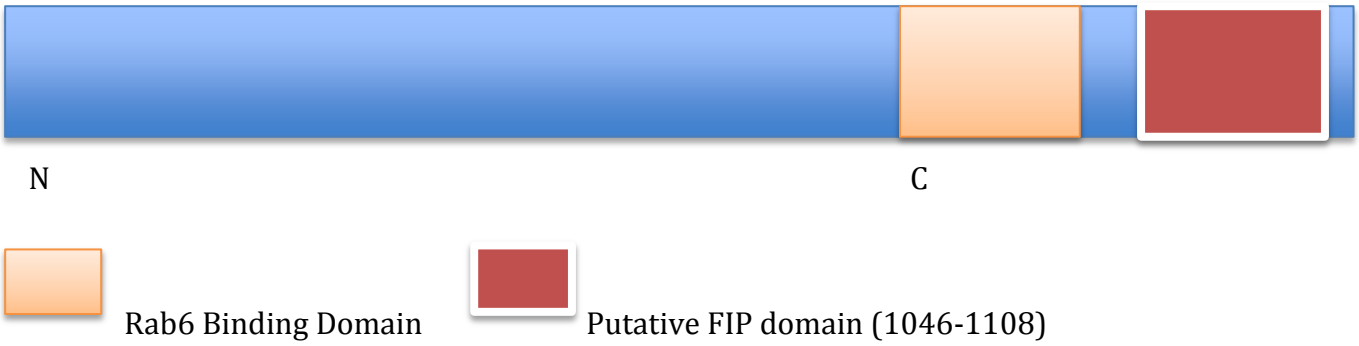
UNDERSTANDING *IN VIVO* ROLES OF ELKS IN INNATE IMMUNITY

Identification of ELKS gene

In papillary thyroid carcinoma tissue samples of Japanese patients, tyrosine kinase domain of proto-oncogene RET was found to be fused to 5` portion of a novel gene due to translocationwhich leads to the constitutive activation of RET. After cloning of cDNA from human brain library, this novel protein was shown to be composed of 948 amino acids predominantly consisting of glutamic acid (E), leucine (L), lysine (K) and serine (S) residues and,therefore, it was named as ELKS (1). Subsequently, four other alternatively spliced ELKS isoforms expressed in different tissues have also been discovered and named as ELKS alpha, beta, delta, gamma and epsilon (2).ELKS epsilon is the longest isoform (1116 aa) in humans. In *musmusculus,*there are four ELKS isoforms longest one having 1120 aminoacids.

ELKS PROTEIN



CHARACTERISTIC FEATURES

- ***Two genes in vertebrates-ELKS1 and ELKS2***
- ***Five alternatively spliced isoforms***
- ***1116 amino acids, around 130kDa***
- ***Mainly composed of coiled coils***
- ***Cytosolic and golgi associated***

In human, among the 5 known alternatively spliced isoforms, ELKS alpha is mainly expressed in the brain and implicated in the release of neurotransmitters in the cytomatrix active zone (CAZ) of the presynaptic neurons (3). In the literature,ELKSis associated with several pathways. In ayeast two-hybrid screen it has been identified as a binding partner for GTP-bound form of small GTPase Rab6 and named as Rab6IP2. Besides, it was proposed to be a regulatory component of IKK complex in NF-KB signaling and this feature is the one we are particularly interested in. Recently it was reported that ELKS has also an important role in NF-KB mediated DNA damage response acting as a scaffold in the cytoplasm (4). However, in spite of its pleitropic functions in several contexts, in vivo role of ELKS is completely unknown. Gene trap homozygous ELKS mutant mice are embryonically lethal because of liver apoptosis (unpublished data). This prevented further analysis of ELKS in mice and prompted scientist to generate a conditionally knock out ELKS mice to understand the specific contribution of ELKS in the innate immune response.

In the immune system intracellular communication is mediated predominantly through cytokines that are small soluble proteins released from the cells upon certain stimuli. Impaired or excessive secretion of cytokines is implicated in the pathogenesis of several

inflammatory diseases (5). Importantly, ELKS has been found to be a core component of the molecular machinery in the release of neurotransmitters in the presynaptic neurons (6) and the secretion of insulin hormone from the beta pancreatic cells (7) both of which are crucial events in the maintenance of tissue homeostatis. Therefore, it is proposed that ELKS isimportant in the membrane trafficking of innate immune cells as well.

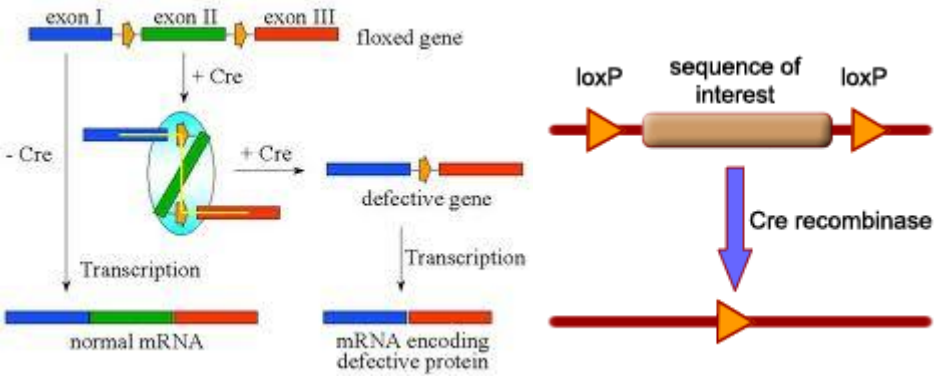
TRANSGENICS

Transgenic animals are extensively used tostudy *in vivo* gene function as well as to model humandiseases. It also permits the evaluation of therapeutic strategies in models of human disease, as well as the investigation of disease progression in a manner not possible in human subjects. Commercial applications include the preparation of recombinant proteins, protection of animals against disease, and introduction of new genetic traits into herds. The technology for producing transgenic animalsexists for a variety of vertebrate and invertebrate species.The mouse is the most utilized organism for research inneurodegenerative diseases. The most commonly usedtechniques for producing transgenic mice involves eitherthe pronuclear injection of transgenes into fertilizedoocytes or embryonic stem cell-mediated gene targeting.Embryonic stem cell technology has been most often usedto produce null mutants (gene knockouts) but may also beused to introduce subtle genetic modifications down to thelevel of making single nucleotide changes in endogenousmouse genes. Methods are also available for inducingconditional gene knockouts as well as inducible control oftransgene expression.

CONDITIONAL/INDUCIBLE GENE INACTIVATION

While gene knockout technology has been invaluable in thestudy of gene function in vivo, there are times when thetechnology has limitations. For example, gene knockoutsmay lead to embryonic lethal phenotypes or result incomplex multisystem abnormalities. The most widely used approachdeveloped to date allow *in vivo* gene inactivation at definedtime points and in a tissue specific manner during developmentor in adult life makes use of the Cre/loxP recombinationsystem.

Cre is a 38 kDa recombinase from the bacteriophage P1that mediates intramolecular and intermolecular sitespecificrecombination between loxP sites (Hamilton andAbremski 1984). The loxP consensus sequence of 34 bpconsists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region. Each inverted repeat binds one Cre molecule and recombination occurs in the spacerregion with the 8 bp spacer determining the directionalityof recombination. Two loxP sequences in opposite orientationinvert the intervening DNA while two sites in thesame orientation mediate excision of the intervening DNAbetween the sites after which only one loxP site remains.To introduce loxP sites in the mouse genome homologousrecombination in ES cells is again exploited. In this case,the targeting construct is designed to have two loxP sitesflanking one or more exons of the gene of interest andpositioned in the surrounding introns so as not to disturb gene expression. Floxed mice created by homologousrecombination (i.e., mice carrying two loxP sites surroundingthe gene of interest) typically show normalexpression of the gene as well as a normal phenotype.Floxed mice are then crossed to mice expressing Crerecombinase leading to permanent inactivation of the gene based on the pattern of Cre expression that can be controlledwith cell type specific promoters.



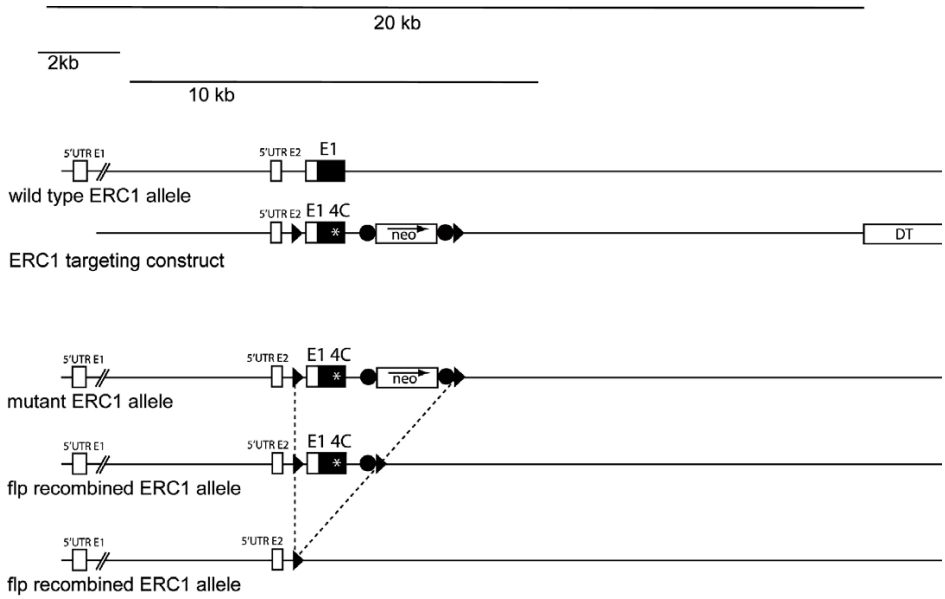
In Cre/loxP technology the gene of interest is flanked by loxP sequences that undergo recombination in the presence of a bacterial enzyme, cre recombinase, excising the sequence in between them. Thus, the gene is only knocked out in the cells that express crerecombinase, which is itself under the control of a specific promoter. Such technology can also be used to knock-*IN* a sequence by inserting a STOP codon flanked by loxP sites upstream of the gene of interest

Hypothesis/Rationale of study

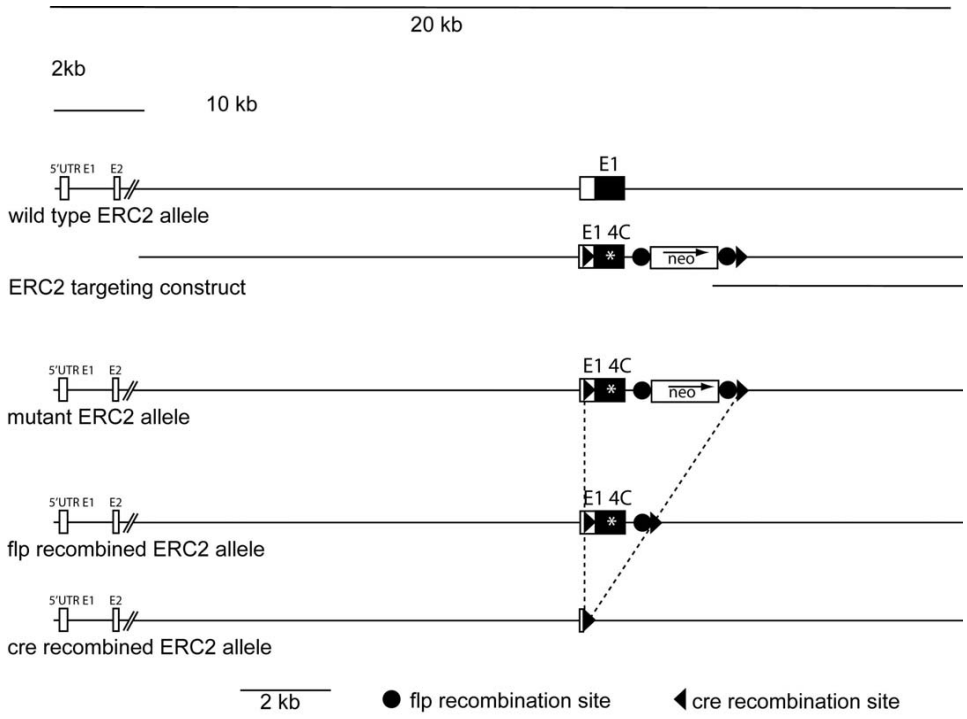
It has been identified that ELKS acts as an essential regulatory subunit of the IKK complex that contains several subunits, including two highly homologous catalytic kinases, IKK1 and IKK2 and an essential regulatory subunit, NEMO and FIP-3. SilencingStudies by RNA interference on ELKS expression showed that it blocked induced expressionof NF-kB target genes, including the NF-kB inhibitor *IkB*aand proinflammatorygenes such as *cyclo-oxygenase 2* and *interleukin 8*. These cells were also notprotected from apoptosis in response to cytokines. Therefore it has been put forth that ELKS may likely function by recruitingIkBato the IKK complex and thus serves a regulatory function for IKK activation. Since ELKS is both important for NF-KB dependent transcription and vesicle fusion, we hypothesized that it may play an important role for cytokine release from immune cells and thereby regulate innate immunity. As it is evident from mice studies that, ELKS whole body knockout renders embryonic lethality, whether it is NF-KB dependent or independent is yet to be determined. Thereby identifying its function in vivo as a regulator of IKK and its modulaory action in the immune system formed the rationale of the study.

GENERATION OF CONDITIONAL KNOCKOUTS

ELKS1 knockout animals



ELKS2knockout animals



RESULTS

GENOTYPING OF ELKS

ERC1 genotyping

PCR Primers

PK05181: 5' GAA CAA GTT TCA GGA CAG CCA AGG 3' (sense)
PK05182: 5' CAG GG ATG ACA ATC TGA AGG C 3' (antisense)

PCR Conditions

Dilute the DreamTaq 2x maser mix (fermantas) with ultrapure water. Run with addition of the specified primers.
Reaction Volume:25ul
1 Sample;
2X Master mix…….12.5ul
DD H_2O……
Primers (100mM)……..0.02ul/each
Sample DNA……..2ul (0.5ug**)**

PCR Cycling Condition…….PCR expected size: WT- 310bp, KO- 360bp

93^0C……10min……1 Cycle
93^0C…..30sec/60^0C……45sec/65^0C…….90sec……..40 Cycles
65^0C……10min……1Cycle
4^0C……Forever

ERC 2 GENOTYPING

PCR PRIMERS

PK04133: 5' GTC CTC AAG GAG CAG ATG AGG G 3' (sense)
PK05183: 5' CAA ATG TTG GCT ATG AGG ATG GC 3' (antisense)

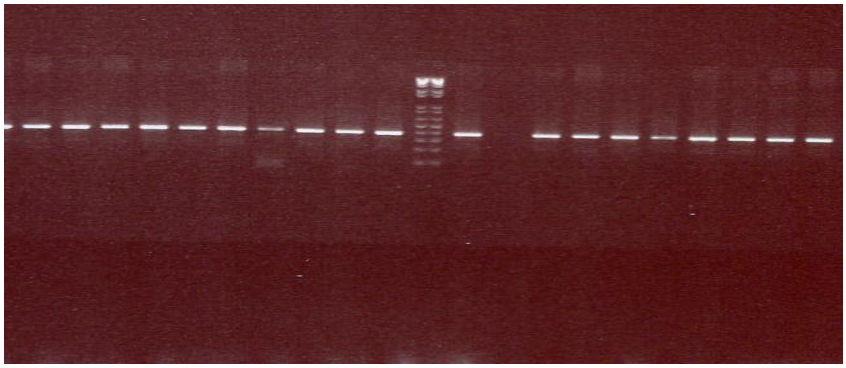
PCR Conditions

Dilute the DreamTaq 2x maser mix (fermantas) with ultrapure water. Run with addition of the specified primers.
Reaction Volume:25ul
1 Sample;
2X Master mix…….12.5ul
DD H_2O…….
Primers (100mM)……..0.02ul/each
Sample DNA……..2ul (0.5ug**)**
PCR cycling conditions……..PCR expected size: WT-- 230bp, KO- 380bp

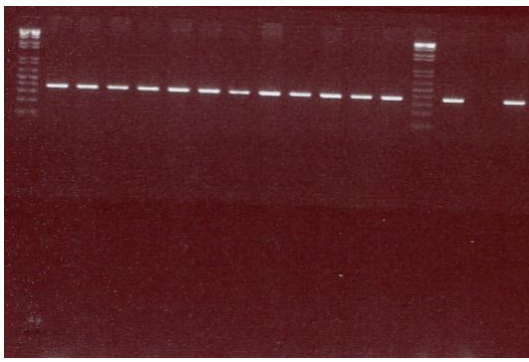
93^0C……..10min……1 Cycle
93^0C…..30sec/60^0C……45sec/65^0C……..90sec……..40 Cycles
65^0C……..10min……..1Cycle
4^0C……Forever

Genotyping of ELKs LysCre Mice

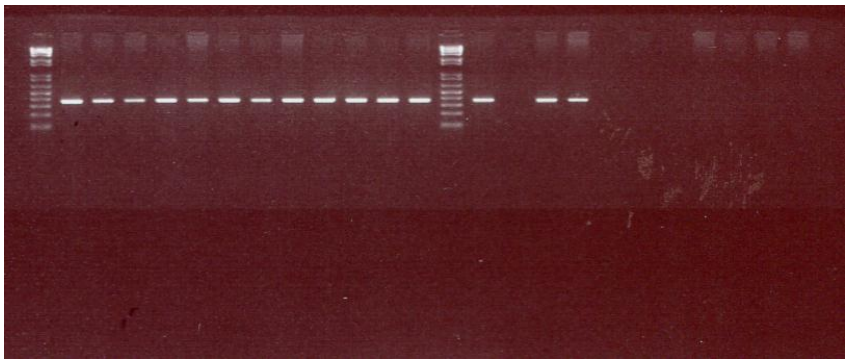
ELKS1



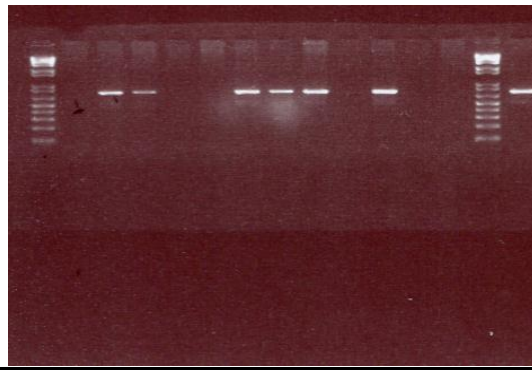
ELKS2



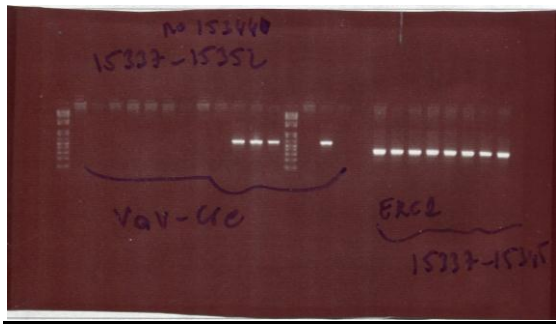
LysCrewildtype

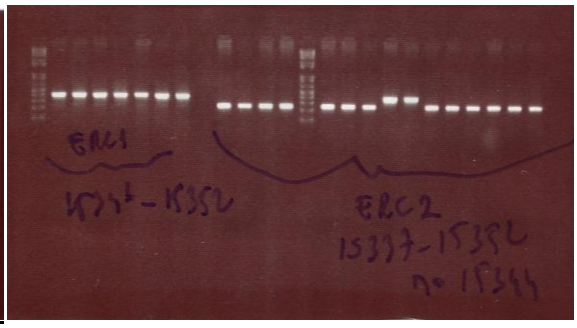


LysCreknockout



Genotyping of ELKs VavCre Mice

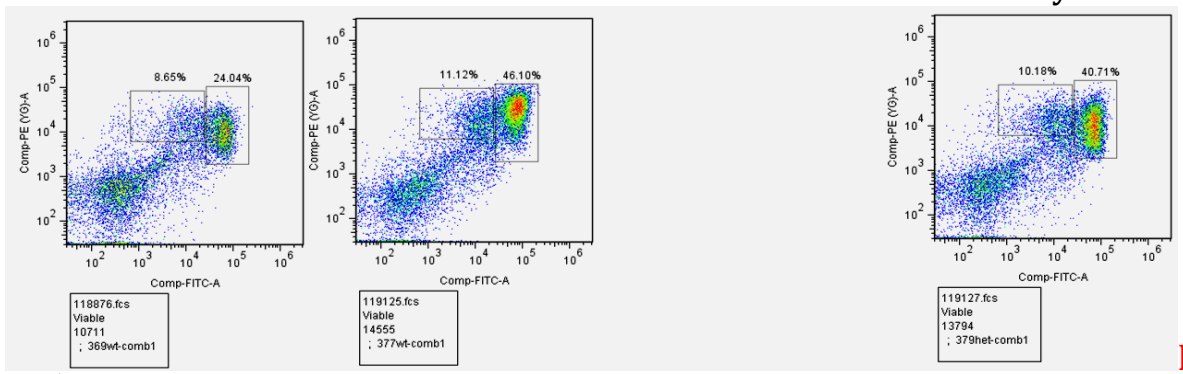




ROLE OF ELKS1 AND ELKS2 IN BONE MARROW DEVELOPMENT

Myeloid cell analysis by flow cytometry

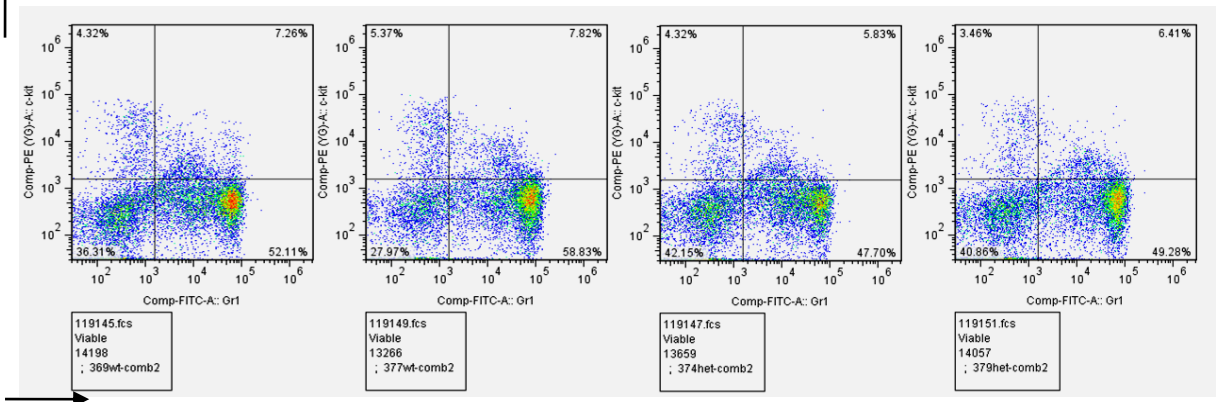
ELKS ELKS2 Floxed **ELKS ELKS2LysCre+**



Mac1

Gr1

ckit



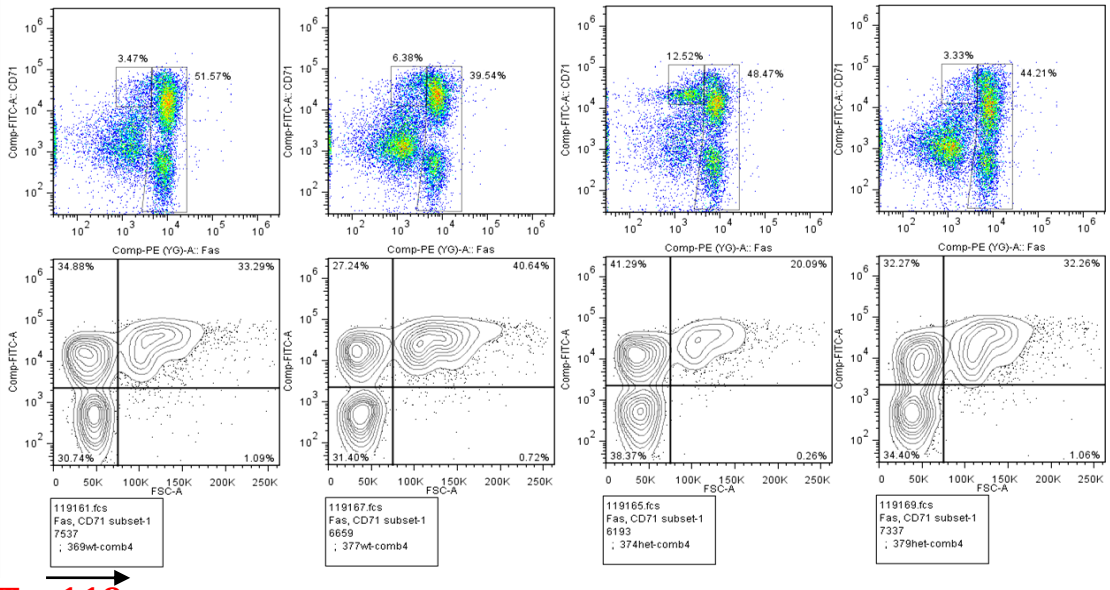
Gr1

There is no change in the number of myeloid cell population in bone marrow of ELKS knockout animals

Erythroid Analysis by Flow cytometry

ELKS ELKS2 Floxed ELKS ELKS2LysCre+

CD71



Ter119

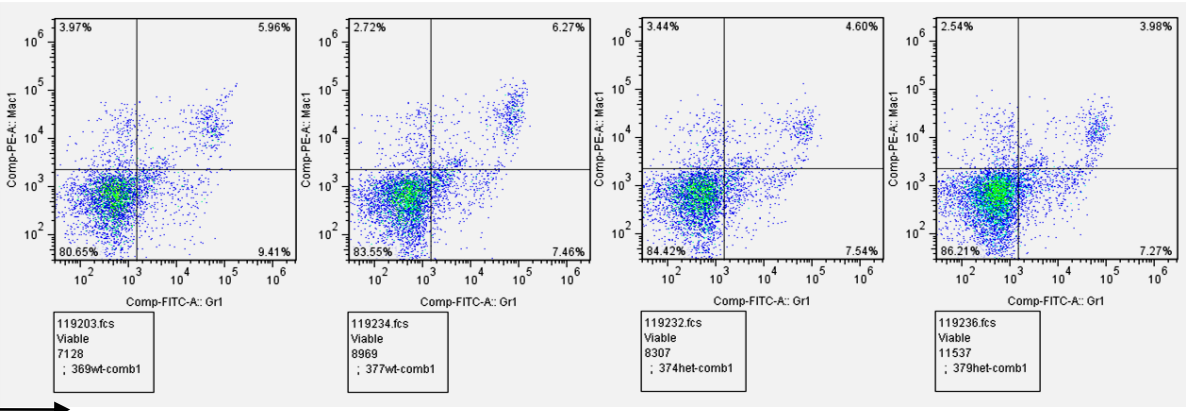
There is no change in the number of erythroid cell population in bone marrow of ELKS knockout animals

ROLE OF ELKS1 AND ELKS2 IN SPLENIC DEVELOPMENT

Myeloid cell analysis by flow cytometry

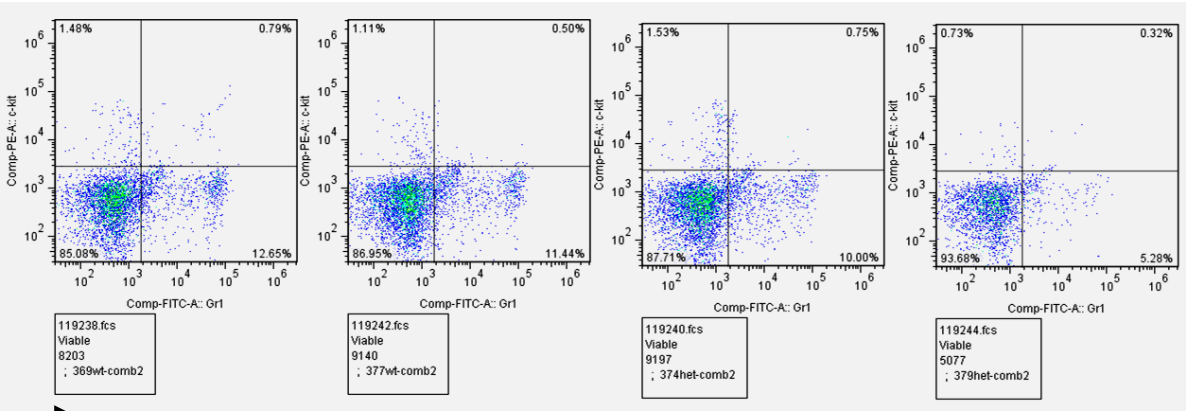
ELKS ELKS2 Floxed **ELKS ELKS2LysCre+**

Mac1



Gr1

ckit



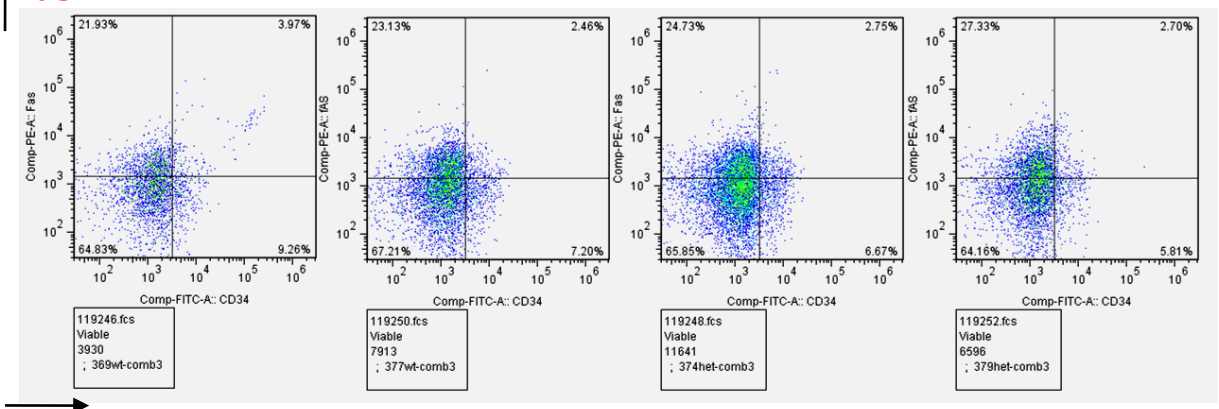
Gr1

There is no change in the number of myeloid cell population in spleen of ELKS knockout animals

B Cell Analysis by Flow cytometry

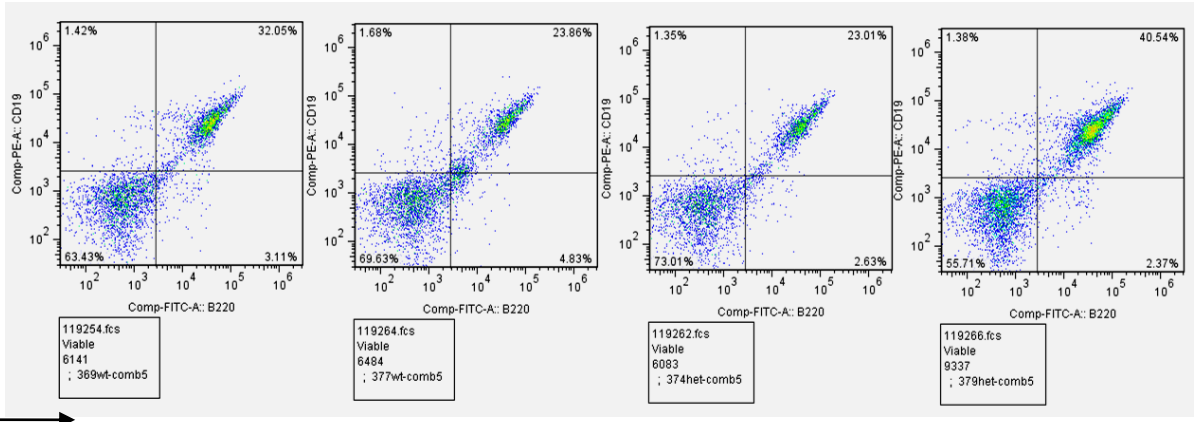
ELKS ELKS2 Floxed **ELKS ELKS2LysCre+**

Fas



CD34

CD19



B220

Bone marrow B cell numbers are increased in ELKS knock out animals.

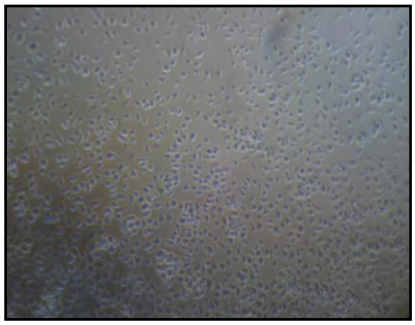
CYTOKINE SECRETION ANALYSIS
Putative Role of ELKS in Cytokine Secretion by Macrophages
BONE MARROW ISOLATION

1 **2** **3**

4 **5** **6**

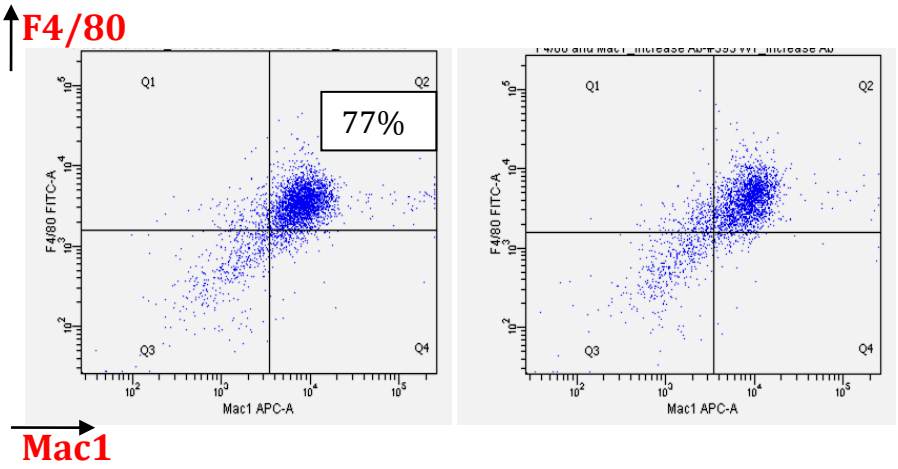
IN VITRO DIFFERENTIATION OF PROGENITORS TO MACROPHAGES USING R-GMCSF

Phase Contrast Image-7days culture

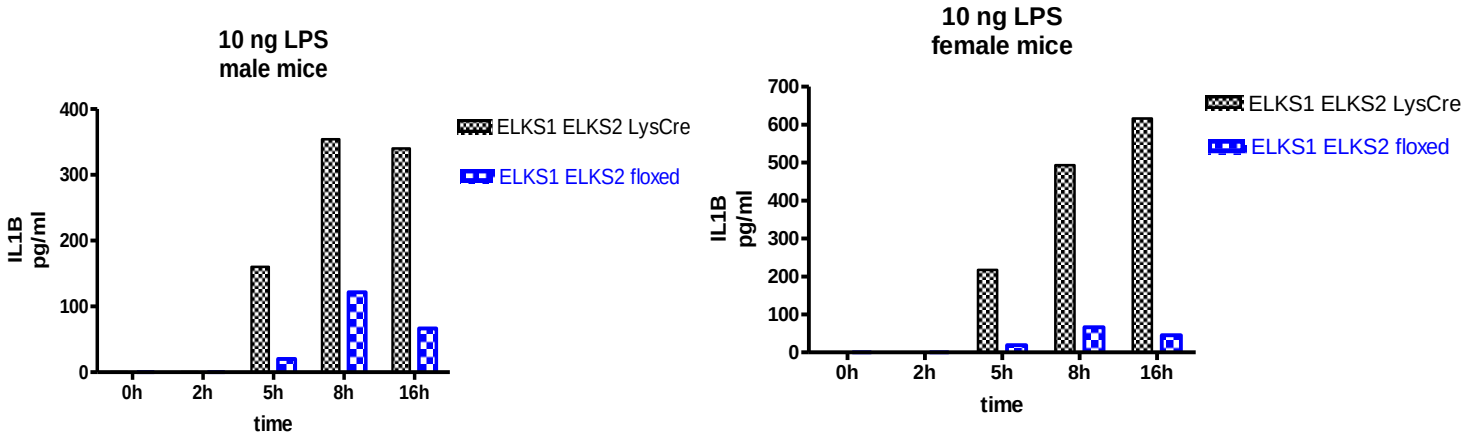


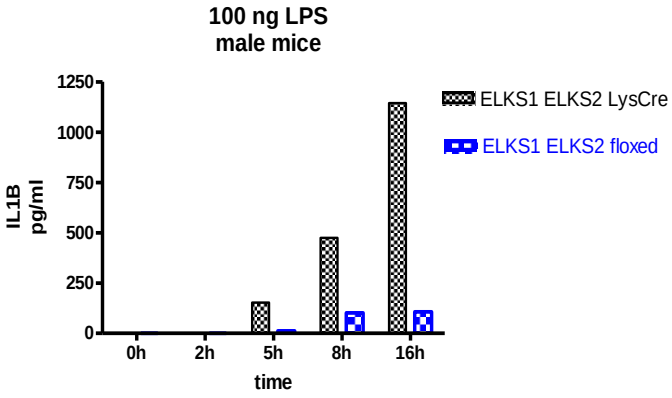
IMMUNOPHENOTYPING OF BONE MARROW DERIVED MACROPHAGES IN ELKS WT & KO

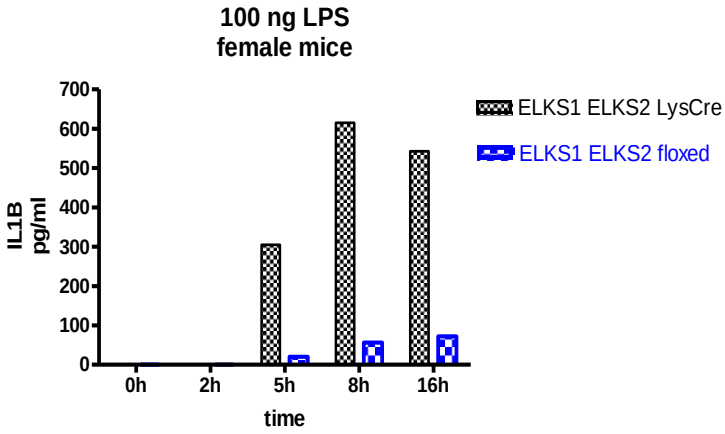
ELKS ELKS2 Floxed **ELKS ELKS2LysCre+**



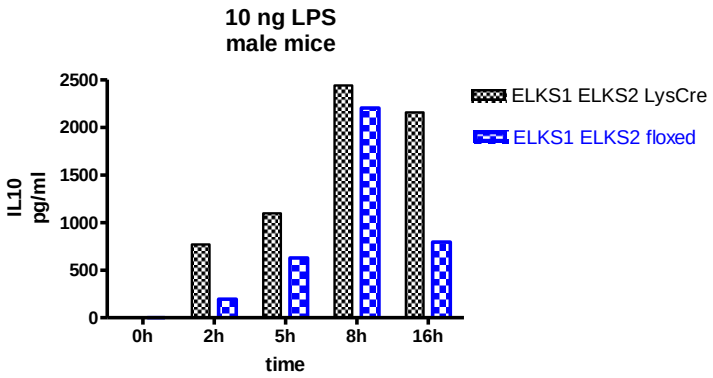
INCREASED IL1B SECRETION BY ELKS KO MACROPHAGES

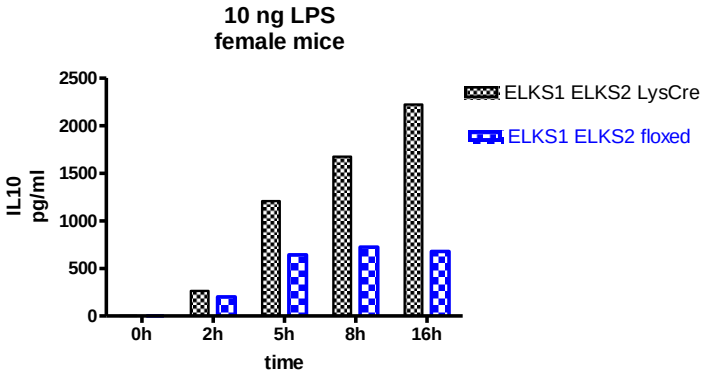


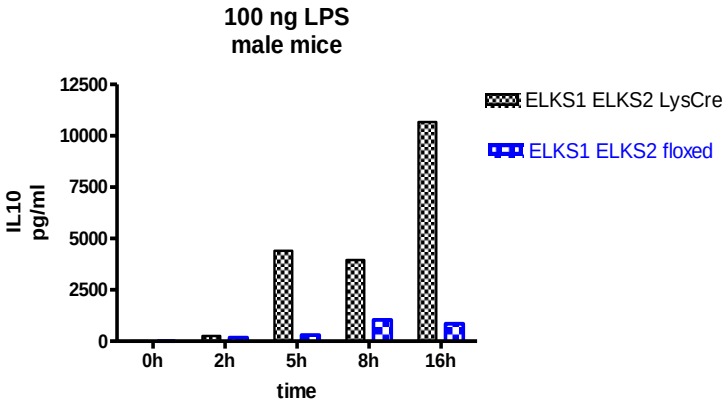


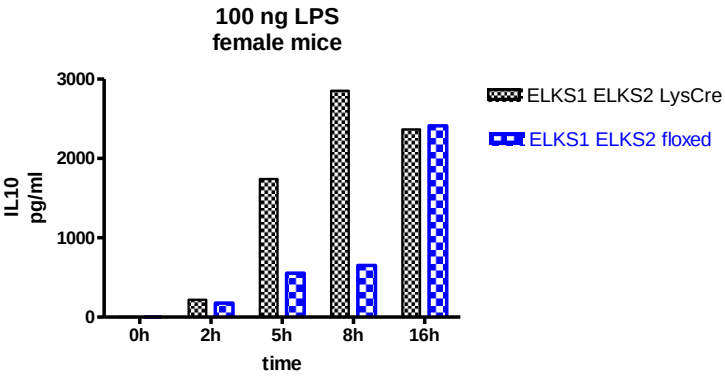


INCREASED IL10 SECRETION BY ELKS KO MACROPHAGES

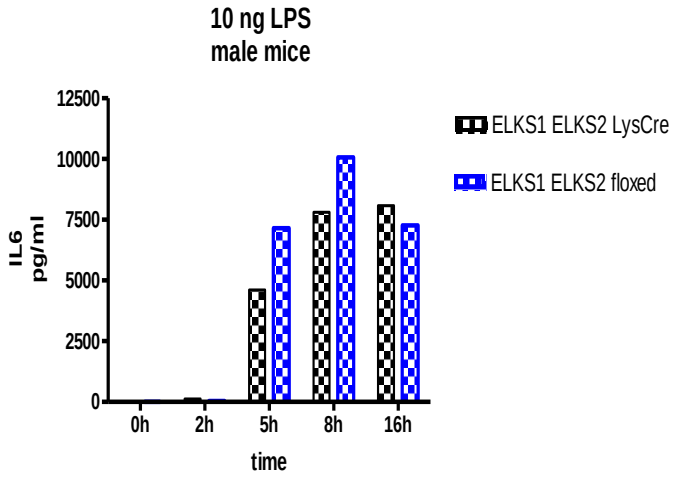


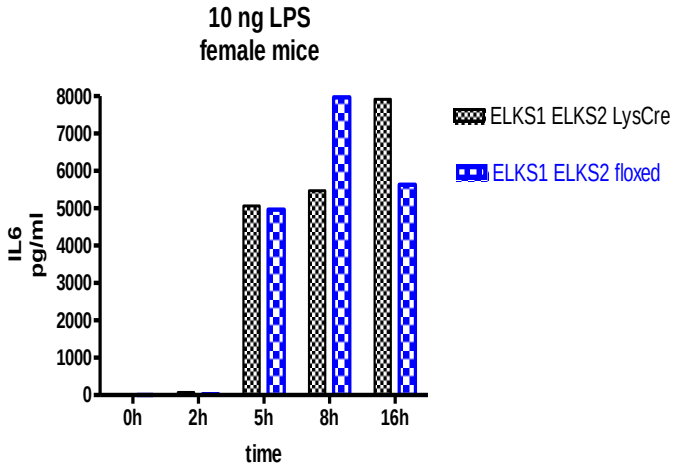


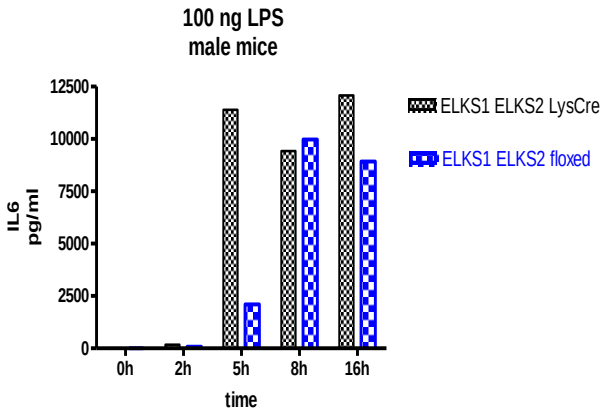


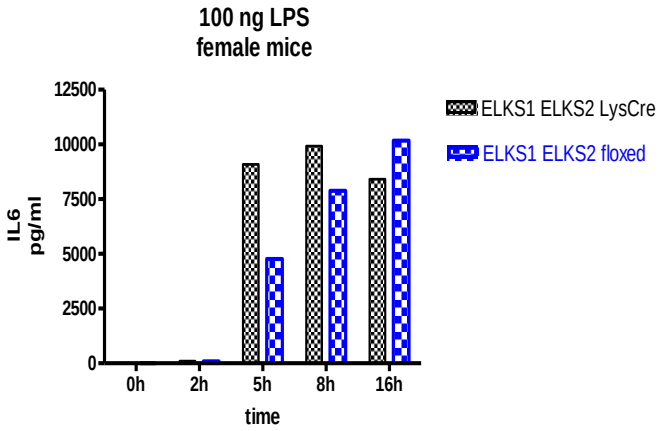


IL-6 SECRETIONBY ELKS WT & KO MACRPPHAGES

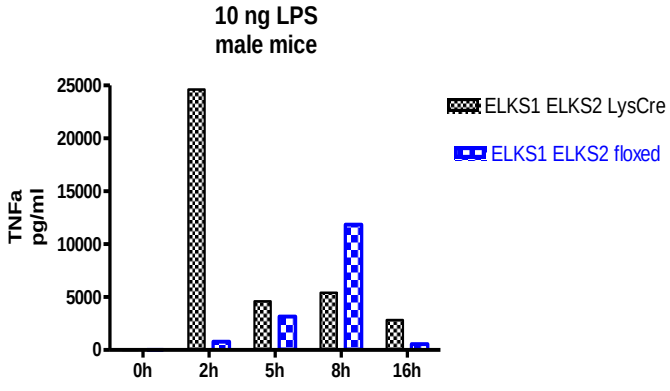


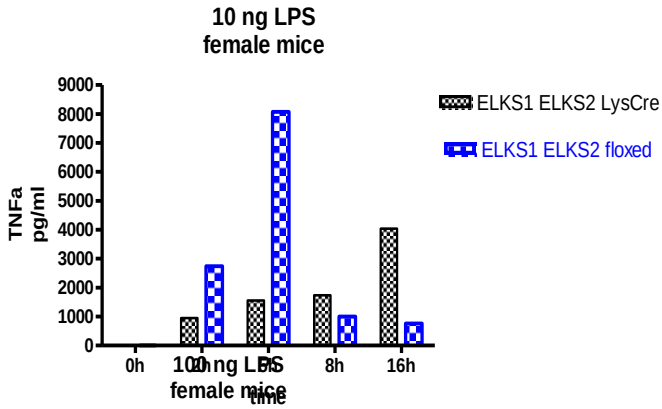


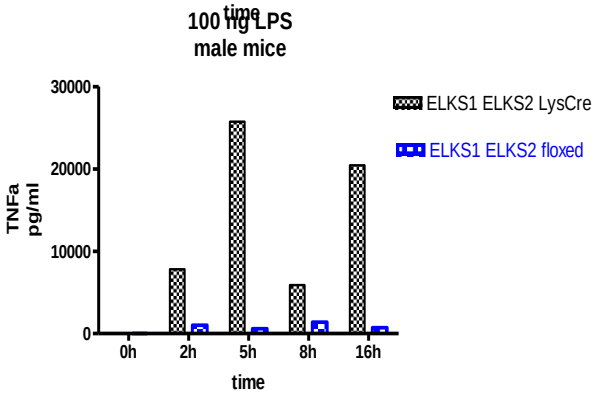


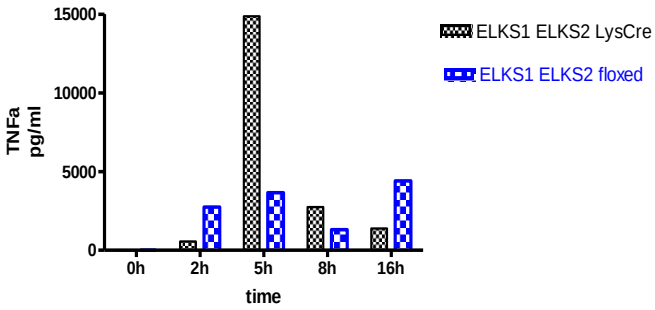


TNF SECRETION BY ELKS WT & KO MACRPPHAGES









ELKS HAS CONSENSUS SUMO SITES

Small ubiquitin-related modifier (SUMO) was discovered as a modifier of mammalianproteins in 1997 by Melchior et al. SUMO has since been demonstrated to be a modifierof many important proteins, giving this modification a vital role in modulating alarge number of important cellular processes. SUMO proteins are very similarto ubiquitin structurally, but sumoylation does not promote degradation of proteinsand instead regulates key functional properties of target proteins. Theseproperties include subcellular localization, protein partnering, and transactivationfunctions of transcription factors, among others. Protein sumoylation plays aparticularly vital role in regulating many important processes occurring in the nucleus, and although sumoylation can be found on proteins that exist in a numberof cellular compartments, most of the sumoylation characterized to date occurs on nuclear proteins.

PREDICTED SUMO SITES FOR ELKS

Position	Peptide	Score	Cutoff	Type
195	PELKKER	2,412	0,17	TypeI: Ψ-K-X-E
444	EQLKEEL	1,322	0,17	TypeI: Ψ-K-X-E
472	GQVKQEL	3,118	0,17	TypeI: Ψ-K-X-E
691	SGLKKDS	3,353	3,33	TypeII: Non-consensus
712	ECLKMES	1,227	0,17	TypeI: Ψ-K-X-E
885	EKVKQEL	3,825	0,17	TypeI: Ψ-K-X-E
927	LEMKQEA	2,687	0,17	TypeI: Ψ-K-X-E
962	AALKREK	2,758	0,17	TypeI: Ψ-K-X-E

ELKS INERACTS WITH SUMO E1 ENZYME AOS1
IMMUNOPRECIPITATION OF ELKS

IgG ELKS IgG ELKS

IP: ELKS
IB: Aos1

IP:
ELKS
IB:
ELKS

ELKS IS SUMO 2\3 CONJUGATED

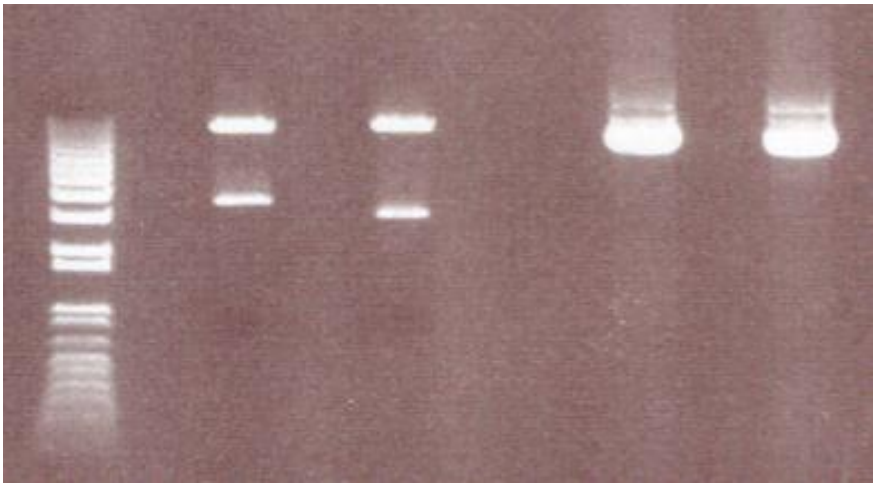
ELKS IgG G beads

IP: ELKS
IB: ELKS
SUMO2\3

CLONING OF ELKS INTO LENIVIRAL VECTOR pBOBI

ELKS 3.4KB

DigestedpBOBIOnlypBOBI



The preliminary results of cloned ELKS however had a mild mismatch probably due to mutaions or incorrect sequence. Need to be repeated

FUTURE PERSPECTIVES

Our preliminary findings confirm that ELKS could probably play an important role in activating the immune system and has an innate role in the inflammatory pathway. As evidenced by cytokine activation studies that ELKS knockout mice secrete more of IL10 and IL1b, may confirm that macrophages challenged by inflammation inducing agents may trigger the immune system and ELKS is essential to modulate them. According to earlier studies, ubiquitination of ELKS in response to genotoxic stress is regulated by the NFkB pathway involving AKT and NEMO. However, sumoylation of ELKS need to be elucidated in detail. In silico studies have reported SUMO binding sites for ELKS. Our preliminary data suggests that ELKS interacts with SUMO E1 binding enzyme AOS1 and immunoprecipiation studies of ELKS protein pave way for the understanding that SUMO2/3 may be conjugated to ELKS. This needs further validation to confirm the hypothesis. Construction of ELKS deletion mutants and SUMO defective mutants may throw more light into the interactions of ELKS and its sumoylation.