Role of Fas in the Hematopoietic Defects Caused by LCMT-1 Knockout in Mice

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biochemistry

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Abstract

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Hematopoiesis is the proliferation and differentiation of hematopoietic stem cells into different blood cells such as erythrocytes and leukocytes. When there are complications in hematopoiesis, various blood disorders such as leukemia and anemia may occur. PP2A is a serine/threonine protein phosphatase that regulates cellular pathways involved in cell development, growth, and differentiation. PP2A is post-translationally regulated by a methyltransferase and a methylesterase. LCMT-1 (leucine carboymethyltransferase-1) is one of the main regulators of PP2A, and it activates PP2A by methylating the PP2A C-subunit carboxy-terminal leucine. Other functions of LCMT-1 include being a positive regulator for the Raf-1 protein, a cellular kinase and proto-oncogene. Previous research shows that Raf-1 down-regulates Fas, a death receptor located on cell membranes that activates caspase pathways and triggers cell death. Keeping this in mind, when LCMT-1, a core regulator of PP2A, is knocked out, various hematopoietic defects result. We hypothesized that the knockout of LCMT-1 causes a down regulation of Raf-1 and in effect causes an up-regulation of the Fas (death receptor) leading to a high amount of cell death and consequental liver and blood defects. In this study, a Fas mutant allele (Lpr) was introduced in order to see if it relieved any of the hematopoietic defects caused by LCMT knockout. LCMT-1 +/- Fas Lpr/Lpr mice were intercrossed with each other to see if LMCT-1 knockout embryos carrying the Fas Lpr allele showed any sign of phenotypic rescue from embryonic lethality, reduced liver size, and increased liver cell death. Two strains of mice, 129S2/SvHsd and C57BL/6J, were used in this study. Results showed that the Fas Lpr allele did not reverse the embryonic lethality, increased cellular death, and reduced liver sizes seen in LCMT-1 knockout mice. Although reduction of Fas by the Fas Lpr allele did not reverse the phenotypic defects caused by the knockout of LCMT-1, other pathways and proteins should be investigated to see if the LMCT-1 knockout mice can be rescued.

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Acknowledgements

I would like to first and foremost thank Dr. Pallas for providing me the opportunity to work in his laboratory. His helpful mentoring, support, and intellect made a great impact on my undergraduate education. I would also like to thank all members of the Pallas Lab for their unending support, patience, and encouragement. I would like to particularly thank Justin Yoo for teaching me all the laboratory techniques and expertise required to carry out my project. I would also like to thank Rhea Bovee for assisting me during the duration of this project. All of the aforementioned people have greatly influenced and inspired me to always work hard, and they have served a great motivation and inspiration for me.

Last but not least, I also thank Dr. Kathleen Campbell and Dr. Megan C. Cole, members of my undergraduate thesis committee, who have been unendingly supportive during my journey in research.

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List of Abbreviations

Fas Lpr	Fas Lymphoproliferation mutant allele
IHC	Immunohistochemistry
HSC	Hematopoietic Stem Cells
KO	Knockout
LCMT-1	Leucine Carboxyl Methyltransferase-1
MAPK	Mitogen-activated protein kinase
PCR	Polymerase Chain Reaction
PP2A	Protein Phosphatase 2A
WT	Wild-type

Introduction

Hematopoiesis and Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are precursors to all blood cells in the human body. They are located in the bone marrow of adults and have the potential to differentiate into erythrocytes (RBCs), megakaryocytes, myeloid cells, lymphocytes, and other types of blood cells (Orkin et al., 2000). In the fetal stage of most vertebrates, HSCs undergo hematopoiesis, or blood cell formation, in the fetal liver. However, in the later stages of embryonic development, HSCs migrate to the bone marrow, and hematopoiesis occurs there until death (Laird et al., 2008). HSCs are capable of differentiating into several different cell lineages, and this process is regulated by many protein factors. One of them is Protein Phosphatase 2A (Chen et al., 2011).

Different hematopoietic diseases are prevalent in today's world such as anemias, leukemias and lymphomas. Viable therapeutic genes have not yet been identified to tackle the many existent blood diseases.

Protein Phosphatase 2A Structure and Function

Protein phosphatase 2A (PP2A) is a highly conserved multifunctional serine/threonine phosphatase. PP2A functions by dephosphorylating various substrates involved in different physiological processes. PP2A is involved in cell growth, apoptosis, cellular development, and is associated with human cancers (Janssen and Goris; 2001).

PP2A is an enzyme made of three different subunits: A, B, and C. The core enzyme of PP2A is a dimer made up of a 65kDa regulatory A subunit, also known as PR65, and a 36kDa catalytic C subunit. The A and C subunits associate with each other

through A subunit's HEAT (huntingtin-elongation-A subunit TOR) repeats 11-15 (Xu et al., 2006). The B-type subunit is a regulatory component of PP2A that associates with the core enzyme of PP2A to create the holoenzyme. There are four major B-type subunit families: B (B55/PR55), B' (B56/PR61), B'' (PR72), and B''' (striatin family). PP2A is mainly thought to exist in the human body as a BAC heterotrimer complex. Given that there are four different B-type subunit families with at least 20 different members overall, many different PP2A holoenzymes exist. Different B-type regulatory subunits associate with the core enzyme of PP2A to form different holoenzymes, and they give specificity to PP2A's localization and activity (Sontag et al., 1995).

Protein Phosphatase 2A Regulation

Many different complexes and substrates, other than the regulatory B subunit, affect the function of PP2A, such as PP2A-specific physiological inhibitors and post-translation modifications (Seschacryulu, 2013). One of the best known mechanisms for regulating PP2A is the reversible methylation and demethylation of the catalytic C subunit carboxy-terminal leucine residue (Leu-309) by leucine carboxyl methyltransferase-1 (LCMT-1) and protein phosphatase methylesterase-1, respectively (Stanevich et al., 2011). Reversible carboxymethlyation of the PP2A catalytic subunit is cell cycle dependent and regulates the formation of certain heterotrimers (Tolstykh et al. 2000; Turowski el al., 1995; Wu et al., 2000; Yu et al., 2001; Wei et al., 2001; Lee and Pallas, 2007). Without LCMT-1, the A and C subunits are not able to bind to certain B-type subunits of PP2A, causing a great reduction in those PP2A heterotrimers (Yu, Moreno, and Pallas, 2001; Lee and Pallas, 2007). Specifically, correct PP2A BAC

holoenzyme formation where the B-type subunit is of the B family is crucially dependent on methylation of the C subunit (Yu et al, 2001 and Lee and Pallas, 2007).

Studies have demonstrated that low levels or knockout of LCMT-1 will result in low levels of these PP2A forms, causing cellular complications such as apoptosis in cultured cancer cells (Lee and Pallas, 2007). Also, loss of LCMT-1 in mice yielded embryos with liver and hematopoietic defects that died after embryonic day 14.5, never making it to live birth, which is around 21 days (Lee and Pallas, 2007).

Raf-1 Kinase

The Raf-1 enzyme is a proto-oncogene serine/threonine protein kinase encoded by the *Raf1* gene and is a member of the Tyrosine-Kinase-Like (TKL) protein family (Ping et al., 1991). Raf-1 phosphorylates the mitogen-activated protein kinase (MAPK) kinase (MEK) protein and regulates the MAPK pathway, which is involved in control of gene expression, cell proliferation, cell survival, apoptosis, and differentiation (Kyriakis et al., 1992). Raf-1 is activated by the binding of activated Ras protein (Daum et al., 1994). Raf-1 and the MAPK pathway play a significant role in hematopoiesis and erythropoiesis (Chang et al., 2002). Raf-1 is critical for keeping the level of the pro-apoptotic protein, Fas, in check (Rubiolo et al., 2006). Loss of Raf-1 leads to high levels of Fas and increased death of hematopoietic cells, reduced embryonic liver size, and early embryonic death (Mikula et al., 2001). This phenotype is very similar to what our lab has observed for LCMT-1 knockout.

Fas Protein and Interaction with Raf-1

The Fas protein, also known as CD95, is a 48 kDa cell surface death receptor and part of the TNF-receptor superfamily. The Fas death receptor is activated by the binding of the Fas ligand and subsequently triggers the death inducing signaling complex (DISC), causing cellular apoptosis (Scott et al., 2009). In one study, Fas was shown to be an upstream regulator of differentiation-associated caspase activation in erythropoiesis and up-regulated in the absence of Raf-1 expression. During blood cell proliferation and development, there is a constant balance between Raf-1 and Fas expression that yields the appropriate amount of proliferation of hematopoietic stem cells and erythrocyte and other blood cell formation (Rubiolo et al., 2006).

Fas Lpr

Fas Lpr is a mutant allele of the Fas gene. It was constructed by insertion of an early transposable element (Etn) containing two poly(A) adenylation sites into intron 2 of the Fas gene (Nagata and Suda, 1995). Fas Lpr gene expression leads to almost no production of the Fas protein, resulting in an almost complete knockout of the Fas protein in homozygous Fas Lpr/Lpr mice (Rubiolo et al. 2006). Reduced levels of Fas protein expression due to the Fas Lpr allele causes autoimmune lymphoproliferative (Lpr) syndrome. Non-malignant proliferation of B and T lymphocytes occurs, causing oversized lymph nodes (Teachey et al., 2010).

In the aforementioned study involving Raf-1 and Fas expression (Rubiolo et al. 2006), Fas protein levels were found to be elevated when Raf-1 gene was knocked out and introduction of one or two Fas Lpr alleles caused a reduction of Fas protein expression, significantly rescuing and restoring liver size and hematopoiesis in Raf-1

deficient mice. The regulatory feedback between Fas and Raf-1 was absent when Raf-1 was made deficient, and this caused an overexpression of Fas, causing liver and hematopoietic defects (Rubiolo et al., 2006).

PP2A regulates Raf-1

Studies have shown that Raf-1 is positively regulated by PP2A (Abraham et al., 2000). When PP2A was inhibited with Okadaic acid, activated Raf-1 protein was also absent (Favre et al., 1997). In addition, in Raf-1 immunoprecipitates, PP2A subunits A and C were also found, and further experimentation confirmed that endogenous Raf-1 associates with PP2A B subunit (Ory et al., 2003).

Hypotheses and Approach

Expression of the Fas Lpr/Lpr gene has been shown to rescue the hematopoietic defects of Raf-1 knockout mice (Rubiolo et al., 2006). Therefore, we hypothesized that LCMT-1 knockout causes increased Fas through mis-regulation of Raf-1 (Figure 1). A prediction of our hypothesis is that embryos with a genotype of LCMT-1 -/- (KO) Fas Lpr/Lpr would exhibit reduced cell death and reduced hematopoietic defects in comparison to LCMT-1 -/- Fas +/+ embryos. We also predicted the possibility of live births of LCMT-1 KO Fas Lpr/Lpr mouse pups.

In summary, this study aims to determine if reduction in Fas protein levels due to the Fas Lpr allele will be able to reverse the hematopoietic defects observed in LCMT-1 knockout mice (Figure 2). To address these questions, flow cytometry and quantitative

immunoblotting were utilized to measure the cellular death, cellular composition of livers, and Fas protein levels in day 14.5 mouse embryos that carried Fas Lpr and in controls.

Methods

Breeding

Pre-existing C57BL/6J LCMT-1 hemizygous knockout (+/-) mice from the Pallas Laboratory were interbred with C57BL/6J LCMT-1 +/+ Fas Lpr/Lpr mice (officially titled "B6.MRL-*Tnfrsf6*^{lpr}*/J")* obtained from Jackson Laboratories. Adult B6.MRL-*Tnfrsf6*^{lpr}*/J* (C57BL/6J) mice exhibit massive lymphadenopathy including aberrant T cell proliferation, arthritis, and immune complex glomerulonephrosis. The mentioned phenotypes closely resemble Autoimmune Lymphoproliferative Syndrome (ALPS) in humans where autoimmune lymphocytes accumulate, causing oversized lymph nodes and hypergammaglobulinemia (http://jaxmice.jax.org/strain/000482.html).

C57BL/6J mice carrying the Lpr allele do not experience lymphoproliferative defects till later stages in life, and Jackson Laboratories reports that C57BL/6J Fas Lpr/Lpr females live to a median 42 weeks (http://jaxmice.jax.org/strain/000482.htmls). For our experiments, C57BL/6J LCMT-1 +/- Fas +/+ (WT) were crossed with C57BL/6 LCMT-1 +/+ (WT) Fas Lpr/Lpr mice to produce either LCMT-1 +/+ Fas +/Lpr or LCMT-1 +/- Fas +/Lpr mice. Then, male and female LCMT-1 +/- Fas +/Lpr mice were crossbred to produce LCMT-1 +/- (Fas +/+, +/Lpr, or Lpr/Lpr) or LCMT-1 +/+ (Fas +/+, +/Lpr, or Lpr/Lpr) mice. C57BL/6J LCMT-1 +/- Fas Lpr/Lpr mice were intercrossed for timed mating dissections to generate data on the effect of introducing two Fas Lpr alleles into the LCMT-1 KO lacking mice.

129S2/SvHsd (129S2) mice were obtained from Harlan Laboratories and crossbred with pre-existing C57BL/6J LCMT-1 +/- Fas WT mice of the Pallas Laboratory. The same breeding patterns as those used for the pure C57BL/6J strain above

were implemented to create 129S2-BL/6 hybrid LCMT-1 +/- Fas +/Lpr mice. 129S2-BL/6 LCMT-1 +/- Fas +/Lpr and 129S2-BL/6 LCMT-1 +/- Fas Lpr/Lpr mice were used for timed matings and dissections.

Mice were cared for and bred under strict guidelines approved by the Institutional Animal Care and Use Committee (IACUC) and regulations of the Emory University School of Medicine Division of Animal Resources.

Genotyping

Tails were clipped from mouse pups 7 to 12 days postnatal and incubated in 150 μL of tail lysis buffer (100 mM Tris-HCL pH 8.5, 5 mM ethylenediaminetetracetic acid (EDTA), 0.2% sodium dodecyl sulfate (SDS), 200 mM NaCL, 100 μg/mL Proteinase K) overnight in a shaking incubator at 56°C. After overnight incubation, tails were mixed by hand and then centrifuged in an Eppendorf microcentrifuge model 5415d for 5 minutes at 14,000 rpm to separate debris. Supernatants from the centrifuged tubes were transferred into separate microcentrifuge tubes and mixed with 150 μL of 100% isopropanol. The tubes were then centrifuged again in an Eppendorf microcentrifuge 5415d for 5 minutes at 14,000 rpm to obtain DNA pellets. Supernatants were removed and DNA pellets were re-suspended in 100 μL of nuclease free water and stored at -20°C until PCR (Polymerase Chain Reaction) genotyping.

When genomic DNA had to be obtained from yolk sacs of mouse embryos, yolk sacs were obtained by dissection and suspended in 1mL of 1x phosphate buffered saline (PBS) solution. They were then spun down at setting 3 in a Fisher Microcentrifgue Model 59A (swinging bucket microcentrifuge) at 4°C. Supernatant was vacuum aspirated and

yolk sacs were re-suspended in 1mL of 1x PBS solution and spun down again. This wash was repeated for a total of three times. Samples were then incubated in PCR lysis buffer (50 mM Tris pH 8.8, 1 mM EDTA, 0.5 Tween 20, 100µg/mL Proteinase K) overnight in a shaking water bath at 56°C. Samples were agitated for 5 minutes on the Super Mixer II (Baxter Scientific) and heated at 99°C for 10 minutes. After cooling for 3 minutes, the samples were spun down in a Eppendorf microcentrifuge 5415d for 5 minutes at 14,000 rpm. Supernatants were collected in separate microcentrifuge tubes and stored at -20°C until PCR genotyping.

For genotypic analysis of embryo torsos, torsos were incubated in 500 µL of 1-Day Torso Lysis buffer (500mM KCl, 100 mM Tris pH 8.3, 0.1 mg/ml gelatin, 1% NP40, 500 µg/µL Proteinase K, and 1% Tween 20) for one hour. After incubation, samples were heated at 99°C for 10 minutes. After, samples were cooled and centrifuged in a Eppendorf 5415d for 5 minutes at 14,000 rpm. Supernatants were transferred to separate microcentrifuge tubes and stored at -20°C until PCR genotyping.

PCR analysis was conducted on the genomic DNA obtained from tails to determine LCMT-1 and Fas genotypes. For LCMT-1 PCR, a primer pair specific for the wild-type LCMT-1 allele (product length ~200 base pairs) and the mutant LCMT-1 allele (product length ~254 base pairs) were used, employing a common sense primer and unique reverse primers. An LCMT-1 wild-type genotype is indicated by the presence of only a bottom band, an LCMT-1 knockout genotype shows a single upper band, while a LCMT-1 +/- genotype shows both bands. For Fas PCR, a three primer set allowing intersection of the wild-type Fas allele (product length ~179 base pairs) and the mutant Fas Lpr allele (product length ~217 base pairs) was used, employing a common sense

primer and unique reverse primers. A Fas wild-type genotype shows only the bottom band, a Fas Lpr/Lpr genotype shows only the top band, while a Fas +/Lpr phenotype shows both bands.

Timed Mating and Breeding

BL/6 and 129S2 strains were both used because previous studies have indicated potential penetrance issues of the Fas Lpr allele in pure BL/6 strains (Jackson Laboratory Information Page). Therefore, BL/6 and 129S2-BL/6 mouse strains that were LCMT-1 +/- and carrying the Fas Lpr allele were bred and used for experiments, and embryos and embryonic livers were observed and analyzed for cell death levels and signs of hematopoietic defect reduction.

Since the LCMT-1 knockout genotype is embryonic lethal, dissections of developing embryos from both C57BL/6J and 129S2-BL/6 mice were conducted approximately 14.5 days post-fertilization (embryonic day 14.5 or E14.5) to provide cells for analysis by flow cytometry. This time frame was used because it had been previously determined by our lab that LCMT-1 knockout embryos die after E15.5 and that they show increased cell death and strikingly smaller liver size by E14.5 due to hematopoietic defects.

For timed matings, two LCMT-1 +/- Fas Lpr/Lpr or LCMT-1 +/- Fas +/Lpr females from either C57BL/6 or 129S2-BL/6 strain backgrounds were put into each male timed mating cage of the identical strain background after 4:30 p.m. The next morning, females were inspected for the presence of a vaginal plug, a sign of copulation. If a vaginal plug was detected, the mouse was presumed to have mated during the night (0.5

days earlier) and was placed in a holding cage apart from male mice. Fourteen days later (E14.5), if the female mouse was confirmed to be pregnant by weight gain and visual inspection, she was sacrificed and dissected to obtain embryos.

Embryo Dissection and Liver Extraction

Pregnant females were dissected approximately 14.5 days after copulation. Females were sacrificed by cervical dislocation, and embryos were dissected out and placed in a petri dish containing PBS. Embryos were immediately flash frozen for western blotting or embryo livers were extracted for flow cytometry.

For western blotting purposes, yolk sacs and embryos were collected and flash frozen with liquid nitrogen and stored at -80°C. Yolk sacs were used for genotypic analysis, and embryos were used to make tissue lysates.

For flow cytometry purposes, torsos were collected for genotypic analysis, and livers were teased out of the torsos using micro-dissection forceps and suspended in 500 µL of 1x Dulbecco's phosphate buffered saline (DPBS) solution for further processing and flow cytometry analysis.

Flow Cytometry

Embryos suspended in 500µL in 1x DPBS were manually dissociated using 25 gauge needles in the existing DPBS solution. Then liver samples were spun down in a swinging bucket microcentrifuge for 1 minute at setting 2.5 (~600 x g). The supernatant was vacuum aspirated, and the liver cells were re-suspended in 1mL of 1x DPBS and spun down again for 1 minute in the swinging bucket centrifuge at setting 2.5(~600 x g).

Cells were resuspended in 1 mL of DPBS. Then 10µL of liver samples were pipetted into a microcentrifuge tube containing 90µL of 1xDPBS and the cells were counted with an Accuri Flow Cytometer.

After genotypes and cell counts were obtained, the appropriate amount of each liver cell suspension was removed from the original liver samples to provide 100,000 cells per staining condition. These cells were placed into microcentrifuge tubes and spun down in a swinging bucket microcentrifuge for 1 minute at setting 2.5. Supernatants were vacuum aspirated and cells were resuspended and blocked in 5% normal mouse serum (NMS) in 1x DPBS (625uL of NMS in PBS per million cells) for 15 minutes.

Antibody staining solutions were prepared using 50 µL of NMS per stain. For antibodies, 3 µL of antibody (Fas PE, BD Pharmingen; Ter119 PE & APC, BD Pharmingen; CD45 FITC, BD Pharmingen) were used for 100uL of NMS. For detection of dead (permeable) cells, 1 µL of 7AAD fluorescent DNA stain (BD Pharminogen) was used. Liver cells blocking in 5% NMS solution were spun down for 1 minute in a swinging bucket centrifuge and vacuum aspirated. Samples were re-suspended in antibody and/or 7-amino-actinomycin D (7AAD) staining solutions and incubated for 15 minutes on ice in the dark. Then they were spun for 1 minute in a swinging bucket centrifuge, the supernatant vacuum aspirated, and the pellet re-suspended in 300 µL of 2% fetal bovine serum (FBS) in 1xDPBS. After pelleting the cells again for 1 minute in a swinging bucket microcentrifuge, the supernatants were vacuum aspirated, and the cell pellet re-suspended in 100 µL of 2% FBS solution. Then the samples were analyzed with a C6 Accuri Flow Cytometer.

Tissue Homogenization for Western Blotting

Flash frozen embryos were homogenized on ice in 10 µL lysis buffer (10% glyercol, 10mM Tris pH 8.0, 70 mM NaCl, 0.025 mM Aprotonin (Sigma-Aldrich), 2mM PMSF (Sigma-Aldrich) per milligram wet weight of embryo, and 100 nM okadaic acid). Afterwards, homogenates were centrifuged in an Eppendorf microcentrifuge 5415d for 10 minutes at 14,000 rpm at 4°C to remove cellular debris. Supernatants were transferred to new microcentrifuge tubes and stored at -20°C.

Fas Immunoblot Assay

A Fas immunoblot assay was performed to determine the level of Fas protein expression in adult mouse splenocytes and mouse embryos carrying the mutant Fas Lpr allele. 50µl of embryo lysates and adult mouse splenocytes were combined with 25 µL of lysis buffer and 25µl GSD sample buffer (4.5 M glycerol, 230 mM SDS, 330 mM dithiothreitol (DTT), 2mM Tris-HCL pH 8.8). Samples were heated for 3 minutes at 99°C prior to analyzing by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Lysates were then separated by SDS-PAGE and the proteins were transferred onto a nitrocellulose membrane (Millipore). Membranes were blocked in 5% non-fat dry milk in 1x TBST solution (20 mM Tris, 0.14M NaCl, 0.1% Tween 20) in order to prevent nonspecific antibody binding. The membrane containing the proteins was immunoblotted with Fas antibody (Millipore; 1:10000) and Tubulin antibody (1:2000). The Fas antibody is a monoclonal rat antibody that recognizes the 45kDa Fas protein. The Tubulin antibody is a monoclonal antibody that was used as a loading control to compare protein levels in

the different lanes. Immunoblots were imaged using a Bio-Rad Fluor S-Max Chemilumimager and Bio-Rad Quantity One Software.

Results

Fas Lpr does not rescue the Embryonic Lethality of LCMT-1 Knockout Mice

Previous findings showed that LCMT-1 KO leads to embryonic lethality and other phenotypic abnormalities (Lee and Pallas, 2007). Under the hypothesis that LCMT-1 KO is making certain PP2A forms nonfunctional and thus causing a Raf-1 defect that results in an up-regulation of Fas protein, leading to liver and hematopoietic defects (Figure 2), we tested whether reduction of Fas protein levels by Fas Lpr would rescue LCMT-1 KO mouse embryos from embryonic lethality, increased cell death, and reduced liver sizes. C57BL/6J LCMT-1 +/- Fas +/Lpr and Fas Lpr/Lpr mice were bred, and cages were checked regularly to see if there were any live births of LCMT-1 KO mice. However, no live births were observed. In addition, no live births of LCMT-1 KO were observed from any of the 129S2-BL/6 LCMT-1 +/- Fas +/Lpr crosses. Therefore, introduction of Fas Lpr allele did not rescue embryos from embryonic death caused by the knockout of LCMT-1.

Introduction of the Fas Lpr Allele in the C57BL/6J mouse strain caused a decreased frequency of live LCMT-1 Knockouts at E14.5

While a complete rescue of embryonic lethality was not seen, it was possible that reduction of Fas by expression of the Fas Lpr allele might cause a partial rescue, extending embryonic life. In a previous Pallas Laboratory study involving C57BL/6J LCMT-1 +/- Fas +/+ mice, there was close to the 1:2:1 distribution of LCMT-1 +/+, +/-, and -/- embryos (Lee, Sambo, and Pallas, unpublished). However, Figure 3 shows that we

found a reduced frequency of LCMT-1 KO Fas Lpr/Lpr embryos at E14.5. Thus, Fas Lpr may induce early death of LCMT-1 -/- embryos in this mouse strain.

With 129S2-BL/6 mice, 33 embryos were dissected and genotyped. Results (Figure 4) show a more Mendelian distribution of LCMT-1 genotypes (almost the predicted 1:2:1 genotypic ratio), suggesting that the negative genetic interaction of Fas Lpr and LCMT-1 KO might be strain specific. Thus, the Fas Lpr allele may affect the survival of LCMT-1 -/- embryos specifically in the C57BL/6 strain. However, the number of embryos analyzed in this strain was lower than that analyzed for the C57BL/6J mice, so analysis of more embryos in this strain will increase the confidence of this conclusion.

Fas Lpr/Lpr does not rescue Embryo Liver Size

Because no live births of LCMT-1 knockout mice were observed, embryo liver sizes were measured by counting fetal liver cells through Flow Cytometry to see if there were any Fas Lpr effects on liver size. The results indicate no rescue of LCMT-1 knockout liver size by the Fas Lpr allele. In C57BL/6J dissections, LCMT-1 +/+ Fas Lpr/Lpr embryos showed an average liver size of 11.1 million cells (Figure 5). In contrast, LCMT-1 -/- Fas Lpr/Lpr embryos showed a much lower cell count of 1.8 million cells, or approximately 6-fold fewer than LCMT-1 WT embryos. When compared with previous studies (Lee, Sambo, and Pallas, unpublished), reduction of Fas through Fas Lpr did not rescue of liver size in C57BL/6J embryos.

Cell counts from the 129S2-BL/6 mice (Figure 6) also showed significantly lower liver cells in LCMT-1 KO embryos compared to those of LCMT-1 WT, regardless of

whether embryos carried a Fas Lpr allele. Moreover, the reduction in liver cell counts due to LCMT-1 KO for Fas +/+ and Fas Lpr/Lpr embryos were similar (2.5 fold vs. 3 fold, respectively), indicating that reduction of Fas through Fas Lpr did not rescue LCMT-1 KO embryo liver sizes at all.

In summary, expression of the Fas Lpr allele did not restore LCMT-1 KO liver sizes to that of a wild-type in either C57BL/6J and 129S2-BL/6 embryos.

Fas Lpr does not decrease Cell Death in LCMT-1 Knockout Embryo Livers

Although no restoration of liver sizes was observed, in order to find out if the Fas Lpr allele lessened the amount of cell death caused by the KO of LCMT-1, liver cells were stained with the DNA-binding molecule 7AAD (BD Biosciences) to indicate dead (permeable) cells, and analyzed using a flow cytometer. In C57BL/6J embryos, 10.7% of LCMT-1 WT Fas Lpr/Lpr livers were stained with 7AAD, while 18.5% of LCMT-1 KO Fas Lpr/Lpr livers stained for 7AAD. These observations, graphed in Figure 7, demonstrate that C57BL/6J LCMT-1 KO embryo livers have a higher average percentage of dead cells in comparison to LCMT-1 WT livers. Compared to the increase in cell death of ~2 fold observed in the Pallas Laboratory for LCMT-1 knockout embryos in a wild-type Fas background, my findings indicate a small rescue at best.

Unlike the results from C57BL/6 dissections, 129S2-BL/6 LCMT-1 KO livers with Fas +/+ or Fas +/Lpr genotypes did not have significantly different levels of cell death, although there was a small, nearly significant ($p=0.09$) increase (Figure 8). This suggests that the amount of cell death induced by LCMT-1 is strain-dependent.

The Fas Lpr allele does not restore normal Erythrocyte levels in LCMT-1 Knockout Embryo Livers

Past studies in the Pallas Laboratory demonstrated decreased live erythrocytes in C57BL/6J LCMT-1 knockout embryos when compared against LCMT-1 wild-type embryos (Lee, Sambo, and Pallas, unpublished). For comparison, erythrocyte levels in the embryonic livers of LCMT-1 KO Fas Lpr/Lpr embryos were measured by staining the liver cells with the erythrocyte-specific antibody, Ter119 (BD Biosciences) to find out if the Fas Lpr allele rescued the previously observed decrease of erythrocytes in LCMT-1 -/- livers. Figure 9 shows a graph of percentage of live erythrocyte cells in C57BL/6J LCMT-1 wild type and knockout embryo livers (all expressing Fas Lpr/Lpr). LCMT-1 KO livers exhibit a lower percentage of live erythrocytes in the livers despite the presence of Fas Lpr, indicating lack of rescue of their LCMT-1 phenotypes by reduction of Fas.

Live erythrocyte levels were also measured in livers from 129S2-BL/6 embryos. LCMT-1 WT, KO, and Hemizygous Fas +/+ and Fas +/Lpr embryos were compared against each other. When LCMT-1 WT Fas +/+ are compared against LCMT-1 KO Fas +/+, erythrocyte levels, results show, like the previous Pallas Laboratory study, a significant reduction of erythrocyte levels in the KO embryos. The Fas Lpr allele shows no positive effects in increasing the erythrocyte levels in LCMT-1 KO embryo livers. Rather, when the Fas Lpr allele is present, there is a general decrease in erythrocyte levels when each pair of Fas genotypes within each LCMT-1 genotype is internally compared with one another (WT vs. WT +/Lpr and KO vs. KO +/Lpr). Reduction of Fas

by the Fas Lpr allele, did not rescue or increase erythrocyte levels in LCMT-1 KO embryo livers.

Fas Protein is Reduced in Mice Carrying the Fas Lpr Allele

One possible explanation for the lack of rescue by the Fas Lpr allele of LCMT-1 KO mice, embryonic lethality, liver cell death, liver size, and erythrocyte numbers is that the Fas Lpr allele might not be having the previously reported effect on Fas protein levels. In particular, this was of concern for C57BL/6J mice where lower penetrance of the Fas Lpr phenotype has been reported (http://jaxmice.jax.org/strain/000482.html). To test for effects of the Fas Lpr allele on Fas protein levels in our mice, lysates were created from adult spleen cells or whole embryos expressing either Fas WT alleles or one or two Fas Lpr alleles. Adult BL/6 spleens and 129S2-BL/6 embryos were homogenized, run on an SDS-Page gel, and immunoblotted with Fas antibody and an antibody to tubulin for a loading control. An image of the immunoblot is shown in Figure 10. For adult BL/6 mice, there is a progressive reduction in Fas protein levels as the genotypes go from Fas WT to Fas +/Lpr to Fas Lpr/Lpr. 129S2-BL/6 embryos wild-type for Fas show a dark band of Fas protein, while Fas Lpr/Lpr embryos have no band, indicating that Fas Lpr greatly reduces Fas protein expression. Therefore, Fas Lpr is having the expected effect on Fas expression in both strains. Thus, Fas is not involved in the hematopoiesis and liver defects observed in the LCMT-1 knockout mice.

Discussion

Through this study we aimed to determine whether the reduction of Fas expression through use of the Fas Lpr allele would be able to reverse the hematopoietic defects caused by the KO of the LCMT-1 gene. When the process of hematopoiesis is not properly regulated or functional, severe diseases and pathology, such as anemia and leukemia, can occur. Additional gene targets for potential therapeutic purposes still need to be identified. This study investigates the role of LCMT-1, a regulator of PP2A, in hematopoiesis. The long-term goal of our studies is to determine if regulation of PP2A methylation might be a viable therapeutic target for blood disorders.

The results of this study showed that reduction of Fas was not able to rescue LCMT-1 KO mice from embryonic lethality. No live births of LCMT-1 KO mice were observed in either the C57BL/6 or 129S2-BL/6 strains. We can infer that the embryonic death caused by the KO of LCMT-1 was not solely due to the Fas death receptor and its relevant caspase death pathway. It can be inferred that another pathway, death-triggering protein, or biochemical factor is partly or wholly responsible for the embryonic lethality. For example, LCMT-1 and PP2A regulate many cellular processes such as modulation of the MAPK and Akt pathways, and another protein regulated by those cascades could be responsible for the hematopoietic defects observed in LCMT-1 KO mice.

The effects of reduction of Fas protein levels through Fas Lpr on LCMT-1 KO embryo livers were investigated by looking at liver sizes and liver cell death levels. Livers of LCMT-1 KO mice that had Fas Lpr/Lpr genotypes showed significantly smaller livers (based on liver cell counts) than their WT counterparts in C57BL/6J mice (an 84% reduction). 129S2-BL/6 LCMT-1 KO Fas +/Lpr embryos showed less severe reductions

(61% reduction) in liver cell counts than C57BL/6J LCMT-1 KO Fas +/+ mice, indicating strain differences. Reduction of Fas protein levels was unable to reverse the abnormally small liver sizes observed in LCMT-1 KO mice in both strains, and strain-specific genes and proteins may be causing different phenotypes in the two strains of LCMT-1 KO mice.

Cell death levels were also measured in both C57BL/6J and 129S2-BL/6 embryo livers. C57BL/6J LCMT-1 KO embryo livers had ~8% more dead liver cells than the LCMT-1 WT embryo livers. 129S2-BL/6 LCMT-1 KO Fas +/Lpr embryo livers had ~11% dead liver cells. The livers had only about ~3% more than their LCMT-1 WT counterparts, and the differences were not significant. This difference in cell death level between these two strains and its correlation with the less severe effect of LCMT-1 KO on liver size in the 129S2-BL/6 embryos brings up the possibility that LCMT-1 KO might be having a more severe effect on C57BL/6J mice than 129S2-BL/6 mice due at least in part to increased cell death.

Erythrocyte levels in C57BL/6J LCMT-1 KO Fas Lpr/Lpr embryo livers were significantly lower (P=0.034) than in LCMT-1 WT Fas Lpr/Lpr embryo livers, and the results were comparable to those obtained in a previous Pallas Laboratory study (Lee, Sambo, and Pallas, unpublished). However, 129S2-BL/6 LCMT-1 WT and KO Fas +/+ embryo livers did not have significantly different percentages of erythrocytes (P=0.235). Therefore, reduction of erythrocyte levels in embryo livers may be strain-specific. Interestingly, when the Fas Lpr allele was introduced, there was a decrease in the percentages of erythrocytes in 129S2-BL/6 LCMT-1 WT and KO embryo livers.

Reduction of Fas through expression of the Fas Lpr allele did not increase erythrocyte levels in LCMT-1 KO mice for both strains.

One of the reasons for the results obtained through this study theoretically could be that Fas Lpr is not having its reported effects on Fas protein levels. Our results showed that the Fas Lpr allele does indeed cause a reduction in Fas protein levels. Although this reduction was reported in 129S2 mouse strains, it was never proven in C57BL/6J strains. My results clearly show a great reduction in Fas protein levels in C57BL/6J mice expressing the Fas Lpr alleles, at least in adults. Our results also show that Fas Lpr allele is reducing Fas protein levels in 129S2-BL/6 embryos.

Although the Fas Lpr allele was able to cause a reduction in Fas protein levels, the reduction was not able to reduce the hematopoietic and liver defects caused by LCMT-1 knockout. We thus can infer that increased levels of the Fas death receptor and increased activation of its relevant caspase pathway are not responsible for embryonic lethality in LCMT-1 KO mice. PP2A, an enzyme that is involved in cell growth, division, and development, and LCMT-1, a regulator of PP2A, are involved in many cellular cascades, such as MAPK and AKT (Andrabi et al., 2007). Therefore, future studies should aim to test other potential pathways in order to identify the gene(s) and protein(s) responsible for the observed hematopoietic defects.

Figures

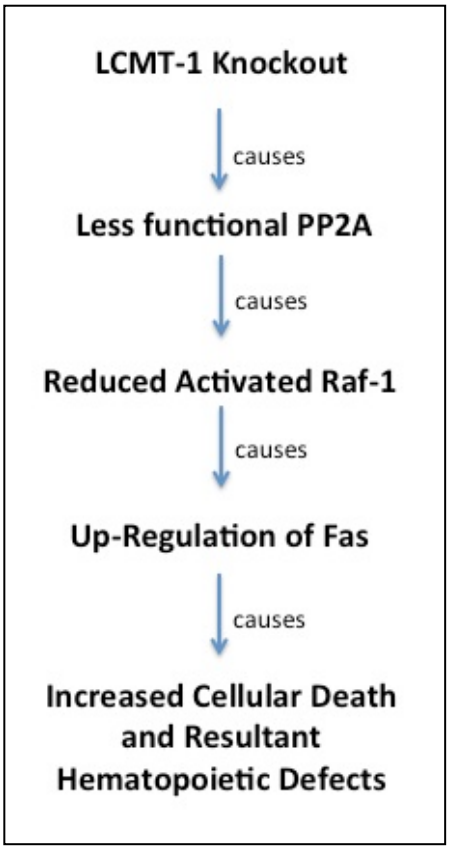

Figure 1: Underlying Hypothesis of Project

LCMT-1 Knockout reduces the amount of functional PP2A, thus yielding defective Raf-1. This will cause up-regulation of Fas death receptor protein, resulting in the observed hematopoietic defects.

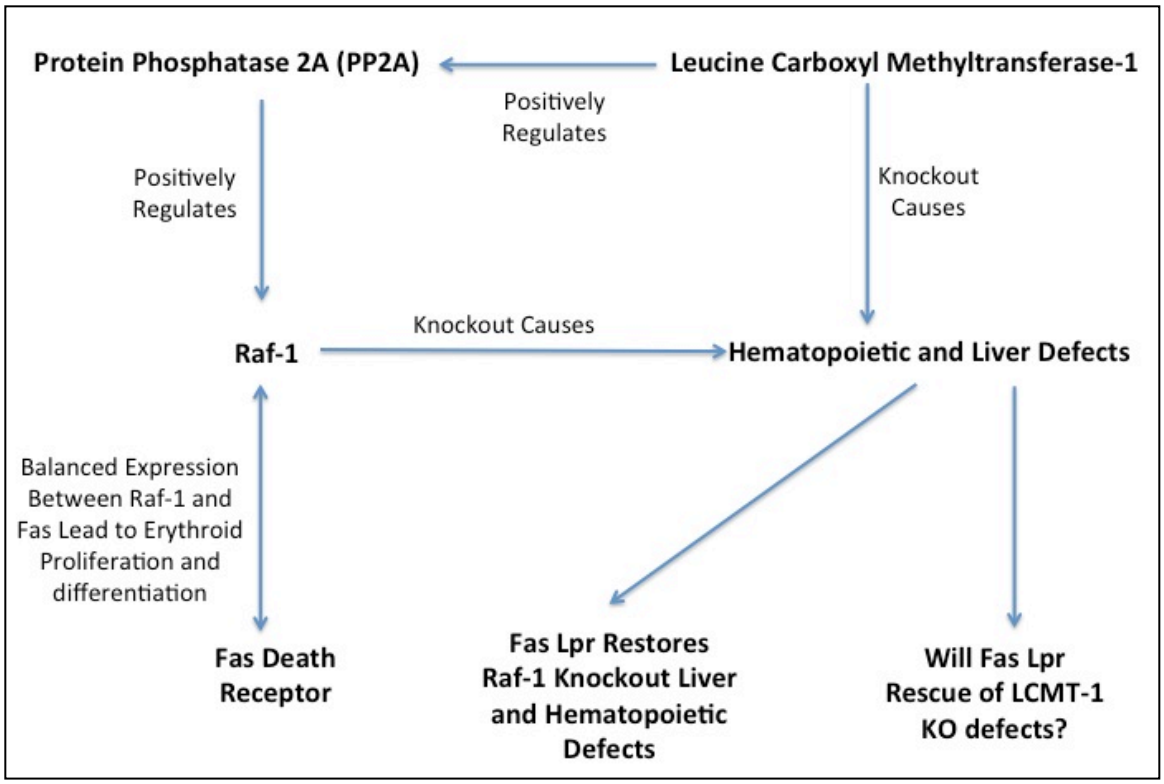

Figure 2: Layout of Major Themes of Project

Relationships of the various proteins in this project are laid out. This study aimed to find out if reduction of Fas caused by the Fas Lpr allele will reverse hematopoietic defects in LCMT-1 Knockout mice.

	LCMT +/+	LCMT +/-	LCMT -/-	Total
Total Number of Embryos	36	72	16	124
Percentage of Total Embryos	29%	58%	13%	

Figure 3: LCMT-1 Genotypes of C57BL/6J Embryos

C57BL/6J LCMT-1 +/- Fas Lpr/Lpr mice were timed mated, so all embryos were Fas Lpr/Lpr. A total of 124 C57BL/6 embryos were dissected. Although the expected ratio of +/+, +/-, and -/- LCMT-1 genotypes was 1:2:1 (25%: 50%: 25%), there were almost two-fold fewer LCMT-1 -/- embryos than expected.

	LCMT +/+	LCMT +/-	LCMT -/-	Total
Total Number of Embryos	8	17	8	33
Percentage of Total Embryos	24%	52%	24%	

Figure 4: LCMT-1 Genotypes of 129S2-BL/6 Embryos

129S2-BL/6 LCMT-1 +/- Fas +/Lpr mice were timed mated, so embryos had varied Fas genotypes. A total of 33 129S2-BL/6 embryos were dissected. The expected ratio of +/+, +/-, and -/- LCMT-1 genotypes was 1:2:1 (25%: 50%: 25%). Results are close to expected genotypes.

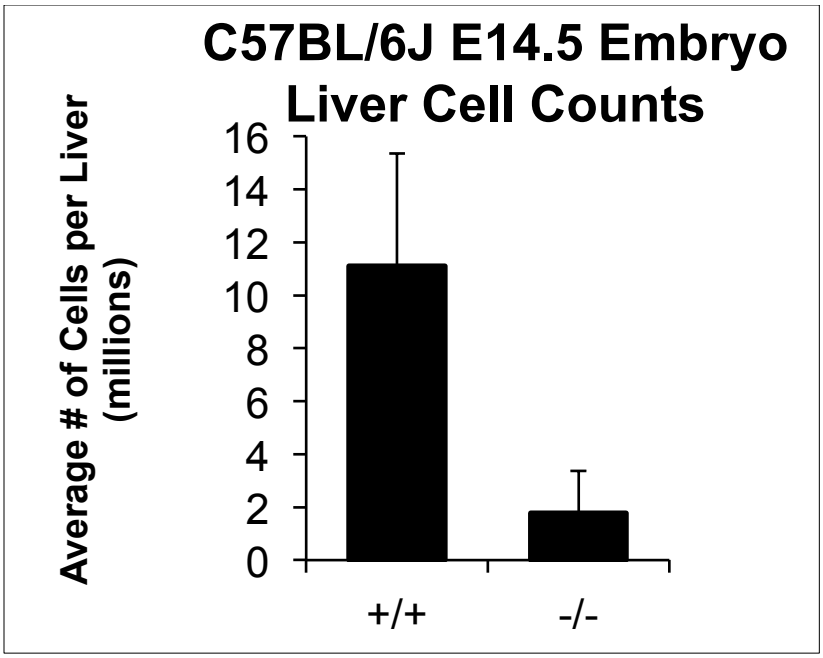

Figure 5: Liver Cell Counts of E14.5 C57BL/6J LCMT-1 +/+ and -/- Fas Lpr/Lpr Embryos

Livers were extracted and their cells were counted with a flow cytometer. LCMT-1 wild-type livers were counted only if, within their litter, there was a KO counterpart, and vice versa. When the average cell counts of LCMT-1 WT and KO Fas Lpr/Lpr were compared by Student's t-test, a P value of .0002 was obtained. When the liver cell counts of the C57BL/6 embryos were compared with those of previous studies, there was no difference between LCMT-1 KO Fas +/+ and Fas Lpr/Lpr embryo livers. Reduction of Fas through the introduction of Fas Lpr was unable to rescue LCMT-1 KO mice from severely reduced liver cell counts.

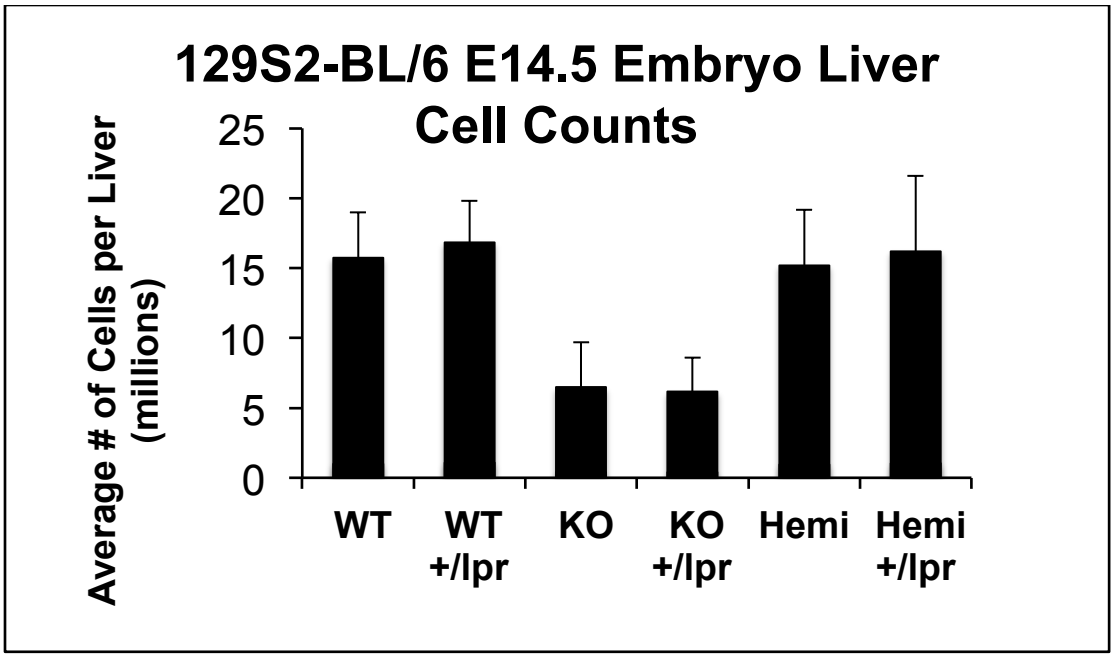

Figure 6: Liver Cell Counts of E14. 5 129S2-BL/6 LCMT-1 WT, Hemi, KO Fas +/+ or Fas +/Lpr Embryos

Livers were extracted and their cells were counted with a flow cytometer. LCMT-1 wild-type livers were counted only if, within their litter, there was a KO counterpart, and vice versa. Student's t-tests were conducted to determine significance of the data. No significant difference was found between LCMT-1 WT Fas +/+ and LCMT-1 WT Fas +/Lpr embryo liver cell counts ($p=0.67$) or between LCMT-1 KO Fas +/+ and LCMT-1 KO Fas +/Lpr embryo liver cell counts ($p=0.304$), indicating that the Fas Lpr allele has no effect on WT levels of embryonic liver cells and that there is no rescue of the LCMT-1 knockout effect on liver size. Instead, LCMT-1 knockout caused an approximately three-fold decrease in the number of liver cells when Fas was WT ($p=2 \times 10^{-5}$) or the Lpr allele ($p=9 \times 10^{-6}$).

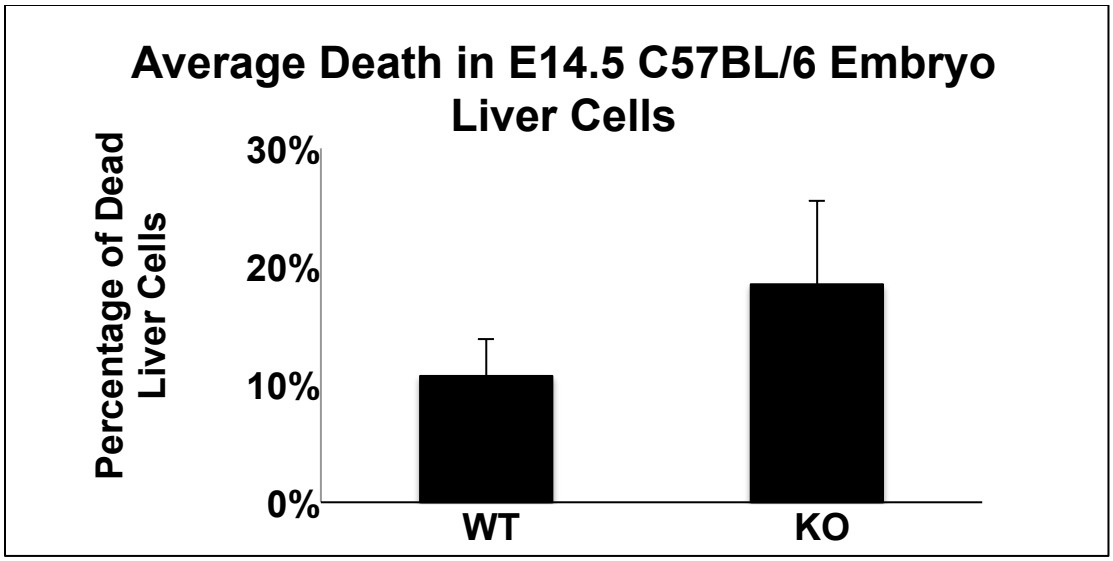

Figure 7: Percentage of Dead Cells in E14.5 C57BL/6J Embryo Livers

Liver cells (from eight LCMT-1 WT and eight KO Fas Lpr/Lpr embryos) were stained with an apoptotic death marker, 7AAD, and analyzed using a flow cytometer. Student's t-test was conducted, and results showed that LCMT-1 KO livers have a higher percentage of dead cells than LCMT-1 WT livers ($p=0.013$). When previous studies were compared to the average death levels of C57BL/6 LCMT-1 WT and KO Fas Lpr/Lpr embryo livers, no reduction of death in LCMT-1 KO Fas Lpr/Lpr livers was apparent.

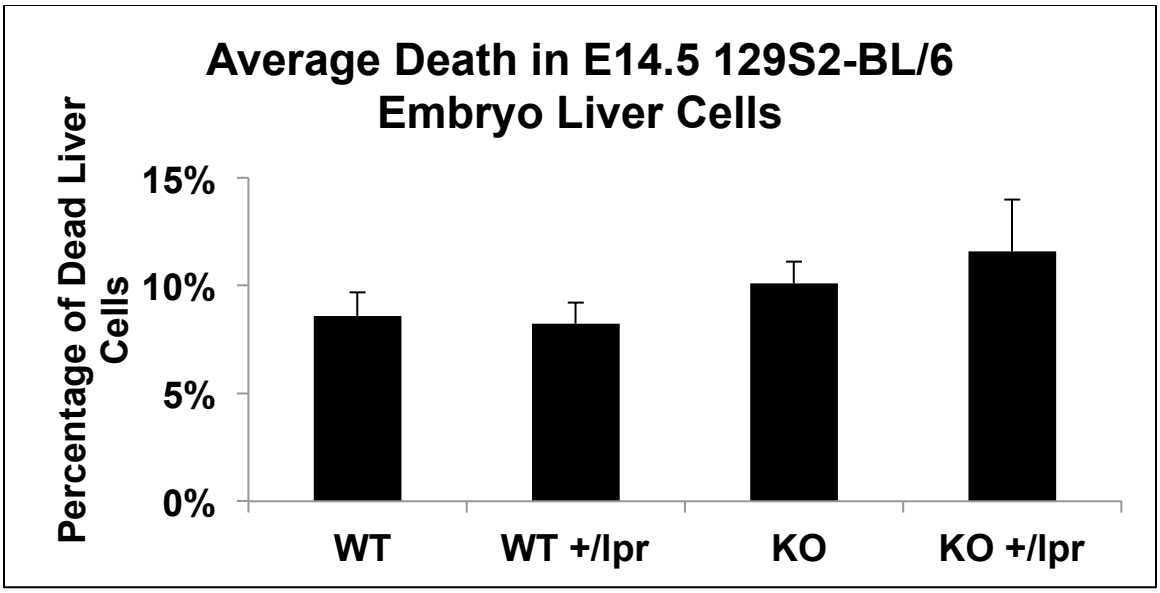

Figure 8: Percentage of Dead Liver Cells in 129S2-BL/6 Embryos

Liver cells from three LCMT-1 WT Fas +/+, four LCMT-1 WT Fas +/Lpr, four LCMT-1 KO Fas +/+, and four LCMT-1 KO embryo livers were stained with 7AAD and analyzed by flow cytometry. Student's t-tests were conducted to determine significance of data. No significant differences were found between the death levels of LCMT-1 WT Fas +/+ and +/Lpr embryo livers ($p=0.66$) and LCMT-1 WT and KO Fas +/+ embryo livers ($p=0.09$). No significant differences in percentages of dead liver cells were found in LCMT-1 WT Fas +/+ and LCMT-1 KO Fas +/Lpr ($p=0.11$). Average percentage of dead cells in embryo livers of both LCMT-1 WT and KO mice were less than those seen in previous studies involving C57BL/6 LCMT-1 WT and KO mice (Lee, Sambo, and Pallas, unpublished).

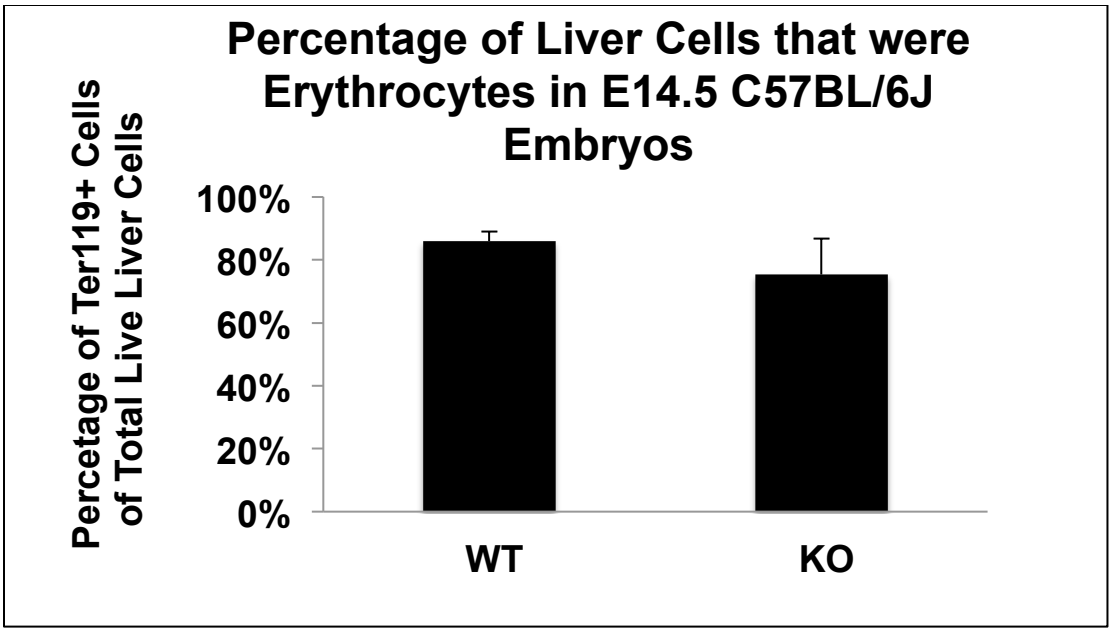

Figure 9: Erythrocyte Levels in E14.5 C57BL/6J LCMT-1 WT and KO Fas Lpr/Lpr Embryo Livers

C57BL/6J LCMT-1 WT and KO Fas Lpr/Lpr livers (from 8 LCMT-1 WT and 8 LCMT-1 KO mice) were stained with an erythrocyte-staining antibody, Ter119 (BD Biosciences). Student's t-test showed a significant difference in Ter119 positive cells from LCMT-1 WT and KO embryo livers ($p=0.034$), indicating no rescue of erythrocyte levels in LCMT-1 KO livers by reduction of Fas through Fas Lpr.

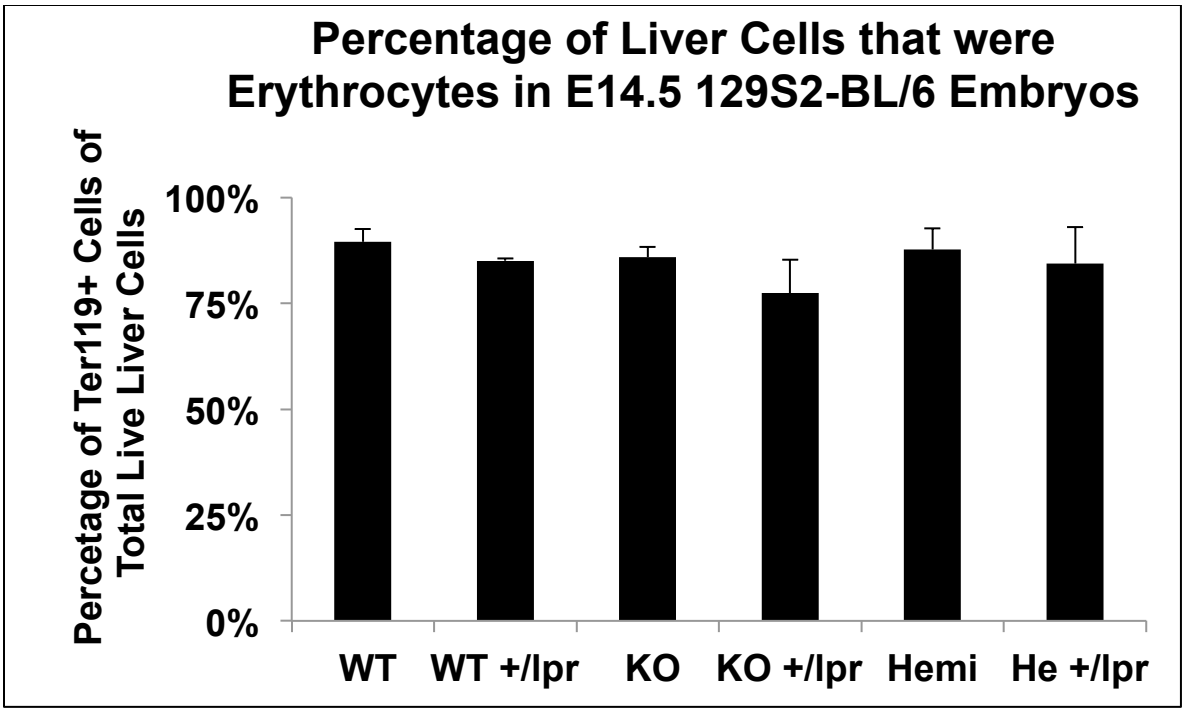

Figure 10: Erythrocyte Levels in E14.5 129S2-BL/6 LCMT-1 WT and KO Fas +/+ or Fas +/Lpr Embryo Livers

129S2-BL/6 LCMT-1 WT Fas +/+ (WT; n=2) and Fas +/Lpr (WT +/Lpr; n=3) embryo liver cells, LCMT-1 KO Fas +/+ (KO; n=3) and Fas +/Lpr (KO +/Lpr; n=3) embryo liver cells, and LCMT-1 +/- Fas +/+ (Hemi; n=2) and Fas +/Lpr (He +/Lpr; n=2) embryo liver cells were stained with an erythrocyte-staining antibody, Ter119 (BD Pharmingen). Error bars show standard deviation or +/- range. No substantial differences were found between the percentages of erythrocytes in liver cells of LCMT-1 WT and KO Fas +/+ embryos, or between LCMT-1 WT Fas +/+ and +/Lpr embryo livers and LCMT-1 WT Fas +/+ and LCMT-1 KO Fas +/Lpr embryo livers. The substantial reduction in erythrocyte levels seen in C57BL/6J mice was not observed in 129S2-BL/6 mice. In addition, Fas reduction by introduction of the Fas Lpr allele did not substantially affect erythrocyte levels.

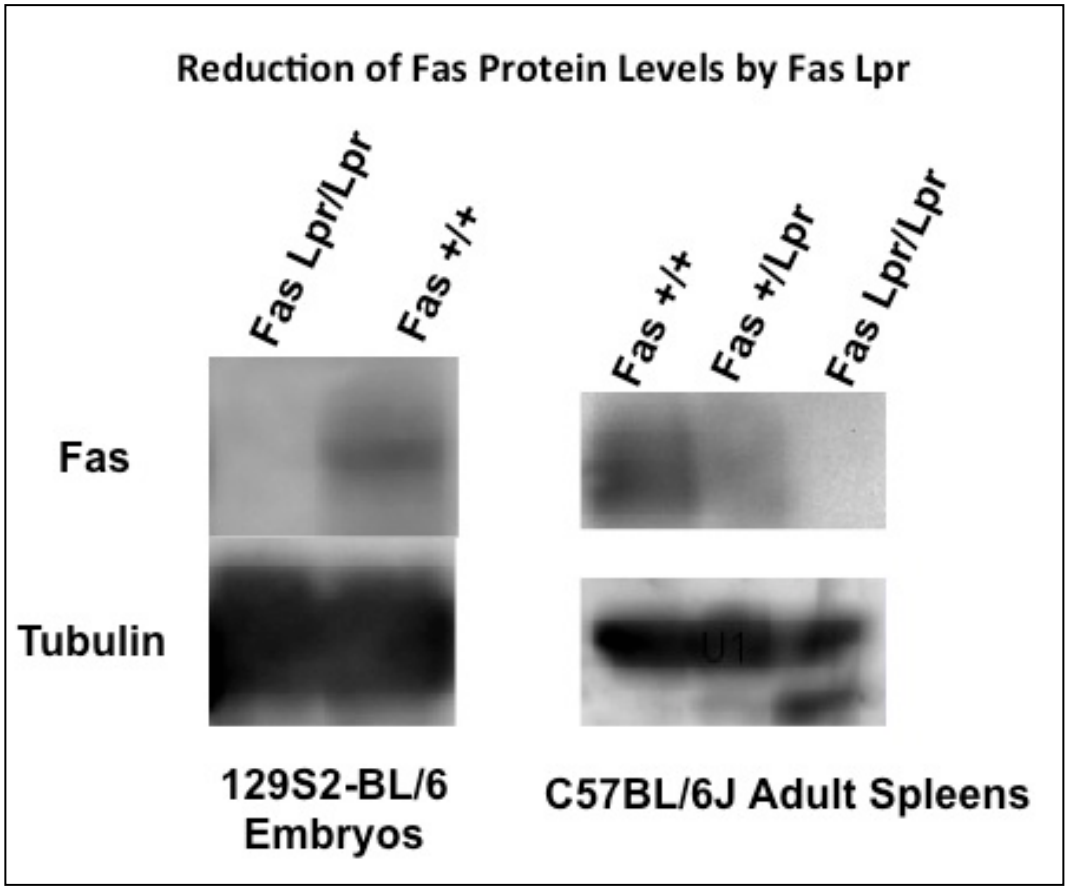

Figure 11: Effects of Fas Lpr on Fas Protein Levels

129S2-BL/6 embryos and C57BL/6J adult spleens were homogenized and run on an SDS-Page gel. 129S2-BL/6 Fas Lpr/Lpr embryo did not have any Fas. However, the Fas +/+ embryo had Fas protein. C57BL/6J Fas +/+, +/Lpr, and Lpr/Lpr adult spleens showed a progressive reduction in Fas protein levels.

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