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Initial Characterization of the Involvement of LPIN2 in Inflammation

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Initial Characterization of the Involvement
of LPIN2 in Inflammation

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science
in Human Genetics

by

Mariela Martinez

2014
ABSTRACT OF THE THESIS

Initial Characterization of the Involvement
of \textit{LPIN2} in Inflammation

by

Mariela Martinez

Master of Science in Human Genetics
University of California, Los Angeles, 2014
Professor Karen Reue, Chair

The innate immune system normally provides the body a first line of defense against infection, but malfunctions in the innate immune system can lead to inappropriate inflammatory responses, giving rise to chronic conditions known as autoinflammatory diseases. Autoinflammatory responses are typically characterized by episodes of fever, joint pain and swelling, or rash and may ultimately lead to damage of vital organs.

Lipin-2 (encoded by the \textit{LPIN2} gene) is an enzyme that acts in the synthesis of glycerolipids, including triacylglycerol and phospholipids. \textit{LPIN2} mutations have been implicated in two distinct autoinflammatory diseases. \textit{LPIN2} homozygous mutations cause the rare autoinflammatory disease Majeed syndrome, which is characterized by episodic fever,
osteomyelitis, and elevated levels of the cytokine interleukin-1β (IL-1β). Recently, heterozygous mutations in LPIN2 have been identified through clinical diagnostic sequencing of four patients of Indian descent with the autoinflammatory disease Familial Mediterranean Fever (FMF). FMF is primarily caused by autosomal recessive mutations in the Mediterranean Fever (MEFV) gene, which encodes pyrin, a modulator of immune response in white blood cells. However, a subset of FMF patients carry only a single MEFV mutant allele, and it is suspected that there are additional genetic factors leading to FMF in these individuals. The mechanism by which a lipid synthetic enzyme such as lipin-2 influences the inflammatory response is not understood. I hypothesized that lipin-2 may have an important role in modulating the inflammatory response in macrophages, and that mutations in LPIN2 may occur in FMF in Mediterranean populations, where FMF is most prevalent.

To assess the role of lipin-2 in inflammation, I characterized gene expression changes induced by inflammatory (endotoxin) and lipotoxic (fatty acid) stimuli in primary macrophages isolated from wild-type and lipin-2-deficient mice. Lipin-2-deficient macrophages exhibited enhanced IL-1β and pyrin gene expression in response to endotoxin, suggesting that lipin-2 may normally have a role in modulating this response. Additional results demonstrated that lipin-2 does not alter macrophage polarization in response to inflammatory stimuli, but may affect the efficiency of autophagy clearance, a process that has been previously associated with levels of IL-1β secretion. These results demonstrate an effect of lipin-2 on macrophage inflammatory response, and provide a starting point for future investigation into the cellular mechanism.

To investigate the presence of LPIN2 mutations in FMF in Mediterranean populations, I obtained DNA samples from twenty FMF patients in Israel who carry a single MEFV missense allele. Sequencing of the LPIN2 coding region identified known LPIN2 variants; however, none
of the variants identified are likely to be pathogenic. Although this is a small sample size, these results suggest that mutations in *LPIN2* are not prevalent in FMF in the Israeli population. Since the patients identified previously with heterozygous mutations in *MEFV* and *LPIN2* were of Indian descent, future sequencing efforts will focus on identifying patient cohorts from India.

Overall, our studies are consistent with a role for lipin-2 in inflammation. Additional efforts of this study will focus on describing the mechanism by which lipin-2 is involved in the inflammatory response.
The thesis of Mariela Martínez is approved.

Esteban C. Dell’Angelica

Jonathan Braun

Karen Reue, Committee Chair

University of California, Los Angeles

2014
To My Family and My Loving Husband
TABLE OF CONTENTS

Abstract of the thesis.................................................................ii
List of Tables and Figures...........................................................ix
Acknowledgements.................................................................x

Chapter 1: Introduction.............................................................1
   LPIN2 mutations and inflammatory disease.............................2
   Inflammation.........................................................................3
   Familial Mediterranean Fever..................................................4
   The dissertation.....................................................................5
References.................................................................................6

Chapter 2: Assessment of the involvement of lipin-2 in inflammation...8
Introduction................................................................................9
   Autoinflammation...................................................................9
   LPIN2 mutations and Majeed syndrome.................................9
   LPIN2 mutations and FMF....................................................10
   The involvement of LPIN2 in inflammation............................10

Methods....................................................................................12
   Mice......................................................................................12
   Peritoneal macrophage isolation...........................................12
   Human monocytic cell line cultures.....................................13
   LPS and fatty acid treatment of macrophages........................13
   Real time RT-PCR................................................................14
   Statistical Analysis..............................................................15
   Assessing autophagy through protein immunoblot..................15

Results......................................................................................17
   Establishment of primary peritoneal macrophage system to study inflammation..............................................17
   Optimization of treatment conditions for further studies..........................................................18
   Lipin-2–deficient macrophages exhibit enhanced induction of IL-1β in response to LPS...........................18
   Lpin1 expression is not altered in lipin-2–deficient macrophages.....................................................19
   Induction IL-1β in Lpin2-deficient peritoneal macrophages is not likely caused by differences in macrophage polarization..............................................................20
   Lpin2-deficient peritoneal macrophages exhibit defective autophagy..................................................21

Discussion.................................................................................23

References.................................................................................34
Chapter 3: \textit{LPIN2} exon sequencing in FMF patients carrying one missense \textit{MEFV} allele

Introduction...........................................................................................................................................38

Experimental Methods..........................................................................................................................43
  Patients.....................................................................................................................................................43
  Primer development.................................................................................................................................43
  PCR amplification.....................................................................................................................................45
  Sequencing...............................................................................................................................................45
  Sequencing analysis.................................................................................................................................46

Results.....................................................................................................................................................47

Discussion...............................................................................................................................................49

References...............................................................................................................................................53
LIST OF TABLES AND GRAPHS

Figure 2-1. Inflammatory gene expression in mouse peritoneal macrophages..................26
Figure 2-2. Inflammatory gene expression in a human monocytic cell line......................27
Figure 2-3. Titration of LPS and fatty acid effects on Lpin2 gene expression levels.........28
Figure 2-4. Pilot analysis of the effect of Lpin2 genotype on inflammatory genes..........29
Figure 2-5. Lpin2-deficient peritoneal macrophages exhibit higher induction
of IL-1β and Mefv.................................................................30
Figure 2-6. Lpin2 deficiency does not alter Lpin1 expression levels............................31
Figure 2-7. Macrophage polarization in Lpin2 wild-type and knockout peritoneal
macrophages.................................................................32
Figure 2-8. Aberrant autophagy in Lpin2-deficient macrophages..............................33
Figure 3-1. Representative PCR amplification of LPIN2 exons.................................51
Figure 3-2. Representative sequence traces of the four variants identified...............52
Table 3-1. Summary of the four LPIN2 variants identified by exon sequencing..........51
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colonies used in these experiments.

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Chapter 1

Introduction
**LPIN2 mutations and inflammatory disease**

Lipin-2 is an Mg\(^2+\)-dependent phosphatidate phosphatase (PAP) enzyme with an essential role in lipid metabolism. Lipin-2 deficiency causes the rare autoinflammatory disease Majeed syndrome, which is characterized by episodic fever, rash, and inflamed bone lesions (osteomyelitis) (Ferguson et. al., 2005). There is no cure for Majeed syndrome, but treatment with antagonists of the cytokine interleukin 1β (IL-1β) showed improvement in one patient (Herlin et al., 2013). The specific role of lipin-2 in inflammation has not been characterized extensively. Previous work suggests that mutations in LPIN2 may result in the loss of PAP enzyme activity in erythrocyte and lymphocytes, potentially contributing to the inflammation observed in Majeed syndrome patients (Donkor et al., 2009). In addition, studies in a murine macrophage cell line and in human blood monocyte-derived macrophages suggest that lipin-2 might have an immune protective role by enhancing cellular triacylglycerol synthesis in response to saturated fatty acid loading (Valdearcos et al., 2012).

Familial Mediterranean fever (FMF) is another autoinflammatory disease in which lipin-2 has also been implicated. FMF is characterized by acute inflammation and recurrent fevers mediated by increased IL-1β secretion. It is caused primarily by autosomal recessive mutations in the *Mediterranean Fever (MEFV)* gene, however, a subset of patients suffering from FMF have been found to be heterozygous for *MEFV* mutations, suggesting that an additional mutation in a separate gene may occur in these cases (Booty et al., 2009). Recently, clinical diagnostic sequencing of four FMF patients of Indian descent identified a single LPIN2 mutated allele in patients also carrying a single mutant *MEFV* allele. These results suggest that there might be a synergistic effect between mutant LPIN2 and MEFV in some cases of FMF. Further study is
required to understand the potential role of lipin-2 in the normal inflammatory response in autoinflammatory diseases such as Majeed syndrome and FMF.

**Inflammation**

Inflammation is a natural response to intracellular and extracellular stimuli, but aberrant immune activation may lead to uncontrolled inflammation, also known as autoinflammation. Autoinflammation is caused by overstimulation of innate immune pro-inflammatory pathways or by impairment of anti-inflammatory pathways (Henderson and Goldbach-Mansky, 2010). The immune system senses danger signals through activation of surface receptors, such as the nucleotide binding oligomerization domain (NOD)-like receptors (NLRs). The NLRs contains N-terminal effector domains, including a pyrin domain (PYD) and a caspase recruitment domain (CARD), which serve as connections to other signaling pathways (Tschopp and Schroder 2010).

One of the members of the NLR family that has been implicated in human inflammatory disease is NLR family pyrin domain-containing 3 [NLRP3, also known as cryopyrin] (Henderson and Goldbach-Mansky, 2010). When activated, the NLRP3 receptor interacts through its N-terminal PYD to form a cytoplasmic complex known as the NLRP3 inflammosome (Tschopp and Schroder 2010; Henderson and Goldbach-Mansky, 2010). The assembly of the NLRP3 inflammosome leads to the maturation of caspase-1, which in turn acts in the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 (Tschopp and Schroder 2010; Martinon, Burns, and Tschopp, 2002; Glaser and Goldbach-Mansky, 2009). IL-1β is potent inducer of fever and inflammation. Overproduction of this cytokine is a hallmark of autoinflammatory diseases, including Majeed syndrome and FMF.
**Familial Mediterranean Fever**

FMF was one of the first Mendelian autoinflammatory diseases to be characterized. The first underlying mutations identified in FMF were recessive mutations in *MEFV*, the gene that encodes pyrin, also known as marenostin (Henderson and Goldbach-Mansky, 2010). Pyrin is mainly found in neutrophils, eosinophils, monocytes, dendritic cells and fibroblasts (Bodar et.al., 2008). It is localized in the cytoplasm and interacts with tubulin in microtubules (Bodar et.al., 2008).

Even though the involvement of pyrin in FMF has been well documented, its physiological role is still not well understood. Some studies suggest a pro-inflammatory role for wild-type pyrin, while others demonstrate an anti-inflammatory role. An *in vitro* transfection model suggested that under some conditions, pyrin forms a complex similar to the NLRP3 inflammasome, leading to activation of IL-1β (Yu et.al., 2007). On the other hand, an anti-inflammatory role for pyrin has been suggested based in increased IL-1β secretion in a murine pyrin-knockout model (Chae et.al., 2003). In addition, *in vitro* studies in murine monocytes showed that transfection of wild-type pyrin suppresses IL-1β secretion (Chae et.al., 2003), potentially by binding to and inhibiting the function of caspase-1 (Chae et al., 2006). The interaction between pyrin and inflammasome components like the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) modulates inflammasome activity by preventing its formation (Chae et al., 2011).

Many mutations in FMF have been found to be located in the caspase binding B30.2 domain of pyrin, the domain through which pyrin binds to inflammasome components such as ASC and caspase-1, leading to decreased binding (Chae et al., 2011). This decreased binding
capacity could potentially lead to uncontrolled activation of the NLRP3 inflammosome and consequently, over-secretion of IL-1β.

The dissertation

The studies described in this thesis aim to establish a foundation on which to build an understanding of the role of lipin-2 in inflammation. In Chapter 2, I capitalized on the availability of a lipin-2–deficient mouse model to assess whether the presence of lipin-2 in macrophages influences the inflammatory response to stimuli such as endotoxin and fatty acids. In Chapter 3, I investigated the possibility that LPIN2 mutations are common in FMF patients that carry a single mutant MEFV allele in individuals of Mediterranean descent, where FMF is most prevalent.
References


Chapter 2

Assessment of the involvement of lipin-2 in inflammation
Introduction

Autoinflammation

Inflammation, the primary response of the immune system against infection, has been termed a “double edged sword” because malfunctions in its mechanism can lead to autoinflammation. Autoinflammation is the process by which an overactive and usually unprovoked innate immune response leads to inflammation of the body’s own tissues. It can be caused by mutations that lead to overstimulation of pro-inflammatory pathways or by the lack of anti-inflammatory pathways to control inflammation (Henderson and Goldbach-Mansky, 2010).

LPIN2 mutations and Majeed syndrome

One of these autoinflammatory disorders is an autosomal recessive disease called Majeed syndrome. This syndrome is characterized by recurrent episodes of fever and inflammation of skin and bone as well as congenital dyserythropoietic anemia (Ferguson et al., 2005; Al-Mosawi et al., 2007). Although there is no known cure for Majeed syndrome, a recent study demonstrated that treatment with interleukin-1β (IL-1β) antagonists was effective in one patient (Herlin et.al., 2013). This suggests that similar to other autoinflammatory disorders, Majeed syndrome is characterized by hypersecretion of IL-1β, a potent pyrogenic pro-inflammatory cytokine. Recently, an unpublished clinical sequencing study suggested that LPIN2 mutations could also be involved in a distinct autoinflammatory disease called Familial Mediterranean Fever (FMF).
**LPIN2 mutations and FMF**

Similar to Majeed syndrome, FMF is characterized by recurrent episodes of fever, uncontrolled inflammation, and hypersecretion of IL-1β. This disease is caused mainly by autosomal recessive mutations in *MEFV*, the gene that encodes pyrin, also known as marenostri (Henderson and Goldbach-Mansky, 2010). However, a subset of patients who have the clinical symptoms of FMF carry only a single mutant *MEFV* allele (Booty et.al., 2009). The clinical sequencing study identified four patients with FMF who carry a single mutant allele for *LPIN2* as well as *MEFV*, the causative gene for FMF. Since both of the diseases in which *LPIN2* mutations have been implicated are autoinflammatory, this suggests that *LPIN2* might have an important role in inflammation.

**The involvement of LPIN2 in inflammation**

*LPIN2* is the gene that codes for the lipin-2 protein, a member of the family of lipin proteins. Lipins are Mg\(^{2+}\) dependent phosphatidatic acid phosphatases (PAP) that generate diacylglycerol (Smith, Weiss, and Kennedy, 1957). The diacylglycerol generated by the lipins is important for the synthesis of membrane phospholipids such as phosphatidylcholine and phosphatidylethanolamine and for the activation of signaling pathways downstream of diacylglycerol kinases (Reue, 2009). Previous studies suggest that in addition to being expressed in tissues such as the liver, *LPIN2* is expressed in lymphoid tissues, such that mutations in this protein may lead to loss of their PAP enzymatic activity in lymphocytes, potentially contributing to the inflammation observed in Majeed syndrome patients (Donkor et.al., 2009).
A recent in vitro study of human blood monocyte-derived macrophages and a murine macrophage cell line suggested that lipin-2 modulates pro-inflammatory signaling by controlling fatty acid detoxification and, ultimately, the induction of pro-inflammatory genes (Valdearcos et. al., 2012). It has been shown that the capacity of macrophages to convert saturated fatty acids to triacylglycerol (TAG) is inversely correlated with their expression of pro-inflammatory genes (Koliwad et. al., 2010; Valdearcos et. al., 2012). This suggests that reducing the intracellular concentration of free fatty acids modulates the inflammatory pathways in macrophages. Valdearcos et.al. showed that lipin-2 deficiency causes the induction of pro-inflammatory genes like interleukin-6 (Il6) and tumor necrosis factor alpha (TNFα) (Valdearcos et.al., 2012). Thus, it appears that lipin-2 enhances cellular triacylglycerol synthesis in response to saturated fatty acid loading, and this could be essential to prevent unregulated inflammation in macrophages. This role of lipin-2 in modulating pro-inflammatory response is relevant to further understanding the etiology of autoinflammatory diseases such as Majeed syndrome and FMF.

The experiments described in this chapter were designed to investigate the hypothesis that lipin-2 is important for the regulation of macrophage inflammatory response. To test this, I utilized Lpin2 knockout mice that were previously generated in the Reue laboratory (Dwyer et. al., 2012) as a source of primary macrophages and challenged them with endotoxin and lipotoxic stimuli. Further understanding if lipin-2 has a role in inflammation could eventually provide critical information for the clinical management of Majeed syndrome and FMF patients carrying LPIN2 mutant alleles.
Methods

Mice

The C57BL/6J Lpin2-deficient mouse strain was previously developed in the Reue laboratory (Dwyer et al., 2012). I established a colony from them and bred mice for the experiments described in this thesis. Lpin2 heterozygous mice (Lpin2+/−) were crossed to generate the mice genotypes: Lpin2 wild-type (Lpin2+/+), Lpin2 heterozygous (Lpin2+/−), and Lpin2 knockout (Lpin2−/−). These mice were genotyped by PCR to detect the wild-type or mutant alleles using primers that span the β-geo coding sequence found within intron 3 of the mutant allele, or amplifying a portion of this intron using primers that amplify only the wild-type allele. The sequences for the genotyping primers were: β-geo Forward 5’-TTATCGATGAGCGTGGTGGTT, β-geo Reverse 5’-GCGCGTACATCGGCAAATAA (mutant) and Lpin2 Intron 3 Forward 5’-CCTGTCTAATGTGCCCTCTCCT, Lpin2 Intron 3 Reverse 5’-AGCAGGTTTAGAGCCATGTGA (wild-type). All mice were maintained under a 12 hour-12 hour light-dark cycle. The Institutional Animal Care and Use Committee of the University of California, Los Angeles approved all animal experimental protocols.

Peritoneal Macrophage Isolation

The peritoneal macrophage isolation was performed by adapting to a previously published protocol (Zhang et al., 2008). Three to four days before the peritoneal macrophage isolation, six to eight week old mice were injected into the peritoneal cavity with 1.5mL of 35 Brewer thioglycollate medium (Hoover and Nacy, 1984). An inflammatory response was elicited for four days, causing the recruitment of macrophages into the peritoneal cavity. Peritoneal macrophages were isolated by peritoneal gavage, washed 3 times, and plated at a
density of $2 \times 10^6$ cells per well in a 6-well plate. Cells were maintained in DMEM/F12 (Cat No.11320-033, Life Technologies) plus 10% fetal bovine serum (FBS), 100 unit/mL of penicillin, and 100µg/mL of streptomycin. Cells were incubated at 37°C in a 5% CO$_2$ humidified incubator.

**Human monocytic cell line cultures**

The THP-1 human monocytic cell line used in these experiments was maintained in RPMI 1640 media (R8758, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL of penicillin, and 100µg/mL of streptomycin. Cells were incubated at 37°C in a 5% CO$_2$ humidified incubator. The macrophage-like phenotype was induced by treatment with 200nM of phorbol-12-myristate-13-acetate (PMA) for 3 days as previously described (Daigneault et.al., 2010).

**LPS and fatty acid treatment of macrophage cultures**

In order to effectively treat cells with fatty acids they need to be complexed to bovine serum albumin (BSA), therefore, I bound both palmitate and oleate to BSA as previously described (Das, Mondal, and Elbein, 2010).

The day after the seeding of $2 \times 10^6$ peritoneal macrophages per well on 6-well plates (or the THP-1 human monocytic cells), 6-hour treatments with 100ng/mL of lipopolysaccharide (Cat.No.L4391, Sigma Aldrich), 300µM of oleic acid, or 300µM palmitic acid were performed. For the first experiments additional treatments with 100ng/mL of LPS combined with 300µM of oleic acid or 300µM of palmitic acid were also performed. Treatment incubation times were
based on a publication comparing inflammatory and lipid metabolic pathways in thioglycollate elicited macrophages versus the RAW264.7 mouse macrophage cell line (Maurya et.al., 2013).

**Real time RT-PCR**

RNA extraction was performed using the TRIzol reagent protocol (Invitrogen). First strand cDNA synthesis with 1µg of total RNA and the iScript Reverse Transcription Mix for RT-qPCR (Cat.No. 170-8840, BioRad). Real time PCR was performed to amplify the cDNA using the SsoFast EvaGreen Super Mix (Cat.No.172-5200) and specific primers for each human or mouse gene. Sequences for the primers used for murine genes were: *Lpin2*-f1845, 5’-AGTTGACCCC-ATCACCCTAG-3’ and *Lpin2*-r2093, 5’CCCCAACGATCAGACTTGGT-3’; *Lpin1*-fL2, 5’-‘CCTTCTATGCTTGTGTGGAACC-3’ and *Lpin1*-fL2, 5’-GTGATCGACCACCTTCG-AAGGC-3’; *mIL1b*-480F, 5’-TCGGACCATATGAGCTGAAAGC-3’ and *mIL1b*-605R, 5’-GAGGCCAAGGCCACAGGTATT-3’ *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’; *Mefv*-F, 5’-AAAGCAAGAAGCCGAAGTG-3’ and *Mefv*-R, 5’-TCTAAAGCCCCGTAGTCCT-3’; *caspase1*-F, 5’-TGCTACCTGGCCAGGA-3’ and *caspase1*-R, 5’-GGGTCCCAGTCAGTCCTGGAAA-3’. The primer sequences for human genes were: *IL-1β*-F, 5’-CTGTCTGCTGCTTGGGAAGA-3’ and *IL-1β*-R, 5’-TTCTGCTTGGAGGCTGTA-3’; *MEFV*-F, 5’-TCCAACCTCCTCCACCAGAAG-3’ and
MEFV-R, 5’-CCTCTCCCACCTGGTCCAA-3’; B2M-F, 5’-GTCTTTCAACAAGAGACTGTC-3’ and B2M-R, 5’-CAAATGCGCATCTTCAAACC-3’; LPIN2-F, 5’-CCTCTCTCTCCGATC-3’ and LPIN2-R, 5’-GGAGAATGCTCCAAAGCA-3’. The relative mRNA abundance for the genes was calculated either by using the Relative Standard Curve Method or ΔΔCT method. Gene expression was normalized to housekeeping genes: ribosomal protein 36b4, Beta 2 microglobulin (B2M), or TATA box binding protein (TbP).

Statistical Analysis

For computation of statistically significant differences in experiments that involved at least three mice (or had triplicate wells per treatment, in the case of the THP-1 cell line), I performed one-way analysis of variance (ANOVA) followed by Student’s t-test. P-values <0.05 were considered statistically significant.

Assessing autophagy through protein immunoblot

Peritoneal macrophages derived from Lpin2 wild type and knockout mice were isolated and cultured as previously described in these methods. The macrophages were cultured for 8 hours either in DMEM with 10% FBS, 100 unit/ml of penicillin, and 100µg/mL of streptomycin (control wells), or with Hanks Balanced Salt Solution (HBSS, Cat. No.14025076, Life Technologies) supplemented with 100ng/mL of LPS (for serum starvation and to induce inflammation). Cellular protein extracts were prepared and quantitated for protein immunoblotting. Lysosomal turnover of autophagosomal marker microtubule associated protein 1A/1B-light chain 3 (LC3-II) was used to monitor starvation-induced autophagy (Tanida, Ueno, and Kominami, 2008). Protein immunoblotting was performed on the protein extracts of serum
starved peritoneal macrophages with anti-LC3 antibody (Cat.No. NB100-2220, Novus Biologicals) and anti-GAPDH (Cat.No.GTX1001118, GeneTex) was used as a protein loading control.
Results

Establishment of primary peritoneal macrophage system to study inflammation

Majeed syndrome (OMIM #609628) is a rare autosomal recessive autoinflammatory disorder caused by mutations in LPIN2, the gene that encodes the lipin-2 protein. Even though the role of lipin-2 in the biosynthesis of lipids has been well described, its role in Majeed syndrome has not been determined. A recent in vitro study suggested that lipin-2 has a protective role in pro-inflammatory signaling by enhancing the cellular capacity of triacylglycerol synthesis in saturated fatty acid-overloaded murine and human macrophages (Valdearcos et.al., 2012).

Inflammation in macrophages can be induced experimentally by treatment with lipopolysaccharide (LPS) or by overloading with saturated fatty acids (Valdearcos et.al., 2012). To establish a system in which to study the role of lipin-2 in inflammation, I isolated thioglycollate-elicited mouse peritoneal macrophages and assessed their response to LPS, oleic acid (OA, a monosaturated fatty acid), palmitic acid (PA, a saturated fatty acid) or the combination of LPS and each fatty acid. As expected, LPS treatment as well as fatty acid treatments substantially induced expression of the gene for the inflammatory cytokine IL-1β (Figure 2-1A). The greatest induction was seen for LPS plus oleic acid.

In addition to IL-1β, I assessed expression of other inflammatory genes including Mefv (also known as pyrin) and caspase-1, an enzyme involved in the cleavage and maturation of IL-1β. There was induction of Mefv and caspase-1 in response to LPS and fatty acids treatments with a profile similar to that of IL-1β (Figure 2-1B,C). Finally, I assessed Lpin2 expression and found that LPS or fatty acid treatments did not alter Lpin2 mRNA levels (Figure 2-1D). This suggests that Lpin2 is not regulated at the transcriptional level by inflammatory stimuli.
To verify that the results observed for the mouse peritoneal macrophages are representative of responses in human cells, I performed the analogous experiment described above in the THP-1 human monocytic cell line. The THP-1 cells were primed with Phorbol 12-myristate 13-acetate (PMA) for 3 days to induce a macrophage-like inflammatory phenotype. The induction of IL-1β and MEFV gene expression was very similar to that observed in the primary mouse macrophages, and LPIN2 expression was not altered (Figure 2-2). Collectively, these results indicate that in our hands, mouse peritoneal macrophages are highly responsive to inflammatory stimuli and that they respond similarly to human monocyte-macrophages, supporting the rationale for using mouse-derived peritoneal macrophages to study the role of lipin-2 in inflammation.

**Optimization of treatment conditions for further studies**

The studies described above used a single concentration of LPS and single time point for fatty acid treatment and showed no significant induction of Lpin2 in response to endotoxin or saturated fatty acids. Since Lpin2 has been shown to be involved in the incorporation of fatty acids into phospholipids and triacylglycerol, it was interesting to not find an induction in response to saturated fatty acids. This led to a pilot experiment with the goal of determining whether Lpin2 expression may be induced under different conditions. In this experiment, LPS concentrations were titrated and different incubation time-points for the fatty acid treatments were tested. The higher concentrations (10 and 100ng/ml) of LPS showed an increase in Lpin2 levels compared to the lower LPS dose (1ng/mL) (Figure 2-3). The 2-hour treatment with palmitic or oleic acid reached the maximal levels of Lpin2 expression, and should therefore be used for subsequent studies.
Lipin-2–deficient macrophages exhibit enhanced induction of IL-1β in response to LPS

Based on the inflammation (including elevated IL-1β levels) observed in Majeed syndrome due to lipin-2 deficiency, I hypothesized that reduced lipin-2 levels may lead to enhanced response to inflammatory stimuli in macrophages. To test this, I initially performed a pilot experiment to determine the effect of lipin-2 deficiency or lipin-2 haploinsufficiency on macrophage induction of IL-1β, Mefv, and caspase-1 under the same treatments administered in the previous experiments with a single mouse of each genotype. Lpin2 knockout (Lpin2−/−) macrophages appeared to have a greater induction of IL-1β in response to LPS compared to the wild-type (Lpin2+/+) or heterozygous (Lpin2+/−) macrophages (Figure 2-4). These results warranted further studies with larger numbers of mice.

I assessed inflammation in peritoneal macrophages derived from four Lpin2 wild-type and four Lpin2 knockout mice. Similar to the pilot experiment, the IL-1β induction in Lpin2-deficient peritoneal macrophages was more than 4-fold higher than for wild-type peritoneal macrophages in response to LPS (Figure 2-5A). There was also a significant induction of IL-1β in Lpin2-deficient macrophages in response to oleic and palmitic acid compared to the same genotype under control treatment. There was a trend for increased Mefv gene expression in Lpin2-deficient macrophages (Figure 2-5B).

To investigate whether the induction of IL-1β observed in Lpin2-deficient macrophages is due to activation of the inflammasome, I assessed the expression levels of two inflammasome components in response to LPS or fatty acids. As described above, caspase-1 has a critical role in the maturation and secretion of IL-1β. Caspase-1 mRNA levels were significantly induced in response to LPS for peritoneal macrophages of both genotypes compared to their untreated
controls (Figure 2-5C). However, no differences occurred in caspase-1 expression between wild-type and Lpin2-deficient macrophages. Another inflammasome component examined was apoptosis-associated speck-like protein containing CARD (ASC). ASC is an adaptor protein that is critical for inflammasome-dependent caspase-1 maturation. There was no significant induction of ASC expression in response to any of the treatments. Collectively, these results indicate that lipin-2 deficiency results in enhanced induction of IL-1β mRNA expression in macrophages exposed to an inflammatory stimulus, and that this is not reflected in induction of inflammasome component gene expression levels. These findings may be relevant to the source of the elevated IL-1β levels that are observed in Majeed patients.

*Lpin1 expression is not altered in lipin-2–deficient macrophages*

Studies suggest that another enzyme from the lipin family, lipin-1, is expressed in macrophages and in some tissues, can functionally compensate for the loss of lipin-2 to maintain glycerolipid homeostasis (Dwyer et.al., 2012). To determine whether lipin-1 levels are influenced by lipin-2 deficiency in peritoneal macrophages, I assessed the expression of Lpin1 in wild-type and lipin-2–deficient peritoneal macrophages under the different treatments. Interestingly, Lpin1 gene expression is regulated quite differently than Lpin2 expression, with a dramatic suppression by LPS and no change in levels in response to fatty acid treatments (Figure 2-6). However, lipin-2 deficiency did not influence Lpin1 gene expression. These results led me to conclude that lipin-1 does not likely compensate for the loss of lipin-2 in macrophages.
Induction IL-1β in Lpin2-deficient peritoneal macrophages is not likely caused by differences in macrophage polarization.

I next assessed whether macrophage polarization was affected by the Lpin2 genotype. Macrophage polarization is the process by which macrophages differentiate into a pro-inflammatory (M1) or an anti-inflammatory (M2) phenotype depending on the stimulus received. Classical activation of macrophages (seen in M1 macrophages) can be stimulated by microbial components such as LPS (Lawrence and Natoli, 2011). These types of macrophages secrete pro-inflammatory cytokines (notably IL-1β) while the M2 type macrophages secrete anti-inflammatory cytokines. Significant induction of the M1-type macrophage marker Nos2 was only observed in wild-type peritoneal macrophages in response to LPS treatment (Figure 2-7A). The trend for Lpin2-deficient macrophages was similar, but did not reach significance due to variation among the samples. On the other hand, interferon gamma (Ifng) was significantly upregulated in peritoneal macrophages from both genotypes compared to their untreated controls (Figure 2-7B). The difference between the levels of Ifng induction in wild-type versus knockout peritoneal macrophages treated with LPS were not significant. Lpin2-deficient macrophages also exhibited a significant upregulation of Ifng in response to palmitic acid treatment when compared to untreated controls. Overall, these results show that M1-macrophage polarization is not likely the cause for the differential induction of IL-1β in response to LPS treatment.

The M2, anti-inflammatory macrophage markers also exhibited similar patterns for wild-type and Lpin2-deficient cells. Arginase-1 (Arg1) was not induced in response to LPS or oleic acid compared to untreated controls (Figure 2-7C). There was a significant decrease in Arg1 expression in both wild-type and knockout macrophages treated with palmitic acid, raising
the possibility that saturated fatty acid loading influences polarization, but a second M2-type macrophage marker, *Ym1*, was not influenced by any treatments or genotype (Figure 2-7D).

Collectively, these results suggest that the induction of IL-1β seen in *Lpin2*-deficient primary mouse macrophages is likely independent of inflammasome activation and macrophage polarization.

**Lpin2-deficient peritoneal macrophages exhibit defective autophagy**

Recently, a study suggested that defective autophagy leads to mitochondria-mediated NLRP3 inflammasome activation, which contributes to hypersecretion of IL-1β (van der Burgh et. al., 2014). Additionally, the Reue laboratory has identified lipin-1 as an important player in autophagy (Zhang et al., 2014). These two findings led us to investigate whether lipin-2-deficient macrophages exhibit a defect in autophagy. To do so, I induced autophagy in peritoneal macrophages from wild-type and lipin-2–deficient mice by serum and glucose starvation in combination with LPS treatment. A reliable method for assessing autophagic flux is to determine levels of the microtubule-associated protein 1A/1B-light chain 3 (LC3) detection through protein immunoblotting (Tanida et.al. 2008). Immunoblotting for LC3 showed increased levels of LC3-I and LC3-II in both the basal (control) condition and after starvation/LPS treatment (Figure 2-8). These results raise the possibility that the IL-1β secretion observed in *Lpin2*-deficient peritoneal macrophages could be related to defective autophagy, and future studies will investigate this possibility.
Discussion

The suggested role of lipin-2 in two distinct autoinflammatory diseases spurred my interest in further understanding the role of this protein in inflammation. To do so, I assessed the response of peritoneal macrophages from wild-type and lipin-2–deficient mice to inflammatory or lipotoxic stimuli, or a combination of the two. I observed a robust induction in macrophages of IL-1β in response to fatty acids as well as LPS. I also observed LPS-mediated upregulation of genes encoding pyrin and caspase-1, an inflammasome component that is essential for maturation and secretion of IL-1β. The responses to LPS and fatty acids were similar in mouse macrophages and the THP-1 human monocytic cell line.

Most importantly, there was a significant enhancement in the expression of IL-1β in response to LPS in macrophages from lipin-2–deficient mice compared to wild-type macrophages. This is relevant to human diseases that are associated with LPIN2 mutations, including Majeed syndrome and potentially FMF, which are both characterized by elevated IL-1β levels. We observed a similar trend for increased Mefv mRNA levels in lipin-2–deficient macrophages, although this did not reach statistical significance due to variation among animals. On the other hand, although caspase-1 mRNA levels were significantly induced in response to LPS, there were no differences between genotypes. These results suggest that the significant induction of IL-1β in lipin-2–deficient peritoneal macrophages is caused by a mechanism that is either independent of caspase-1, or that there is a posttranslational effect leading to this induction.

Having observed an enhanced inflammatory response in lipin-2–deficient macrophages, we hypothesized that there may be differences in macrophage polarization. However, analysis of M1 and M2 macrophage markers did not reveal a clear difference between genotypes, and we
conclude that this is not likely the mechanism for the enhanced IL-1β levels in lipin-2–deficiency.

Another process that may influence IL-1β levels is autophagy. A recent publication demonstrated that impairments in autophagy clearance are associated with hypersecretion of IL-1β (van der Burgh, 2014). Additionally, recent studies from the Reue laboratory have suggested that lipin-1, a close relative of lipin-2, has a critical role in autophagy in skeletal muscle (Zhang et al., 2014). This sparked my interest in assessing whether lipin-2 could also be involved in autophagy. We evaluated the induction of autophagy in macrophages of wild-type and lipin-2–deficient mice under basal and starvation conditions by assessing the accumulation of the LC3 protein, levels of which are influenced by both autophagosome formation and autophagy clearance. Lipin-2–deficient macrophages had substantially increased levels of LC3 under both basal and starvation conditions, indicating an effect of lipin-2 on this pathway. At present it is not clear if the increased levels of LC3 are related to enhanced initiation of autophagy, or impaired autophagy clearance, both of which lead to accumulation of LC3. We suspect that lipin-2 deficiency in macrophages may have an effect similar to lipin-1 deficiency in muscle, where autophagy initiation was normal, but autophagy clearance is diminished (Zhang et al., 2014). Future studies can distinguish between effects on autophagy initiation or clearance through the use of reporters that allow microscopic visualization of specific steps of the autophagy process (Zhang et al., 2014). Additional studies must also examine whether secretion of the IL-1β protein is enhanced in lipin-2–deficient macrophages, and which stimuli promote this.

Overall, these studies provide preliminary evidence for a mechanism by which lipin-2 may influence inflammation in macrophages. Further investigation is warranted to determine
whether this plays a role in the inflammatory process that is evident in Majeed syndrome, as well as a potential role in FMF.
Figures

Figure 2-1. Inflammatory gene expression in mouse peritoneal macrophages

Peritoneal macrophages from four C57BL/6J mice were isolated by thioglycollate-induced recruitment of macrophages to the peritoneal cavity followed by peritoneal gavage. The cells were plated in 6-well plates at a density of 2x10^6 cells per well and were treated in triplicates for six hours with: 100ng/mL of lipopolysaccharide (LPS), 300µM of oleic acid (OA), 300µM of palmitic acids, and 100ng/mL of LPS in combination with 300µM of OA or PA (LPS+OA, LPS+PA, respectively). Cells were harvested and gene expression was quantified by real-time RT-PCR. Gene expression levels were normalized to the mRNA levels of the TATA box binding protein (TbP) housekeeping gene.

(A) IL-1β mRNA levels.
(B) Mefv (pyrin) mRNA levels.
(C) Caspase-1 mRNA.
(D) Lpin-2 mRNA levels.

In all panels, m, mouse. *, p < 0.05; **, p<0.01; ***, p < 0.001; ****, p < 0.0001 vs. untreated control.
Figure 2-2. Inflammatory gene expression in a human monocytic cell line

The THP-1 human monocytic cell line was treated with Phorbol 12-myristate 13-acetate (PMA) for 3 days to induce a macrophage-like phenotype. Then, the cells were treated for six hours with: 100ng/mL of lipopolysaccharide (LPS), 300µM oleic acid (OA), 300µM palmitic acid (PA), and 100ng/mL of LPS in combination with 300µM of OA or PA (LPS+OA, LPS+PA, respectively). Cells were harvested and gene expression was quantified by real-time RT-PCR. Gene expression levels were normalized to Beta 2 microglobulin (β2M) housekeeping gene.

(A) IL-1β mRNA levels.
(B) MEFV (pyrin) mRNA levels.
(C) LPIN2 mRNA.

In all panels, h, human. *, p < 0.05; **, p<0.01; ***, p < 0.001; ****, p < 0.0001 vs. untreated control.
Figure 2-3. Titration of LPS and fatty acid effects on \textit{Lpin2} gene expression levels

\textit{Lpin2} expression levels were assessed in response to various concentrations of LPS (2-hour treatments) and 300µM treatments for oleic and palmitic acids for the times indicated on the figure. Results shown are representative of peritoneal macrophages derived from a single mouse. Cells were harvested and gene expression was quantified by real-time RT-PCR. Gene expression levels were normalized to Beta 2 macroglobulin (\(\beta2M\)) and 36b4 housekeeping genes.
Figure 2-4 Pilot analysis of the effect of Lpin2 genotype on inflammatory genes

Lpin2 wild-type (Lpin2 +/-, shown in black), Lpin2 heterozygous (Lpin2 +/-, shown in grey), and Lpin2 knockout (Lpin2 /-, shown in white) peritoneal macrophages were isolated by thioglycollate-induced recruitment of macrophages to the peritoneal cavity followed by peritoneal gavage (for one mouse per genotype). The cells were plated in 6-well plates at a density of 2x10^6 cells per well and were treated for six hours with: 100ng/mL of lipopolysaccharide (LPS), 1ng/mL of lipopolysaccharide (LPS), 300µM of oleic acid (OA), and 300µM of palmitic acid (PA). Treatments were done in duplicates. Cells were harvested and gene expression was quantified by real-time RT-PCR. Gene expression levels were normalized to 36b4 mRNA levels.

(A) IL-1β mRNA levels.
(B) Mefv (pyrin) mRNA levels.
(C) Caspase-1 mRNA.
Figure 2-5. *Lpin2*-deficient peritoneal macrophages exhibit higher induction of *IL-1β* and *Mefv*.

*Lpin2* wild-type (*Lpin2* +/+, shown in black) and *Lpin2* knockout (*Lpin2*−/−, shown in white) peritoneal macrophages were isolated from four mice per genotype by thioglycollate-induced recruitment of macrophages to the peritoneal cavity followed by peritoneal gavage. The cells were plated in 6-well plates at a density of 2x10^6 cells per well and were treated for six hours with: 100ng/mL of lipopolysaccharide (LPS), 300µM of oleic acid (OA), and 300µM of palmitic acids (PA). In this experiment, the treatments were done on one well per treatment per mouse (n=4 mice per genotype). Cells were harvested and gene expression was quantified by real-time RT-PCR. Gene expression levels were normalized to 36b4 and B2M mRNA levels.

(A) *IL-1β* mRNA levels.
(B) *Mefv* (pyrin) mRNA levels.
(C) *Caspase-1* mRNA.
(D) *ASC* (apoptosis speck-like protein containing a CARD domain) mRNA levels.

+, p < 0.05; *, p < 0.05; **, p<0.01; ***, p < 0.001 vs. untreated control levels for the same genotype.
Figure 2-6. *Lpin2* deficiency does not alter *Lpin1* expression levels

*Lpin1* mRNA levels were assessed by real-time RT-PCR in the same samples as Figure 2-5. Gene expression levels were normalized to 36b4 and B2M mRNA levels. *Lpin2* +/+ , shown in black; *Lpin2* -/- , shown in white

*, p < 0.05; **, p<0.01 vs. untreated control levels for the same genotype
Macrophage polarization was assessed by expression of M1-type (pro-inflammatory) and M2-type (anti-inflammatory) macrophage markers. The M1-type macrophage markers in this experiment were nitric oxide synthase 2 (Nos2) and Interferon gamma (Ifng) and the M2-type macrophage markers were arginase-1 (Arg1) and chitinase 3-like-3 protein (Ym-1). *Lpin2+/+, shown in black;Lpin2−/−, shown in white. The mRNA levels for these genes were assessed by real-time RT-PCR in the same samples as Figure 2-5. Gene expression levels were normalized to 36b4 and B2M mRNA levels.

(A) Nos2 mRNA levels.
(B) Ifng mRNA levels.
(C) Arg1 mRNA levels.
(D) Ym-1 mRNA levels.

*, p < 0.05; **, p<0.01 vs. untreated control levels for the same genotype.
Figure 2-8. Aberrant autophagy in \textit{Lpin2}-deficient macrophages

Peritoneal macrophages isolated from \textit{Lpin2} wild-type and knockout mice were isolated by thioglycollate-induced recruitment of macrophages to the peritoneal cavity followed by peritoneal gavage. The macrophages were then treated with DMEM plus 10% fetal bovine serum (control) or in starvation conditions (Hank’s balanced salt solution) with LPS to induce inflammation. Immunoblot analysis for LC3 and GAPDH protein loading control is shown.
References


Chapter 3

_LPIN2_ exon sequencing in FMF patients carrying one missense _MEFV_ allele
Introduction

Familial Mediterranean fever (FMF; OMIM no. #249100) is an autoinflammatory disease of Mendelian autosomal recessive inheritance characterized by episodes of periodic fever. It affects approximately 100,000 people worldwide, making it one of the most common periodic fever syndromes (Lidar and Livneh, 2007; Glaser and Goldback-Mansky, 2008). There is high prevalence of FMF among ethnic groups from the Middle East, including non-Ashkenazi Jews, Ashkenazi Jews, Turks, Armenians, and Arabs (Booty et al., 2009). FMF is also not uncommon in other Mediterranean populations such as Italians, Spanish, Portuguese, French, and Greeks.

In addition to unprovoked episodes of fever, FMF patients also experience abdominal pain, arthritis, synovitis, serositis, peritonitis, and skin inflammation (Booty et al., 2009; Heymann and Rösen-Wolff, 2013; Hoffman and Simon, 2009; Hofman et al., 2011). There is typically an elevation of inflammatory markers (leukocyte count, C-reactive protein, sedimentation rate, and serum amyloid A) that may persist even in asymptomatic periods between attacks (Glaser and Goldback-Mansky, 2008). The episodes of inflammation are generally short, lasting one to three days and usually end abruptly. FMF patients may experience episodes at intervals ranging from once a week to once a decade (El-Shanti, Majeed, and El-Khateeb, 2006). In general, episodes begin during childhood presenting before 20 years of age for more than 80% of the patients (El-Shanti, Majeed and El-Khateeb, 2006). The most severe complication associated with FMF is amyloidosis, which is caused by prolonged elevation of serum amyloid A levels. This complication primarily occurs in the kidneys for 11.4% of FMF patients and can lead to renal failure (Glaser and Goldback-Mansky, 2008; Touitou et al., 2007). The most effective treatment for most FMF patients is colchicine, which has proven effective in preventing amyloidosis in most cases.
**MEFV** (MEditerranean FeVer), the gene responsible for FMF, is located in the short arm of chromosome 16 (El-Shanti, Majeed and El-Khateeb, 2006; French FMF Consortium and International FMF Consortium). **MEFV** encodes a 781–amino acid protein known as pyrin (alternatively, marenostin) (Booty et.al., 2009). It is mainly expressed in cytokine-activated monocytes, dendritic cells, synovial fibroblasts, and polymorphonuclear cells (Booty et.al., 2009; Centola et.al., 2000; Diaz et.al., 2004). The exact role of pyrin in inflammatory response is still to be clearly elucidated since it has been shown to either activate or modulate IL-1β depending on the experimental setting (Booty et.al., 2009; Chae et.al., 2003; Papin et.al., 2007; Seshadri et.al., 2007).

The most common mutations associated with FMF are missense mutations in **MEFV**, particularly M694V, V726A, M694I, M680I, and E148Q (El-Shanti, Majeed and El-Khateeb, 2006; French FMF Consortium and International FMF Consortium; Henderson and Goldbach-Mansky, 2011; Booth et.al., 1998; Bernot et.al., 1998; Touitou, 2001). The carrier frequency for FMF could be as high as one in four for the commonly affected ethnic groups (individuals of Mediterranean origin). It can range from 37–39% in Armenians and Iraqi Jews to 20% in Turks, North African and Ashkenazi Jews, and Arabs (Booty et.al., 2009). This relatively high carrier frequency has raised the possibility that there is a selective heterozygote advantage (Booty et. al., 2009; El-Shanti, Majeed, and El-Khateeb, 2006). It has been shown that the prevalence of FMF is less than expected based on the high carrier frequency.

On the other hand, the E148Q variant has an allele frequency of up to 23% in Japanese and ranges from 0.5-5% in Caucasians (Henderson and Goldbach-Mansky, 2011; Topaloglu et.al., 2005). This variant has also been found to confer nonspecific inflammatory phenotype susceptibility in homozygous individuals from Greek and Turkish populations (Henderson and
The most common mutations \textit{MEFV} (M694V, V726A, M694I, M680I, and E148Q) account for approximately 80% of the patients from the classically affected populations. However, it has been shown that there are a substantial number of patients (up to 30% depending on the population) with only one \textit{MEFV} mutation found in the entire coding region (Booty et. al., 2009; Medlej-Hashim et. al., 2000; Booth et. al., 2000; Aldea et. al., 2004; Marek-Yagel et. al., 2009). These results led to a study in which a second \textit{MEFV} mutation was assessed by sequencing of all exons in 46 clinically diagnosed FMF patients carrying only one high penetrance mutation in \textit{MEFV} (Booty et. al., 2009). The study showed that a second \textit{MEFV} mutation could not be identified in these FMF patients. A haplotype analysis performed in this study did not demonstrate a common haplotype associated to transmission of the second FMF allele. Interestingly, in a small subset of patients the investigators identified mutations in pyrin interacting proteins, suggesting that the combination of one mutant pyrin and one mutant allele of another protein could lead to inflammation that is sufficient to cause the disease. Overall, these results suggest the possibility of digenic inheritance as a cause for a proportion of FMF patients (Booty et. al., 2009).

Another autoinflammatory disease for which overly active inflammation has been suggested to be crucial is Majeed syndrome (Ferguson et. al., 2005). Similar to FMF, Majeed syndrome also affects primarily individuals from Mediterranean populations. Elevated levels of IL-1\(\beta\) and recurrent fever characterize both Majeed syndrome and FMF. Majeed syndrome is also characterized by recurrent fever, chronic recurrent multifocal osteomyelitis (CRMO), congenital dyserythropoietic anemia (CDA) and inflammatory dermatosis. (Ferguson et. al., 2005; Glaser and Goldbach-Mansky, 2009). Episodes generally recur every one to two times a
month and can last three to four days (Bodar et al., 2008). This autoinflammatory syndrome is caused by mutations in LPIN2, the gene that encodes for the lipin-2 protein (Glaser and Goldbach-Mansky, 2009; Ferguson et al., 2005).

LPIN2 is a gene composed of 20 exons that span approximately 95kb and produces an 896 amino acid protein (Peterfy et al., 2001). Lipin-2 is a member of a family of Mg$^{2+}$ phosphatidate phosphatase (PAP) enzymes, which catalyze the conversion of phosphatidate (PA) to diacylglycerol, an important step in glycerolipid biosynthesis (Donkor et al., 2009). In addition to being expressed in tissues such as the liver, lipin-2 is also expressed in sites of lymphopoiesis such as bone marrow, spleen, and lymph nodes (Donkor et al., 2009). This suggests that lipin-2 activity in these tissues might contribute to the inflammation observed in patients with Majeed syndrome. To further support this possibility, an in vitro study demonstrated a protective role for lipin-2 in pro-inflammatory signaling induced by saturated fatty acids that occurs concurrent with an enhanced triacylglycerol synthesis (Valdearcos et al., 2012).

The Reue laboratory was recently presented with the case of a 13-year-old female of Indian background who had been clinically diagnosed with FMF. At 11 years of age, the patients began to experience six to seven fevers per year, possibly triggered by stress. In addition to this symptom, she also experienced nephritis and was found to be unlikely to have lupus. A genetic diagnostic panel of 7 genes for periodic fever syndromes identified that she has a Glutamic acid (Glu, E) to Glutamine (Gln, Q) change in residue 148 (E148Q) within the MEFV gene and the Serine (Ser, S) to Phenylalanine (Phe, F) change at residue 203 (S203F) of the LPIN2 gene. In addition, clinical diagnostic sequencing identified three other unrelated subjects of Indian heritage with periodic fever syndrome who have a mutation in a single allele of MEFV in
combination with S203F mutation in a single allele of LPIN2. This sparked my interest to further assess the distribution of LPIN2 mutations in FMF patients who are heterozygous for MEFV mutant alleles.

We obtained clinical DNA samples from a set of clinically diagnosed FMF patients from Israel for which a heterozygous MEFV mutant allele was confirmed. My hypothesis was that sequencing the entire coding region of the LPIN2 gene in clinically diagnosed MEFV heterozygous patients would lead to the identification of LPIN2 mutant alleles.

These findings could provide additional evidence for the role of LPIN2 in inflammation and would help further understand FMF etiology for these MEFV heterozygous patients. Identifying LPIN2 mutations in FMF patients that are heterozygous for MEFV mutations could also inform in a near future about the incidence of digenic inheritance in FMF. Ultimately, this knowledge could lead to new avenues for the clinical management of this disease.
Methods

Patients

Twenty patient DNA samples were obtained from Dr. Mordechai Shohat and Lior Cohen at the Department of Medical Genetics Rabin Medical Center, Beilinson Campus in Petah-Tikva, Israel. No patient identifying information was provided with the samples, which were provided as part of the clinical diagnosis. All patient samples had been clinically diagnosed with FMF and are heterozygous for the pyrin allele M694V. DNA samples were stored at -80°C until used.

Primer Development

Sequencing primers were developed using Primer3 frodo: http://bioinfo.ut.ee/primer3-0.4.0/. Considerations for primer design were as follows: the primer sequence didn’t overlap with regions where common SNPs are found, a GC content of <50%, no predicted hairpin formation, and melting temperature (Tm) between 57 and 62°C, ideally there should be at least one C or G at the beginning and end of the primer, primer length approximately 18bp, and PCR amplification size should be of 600bp. OligoCalc software (http://www.basic.northwestern.edu/biotools/OligoCalc.html) was used to check for self-complementarity within primer sequences. In addition, a Blat search of each primer sequence was done on UCSC Genome Browser and in silico PCR was performed to ensure the primer was specific to the exon of interest. The specificity of each primer was tested prior to use with experimental samples. The information for each primer used in this pilot experiment is summarized in the table on the next page.
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**PCR amplification**

The 20 exons in *LPIN2* were amplified by polymerase chain reaction (PCR). HT29 human cell line DNA was used a positive control in each PCR reaction and DNase free water was used as a negative control. The PCR reagents used for the PCR reaction were from Bioline (Cat.No.BIO-21042): Biolase DNA polymerase, 10x NH₄ Reaction Buffer, and 50mM MgCl₂ Solution. The PCR reaction conditions for 20µl reaction were: 2µl of 10X NH₄ Reaction Buffer, 1.2µl 50mM MgCl₂ Solution, 1.6µl 100mM dNTP Mix, 1µl Forward Primer, 1µl Reverse Primer, Biolase enzyme 0.3µl, and 11.9µl of RNAse free H₂O. The PCR touchdown program used for these reactions was: 1) 94°C for 3:00 minutes, 2) 92°C for 0:40 seconds, 3) 63°C for 0:40 seconds, 4) -0.5°C per cycle, 5) 72°C for 1:00 minute, 6) go to step 2 for 19 times, 7) 92°C for 0:40 seconds, 8) 53°C for 0:40 seconds, 9) 72°C for 1:00 minute, 10) +5 seconds per cycle, 11) go to 6, 20 times, 12) 72°C for 5:00 minutes, 13) 4°C for ever. Gel electrophoresis was used to visualize the PCR products. A representative example of PCR products for *LPIN2* exons visualized by gel electrophoresis is shown in Figure 3-1.

**Sequencing**

Prior to sequencing, PCR products for each exon were treated with Exonuclease I and Shrimp Alkaline Phosphatase (SAP) at 37°C for 15 minutes and then 80°C for 15 minutes. The sequencing reagents per reaction were: 5µl of purified PCR product, 1µl of 5µM Forward or Reverse primer, 2µl of Big Dye Terminator Mix (Cat.No. 4337455), and 2µl of Seq Saver. The sequencing reaction conditions were: 1) 96°C 0:10 seconds, 2) 50°C for 0:15 seconds, 3) 60°C for 4:00 minutes, go to step 1 twenty four times and then 4°C forever. The samples were then analyzed at the UCLA GenoSeq Core using the
Applied Biosystems 3730 Capillary DNA Analyzers:

(http://www.genoseq.ucla.edu/action/view/Sequencing).

**Sequencing analysis**

The sequence of each exon was aligned to *LPIN2* sequence obtained from the UCSC genome browser with A plasmid Editor (ApE) Software (http://biologylabs.utah.edu/jorgensen/wayne/ap/). Putative sequence variants were confirmed by sequencing both DNA strands.
Results

The sequencing of LPIN2 exons was performed in 20 FMF patients from Israel that carry the M694V missense mutation in a single MEFV allele. LPIN2 exon sequencing in twenty Israeli FMF patients identified four variants. These include two missense amino acid substitutions, Cys874Phe (found in two patients) and the Glu601Lys (found in one patient). Additional variants were identified in the 3’ untranslated region (3’UTR) of the LPIN2 gene (found in 7 out of 20 patients) and a synonymous variant Thr180Thr (found in one patient). All the variants identified in this study were present in dbSNP (Table 3-2). Representative sequence traces for the four variants identified can be found on Figure 3-2.

Since there was not access to a control group, I assessed the 1,000 genomes data to observe the frequency of the missense alleles identified in this study in the general population. The first variant involved the cysteine (C) to phenylalanine (F) substitution in residue 874 (rs201160155) (Table 3-2). According to the SIFT and PolyPhen algorithms found in Ensembl, 0.58 and 0.009 respectively, this variant is not predicted to be disease causing. However, the genotype frequency of this variant in Europeans was 0.002 for the A/C change and 0.998 for the C/C genotype. The frequency of allele A was 0.001 while the frequency of C was 0.999. The average heterozygosity found in Europeans was 0.002 with a standard error of +/- 0.032. This low frequency for the A allele suggests that this change may have an effect in gene function. However, not enough information about allele frequencies in other populations besides Europeans was found in 1,000 genomes.

On the other hand, the glutamine to lysine change in the 601 residue of LPIN2 had a genotype frequency for C/C of 0.984 and 0.016 for C/T. The frequency for the C allele is 0.992 (major allele) and for the T allele it is 0.008 (minor allele). This frequency was similar across
different ethnic groups in 1,000 genomes project. The fact that this missense allele occurs at a low frequency in the general population raises the possibility that it has an effect in gene function. These results suggest that additional functional studies would be necessary to conclude whether these two missense variants have an effect in \textit{LPIN2} gene function.
Discussion

The *LPIN2* exon sequencing experiment sought to determine if *LPIN2* mutations are present in individuals of Mediterranean descent with FMF and a mutation in a single *MEFV* mutant allele. Identifying *LPIN2* variants in these patients could provide support for the hypothesis that some cases of FMF could be caused by the synergistic effects of *LPIN2* and *MEFV* mutations. Two *LPIN2* missense variants were identified among the FMF patients (Cys874Phe and Glu601Lys). Both result in non-conservative amino acid substitutions. The SYFT and PolyPhen algorithms do not predict these to be pathogenic alterations, but it is well known that these predictions are often unreliable. The true effect of these amino acid substitutions would need to be verified experimentally by observing their effects, for example, in lipin-2 PAP enzyme activity and subcellular localization.

One important aspect of the patient cohort is that all individuals carried the M694V *MEFV* mutant allele. The M694V variant has been associated with more severe disease phenotype and higher risk of amyloidosis in homozygous patients (Dewalle, 1998; Gershoni-Baruch et al., 2002). It is therefore possible that in these patients, the M694V mutant allele in *MEFV* is adequate to elicit the FMF phenotype. The finding that *MEFV* heterozygous patients have a similar clinical phenotype to homozygous patients could be explained by the involvement of additional genes or environmental factors that potentiate the effects of the *MEFV* mutant.

Indeed, there is some evidence that modifier genes influence FMF etiology in *MEFV* heterozygous patients. The presence of modifier genes such as specific alleles of the MHC class I Chain-Related Protein A (MICA) and serum amyloid A (SAA) have been associated with FMF phenotype severity and risk of amyloidosis (Cazeneuve et al., 2000 and Touitou et al., 2001). It
is also important to consider that a larger sample of patients will be needed to determine the prevalence of \textit{LPIN2} variants in Mediterranean FMF patients.

It is likely that the patients’ ethnicity is important for the detection of \textit{LPIN2} variants. This is supported by the fact that the FMF patients identified to carry \textit{LPIN2} variants in the clinical sequencing samples were all of Indian descent. In addition, these patients carried the E148Q \textit{MEFV} variant instead of the M694V variant. Ideally, we would like to assess prevalence of \textit{LPIN2} mutations in FMF patients of Indian decent that are heterozygous for \textit{MEFV} mutations. Unfortunately, at the time of this exploratory sequencing study, no such samples were available.

To further confirm if the \textit{LPIN2} variants found in FMF heterozygous patients are likely influencing the disease, a healthy control group from the same ethnicity should be sequenced as well. This control group could help distinguish common \textit{LPIN2} variants from pathogenic variants. Identifying additional FMF patients carrying \textit{LPIN2} variants will provide additional evidence for the role of \textit{LPIN2} in inflammation, and further evidence of a digenic mechanism in FMF. Collectively, this information will be useful for the management and diagnosis of patients suffering from FMF.
Table 3-1. Summary of the four LPIN2 variants identified by exon sequencing.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Wild-Type</th>
<th>Amino Acid Change</th>
<th>Exon</th>
<th>Predicted to be damaging</th>
<th>Coding</th>
<th>Found on dbSNP</th>
<th>Occurrence</th>
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<tbody>
<tr>
<td>Cys874Phe</td>
<td>TGC (Cys)</td>
<td>TTC (Phe)</td>
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<td>ACC (Thr)</td>
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<td>Glu601Lys</td>
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<td>AAG (Lys)</td>
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hLipin2 Exons 1-10 Primer Test

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</table>

All ten Lipin2 primer sets seem to amplify the product of interest effectively.

Figure 3-1. Representative PCR amplification of LPIN2 exons.
Figure 3-2. Representative sequence traces for the four variants identified.

(A) Sequence trace for the adenine (A) to cysteine (C) change in residue 874 that leads to cysteine to a phenylalanine change.

(B) Sequence trace for the variant found in the 3’ untranslated region.

(C) Sequence traces for the adenine (A) to cytosine (C) change that leads to a synonymous variant in residue 180.

(D) Sequence traces for the guanine (G) to adenine (A) substitution in residue 601 that leads to a glutaminic acid to lysine change.
References


Kone-Paut I, Hentgen V, Guillaume-Czitrom S, Compeyrot-Lacassagne S, Tran TA, and Touitou


Valdearcos M, Esquinas E, Meana C, Peña L, Gil-de-Gomez L, Balsinde J, and Balboa MA. (2012) Lipin-2 reduces proinflammatory signaling induced by saturated fatty acids in