of prey alone and bait alone (negative controls) as well as MyoD, Id (positive controls). A light signal in experimental wells above negative control levels correlates to a positive interaction of the fusion proteins. We discovered our system seemed to have a slight preference for Vav1 as the bait and SelK as prey, as indicated by reaction pACT-SelK, pBIND-Vav1, however due to relatively low levels of light intensity and high level of negative control, this conclusion could not be significantly reached.

Overall the two-hybrid system has strong potential for discovering prospective binding partners for proteins of unknown function. The mammalian cell model also has advantages over the traditional yeast two-hybrid by interactions resembling more accurate conditions of mammalian protein affinity. We concluded that due to our fusion protein design of using the putative functional binding portions of each protein, optimal conformations required for a proper protein-protein interaction may not have been achieved. Full-length SelK or Vav proteins may be required for interactions to occur. Alternatively, proper localization of the fusion proteins also may not have been achieved resulting in a negative signal. In particular, since SelK is a transmembrane protein, its location in the ER membrane (not included in the mammalian two-hybrid screen) may also be required for interactions to occur. Despite confirmed sequence analysis, there is the possibility that each construct is not properly expressed. An improper sequence may lead to mRNA degradation or the resulting protein being labeled for degradation. Finally, we must also consider the possibility that SelK does not actually interact with Vav1 or Vav2, although the co-immunoprecipitation data would suggest otherwise.

Immunoprecipitation of V5 tagged SelK yields prospective binding partners.

As a complementary and alternative approach to identify a binding partner for SelK, co-immunoprecipitation was performed in a non-biased fashion with the implementation of tagged SelK. The V5 protein tag is a common epitope used for a multitude of research techniques such as immunoprecipitation, ELISA, and Western blot. The peptide sequence is derived from the V protein of the Simian parainfluenza virus 5, with the short amino acid sequence GKPIPNPLLGLDST (35). PCR was implemented to introduce the V5 sequence into SelK pcDNA3.1 vector as described in materials and methods courtesy of colleague Jeffrey Squires, and confirmation of its expression in was detected in transfected macrophage cell line RAW by Western blot analysis (Fig 16).

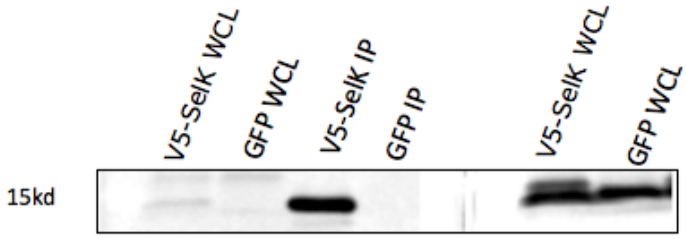

Figure 16. RAW macrophage cell lines express V5 tagged SelK. Overexpressed V5-SelK shows a light band at the predicted size of 15kDa when blotted for anti-V5, left panel. V5-SelK was also detected from immunoprecipitation supernatants samples. WCL also show V5-SelK and endogenous SelK when blotted with anti-SelK antibody.

Once our V5-SelK construct proved to be properly expressed we continued with our immunoprecipitation and identification of binding partners. Our next experimental procedure was to perform an immunoprecipitation pull-down in order to stain the protein gel with Coomassie blue to identify any protein bands co-immunoprecipitating with V5-SelK. SelK KO BMDM and RAW cell line macrophages were transfected to overexpress V5-SelK or GFP for control. Pull-down supernatants were separated by gel electrophoresis and stained with Coomassie blue.

Figure 17 shows similar banding patterns between the V5-SelK pull-down and the GFP control, suggesting the presence of many non-specific proteins in both lanes. However, we detected a faint protein band between the 50 and 75kDa markers that were present in the V5-SelK lane but not in the GFP control. These areas are indicated in figure 17 by the red arrow (left panel), and grey box (right panel). These areas for both lanes were excised from the gel and slices were sent for amino-acid microsequenceing using the Low Complexity/ Mass Spectrophotometry (LC/MS) process by Applied Biomics.

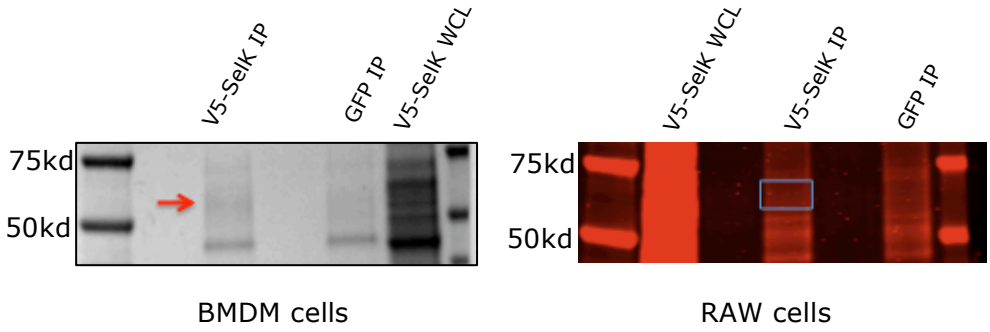

Figure 17 Coomassie stain of immunoprecipitation supernatants. Faint bands appear after destaining of Coomassie blue between the 50 and 75kDa markers. Left panel shows SelK KO BMDM transfection and RAW macrophage cells line in right panel.

The sensitivity of Coomassie staining must also be considered in such a procedure, the lower limit is reported to be at 9ng of protein (36). As a secondary method to visualize a faint protein band, we used a more sensitive silver stain assay. Silver staining has a reported sensitivity of 0.5ng (37), by the reaction of silver nitrate to metallic silver at the location of a protein band.

Figure 18 shows the results of a silver stained protein from RAW cells separated by SDS gel electrophoresis. Outlined in black is the approximate region that was excised for LC/MS. Despite the increased sensitivity of silver stain, no obvious protein band was visible at the corresponding location of the Coomassie stained gel. This maybe attributed to the inability of silver stain to bind to certain glycoproteins or lipoproteins. The silver stain may have also increased the background, making this more sensitive detection approach less effective at identifying proteins more prevalent at the 10-100ng level. Alternatively, the proteins detected by Coomassie in the first experiments may not have been pulled down in the second experiments.

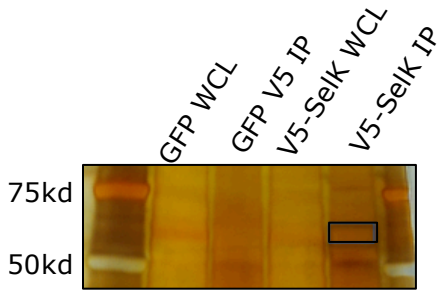

Figure 18. Silver stained SDS gel of V5-SelK Immunoprecipitation. RAW cell line pull-down supernatants were separated by gel electrophoresis and silver stained in order to visualize protein bands. The black box region in the approximate area ~60kDa, to excised gel bands in coomassie stained protein gels.

Protein identification results were obtained from Applied Biomics, and comparison of the V5-SelK to control only yielded two potential binding partners that were unique to the V5-SelK. These proteins were the Pyruvate Kinase M1/M2, and Arf-GAP protein 2, which does contain an SH3 domain.

Pyruvate kinase is an enzyme within the glycolysis pathway that catalyzes a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to yield pyruvate and adenosine triphosphate (ATP)(38). Arf-GAP protein 2 has a known SH3 domain and associates with the cytosolic leaflet of the plasma membrane and helps to mediate intracellular traffic and vesicle transport out of the cell (39).

DISCUSSION

The cellular function of SelK continues to be studied, and interpretations by various researchers foster discussion. The functional aspects of SelK have begun to emerge from recent publications examining the phenotype of SelK KO mice and various cell types (21, 22, 27), and particularly the roles that SelK plays in activation of immune cells (26). Ca^{2+} flux is impaired in SelK deficient cells and is a critical step for immune cell activation, and facilitates many downstream mediators. SelK deficiency results in impaired motility in T cells, neutrophils, and macrophages (22).

After stimulation through Fcγ receptors, we found that low output of nitric oxide was significantly reduced in SelK KO macrophages compared to WT controls after stimulation through Fcγ receptors (26). We also found that SelK was required for calcium dependent ERK activation and subsequent transcription of nNOS (26). Figure 19 is a schematic model representation of the role SelK may play in low output NO as a result of Fcγ receptor stimulation, however the exact mechanism in which SelK may exert its effects on Ca^{2+} flux remains unknown.

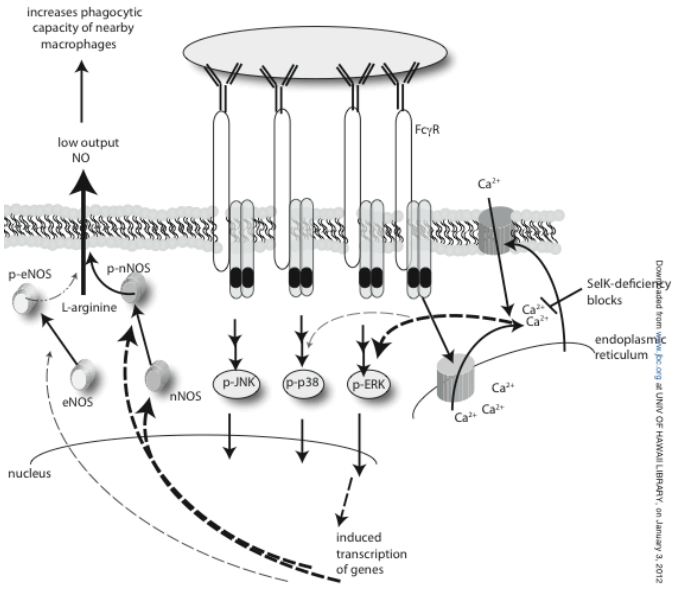

Figure 19. Proposed model mechanism of NO secretion by macrophages after Fcγ receptor activation. Fcγ Receptor engagement induces SOCE, which in turn phosphorylates ERK. ERK activation regulates JNK activation and gene transcription of nitric oxide synthases (26).

The structural features of SelK led us to implement several research techniques, and explore the many possibilities of this unique protein. The lack of a redox motif that is present in many other selenoproteins gives us alternative avenues to explore the function of SelK. As previously stated, SelK contains two acidic residues within the hydrophobic region of the lipid bilayer. As this is a highly energetically unfavorable conformation for the transmembrane region, this may suggest a possible protein-protein interaction, or dimerization through this domain. Studies of this feature of SelK have yet to come to light, and may provide insight toward this proteins function.

As a consequence of the proline rich structure of SelK, the SH3 binding domain is a prime candidate to resolve a mechanistic function of SelK. We implemented several research techniques to exploit this domain. Our Mammalian Two-hybrid system did not provide us with confirmation of an association with the SH3 domains of Vav proteins after several attempts in several cell lines. As stated earlier, only functional domain regions of SelK and Vav proteins were included in the design of the fusion proteins, and the structure of the fusion proteins may not have accurately resembled physiological proteins. In addition, we cannot confirm that proper localization of each fusion protein occurred, which would prevent an interaction. Finally, the SelK fusion proteins were not designed for transmembrane configuration, and this may also be required for interaction to occur with the binding partner fusion protein. Full length SelK and Vav proteins may be required in order for a protein-protein interaction, and successful molecular cloning of each sequence into their respective plasmids may prove a positive interaction. The Mammalian Two-hybrid system was a high-risk experiment, and other possible methods such as Tandem Affinity Purification, or traditional yeast two-hybrid may provide positive identification of binding partners for SelK.

Immunoprecipitation results yielded interesting potential binding partners after mass spectrophotometry. The protein Arf-GAP that co-immunoprecipitated with tagged SelK contains an SH3 binding domain and is s strong candidate for a binding partner. Arf-GAP proteins are GTP binding proteins, and regulate intracellular traffic. Arf-GAP proteins hydrolyze GTP to GDP and are localized on the cytosolic leaflet of the lipid bilayer, but their complete characterization has not been resolved (39). As of yet SelK has not been implicated in vesicle formation as Arf-GAP proteins have, and it can only be speculated that Arf-GAP proteins may be involved in cytoskeletal rearrangement and association with GEF proteins such as Vav1.

The other protein identified from immunoprecipitation, pyruvate kinase, is an enzyme in the glycolysis pathway, and was of particular interest due to the high number of peptides, which corresponds to the amount of protein in the excised gel band that was identified during mass spectrophotometry. Further evaluation revealed that pyruvate kinase has been shown as a binding partner to Fyn (40). Fyn is an oncogene member of the Src family kinases. Fyn contains an SH3 domain and functions to activate protein Ras, and FAK for cell adhesion and mobilization, and also PI3K (41). The interaction of Fyn with pyruvate kinase has been suggested as a result of localized cellular functions that require high-energy consumption (40). This protein interaction may be involved in cytoskeletal rearrangement, or ER mobilization. The specific metabolism of SelK KO cells has yet to be compared to those of Wild-type, and examination of the glycolysis pathway may provide another role to the function of SelK.

However, the calcium dependent immune cell functions we have identified do not rule out the role SelK may play with Derlin association in the guiding of misfolded proteins out of the ER to the proteasome for degradation. Like several other proteins, SelK may provide multiple functions, particularly in immune cells, which may also explain the increased expression of SelK we find in immune cells. Studies in

cell lines have provided binding partners of SelK in ERAD complexes, however we utilize immune cells to identify protein interactions with SelK to determine its function. In this manner, the 'housekeeping' role that SelK plays in most tissues may involve mitigating ER stress and this may explain the low abundance but wide tissue distribution. In contrast, the receptor-mediated calcium flux may reflect an additional, specialized role for SelK in immune cells. There is a similar duality of roles for another selenoprotein: GPx4 (42). This important antioxidant enzyme plays a structural role in sperm cells, and this moonlighting situation may be similar for SelK.

Continuing research is attempting to confirm binding partners of immunoprecipitation with the deletion and mutation of the SH3 binding domain of SelK. Although the exact mechanisms and functions of Selenoprotein K remain elusive, progress has been made to better understand this unique member of the selenoprotein family. The high invest of cellular energy to synthesize this SelK is a key component that drives research. These findings need to be further developed in follow-up studies, and interactions with the putative binding partners will need to be confirmed. However, these studies represent an important first step towards gaining insight into the function of SelK in immune cells, and the effects of its deletion after an immunogenic challenge. Further research is warranted to determine a more complete role of this unique protein within a unique family of proteins.

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