ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

ANALYSIS OF MEFV GENE ALTERNATIVELY SPLICED TRANSCRIPTS EXPRESSION PATTERNS IN CELL CULTURE MODELS

M.Sc. THESIS

İrem ABACI

Department of Advanced Technologies

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Thesis Advisor: Assoc. Prof. Dr. Eda TAHİR TURANLI

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HÜCRE KÜLTÜRÜ MODELLERİNDE MEFV GENİ ALTERNATİF KIRPILMIŞ TRANSKRİPTLERİNİN EKSPRESYON MODELLERİNİN ANALİZİ

YÜKSEK LİSANS TEZİ

İrem ABACI
(521101107)

İleri Teknolojiler Anabilim Dalı

Moleküler Biyoloji Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Doç. Dr. Eda TAHİR TURANLI

EYLÜL 2013

İrem Abacı, a **M.Sc.** student of ITU **Graduate school of Science Engineering and Technology** 521101107, successfully defended the **thesis** entitled "**ANALYSIS OF MEFV GENE ALTERNATIVELY SPLICED TRANSCRIPTS EXPRESSION PATTERNS IN CELL CULTURE MODELS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Assoc. Prof. Dr. Eda TAHİR TURANLI**
İstanbul Technical University

Jury Members : **Prof. Dr. Arzu KARABAY KORKMAZ**
İstanbul Technical University

Prof. Dr. Aslı TOLUN
Boğaziçi University

Date of Submission : 3 May 2013
Date of Defense : 13 September 2013

To my family,

FOREWORD

I would like to appreciate deeply my supervisor Assoc. Prof. Dr. Eda TAHİR TURANLI for her inspiring support and guidence. It was a great experience to me to complete this study under her supervision. I want to thank her for encouraging me to establish and complete this thesis.

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And also, I want to thank to my dear family for their patience, undeterred support and endless love.

2013, Fall **İrem ABACI**

(Molecular Biologist)

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ABBREVIATIONS

µL	: Microliter
ASC	: Apoptosis-associated speck-like protein
cDNA	: Complementary DNA
dH2O	: Distilated water
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide
FMF	: Familial Mediterranean Fever
g	: Gram
IL-1β	: Interlukin-1 Beta
IL-18	: Interleukin- 18
IL-33	: Interleukin-33
LPS	: Lipopolysaccharide
mM	: Milimolar
M	: Molar
MEFV	: Mediterranean Fever gene
MEFV-fl-GFP	: MEFV full length green flourescent protein
MEFV-2Δ-GFP	: MEFV exon 2 deleted isoform green fluorescent protein
mRNA	: Messenger Ribonucleic Acid
NF-κB	: Nuclear Factor Kappa B
NLRP	: NOD- Like Receptor Protein
NLS	: Nuclear Localization Signal
NS	: Non Significant
PBS	: Phosphate Buffered Saline
PMA	: Phorbol 12-myristate 13-acetate
PCR	: Polymerase Chain Reaction
PSTPIP1	: Proline-Serine-Threonine Phosphatase-Interacting Protein 1
P/M	: Pyrin / Marenostrin
QRT-PCR	: Quantitative Real Time Polymerase Chain Reaction
RNA	: Ribonucleic Acid
RT-PCR	: Real Time-Polymerase Chain Reaction
TNF-α	: Tumor Necrosis Factor - alpha
UV	: Ultra-violet

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ANALYSIS OF MEFV GENE ALTERNATIVELY SPLICED TRANSCRIPTS EXPRESSION PATTERNS IN CELL CULTURE MODELS

SUMMARY

Familial Mediterranean Fever (FMF) is an autoinflammatory disease of our region with a frequency of 1/200 to 1/1073. Main symptoms include periodicly recurrent fever attacks with painful inflammation in peritoneum, pleura, synovium and pericardium. These FMF attacks may result as amyloid formation and aggregation, if not treated, aggregation may lead to renal failure. The gene of FMF is MEFV, in which to date more than 200 variations were identified in patients. Although hereditary mode of inheritance of FMF was accepted as autosomal recessive, dominant or compound heterozygous cases as well as patients without any MEFV pathological mutations were identified. Furthermore there is evidence of MEFV involved in other inflammatory immune diseases such as Behcet syndrome, Crohn's Disease and Gut.

MEFV gene is an inflammatory/regulatory gene, codes for Pyrin/Marenostrin (P/M) protein. P/M is involved in inflammatory pathway through interacting with pro-inflammatory elements, forms a part of the "inflammasome" complex. Inflammasome assembled in the cytoplasm causes the secretion of proinflammatory cytokines and leads to inflammatory response. MEFV gene is transcripted in several alternatively spliced forms. The most common MEFV protein isoforms found in peripheral blood leukocytes and synovial fibroblasts are MEFV-8ext (additional extension of exon 8), MEFV-4a (lacking exon 5, 6, 7, 8, 9 and 10, with the addition of part of exon 4), MEFV-2a (with part of indicated exon) and MEFV-2Δ (exon 2 deleted transcript). Especially exon 2 is thought to be important in localization estimation. It was shown that, deletion of exon 2, leads to localization errors even if the localization signals exist in the environment . Previous study of our group indicated that MEFV-2Δ is expressed higher in FMF cases rather than healthy controls ($p= 0.026$) in total blood samples.These findings leaded to the assumption that there can be a connection between inflammatory pathway and MEFV-2Δ transcript levels.

Our main hypothesis is to test the exact role of MEFV in inflammatory pathways. To understand the relationship, first we designed an inflammation model with primary human peripheral blood leukocyte (PBL) cell culture. We used lipopolysaccharide (LPS) induction to model inflammation and measured inflammatory cytokine levels. Our initial findings indicated a low cytokine levels in primary cell culture model. Therefore we constructed another inflammation model with HL-60 premyelocytic cell line cells. LPS induction of Phorbol 12-myristate 13-acetate (PMA) treated HL-60 cells produced excess amount of proinflammatory cytokine interleukin 1-beta (IL-1β).

We further compared MEFV-2Δ expression with oher alternatively spliced transcripts in synovial fluid and blood samples of FMF patients in their attack period. To do this, we isolated RNA molecules from the samples and applied Reverse Transcription Polymerase Chain Reaction (RT-PCR) in combination with Quantitatvie Real Time PCR (QRT-PCR). Due to MEFV-2Δ's being a rare product in all other RNA molecules, two different cDNA synthesis strategies were applied and compared to detect the accurate relative amounts of MEFV-2Δ and other MEFV transcripts. These strategies were cDNA synthesis with random priming and cDNA synthesis with target specific priming of MEFV aternatively spliced transcripts. We checked inflammation status with pro-IL-1β and TNF-α mRNA levels in each set up and we compared MEFV alternatively spliced transcript levels in stimulated versus untreated cells, additionally in synovial fluid and blood samples of FMF cases. We constructed inflammation model succesfully with LPS induction after phorbol 12-myristate 13-acetate (PMA) differentiation. Furthermore, target specific cDNA synthesis resulted more accurately than random primed cDNA synthesis. Ct values of randomly primed MEFV alternatively spliced form cDNAs were around 40, while target specifically primed cDNA Ct values were in interval of 20-25 especially in inflammation cases.

In conclusion, our findings indicated that MEFV-2Δ is highly expressed in inflammation conditions (in inflammation model $p=0.024$; in synovial fluid samples $p=0.037$), and this transcript may have a putative function in inflammatory pathway and in FMF pathology.This result was confirmed with acute FMF synovial fluid and blood samples and the hypothesis of MEFV-2Δ transcript's being important for inflammatory pathway was supported. Further studies are required in higher number of patients during attack and remission periods, as well proteins interacting with MEFV-2Δ isoform and fulllenght pyrin need to be identified.

HÜCRE KÜLTÜRÜ MODELLERİNDE MEFV GENİ ALTERNATİF KIRPILMIŞ TRANSKRİPTLERİNİN EKSPRESYON MODELLERİNİN ANALİZİ

ÖZET

Ailevi Akdeniz Ateşi (AAA), bölgemizdeki görülme sıklığı 1/200 ila 1/1073 arasında olan otoenflamatuvar bir hastalıktır. Temel semptomları, periyodik olarak tekrarlanan ağrılı karın zarı, akciğer zarı, eklem sıvısı ve kalp dış zarı enflamasyonlarıdır. Bu ataklar sonucu "amiloid" denilen protein çökeltilerinin oluşma olasılığı yüksektir, tedavi edilmezse amiloid birikimi böbrek yetmezliğine bağlı olarak ölüme sebebiyet verebilir. FMF hastalığıyla ilintili olan gen MEFV olup FMF hastalarında bu genin 200'den fazla varyasyonu tanımlanmıştır. FMF hastalığının kalıtım modeli otozomal resesif olarak kabul edilmesine rağmen bazı durumlar baskın geçişli ya da bileşik heterozigot geçişli olarak raporlanmış olup, bazılarında hiç mutasyon gözlenmemiştir. MEFV geni enflamatuvar/regülatör gen olup "Pyrin/Marenostrin (P/M)" adı verilen bir proteini kodlar. P/M, pro-enflamatuvar elementlerle etkileşim halinde enflamazom kompleksinin bir parçasını oluşturarak enflamasyon yolizine dahil olur. Bununla birlikte MEFV geninin Behcet Sendromu, Crohn's hastalığı ve gut gibi diğer otoenflamatuvar hastalıklarla da ilgili olduğu kanıtlanmıştır.

Alternatif kırpılma, transkripsiyon aşamasında gen ekzonlarının farklı sıralarda birleştirilmesi sonucu aynı genden türetilmiş çeşitli protein izoformlarının oluşmasını sağlayan bir süreçtir. "Anlamsızlık aracılıklı yıkım (AAY)" adı verilen bir mekanizma ile birlikte alternatif kırpılma, gen ekspresyonu ve protein üretiminin düzenlenmesi ve çeşitli protein yapılarının evrilmesi süreçlerinin bir parçasıdır. Aynı genden üretilen çeşitli protein izoformlarından, hücre gelişim seviyesine göre işlevsel olmayan protein AAY aracılığıyla parçalanır. Hücrede bu mekanizmalar, ihtiyaç duyulan proteinin üretilmesinde düzenleyici olarak işlev görür. Kompleks hastalık patofizyolojilerinde önemli bir rol oynadığı düşünülen bu süreçler, aynı zamanda gelişimsel farklılaşma yolizlerinde de oldukça etkin bir rol üstlenir. Alternatif kırpılmanın kanser, Parkinson, Alzheimer ve Kistik fibrozis gibi hastalık patolojilerinde görülen moleküler etkileşimlerde önemli rolü bulunduğu bilinmektedir.

MEFV geni transkriptleri çeşitli alternatif kırpılmış formlarda üretilmektedir. Özellikle lökositlerde ve eklem sıvısında en yaygın olarak gözlenen MEFV alternatif kırpılmış formları şunlardır: MEFV-8ext (8. ekzonun ek bir kısmını içeren izoform), MEFV-4a (5, 6, 7, 8, 9 ve 10. ekzonları içermeyen, 4. ekzonun bir kısmını içeren form), MEFV-2a (2. ekzonun bir parçasını içeren form) ve MEFV-2Δ (2. ekzonu içermeyen transkript). MEFV geninin 2. ekzonunun proteinin lokalizasyon tayininde önemli rol oynadığı düşünülmektedir. 2. ekzonun delesyonunun, ortamda nükleer lokalizasyon sinyalleri bulunmasına rağmen molekülün hatalı lokalizasyonuna yol açtığı bilinmektedir. Grubumuzca yapılan bir çalışmada da MEFV-2Δ izoformunun FMF hastalarında sağlıklı kontrollere göre daha yüksek oranda bulunduğu gösterilmiştir (p=0,026). Bu buluntular, enflamasyon yolizi ile MEFV-2Δ izoformunun ekspresyon seviyesi arasında bir bağlantı olasılığını işaret etmektedir.

Gen ekspresyon seviyesi belirleme ve karşılaştırma çalışmalarında sıklıkla kullanılan yöntemlerden biri ters transkripsiyon - relatif kantitasyondur. Bu tip çalışmalarda, öncelikle örneklerden RNA izolasyonu yapılır ve RNA'dan, ters transkripsiyon polimeraz zincir reaksiyonu ile komplementer DNA sentezlenir. Komplementer DNA sentezi için rastlantısal primerler, "oligo-dT" primerleri veya hedefe özgü dizayn edilmiş primerler kullanılabilir. Bu üç yöntemden rastlantısal primerler ve oligo-dT primerleri, izole edilmiş RNA havuzu içinden herhangi bir RNA'ya bağlanarak hedef gözetmeksizin reaksiyon sonunda karışık bir komplementer DNA içeriği oluşmasını sağlarlar. Hedefe özgü primerler ise belirli bir RNA molekülüne bağlanarak özellikle bu RNA'ya ait bir komplementer DNA sentezini sağlarlar. Rastlantısal ve oligo-dT primerleri, tek reaksiyon ile birden fazla çeşitte komplementer DNA sentezine olanak verirken hedefe özgü primerler, bir reaksyonda tek tip komplementer DNA sentezini sağlar. Hedefe özgü primerler ile nadir olarak üretilen bir transkriptin tespiti sağlanabilir. Literatürde yer alan çalışmalarda, gen havuzu içinde nadir olarak üretilen transkriptlerin tespitinde hedefe özgü primerlerin daha doğru sonuç verdiği belirtilmiştir.

Çalışmamızın temel hipotezi enflamatuvar yolizlerinde MEFV'in kesin rolünün belirlenmesidir. Enflamasyonda MEFV geninin rolünü anlamak için öncelikle insan primer lökosit kültüründe bir enflamasyon modeli tasarladık. Lökositleri aktive etmek için lipopolisakkarit (LPS) kullandık ve sonuç enflamatuvar sitokin seviyesini ölçtük. İlk buluntularımız primer kültür enflamasyon modelinde düşük sitokin seviyesini göstermekteydi. Bunun üzerine HL-60 premiyelotik hücre hattı hücrelerini kullanarak başka bir enflamasyon modeli hazırladık. Sonuç olarak Forbol 12-miristat 13-asetat (PMA)

uygulanmış HL-60 hücrelerinin LPS ile uyarımı, proenflamatuvar bir sitokin olan İnterlökin 1-beta (IL-1β)'nın yüksek oranda üretimine yol açtı.

Daha sonra, MEFV-2Δ üretimini atak dönemindeki AAA hastalarının eklem sıvısı ve kan örneklerindeki diğer transkriptlerle karşılaştırdık. Bunun için örneklerden RNA izolasyonunu gerçekleştirdikten sonra Reverse Transkripsiyon Polimeraz Zincir Reaksiyonu (RT-PCR) yöntemini Kantitatif Gerçek Zamanlı Polimeraz Zincir Reaksiyonu (Q-RT PCR) yöntemi ile birlikte kullandık. MEFV-2Δ transkriptinin diğer RNA'lar içinde nadir üretilen bir ürün olması sebebiyle, ekspresyon profilinin doğru karşılaştırılabilmesi için iki farklı cDNA sentez stratejisi uyguladık. Bu stratejiler MEFV alternatif kırpılmış transkriptlerinden rastlantısal primerler ile komplementer DNA(cDNA) sentezi ve hedefe özgü primerler ile cDNA sentezi idi. Enflamasyon durumunu deneyin her bir basamağındaki enflamatuvar sitokinler pro-IL-1β ve Tumor Necrosis Factor-Alpha (TNF-α) üretimi seviyesiyle ve sağlıklı kontrollerle karşılaştırdık. Enflamasyon modelini PMA ve LPS uygulanmış hücrelerde başarı ile gerçekleştirdik. Ayrıca hedefe özgü primerler ile sentezlenen cDNA'lar ile rastlantısal primerlerle sentezlenmiş cDNA'lara göre daha doğru sonuçlar alınmıştır. Buna göre hedefe özgü primerlerle sentezlenmiş cDNAnın taslak DNA olarak kullanıldığı durumda, gerçek zamanlı PCR'da referansa göre belirlenen eşik değer floresan sinyalinin aşıldığı döngü sayısı (Ct) ortalama 25 iken rastlantısal primerle sentezlenmiş cDNA'nın taslak olarak kullanıldığı reaksiyonlarda bu değerler ortalama 40 olarak bulunmuştur.

Sonuç olarak, enflamasyon durumunda diğer transkriptlerin ekspresyon seviyesinin düşmesine rağmen MEFV-2Δ transkriptinin ekspresyonunun arttığı gözlenmiştir (enflamasyon modelinde $p=0.024$, sinoviyal sıvı örneklerinde $p=0.037$). Bu artış, özellikle hedefe özgü primerlerle yapılan sentezlenmiş komplementer DNA karşılaştırmalarında belirgin olarak gösterilmiştir. Enflamasyon ile paralel olarak gözlenen MEFV-2Δ ekspresyon artışı akut FMF durumundaki hastaların sinoviyal sıvı ve kan örneklerinden alınan sonuçlarla doğrulanmış olup, MEFV-2Δ transkriptinin enflamatuvar yolizinde önemli bir rolü olabileceği hipotezi desteklenmiştir. Daha yüksek sayıda hasta örneklemesi ile MEFV-2Δ izoformu ve pyrin ile etkileşen proteinlerin tanımlaması çalışmanın daha sonraki basamakları için gereklidir.

1. INTRODUCTION

1.1 Mediterranean Fever Gene (MEFV) and Familial Mediterranean Fever (FMF)

MEFV (Mediterranean FeVer) gene was identified in 1997 as the gene related with FMF. It is located in the short arm of 16th chromosome (Figure 1.1). Its position was defined by positional cloning (position of 13.3, between base pairs of 3,232,028 - 3,246,627) [1]. MEFV gene is known as an inflammatory regulatory gene. MEFV contains 10 exons and encodes "Pyrin/Marenostrin (P/M)". There are more than 200 variants of MEFV gene to date, which are related to FMF. Some of the mutations are deletion mutations, but the most effective ones are missense or frameshift mutations [2]. In FMF cases; M694V, V726A, M694I, M680I in exon 2 and E148Q in exon 10 are most frequent mutations. MEFV mutations may lead to production of malformed or reduced levels of P/M. Loss of function of P/M protein results as inappropriate or prolonged inflammations [3].

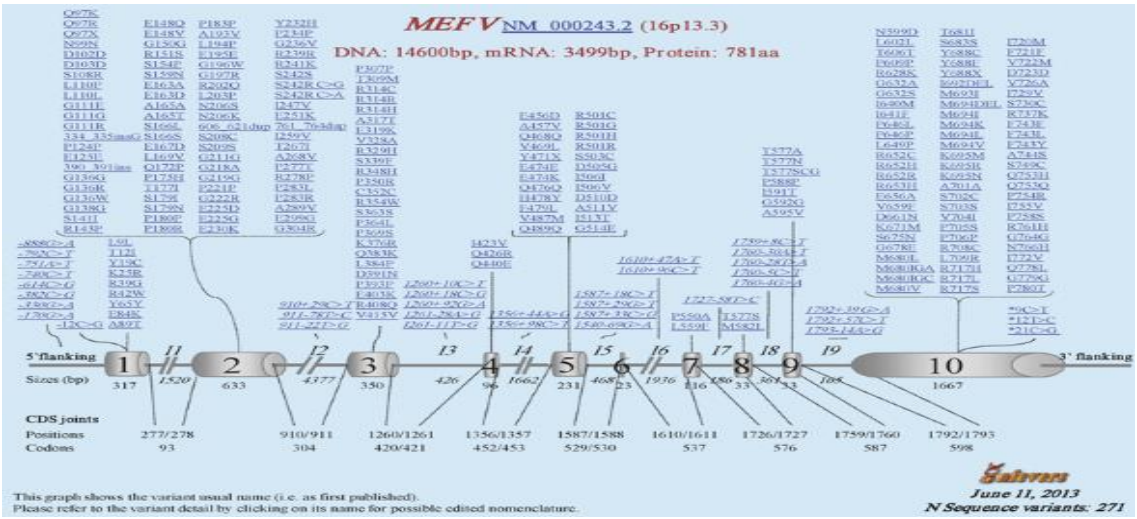

Figure 1.1 : MEFV gene sequence variants determined until 06.11.2013, retrieved from ***Infevers:*** *an online database for autoinf. mutations (http://fmf.igh.cnrs.fr/ISSAID/infevers/*) [4].

Familial Mediterranean Fever (FMF) is a hereditary autoinflamatory condition which is characterized with periodicly recurrent fever attacks with painful inflammation in peritoneum, pleura, synovium and pericardium. Mediterranean populations (Turk, Arab, Armenian, and

Sephardic Jewish) are especially affected with the disorder. FMF attacks may result as amyloid formation and aggregation, if disease is not treated, it may lead to renal failure. Only effective treatment currently is colchicine against amyloid formation; however, it has serious side effects. Although hereditary penetrance pattern of FMF was accepted as autosomal recessive, some cases are reported as dominant or compound heterozygous and there are patients without MEFV have no mutations. The presence of FMF patients without major MEFV mutations indicate an involvement of other genetic or epigenetic mechanisms in FMF etiology [5].

1.1.1 MEFV Gene Expression and Alternatively Spliced Transcripts

Alternative Splicing is the process of reconnection of exons in different ways. At the end of the splicing, several different products form from the single gene. In human, over 80% genes are alternatively spliced as a part of natural transcription process [6].

MEFV is primarily expressed in white blood cells (especially in monocytes, neutrophils and eosinophils), synovial fibroblasts and dendritic cells [7]. MEFV expression is increased by pro-inflammatory agents such as Interferon γ (IFN-γ), Tumour necrosis factor α (TNF α), lipopolysaccharide (LPS) and Interleukin 1-beta (IL-1β). MEFV expression leads to increase in leukocyte level through inflammatory regulation [8].

MEFV has several alternatively spliced forms**.** The most common transcripts found in peripheral blood leukocytes and synovial fibroblasts are (Figure 1.2) exon 8ext (an extension of exon 8), exon 4a (lacking exon 5,6,7,8, 9 and 10, with the addition of part of exon 4), exon 2a (with part of indicated exon) and exon 2Δ (exon 2 deleted transcript) [2]. MEFV-2Δ isoform is expressed in leukocytes and translated as a 570 residues length protein. This product is located primarily in nucleus while the full-length form of (P/M) is located in cytoplasm. Exon 2 is thought contain a localization regulator since the deletion of this region leads to localization errors even in the presence of Nuclear Localization Signals (NLS) [9]. However, the nature and the mechanism of this regulation is still unknown.

Figure 1.2: Alternatively spliced transcripts of MEFV gene in PBL and Synovial Fibroblasts [adapted from 2].

1.1.2 MEFV product: Pyrin (P/M) and protein isoforms

MEFV expression patterns are important in the pathophysiology of FMF. As it is stated in the study of Grandemange et.al, beside the regular full-length P/M protein, there are several protein isoforms of P/M especially in leukocytes, translated from alternatively spliced transcripts of the MEFV gene. These forms are thought to be localized in different sub-cellular compartments according to their functions in inflammatory pathway [10]. The study indicated that MEFV expression is regulated post-transcriptionally via the mechanism of Nonsense Mediated Decay (NMD). According to the study, MEFV-fl, MEFV-2Δ and MEFV-2a (Figure 1.2) is not subject of NMD and they form as protein isoforms. However especially transcripts that containing -4a are degraded via NMD. Transcripts that containing -8ext are regulated in a cell specific way; they are degraded in monocytes but not in neutrophils [10].

Complete P/M molecule has five functional domains (Figure 1.3). N-terminal (P/M) domain captivating 1-95 residues (alternative names are: DAPIN, PYD, PAAD), bZIP transcription factor basic domain (residues 266-280), B-Box zinc finger domain (375-407), α-helical (coiled coil) domain (408-594) and C terminal B30.2 domain (also known as PRYSPRY) (598-774) [11].

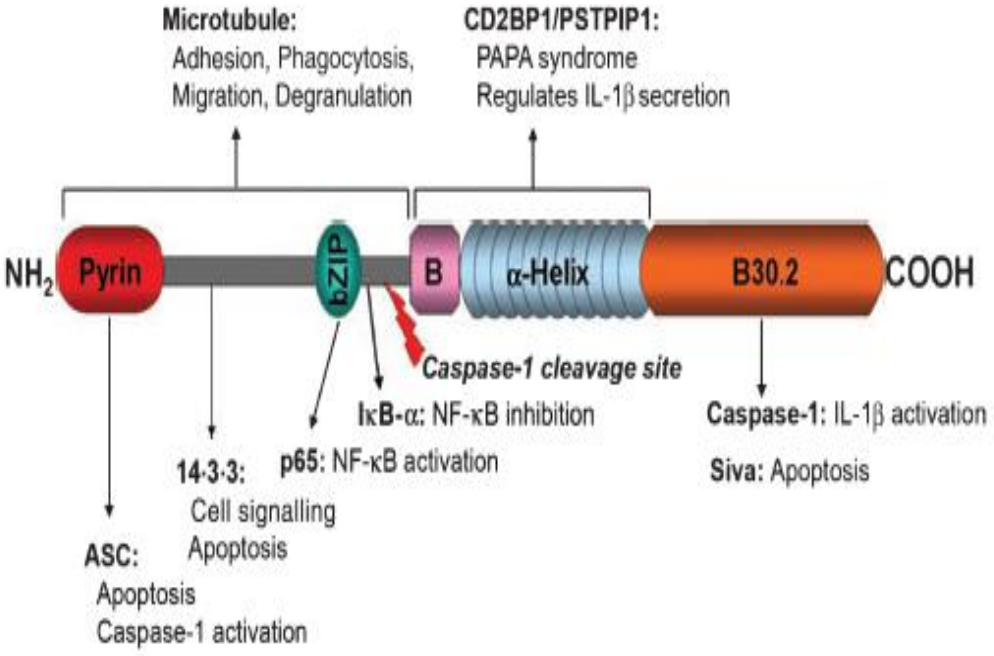

Figure 1.3 : P/M molecule and domain interactions [8].

The most important mutations are generally affecting B30.2 domain, where (P/M) interacts with caspase-1 [8]. These mutations found to be related with other inflammatory diseases such as Behcet's disease, Rhematouid arthiritis and inflammatory bowel disease (IBD) [12].

According to the study of Ustek *et.al.* low mRNA levels of MEFV is related to FMF [11]. Notarnicola *et.al.* also showed the relationship between mRNA level of MEFV gene and MEFV mutations. According to the results, M694V mutations seen in FMF patients is associated to low MEFV expression levels [4]. However in the study of Booty *et.al.* MEFV expression was slightly higher in FMF patients than healthy people [15].

1.2 Pyrin (P/M) and Inflammation

(P/M) associates Apoptosis-associated Speck-like Protein (ASC), adaptor molecule of inflammasome (Figure 1.4). It has PYD domain at its N-terminal, that interacts with (P/M) and CARD domain at C-terminal which associates with caspase-1. Activated caspase-1 induce the production of cytokines IL-1β, IL-18, IL-33, which results in interaction with ASC. The other activities of (P/M) is thought to be maturation of IL-1β and activation of NF-κB to regulate caspase-1 [11]. According to the study of Chae *et.al*, the wild type (P/M) domain of B30.2 interacts the inflammasome subunits of p20 and p10, which prevent these two

molecules to form a heterodimeric assembly after interaction. However, in FMF condition mutant (P/M) cannot interact with these molecules that would recruit assembling of them and this p20/p10 heterodimer induces IL-1β activation that may result as inflammation. This active heterodimer than cleaves (P/M) at Asp330 that is located between B-box zinc finger domain and bZIP basic domain. After cleavage N-terminal, cleaved part of the (P/M) interacts with p65 and other inflammation inducing factors while second part is interacted NF-κB in terms of inducing expression of inflammatory genes [8].

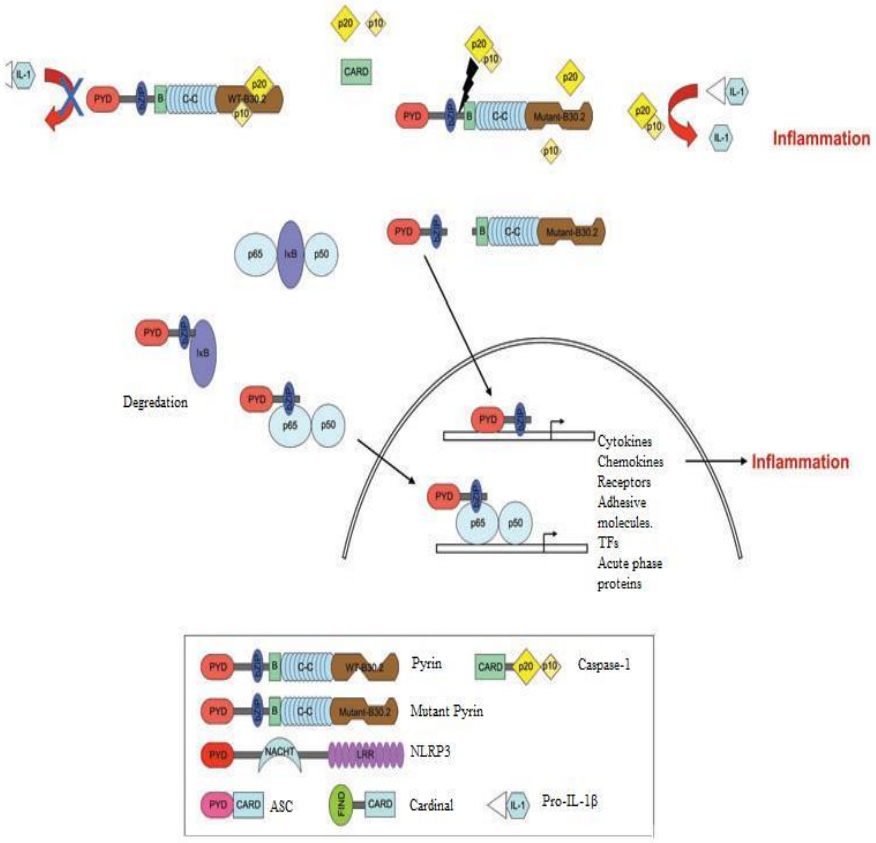

Figure 1.4 : P/M and Inflammation [5].

It is shown that expression of (P/M) or its corresponding disease-associated mutants could induce ASC oligomerization and activation of caspase-1, but not NF-κB. On the other hand (P/M)-ASC interaction for caspase-1 activation is still unclear since MEFV knock-out mice has not been done to our knowledge [13].

FMF pathogenesis was thought to be based on mutation, but new studies revealed that it is not completely mutation oriented. On the other hand deficiency of relevant studies and samples in the studies, it is hard to come to an accurate conclusion.

1.3 Establishment Of Long Term Cell Culture and Inflammation Modelling

Cell culture is the process of controlled growth of the certain type of cells in appropriate environmental conditions. To understand the features and processes of *in vivo systems, in vitro* model with cell culture systems are used. Cell culture can be established from primary cells (the cells that directly isolated and cultured from any organism) or leukemic (infinitely proliferating) cell lines [16].

Cultured cells need sterile environment with a stable pH and nutrients to grow and proliferate. Balanced salt solutions are minimal required media, defined for primary cell cultures and these solutions are commercially avaliable. These media is supplied with lipids, vitamins, amino acids and fatty acids to sustain the culture. Each cell type has special needs of medium contents and optimization is needed to establish long-term culture of desired cell type [16].

Primary cell culture establishment is a process, which has limitations of providing purity of the desired cell type, vitality and proliferation without difference in culture characteristics. Since primary cells are quietly fragile, it is needed use of growth factors and some other supplementary chemicals to maintain culture for long term [16].

Peripheral blood leukocytes are the cells, which are the parts of inflammation process. These cells express inflammatory regulatory MEFV gene transcripts and take part in inflammation actively. Pathogens or external molecules are recepted as pathogen associated molecular patterns (PAMPs). They stimulate inflammatory pathway and lead to cytokine production [13]. Lipopolysaccharide (LPS) is one of the stimulatory factors (Figure 1.5). This molecule is produced from inner membrane of Gram-negative bacteria (e.g. *E.coli*) and recruits inflammatory assembly formation: inflammasome and cytokine secretion [17].

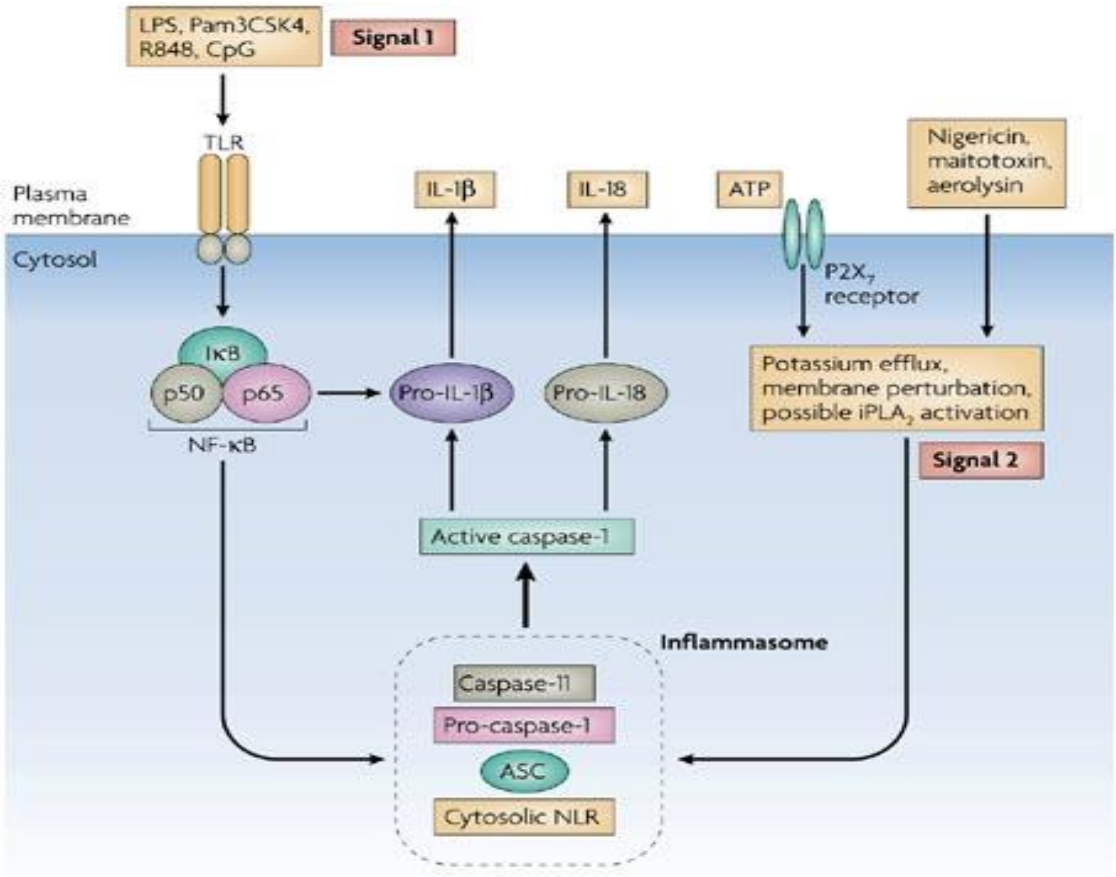

Figure 1.5 : LPS structure and inflammation [17].

Another cell culture type is infinite cell culture with leukemic cell line cells. The promyelocytic leukemia cell line HL60 is one of the most used cell type for inflammation studies. It is possible to differentiate these cells into macrophage-like cells by stimulating them with the chemical called Phorbol 12-Myristate 13-Acetate (PMA) or into granulocytic cells by incubating them with Retinoic Acid (RA) [18]. PMA affects cells with activation of Protein kinase C that leads to production of transcription factors [19]. Retinoic acid RA's biochemical effects are unclear. RA binds to specific nuclear receptors (RAR) and RA-RAR complex regulates target gene transcription, which are key regulators for differentiation [16].

Immune system is recruited with pathogen associated molecular patterns such as lipopolysaccharide (LPS) and this recruitment activates inflammatory pathway (Figure 1.6) [11].

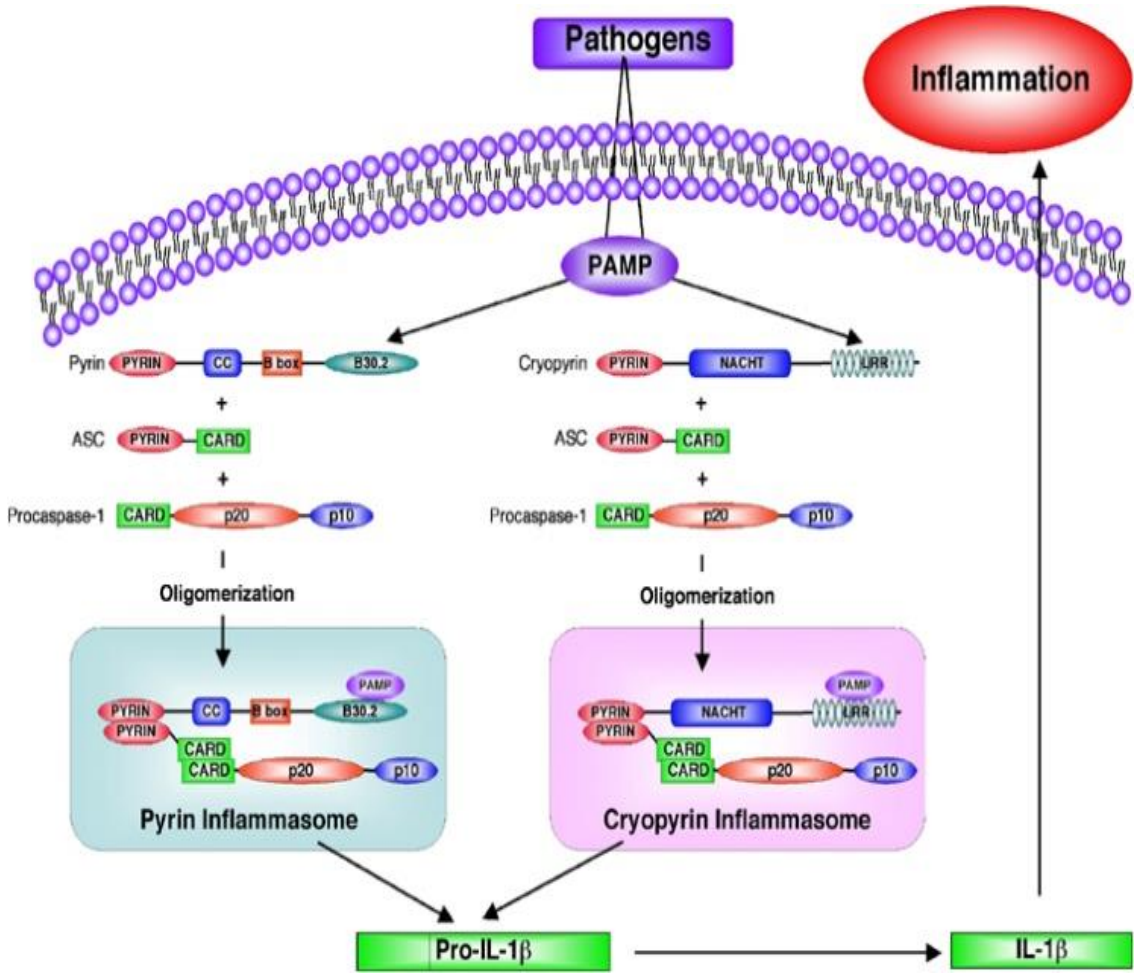

Figure 1.6 : PAMP and Inflammation [11].

PAMPs interact with B30.2 domain of Pyrin and URR domain of Cryopyrin (Fgure 1.6). Both of these interactions lead to ASC oligomerization process and inflammasome assembly. Inflammasome formation recruits cytokine production such as Pro-IL-1β, it is formed as IL-1β after maturation. Inflmmation is a general term of cytokine activity [11].

Inflammation status of the cells can be detected with inflammatory cytokine mRNA levels in the culture or tissue. The most common cytokines produced in inflammatory pathway are interleukin 1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α). These cytokines are commonly used as inflammation markers in molecular biology experiments [11].

1.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Complementary Deoxyribonucleicacid (cDNA) Synthesis Strategies

The most common RNA expression level detection method is Reverse Transcription Polymerase Chain Reaction (RT-PCR). In this technique, isolated RNA is converted into

double stranded complementary DNA (cDNA) with the activity of reverse transcriptase enzyme. cDNA is conventionally synthesized by using random (oligo-dT) primers. These primers match with poly-A tail of each RNA randomly. Finally a pool is constructed with randomly synthesized cDNA. Other priming strategies include use of hexamers or target specific primers [22].

In relative quantitaton studies, random oligo-dT primers are preferred because of the ability of synthesizing plenty different cDNAs in one reaction with these primers. In target specific priming, only one type of cDNA (specific sequence) is synthesized and to compare relative amounts with other RNA expressions, single specific reactions are needed. In the study of Deprez R. H. it is revealed that, expression analysis with target specific cDNA synthesis results are more accurate and sensitive than other priming strategies, especially for rare transcripts [23]. Since gene specific primers only bind to its complementary sequence, rare products can be captured from the pool as a result of the specific reaction. The rare product could possibly elimimated in the pool, however it is impossible to miss the rare product with gene specific priming. Target specific priming technique is uniplex, so to achieve a relative quantitation, the data should be normalized with a proper reference gene with the same reaction conditions and efficiency of the reactions should be took in consideration for proper computation [23].

1.5 Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

Real Time Polymerase Chain Reaction is a technique that enables detection and quantitative analysis of targeted DNA molecule or molecules. The most important property of the technique is its providing the possibility of amplification detection in its “real time”, while it is occuring instead of determining quantity at the end [21]. To this purpose, detection is provided by monitoring targets via two methods; first one is double-strand DNA dying with non-specific intercalating dyes (such as SYBR Green). The second one is hybridization based fluorescent labeled oligonucleotide consisting DNA probs which enables detection after hybridization with its complementary –target- DNA.

In this study, Real Time PCR applied as combined with Reverse Transcription and with SYBR Green intercalating dye, in terms of determining expression analysis of spliced forms of MEFV and inflammation related cytokines (interleukin 1-beta and tumor necrosis-alpha) [24].

Intercalating dyes bind to all DNA molcules without regarding any specificity, this feature leads to detection of all DNA molecules in the samples which includes both target and non-target items[25]. As amplification occurs intercalating dye binds to more amount of DNA which result as more fluorescent signals that show directly the amount of amplified DNA in the sample . Although using intercalating dyes is an effective way for estimating amount of amplified DNA molecules in a sample it is stil problematique these type of dyes' being unspesific. Thus, the fluorescence signals may refer to unspecific-unwanted amplifications such as primer dimers[26].

These type of reactions are done by adding dsDNA intercalating dye to the PCR mix which contains sample, at the beginning of the reaction. Then, the mix is run in a thermocycler. A reference to a standard dilusion is used for quantification of concentration. Each cycle, fluorescence signals are measured by a light detector and after detection is completed, achieved data is used in further analyses [27].

Quantification is effectuated by plotting cycle number against fluorescent output on a logaritmic scale. The cycle that sample's fluorescence passes above the determined treshold (cycle treshold (Ct)) is used for quantifying template amount. Final Ct values enables comparison of template amounts[28]. For quantifying relative gene expression, evaluated level of target cDNA is divided to a known amount of housekeeping gene's cDNA which is approximately same in each tissue and condition. Finally two methods can be held for achieving result of quantification which are absolute and relative quantifications[29]. Absolute quantification is accurate number of target molecules existing in the sample while relative quantification gives information about levels (as folds of each other) of expression of interested genes according to each other, instead of direct measurement[30].

1.6 Aim Of The Study

We previously obeserved an increased MEFV exon 2 deleted mRNA in FMF patients leukocytes compared to healthy controls, which led us to hypotesize exon 2 splicing may have a role in inflammation and FMF pathology [20]. To this end, here we aimed to construct an inflammation model in human PBL primary cell culture and HL60 cell line cell culture to detect and analyse MEFV alternative spliced forms expression levels in inflammatory conditions *in vitro*. We also examined the spliced form transcript levels in different tissues (blood and synovial fluids) of FMF patients during their inflammatory attack period. To understand the expression differences, two different *in vitro* inflammation models were

constructed with primary cell culture (peripheral blood leukocytes) and HL-60 cells. LPS induction is used as inflammatory agent with and without PMA and RA. For the consistency of the *in vitro* models with *in vivo* mechanisms, synovial fluid and blood tissues from FMF patients in their attack period were collected to construct a comperative study. After total RNA isolation for each culture and tissues, random and target specific priming strategies were applied to the aim of detecting MEFV-2Δ expression, rare MEFV transcript. Finally Quantitative Real Time PCR analysis was done to detect expression levels of the transcripts. The results were analysed statistically with using SPSS program (v. 21) for student's t test and chi-square analysis.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipment

2.1.1 Used equipments

The laboratory equipment used in this study listed in Appendix A.

2.1.2 Used chemicals, enzymes, markers and kits

The chemicals, enzymes, markers and kits used given in Appendix B with their suppliers.

2.2 Methods

2.2.1 Primary PBL cell culture

To construct the long term cell culture, different culture media were used. Each medium was prepared with sterilized materials and filtered with 0,22µm sterile microfilter. Cells were incubated in sterile fresh medium containing flasks, in 37ºC incubator with %1 CO_2. The culture was maintained by refreshment of the media for each 3 days.

Counting of living cells was done with Trypan Blue method. Firstly, Trypan Blue solution was prepared by adding %0.4 Trypan Blue stain in PBS. Cells were diluted with Trypan Blue solution (1:1). 10µl from this homogenized mix was transferred on a haemocytometer to count cell number/ml. Cover slip was put on the haemocyometer. Number of the cells in the large square of haemocytometer was counted in light microscope.

The cell count of sample was calculated using the formula:

Average cell number of in the sample:

count of the cells in large square of haemocytometer x dilution factor x 10^4**.** **(3.1)**

2.2.1.1 Isolation of peripheral blood leukocytes from whole blood

Whole blood samples for primary PBL culture collected from healthy volunteers. Pooled blood sample collection was diluted 1:1 using sterilized Dulbecco's Phosphate-Bovine-Saline (PBS). 15 ml Biocoll Seperation Medium for each 25ml of diluted blood was used to formate density gradient for separation of the leukocytes from other blood layers. For this process, Falcon tubes (50ml) were used. First, 15ml of Biocoll was added to empty Falcon tube then 25 ml of 1:1 diluted blood sample was added upon the Biocoll carefully without disturbing separation layer of two phases. The whole tube was centrifuged at 20°C, 2300 RPM for 15 minutes. After centrifugation, leukocytes were collected as a "cloudy interphase" with 1000µl pipette into a new clean Falcon tube.

10 mL of PBS were added upon collected leukocyte to wash the cells. Then tube was centrifuged for 5 minutes at 2300 RPM. This step was done twice, the final pellet was dissolved in the medium (5 mL) which will be used further and incubated in 37ºC incubator with %1 CO_2.

2.2.1.2 Cell culture medium preparation

For the establishment of long term primary cell culture, different mixtures was prepared from literature. According to culture stability, the everlast culture medium ingredient was chosen to maintain the culture for the next stages of the study.

Each medium content was prepared in sterile conditions and cell growth was provided in T25 flasks with refreshing media in each 3 days, in %1 CO_2 incubator at 37º C.

Table 2.1 : Establishment of Long Term Primary PBL Culture.

COMPONENT	DMEM Med.	RPMI Med. 1	RPMI Med. 2	RPMI Med. 3	RPMI Med. 4	RPMI Med. 5
DMEM (mL)	79.79					
RPMI (mL)		133.2	150	100	135	120
10% Horse Serum (mL)	10					
10% Fetal Bovine Serum (mL)	10	15		10	15	30
HEPES (mg)	476.6					
Glutamine (mM)		4	4	4	4	4
Penicillin/Sterptomycin (µL)	100	300	300	200		
Non-essential aminoacids (µL)	200			100		

2.2.1.3 Leukocyte activation with Lipopolysaccharide (LPS) stimulation

For LPS Stimulation, PBL cells were transferred into 6-well plate (1ml culture/well), and plate was incubated for 24 hours. Then, different concentrations of LPS were added to each group (10ng/µl, 50ng/µl and 100 ng/µl). Each well was studied as triplicate. The cells were incubated for another 24 hour. Before / after each treatment, cell number of the wells were calculated with Trypan Blue method and total RNA isolation was done separately for each group.

2.2.2 HL60 cell culture and LPS induction

Since primary cell culture is quietly fragile, to maintain proliferation and healthy growth of the cells a more reliable cell culture was constructed with HL60 cell line cells. To obtain inflammation and MEFV expression relationship an inflammation program was applied to cells as indicated in the figure 2.1.

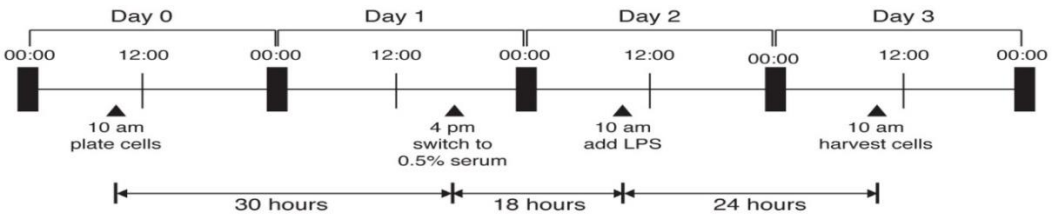

Figure 2.1 LPS Induction of HL60 cells [32].

After isolation from total blood, cells were incubated in proper medium for 18 hours before serum deprivation. For serum deprivation 18 hours incubated cells were centrifuged at 1000 X g for 1 minute. The pellet was dissolved in serum free or %0.05 serum containing medium and culture was incubated for another 18 hours before LPS addition. Then the culture was stimulated with LPS and incubated for 24 hours. In the "Day 3" cells were harvested and RNA isolation was done separately for each group.

2.2.2.1 HL60 cell differentiation

HL60 cells differentiated into macrophage-like cells by stimulation with PMA (10 ng/ml) for 5 hours before LPS addition induction. For differentiation into granulocytic lineage, RA incubation was applied as in a concentration of 0.2nM for overnight before LPS addition. The same LPS induction protocol was applied as described above.

2.2.2.2 Inflammation modelling with Lipopolysaccharide (LPS) induction of HL60 cells

LPS Induction was applied according to the figure 2.1 with several different concentrations of LPS (10 ng/ml, 100 ng/ml, 1µg/ml, 10 µg/ml).

2.2.3 Sample collection for FMF study

To compare MEFV alternatively spliced forms expression levels, FMF patients synovial fluid and blood tissues were collected from Rheumatology Department of Cerrahpaşa University Medical Faculty. Each patient was in attack period of the disease. Healthy control samples were collected from other patients of the hospital who has no any inflammatory disease.

Ethics Review Committee of Istanbul University Cerrahpaşa Medical Faculty approved the study. Consent form was obtained from each patient. Blood and synovial fluid samples were collected in 4 cc vacuum tubes containing EDTA and were kept at 4 °C for maximum two hours for prevention of RNA degradation.

2.2.4 Total RNA isolation

2.2.4.1 Total RNA isolation from cell culture

Total RNA isolation from primary PBL and HL60 cell culture was done with High Pure RNA Isolation Kit (Roche). First, culture samples were transferred into 1.5 mL Eppendorf tubes. The tubes were centrifuged at 500 X g for 3 minutes and the supernatant was removed carefully. 200 µl PBS was added into white pellet and resuspended. Also 400 µl Lysis/Binding

Buffer was added and mixed by vortex for 15 seconds. The sample was transferred to filter tube and centrifuged at 8.000 X g for 15 seconds. After centrifugation, fluid was discarded. For each sample 90 µl DNase incubation buffer and 10 µl DNase I were mixed in a separate tube. The mixture was added into filter tube and incubated at room temperature for 15 minutes. After incubation, 500 µl Wash I Buffer was added into filter tube and centrifuged at 8000 X g for 15 seconds and the fluid were discarded. Then 500 µl Wash II Buffer was added into filter tube and centrifuged at 8.000 X g for 15 seconds and the fluid were discarded. 200 µl Wash II Buffer was added again into filter tube and centrifuged at maximum speed for 2 minutes and the fluid was discarded with collection tube. The filter tubes were inserted 1.5 ml micro centrifuge tubes and 30 µl Elution Buffer was added. After incubation for 30 minutes in 4° C The tubes were centrifuged at 8.000 X g for 1 minute. The eluted RNAs were used for cDNA synthesis immediately since freeze-thaw action is degrading 60% of RNA.

2.2.4.2 Total RNA isolation from whole blood

Total RNA isolation from whole human blood was done by High Pure RNA Isolation Kit (Roche). 2 ml of Red Blood Cell Lysis Buffer was added to a sterile 1.5 ml reaction tubes. 500 µl peripheral blood was added into tubes. The tubes were shaken at room temperature for 10 minutes. Then the tubes were centrifuged at 500 X g for 5 minutes. After centrifugation, red supernatant was removed carefully with a sterile pipette. Again 1 ml Red Blood Cell Lysis Buffer was added to white pellet and mixed gently. The tubes were centrifuged again at 500 X g for 3 minutes and the supernatant was removed carefully. 200 µl PBS was added into white pellet. Also 400 µl Lysis/Binding Buffer was added and mixed by vortex for 15 seconds. The sample was transferred to filter tube (spin column) and centrifuged at 8.000 X g for 15 seconds. After centrifugation, fluid was discarded. For each sample 90 µl DNase incubation buffer and 10 µl DNase I were mixed in a separate tube. The mixture was added upon the filter tube. The tubes were incubated at room temperature for 15 minutes. After incubation, 500 µl Wash I Buffer was added into filter tube and centrifuged at 8000 X g for 15 seconds and the fluid were discarded. Then 500 µl Wash II Buffer was added into filter tube and centrifuged at 8.000 X g for 15 seconds and the fluid were discarded. 200µl Wash II Buffer was added again into filter tube and centrifuged at maximum speed for 2 minutes and the fluid was discarded with collection tube. The filter tubes were inserted 1.5 ml microcentrifuge tubes and 30 µl Elution Buffer was added. After incubation for 30 minutes in 4° C. The tubes were centrifuged at 8.000 X g for 1 minute. The eluted RNA samples were used for cDNA synthesis immediately.

2.2.4.3 Total RNA isolation from synovial fluid

Total RNA isolation from synovial fluid was done with High Pure RNA Isolation Kit (Roche). First, synovial fluid samples were diluted (1:1) with PBS. Diluted samples were transferred into 50 mL sterile Falcon tubes. The tubes were centrifuged at 500 X g for 3 minutes and the supernatant was removed carefully. 200µl PBS was added into white pellet and resuspended. Also 400 µl Lysis/Binding Buffer was added and mixed by vortex for 15 seconds. The sample was transferred to filter tube and centrifuged at 8.000 X g for 15 seconds. After centrifugation, fluid was discarded. For each sample 90 µl DNase incubation buffer and 10 µl DNase I were mixed in a separate tube. The mixture was added into filter tube and incubated at room temperature for 15 minutes. After incubation, 500 µl Wash I Buffer was added into filter tube and centrifuged at 8000 X g for 15 seconds and the fluid were discarded. Then 500 µl Wash II Buffer was added into filter tube and centrifuged at 8.000 X g for 15 seconds and the fluid were discarded. 200 µl Wash II Buffer was added again into filter tube and centrifuged at maximum speed for 2 minutes and the fluid was discarded with collection tube. The filter tubes were inserted 1.5 ml microcentrifuge tubes and 30 µl Elution Buffer was added. After incubation for 30 minutes in 4°C. The tubes were centrifuged at 8.000 X g for 1 minute. The eluted RNA samples were used for cDNA synthesis immediately since freeze-thaw process is degrading 60% of RNA.

2.5 Complementary DNA (cDNA) Synthesis

Since RNA is a fragile molecule, to prevent degredation of RNA, complementary DNA (cDNA) synthesis was done immediately after isolation with reverse transcription polymerase chain reaction (RT-PCR) by using ABI High Capacity Reverse Transcription Kit®. For cDNA synthesis two different priming strategies were used which are random priming and target specific priming.

2.5.1 cDNA synthesis with random priming

Master mix preparation: 2 µl of 10x RT Buffer, 0,8 µl of 25x dNTP Mix (100 mM), 2 µl of RT Random Primers 1 µl of Reverse Transcriptase enzyme, 4,2 µl of Nuclease-free H_2O per sample was added to compose a master mix. Then, 10 µl of the mix was distributed to PCR tubes and 10 µl RNA per tube was added for each PCR tube. (Final reaction volume is 20 µl).

PCR tubes were transferred to a thermal cycler, the PCR program was edited as indicated in Table 2.2.

Table 2.2 cDNA Synthesis Reaction Protocol.

Tempeature (°C)	Time
25	10
37	120
85	5
4	∞

2.5.2 cDNA synthesis with target specific priming

Same reaction volumes and conditions were applied using the primer sets below, instead of random primers. Target specifically designed primers were used to detect each RNA expression in separate reactions.

Table 2.3 : Oligonucleotide primer sequences.

Oligonucleotide primer name	Oligonucleotide primer sequence
MEFV 1-3 Forward	CAT TCA GGG AAG GCC ACC AG
MEFV 1-3 Reverse	TTC CTT TCA TGG GAG TCC TG
MEFV 2-3 Forward	GAA ATC CAG AAC ATT CGG TCA
MEFV 2-3 Reverse	ACC GTC AAC TGG GTC TCC TT
MEFV 10 Forward	GGA GGG AAG AAC ACA GCT CC
MEFV 10 Reverse	GGA GGG AAG AAC ACA GCT CC

2.6 Quantitative Real Time PCR MEFV Gene Expression Analysis

The real – time PCR experiments was done by using LightCycler ® 480 Master Kit (Roche) and performed at Roche LightCycler ® 480 instrument. Beta Actin gene was used as reference gene.

Real Time PCR mix was prepared as indicated: 10µl of Roche Light Cycler 480 Real Time PCR Master mix, 1,25 µl of forward primer (10 µM) and 1,25 µl of reverse primer (10 µM) were mixed for each sample that would be exposed to Real Time PCR application. 5,5 µl of dH_2O and 2 µl of template DNA (100 ng/µl) were added.

These samples transferred to light cycler and Real-Time PCR protocol was applied as indicated in the Table 2.4.

Table 2.4 : Quantitative Real Time PCR Protocol.

Program	Temperature (°C)	Time (min:sec)	
Pre-incubation	5	10:00	
Denaturation	95	10:00	
Annealing	59	00:30	45 cycles
Extension	72	00:01	

To check reaction specifity in SYBR-Green I based real time PCR reactions, melting curve analysis is used after real time PCR reaction.

Table 2.5 : Quantitative Real Time PCR Protocol for Melting Curve Analysis.

Temperature (°C)	Time (min:sec)
95	00:05
65	00:05
97	00:05
40	00:30

Specific oligonucleotide primers given in the table 2.6, were designed using Premier Biosoft primer designing software. MEFV 1-3 and 2-3 primers were designed to target exon-exon junction regions. MEFV 1-3 primer set captivates MEFV region which excludes exon 2. MEFV 2-3 primer set was designed to captivate exon 2. MEFV 10 primer set binds to a specific region in exon 10, this set is designed to capture all other spliced MEFV transcripts since exon 10 is a common part of the spliced transcripts. Interleukin 1β and TNF-α sets are specific to indicated cytokines. β-Actin primer set was used to detect β-Actin as reference – house-keeping gene.

Table 2.6 Oligonucleotide primer sequences.

Oligonucleotide primer name	Oligonucleotide primer sequence
MEFV 1-3 Forward	CAT TCA GGG AAG GCC ACC AG
MEFV 1-3 Reverse	TTC CTT TCA TGG GAG TCC TG
MEFV 2-3 Forward	GAA ATC CAG AAC ATT CGG TCA
MEFV 2-3 Reverse	ACC GTC AAC TGG GTC TCC TT
MEFV 10 Forward	GGA GGG AAG AAC ACA GCT CC
MEFV 10 Reverse	GGA GGG AAG AAC ACA GCT CC
Interleukin 1β Forward	GTC CTC AAT CTG CAC TCA ATC C
Interleukin 1β Reverse	TGA CAG GCG AGG CTA TCT CAG
β-Actin Forward	ACC GAG GAC TAT GAT TGC AC
β-Actin Reverse	TCC ATA AGC CGT CAT ACA TAT
TNF-α Forward	GCA GGC AGC CAG ATC ATC TGC
TNF-α Reverse	CGA GCT ACC CCT CTG ATT

2.7 Relative Quantification and Statistical Analysis

Relative Quantification was determined via comparative C_T (ΔC_T) method that calculates relative gene expression. This method was applied by substracting C_T value of target gene from reference gene's C_T value.

$$(C_T \text{ of MEFV transcript} - C_T \text{ of Beta Actin Gene (ACTB)}) = \Delta C_T \quad \textbf{(3.2)}$$

Then, this ΔC_T value was exposed to "$2^{-\Delta CT}$" process to evaluate relative expressions.

T value indicates the fold of expression that change between target gene (MEFV) and reference gene (Beta Actin).

The $2^{-\Delta CT}$ value of FMF patients group and healthy control group were analyzed statistically by using *Student's t test*, Chi square analyses using SPSS statistical analysis program (v.21).

3. RESULTS

3.1 Primary Cell Culture Maintenance

Primary cell culture maintenance success was evaluated according to the rate of vitality, proliferation and apperance of cells in the culture. According to these criteria, the results are summarized in the Table 3.1, Figure 3.1 and Figure 3.2.

Table 3.1 : Primary PBL culture maintenance and characteristics.

CELL TYPE	ISOLATN. METHOD	MEDİUM	VİTALİTY	CELL CHARACT.
PBL	Density Gradient	DMEM Medium: DMEM/HS/FBS/Glutamine PenStrep/ non essential aa mix/HEPES	7-9 days	Small cells, proliferation in low rate, cluster formation
		RPMI Medium 1: RPMI 1640/Glutamine/PenStrep	5-7 days	Small cells, cluster formation
		RPMI Medium 2: RPMI 1640/10%FBS/Glutamine/PenStrep	7-9 days	Small cells, fast proliferation
		RPMI Medium 3: RPMI 1640/Glutamine/PenStrep/aminoacid mix	10-13 days	Small cells, cluster formation
		RPMI Medium 4: RPMI 1640/10%FBS/Glutamine/PenStrep	7-9 days	Small cells, proliferation in low rate
		RPMI Medium 5: RMI 1640/10%FBS/Glutamine	9-11 days	Small cells, proliferation in low rate.
		RPMI Medium 6: RPMI1640/20%FBS/Glutamine/	10-12 days	Small cells, proliferation in low rate.

RPMI Medium 3: Enriched RPMI 1640 medium with Glutamine, Penicillin-Streptomycin and non-essential aminoacid mix is the best medium for minimal primary PBL culture with long living cells, high proliferation rate and late formation of clusters (Figure 3.1 and Figure 3.2).

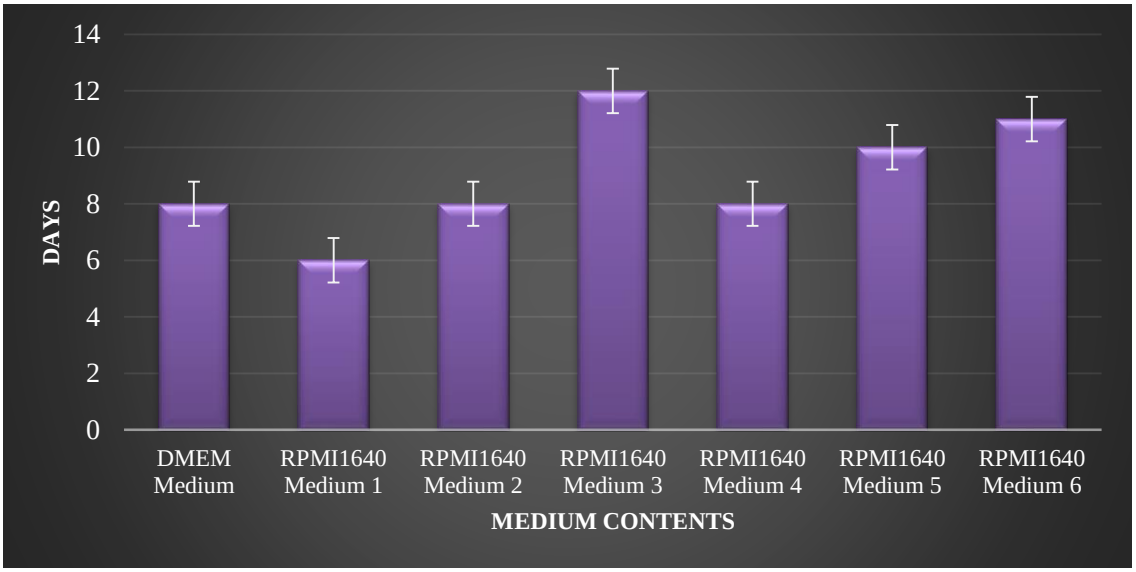

Figure 3.1 : PBL primary cell culture vitality rates of different medium contents (days).

The other medium contents leaded to fast cell death, low growth and proliferation profile. On the other hand, all culture apperance were similar, rare, small cells after isolation and after incubation, increase in cell number, dead cells in the culture with cluster formation (Figure 3.2)

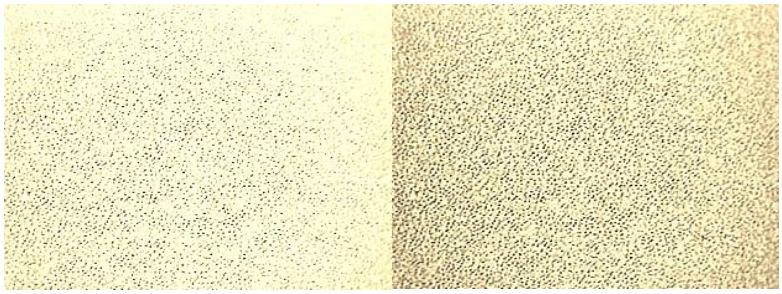

Figure 3.2 : Culture view right after isolation & after incubation (sample figure).

3.1.1 PBL culture MEFV alternatively spliced transcripts expression in terms Of LPS activation

To understand inflammation and MEFV alternatively spliced forms expression relationship PBL culture was activated with LPS and MEFV alternatively spliced forms expression levels were detected with real time PCR method. Data analysis was done with Student's t test.

In the data figured (Figure 3.3), LPS (10) cells were incubated with 10 ng/µl LPS and LPS (100) cells incubated with 100ng/µl LPS. LPS-PM cells were incubated with a combination of 10 ng/µl PMA and100ng/µl LPS. Control / untreated cells were only exposed to serum deprivation.

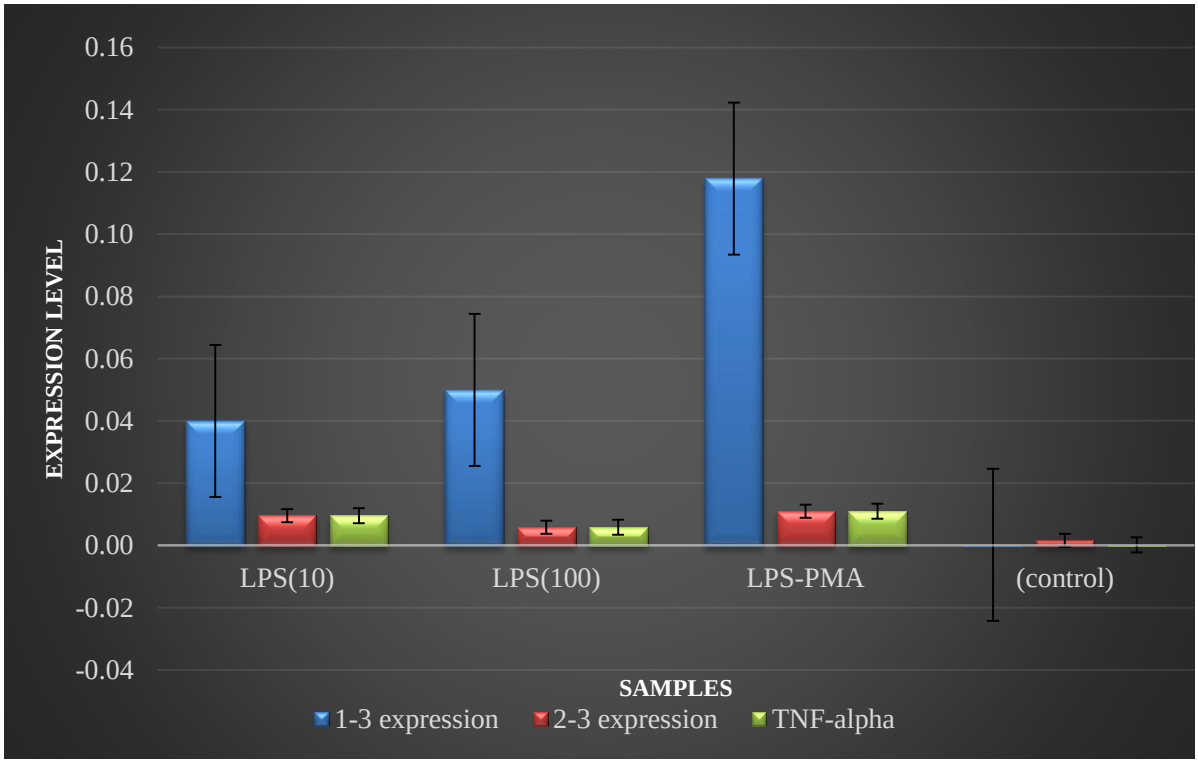

Figure 3.3 : LPS induced PBL MEFV alternative spliced transcripts expression levels.

It is seen that an inflammation indicator TNF alpha expression is not statistically significant, due to LPS induction ($p>0.05$). On the other hand, MEFV 1-3 expression (MEFV-2Δ) is significantly higher than MEFV 2-3 expression ($p<0.05$).

3.2 HL60 Cell Culture Maintenance

3.2.1 Cell vitality after PMA and LPS incubation

According to literature review, PMA stimulation was done for different time intervals. PMA and LPS treatment effects to cell vitality is summarized in Figure 3.4 and Figure 3.5.

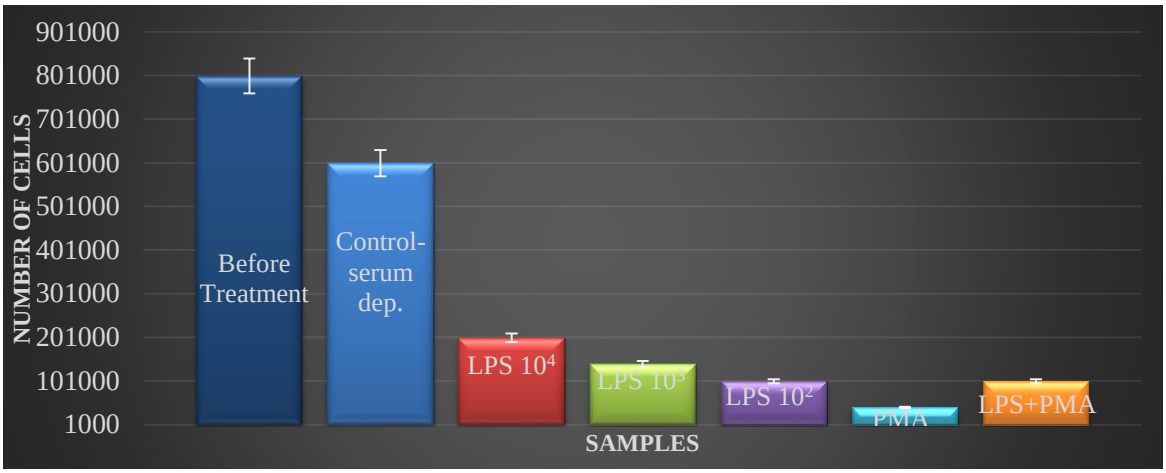

Figure 3.4 : PMA Stimulation with LPS induction (simultaneously) effects on cell number of the culture.

The most destructive effect was observed with PMA application in cell vitality (5% cell vitality). To enhance PMA effect, PMA was applied 5 hours before LPS, and cell vitality was affected more than simultaneous incubation (Figure 3.5).

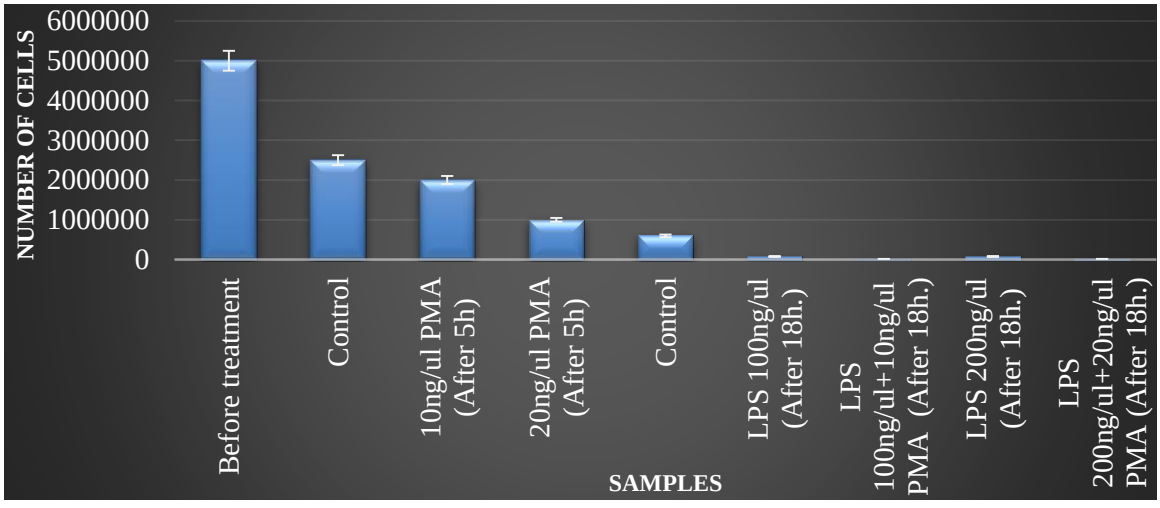

Figure 3.5 : PMA stimulation before/in combination with LPS induction effects of cell number of the culture (PMA stimulation was applied 5h prior to LPS induction).

Since the desired inflammation profile is observed with prior PMA stimulation, next steps of the experiments were done with prior PMA stimulaton to LPS treatment.

3.2.2 Quantitative Real Time PCR results of HL60 LPS induction

HL60 cell culture MEFV expression was detected with two different strategies: random priming and target specific priming. According to results, LPS alone did not alter IL-1β production. However, it enhanced MEFV mRNA expression. On the other hand, LPS with PMA decreased MEFV other spliced forms expression but increased production of MEFV-2Δ mRNA. The random priming and target specific priming results were summarized in figures 3.6 and 3.7.

In the Figure 3.6, data schematized in logatitmic scale, LPS 10 refers to cells incubated with 10 ng/µl LPS. PMA refers to cells incubated with 10 ng/µl PMA LPS 100: cells incubated with 100 ng/µl LPS. LPS1000 cells incubated with 1000 ng/µl LPS. LPS10000 cells incubated with 10000 ng/µl LPS. LPS-PMA: cells incubated with a combination of 10 ng/µl PMA and100ng/µl LPS LPS 200: cells incubated with 200 ng/µl LPS. LPS100+ATRA: cells incubated with 0.02 nM Retinoic acid and 100 ng/µl LPS.

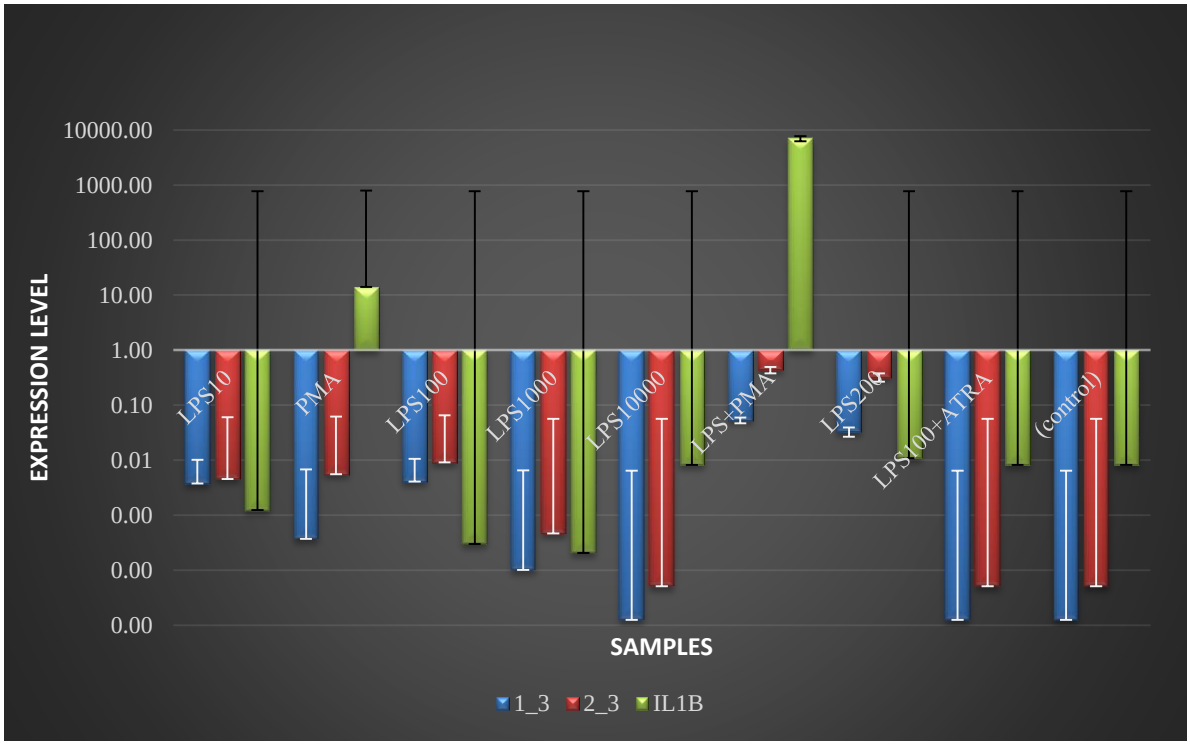

Figure 3.6 :MEFV alternatively splicing transcripts expression levels after LPS induction in HL60 cells / random priming.

It is seen that LPS-PMA combination leaded to expression of IL-1β RNA, inflammation indicator, in excess amount and significantly higher than untreated control sample. However, MEFV1-3 expression is not as high as expected.

In the Figure 3.7, L100 refers to the cells incubated with 100 ng/µl LPS. L200 shows the cells incubated with 200 ng/µl LPS. LA cells were incubated with 0.02 nM Retinoic acid and 100 ng/µl LPS LP cells were incubated with a combination of 10 ng/µl PMA and100ng/µl LPS Control shows the untreated cells and RA treatment was incubation with only 0.02 nM retinoic acid . ($p<0.5$for L200, LA and LP)

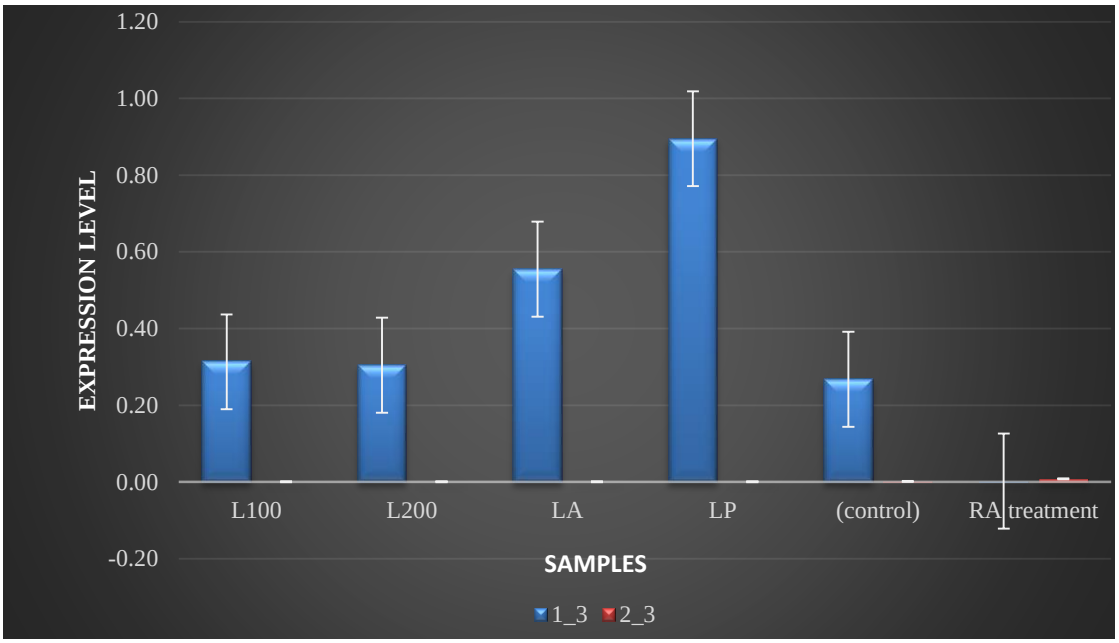

Figure 3.7 MEFV alternatively splicing forms expression levels after LPS induction in HL60 cells / target specific priming.

It is observed in Figure 3.7, LPS treatment with high amounts leading to increase of MEFV-2Δ expression, but not for the other spliced forms that containinf exon 2 of MEFV. On the other hand the most dramatical increase is seen with LPS and PMA combination. Target specific primed cDNA data was more accurate and repeatable than random primed cDNA study.

3.3 MEFV Alternatively Spliced Transcripts Expression Comparison Of Synovial Fluid and Blood Tissues in Attack Period Of FMF Disease

MEFV Spliced forms expression levels compared in blood and synovial fluid tissues of FMF patients. Again, two different cDNA synthesis strategies were applied to the cDNAs as random priming and target specific priming.

Figure 3.8 shows seperate patient samples with the comparison of MEFV isoforms expression. (P2-Blood/Random: Acute FMF Patient no. 2-blood sample P2-SF/Random: Acute FMF Patient no. 2-synovial fluid sample , P3-SF/Random: Acute FMF Patient no. 3-synovial fluid sample, P4-Blood/Random: Acute FMF Patient no.4-blood sample, P4-SF/Random: Acute FMF Patient no. 4-synovial fluid sample, template cDNA synthesized with Random priming). MEFV-2Δ expression is detected as less than other forms for each FMF patient in acute phase, in blood and synovial fluid. Also, IL-1β expression level is not as high as expected. The results with random priming was different for each sample sets which indicates unrepeatability.

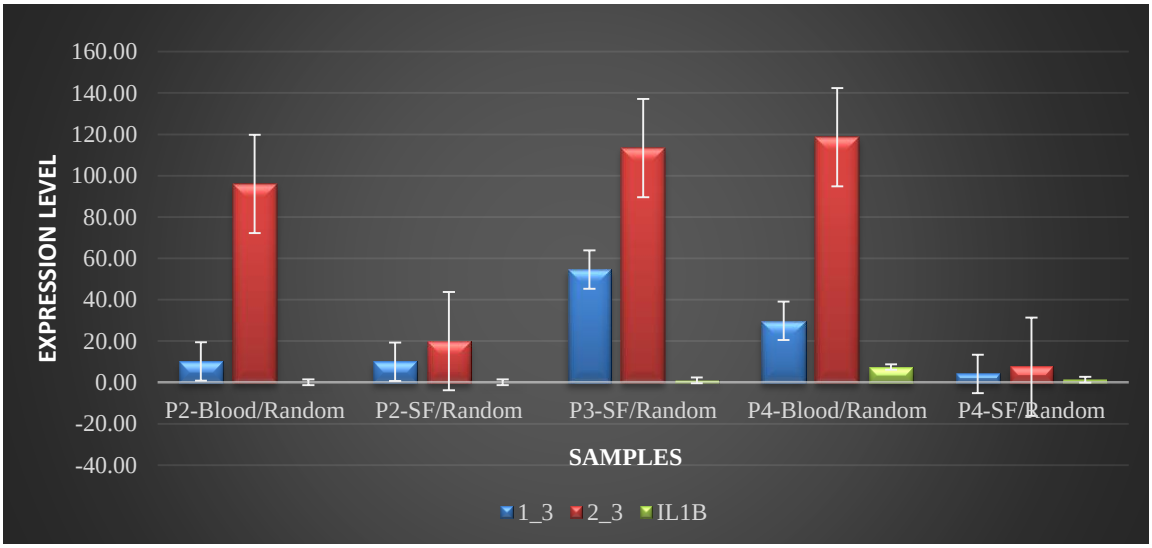

Figure 3.8 MEFV alternatively splicing forms expression levels in FMF acute phase cases/random priming.

Differently from random priming results The results of target specifically primed transcripts expression analysis revealed that, MEF-2Δ expression is significantly higher than exon 2 containing transcript levels as shown in the figures 3.9 and 3.10.

In the figure 3.9, P1-Blood/T indicates the sample of acute FMF patient no. 1-blood sample. P1-SF/T shows the same patient's synovial fluid sample. Similarly, P2-Blood/T: Acute FMF Patient no. 2-blood sample P2-SF/T: Acute FMF Patient no. 2-synovial fluid sample; P3-

SF/T: Acute FMF Patient no. 3-synovial fluid sample, P4-Blood/T: Acute FMF Patient no.4-blood sample, P4-SF/T: Acute FMF Patient no. 4-synovial fluid sample, for all of the data set, template cDNA synthesized with target specific priming. ($p<0.05$ for P1, P2, P3, P4, $p>0.05$ for P2)

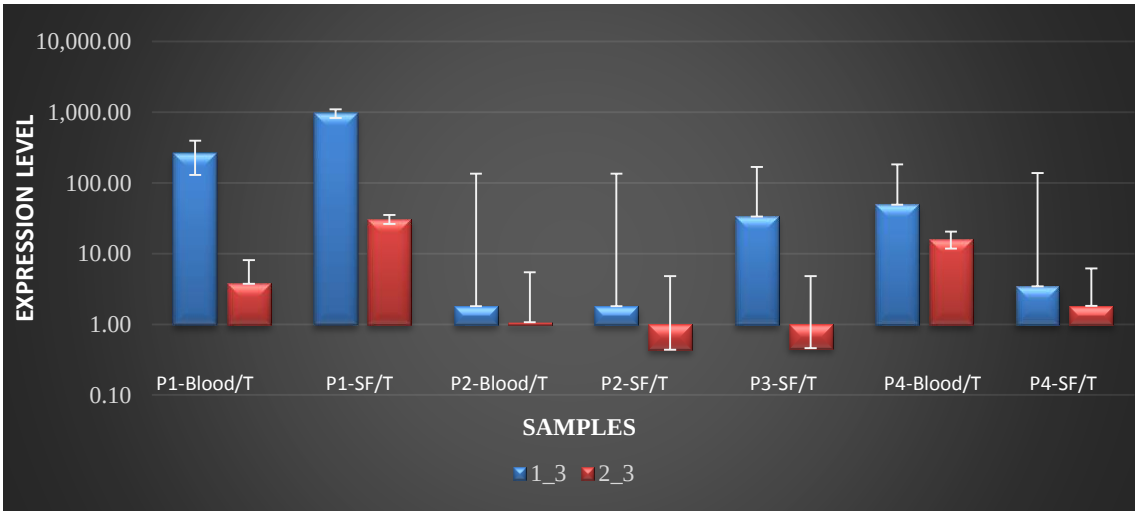

Figure 3.9 : MEFV Alternatively Splicing Forms Expression Levels in FMF acute phase cases/target specific priming.

In the figure 3.10 there is a general comparison of average expression levels of MEFV alternatively spliced transcripts. In this data the templates were synthesized with target specific priming. Figure indicates MEFV-2Δ expression being higher than other transcripts of MEFV that contains exon 2. (*For both of blood and synovial fluid data sets $p<0.05$)

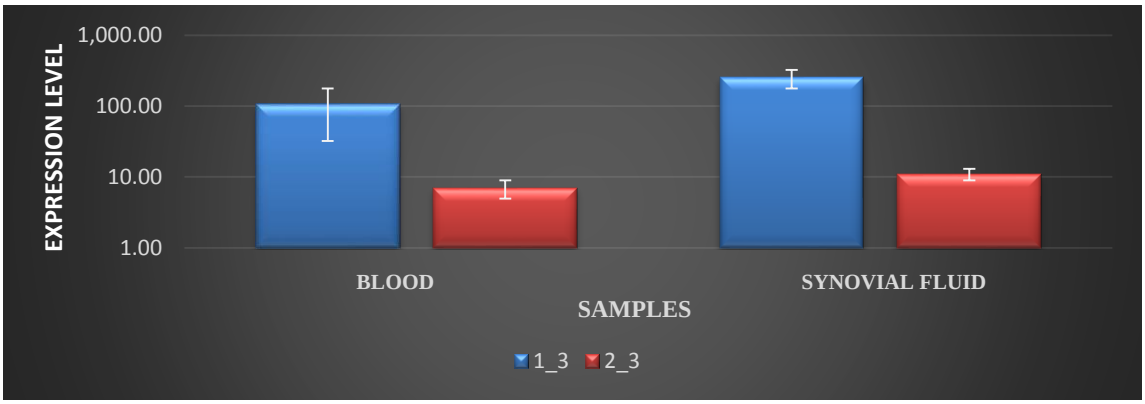

Figure 3.10 : MEFV Alternatively Splicing Forms Expression Levels in FMF acute phase cases/target specific priming.

These data reveals that, exon 2 lacking transcript of MEFV is abundantly expressed in inflammation cases and target specific priming is a suitable method to detect this rare mRNA expression levels.

4. DISCUSSION

Since in our study, we wanted to determine inflammation parameters, using *in vitro* inflammation model; to construct a minimal culture medium was important. It was possible to elongate culture vitality with supplements like hydrocortisone and other growth factors, but it is known that, these materials have putative effects on molecular interactions of inflammatory pathway [16]. Thus the medium contents were chosen as providing the minimal needs as possible. Results indicated that the medium containing non-essential aminoacid mix with 10% FBS was the most suitable medium for primary PBL cell culture according to relatively less cluster formation and slow death rate of the cells than other media. This medium was chosen to be used further studies of inflammation model.

Both *in vitro* inflammation model and synovial fluid sample study was designed to reveal MEFV-2Δ isoform expression pattern in inflammation cases. Alternative splicing is a process of reconnection of exons, known as a putative process in gene expression and also have important effects in complex disease pathophysiologies. It is a biological trade-off to arrange developmental differentiations. Beside nonsense mediated decay (NMD), it also leads to evolution of preferred combinations of protein structures. Cells prefer to produce each alternatively spliced form as a regulatory mechanism whether it is useful or not for cellular activities. Unuseful molecules are degraded with NMD [9]. Also it is known that alternative splicing is involved in many disease pathogenesis such as cancer, Alzheimer's Disease, Parkinson, Cystic Fibrosis [32]. Specifically MEFV alternative splicing is important in several disease pathologies such as Behcet's syndrome, Chron's disease and gut [9].

In practical applications of the designed experimental flow, it was hard to detect MEFV-2Δ mRNA since it is rarely produced relative to all other mRNA expression levels in leukocytes, synovial fluid and cultured cells. To detect this rare transcript, cDNA synthesis methods with random or oligodT priming were neither unreliable, inaccurate, nor repeatable. To reveal the expression rate relative to other spliced forms, we decided to use target specificly designed primers instead of random primers. After analysis, we compared both of expression detection patterns with each other for each data set. According to results, target specific priming, as

indicated in literature [23], is the most reliable method to detect rare products in real time PCR analysis. Expression of the MEFV alternatively spliced transcript MEFV-2Δ is rare, but it is increasing in inflammatory states(p=0.024 in HL-60 cells and p=0.037 in synovial fluid samples) as we have shown before (p=0.026) [36]. To detect the inflammation status of the samples, different cytokine mRNA expression levels such as TNF-alpha and Interleukin-1beta was evaluated. IL-1β amplification was shown in LPS+PMA treated HL-60 cells. Thus we can say the inflammation was succeeded with both LPS and PMA treatment, and inflammation triggers MEFV-2Δ production in HL-60 cells rather than the other alternative spliced forms of MEFV such as MEFV full length and other exon 2 containing transcripts. Our findings indicated that MEFV-2Δ is highly expressed in inflammation conditions, and this isoform may have a putative function in inflammatory pathway and in FMF pathology. This result was confirmed with acute FMF synovial fluid and blood samples and the hypothesis of MEFV-2Δ transcript's being important for inflammatory pathway was supported.

Furthermore importance of MEFV exon 2 in localization estimation is shown in a different study of our group. The study indicated that in neutrophil-like differentiated HL-60 cells and LPS/PMA treated HL-60 cells MEFV full length green flourescent protein (MEFV-fl-GFP) and exon 2 deleted MEFV isoform green flourescent protein (MEFV-2Δ-GFP) transcripts are located in the cytoplasm. However in non-treated HL-60 cells MEFV-fl-GFP was located in cytoplasm and MEFV-2Δ-GFP was in nucleus [33]. Combining this data with IL-1β increase being alongside with increase in MEFV-2Δ expression in inflammatory cases strengthens our hypothesis of MEFV-2Δ isoform's impotant role in inflammatory pathway. To understand detailed role of MEFV-2Δ in inflammatory pathway and disease pathogenesis, further studies required with increased numbers of patient samples and additionally protein interaction comparisons of MEFV-fl and MEFV-2Δ isoforms.

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APPENDICES

APPENDIX A: Used Laboratory Equipments

APPENDIX B: Used Chemicals and Kits

APPENDIX A: Used Laboratory Equipments

Centrifuges

Sigma 1-13 B. Braun International

Allegra 25R Centrifuge Beckman Coulter

Pipettes

Gilson Pipetman 20 µL 200 µl, 1000 µl

Spectrophotometer

Thermo NanoDrop2000

Thermal cycler

Techne FTGENE 5D

Light Cycler

Roche Light Cycler 480

Light Microscope

Olympus CH30

APPENDIX B: Used Chemicals and Kits

Chemicals:

dNTP	Fermentas, iNtRON Biotechnology
Ethanol	Riedel-de Hanen
MgCl2	Fermentas, iNtRON Biotechnology
NaOH	2008
Primers	IDT, Sentromer
i-Taq DNA Polymerase	iNtRON Biotechnology
Tris Base	Amresco
10X PCR Buffer	Fermentas
Taq DNA Polymerase	Fermentas

Kits:

cDNA Synthesis	High Capacity Reverse Transcription Kit (Applied Biosystems)
DNA Isolation	DNA Isolation Kit from Mammalian Blood (Roche)

RNA Isolation	High Pure RNA Isolation Kit (Roche)
Real-Time PCR	LightCycler 480 Master Mix (Roche)
PCR	High Fidelity Polymerase Chain Reaction

CURRICULUM VITAE

Name Surname: İrem Abacı

Place and Date of Birth: Antakya, 07/07/1988

Address: Tarabya Şenevler Cad. Güler Sk. No: 16/1 Kat:1, Sarıyer - İSTANBUL

E-Mail: irem.abaci@gmail.com

B.Sc.: Molecular Bology and Genetics

Professional Experience and Rewards:

Sentromer DNA Technologies LLC, from April 2012, Production Manager

PUBLICATIONS/POSTER PRESENTATIONS

Celikyapi G., Erdemir Ş., Abaci İ., Seyahi E., Uğurlu S., Turanli E. T.,
"Investigation of MEFV Transcript Levels and Pyrin Isoforms Localization in Induced Inflammatory Cell Culture Models". Poster presented as part of the Molbiyokon 2013, İstanbul, TR, 22-23 November 2013. Abstract published in Molbiyokon 2013. Current Abstracts. , NY: Springer; 2013:101.