Defining the function of Pyrin, the Familial Mediterranean Fever-associated protein, in inflammation

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ABSTRACT

PAMELA ROSE HESKER: Defining the function of Pyrin, the Familial Mediterranean Fever-associated protein, in inflammation.
(Under the direction of Dr. Beverly H. Koller, Ph.D.)

Every day, the immune system makes decisions to differentiate harmful and inert stimuli, which allows protection against pathogens and prevents unnecessary or uncontrolled inflammation. Perturbations to the innate immune system can lead to autoinflammatory disorders such as Familial Mediterranean Fever (FMF), which is an inherited disorder characterized by unprovoked episodes of inflammation and fever. The genetic abnormality underlying FMF disorder is mutations in the gene *MEFV* (Mediterranean Fever), which encodes the protein Pyrin. Previous research indicates that Pyrin alters function of the inflammasome multiprotein complex that mediates post-translational IL-1 family cytokine processing. This work has led to disparate conclusions about the function of Pyrin. Interpretation of these results is ambiguous, in part, because mutations within the Pyrin protein are not defined as gain or loss of function. Previous research also indicates that the expression of *MEFV* is abundant in neutrophils, and that neutrophils from FMF patients display altered immune function and survival in comparison to neutrophils from healthy donors. However, there is no direct evidence that mutations in Pyrin affect neutrophil cell processes. We postulated that mice lacking Pyrin (*Mefv*$^{-/-}$) would allow us to clarify the function of Pyrin in the regulation of the immune response and FMF pathophysiology. Characterization of naïve *Mefv*$^{-/-}$ mice revealed no deficits in immune cell development or distribution and no indication of unprovoked inflammation. In response to immune challenge *in vitro*, IL-1β cytokine

levels were increased by the absence of Pyrin. However, neutrophil recruitment and survival were not affected by the loss of Pyrin. *In vivo* models of peritonitis demonstrated that *Mefv*$^{-/-}$ mice generate a normal hypothermic response and recruit and retain inflammatory cells normally. No difference in the physiological outcome of immune challenge was detected. These studies indicate that Pyrin negatively regulates the immune response by altering IL-1β secretion. Since IL-1β is at least in part responsible for FMF-associated inflammation, our findings support a model in which loss-of-function mutations in Pyrin can cause FMF.

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AIM2	Absent in melanoma 2
ATP	adenosine 5'-triphosphate
Arf	ADP-ribosylation factor
Alum	aluminum hydroxide
Amp^r	ampicillin resistance
ANOVA	analysis of variance
An V	Annexin V
ASC	apoptosis-associated speck-like protein containing a CARD
LT	*Bacillus anthracis* lethal toxin
BAC	bacteria artificial chromosome
BB	B-box domain
BMMΦ	bone marrow-derived macrophages
BMMC	bone marrow-derived mast cells
bZ	bZip domain
CPPD	calcium pyrophosphate dihydrate
COP	CARD-only protein
CARD	Caspase activation and recruitment domain
CASP-1	Caspase-1

cPOP2	Cellular pyrin-only protein 2
CD	cluster of differentiation
C-C	coiled-coiled domain
CFU	colony forming units
cDNA	complementary DNA
CRP	C-reactive protein
CAPS	Cryopyrin-associated periodic syndromes
Ct	cycle number
Cre	Cyclization recombinase
DAMP	danger-associated molecular pattern
del	deletion
DNA	deoxyribonucleic acid
NATCH	domain present in NAIP, CIITA, HET E, and TP1
dsDNA	double-stranded deoxyribonucleic acid
elic pMΦs	elicited peritoneal macrophages
ESC	embryonic stem cell
ESR	erythrocyte sedimentation rate
EDTA	ethylenediaminetetraacetic acid

ext	extension
FMF	Familial Mediterranean Fever
FAF1	Fas-associated factor 1
FACS	fluorescently activated cell sorting
FIIND	Function to find domain
GM-CSF	Granulocyte/moncyte-colony stimulating factor
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
TH1	helper T cell subset 1
TH2	helper T cell subset 2
HMGB1	High mobility group box protein 1
HIN	HIN-200/IF120x domain
IκB	Inhibitor of kappa B
INCA	Inhibitory CARD
IFN	Interferon
IL	Interleukin
IL-1R	Interleukin-1 receptor
i.p.	intraperitoneal

JAK-STAT	Janus kinase-signal transducers and activators of transcription
Kan^r	kanomycin resistance
kb	kilobase
KO	knock-out
LDH	Lactate dehydrogenase
LRR	leucine rich repeat
LTB_4	Leukotriene B 4
LPS	lipopolysaccharide
LYM	lymphocytes
MΦ	macrophage
M-CSF	Macrophage colony-stimulating factor
MIP-2	Macrophage inflammatory protein-2
MEFV	Mediterranean fever gene
Mefv	Mediterranean fever gene
MCP-1	Monocyte chemoattractant protein-1
MSU	monosodium urate
$Mefv^{trunc/trunc}$	mouse line carrying a targetted disruption of *Mefv*
$Mefv^{-/-}$	mouse line carrying null *Mefv* alleles

MDP	muramyl dipeptide
MPO	Myeloperoxidase
Neo^r	neomycin resistance
NET	neutrophil extracellular trap
NLRC	NLR containing a CARD domain
NLRP	NLR containing a pyrin domain
NAIP	NLR family, apoptosis inhibitory protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NBD	nucleotide-binding domain
NLR	nucleotide-binding domain, leucine-rich repeat
OCT	octacalcium phosphate
OD	optical density
PAMP	pathogen-associated molecular pattern
PGN	peptidoglycan
PFAPA	periodic fever, aphthous stomatitis, pharyngitis, and adenitis
pMΦ	peritoneal macrophages
PMA	Phorbol myristate acetate
PBS	phosphate-buffered saline

PSTPIP1	Proline-serine-threonine phosphatase-interacting protein 1
PI	propidium iodide
PKC	Protein kinase C
$P2rX_7$	Purinergic receptor P2X, ligand-gated ion channel, 7
PAPA	Pyogenic sterile arthritis, pyoderma gangrenosum, and acne
PYD	pyrin domain
P/M-IP1	Pyrin/marenostrin interacting protein 1
POP	Pyrin-only protein
POP1	Pyrin-only protein 1
rpMΦ	resident peritoneal macrophages
RNA	ribonucleic acid
PAI-2	Serpin plasminogen activator inhibitor 2
PI-9	Serpin proteinase inhibitor 9
SAA	Serum amyloid A
SEM	standard error of the mean
TiO2	titanium dioxide
TRAPS	TNF-Receptor Associated Periodic Syndrome
TLR	Toll-like receptor

TGF	transforming growth factor
TRIM20	tripartite motif-20
trunc	truncated
TNF	Tumor necrosis factor
UTR	untranslated region
VASP	Vasodilator-stimulated phosphoprotein
WT	wild type

CHAPTER 1

Introduction

Familial Mediterranean Fever (FMF) is a disorder of the innate immune system

Familial Mediterranean Fever (FMF) is an inherited autoinflammatory disease characterized by sudden episodes of fever and inflammation that typically last 1 – 3 days. Fever and acute abdominal pain are the most common symptoms, but symptoms can also include pain in the joints or lungs or lesions in the skin. The location and the severity of pain and inflammation differ between patients and between attack episodes within each patient. Acute attack periods are interspersed with remission periods of either low-grade or undetectable inflammation. FMF has long-term consequences that include tissue scarring, which is secondary to inflammation, and amyloid deposition within organs, especially the kidneys. Severe cases result in kidney failure and subsequent death. Disease onset and diagnosis usually occurs in children before age eight (1, 2).

The innate, but not the adaptive, arm of the immune system mediates FMF pathologies. Local tissue inflammation is due, in part, to considerable deposition of neutrophils. Neither T lymphocyte infiltration, nor auto-reactive antibodies have been detected, indicating that T and B lymphocytes are unlikely to be involved in mediating inflammation. Patients have an increase in the acute phase reactants: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), serum amyloid A (SAA), β-2 microglobulin, and fibrinogen. They also have increased levels of

the Interleukin (IL) cytokines 6 and 10 (3), and the cytokine SA100A12 (4) in comparison to healthy controls. IL-1β cytokine levels are also elevated in some patients (3). IL-1β is of particular interest because it is elevated in patients with several different overactive immune system disorders, and blocking the signaling of IL-1β can decrease inflammation. IL-1β is produced predominantly by macrophages.

FMF was associated with mutations in Mefv (Mediterranean Fever) based on linkage analysis and was confirmed by positional cloning (5, 6). Mefv encodes the protein Pyrin. Consistent with IL-1β-mediated inflammatory responses and neutrophil accumulation in FMF, Pyrin expression is highest in macrophage and neutrophil cells (7, 8). Thus, the symptoms of FMF, clinical tests, and the properties of the Pyrin protein indicate that the innate immune system mediates disease. Specifically, neutrophils and macrophages, are likely to play an important role in FMF-associated inflammation that is caused by mutations in the Mefv gene. Accordingly, previous laboratory research demonstrates a role of Pyrin in macrophage-mediated IL-1β production and in survival, chemotaxis, and phagocytosis processes of neutrophils. In this chapter, I will summarize previous research on the regulation of IL-1β production and neutrophil functions, as they pertain to Mefv and FMF, and then discuss the features of the MEFV gene and Pyrin protein. These data fit together nicely to provide support for a role of Pyrin in IL-1β production and neutrophil physiology, although the specific contribution of Pyrin is unclear. One of the complications in interpreting the contribution of Pyrin is the limited understanding of the genetic inheritance of FMF and the consequences of FMF-associated mutations on Pyrin's function. Conflicting data suggests that mutations cause a loss-of-function or a gain-of-function. This will be presented later in this chapter. The central aim of this work was to use the mouse as a model organism, capable of genetic manipulation, to isolate and differentiate Pyrin's role in innate immune responses in an endogenous system.

Regulation of the innate immune system

Immune responses are normal physiological processes that are required for defense against invading pathogens, but must be limited to prevent excessive tissue damage. Appropriate activity is partially accomplished by coordinated processes of two phases of the immune response: the innate immune response and the adaptive immune response. The innate immune system orchestrates a relatively non-specific response against pathogens. This phase begins immediately at the initial sensing of infection or tissue damage and clears most pathogens. Protection is conferred by production and release of molecules such as proteases and free oxygen radicals that damage pathogens and through coordinated death of infected cells by apoptosis or phagocytosis. Although critical to protect the organism as a whole, the innate immune response is damaging to local healthy tissue at the site of infection. It is important for the immune response to be as weak as possible in order to limit healthy tissue damage while effectively preventing pathogenesis. When the pathogen is persistent, and perhaps a less immediate threat to survival of the whole organism, activation of the adaptive immune system complements the limitations of the innate immune system. The adaptive response phase is slower, but responds very specifically to the pathogen so that a more robust defense against the pathogen is possible without causing too much damage to healthy tissues. Disruptions to the mechanisms controlling these responses can cause either unproductive responses that allow the spread of infection, or overactive immune responses that damage healthy tissue. These are referred to as autoinflammatory disorders, allergy, autoimmune disorders, or tissue graft rejection, depending on the tissues mediating the immune system activity. Since FMF is an autoinflammatory disorder caused by misregulation of the innate immune system, the innate immune system will be the focus for the rest of this work.

Appropriate pathogen recognition is critical for the prevention of autoinflammatory disorders. The innate immune system must make quick decisions about whether or not a pathogen or foreign particle threatens the survival of the organism. To confer this ability, cells often have co-stimulatory requirements or two independent pathways that converge and activate an immune response. In 1994, Polly Matzinger proposed "the danger hypothesis" to account for some of the gaps in the current model describing the process of self versus non-self decisions. "The danger hypothesis" proposes that the immune system does not directly detect self versus non-self stimuli to guide appropriate immune responses, but instead detects and protects against danger. Indications of danger are provided by a network of extrinsic communication signals from damaged tissues and intrinsic cell stress signals. Thus, the presentation of a pathogen in the context of a danger signal triggers the innate immune response. These independent signals come together to tailor the breadth and magnitude of the response (9-11).

Interleukin-1β production

Cytokine and chemokine signaling molecules circulate throughout the body and coordinate the strength and timing of pathogen responses. The cytokine IL-1β is released very early during the innate immune response and is an important mediator of the overall magnitude of the immune response. Its importance in the immune response is underscored by its contribution to sepsis, the autoimmune disorders rheumatoid arthritis, Crohn's disease, and multiple sclerosis, and the autoinflammatory disorders CAPS, PAPA syndrome, and FMF. Controlled experiments implicate Pyrin, the protein mutated in FMF, in the regulation of IL-1β production. IL-1β is produced and secreted, predominantly by macrophages, in response to pathogen and danger signals. For example, in response to simulation with sterile agents, such as necrotic cells which are a source of cellular damage or danger signals, IL-1β mediates a pro-inflammatory response

that can be quantified by neutrophil recruitment (12). IL-1β binds to the IL-1 receptor (IL-1R) on the external surface of cells and promotes further downstream pro-inflammatory cytokine signaling, cell recruitment, fever, and overall metabolic changes (13).

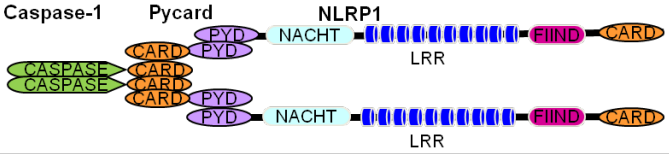
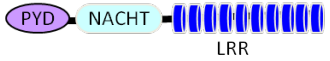
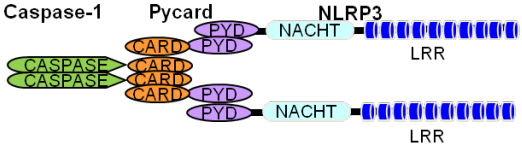
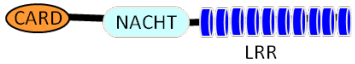
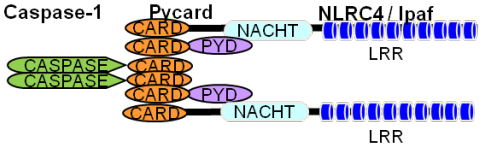
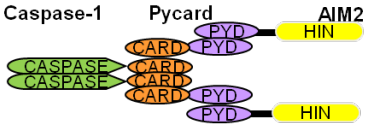
IL-1β production involves two signaling pathways: 1) Pro-IL-1β transcripts are produced in a pathogen-stimulated and NF-kB-dependent manner, and 2) Caspase-1 processes pro-IL-1β protein to mature, or active, IL-1β cytokine (14). Another IL-1 family cytokine, IL-18, is cleaved by Caspase-1 in an analogous manner. Caspase-1, a cysteine-aspartic acid protease, is activated by autocatalytic cleavage, which is promoted by inflammasome complexes in response to cellular damage (15, 16). The vast majority of IL-1β is produced in a Caspase-1-dependent manner, although other proteases, produced by neutrophils and mast cells, can generate an active form of IL-1β with relatively low efficiency. Pyrin is implicated in the regulation of IL-1β production during both the transcriptional and the inflammasome-dependent processing stages.

The inflammasome complexes

There are at least six inflammasome complexes that activate Caspase-1. Five are nucleated by a NLR family protein and the last contains the HIN-200 family member Absent in Melanoma 2 (AIM2). The NLR/AIM2 protein binds either directly or indirectly to Caspase-1 through their pyrin domain (PYD) or caspase activation and recruitment domain (CARD) domain. Unfolding of the NLR protein is the “ignition switch” to inflammasome complex formation, and each is triggered by different stimuli. In other words, the preferred inflammasome complex is dictated by the stimulus initiating the immune response, in order to allow some tailoring of the innate immune response. Immune stimuli have been tested primarily *in vitro* using overexpression or knock-out experimental systems to access the contribution of

the indicated protein to IL-1β production. It is unclear whether heterocomplexes with multiple NLRs form functional inflammasomes to further tailor a response to different stimuli. Table 1.1 shows the functional domain structures of the four proteins that nucleate inflammasomes, the predicted inflammasome complex structures, and the stimuli known to activate each of them.

Table 1.1 Inflammasome complexes.

Protein Structure	Inflammasome Structure	Stimuli
 NLRP1		*Bacillus anthrax* lethal toxin, MDP
 NLRP3		*Staphylococcus aureus*, *Listeria monocytogenes*, *Shingella flexneri*, ATP, large particles (asbestos, monosodium urate (MSU), calcium pyrophosphate, silica, nanoparticles), aluminum hydroxide (Alum), imidazoquinoline compounds (R837, R848), pore-forming toxins (nigericin, maitotoxin), MDP, oxygen radicals (titanium dioxide, H_2O_2), acetominephen, skin irritants (TNP-Cl, TNCB, DNFB)
 NLRC4/ Ipaf		Flagellin of *Salmonella typhimurium*, *Legionella pneumophila*, and *Pseudomonas aeruginosa; Shigella flexneri*
 AIM2		*Listeria monocytogenes*, vaccinia virus, mouse cytomegalovirus, dsDNA (cytosolic)

PYD, pyrin domain; LRR, leucine-rich repeat; FIIND, function to find; HIN, HIN-200/IF120x.

The inflammasome structures are depicted as dimers, but also form higher-order molecular complexes.

The indicated stimuli have been tested primarily in vitro, using a readout of Caspase-1 cleavage, Caspase-1 activity, or IL-1β production as a readout of inflammasome-mediated immune system activation.

The protein domain structure of the NLRs is critical to their function. NLRP1 – 3 and 6 and NLRC4 are members of the NLR protein family, based upon the presence of two domains for which they are named: the nucleotide-binding domain (NBD or NATCH) and the leucine-rich repeat (LRR). These proteins also contain a pyrin domain (PYD) and/or a caspase recruitment domain (CARD). NLRP1 contains an additional domain, a function to find (FIIND) domain. AIM2 contains a PYD and HIN-200/IF120x domain (HIN) and thus shows little structural similarity to the NLRs, but functional analysis indicates that it nucleates inflammasome complexes. Unfolding of the NLR/AIM2 exposes the binding surfaces of the PYD and CARD domains and is regarded as the "ignition switch" for inflammasome complex formation. The PYD and CARD form homodimers with cognate domains present in ASC (apoptosis-associated speck-like protein containing a CARD) or Caspase-1 to bring the inflammasome complexes together. The inflammasome complexes are often depicted as dimers, but electron micrographs of *in vitro* reconstituted NLRP1 protein complexes show that they actually form higher order complexes, similar to apoptosomes, with 7 NLRP1 proteins forming a circular perimeter around the ASC and Caspase-1 proteins at the core (17).

The NLRP1 inflammasome was the first inflammasome to be described (18), and it remains the only inflammasome complex verified by reconstitution using purified human recombinant proteins (17). The *Nlrp1* gene was identified in mice based upon differential susceptibility of inbred mouse strains to *Bacillus anthrax* lethal toxin (LT) induced death. The susceptibility region was mapped to a locus containing three orthologs analogous to the human *NLRP1* gene: *Nlrp1a, Nlrp1b*, and *Nlrp1c* (19). Further studies using genetically-deficient mice have verified that *Nlrp1b* is indeed necessary for LT-induced Caspase-1 activation and IL-1β production.

NLRP3, formerly referred to as NALP3, Cias1, Cryopyrin, and PYPAF1, responds to the broadest range of stimuli tested so far (20), and signaling through the NLRP3 inflammasome is most well-characterized. Deletion of the NLRP3 gene in mice provides confirmation that NLRP3

is required for IL-1β production in response to pathogens and toxins (20). NLRP3 mediates IL-1β production following exposure to a diverse set of stimuli, including some bacteria, ATP, uric acid crystals common in gout, the vaccine adjuvant alum, antiviral drug compounds, reactive oxygen species, and cell membrane permeabilizing toxins. It remains unclear how such a diverse set of stimuli can activate NLRP3, and it will be interesting for future studies to determine where the danger response pathways converge to trigger NLRP3 inflammasome complex formation. Under physiological conditions, NLRP3 folds upon itself to prevent activation. NLRP3 unfolds in response to low intracellular potassium levels induced by hypotonic cell medium or treatment with a toxin that permeabilizes the cell membrane. These treatments favor potassium efflux (21-23). It is likely that NLRP3 unfolding requires interaction between a phosphorylated nucleotide and the NACHT domain to transfer energy that enables protein unfolding. Once unfolded, NLRP3 forms an inflammasome complex that brings together NLRP3 and Caspase-1 through the adaptor protein ASC (gene: *Pycard*). Inflammasomes aggregate to form larger protein complexes called ASC specks. Proximity of multiple Caspase-1 molecules causes autocatalytic cleavage and activation of Caspase-1. Active Caspase-1 cleaves pro-IL-1β (and pro-IL-18) to their mature forms, and these molecules are released from the cell (24). The NLRP3-mediated response to a combined exposure of LPS and ATP is well described. LPS activates NF-κB-dependent transcription of IL-1β, and ATP triggers NLRP3 unfolding (Figure 1.1). Current research efforts also continue to delineate the role of NLRP3 inflammasomes in inflammation-induced cell death and in shaping the adaptive immune response.

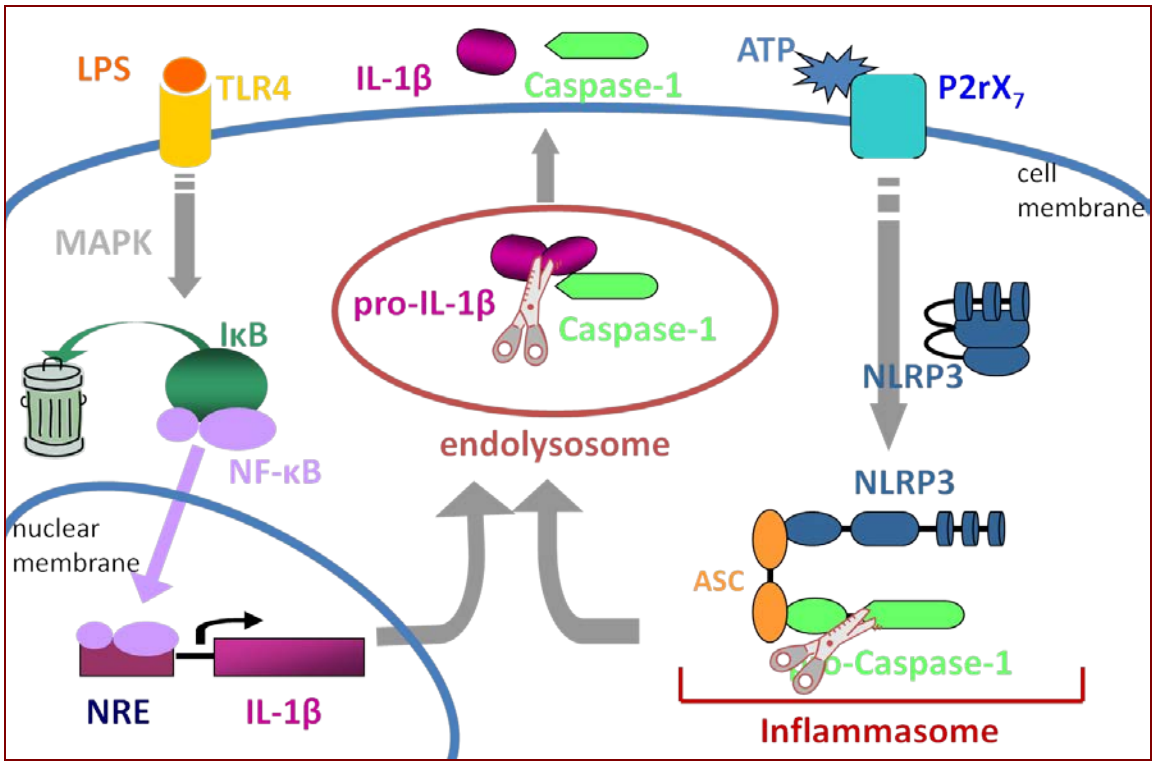


Figure 1.1. LPS and ATP stimulated IL-1β production. IL-1β production is mediated by 1) NF-κB dependent transcription, and 2) Caspase-1 mediated cleavage of pro-IL-1β to form a mature protein capable of signaling. The pathogen-associated molecular pattern (PAMP) endotoxin (LPS) is recognized by Toll-like receptor 4 (TLR4). Signaling through TLR4 results in NF-κB activation and induced transcription of IL-1β. IL-1β production is also regulated at the protein level by the NLRP3 inflammasome. The NLRP3 inflammasome is activated by the danger-associated molecular pattern (DAMP) of high extracellular ATP (concentrations >500 times the physiological level) (25). ATP binds to $P2rX_7$ (purinergic receptor P2X, ligand-gated ion channel, 7), which is an ATP-dependent potassium channel. $P2rX_7$ allows rapid efflux of K^+ from the cell. Subsequently, NLRP3 unfolds, and its exposed pyrin domain forms homodomain interactions with the pyrin domain of ASC. ASC is an adaptor protein that also binds pro-Caspase-1 through CARD homodomain interactions. Pro-Caspase-1 is autocatalytically cleaved and activated Caspase-1 then cleaves pro-IL-1β inside of intracellular vesicles. IL-1β and Caspase-1 are released from the cell when the vesicles fuse with the plasma membrane (24).

NLRC4, or Ipaf, responds to intracellular bacterial virulence factors and nucleates an inflammasome that contains either NLRC4 and Caspase-1 with ASC, or NLRC4 and Caspase-1 alone and requires NAIP5 for activation. *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Shingella flexneri* activate a ASC-dependent NLRC4 inflammasome, whereas Caspase-1 activation in response to *Legionella pneumophila* requires NAIP5, but not ASC (26). Purified recombinant flagellin has also been shown to increase IL-1β production in an NLRC4-dependent manner, indicating that flagellin alone can activate the NLRC4 inflammasome. Correspondingly, *S. typhimurium* and *L. pneumophila* that are deficinent in flagellin have a reduced ability to activate Caspase-1. However, *S. flexneri* does not express flagellin; its recognition factor is unknown. The intracellular delivery of virulence factors through a Type III or Type IV secretion system is critical for NLRC4 activation. This finding helps to separate the NLRC4 inflammasome pathway as a virulence factor-sensing pathway that is independent of TLR5, which senses flagellin that is presented extracellularly (21, 27).

AIM2 is not an NLR protein, but nevertheless it contains a PYD that is capable of binding ASC and forming an inflammasome complex involved in both IL-1β and IL-18 production, as well as inflammasome-mediated cell death. Elicited peritoneal and bone marrow-derived macrophages from AIM2-deficient mice have impaired Caspase-1 cleavage and IL-1β and IL-18 release, and they are protected from cell death following transfection with double-stranded DNA (dsDNA). Biologically, this translates to AIM2 mediating inflammasome activation in response to some bacteria and viruses, specifically *Francisella tularensis*, *L. monocytogenes*, vaccinia virus, and mouse cytomegalovirus (28). Immunofluorescence micrographs of macrophages infected with *F. tularensis* brilliantly show cellular localization between dsDNA, AIM2, and ASC (29). This result provides initial evidence that an inflammasome complex can directly perceive pathogen virulence factors (30). The absence of the NACHT and LRR domain also confirms the capacity for the PYD to mediate inflammasome complex formation, at least for AIM2.

The NLRP6 inflammasome was recently described. NLRP6 complexes with ASC and Caspase-1 to form an inflammasome which mediates pro-IL-18 cleavage (31, 32). In vivo, *Nlrp6*-deficient mice have exacerbated intestinal inflammation and an increased propensity for tumors in dextran sodium sulfate models of colitis (31-33). Phenotypes are similar to those of *Pycard*-deficient mice (31).

The inflammasomes differ slightly between humans (depicted in Table 1.1) and mice. The PYD of mouse NLRP1b is non-functional, and instead the C-terminal CARD binds directly to Caspase-1, whereas the CARD of human NLRP1 binds to caspase-5, another pro-inflammatory caspase. Human NLRP3 inflammasome complex may also contain CARD8/CARDINAL. The molecular contribution of CARD8 towards Caspase-1 activation and IL-1β production is not well-described, and CARD8 is absent from the mouse genome (34). However mutations in CARD8 are associated with increased risk for Crohn's disease and ulcerative colitis and increased IL-1β levels in some populations (35, 36). However, despite these differences, the function of the inflammasomes is well-conserved between humans and mice.

The inflammasome complex provides a unique signaling pathway that is amendable to manipulation to specifically affect IL-1β and IL-18 production without affecting production of other cytokines. Previous research indicates that both endogenous and pathogen-derived molecules can interfere with this pathway. This is important on a clinical level, because it suggests that continued research will help to direct production of pharmaceutical agents that can interfere with IL-1β production in a more specific manner. In theory, the inhibition of aberrant, disease-associated IL-1β production will be possible without limiting crucial pathogen-stimulated IL-1β and IL-18 signaling. Pyrin regulates inflammasome-mediated IL-1β production. Contradictory models suggest that pyrin nucleates its own Caspase-1-activating inflammasome, or that Pyrin inhibits activity of the NLRP3, or perhaps all, inflammasome complexes. Directed

therapies to influence Pyrin's molecular functions are a promising avenue for improved therapies for immune disorders.

Inflammasome-mediated autoinflammatory disorders

Autoinflammatory disorders, by definition, are innate immune responses in the absence of an identifiable stimulus. Familial Mediterranean Fever (FMF) is part of a group of hereditary autoinflammatory disorders. It was originally identified as a unique disorder in 1948, and named benign paroxysmal peritonitis (37) and later periodic disease (38), periodic fever, and Siegal-Cattan-Mamou syndrome. Today, periodic disease describes a family of genetic autoinflammatory disorders that include: Cryopyrin-Associated Periodic Syndromes (CAPS), PAPA syndrome (Pyogenic sterile arthritis, pyoderma gangrenosum, and acne), Blau Syndrome, early-onset Sarcoidosis, Familial Hibernian Fever (also known as TNF-Receptor Associated Periodic Syndrome, or TRAPS), Hyperimmunoglobulinemia D with recurrent fever syndrome (also known as mevalonate kinase deficiency), PFAPA syndrome (Periodic fever, aphthous stomatitis, pharyngitis, and adenitis), and Deficiency of the Interleukin-1–receptor antagonist syndrome.

FMF, CAPS, and PAPA diseases are all characterized by spontaneous inflammation or inflammation in response to innocuous stimuli, such as cold temperatures (39), and have similar symptoms, including elevated levels of IL-1β and neutrophil deposition within inflamed tissues (Table 1.2). Mutations in Pyrin, NLRP3, and PSTPIP1 cause FMF, CAPS, and PAPA syndrome, respectively, and have all been shown to influence IL-1β production. NLRP3 enhances IL-1β production. The vast majority of CAPS-related mutations in NLRP3 are missense mutations within the NATCH domain, and it is hypothesized that they result in constitutive unfolding of NLRP3 and increased sensitivity to stimuli. Thus, these mutations provide a gain-of-function with respect to IL-1β production (34). Studies have contradictorily shown that Pyrin increases or decreases IL-1β production. How mutations affect Pyrin's

function, and why there are discrepancies in the results of various studies, is unclear. The proposed mechanisms by which Pyrin alters IL-1β production will be discussed further in the following sections. PSTPIP1 (also known as CD2BP1) binds to Pyrin, and this interaction influences the binding of Pyrin to ASC. Mutations in PSTPIP1 increase PSTPIP1's binding affinity for Pyrin (40, 41). In one model, an interaction between Pyrin and PSTPIP1 leads to decreased binding between Pyrin and ASC. In this model, Pyrin and ASC interaction functions to decrease IL-1β production (41). However, an alternative model depicts that PSTPIP1-Pyrin interaction unfolds Pyrin and reveals the domains necessary for Pyrin to bind ASC. In this model, Pyrin and ASC interaction forms a pyroptosome that promotes IL-1β production and cell death (40). Despite the differences in these two models, they both posit that PAPA syndrome-associated mutations in PSTPIP1 increase IL-1β production through a mechanism mediated by Pyrin and the inflammasome.

Table 1.2. Symptoms and genetic alterations of the inflammasome-mediated autoinflammatory disorders. .

Disorder	Symptoms	Genetic Alteration
Familial Mediterranean Fever (FMF)	Fever, Peritonitis, pleuritis, Arthritis, Skin rashes, polymorphonuclear infiltration at sites of inflammation, Amyloidosis, Elevated IL-1β	Missense mutations in Mefv; Autosomal recessive
Cryopyrin-associated Periodic Syndromes (CAPS) • Familial cold autoinflammatory syndrome • Muckle-Wells syndrome • Neonatal-onset multisystem inflammatory disorder	Skin rashes (uticaria) in response to cold, polymorphonuclear infiltration at uticarial sites, Fever, Hearing loss, Arthritis, Amyloidosis, Meningitis, Inflammation of the eyes, Elevated IL-1β	Missense mutations in NLRP3 (formerly Cryopyrin); Autosomal dominant
PAPA syndrome (Pyogenic sterile arthritis, pyoderma gangrenosum, and acne)	Arthritis, polymorphonuclear infiltration at sites of inflammation, Skin rashes with ulcerative lesions, cystic acne, Elevated IL-1β	Point mutations in PSTPIP1; Autosomal dominant

The similarities among these diseases, and especially the common pathologies of elevated IL-1β and neutrophilia support a model that NLRP3, Pyrin, and PSTPIP1 function within the same signaling pathway to affect innate immune responses. Further evidence to support a function for Pyrin in IL-1β production and neutrophil dynamics will be discussed later in this chapter. First, I will provide a conceptual framework for these function of Pyrin by describing the expression pattern of *Mefv* and the molecular interactions previously reported for Pyrin.

The FMF-associated gene, Mefv, encodes the protein Pyrin

The Mefv gene is part of chromosome 16 in the human (5, 6), chimpanzee, and mouse genomes, chromosome 6 in the dog, and chromosome 10 in the rat genome. In both mouse and human, the genomic region contains 10 exons, which are all part of the coding region (Figure 1.2). The corresponding human and mouse Pyrin proteins are also of similar length: 781 and 767 amino acids, respectively.

The promoter region of human Mefv contains regulatory elements associated with inflammation-dependent transcription. Specifically, C/EBPα and NF-κB response elements are necessary for mediating the increase in *MEFV* expression induced by tumor necrosis factor (TNF)-α (42). In mice, cytokine-induced Pyrin expression is prevented by the genetic loss of *Stat6* and *NF-κB*, indicating that these elements are critical to the regulation of *Mefv* expression (43), although the regulation of *Mefv* may be a downstream effect of another cytokine that is regulated by Stat6 and NF-κB elements, such as IL-4 or IFNγ. Thus, it appears that *Mefv* expression is regulated somewhat differently in humans and mice.

Alternative splicing of both the human and mouse transcripts has been detected. In humans, there are isoforms lacking one or more exons: exon 2 (del2), exons 3 and 4 (del34), exons 2,3,4 (del234), exons 2,3,4,5 (del2345), exon 7 (del7), and exon 7 and 8 (del78). These are found in both FMF-patients and healthy controls, suggesting that mutations in *MEFV* may not influence splicing, and that alternative splicing may not have a pathological effect (44). In peripheral blood leukocytes and sonovial fibroblasts, splice variants with alternative exons 2a and 4a in place of 2 and 4, respectively, have been identified. The 4a exon substitution creates a frame-shift and a predicted truncated protein ending in exon 5. A transcript that contains an extended version of exon 8 (8ext) and lacks exons 9 and 10 is induced by LPS and accounts for 27% of transcripts in sonovial fibroblasts. Transcripts with del2 or 2a combined with 4a or 8ext were also detected (45). In mice, alternative splicing replaces exon 9 with 9a. It remains unclear if these splice variants provide wild-type function in a similar, increased, or decreased capacity, or if they have novel function or no function at all.

Structural analysis of the Pyrin protein places it in several protein families. The human Pyrin protein, also known as tripartite motif-20 (TRIM20) and marenostrin, contains five functional domains: a pyrin domain (PYD), a bZip domain (bZ), a B-box domain (BB), a coiled-coiled domain (C-C), and a B30.2 domain (Figure 1.2). The PYD is common to death-domain

family proteins (46). Within this family are NLRs, several of which function in innate immunity, and specifically, IL-1β production. As previously mentioned, the PYD mediates homodomain interactions that are essential to the formation of the inflammasome complexes (47). However, in contrast to NLRs which contain a NBD and a LRR domain, the only common domain of Pyrin is the PYD. Thus, Pyrin is part of a family of POPs (PYD-only proteins) that inhibit inflammasome-dependent IL-1β production (48). Pyrin also has a B30.2 region with a PRY-SPRY domain, which places it in the TRIM (tripartite motif) protein family. Nearly 150 proteins contain a SPRY domain. It forms an immunoglobulin-like structure that mediates protein-ligand and protein-protein interactions and is implicated in pathogen detection. For example, allelic differences in TRIM5α change the capacity for TRIM5α to recognize the HIV viral capsid (49). The majority of FMF-associated mutations are found in the SPRY domain, suggesting that the SPRY domain provides an essential function. However, conservation of the Pyrin protein between mice and rats, which both lack the PRY-SPRY domain (7), indicate that genetic selection pressure remains, despite the loss of this region. Therefore, the PRY-SPRY domain is unlikely to account for all of the functions of Pyrin.

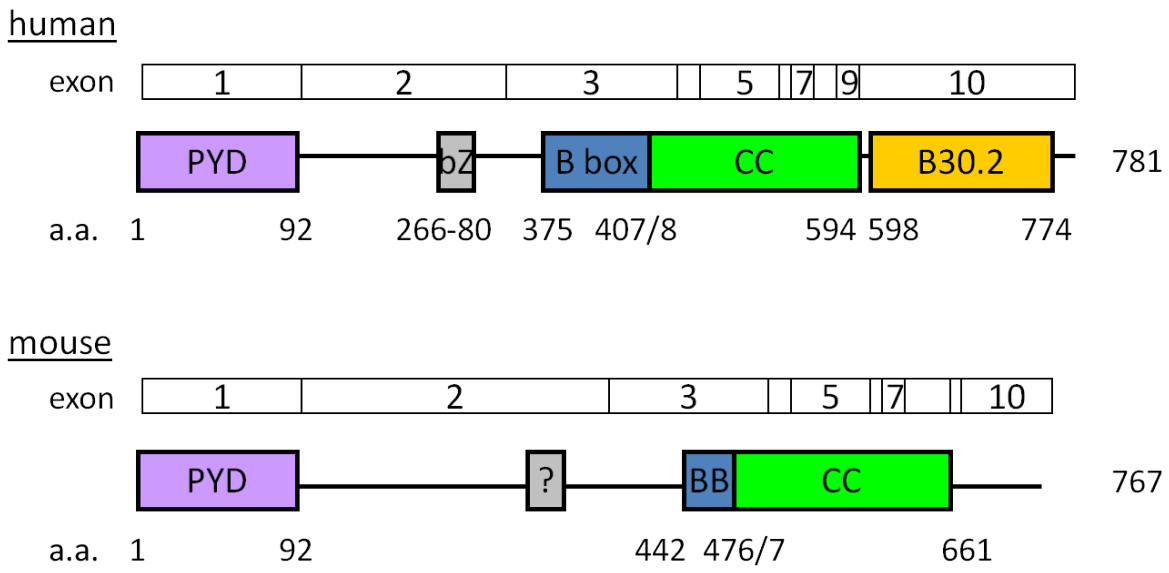


Figure 1.2. Mefv encodes Pyrin. The orthologous Mefv genes of the human and mouse genomes both contain ten exons and encode proteins with a high degree of homology. Gene exons are shown as unfilled boxes and align with protein domains (filled boxes) as indicated. Exons 1-8, except exon 2, are especially similar between humans and mice. The B30.2 domain of the human protein is missing from the mouse. The mouse protein contains the bZip domain (bZ) sequence, but it is unclear if its function is conserved. PYD, pyrin domain; BB, B-box; C-C, coiled-coiled domain.

The human and mouse proteins contain a high degree of similarity: 47.6% identity, 65.5% similarity. The PYD is encoded by the first exon and is especially well-conserved between human and mouse, providing further evidence of the importance of the PYD in overall Pyrin function. The BB and CC are also conserved between human and murine Pyrin, and the bZ is likely present in the mouse protein as well as the human protein. The B30.2 domain that is present in human Pyrin, is however, absent from the mouse and rat protein (7). Based upon the high degree of identity at the nucleotide level, 59%, it is believed that the B30.2 domain is absent due to a pre-mature stop codon created by an ancient frame-shift mutation (7). As will be discussed in detail later, most of the common FMF-associated mutations in Pyrin cluster

within the B30.2 region of the human protein, creating a conundrum as to how function is conserved between mice and humans. It is, however, clear that there are disease associated mutations which lie outside of the B30.2 region (50-54).

Expression of Mefv

Mefv expression is highest in immune tissues in both humans and mice. In humans, significant expression can be detected in the spleen, lung, and muscle tissues and very low levels of expression are detected in several other human organs by RT-PCR (8). At the cellular level, expression is highest in neutrophils and macrophages, and there is low expression in B220+ B cells, CD3+ T cells, eosinophils, dendritic cells, and epithelial cells of the lung, peritoneum, and synovium (8, 45, 55). Rodents may have a more restricted expression pattern of *Mefv* in immune tissues. By northern blot analysis, expression was detected in the spleen of mice and rats and also in the lung and kidney of rats. Expression was not detected during mouse embryonic development. In the murine spleen, in situ hybridization showed that expression was concentrated in the primary follicle and marginal zone regions, which contain mostly granulocytes and B cells. At the cellular level, Pyrin is expressed in granulocytes and macrophages, but not lymphocytes (7).

At the sub-cellular level, human Pyrin has been detected in both the nucleus and cytoplasm of cells. The intracellular localization of Pyrin appears to be affected by cell type, differential splicing, and Caspase-1 cleavage (45, 56, 57). Full-length Pyrin is predominantly found in the cytosol (8), and it co-localizes with perinuclear ASC-specks (47), and cytoskeletal elements (58, 59). The del2 splice variant (56), and the N-terminal Caspase-1-mediated cleavage fragment of Pyrin, however, appears to localize to the nucleus. It is unclear if any mutations within the coding region, such as those associated with FMF, affect Pyrin localization. In COS-7 cells

overexpressing human Pyrin, localization was unaffected by M694V or V726A mutations (8). Less is known about the cellular localization of murine Pyrin. Since there are fewer splice variants described for the mouse transcript and protein cleavage is not described, protein localization may be less complex in mouse cells.

Pyrin expression in humans and rodents is regulated by sterile immune stimuli and cytokines. Lipopolysacchride (LPS), interferon (IFN)-γ, and IFN-α increase expression of *MEFV* in human macrophages (55), while IL-4, IL-10, and transforming growth factor (TGF)-β can abrogate the effect of increased expression following a combined treatment of LPS and IFN-γ (55). There are some differences between humans and mice in the induction of Pyrin expression. IFN-γ dose-dependently increases expression at the transcript and protein level in human macrophages (55, 57). In mice, IFN-γ increases *Mefv* expression at the transcript level, although it has also been reported that a difference in Pyrin expression was not seen at the protein level in murine macrophages (43). LPS, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12 increase expression of Pyrin in murine macrophages. The T_H2-inducing cytokines IL-4 and IL-10 are the strongest inducers of mouse Pyrin expression (43), but perhaps not human Pyrin. Instead, IL-4 treatment inhibits expression at the transcript level, and IL-10 induces cleavage of the human Pyrin protein (57). *In vivo* intratracheal administration of LPS or silica increased levels of *Mefv* in rats (60). Thus, Pyrin expression is regulated by endogenous and exogenous immune stimulants, both *in vitro* and *in vivo*. Mutations in Mefv that are associated with FMF may increase, decrease, or have no effect on levels of Pyrin expression.

Molecular interactions

Previous research has demonstrated binding between Pyrin and several other proteins, cytoskeletal elements, and itself (Figure 1.3). These interactions have fueled multiple

hypotheses for the function of Pyrin. In this section, I will describe the previously-reported molecular interactions of Pyrin. The functional relevance of these interactions will be discussed in subsequent sections, as they pertain to IL-1β production and neutrophil physiology.

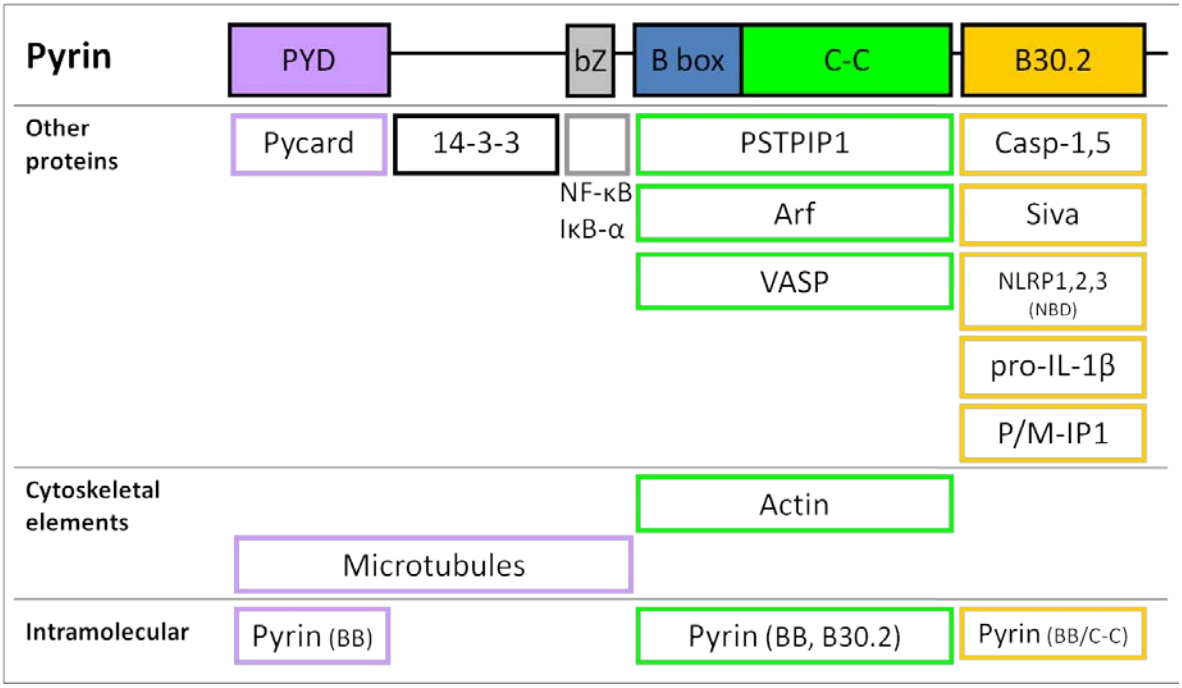


Figure 1.2. Pyrin's molecular interactions. Through co-immunoprecipitation experiments and/or co-localization experiments using cells overexpressing Pyrin, the human Pyrin protein has been shown to interact with several proteins, itself, and cytoskeletal elements. These are shown as unfilled boxes placed under the domains in Pyrin that are necessary for the molecular interaction. This figure summarizes findings from several groups using four different antibodies (43, 47, 57, 59, 61-65). The interaction of Pyrin with ASC, NLRP3, and Caspase-1 is reported by more than one laboratory. Published work indicates that the mouse Pyrin protein also interacts with ASC, but the other interactions have not been demonstrated for the mouse protein. Murine Pyrin does not contain the B30.2 domain, so these interactions are especially unclear. PYD, pyrin domain; bZ, bZip domain; C-C, coiled-coiled domain.

Protein interactions

An interaction between Pyrin and ASC was demonstrated through a yeast two-hybrid screen of the human genome and through immunofluorescence microscopy and coimmunoprecipitation of endogenous human and mouse Pyrin with ASC proteins (47). Several studies indicate that Pyrin's interaction with ASC affects production of IL-1β (40, 43, 61, 66, 67) and inflammation-associated cell death (40, 43, 47).

The SPRY domain of Pyrin interacts with the NACHT domain of NLRPs 1-3 the pro domain of pro-IL-1β, and Caspase-1 based upon co-immunoprecipitation experiments (61, 62). These interactions support multiple models of ASC-independent contributions of Pyrin to IL-1β regulation.

An interaction between Pyrin and 14-3-3 has also been shown. 14-3-3 is implicated in multiple signal transduction pathways and in protein shuttling across the nuclear membrane (64). It is reasonable to hypothesize that 14-3-3 mediates cytoplasmic-nuclear shuttling of Pyrin.

A co-immunoprecipitation experiment identified an interaction between Pyrin and Siva1. Siva1 is proapoptotic protein, and its interaction with Pyrin reduced the ability of Siva1 to induce apoptosis (63). However, in a screen of gene networks involved in FMF pathogenesis, Siva1-mediated apoptosis was not linked to FMF. Gene network analysis in THP-1 human monocytes supports an interaction of *Mefv* with *Pycard*, *Caspase-1*, *Nlrp3*, *Pstpip1*, *NF-κB*, and *Interferon-α*, but the gene networks for *Mefv* and *Siva1* do not overlap (68).

Cytoskeletal interactions

In an initial yeast two-hybrid screen for proteins that interact with Pyrin, human Pyrin was shown to interact with a novel protein, dubbed Pyrin/marenostrin interacting protein 1 (P/M-IP1). M694V and V726A mutations decreased, but did not eliminate, protein interaction in yeast cells.

P/M-IP1 and Pyrin co-localized to perinuclear structures in Cos-7 cells (69). Based upon these findings, Pyrin was believed to have a role in Golgi-mediated transport. A direct interaction in human cells has not been demonstrated by coimmunoprecipitation experiments. Further experiments have failed to demonstrate co-localization of Pyrin with Golgi structures. In fact, paclitaxel or colchicine treatment of Cos-7 cells, at a dose capable of diffusing Golgi structures, did not disrupt the cellular distribution of Pyrin (58).

Immunofluorescent co-localization and an *in vitro* binding assay were used to show that Pyrin associates with microtubules. However, the common FMF-associated mutations M680I, M694V and V726A mutations did not affect binding, which limits support for the hypothesis that Pyrin's association with microtubules has functional consequences that are important in FMF pathophysiology. In the same study, Pyrin also co-localized with actin depolymerization ends located at cell membrane ruffles on the lagging end of HeLa cells (58). Subsequently, another group found that Pyrin co-localized with actin, but not microtubules, and α-actin coimmunoprecipitated with Pyrin (59). Furthermore, the toxin Cytochalasin D, which inhibits actin polymerization, disrupted the cellular distribution of both actin and Pyrin. However, in this study, Pyrin and ASC co-localized in HeLa cells to the leading edge of cell membrane ruffles that are rich in polymerizing actin. VASP and Arp3 proteins are important in actin polymerization, and a coimmunoprecipitation experiment using myc-labeled Pyrin and GFP labeled VASP or Arp3 showed that these proteins can bind to Pyrin. VASP and Arp3 also co-localized to ASC specks in the presence but not the absence of Pyrin (59). Thus, these studies suggest that Pyrin may coordinate interaction between ASC specks and the polymerizing tails of actin at an immunological synapse.

Pyrin binds to proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1), which is associated with PAPA syndrome, as previously mentioned. The B box and coiled-coiled region of Pyrin is necessary and sufficient for an interaction with PSTPIP1 (40, 41). PSTPIP1

may influence Pyrin's function with cytoskeletal elements. PSTPIP1 binds to PTP-PEST, which is a scaffolding protein that associates with actin through the protein WASP (70). Pyrin can co-localize with actin even in the absence of ASC and PSTPIP1, indicating that Pyrin's interaction with actin is not mediated by ASC or PSTPIP1 (59). Rather, Pyrin recruited PSTPIP1 to ASC specks. The PAPA-associated mutation W232A in PSTPIP1 prevented its recruitment to ASC specks (59), but the A230T and E250Q mutations caused an increase in recruitment (71). In summary, through both direct interactions and indirect protein-protein mediated interactions, Pyrin is implicated in cytoskeletal function and could influence cell shape, cell migration, and organization of an immunological synapse (70).

Intramolecular interactions

In one study, IL-1β interacted more strongly with independent domains of Pyrin compared to full-length Pyrin. Further research based upon this observation showed that the SPRY domain interacts with the BB and C-C domains of Pyrin, and masks the interaction of the SPRY domain with pro-IL-1β in coimmunoprecipitation studies. However, M694V mutation did not affect intermolecular binding or intramolecular binding, so the functional significance of these interactions remains unclear (62). The BB region of Pyrin has also been shown to bind the PYD of Pyrin and interactions of these domains forms Pyrin homotrimers (40). In contrast, however, a yeast two-hybrid assay failed to detect intramolecular interactions of human Pyrin, when expressed in yeast cells (69).

Previous research implicates Pyrin in IL-1β production

Several lines of evidence indicate that Pyrin affects IL-1β production. First, some FMF patients have elevated serum IL-1β during inflammatory attacks and blocking IL-1β signaling is an effective prophylactic treatment (61). Second, controlled experiments indicate a role of Pyrin

in IL-1β production in human and mouse experimental systems. Third, Pyrin interacts with proteins of the inflammasome complex, NF-κB and IκB-α, and IL-1β itself. However, there are still outstanding questions. First, it is unclear if WT Pyrin acts to positively or negatively regulate IL-1β production. Second, do mutations in Pyrin cause a loss or gain of function relative to WT Pyrin that affects IL-1β production? Does the mutant Pyrin protein also have novel functions? Third, does Pyrin affect IL-1β transcription, pro-IL-1β protein processing that is either dependent or independent of inflammasome complexes, and/or IL-1β cytokine release from the cell?

In the sections below, I will detail the evidence that supports several hypotheses as to how Pyrin affects IL-1β production. Some of the proposed models directly conflict with one another as to the function of Pyrin, and are thus unlikely to occur simultaneously. The validity of these models is not necessarily mutually exclusive and may be context (i.e. pathogen) dependent. It is also possible that more than one of these mechanisms facilitate concurrent and/or synergistic effects.

Pyrin is a POP that negatively regulates IL-1β production

Based upon a high percent of amino acid sequence identity within predicted CARD and pyrin (PYD) domains, a group of proteins was identified within the human proteome which contain a CARD or PYD, but lack other domains necessary to propagate inflammasome activity. These proteins are predicted to bind ASC, Caspase-1 or the NLR protein through cognate domain interactions, and sequester them to prevent formation of productive inflammasomes. These proteins are called COPs (CARD-only proteins) and POPs (PYD-only proteins). Structurally, Pyrin is considered a POP. Results using *in vitro* overexpression experimental systems support the COP and POP model for the CARD-containing inhibitors COP (CARD-only protein), INCA (inhibitory CARD) and ICEBERG (72-74) and the pyrin-containing inhibitor cPOP2 (cellular pyrin-only protein 2). POP1 (pyrin-only protein 1) was originally shown to increase IL-1β production, but a recent protein binding study suggests that POP1 may also

decrease NLR-dependent IL-1β production (75-78). The *in vivo* function of these genes has not been examined, because they are absent from the mouse genome. Conversely, *Caspase12* deficient mice display increased IL-1β cytokine production, suggesting that it negatively regulates inflammasome activity *in vivo*. Thus, there is support for the model of COPs in an *in vivo* system. However, the significance of Caspase-12 in humans is questioned because the gene structure varies between mouse, dog, and human genomes (79). Pyrin is the only COP or POP that has been shown to negatively regulate IL-1β production *in vitro* and *in vivo* in both humans and mice.

More specifically, the POP model proposes that Pyrin competes with NLRs, specifically NLRP3, for interaction with ASC, resulting in less Caspase-1 activity and less mature IL-1β production. In a mixed lymphyocyte system using lysates from PT67 mouse epithelial cells overexpressing human Pyrin, ASC, and CASP-1, CASP-1 interacted with ASC in the absence, but not the presence of Pyrin. Pyrin coimmunoprecipitated with ASC regardless of CASP-1 expression. Together, these findings indicate that Pyrin competes with CASP-1 for binding to ASC (43). Using reconstituted HEK293T human epithelial cells, Pyrin was also shown to compete with NLRP3 for binding to ASC (65). In another study, Pyrin was shown to dose-dependently reduce IL-1β production in transiently-transfected HEK293T (80). U937 human monocytes overexpressing Pyrin also had decreased IL-1β production in response to LPS compared to cells treated with empty vector. The M694V mutation reduced but did not eliminate the regulatory effect of Pyrin (43). Correspondingly, a knockdown of endogenous Pyrin expression by THP-1 cells was shown to increase IL-1β production in response to LPS (61), peptidoglycan (PGN) and uric acid crystals (MSU) (62).

Pyrin forms an inflammasome which positively regulates IL-1β production

An antithetical model proposes that Pyrin's interaction with ASC enables the formation of a Pyrin inflammasome that activates Caspase-1 and leads to increased IL-1β production. This

model draws upon the similarity in structure between Pyrin and NLR proteins. The PRY/SPRY domain of human Pyrin forms an immunoglobulin receptor-like structure that could facilitate pathogen recognition in a manner analogous to the predicted receptor function of the LRR of NLR proteins. In a HEK293T cell overexpression system, increasing levels of Pyrin dose-dependently increased Caspase-1 processing and IL-1β cleavage. SPRY domain mutants M694V and V726A were also able to increase Caspase-1 processing at levels similar to WT Pyrin. Overexpression of WT or M694V, but not the PYD mutant L16P/F24S, also induced ASC oligomerization. Furthermore, a Pyrin-ASC complex was shown to coimmunoprecipitate Caspase-1. These data are consistent with a model in which PYD-PYD interactions between Pyrin and ASC mediate the formation of an inflammasome complex (67). Another group also showed that Pyrin dose-dependently increased Caspase-1 activity and IL-1β release from HEK293T cells reconstituted with Pyrin, ASC, and Caspase-1, with or without IL-1β, and without an NLR. A knock-down of Pyrin expression in the human monocytic cell line THP-1 and in human peripheral blood mononuclear cells resulted in decreased IL-1β release in response to LPS (66). In support of this model, there is positive selection for mutations within the B30.2 domain that is likely to function as a ligand receptor, suggesting that mutations in Pyrin may provide some selective advantage against a pathogen. The biggest caveat of this model is a lack of evidence to support a pathogen that requires Pyrin for an efficient immune response, and specifically, IL-1β production.

It is also reasonable to imagine that Pyrin self-regulates its function by masking functional domains through intramolecular interactions. In this model, Pyrin increases IL-1β production through its PYD domain, but the PYD domain must be exposed. The B30.2 domain inhibits this function by hiding the PYD domain until a ligand causes unfolding of Pyrin. If this were true, a mutation in the B30.2 domain could permanently expose the PYD domain and lead to increased IL-1β production in the absence of a ligand. This model follows a paradigm proposed for

NLRP3 that suggests that mutations in the NATCH domain cause its PYD domain to be permanently exposed. It is important to note that NLRP3 is a positive regulator of IL-1β production. By analogy then, it is also possible to explain how Pyrin could be a positive regulator of IL-1β production, and mutations in Pyrin lead to increased IL-1β production.

Pyrin forms a pyroptosome

The Pyrin-ASC interaction is the central evidence for yet a third model. In this model, Pyrin and ASC complex into a "pyroptosome" that increases apoptosis during inflammation-induced cell death processes. IL-1β release may increase during this process, but is not produced in an active process, and is instead a by-product of cell death (40). HEK293T cells transfected with full-length Pyrin or the PYD only, but not Pyrin with a deleted PYD, showed increased apoptosis (65). In another sudy, however, Pyrin increased ASC-speck formation, which is associated with inflammasome-mediated cell death, but the ASC-speck positive cells actually had increased survival (47). Additionally, Pyrin has been shown to interact with Siva, a proapoptotic protein (63); however, there is no evidence to support a functional outcome of this interaction (68).

Pyrin's interaction with Caspase-1 could lead to multiple outcomes

Pyrin has been shown to interact with the catalytic subunits of Caspase-1 in the absence or presence of ASC (61, 62). This interaction is suggested to have three different outcomes. First, it may function to sequester Caspase-1 and prevent Caspase-1-mediated IL-1β cleavage. This model is similar to the POP model, except in this case Pyrin binds Caspase-1 rather than ASC, and the interaction could occur before or after inflammasome-mediated Caspase-1 cleavage. In HEK293T cells overexpressing proIL-1β and Caspase-1, the addition of full-length Pyrin expression inhibited inflammasome-dependent IL-1β production induced by cold temperature (30°C). Deletion of the PYD did not prevent the inhibitory effect, and the SPRY domain alone was sufficient for Pyrin's inhibitory effect. In a dose-dependent manner, expression of the SPRY domain alone increased the amount of pro-IL-1β that co-immunoprecipitated with

Caspase-1, but decreased the amount of processed IL-1β. Therefore, Pyrin probably does not compete with proIL-1β for binding to Caspase-1, but inhibits the catalytic activity required for Caspase-1 to process proIL-1β (62). Correspondingly, another group showed in a PT67 overexpression system that deletion of the B30.2 region made the inhibitory effect of Pyrin on IL-1β production less pronounced than the inhibitory activity of full-length Pyrin. FMF-associated mutations in the B30.2 domain reduced binding of the C-terminal region to Caspase-1. Crystal structure modeling indicated that amino acids 680 and 694 are important in Pyrin-Caspase-1 interaction (61).

Alternatively, in the second model, Pyrin does not inhibit Caspase-1 activity, but instead is a substrate for Caspase-1-mediated cleavage (57, 67). The cleavage site was mapped to amino acids 330-331 in human Pyrin, which is between the PYD and BB, but the C-terminal B30.2 region was important in mediating the interaction of Pyrin with Caspase-1. FMF-associated mutations M690I, M694V, and V726A in the B30.2 domain increased Pyrin cleavage (57). However, another group demonstrated that the M694V mutation does not affect the ability of Pyrin to bind Caspase-1 (62). IFNγ, IL-4, and IL-10 induce cleavage of Pyrin (57). A panel comparing 10 healthy and 10 FMF patients showed an increased relative abundance of cleaved versus uncleaved Pyrin in FMF patients, and this correlated with increased IκB-α proteolysis. Treatment of HeLa cells with colchicine dose-dependently reduced IκB-α proteolysis (57).

The N-terminal 330 amino acid Caspase-1 cleavage fragment of Pyrin localizes to the nucleus, and the bZip domain and a short, adjacent C-terminal region bind to NF-κB and IκB-α, respectively. These interactions result in NF-κB and IκB-α translocation to the nucleus, and IκB-α proteolysis (57). In theory, this should result in increased NF-κB-dependent transcription, and since the IL-1β promoter contains an NF-κB response element, the end result should be increased IL-1β transcription. However, in a HEK293T cell overexpression system, neither WT nor M694V or V726A Pyrin expression had an effect on NF-κB-dependent luciferase activity

(67). In another study using HEK293T cells, full-length Pyrin and the PYD alone actually decreased NF-κB-dependent luciferase activity, but expression of Pyrin with a PYD deletion had no effect (65). Thus, an interaction between Pyrin and Caspase-1 has been investigate by several group and findings suggest that there are two functional outcomes. The first is sequestration of Caspase-1 to prevent cleavage of IL-1β by Caspase-1. This interaction depends on the B30.2 domain of Pyrin. The second is Caspase-1-mediated cleavage of Pyrin. Cleaved Pyrin has a subsequent function of increasing NF-κB translocation to the nucleus. Importantly, cleaved Pyrin inself is also translocated to the nucleus, which would limit interactions of the PYD of cleaved Pyrin and elements, such as inflammasome complexes and the cytoskeleton, which are found in the cytoplasm. The different functions of Pyrin could have context-dependent actions, or they could occur simultaneously. It is important to note that the first and second functions require different sub-cellular locations of the N-terminal, but not the C-terminal, region of Pyrin. Since the SPRY domain mediates Pyrin's interaction with Caspase-1, it is possible for the Caspase-1 inhibitory function of Pyrin to occur before, after, and/or simultaneously to Pyrin cleavage.

Pyrin's interaction with the cytoskeleton may mediate IL-1β production

Pyrin's interaction with cytoskeletal elements may also mediate IL-1β production. Colchicine and nacodazole, cytoskeletal disrupting agents, inhibit the ability of Pyrin to activate Caspase-1 (40). Pyrin may also regulate formation or stability of an immunological synapse of inflammasome complexes and the actin cytoskeleton. This provides a nice explanation for the therapeutic benefit of colchicine in FMF patients.

Evidence using mouse models support conflicting hypotheses

So far, the evidence detailed above comes from *in vitro* experiments, mostly using overexpression systems in immortalized cell lines. There is also evidence to support a role of Pyrin *in vivo* using three mouse model systems. In the first mouse model ($Mefv^{trunc/trunc}$), a

disruption of Pyrin in exon 3 leaves the PYD intact while removing the BB and C-C domains, and the C-terminal region corresponding to the B30.2 domain of human Pyrin. The mutation was designed to mimick FMF-associated mutations in the human B30.2 domain, and is based upon the assumption that the function of the B30.2 domain is lost as a result of FMF-associated point mutations. These mice were reported to display exacerbated innate immune responses. Macrophages had increased Caspase-1 cleavage and production of IL-1β following treatment with LPS or LPS and IL-4. Macrophages also had impaired apoptosis following LPS/IL-4 treatment. Furthermore, $Mefv^{trunc/trunc}$ mice displayed exacerbated hypothermia and an increased susceptibility to septic shock induced by LPS. Importantly, IL-1β release, macrophage apoptosis, and LPS-induced lethality of heterozygotes was similar to WT, indicating that two mutant alleles are necessary to produce an altered phenotype. The authors concluded that the truncation of the Pyrin protein resulted in hypomorphic Pyrin function that let to heightened sensitivity and response to pathogens. Thus, a biological basis for selection of allelic variants is an increased resistance to pathogens. A significant conclusion was made based upon these studies: FMF-associated mutations are hypomorphic. However, there are two major caveats to this study. First, the *Mefv*^{trunc} allele expresses a partial protein, and furthermore, expression of the PYD domain appears to be increased in these mice (43). Therefore, the Pyrin truncation mouse could represent a functional hypermorph, rather than a hypomorph, as the authors suggest. Second, the endogenous mouse protein lacks the B30.2 domain, so correlating the function of mouse Pyrin back to humans is a controversial matter.

Another mouse model system, reported recently, was designed to specifically address the role of the B30.2 domain. Knock-in mouse lines were constructed to add the B30.2 domain of human Pyrin onto the end of the mouse Pyrin protein. Specifically, exons 7–10 of mouse Mefv were replaced with exons 7–10 of human Mefv. Four mouse lines were created to introduce the WT B30.2 sequence and 3 FMF-associated B30.2 domain mutants: M964V, M680I, and

V726A. The system was designed to test the hypothesis that mutations in the B30.2 region cause a gain-of-function that leads to FMF disease. Mice with two B30.2 mutant alleles showed severe spontaneous inflammation and even developmental retardation. Pathology indicated a substantial increase in $CD11b^+$ innate immune cells in the peripheral blood and spleen of naïve homozygous V726A mutants compared to WT mice. Furthermore, homozygous mice showed aberrant Caspase-1 cleavage and IL-1β production in response to LPS alone. *Pycard* (ASC) deficiency largely rescued the phenotype of homozygous Pyrin B30.2 mutant mice so that $CD11b^+$ populations and IL-1β production was similar to WT mice. NLRP3-deficiency, however, did not rescue mice from spontaneous inflammation. Thus, the inflammatory phenotype in these mice was dependent upon inflammasome-mediated IL-1β production, but not the NLRP3 inflammasome in particular.

However, heterozygous $Mefv^{V726A/+}$ mice were healthy and had $CD11b^+$ cell populations and IL-1β production similar to WT mice, which is inconsistent with a model in which mutations promote FMF via a gain-of-function. Even more puzzling, mice homozygous for WT B30.2 were not born, suggesting that developmental retardation is too severe to be compatible with life. Interestingly, another group has observed that THP-1 cells infectected with retrovirus to express only the SPRY domain of Pyrin were not viable, whereas, in the same system, full-lenth Pyrin was not toxic (62). Furthermore, the severity of homozygous B30.2 mutant mice is inversely proportional to the severity of FMF-phenotypes for the three mutations tested. These findings are actually consistent with the hypothesis that murine Pyrin containing a wild-type B30.2 domain causes aberrant function that is incompatible with life, and mutations in the B30.2 domain cause a loss-of-function of these aberrant wild-type B30.2 domain mutations. Thus, FMF is inherited in a phenotypically recessive manner, which is typical of diseases caused by a loss of protein function. To address this possibility, Mefv-deficient mice were generated. $Mefv^{-/-}$ mice did not display spontaneous inflammation nor statistically significant increases in $CD11b^+$

cell populations or in IL-1β production detected by western blot analysis. The conclusion of this work was that B30.2 mutations cause a gain-of-function, but in a dose-dependent manner. It is important to recognize that the inability to characterize mice homozygous for the WT B30.2 domain means that this conclusion is predicated on a lack of a phenotype of the Mefv-deficient mice. While the severe spontaneous inflammation of the homozygous B30.2 domain mutant mice is an overt phenotype that would be easily identified in the $Mefv^{-/-}$ mice if it were present, it remains possible that $Mefv^{-/-}$ mice have an innate immune system phenotype that could not be distinguished by the experiments chosen to test these mice.

In summary, evidence from these studies support conflicting models of Pyrin's function, and further experiments are necessary to clarify both the contribution of Pyrin to the innate immune system and the inheritance pattern of FMF.

Evidence from human patients supports conflicting hypotheses

Evidence shows that IL-1β production is altered during FMF-associated inflammatory attacks in at least some patients. PBMC from patients with FMF have increased IL-1β production (43). Treatment with IL-1β blockers helps prevent and lessen the severity of inflammatory attacks. However, the role that mutations in Pyrin play in IL-1β production in FMF patients is less clear. The expression level of Pyrin in patients compared to healthy controls is increased, decreased, or unchanged, depending upon the patient, and is not correlated with specific mutations within *Mefv* in a qualitative or quantitative way. Moreover, mutations may affect alternative splicing, post-translational modifications, cellular localization, and/or Caspase-1 cleavage of Pyrin, and one or more of these effects indirectly changes IL-1β levels. It is most likely, however, that mutations directly affect the functional activity of Pyrin, since the majority of mutations are located in functionally significant exonic regions. In summary, it is uncertain if

mutations mediate a gain or loss of Pyrin expression, localization, or function, and thus it is unclear if WT Pyrin positively or negatively regulates IL-1β production.

The role of neutrophils in FMF

Early studies to determine the pathophysiology of FMF focused on neutrophils for three reasons: 1) Inflamed tissues in FMF patients have considerable neutrophil deposition (81), indicating that the autoinflammatory response in these patients is mediated, at least in part, by neutrophils. 2) Colchicine drug therapy can prevent FMF attacks, and while its mechanism of action in FMF is unclear, previous research indicates that colchicine is sequestered mainly by neutrophils (82, 83), so its therapeutic effects are likely to be on this cell type. 3) Neutrophils express *Mefv* at high levels (7, 55). These data support the hypothesis that mutations in *Mefv* cause altered neutrophil physiology, and the correlative nature of these data leave open many possible processes of neutrophils that could be affected by Pyrin. In the next section, the normal functions of neutrophils are reviewed, and in the following section, I will detail some of the previous research to assess the function of neutrophils in FMF patients.

Neutrophil physiology

Neutrophil Production

Neutrophils differentiate in the bone marrow from hematopoietic stem cells, through the myeloid lineage, and into the terminally-differentiated neutrophil cell type. Concurrent with development, neutrophils migrate from the marrow region across sinusoidal endothelium into sinusoid vessels within the bone. From there, neutrophils enter general circulation through the bloodstream (84). The cytokine granulocyte-colony stimulating factor (G-SCF) regulates both

the differentiation of neutrophils from myeloid precursor cells and release of neutrophils from the bone marrow (85). Neutrophils are maintained in high numbers (2.0 – 7.5 x 10^9 cells/L) within the blood under normal physiological conditions (86), but their circulatory half-life averages only 6 hours in humans and 8 hours in mice (85). The average human body produces greater than 5 – 10 x 10^{10} neutrophils each day to counter the rapid turnover (87). Neutrophil production is amplified during inflammation, in response to the cytokines G-CSF, GM-CSF, and IL-3 produced in the bone marrow, and GM-CSF, IL-6, and IL-17 produced by peripheral tissues (85). The cell surface marker Gr-1 is used to detect the differentiation of myeloid precursor cells to neutrophils. Gr-1 belongs to the Ly-6 gene family of adhesion molecules, and its expression is restricted to differentiated granulocytes, whereas it is absent from myeloid precursor cells and other hematopoietic-lineage cells (88).

Neutrophil recruitment

In the absence of infection, the location of neutrophils is limited to the bone marrow, bloodstream, spleen, and liver. Neutrophils are rapidly recruited to sites of inflammation by cytokine/chemokine concentration gradients. TNF-a, IL-1β, Leukotriene B4 (LTB_4), IL-8, and monocyte chemoattractant protein-1 (MCP-1) have all been shown to promote neutrophil recruitment to peripheral tissues (89-91). Chemotactic stimuli activate signaling pathways to alter cell surface marker expression and to facilitate cytoskeletal rearrangements. These changes result in migration of neutrophils from the bone marrow through the bloodstream and into peripheral tissues, where they help control local infections (87). *In vitro* studies to mimic the movement of neutrophils in the bloodstream have shown that neutrophils begin rolling along an endothelial cell layer within two minutes of thrombin-induced endothelial cell damage (92). *In vivo*, neutrophils can be visualized within infected tissues by 2 hours post-infection. The presence of neutrophils in peripheral tissues is typically accompanied by signs of their activation.

Neutrophil effector functions

When neutrophils reach the source of the chemoattractant, (i.e. the pathogen or the highest cytokine/chemokine concentration), their cellular profile changes. The once-migratory cells are immobilized and neutrophil effector functions initiate (87). Neutrophils propagate a localized host response at infected or damaged tissues through several effector mechanisms: 1) the release of microbicidal agents and proteolytic enzymes, including myeloperoxidase, defensins, and hydrolases, from cytoplasmic granules, 2) the release of pro-inflammatory cytokines such as IL-8 and TNFα, which recruit and activate immune cells, 3) a respiratory burst that produces reactive oxygen species that kill pathogens, 4) the phagocytosis of pathogens, and 5) the extrusion of neutrophil extracellular traps (NETs), which help immobilize pathogens.

Neutrophil lifespan

Neutrophils are short lived cells. Non-activated neutrophils survive in the bloodstream for an average of 5.4 days (86), and activated neutrophils which have migrated to peripheral tissues survive for an even briefer time period of 1 – 3 days.

As human neutrophils age, they show signs of reduced chemotaxis *in vivo*, and reduced phagocytosis and respiratory burst *in vitro*. This is likely caused by a reduction in several receptor-mediated signaling pathways, including protein kinase B, phosphoinositide-3-kinase, and JAK-STAT (Janus kinase – signal transducers and activators of transcription) signaling (93). Non-activated neutrophils are removed from circulation by migrating to the bone marrow, spleen, or liver, where they apoptose. It is suggested that age-dependent removal is important for maintaining adequate overall anti-microbial function (87).

Removal of activated, peripheral neutrophils is an important step in the resolution of inflammation. While neutrophil functions mitigate risk of the spread of infection, their release of oxidizing agents and proteases is also damaging to neighboring healthy tissue. Neutrophil

clearance from peripheral tissues occurs via apoptosis, necrosis, or phagocytosis by neighboring macrophages. Neutrophils also die via uncontrolled necrosis induced by the pathogen. A balance in the rate and extent of neutrophil influx, anti-microbial activity, and tissue clearance is critical to allow pathogen defense and prevent extensive tissue damage and chronic inflammation (94).

Evidence for altered neutrophil physiology in FMF

Neutrophil activation is altered in FMF patients

Differences have been detected in the *ex vivo* activation of neutrophils from patients with FMF compared to healthy controls. Oxidative burst in response to either phorbol myristate acetate (PMA) or monosodium urate crystals (MSU) is increased in neutrophils from FMF patients compared to healthy controls (95). Furthermore, spontaneous free radical production and chemotaxis-, phagocytosis-, and PKC- induced respiratory burst by neutrophils from FMF patients is greater compared to neutrophils from healthy controls (96). Neutrophils from FMF patients are also more effective at phagocytosing bacteria than neutrophils from healthy controls (97). Interestingly, activation of the oxidative burst and phagocytosis in neutrophils demonstrates a biphasic phenotype in which activation of neutrophils collected from FMF patients in remission is more intense than in neutrophils harvested from FMF patients during an attack period (97-99). This suggests that the inflammatory actions of neutrophils are quite variable during the course of FMF disease.

Neutrophil survival is altered in FMF patients

As previously mentioned, extreme neutrophilia at inflamed tissues during FMF attacks has been observed by histological staining, and it could be a result of increased neutrophil production, chemotaxis, survival, or retention at sites of inflammation. It is interesting to note that FMF attacks last 1 – 3 days, which corresponds to the deposition and clearance of

neutrophils from inflamed tissues. This begs the question of whether or not resolution of FMF attacks is influenced by direct alterations of the kinetics of neutrophil apoptosis. Indeed, circulating neutrophils from FMF patients have altered survival in experimental systems. Neutrophils from humans or rodents quickly die in culture. Exposure to cytokines or endotoxin (LPS) reduces the rate at which neutrophils from healthy human donors or wild-type mice spontaneously apoptose (100, 101). Neutrophils from FMF patients, however, show an increase in apoptosis following exposure to endotoxin (100). If anything, these data suggest that the accumulation of neutrophils in FMF patients is not due to increased survival or impaired apoptosis of neutrophils, but data are too limited to make a clear statement regarding the survival kinetics of neutrophils FMF patients compared to healthy controls.

Treatment of FMF with colchicine

Some of the most convincing evidence that neutrophils are directly altered in FMF is actually based on successful treatment of FMF with colchicine. Colchicine is the main therapeutic agent prescribed for patients with FMF. Colchicine is sequestered mainly by neutrophils *in vivo*, suggesting that its beneficial effects are through modulation of neutrophil physiology (82, 83). Colchicine treatment modulates chemotaxis, oxidative burst, and phagocytosis functions of neutrophils. Colchicine inhibits neutrophil chemotaxis by decreasing expression of both E- and L- selectin, which are required for neutrophil migration to inflamed tissues (102). Colchicine also inhibits release of chemotactic substances from neutrophils following phagocytosis of urate crystals (103). In neutrophils from FMF patients, colchicine blocks initiation of the spontaneous respiratory burst. Once induced, however, colchicine actually increases the intensity of the respiratory burst (104). Colchicine inhibits phagocytosis of bacteria by neutrophils from FMF patients in a dose-dependent and time-dependent manner. Suppression of phagocytosis becomes more effective as the bacterial burden decreases (97). Evidence that colchicine alters neutrophil physiology suggests that FMF inflammatory attacks

are mediated at least in part by neutrophils, and thus mutations in *Mefv* may directly affect neutrophil function.

The genetics of FMF

Polymorphisms within *Mefv* were identified by linkage analysis and positional cloning as the underlying genetic defect associated with FMF. Mutations M680V, M694I, M694V and V726A were identified in the initial analysis and are all associated with one founding haplotype (5, 6). In a later study, another founding haplotype containing the mutation E148Q was associated with FMF, adding further support for *Mefv*'s association with FMF (105). To date, these five mutations account for the vast majority of FMF patients. The first four, as well as the majority of all polymorphisms, lie within the B30.2 domain, which suggests that this domain is especially important for Pyrin's function. Figure 1.4 summarizes the polymorphisms within the Mefv gene. There are 211 sequence variants within a relatively small 2.3 kb mRNA. Ninety-one are associated with a phenotype. The polymorphisms are all missense mutations or deletion point mutations (51-54) and infers that non-sense mutations or copy number variations either have no phenotype, or conversely, are incompatible with life.

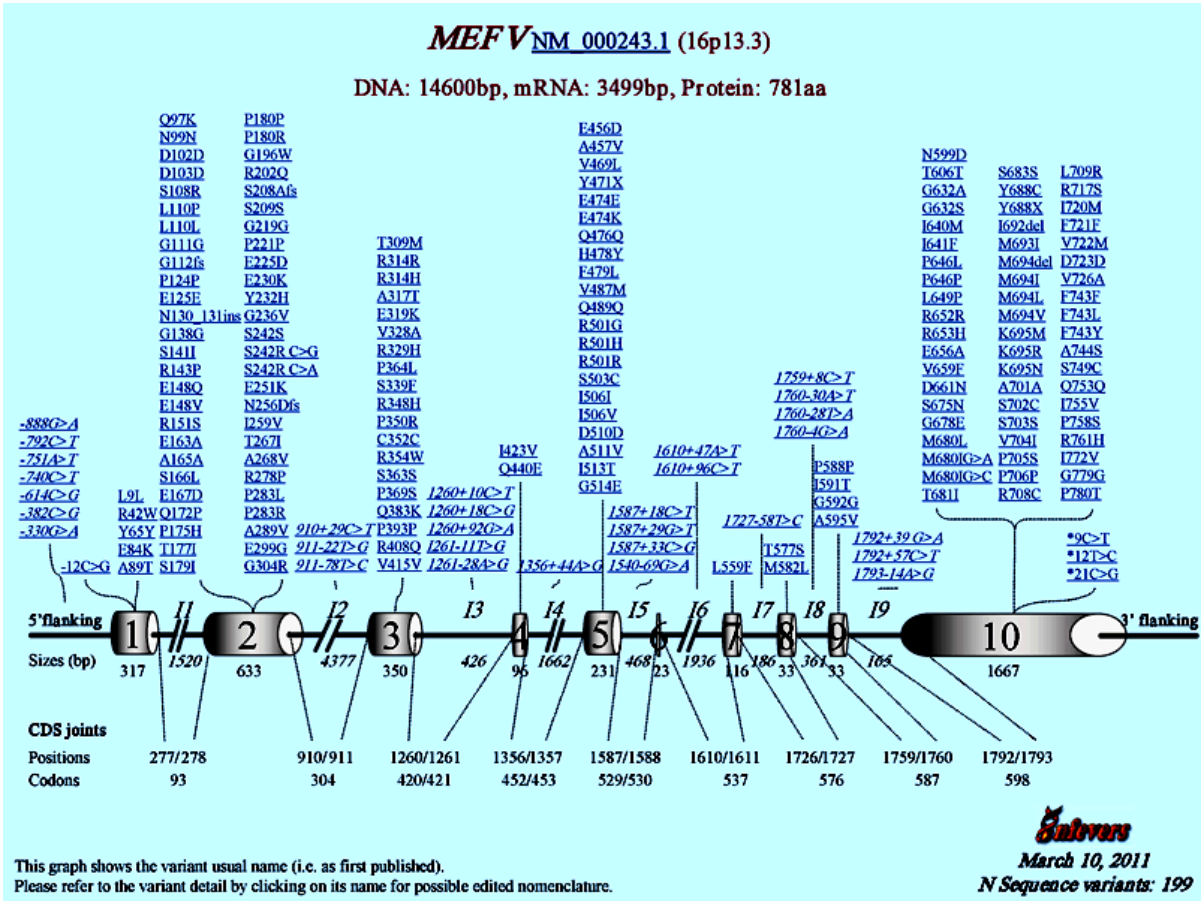


Figure 1.4. Mefv sequence variants. Polymorphisms within the Mefv gene are found primarily within exons, especially exon 10, and are single nucleotide polymorphisms that cause missense mutations or single amino acid deletions or duplications.

Molecular and pedigree analyses support a pattern of predominantly recessive inheritance of FMF. Most patients have mutations within the coding region of both *Mefv* alleles, however some individuals that fulfill Tel-Hashomer criteria (106) for FMF disease have only one mutated allele. There is a wide breadth of phenotypic variation among patients in both the severity of FMF and the penetrance of FMF-associated mutations. For example, pedigree analysis of one family indicates that 1 asymptotic grandmother and 1 asymptotic father carry the same allelic variants (M694V/V726A) as 4 grandchildren with childhood disease onset (107). M694V and

ΔM694 are associated with aggressive renal amyloidosis and poor prognosis in patients with two allelic variants, especially M694V/M694V (2). These mutations also exhibit some dominant penetrance. At least two patients with one V726A mutation and no other detectable mutations also have FMF symptoms (108). In a population genetics study in Crete, 83.1% (59/71) of patients carried 2 mutant alleles (109), but in a more recent study with 2,067 Turkish patients, only 23.7% (490/2,067) had 2 mutant alleles (110). Evidence of dominant penetrance is limited, however, by the ability to detect a second allelic variant. Genetic testing is largely confined to detection of the common mutations and/or sequencing of exon 10, which encodes the B30.2 domain (111). The studies in Crete and Turkey, for example, were limited to the 12 most common *MEFV* mutations. Many patients are compound heterozygotes, so there is an extended range of potential activity of Pyrin created by a large number of possible genotype combinations.

The five most common FMF-associated mutations originated 3,000 – 30,000 years ago in people descendent from ethnic groups originating from the Mediterranean region, especially Iraqi and Ashkenazi Jews, Armenians, and Druze populations, and have been maintained through a founding effect (112). Accordingly, FMF primarily affects ethnic groups that originate from the Mediterranean region, especially: Armenians, Sephardic Jews, Turks, North Africans, Arabs, and, less commonly, Greeks and Italians. The incidence of FMF in Armenians is 1:500. The carrier frequency is especially high in these populations as well (1). A clear association between the prevalence of disease-associated haplotypes and geographic origin suggests that an environmental stress caused the founding mutations (6).

Evolution studies indicate that there is positive selection for allelic variants of *Mefv*. Statistical analysis of the ratio of the frequency of nonsynonymous to synonymous mutations confirms a model of episodic positive selection for allelic variants of *MEFV* (113). Increased incidence of carriers in the Mediterranean area compared to other parts of the world suggests

that positive selection pressure maintains heterozygous carriers within this region due to an environmental stressor. Carrier frequencies are astonishingly high, reaching 1:3 in some sub-populations within the Mediterranean region. While the five most common FMF-associated mutations show a founding affect in genetically-isolated populations, mutations also repeatedly arise *de novo*, indicating that there is a selection for allelic variants.

Genetic evidence clearly defines *Mefv* as the FMF-associate gene, but complexities of the underlying genetics makes interpretations of functional studies difficult. Recessive inheritance of FMF suggests that a functional loss of both Mefv alleles is necessary for disease pathology. However, reports of dominant penetrance of disease argues that mutations lead to a gain-of-function. This implication is limited, however, by confined genetic testing to identify a second allelic variant. In summary, further molecular genetic, population, and functional studies are fundamental to elucidate what appears to be a complex role for Mefv in innate immunity.

Mice and Man: Using the mouse as a model organism

The immune response is generally well-conserved between human and mice, which allows the mouse to be used as a small animal model system for inflammation that is amenable to genetic manipulations and *in vivo* studies. Cross-species infection models allow for direct comparison of the dynamics of the immune response between mice and humans. Inflammatory mediators that regulate the innate immune response to these pathogens are fairly well-conserved on both a genetic and functional level. Specifically, IL-1β production and signaling can be correlated between mice and humans. For example, mouse models implicating IL-1β in disease progression have been translated to successful treatment of the corresponding disease in humans using IL-1β blocking therapies (13).

The *in vivo* immune response is an orchestrated effort of many cell types that involves cell migration, extravasation, activation, and resolution. The dynamics of these processes cannot be appreciated by any *in vitro* experiment. It is especially important to recognize the overall *in vivo* process of the immune system in the case of an autoinflammatory disorder such as FMF. Since these disorders stem from unprovoked responses in the absence of a pathogen, it is necessary to develop a mouse model system in which aberrant immune activation can be tested in the context of an endogenous system. By harnessing the power of manipulation to the mouse genome, we were able to create a system to allow for removal of the *Mefv* gene and stable *Mefv* expression. Specifically for *Mefv*, this is an important point since various overexpression and knock-down experimental systems have provided inconclusive results.

Summary and Significance of this work

The literature described above details some of the incredible work that has shaped our understanding of the recognition stage of the innate immune response and how danger signals can help guide the magnitude and duration of the immune response in order to promote necessary immune system activation and prevent autoinflammatory disorders such as FMF. Familial Mediterranean Fever (FMF) is an autosomal recessive autoinflammatory disease characterized by unprovoked fevers and acute inflammation leading to peritonitis, arthritis, pleuritis, skin rashes, and amyloidosis. In the Mediterranean region, the incidence of FMF is estimated at 1:500 Armenians (1) and between 1:3000 and 1:6000 Iranian Jews (114), indicating that FMF is a major health concern in some populations.

Current therapeutic regimes for FMF patients involve nonsteroidal anti-inflammatory drugs to alleviate acute inflammatory episodes and life-long prophylactic treatment with colchicine or IL-1β blockers (2). Colchicine drug therapy is the treatment of choice for FMF patients. Its

mechanism of action is unclear, and some patients do not respond to colchicine treatment or cannot tolerate its side effects. A better understanding of the function of Pyrin may shed light on the mechanism of action of colchicine and lead to the ability to predict whether or not colchicine will be beneficial to individual patients. Furthermore, the IL-1β blocker anakinra has been successfully used to treat colchicine-nonresponsive patients (115). This indicates that IL-1β is at least partially responsible for the symptoms of FMF.

IL-1β is a critical mediator of not just in FMF pathology, but of many innate immune responses. Its production is regulated by the inflammasome signaling pathway, which is activated by danger signals. IL-1β/IL-1R interaction drives a pro-inflammatory immune response, and approaches to block this interaction effectively decrease inflammation. Lifelong therapeutic use of IL-1β-blockers has dangerous side effects, as long-term, broad immune suppression leaves the body susceptible to dangerous pathogens. Further knowledge of the regulation of the inflammasome pathway should help in the design of tailored therapeutic approaches to more precisely control aberrant IL-1β production. Several chronic autoimmune disorders, including rheumatoid arthritis and multiple sclerosis, as well as the autoinflammatory CAPS and FMF, are treated with IL-1β blockers. While this proposal focuses primarily on characterizing the function of Pyrin in disease symptoms related to FMF, delineating the role of Pyrin will provide insight into the causes and potential treatments of all inflammatory disorders.

The overall aim of this dissertation was to assess the role of Pyrin in regulating innate immune responses. Our approach was to generate Pyrin-deficient mice and characterize these mice in a naïve state and during innate immune responses. We addressed the contribution of Pyrin to immune cell development, recruitment, activation, and survival. The first specific aim focused on determining if Pyrin modulates inflammasome-mediated IL-1β production. Although previous work has indicated that Pyrin can modulate IL-1β production, its contribution as a positive or negative regulator of IL-1β production is controversial. Here, we address this

question using a genetic approach and *in vitro* and *in vivo* experimental systems. The second specific aim of this work addresses the direct contribution of Pyrin to neutrophil physiology. We used a genetic approach and *in vitro* and *in vivo* studies to detect differences in neutrophil development, recruitment, and/or survival *in vitro* and *in vivo*. Our findings provide a framework for us to think about how mutations in Pyrin lead to misregulation of the inflammatory responses and contribute to spontaneous activation of the innate immune system in FMF.

Research presented in this dissertation

In this dissertation, I present our investigations into how Pyrin regulates innate immune responses. We generated Mefv-deficient mice, as described in chapter 2, that we used throughout this study to provide a novel *in vitro* and *in vivo* genetic system for assessing the consequences of a loss of Pyrin function. Chapter 3 describes our studies to characterize the naïve state of Pyrin-deficient mice in order to determine if these mice had altered immune cell development or indications of spontaneous inflammation. Chapter 4 details the role of Pyrin in IL-1β production. Chapter 5 discusses our work to characterize the contribution of Pyrin to neutrophil physiology. In chapter 6, we describe *in vivo* experimental models that we used to ascertain a role of Pyrin in fever and peritonitis, common symptoms of FMF. Together, this work demonstrates that a loss of Pyrin function can alter the innate immune response, and specifically, inflammasome-dependent IL-1β production. This finding suggests that a loss of Pyrin function is responsible for at least some of the pathological phenotypes seen in FMF patients.

References

1. Rosenberg, P. M., and S. E. Goldfinger. 2011. Pathophysiology of familial Mediterranean fever. In *UpToDate.com*, 2011 ed.

2. Shohat, M., and G. J. Halpern. 2011. Familial Mediterranean fever-A review. *Genet Med* 13:487-498.

3. Manukyan, G., K. Ghazaryan, Z. Ktsoyan, Z. Khachatryan, D. Kelly, M. Tatyan, M. Agababova, and R. Aminov. 2010. Comparative analysis of cytokine profiles in autoinflammatory and autoimmune conditions. *Cytokine* 50:146-151.

4. Kallinich, T., H. Wittkowski, R. Keitzer, J. Roth, and D. Foell. 2010. Neutrophil-derived S100A12 as novel biomarker of inflammation in familial Mediterranean fever. *Ann Rheum Dis* 69:677-682.

5. Consortium, F. F. 1997. A candidate gene for familial Mediterranean fever. *Nat Genet* 17:25-31.

6. Consortium, T. I. F. 1997. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. *Cell* 90:797-807.

7. Chae, J. J., M. Centola, I. Aksentijevich, A. Dutra, M. Tran, G. Wood, K. Nagaraju, D. W. Kingma, P. P. Liu, and D. L. Kastner. 2000. Isolation, genomic organization, and expression analysis of the mouse and rat homologs of MEFV, the gene for familial mediterranean fever. *Mamm Genome* 11:428-435.

8. Tidow, N., X. Chen, C. Muller, S. Kawano, A. F. Gombart, N. Fischel-Ghodsian, and H. P. Koeffler. 2000. Hematopoietic-specific expression of MEFV, the gene mutated in familial Mediterranean fever, and subcellular localization of its corresponding protein, pyrin. *Blood* 95:1451-1455.

9. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:991-1045.

10. Kono, H., and K. L. Rock. 2008. How dying cells alert the immune system to danger. *Nat Rev Immunol* 8:279-289.

11. Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425:516-521.

12. Chen, C. J., H. Kono, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* 13:851-856.

13. Hallegua, D. S., and M. H. Weisman. 2002. Potential therapeutic uses of interleukin 1 receptor antagonists in human diseases. *Ann Rheum Dis* 61:960-967.

14. Chin, J., and M. J. Kostura. 1993. Dissociation of IL-1 beta synthesis and secretion in human blood monocytes stimulated with bacterial cell wall products. *J Immunol* 151:5574-5585.

15. Pomerantz, B. J., L. L. Reznikov, A. H. Harken, and C. A. Dinarello. 2001. Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1beta. *Proc Natl Acad Sci U S A* 98:2871-2876.

16. Westphal, E., M. Herzberg, I. Neumann, L. Beibei, C. Pilowski, C. Li, K. Werdan, and H. Loppnow. 2006. Neutrophils process interleukin-1beta and interleukin-18 precursors in a caspase-1-like fashion--processing is inhibited by human vascular smooth muscle cells. *Eur Cytokine Netw* 17:19-28.

17. Faustin, B., L. Lartigue, J. M. Bruey, F. Luciano, E. Sergienko, B. Bailly-Maitre, N. Volkmann, D. Hanein, I. Rouiller, and J. C. Reed. 2007. Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell* 25:713-724.

18. Hlaing, T., R. F. Guo, K. A. Dilley, J. M. Loussia, T. A. Morrish, M. M. Shi, C. Vincenz, and P. A. Ward. 2001. Molecular cloning and characterization of DEFCAP-L and -S, two isoforms of a novel member of the mammalian Ced-4 family of apoptosis proteins. *J Biol Chem* 276:9230-9238.

19. Martinon, F., A. Mayor, and J. Tschopp. 2009. The inflammasomes: guardians of the body. *Annu Rev Immunol* 27:229-265.

20. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.

21. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10:417-426.

22. Kostura, M. J., M. J. Tocci, G. Limjuco, J. Chin, P. Cameron, A. G. Hillman, N. A. Chartrain, and J. A. Schmidt. 1989. Identification of a monocyte specific pre-interleukin 1 beta convertase activity. *Proc Natl Acad Sci U S A* 86:5227-5231.

23. Petrilli, V., S. Papin, C. Dostert, A. Mayor, F. Martinon, and J. Tschopp. 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14:1583-1589.

24. Wewers, M. D. 2004. IL-1beta: an endosomal exit. *Proc Natl Acad Sci U S A* 101:10241-10242.

25. Martinon, F. 2008. Detection of immune danger signals by NALP3. *J Leukoc Biol* 83:507-511.

26. Lightfield, K. L., J. Persson, N. J. Trinidad, S. W. Brubaker, E. M. Kofoed, J. D. Sauer, E. A. Dunipace, S. E. Warren, E. A. Miao, and R. E. Vance. 2011. Differential requirements for NAIP5 in activation of the NLRC4 inflammasome. *Infect Immun* 79:1606-1614.

27. Lamkanfi, M., T. D. Kanneganti, L. Franchi, and G. Nunez. 2007. Caspase-1 inflammasomes in infection and inflammation. *J Leukoc Biol* 82:220-225.

28. Rathinam, V. A., Z. Jiang, S. N. Waggoner, S. Sharma, L. E. Cole, L. Waggoner, S. K. Vanaja, B. G. Monks, S. Ganesan, E. Latz, V. Hornung, S. N. Vogel, E. Szomolanyi-Tsuda, and K. A. Fitzgerald. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11:395-402.

29. Jones, J. W., N. Kayagaki, P. Broz, T. Henry, K. Newton, K. O'Rourke, S. Chan, J. Dong, Y. Qu, M. Roose-Girma, V. M. Dixit, and D. M. Monack. 2010. Absent in melanoma 2 is required for innate immune recognition of Francisella tularensis. *Proc Natl Acad Sci U S A* 107:9771-9776.

30. Kanneganti, T. D. 2010. Central roles of NLRs and inflammasomes in viral infection. *Nat Rev Immunol* 10:688-698.

31. Elinav, E., T. Strowig, A. L. Kau, J. Henao-Mejia, C. A. Thaiss, C. J. Booth, D. R. Peaper, J. Bertin, S. C. Eisenbarth, J. I. Gordon, and R. A. Flavell. 2011. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 145:745-757.

32. Chen, G. Y., M. Liu, F. Wang, J. Bertin, and G. Nunez. 2011. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol* 186:7187-7194.

33. Normand, S., A. Delanoye-Crespin, A. Bressenot, L. Huot, T. Grandjean, L. Peyrin-Biroulet, Y. Lemoine, D. Hot, and M. Chamaillard. 2011. Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci U S A* 108:9601-9606.

34. Agostini, L., F. Martinon, K. Burns, M. F. McDermott, P. N. Hawkins, and J. Tschopp. 2004. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20:319-325.

35. Yang, S. K., H. Kim, M. Hong, J. Lim, E. Choi, B. D. Ye, S. K. Park, and K. Song. 2011. Association of CARD8 with inflammatory bowel disease in Koreans. *J Hum Genet* 56:217-223.

36. Franke, A., P. Rosenstiel, T. Balschun, O. Von Kampen, S. Schreiber, C. Sina, J. Hampe, T. H. Karlsen, M. H. Vatn, and C. Solberg. 2007. No association between the TUCAN (CARD8) Cys10Stop mutation and inflammatory bowel disease in a large retrospective German and a clinically well-characterized Norwegian sample. *Gastroenterology* 132:2080-2081.

37. Siegal, S. 1949. Benign paroxysmal peritonitis. *Gastroenterology* 12:234-247.

38. Reimann, H. A. 1948. Periodic disease; a probable syndrome including periodic fever, benign paroxysmal peritonitis, cyclic neutropenia and intermittent arthralgia. *J Am Med Assoc* 136:239-244.

39. Inohara, N., and G. Nunez. 2003. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat Rev Immunol* 3:371-382.

40. Yu, J. W., T. Fernandes-Alnemri, P. Datta, J. Wu, C. Juliana, L. Solorzano, M. McCormick, Z. Zhang, and E. S. Alnemri. 2007. Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants. *Mol Cell* 28:214-227.

41. Shoham, N. G., M. Centola, E. Mansfield, K. M. Hull, G. Wood, C. A. Wise, and D. L. Kastner. 2003. Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. *Proc Natl Acad Sci U S A* 100:13501-13506.

42. Papin, S., C. Cazeneuve, P. Duquesnoy, I. Jeru, D. Sahali, and S. Amselem. 2003. The tumor necrosis factor alpha-dependent activation of the human mediterranean fever (MEFV) promoter is mediated by a synergistic interaction between C/EBP beta and NF kappaB p65. *J Biol Chem* 278:48839-48847.

43. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* 11:591-604.

44. Medlej-Hashim, M., N. Nehme, E. Chouery, N. Jalkh, and A. Megarbane. 2010. Novel MEFV transcripts in Familial Mediterranean fever patients and controls. *BMC Med Genet* 11:87.

45. Diaz, A., C. Hu, D. L. Kastner, P. Schaner, A. M. Reginato, N. Richards, and D. L. Gumucio. 2004. Lipopolysaccharide-induced expression of multiple alternatively spliced MEFV transcripts in human synovial fibroblasts: a prominent splice isoform lacks the C-terminal domain that is highly mutated in familial Mediterranean fever. *Arthritis Rheum* 50:3679-3689.

46. Harton, J. A., M. W. Linhoff, J. Zhang, and J. P. Ting. 2002. Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains. *J Immunol* 169:4088-4093.

47. Richards, N., P. Schaner, A. Diaz, J. Stuckey, E. Shelden, A. Wadhwa, and D. L. Gumucio. 2001. Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem* 276:39320-39329.

48. Stehlik, C., and A. Dorfleutner. 2007. COPs and POPs: modulators of inflammasome activity. *J Immunol* 179:7993-7998.

49. Woo, J. S., J. H. Imm, C. K. Min, K. J. Kim, S. S. Cha, and B. H. Oh. 2006. Structural and functional insights into the B30.2/SPRY domain. *Embo J* 25:1353-1363.

50. Topaloglu, R., F. Ozaltin, E. Yilmaz, S. Ozen, B. Balci, N. Besbas, and A. Bakkaloglu. 2005. E148Q is a disease-causing MEFV mutation: a phenotypic evaluation in patients with familial Mediterranean fever. *Ann Rheum Dis* 64:750-752.

51. 2011. Infevers: an online database for autoinflammatory mutations. In *Infevers.com*, 2008 ed.

52. Milhavet, F., L. Cuisset, H. M. Hoffman, R. Slim, H. El-Shanti, I. Aksentijevich, S. Lesage, H. Waterham, C. Wise, C. Sarrauste de Menthiere, and I. Touitou. 2008. The infevers autoinflammatory mutation online registry: update with new genes and functions. *Hum Mutat* 29:803-808.

53. Sarrauste de Menthiere, C., S. Terriere, D. Pugnere, M. Ruiz, J. Demaille, and I. Touitou. 2003. INFEVERS: the Registry for FMF and hereditary inflammatory disorders mutations. *Nucleic Acids Res* 31:282-285.

54. Touitou, I., S. Lesage, M. McDermott, L. Cuisset, H. Hoffman, C. Dode, N. Shoham, E. Aganna, J. P. Hugot, C. Wise, H. Waterham, D. Pugnere, J. Demaille, and C. Sarrauste de Menthiere. 2004. Infevers: an evolving mutation database for auto-inflammatory syndromes. *Hum Mutat* 24:194-198.

55. Centola, M., G. Wood, D. M. Frucht, J. Galon, M. Aringer, C. Farrell, D. W. Kingma, M. E. Horwitz, E. Mansfield, S. M. Holland, J. J. O'Shea, H. F. Rosenberg, H. L. Malech, and D. L. Kastner. 2000. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood* 95:3223-3231.

56. Papin, S., P. Duquesnoy, C. Cazeneuve, J. Pantel, M. Coppey-Moisan, C. Dargemont, and S. Amselem. 2000. Alternative splicing at the MEFV locus involved in familial Mediterranean fever regulates translocation of the marenostrin/pyrin protein to the nucleus. *Hum Mol Genet* 9:3001-3009.

57. Chae, J. J., G. Wood, K. Richard, H. Jaffe, N. T. Colburn, S. L. Masters, D. L. Gumucio, N. G. Shoham, and D. L. Kastner. 2008. The familial Mediterranean fever protein, pyrin, is cleaved by caspase-1 and activates NF-kappaB through its N-terminal fragment. *Blood* 112:1794-1803.

58. Mansfield, E., J. J. Chae, H. D. Komarow, T. M. Brotz, D. M. Frucht, I. Aksentijevich, and D. L. Kastner. 2001. The familial Mediterranean fever protein, pyrin, associates with microtubules and colocalizes with actin filaments. *Blood* 98:851-859.

59. Waite, A. L., P. Schaner, C. Hu, N. Richards, B. Balci-Peynircioglu, A. Hong, M. Fox, and D. L. Gumucio. 2009. Pyrin and ASC co-localize to cellular sites that are rich in polymerizing actin. *Exp Biol Med (Maywood)* 234:40-52.

60. Rao, K. M., and T. Meighan. 2006. Exposure in vivo to silica or lipopolysaccharide produces transient or sustained upregulation, respectively, of PYPAF7 and MEFV genes in bronchoalveolar lavage cells in rats. *J Toxicol Environ Health A* 69:481-490.

61. Chae, J. J., G. Wood, S. L. Masters, K. Richard, G. Park, B. J. Smith, and D. L. Kastner. 2006. The B30.2 domain of pyrin, the familial Mediterranean fever protein, interacts directly with caspase-1 to modulate IL-1beta production. *Proc Natl Acad Sci U S A* 103:9982-9987.

62. Papin, S., S. Cuenin, L. Agostini, F. Martinon, S. Werner, H. D. Beer, C. Grutter, M. Grutter, and J. Tschopp. 2007. The SPRY domain of Pyrin, mutated in familial Mediterranean fever patients, interacts with inflammasome components and inhibits proIL-1beta processing. *Cell Death Differ* 14:1457-1466.

63. Balci-Peynircioglu, B., A. L. Waite, C. Hu, N. Richards, A. Staubach-Grosse, E. Yilmaz, and D. L. Gumucio. 2008. Pyrin, product of the MEFV locus, interacts with the proapoptotic protein, Siva. *J Cell Physiol* 216:595-602.

64. Jeru, I., S. Papin, S. L'Hoste, P. Duquesnoy, C. Cazeneuve, J. Camonis, and S. Amselem. 2005. Interaction of pyrin with 14.3.3 in an isoform-specific and phosphorylation-dependent manner regulates its translocation to the nucleus. *Arthritis Rheum* 52:1848-1857.

65. Dowds, T. A., J. Masumoto, F. F. Chen, Y. Ogura, N. Inohara, and G. Nunez. 2003. Regulation of cryopyrin/Pypaf1 signaling by pyrin, the familial Mediterranean fever gene product. *Biochem Biophys Res Commun* 302:575-580.

66. Seshadri, S., M. D. Duncan, J. M. Hart, M. A. Gavrilin, and M. D. Wewers. 2007. Pyrin levels in human monocytes and monocyte-derived macrophages regulate IL-1beta processing and release. *J Immunol* 179:1274-1281.

67. Yu, J. W., J. Wu, Z. Zhang, P. Datta, I. Ibrahimi, S. Taniguchi, J. Sagara, T. Fernandes-Alnemri, and E. S. Alnemri. 2006. Cryopyrin and pyrin activate caspase-1, but not NF-kappaB, via ASC oligomerization. *Cell Death Differ* 13:236-249.

68. Goulielmos, G. N., E. Petraki, D. Vassou, E. Eliopoulos, D. Iliopoulos, P. Sidiropoulos, I. Aksentijevich, D. Kardassis, and D. T. Boumpas. 2010. The role of the pro-apoptotic protein Siva in the pathogenesis of Familial Mediterranean fever: A structural and functional analysis. *Biochem Biophys Res Commun* 402:141-146.

69. Chen, X., Y. Bykhovskaya, N. Tidow, M. Hamon, Z. Bercovitz, O. Spirina, and N. Fischel-Ghodsian. 2000. The familial mediterranean fever protein interacts and colocalizes with a putative Golgi transporter. *Proc Soc Exp Biol Med* 224:32-40.

70. Badour, K., J. Zhang, F. Shi, M. K. McGavin, V. Rampersad, L. A. Hardy, D. Field, and K. A. Siminovitch. 2003. The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. *Immunity* 18:141-154.

71. Waite, A. L., P. Schaner, N. Richards, B. Balci-Peynircioglu, S. L. Masters, S. D. Brydges, M. Fox, A. Hong, E. Yilmaz, D. L. Kastner, E. L. Reinherz, and D. L. Gumucio. 2009. Pyrin Modulates the Intracellular Distribution of PSTPIP1. *PLoS One* 4:e6147.

72. Druilhe, A., S. M. Srinivasula, M. Razmara, M. Ahmad, and E. S. Alnemri. 2001. Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. *Cell Death Differ* 8:649-657.

73. Lamkanfi, M., G. Denecker, M. Kalai, K. D'Hondt, A. Meeus, W. Declercq, X. Saelens, and P. Vandenabeele. 2004. INCA, a novel human caspase recruitment domain protein that inhibits interleukin-1beta generation. *J Biol Chem* 279:51729-51738.

74. Lee, S. H., C. Stehlik, and J. C. Reed. 2001. Cop, a caspase recruitment domain-containing protein and inhibitor of caspase-1 activation processing. *J Biol Chem* 276:34495-34500.

75. Bedoya, F., L. L. Sandler, and J. A. Harton. 2007. Pyrin-only protein 2 modulates NF-kappaB and disrupts ASC:CLR interactions. *J Immunol* 178:3837-3845.

76. Dorfleutner, A., N. B. Bryan, S. J. Talbott, K. N. Funya, S. L. Rellick, J. C. Reed, X. Shi, Y. Rojanasakul, D. C. Flynn, and C. Stehlik. 2007. Cellular pyrin domain-only protein 2 is a candidate regulator of inflammasome activation. *Infect Immun* 75:1484-1492.

77. Srimathi, T., S. L. Robbins, R. L. Dubas, H. Chang, H. Cheng, H. Roder, and Y. C. Park. 2008. Mapping of POP1-binding site on pyrin domain of ASC. *J Biol Chem* 283:15390-15398.

78. Stehlik, C., M. Krajewska, K. Welsh, S. Krajewski, A. Godzik, and J. C. Reed. 2003. The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and pro-caspase-1 regulation. *Biochem J* 373:101-113.

79. Saleh, M., J. C. Mathison, M. K. Wolinski, S. J. Bensinger, P. Fitzgerald, N. Droin, R. J. Ulevitch, D. R. Green, and D. W. Nicholson. 2006. Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice. *Nature* 440:1064-1068.

80. Stehlik, C., S. H. Lee, A. Dorfleutner, A. Stassinopoulos, J. Sagara, and J. C. Reed. 2003. Apoptosis-associated speck-like protein containing a caspase recruitment domain is a regulator of procaspase-1 activation. *J Immunol* 171:6154-6163.

81. Ben-Zvi, I., and A. Livneh. 2011. Chronic inflammation in FMF: markers, risk factors, outcomes and therapy. *Nat Rev Rheumatol* 7:105-112.

82. Ben-Chetrit, E., and M. Levy. 1998. Does the lack of the P-glycoprotein efflux pump in neutrophils explain the efficacy of colchicine in familial Mediterranean fever and other inflammatory diseases? *Med Hypotheses* 51:377-380.

83. Nuki, G. 2008. Colchicine: its mechanism of action and efficacy in crystal-induced inflammation. *Curr Rheumatol Rep* 10:218-227.

84. Yin, T., and L. Li. 2006. The stem cell niches in bone. *J Clin Invest* 116:1195-1201.

85. Rankin, S. M. 2010. The bone marrow: a site of neutrophil clearance. *J Leukoc Biol* 88:241-251.

86. Contributors, W. 2011. Neutrophil granulocyte. Wikipedia, The Free Encyclopedia.

87. Cowburn, A. S., A. M. Condliffe, N. Farahi, C. Summers, and E. R. Chilvers. 2008. Advances in neutrophil biology: clinical implications. *Chest* 134:606-612.

88. Fleming, T. J., M. L. Fleming, and T. R. Malek. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol* 151:2399-2408.

89. Monteiro, A. P., C. S. Pinheiro, T. Luna-Gomes, L. R. Alves, C. M. Maya-Monteiro, B. N. Porto, C. Barja-Fidalgo, C. F. Benjamim, M. Peters-Golden, C. Bandeira-Melo, M. T. Bozza, and C. Canetti. 2011. Leukotriene B4 mediates neutrophil migration induced by heme. *J Immunol* 186:6562-6567.

90. Ribeiro, R. A., C. A. Flores, F. Q. Cunha, and S. H. Ferreira. 1991. IL-8 causes in vivo neutrophil migration by a cell-dependent mechanism. *Immunology* 73:472-477.

91. Ajuebor, M. N., A. M. Das, L. Virag, R. J. Flower, C. Szabo, and M. Perretti. 1999. Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10. *J Immunol* 162:1685-1691.

92. Ostrovsky, L., J. Carvalho-Tavares, R. C. Woodman, and P. Kubes. 2000. Translational inhibition of E-selectin expression stimulates P-selectin-dependent neutrophil recruitment. *Am J Physiol Heart Circ Physiol* 278:H1225-1232.

93. Shaw, A. C., S. Joshi, H. Greenwood, A. Panda, and J. M. Lord. 2010. Aging of the innate immune system. *Curr Opin Immunol* 22:507-513.

94. Reddy, R. C., and T. J. Standiford. 2010. Effects of sepsis on neutrophil chemotaxis. *Curr Opin Hematol* 17:18-24.

95. Gogus, F., I. Fresko, Y. Elbir, E. Eksioglu-Demiralp, and H. Direskeneli. 2005. Oxidative burst response to monosodium urate crystals in patients with Behcet's syndrome. *Clin Exp Rheumatol* 23:S81-85.

96. Avetisian, S. A., T. K. Davtian, G. S. Akopian, A. M. Manukian, and D. A. Pogosian. 2006. [Spontaneous and inducible respiratory burst of monocytes and neutrophils in periodic autoinflammatory fever]. *Patol Fiziol Eksp Ter*:11-12.

97. Akopian, V. P., S. A. Avetisian, and T. K. Davtian. 2005. [The effect of colcichine and iodine-lithium-alpha-dextrin on the phagocytosis of granulocytes and monocytes in patients with familial Mediterranean fever]. *Eksp Klin Farmakol* 68:36-39.

98. Davtian, T. K., S. A. Avetisian, G. S. Akopian, and V. M. Arutiunian. 2005. [A respiratory splash of monocytes and neutrophils in familial Mediterranean fever]. *Ter Arkh* 77:39-43.

99. Davtyan, T. K., G. S. Hakopyan, S. A. Avetisyan, and N. R. Mkrtchyan. 2006. Impaired endotoxin tolerance induction in patients with familial Mediterranean fever. *Pathobiology* 73:26-39.

100. Davtyan, T. K., V. A. Harutyunyan, G. S. Hakobyan, and S. A. Avetisyan. 2008. Heightened endotoxin susceptibility of monocytes and neutrophils during familial Mediterranean fever. *FEMS Immunol Med Microbiol* 52:370-378.

101. Ledford, J. G., M. Kovarova, and B. H. Koller. 2007. Impaired host defense in mice lacking ONZIN. *J Immunol* 178:5132-5143.

102. Cronstein, B. N., Y. Molad, J. Reibman, E. Balakhane, R. I. Levin, and G. Weissmann. 1995. Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. *J Clin Invest* 96:994-1002.

103. Phelps, P. 1970. Polymorphonuclear leukocyte motility in vitro: IV. Colchicine inhibition of chemotactic activity formation after phagocytosis of urate crystals. *Arthritis Rheum* 13:S25-33.

104. Akopian, V. P., S. A. Avetisian, and T. K. Davtian. 2005. [The effect of colchicine on the periodicity of the spontaneous and induced respiratory burst of granulocytes and monocytes during familial Mediterranean fever]. *Eksp Klin Farmakol* 68:36-39.

105. Bernot, A., C. da Silva, J. L. Petit, C. Cruaud, C. Caloustian, V. Castet, M. Ahmed-Arab, C. Dross, M. Dupont, D. Cattan, N. Smaoui, C. Dode, C. Pecheux, B. Nedelec, J. Medaxian, M. Rozenbaum, I. Rosner, M. Delpech, G. Grateau, J. Demaille, J. Weissenbach, and I. Touitou. 1998. Non-founder mutations in the MEFV gene establish

this gene as the cause of familial Mediterranean fever (FMF). *Hum Mol Genet* 7:1317-1325.

106.Mor, A., Y. Shinar, N. Zaks, P. Langevitz, A. Chetrit, S. Shtrasburg, E. Rabinovitz, and A. Livneh. 2005. Evaluation of disease severity in familial Mediterranean fever. *Semin Arthritis Rheum* 35:57-64.

107.Gershoni-Baruch, R., M. Shinawi, H. Shamaly, L. Katsinetz, and R. Brik. 2002. Familial Mediterranean fever: the segregation of four different mutations in 13 individuals from one inbred family: genotype-phenotype correlation and intrafamilial variability. *Am J Med Genet* 109:198-201.

108.Medlej-Hashim, M., E. Chouery, N. Salem, V. Delague, G. Lefranc, J. Loiselet, and A. Megarbane. 2010. Familial Mediterranean fever in a large Lebanese family: multiple MEFV mutations and evidence for a Founder effect of the p.[M694I] mutation. *Eur J Med Genet* 54:50-54.

109.Fragouli, E., E. Eliopoulos, E. Petraki, P. Sidiropoulos, I. Aksentijevich, E. Galanakis, H. Kritikos, A. Repa, G. Fragiadakis, D. Boumpas, and G. N. Goulielmos. 2008. Familial Mediterranean Fever in Crete: a genetic and structural biological approach in a population of 'intermediate risk'. *Clin Genet* 73:152-159.

110.Dundar, M., E. F. Emirogullari, A. Kiraz, S. Taheri, and M. Baskol. 2010. Common Familial Mediterranean Fever gene mutations in a Turkish cohort. *Mol Biol Rep*.

111.Berdeli, A., S. Mir, S. Nalbantoglu, N. Kutukculer, B. Sozeri, Y. Kabasakal, S. Cam, and M. Solak. 2011. Comprehensive Analysis of a Large-Scale Screen for MEFV Gene Mutations: Do They Truly Provide a "Heterozygote Advantage" in Turkey? *Genet Test Mol Biomarkers*.

112.Jalkh, N., E. Genin, E. Chouery, V. Delague, M. Medlej-Hashim, C. A. Idrac, A. Megarbane, and J. L. Serre. 2008. Familial Mediterranean Fever in Lebanon: founder effects for different MEFV mutations. *Ann Hum Genet* 72:41-47.

113.Schaner, P., N. Richards, A. Wadhwa, I. Aksentijevich, D. Kastner, P. Tucker, and D. Gumucio. 2001. Episodic evolution of pyrin in primates: human mutations recapitulate ancestral amino acid states. *Nat Genet* 27:318-321.

114.Shinar, Y., I. Kuchuk, S. Menasherow, M. Kolet, M. Lidar, P. Langevitz, and A. Livneh. 2007. Unique spectrum of MEFV mutations in Iranian Jewish FMF patients--clinical and demographic significance. *Rheumatology (Oxford)* 46:1718-1722.

115.Guz, G., M. Kanbay, and M. A. Ozturk. 2009. Current perspectives on familial Mediterranean fever. *Curr Opin Infect Dis* 22:309-315.

CHAPTER 2

Expression of *Mefv*

Familial Mediterranean Fever (FMF) is characterized by aberrant activation of the immune system. Thus, it not surprising that *MEFV* expression levels are high in immune tissues in both humans and mice. To further clarify which immune tissues of the mouse express *Mefv*, real-time PCR was used to assess expression. *Mefv* expression was detected in several organs and it was particularly high in bone marrow. High expression was found in cells of the myeloid lineage and increased as cells matured along this lineage. Expression of *Mefv* in macrophages was induced after exposure to lipopolysaccharide (LPS) and Interleukin-4 (IL-4). The detection of *Mefv* in immune tissues of naïve mice, and the increased expression of *Mefv* during immune cell maturation and activation suggests that Pyrin function is important to the immune system during normal physiological states and is increasingly important during activation of the immune system.

Introduction

FMF is characterized by sudden inflammatory episodes associated with neutrophil influx and elevated acute response proteins and cytokines (1). Previous research suggests that FMF is, at least in part, due to problems with intrinsic neutrophil and monocyte/macrophage functions (2-5). Accordingly, expression of *MEFV* can be detected in neutrophils and macrophages from humans and mice (6, 7). However, several conundrums in the proposed models by which mutations in Pyrin causes FMF leave open the possibility that Pyrin may contribute to processes in additional cell types that have not been examined.

Neutrophils and macrophages differentiate from a common myeloid precursor cell in the bone marrow. The profile of neutrophils and macrophages changes as they mature and following exposure to immune stimuli and cytokines. For example, the expression of surface antigen receptors changes during the 24 h lifespan of neutrophils, and the expression profiles correlates with the extent of activation, extravasation, recruitment, and apoptosis of neutrophils (8). Macrophages develop from immature macrophages, or monocytes, give rise to other cell types: dendritic cells and osteoclasts, and to 4 subsets of macrophages: innate activated, classically activated, alternatively activated, and deactivated. Each of these cell types differs in immune function (9). Differences between monocytes and macrophages are particularly well-characterized and include differences in phagocytosis, cytokine release, cell migration, and cell survival mechanisms (9-11). Specifically, the magnitude and kinetics of IL-1β cytokine release varies with monocyte/macrophage cell maturity (11). This is of particular interest since human *MEFV* expression changes following maturation of and activation of neutrophils and macrophages (2, 11, 12).

We examined the expression levels of *Mefv* in immune tissues collected from mice. *Mefv* expression was high in the spleen and bone marrow, but was also detected, at a much lower

level, in other immune organs. *Mefv* expression was high in neutrophils and macrophages and was increased by cell maturation and activation.

Materials and Methods

Sources of RNA

Whole organs (lung, iguanal lymph nodes, thymus, and spleen) were snap-frozen and smashed to collect tissue for RNA preparation. Whole bone marrow was collected by flushing femurs and tibias with phosphate-buffered saline (PBS; Gibco). Bone marrow was cultured with appropriate cytokines to derive mast cells (interleukin-3, stem-cell factor) and macrophages (L-cell conditioned media as a source of M-CSF). Bone-marrow neutrophils were isolated from total bone marrow by centrifugation through a Histopaque density gradient (density 1.083 g/mL and 1.119 g/mL; Sigma-Aldrich) per manufacturer's instructions. Greater than 90% of cells were classified as neutrophils based upon morphological analysis of cytospun cells stained with Hema-3 (Protocol, Fisher Scientific). Resident peritoneal macrophages were collected by PBS lavage from the peritoneal cavity of naïve mice. Peritoneal cells were cultured in media without serum for 2 h to isolate adherent macrophages from other cell types. Recruited/elicited peritoneal macrophages were collected by peritoneal lavage, as above, from mice treated 3 d prior with intraperitoneal (*i.p.*) injection of 3 mLs of 3% Brewer's thioglycolate medium. Elicited neutrophils were collected from the peritoneal cavity of mice at 4 h following *i.p.* injection of 1 mL of 1 mg/mL Zymosan-A in PBS.

Total RNA was isolated using RNA Bee (Tel-test) as instructed by the manufacturer. RNA was eluted in nuclease-free water. The optical density (OD) at 260 nm was used to determine RNA concentration, and the 260/280 nm ratio was 1.7 – 2.0 for all samples.

Real-time PCR

Total RNA was converted to cDNA per manufacturer's protocol (high capacity cDNA Reverse Transcription Kit; Applied Biosystems). For neutrophils, 500 ng of total RNA was used. Expression of *Mefv, Nlrp3, Pycard, Il1b, Gapdh, β-actin*, and *Hprt* were detected with TaqMan

universal PCR master mix and probes Mm00490258_m1 (*Mefv*), 4352932-070313 (*Gapdh*), 4352664-0608007 (*β-actin*), and Mm00446968_m1 (*Hprt*) by an ABI Prism 7900HT detection system according to manufacturer protocol (all from Applied Biosystems). The *Mefv* probe detects all known splice variants. Relative gene expression values were calculated from the cycle number (Ct) data by the equations below.

1. Ct *Mefv* – Ct *Gapdh* = Δ Ct (normalizes *Mefv* to the *Gapdh* internal sample control)
2. Δ Ct of sample - Δ Ct of control sample = ΔΔ Ct (normalizes values to control sample)
3. 2 ^ -ΔΔ Ct = fold change (converts values to relative expression ratio)

Northern blot expression analysis

Total neutrophil RNA was electrophoresed on a 1.1% formaldehyde, 1.2% agarose gel containing formamide and transferred to a nylon membrane (Bright Star Plus Positively-charged nylon membrane, Ambion). Full-length cDNA corresponding to *Mefv* was radiolabeled with [α-^{32}P]dCTP per manufacturer's instructions (Random Primed DNA Labeling Kit; Roche AppliedScience). RNA blots were probed with radiolabled cDNA probe in hybridization buffer following manufacter's protocol (ULTRAhyb Ultrasensitive Hybridization Buffer, Applied Biosystems). Membranes were hybridized and washed at 42°C. RNA was visualized on autoradiography film (BioMax, Kodak) following overnight storage with the membrane at -80°C. Membranes were probed with a β-actin probe following the same protocol.

Statistical Analyses

One-way ANOVA and Tukey's post-test were used to determine significance.

Animal Care and Use

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of UNC Chapel Hill.

Results

Cells of the myeloid lineage express high levels of *Mefv*

Broader expression of *MEFV* has been detected in humans by RT-PCR (12) than in mice by northern blot (7). We reasoned that *Mefv* might have a broader expression pattern in mice at a low-level that limits detection by northern blot. *Mefv* expression in immune tissues was analyzed using real-time PCR. Expression was detected in spleen as expected, but also in lymph node and bone marrow and to a lesser extent in lung and thymus (Figure 2.1*A*). Based upon northern blot analysis, expression was highest in myeloid cells, and expression in neutrophils was markedly increased compared to macrophages (Figure 2.1*B*). Expression of *Mefv* was undetectable in bone marrow-derived mast cells (data not shown).

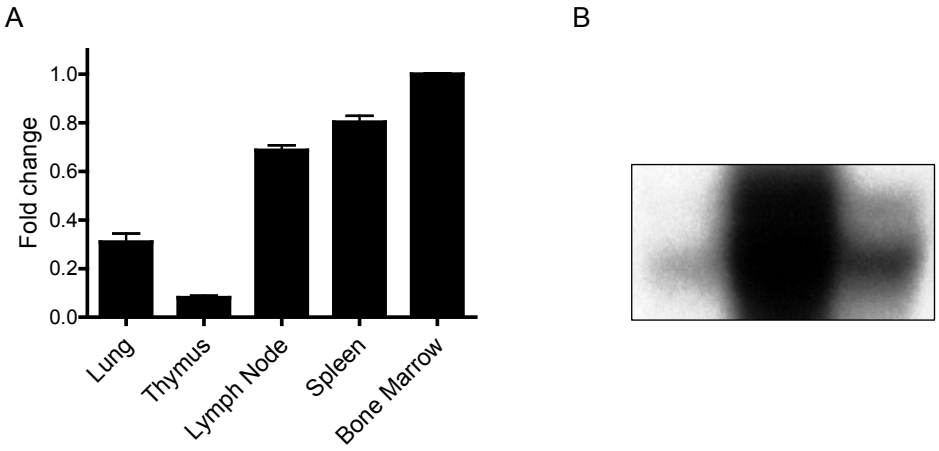


Figure 2.1. *Mefv* is expressed in immune tissues. *A*, Real-time PCR analysis of *Mefv* expression in WT tissues. Ct values are normalized to *Gapdh* and expressed as fold change relative to bone marrow. The raw Ct value of *Mefv* in all tissues was below 32 for all tissues shown. The Ct value of neutrophils from *Mefv*$^{-/-}$ mice was undetermined (>40). *B*, Northern blot analysis to detect *Mefv* transcripts in bone marrow, recruited peritoneal neutrophils, and recruited peritoneal macrophages. Full-length *Mefv* cDNA was used as a probe. Equal amounts of RNA for each sample were loaded.

Mefv expression is increased upon maturation of neutrophils and macrophages

To further characterize the expression of *Mefv* in myeloid cells, neutrophils and macrophages were harvested from different tissues within the mouse. Neutrophils were isolated from total bone marrow or peritoneal cells were collected at 4 h following treatment with Zymosan A. At the mRNA transcript level, *Mefv* expression was 23-fold higher in neutrophils recruited to the peritoneal cavity than in neutrophils isolated from the bone marrow (Figure 2.2*A*). In macrophages, *Mefv* expression was easily detected in bone marrow-derived monocyte/macrophages. Bone marrow-derived macrophages were cultured for 8 – 12 days before expression analysis, and no significant differences were seen in Mefv expression. However, *Mefv* expression was markedly higher in both resident peritoneal macrophages (rpMΦ) and in macrophages collected from the peritoneal cavity at 72 h after the induction of peritonitis with the sterile immunostimulant, thioglycolate (Figure 2.2*B*).

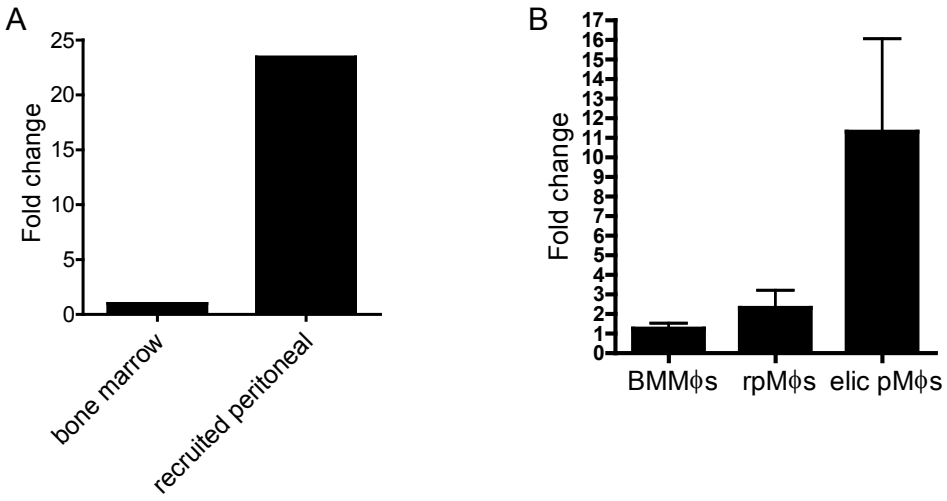


Figure 2.2. *Mefv* expression increases upon matuaration of neutrophils and macrophages collected from mice. Relative expression of *Mefv* was determined by real-time PCR analysis for neutrophils, *A*, and macrophages, *B*. *A*, Neutrophils were isolated from bone marrow or peritoneal cells were harvested at 4 h after treatment with 1 mg of Zymosan-A *i.p.* Greater than 90% of cells were considered neutrophils by differential cell staining. Data are expressed as fold change relative to bone marrow neutrophils. RNA from several wild-type B6;129S6 mice was pooled. Results are representative of two experiments. *B*, Macrophages were derived from whole bone marrow (BMMΦs) or were collected from the peritoneal cavity of naïve mice (resident peritoneal macrophages, rpMΦs) or mice at 72 h after treatment with thioglycolate (elic pMΦs). Expression of myeloperoxidase, a gene expressed in neutrophils but not macrophages, was measured to ensure that differences in expression were not due to the presence of neutrophils. Data are expressed as fold change relative to BMMΦs.

Mefv expression is increased upon activation of macrophages

Previous reports indicate that Pyrin expression is affected by treatment of macrophages with LPS and cytokines. However, previous studies show that Interleukin (IL)-4 and Interferon-gamma (IFN-γ) have opposing affects on *Mefv* expression in mice and humans. Whereas IL-4 increases Pyrin protein expression in mice, it inhibits expression in humans. IFN-γ does not affect expression of mouse Pyrin but increases Pyrin protein levels in humans (2, 5). Here, the

abundance of *Mefv* transcripts was measured in macrophages cultured for 24 h in the presence of LPS, LPS and IL-4, or left untreated. Consistent with previous findings at the protein level (5), *Mefv* expression increased after treatment with LPS and LPS and IL-4. Expression levels of inflammasome proteins *Nlrp3* and *Pycard*, which are involved in IL-1β production, and expression of *Il1β* itself were also determined. Similar to *Mefv*, *Nlrp3,* and *Il1β* expression were increased after treatment with LPS. The addition of IL-4 resulted in a further increase in *Nlrp3* and *Il1β* expression (Figure 2.3).

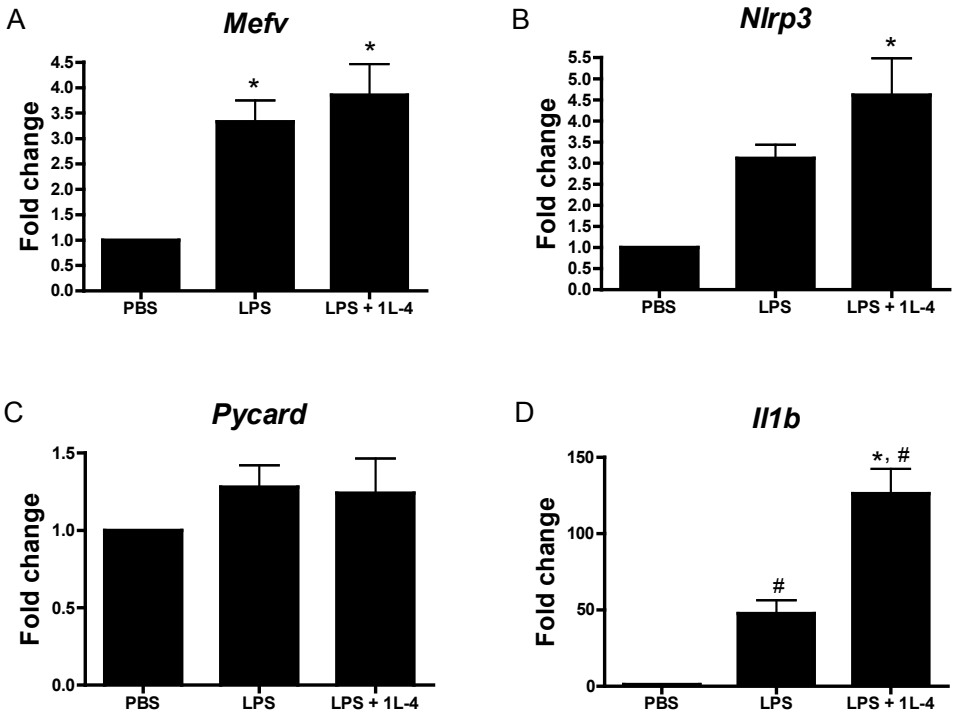


Figure 2.3. *Mefv* expression in macrophages is increased after pathogen and cytokine treatment. Thioglycolate-elicited peritoneal macrophages were cultured for 22 h with 1 μg/mL lipopolysaccharide (LPS) and 12.5 ng/mL as indicated. Relative expression of *A*, *Mefv, B, Nlrp3, C, Pycard,* and *D, Il1β* was detected by real-time PCR. Results are expressed as fold change compared to PBS-treated samples. n = 3. *, $p < 0.05$ for LPS or LPS + IL-4 treatment compared to PBS treatment. #, $p < 0.05$ for LPS + IL-4 treatment compared to LPS.

Discussion

Here, we describe our analysis of *Mefv* expression in wild-type mice. Previous studies indicate that *Mefv* is expressed in a broad set of tissues in humans (12), and we hypothesized that expression in the mouse might be broader than previously appreciated due to the detection limit of northern blot analyses. The pathology of FMF is especially complex and many different tissue and cell types may be involved in inflammatory attacks.

Our studies indicate that *Mefv* expression is highest in the bone marrow, spleen, and lymph nodes, but can also be detected at low levels in the lung and thymus. On a cellular level, expression was highest in neutrophils and macrophages and was undetectable in bone marrow-derived mast cells. By using cDNA derived from *Mefv*-null neutrophils as a negative control, a difference between low-level expression and no expression could be resolved. Our findings extend the panel of tissues in mice that express *Mefv*, although these results should be confirmed at the protein level once a Pyrin antibody is commercially available. Among the panel of tissues previously characterized by northern blot analysis, expression was detected in the spleen but not the thymus, lung, brain, heart, kidney, liver, muscle, ovary, or testis (7). In humans, significant expression of *MEFV* can be detected in the spleen, lung, consistent with the profile of mouse *Mefv.* In contrast, human *MEFV* is also expressed in muscle, and previous results of semi-quantitative RT-PCR analysis also showed a low number of MEFV transcripts in cDNA collected from heart, kidney, and liver organs. *MEFV* transcripts were not detected in the small intestines, brain, prostate, or ovary (12), and the later three organs in the mouse also lack *Mefv* expression (7). At the cellular level, human *MEFV* expression is highest in neutrophils and macrophages, and there is low expression in B220+ B cells, CD3+ T cells, eosinophils, dendritic cells, and epithelial cells of the lung, peritoneum, and synovium (6, 12, 13). Previous reports suggest that murine *Mefv* expression is limited to neutrophils and monocytes/macrophages. Expression of *Mefv* in whole bone marrow or lymph nodes was not examined previously. High

expression in bone marrow was expected, since a large portion of bone marrow cells are developing/maturing neutrophils. It is not surprising to see low level of expression in the lung, since expression of *Mefv* in the lung has been reported for rats and humans. The low level of expression in that was detected in the lung and thymus in our studies using real-time PCR is likely below the detection limit of northern blot analysis that was used in the previous study (7). Low expression in human T cells may correspond with the low level of *Mefv* expression that we detected in the thymus of mice.

We found that *Mefv* expression was increased in neutrophils and macrophages from the peritoneal cavity in comparison to neutrophils collected from the bone marrow or monocytes/macrophages derived from bone marrow precursors. Since expression is also increased in resident or resting peritoneal macrophages compared to bone marrow-derived macrophages, the increased expression is likely to be a function of cell maturity in the myeloid lineage. In humans, on the other hand, the expression of Pyrin decreases as peripheral blood mononuclear cells are differentiated from monocytes to macrophages (11). However, our findings for neutrophils are consistent with previous reports that expression of *MEFV* increases in HL-60 promyeloblast cells and peripheral blood hematopoietic precursors as they differentiate along a granulocytic lineage (12). The influence of cell maturity on Mefv expression may be a species and/or cell type –specific contribution, but further research is necessary to examine this possibility.

Consistent with previous reports (5), our results indicate that expression of the mouse Mefv gene is enhanced following exposure to LPS and IL-4, at least in macrophages. Expression levels of inflammasome complex genes and *Il1β* were also assessed in parallel to provide context for future studies to assess the role of Pyrin in inflammasome-dependent IL-1β production, which will be discussed in Chapter 4. Expression of the human *MEFV* gene is also increased after exposure of monocytes and macrophages to LPS (2, 6, 11), however, IL-4 does

not increase human *MEFV* expression in peripheral blood mononuclear cells and instead decreases *MEFV* expression (2). IL-4 stimulates an alternative activation phenotype of macrophages (14) and polarizes T cells towards a T helper type 2 response (9, 15). Interestingly, Interferon-gamma, which polarizes T cells towards T helper type 1, increases *MEFV* expression in humans and mice (2, 5, 9). One previous study found that FMF patients have higher levels of cytokines associated with T helper type 1 responses (16). Further studies are needed to clarify the role of wild-type and mutant Pyrin proteins in T cell polarization and alternative versus classical activation of macrophages.

It is unclear whether or not FMF-associated mutations within *MEFV* have an effect on *MEFV* expression levels. Since the majority of these mutations lie within the coding region rather than the promoter or intronic regions, they are more likely to affect protein function or protein stability rather than transcript or protein expression levels. Previous studies have demonstrated increased, decreased, or unchanged expression of *MEFV* in FMF patients compared to healthy controls (17, 18). Alternative splicing of the *MEFV* transcript has been well-described (19), so conclusions regarding the effect of mutations on expression levels are complicated by the ability to detect splice variants of the *MEFV* transcript or isoforms of the Pyrin protein they encode. A recent study has also shown that non-sense mediated decay is involved in the regulation of expression of *MEFV* transcripts (18).

Information from these expression analyses extends the rationale for future studies using *Mefv*-deficient mice. Since a difference due to the loss of *Mefv* will be more apparent in cells with high levels of expression, future studies will focus on neutrophils and macrophages isolated from the peritoneal cavity.

References

1. Rosenberg, P. M., and S. E. Goldfinger. 2011. Pathophysiology of familial Mediterranean fever. In *UpToDate.com*, 2011 ed.

2. Chae, J. J., G. Wood, K. Richard, H. Jaffe, N. T. Colburn, S. L. Masters, D. L. Gumucio, N. G. Shoham, and D. L. Kastner. 2008. The familial Mediterranean fever protein, pyrin, is cleaved by caspase-1 and activates NF-kappaB through its N-terminal fragment. *Blood* 112:1794-1803.

3. Kallinich, T., H. Wittkowski, R. Keitzer, J. Roth, and D. Foell. 2010. Neutrophil-derived S100A12 as novel biomarker of inflammation in familial Mediterranean fever. *Ann Rheum Dis* 69:677-682.

4. Manukyan, G., K. Ghazaryan, Z. Ktsoyan, Z. Khachatryan, D. Kelly, M. Tatyan, M. Agababova, and R. Aminov. 2010. Comparative analysis of cytokine profiles in autoinflammatory and autoimmune conditions. *Cytokine* 50:146-151.

5. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* 11:591-604.

6. Centola, M., G. Wood, D. M. Frucht, J. Galon, M. Aringer, C. Farrell, D. W. Kingma, M. E. Horwitz, E. Mansfield, S. M. Holland, J. J. O'Shea, H. F. Rosenberg, H. L. Malech, and D. L. Kastner. 2000. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood* 95:3223-3231.

7. Chae, J. J., M. Centola, I. Aksentijevich, A. Dutra, M. Tran, G. Wood, K. Nagaraju, D. W. Kingma, P. P. Liu, and D. L. Kastner. 2000. Isolation, genomic organization, and expression analysis of the mouse and rat homologs of MEFV, the gene for familial mediterranean fever. *Mamm Genome* 11:428-435.

8. Cowburn, A. S., A. M. Condliffe, N. Farahi, C. Summers, and E. R. Chilvers. 2008. Advances in neutrophil biology: clinical implications. *Chest* 134:606-612.

9. Gordon, S., and P. R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5:953-964.

10. Herzyk, D. J., J. N. Allen, C. B. Marsh, and M. D. Wewers. 1992. Macrophage and monocyte IL-1 beta regulation differs at multiple sites. Messenger RNA expression, translation, and post-translational processing. *J Immunol* 149:3052-3058.

11. Seshadri, S., M. D. Duncan, J. M. Hart, M. A. Gavrilin, and M. D. Wewers. 2007. Pyrin levels in human monocytes and monocyte-derived macrophages regulate IL-1beta processing and release. *J Immunol* 179:1274-1281.

12. Tidow, N., X. Chen, C. Muller, S. Kawano, A. F. Gombart, N. Fischel-Ghodsian, and H. P. Koeffler. 2000. Hematopoietic-specific expression of MEFV, the gene mutated in familial Mediterranean fever, and subcellular localization of its corresponding protein, pyrin. *Blood* 95:1451-1455.

13. Diaz, A., C. Hu, D. L. Kastner, P. Schaner, A. M. Reginato, N. Richards, and D. L. Gumucio. 2004. Lipopolysaccharide-induced expression of multiple alternatively spliced MEFV transcripts in human synovial fibroblasts: a prominent splice isoform lacks the C-terminal domain that is highly mutated in familial Mediterranean fever. *Arthritis Rheum* 50:3679-3689.

14. Martinez, F. O., L. Helming, and S. Gordon. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* 27:451-483.

15. Stanley, A. C., and P. Lacy. 2010. Pathways for cytokine secretion. *Physiology (Bethesda)* 25:218-229.

16. Simsek, I., S. Pay, A. Pekel, A. Dinc, U. Musabak, H. Erdem, and A. Sengul. 2007. Serum proinflammatory cytokines directing T helper 1 polarization in patients with familial Mediterranean fever. *Rheumatol Int* 27:807-811.

17. Chae, J. J., Y. H. Cho, G. S. Lee, J. Cheng, P. P. Liu, L. Feigenbaum, S. I. Katz, and D. L. Kastner. 2011. Gain-of-Function Pyrin Mutations Induce NLRP3 Protein-Independent Interleukin-1beta Activation and Severe Autoinflammation in Mice. *Immunity*.

18. Grandemange, S., S. Soler, and I. Touitou. 2009. Expression of the familial Mediterranean fever gene is regulated by nonsense-mediated decay. *Hum Mol Genet* 18:4746-4755.

19. Medlej-Hashim, M., N. Nehme, E. Chouery, N. Jalkh, and A. Megarbane. 2010. Novel MEFV transcripts in Familial Mediterranean fever patients and controls. *BMC Med Genet* 11:87.

CHAPTER 3

Creation of *Mefv* null mice and characterization of naïve mice

The ability to engineer mouse lines with mutations in genes suspected to contribute to a particular signaling pathway has facilitated our understanding of the immune response. In particular, mice with mutations in genes involved in inflammasome signaling have extended our knowledge of the involvement of these genes during *in vivo* pathogen responses. In this chapter, I describe the creation of a mouse line that lacks the *Mefv* gene, which encodes the Pyrin protein. We used a unique targeting strategy to remove the coding region and all known regulatory elements. The loss of *Mefv* did not impact the growth or survival of mice, nor did it impact the size or cellular composition of immune organs. These results suggest that mice in a naïve state can compensate for the loss of Pyrin function.

Introduction

Genetically altered mice provide a system to isolate the contribution of a gene in a physiologically relevant *in vivo* system. The development of constitutive knock-out, conditional knockout, and transgenic mouse models, as well as selective breeding strategies to maintain spontaneous mutations, have helped to elucidate the contribution of a gene or cell type towards inflammation. Specific to the inflammasome pathway, mice deficient in *Nlrp1b, Nlrp3, Nlrc4, Aim2, Pycard* (ASC), and *Caspase-1* have been developed (1-4). Bone marrow-derived macrophages and macrophages collected from the peritoneal cavities of these animals have impaired IL-1β production, demonstrating that these proteins enhance IL-1β production (1-5). Caspase-12 deficient mice, on the other hand, display elevated levels of IL-1β, indicating that Caspase-12 negatively regulates IL-1β production in mice (6). Treatment of genetically deficient mice provides evidence that the importance of these proteins for IL-1β production and pathogen resistance is maintained *in vivo.* (1-4, 6-9). Thus, it is reasonable to anticipate that a contribution of *Mefv* (Pyrin) towards inflammasome-dependent IL-1β production, either enhancing or inhibiting IL-1β production, will be detectable using *in vitro* and *in vivo* experimental systems.

Targeting constructs designed to generate null alleles can result in modified loci, which because of alternative splicing, continue to direct the production of a protein with some activity. Perhaps even more problematic, in some cases, integration of a targeting vector generates an allele that produces transcripts encoding proteins with novel function. These possibilities are of particular concern when alternative splicing has been well documented, as is the case for the *Mefv* gene (10, 11). Alternative splicing of Mefv transcripts may significantly alter Pyrin function (10).

The inflammatory attacks of Familial Mediterranean Fever (FMF) occur in the apparent absence of a pathogen, thus, stimulation of the immune response with a pathogen is not

required to detect the immune system defect in these patients. Consistent with this, as previously described in Chapter 2, *Mefv* is expressed in immune organs of naïve, or untreated mice, which suggests that Pyrin functions in the development or maintenance of the immune system in a naïve, or homeostatic, state.

To enable further study of the contribution of Pyrin to IL-1β production and FMF pathophysiology, we created a Mefv-defecient mouse line. A targeting construct capable of removing the entire coding sequence and the promoter region of the gene was developed to ensure that the mutation introduced into the *Mefv* locus creates a null allele. The survival and growth of these mice, and the cell profiles of immune organs were maintained despite the loss of Pyrin.

Materials and Methods

Creation of Mefv^{Del} targeting plasmid

A bacterial artificial chromosome (BAC) containing the *Mefv* genomic region of the mouse strain 129S7/SvEv (clone ID bMQ133e1, Geneservice, United Kingdom) was altered by Red/ET recombination in bacteria to generate a targeting vector (Mefv^{Del}). The entire coding region and 5 kb upstream and downstream was removed and replaced with a deletor cassette. Bioinformatic websites http://genome.ucsc.edu/ and http://genome.lbl.gov/vista/index.shtml were used to assign the gene region and assess possible regulatory regions within the promoter and 3' intergenic region. The deletor cassette contains a Neo^r,Kan^r gene that was used for selection, a loxP site and the 3' end of the *Hprt* gene that were used for a later genetic manipulation of the *Mefv* locus, and Bam HI and Xba I restriction digest sites that allowed for screening of targeting events by Southern blot. The region downstream of the *Mefv* gene in the BAC was shortened and replaced with an ampicillin resistance cassette (Amp^r), but proper recombination in ESCs prevents incorporation of Amp^r into the mouse genome. Mefv^{Del} was linearized and electroporated into embryonic stem cells (ESCs) derived from 129Sv/Ev mice. Table 2.2 lists the PCR primers used in construction of Mefv^{Del}.

Detection of ESC transformants and mice carrying a targeted allele

ESC transformants were isolated by selection using standard methodologies (12) ESC lysates were prepared and used for PCR analysis with the primers listed in Table 2.2. For Southern blotting, DNA was isolated from ESCs using standard methodologies. DNA was digested with Bam HI or Xba I and electrophoresed through a 0.8% agarose gel and transferred to a Nitrocellulose (Hybond-N) membrane. A 527-bp genomic fragment, created by PCR using the primers shown in Table 2.2, hybridizes downstream of the targeted region and was used as a probe. The probe was radiolabeled with [α-^{32}P]dCTP per manufacturer's instructions (Random Primed DNA Labeling Kit; Roche AppliedScience) and hybridized to the nylon

membrane as instructed (Rapid-hyb, GE Healthcare Life Sciences). DNA fragment length was visualized on autoradiography film (BioMax, Kodak) following overnight storage with the membrane at -80°C. A custom-made real-time PCR probe (Applied Biosystems) was engineered to detect the copy numbers of exon 1 of *Mefv* in the genome relative to the number of copies of the *Hprt* gene. ESCs that contained a targeted *Mefv* allele were injected into C57BL/6 blastocysts, and the chimeric blastocysts were implanted into C57BL/6 foster moms. Chimeric mice were identified by coat color and mated with both C57BL/6 and 129/SvEv mice. Transmission of the targeted allele was identified by Southern blot.

Northern blotting

Total RNA was isolated from neutrophils using RNA Bee (Tel-test) as instructed by the manufacturer. RNA was electrophoresed through a 1.1% formaldehyde, 1.2% agarose gel containing formamide and transferred to a nylon membrane (Bright Star Plus Positively-charged nylon membrane, Ambion). Full-length cDNA corresponding to Mefv was radiolabeled with [α-^{32}P]dCTP per manufacturer's instructions (Random Primed DNA Labeling Kit; Roche AppliedScience). RNA blots were probed with radiolabled cDNA probe in hybridization buffer following manufacter's protocol (ULTRAhyb Ultrasensitive Hybridization Buffer, Applied Biosystems). Membranes were hybridized and washed at 42°C. RNA was visualized on autoradiography film (BioMax, Kodak) following overnight storage with the membrane at -80°C. Membranes were probed with a β-actin probe following the same protocol.

Real-time PCR to detect Mefv expression

Total RNA was isolated from the bone marrow and spleen, and the optical density (OD) at 260 nm was used to determine RNA concentration. RNA was converted to cDNA per manufacturer's protocol (high capacity cDNA Reverse Transcription Kit; Applied Biosystems). TaqMan universal PCR master mix and probes corresponding to Mefv and β-actin mRNA transcripts were used to quantify fluorescence intensity by an ABI Prism 7900HT detection

system according to manufacturer protocol (all from Applied Biosystems). The Mefv probe detects all known splice variants. Relative expression values were calculated from the cycle number (Ct) data.

Mouse genotyping

Mice used in experiments were genotyped by PCR and/or Southern blot. PCR primer sequences listed in Table 2.2. PCR product sizes were 351 bases for the WT allele and 594 bases for the targeted allele.

Flow cytometry

Single-cell suspensions of thymocytes, splenocytes, and lymph node cells were prepared using standard methodologies. Cells were incubated in PBS containing 0.1% BSA during staining. Fc-block, Gr-1-APC, CD11b-PE, CD-11c-FITC, CD-3-FITC, CD-4-PE, CD-8-PE Abs (BD Biosciences) were used as recommended by the manufacturer and compared with isotype-matched Ab controls. Samples were scanned using a Beckman Coulter CyAn ADP Analyzer, and data were analyzed using FloJo (TreeStar) software.

Peritoneal lavage

The peritoneal cavity was lavaged with 4 mL phosphate-buffered saline (PBS) and the concentration of cells was determined using a hemocytometer.

Blood analysis

Blood was collected by cardiac puncture. Whole blood containing 5mM EDTA was used for analysis on the Heska Hematology Analyzer by the Animal Clinical Chemistry and Gene Expression Laboratory at UNC-Chapel Hill.

Use of animals

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of UNC Chapel Hill.

Table 2.1. PCR primers.

Construction of Mefv^{Del} vector:	
3' Red/ET arm upper (56624):	gagaggtacCAAGAGGGGCAAGATTTGAA
3' Red/ET arm 5' junx lower (56621):	GTGCTCGAAGGAGTTTCCTGcaattggctagc acgcgtggatcctctagaGCCCTGTGTTGCCATA ACTT
5' Red/ET arm upper (56623):	gagaggtacCTCATGGCTCTTGCTGCATA
5' Red/ET arm lower (56622):	CAGGAAACTCCTTCGAGCAC
Amp 3' deletion (56719):	CTTTGGCCGCCGCCCAGTCCTGCTCGCT TCGCTACTTGGAGCCACTATCGATGATCT TTTCTACGGGGTCTGACG
Screen for targeted ESCs:	
Upper	Common BAC primer
Lower:	AGATCCACGCCTAGCTTTCA
3' probe for Southern blot:	
Upper:	GTGGTGGGTCTTCCTGTGTT
Lower:	GGTCTCTGTATGGGGTTGAAA
Mouse genotyping primers:	
Endogenous:	CTTTGGAGATTGCTGGCTGT
Common:	TCCAGGAAATGGAGAGATGG
Targeted:	AAATGCCTGCTCTTTACTGAAGG

PCR primers are listed in forward orientation.

Results

Design of the Mefv targeting vector

The mouse *Mefv* gene is located within a 10 kb region on chromosome 16 in the mouse. The primary transcript contains 10 exons, with the translational start site located in exon 1. To generate a mouse line carrying a null Mefv allele, we designed a targeting construct capable of deleting the entire Mefv locus. Homologous recombination of this vector with the endogenous gene resulted in a 21 kb deletion at the *Mefv* locus, replacing the gene with a selectable marker gene. This deletion includes all identified exons as well as the 5 kb promoter region and the intergenic region immediately 3' of the 3'UTR (Figure 3.1*A*). A Vista plot of the genomic region was used to ensure that regions which are conserved between the human, mouse, and rat genomes and are likely to represent regulatory regions were removed upon homologous recombination (13). The genomic region was replaced with a cassette containing: 1) a neomycin resistance gene (Neo^r) that was used for selection, 2) Bam HI and Xba I restriction digest sites that were used to screen for recombinants, and 3) elements for a later genetic manipulation at the targeted locus.

Targeted mouse ESCs were identified

Correctly targeted ES cell lines were identified by three methods. PCR was used as a preliminary screen and detected a fragment length consistent with incorporation of the targeting vector into the mouse genome in some cell lines. These clones were screened by Southern blot analysis using the 3' probe (Figure 3.1*A*). Proper targeting of the *Mefv* locus created Bam HI and Xba I fragments that were shorter than those corresponding to the endogenous allele (Figure 3.1*B*). Finally, real-time PCR detected a 50% decrease in the relative copy number of *Mefv* in the mouse genome (Figure 3.1*C*). Proper recombination at the Mefv locus occurred at a frequency of 0.15% (2 of 672 ESC colonies screened).

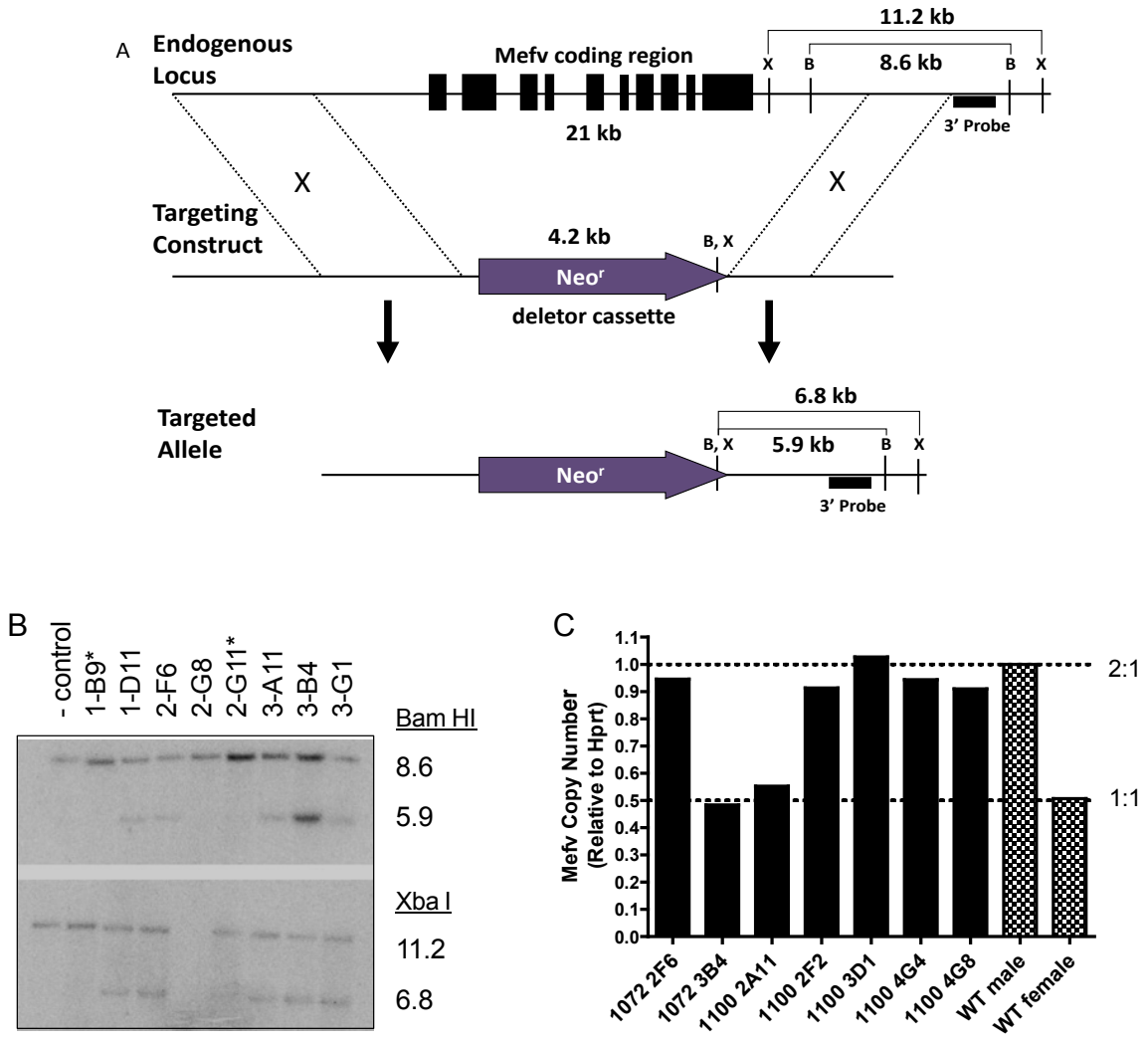


Figure 3.1. Targeting of the *Mefv* locus and screening of ESC clones. *A*, Schematic showing the endogenous *Mefv* locus, gene-targeting construct, and targeted locus after homologous recombination with the targeting construct. Upon homologous recombination, the entire coding region and 5 kb on either side of the *Mefv* gene was replaced with a selectable neomycin resistance (neo^r) gene. Exons are depicted as filled boxes. Arrow indicates directionality of the open reading frames. Bam HI and Xba I restriction sites and the genomic fragment (3' probe) used in Southern blot analysis are indicated. *B*, Southern blot of DNA collected from ES cell lines and digested with Bam HI (top) or Xba I (bottom). Labels identify individual ES cell lines. *C*, Genomic copy number of the *Mefv* gene relative to the *Hprt* gene. Real-time PCR on genomic DNA collected from ES cell lines detects exon 1 of *Mefv* and a unique genomic region within *Hprt*. Since *Hprt* is located on the X chromosome, a wild-type male has 2 copies of *Mefv* per *Hprt*. Targeted male ES cell lines and wild-type female ES cell

lines have a 1:1 of *Mefv* to *Hprt*. A comparison between DNA from a wild-type male and wild-type female served as an assay control (checkered boxes). Results are normalized to a wild-type male. Labels identify individual ES cell lines. Results for clones 1072-3B4 and 1100-2A11 were confirmed by at least two independent experiments.

Mice carrying a null *Mefv* allele were generated

Chimeras generated from targeted 129S6 ES cell lines were crossed with both C57BL/6 and 129S6 mice. As the ES cells were generated from the 129 substrain, breeding with 129S6 mice generated 129 $Mefv^{-/-}$ co-isogenic mice, which were used in all functional assays. $Mefv^{+/-}$ mice were intercrossed, and DNA collected from the offspring was screened by Southern blot analysis and identifed pups that inherited the null allele (Figure 3.2*A*). Inheritance of the null *Mefv* allele followed a Medelian pattern. DNA from animals homozygous for the targeted locus ($Mefv^{-/-}$) was subjected to additional Southern blot analysis to verify the absence of the *Mefv* gene. As expected, DNA from these pups failed to hybridize with probes corresponding to various regions of the *Mefv* gene.

Consistent with our Southern analysis, northern blot analysis using a full length *Mefv* cDNA probe failed to detect *Mefv* transcripts in mRNA prepared from $Mefv^{-/-}$ neutrophils. *Mefv* transcripts present in the wild type cells were absent, and no aberrant transcripts could be detected, confirming that the targeting strategy generated a null *Mefv* allele. A β-actin cDNA probe was used to verify equivalent loading of RNA (Figure 3.2*B*).

To further define the mutation introduced into the *Mefv* locus, RNA was prepare from the bone marrow and spleen of $Mefv^{+/+}$, $Mefv^{+/-}$ and $Mefv^{-/-}$ animals, and *Mefv* expression was assessed by quantitative PCR using primers specific for mRNA transcripts of the WT gene. Expression of *Mefv* is undetectable in $Mefv^{-/-}$ animals. A 50% decrease in expression is

observed in heterozygous animals compared to *Mefv*$^{+/+}$ animals, indicating that there is little or no compensatory transcription from the remaining wild-type *Mefv* allele (Figure 3.2*C*).

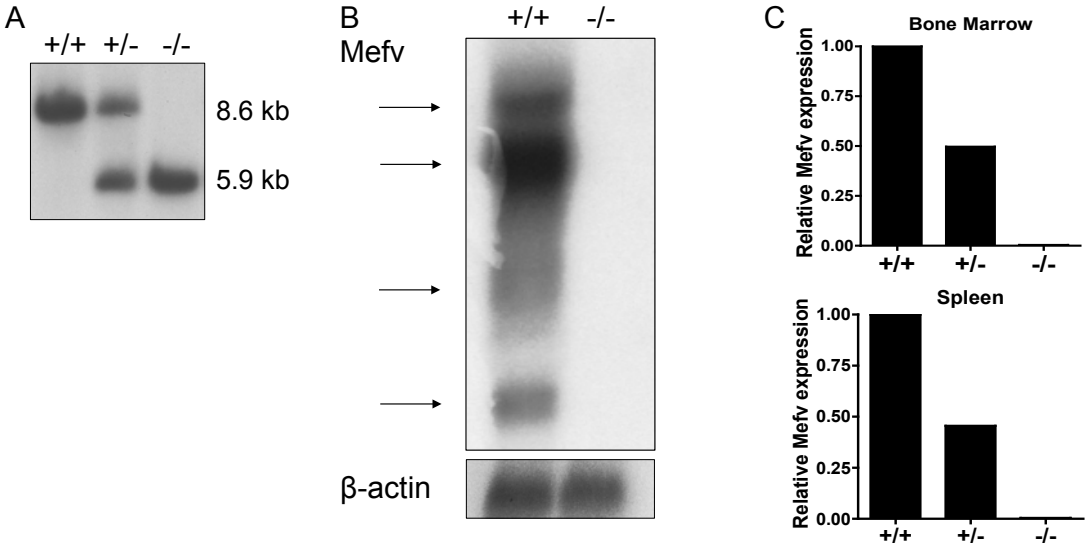


Figure 3.2. Verification of *Mefv*$^{-/-}$ mice. *A*, Southern blot analysis of DNA from offspring generated by the intercross of mice heterozygous for the *Mefv* mutant allele. The 8.6 kb BamHI restriction fragment is generated by the endogenous WT *Mefv* alelle and the 5.9 kb fragment is generated by the targeted *Mefv* null allele. *B*, Northern blot analysis of RNA collected from recruited peritoneal neutrophils of *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ mice. A full-length *Mefv* cDNA probe was used to detect transcripts corresponding to the *Mefv* gene. Several bands were detected in the WT sample, indicating the presence of multiple splice variants of *Mefv*. All bands are absent in *Mefv*$^{-/-}$ mice. A probe specific to *β-actin* confirms equal loading of the RNA samples. *C*, Relative expression of *Mefv* in bone marrow and spleen tissues collected from *Mefv*$^{+/+}$, *Mefv*$^{+/-}$, and *Mefv*$^{-/-}$ mice.

Naïve *Mefv*-deficient mice showed no developmental abnormalities

The loss of Pyrin did not impact the survival, growth, or reproductive performance of the mice. Intercrossing heterozygous animals produced litters with *Mefv*$^{-/-}$ mice at expected

Mendelian ratios, and the size and frequency of litters from *Mefv*$^{-/-}$ mating pairs were similar compared to coisogenic *Mefv*$^{+/+}$ (WT) 129/S6 mice. Total body weight (Figure 3.3*A*) and organ weights (Figure 3.3*B*) were similar between age-matched 10-week-old WT and KO mice. Furthermore, *Mefv*$^{-/-}$ mice be distinguished from littermates based on morphological or behavioral criteria. *Mefv*-deficient mice displayed no overt signs of acute or chronic disease, such as lethargy or sudden death.

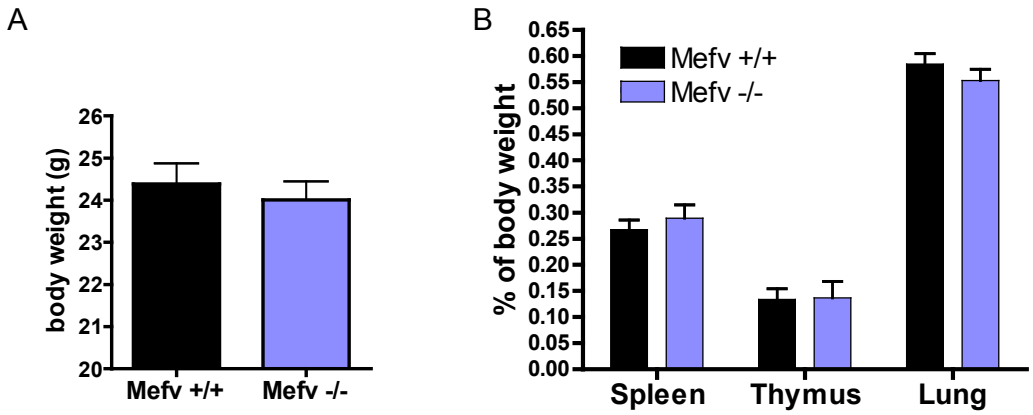
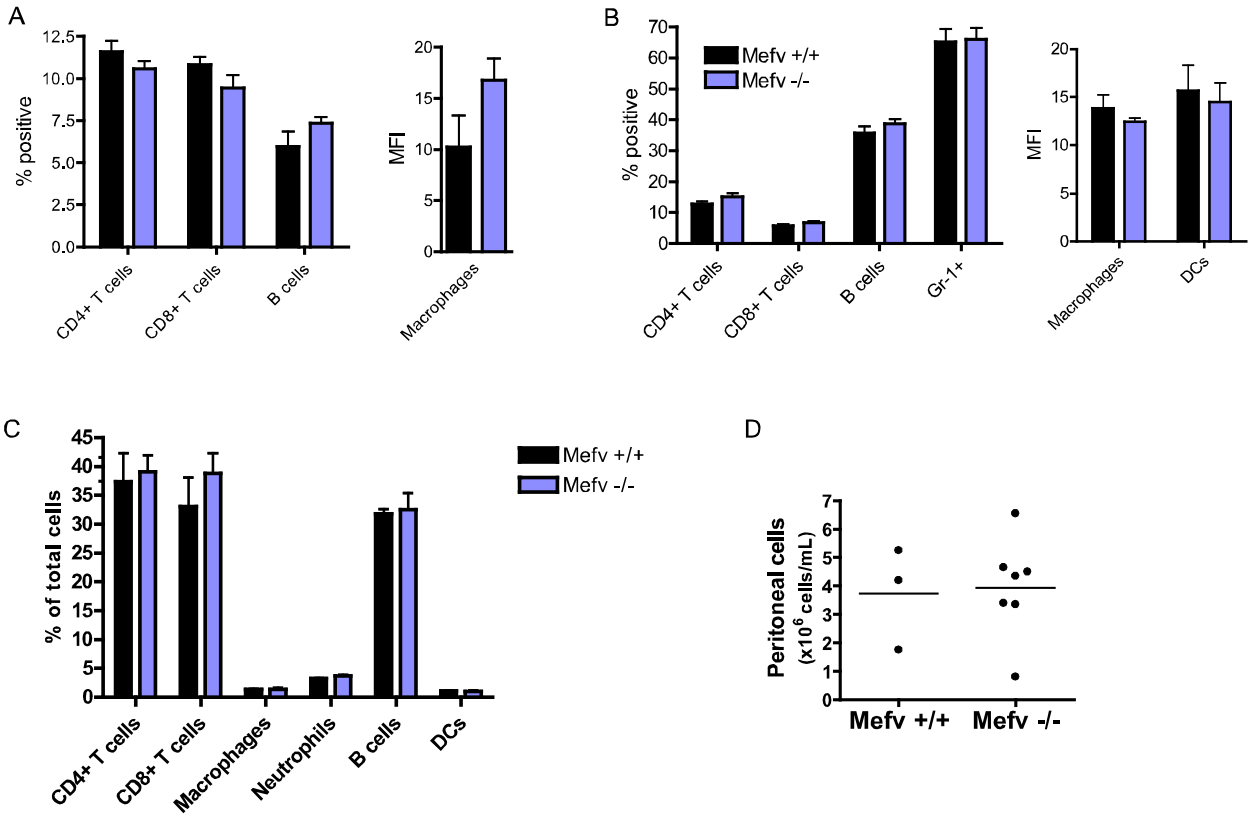


Figure 3.3. *Mefv*$^{-/-}$ mice had normal body and organ weights. *A*, Body weight of *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ mice at 10 weeks of age. n = 18 *Mefv*$^{+/+}$, 19 *Mefv*$^{-/-}$ mice, p = 0.57. *B*, Weight of spleen, thymus, and lung organs is expressed as percent of total body weight. n = 3 mice per genotype.

Since Mefv expression was detected in immune organs of untreated mice, and FMF is an autoinflammatory disorder characterized by episodes of spontaneous inflammation, we hypothesized that the loss of Mefv might affect the resting, or naïve, state of the immune system. We characterized naïve animals maintained in a sterile and pathogen-free environment

for indications of altered immune system development or signs of inflammation. Cell populations within the primary immune organs of the thymus, spleen, and mesenteric lymph nodes from naïve mice were characterized by flow cytometry. No change in cellularity of the thymus, spleen, or lymph nodes was apparent. Furthermore, analysis of the cellular composition of these organs failed to identify a role for *Mefv* in the development of lymphocytes or cells of myeloid lineage, despite the high level of expression of *Mefv* in this later population (Figure 3.4*A*-*C* and data not shown). To examine a large population of innate immune cells, cells residing in the peritoneal cavity were collected by lavage. The total number of cells was similar between WT and KO animals (Figure 3.4*D*). Additionally, blood collected from 6-month-old WT and *Mefv*$^{-/-}$ animals also indicated a similar concentration of circulating lymphocytes, monocytes, and granulocytes between cohorts (Figure 3.4*E*).



E

	WBC $x10^3$/uL	RBC $x10^6$/uL	PLT $x10^3$/uL	LYMPH %	GRAN %	MONO %	HCT %	HGB g/dL
WT 129	1.71 ± 0.28	8.29 ± 0.16	238.10 ± 29.55	73.47 ± 3.29	17.08 ± 2.13	10.50 ± 0.65	45.29 ± 0.98	13.79 ± 0.25
Mefv -/-	2.12 ± 0.35	8.34 ± 0.24	285.50 ± 31.61	67.25 ± 6.08	23.72 ± 6.97	10.03 ± 0.65	45.73 ± 1.09	13.90 ± 0.30

Figure 3.4. Naïve *Mefv*$^{-/-}$ mice have a normal immune system profile. *A-C*, FACS analysis shows the percentage of thymocytes, *A*, splenocytes *B*, and lymph node cells, *C*, that are positive for T lymphocyte (CD3/CD4 and CD3/CD8), B lymphocyte (B220), macrophage (CD11b), dendritic cell (CD11c), and neutrophil (Gr-1) cell receptors. n = 3 – 6 mice per genotype for each tissue. *D*, Total number of resident peritoneal cells collected in 4 mL of lavage fluid. Each dot represents one mouse. *E*, Analysis of blood collected from WT and *Mefv*$^{-/-}$ mice. n = 13 mice per genotype.

Discussion

We describe here the generation of a mouse in which the entire *Mefv* gene is excised. Although a more difficult mutation to generate, this strategy ensures the loss of not only the primary *Mefv* transcript, but also transcripts initiated from internal promoters and splice variants from the *Mefv* locus. Thus, proteins containing all or a subset of the many functional domains of Pyrin cannot be generated from this modified locus. Pyrin null mice were born at a normal Mendelian ratio and displayed normal growth and development. Immune system development was unaffected by the loss of Pyrin, and naïve mice showed no signs of inflammation or illness. Our results suggest that there may be a mechanism to prevent a functional deficit caused by the loss of Pyrin, at least in a naïve immune state.

Our northern blot analysis indicates that alternative splicing of murine *Mefv* is more extensive than previously described. Although multiple splice variants of the human *MEFV* gene have been described (10), in mice and rats, only one transcript for the *Mefv* gene was detected by previous northern blot analysis (14). Using RT-PCR, one splice variant of the mouse gene was identified in which exon 9 is replaced with an alternative exon a found in intron 9 (11). Nucleotide sequencing of the bands detected by our northern blot analysis is necessary to confirm the identity of these bands as transcripts of the *Mefv* gene, however their absence in *Mefv* null mice provides preliminary support.

It is not too surprising that naïve mice showed no signs of inflammation or illness. The contribution of many genes cannot be detected in knock-out mice in a naïve state, but following treatments to induce an innate immune response, the contribution of these genes to inflammation is striking. Many other mouse lines with deficiencies in genes that contribute to the innate immune response do not display a phenotype. Specific to IL-1β production, *Il1β*, *Caspase-1*, *Nlrp1b*, and *Nlrp3* –deficient mice do not show overt signs of spontaneous inflammation or developmental deficits. Furthermore, immune cell composition of naïve mice

was unaltered by the loss of *Aim2* (15). Moreover, analogous to these previous studies, the lack of a detectable phenotype in naïve mice leaves open the possibility that a functional role of Pyrin may be distinguished following treatment to Pyrin-deficient mice to induce inflammation.

Mice carrying a disrupted *Mefv* allele were previously generated. These mice carry an allele in which exon 3 is disrupted to place eGFP and pgk-Neo^r cassettes in frame, and a stop codon terminates translation within the altered third exon. These mice produce a protein with an intact pyrin domain (PYD), but the rest of the protein is deleted. Anecdotal notes of exacerbated immune function were reported in naïve mice and significant function differences were seen upon treatment of homozygous mutant mice with immune stimulants. Immunological phenotypes including fever, increased IL-1β production, increased lethality in response to LPS, increased cellularity after the induction of peritonitis, and impaired apoptosis of macrophages were observed (11). However, extrapolating the results of these studies to define the function of Pyrin is complicated by the presence of a truncated Pyrin protein in these animals, especially since individual domains within Pyrin appear to be functionally competent. The authors based the interpretations of their studies on the idea that these mice express proteins with hypomorphic function. However, expression of the truncated Pyrin protein appears to be greater than that of endogenous Pyrin. This finding is more consistent with a model in which the disrupted gene produces a protein with increased function. Furthermore, mutations in human Pyrin may not confer a loss-of-function to the Pyrin protein. Intramolecular interactions between the domains of Pyrin have been seen (16), and it is reasonable to hypothesize that a mutation might alter these protein interactions and influence Pyrin to lead to a gain-of-function. The PYD of the truncated mouse protein may function very differently than the wild-type Pyrin protein and/or mutant Pyrin generated by FMF-associated alleles. Mutations could even result in hypermorphic function with respect to some interactions and hypomorphic function with respect to others. Therefore, to address the overall function of Pyrin, we generated mice missing the

entire Pyrin protein. Our strategy to remove the entire coding region and eliminate all potential splice variants will help to clarify what appears to be a quite complex contribution of Pyrin to innate immune responses.

A recent report describes the generation of mice expressing a chimeric Pyrin protein. The majority of the protein is of endogenous origin, but a short region of the C-terminal end is removed and replaced with a mutated C-terminal region of human Pyrin, which includes the B30.2 domain. Homozygous mice displayed delayed growth, visual signs of inflammation including dermatitis, and an increased number of innate immune cells, even in a resting state. Similar to studies using mice expressing a truncated Pyrin protein, work with these mice suggests that disruption to the Pyrin protein leads to inflammatory phenotypes. However, again, discerning the contribution of Pyrin to the innate immune system is complicated by the presence of an altered protein which could have novel functions. Consistent with this hypothesis, mice in which the wild-type B30.2 sequence was incorporated into the *Mefv* locus could not be generated, perhaps due to a novel protein function(s) that is incompatible with embryonic development.

While the majority of the *Mefv* gene is orthologous between mice and humans, a point mutation within the mouse gene forms a stop codon and causes early termination of transcription. Thus, Pyrin encoded by the mouse gene lacks the C-terminal domain, termed B30.2, present in the human protein. This complicates comparison of gene function, not only because numerous functions including interaction with Caspase-1, NLRPs1-3, and IL-1β have been assigned to this domain (16), but also because the majority of the FMF mutations are located in this C-terminal region (17). In addition, major differences in the regulation of the gene have been noted between human and mouse: while the mouse gene is induced by IL-4 (11), this cytokine inhibits expression of the human ortholog (18). These differences raise the possibility that one or more functions of Pyrin is unique to the human protein and is therefore

difficult to model in the mouse. Thus, perhaps for no other autoinflammatory disorder is the validity of extrapolating results from mouse studies more subject to scrutiny than for FMF. To address this problem, the targeted Mefv locus is designed to allow a further recombination event to introduce the human *MEFV* gene and its regulatory elements into the mouse genome. Future studies, beyond the scope of this work, will examine the contribution of the B30.2 domain to the overall function of the Pyrin protein.

References

1. Hornung, V., A. Ablasser, M. Charrel-Dennis, F. Bauernfeind, G. Horvath, D. R. Caffrey, E. Latz, and K. A. Fitzgerald. 2009. AIM2 recognizes cytosolic dsDNA and forms a Caspase-1-activating inflammasome with ASC. *Nature* 458:514-518.

2. Li, P., H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, and et al. 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* 80:401-411.

3. Sutterwala, F. S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G. S. Lichtenberger, E. P. Grant, J. Bertin, A. J. Coyle, J. E. Galan, P. W. Askenase, and R. A. Flavell. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of Caspase-1. *Immunity* 24:317-327.

4. Mariathasan, S., K. Newton, D. M. Monack, D. Vucic, D. M. French, W. P. Lee, M. Roose-Girma, S. Erickson, and V. M. Dixit. 2004. Differential activation of the inflammasome by Caspase-1 adaptors ASC and Ipaf. *Nature* 430:213-218.

5. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.

6. Saleh, M., J. C. Mathison, M. K. Wolinski, S. J. Bensinger, P. Fitzgerald, N. Droin, R. J. Ulevitch, D. R. Green, and D. W. Nicholson. 2006. Enhanced bacterial clearance and sepsis resistance in Caspase-12-deficient mice. *Nature* 440:1064-1068.

7. Solle, M., J. Labasi, D. G. Perregaux, E. Stam, N. Petrushova, B. H. Koller, R. J. Griffiths, and C. A. Gabel. 2001. Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 276:125-132.

8. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.

9. Allen, I. C., M. A. Scull, C. B. Moore, E. K. Holl, E. McElvania-TeKippe, D. J. Taxman, E. H. Guthrie, R. J. Pickles, and J. P. Ting. 2009. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 30:556-565.

10. Medlej-Hashim, M., N. Nehme, E. Chouery, N. Jalkh, and A. Megarbane. 2010. Novel MEFV transcripts in Familial Mediterranean fever patients and controls. *BMC Med Genet* 11:87.

11. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* 11:591-604.

12. Mohn, A. a. K., B. H. 1995. Genetic Manipulation of Embryonic Stem Cells. In *DNA Cloning 4*, 2nd ed. G. D. a. H. BD, ed. Oxford, New York. 143-182.

13. Frazer, K. A., L. Pachter, A. Poliakov, E. M. Rubin, and I. Dubchak. 2004. VISTA: computational tools for comparative genomics. *Nucleic Acids Res* 32:W273-279.

14. Chae, J. J., M. Centola, I. Aksentijevich, A. Dutra, M. Tran, G. Wood, K. Nagaraju, D. W. Kingma, P. P. Liu, and D. L. Kastner. 2000. Isolation, genomic organization, and expression analysis of the mouse and rat homologs of MEFV, the gene for familial mediterranean fever. *Mamm Genome* 11:428-435.

15. Rathinam, V. A., Z. Jiang, S. N. Waggoner, S. Sharma, L. E. Cole, L. Waggoner, S. K. Vanaja, B. G. Monks, S. Ganesan, E. Latz, V. Hornung, S. N. Vogel, E. Szomolanyi-Tsuda, and K. A. Fitzgerald. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11:395-402.

16. Papin, S., S. Cuenin, L. Agostini, F. Martinon, S. Werner, H. D. Beer, C. Grutter, M. Grutter, and J. Tschopp. 2007. The SPRY domain of Pyrin, mutated in familial Mediterranean fever patients, interacts with inflammasome components and inhibits proIL-1beta processing. *Cell Death Differ* 14:1457-1466.

17. Shohat, M., and G. J. Halpern. 2011. Familial Mediterranean fever-A review. *Genet Med* 13:487-498.

18. Centola, M., G. Wood, D. M. Frucht, J. Galon, M. Aringer, C. Farrell, D. W. Kingma, M. E. Horwitz, E. Mansfield, S. M. Holland, J. J. O'Shea, H. F. Rosenberg, H. L. Malech, and D. L. Kastner. 2000. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood* 95:3223-3231.

CHAPTER 4

Genetic loss of murine Pyrin, the Familial Mediterranean Fever protein, increases Interleukin-1β levels

Familial Mediterranean Fever (FMF) is an inherited autoinflammatory disorder characterized by unprovoked episodes of fever and inflammation. The associated gene, *MEFV* (Mediterranean Fever), is expressed primarily by cells of myeloid lineage and encodes the protein Pyrin/TRIM20/Marenostrin. The mechanism by which mutations in Pyrin alter protein function to cause episodic inflammation is controversial. To address this question, we have generated a mouse line lacking the *Mefv* gene by removing a 21 kb fragment containing the entire *Mefv* locus. We show enhanced IL-1β release by *Mefv*$^{-/-}$ macrophages in response to a spectrum of inflammatory stimuli, including stimuli dependent on IL-1β processing by the NLRP1b, NLRP3 and NLRC4 inflammasomes. These results are consistent with a model in which Pyrin acts to limit the release of IL-1β generated by activation and assembly of inflammasomes in response to subclinical immune challenges.

Introduction

Mutations in *MEFV* predispose humans to Familial Mediterranean Fever (FMF), a disease characterized by spontaneous activation of the innate immune system in the absence of a detectable pathogenic stimulus. Fever and acute abdominal pain are the most common symptoms, but disease manifestations also include arthritis, pleuritis, localized erythema, and amyloidosis of the kidneys. The inflammation observed in FMF patients is characterized by neutrophil influx to peripheral tissues and increased serum levels of acute-phase reactant proteins and cytokines (1). The efficacy of pharmacological inhibitors of IL-1β/IL-1 receptor signaling in treatment of some patients supports the hypothesis that pathophysiology of FMF is mediated in part by this cytokine (2).

MEFV encodes a 781 a.a. cytoplasmic protein comprised of 5 domains: pyrin, b-Zip, B-Box, coiled-coiled, and PRY/SPRY (B30.2), although this later domain is absent in the mouse protein. The pyrin domain is of particular interest since this domain is found in several proteins critical in IL-1β production (3). Similar to most cytokines, *Il1β* transcript levels are enhanced by cellular stimulation with cytokines or pathogen-associated molecular patterns (PAMPs) (4). However, the *Il1β* gene does not encode a leader peptide to facilitate transport and secretion of the protein (5). Instead, it is synthesized as an inactive pro protein which is cleaved by the cysteine-aspartic acid protease Caspase-1 (CASP-1) to form mature IL-1β. Catalytic activity of CASP-1 also requires removal of a regulatory domain, although in this case, this is achieved through autocatalytic cleavage. Activation of CASP-1 is observed after exposure of cells to both host-derived signals of cellular stress or damage, called danger-associated molecular patterns (DAMPs) and to PAMPS. These signals trigger the assembly of inflammasomes, protein scaffolds nucleated by a nucleotide-binding domain, leucine-rich repeat family (NLR) protein. A number of NLRs are capable of assembling an inflammasome in response to unique but overlapping DAMPS and PAMPS. To date, these include NLRP3, NLRP1b, NLRC4, NLRP2

and NLRP6 (6-11). However, assembly of a CASP-1 activating inflammasome complex is not restricted to the NLR family, as more recently, the protein absent in melanoma 2 (AIM2) has been shown to contribute to CASP-1 activation and IL-1β release in response to dsDNA (12).

In addition to the NLR/AIM2 protein, the inflammasome often contains the protein adaptor ASC which facilitates the recruitment of pro-Caspase-1 to the complex (3). Interaction between the pyrin and card domains of various proteins is critical for inflammasome assembly, both the self association of the NLR proteins and the recruitment of ASC and CASP-1 to the complex. It is, therefore, not surprising that it has been suggested that proteins which contain a PYD or CARD domain, yet lack other critical functional domains, act as dominant negative regulators of inflammasome assembly (13). Furthermore, a number of studies indicate that Pyrin might function in a similar manner, with disease-associated mutations resulting in reduced ability to limit inflammasome assembly (14-17). Such a model is consistent with the recessive pattern of inheritance of the disease (18, 19) and with previous findings that Pyrin can bind ASC, CASP-1, NLRPs 1-3, and IL-1β (15, 17, 20, 21).

Not all reports, however, support this model. Molecular genetic reports of both the absence of individuals carrying null *MEFV* alleles and the observation of disease in some individuals believed to carry a wild-type allele, have led to the suggestion that FMF is an autosomal dominant disease with variable penetrance (22, 23). It has been posited that Pyrin itself can assemble a CASP-1 activating inflammasome, with the assembly of the mutant Pyrin inflammasome triggered by lower, subclinical, levels of PAMPs/DAMPs (21, 24-27). Transfection and knockdown studies provide limited clarification of the function Pyrin, as in some studies, Pyrin increased IL-1β release, while in others, using similar strategies, cytokine production was decreased (14-17, 21, 25-27).

To further examine the function of Pyrin, we generated a mouse carrying a null *Mefv* allele. This mouse lacks the entire 21 kb locus. Using primary cells from this mouse line, we show that Pyrin negatively regulates IL-1β production in response to inflammasome-dependent stimuli.

Materials and Methods

In vitro macrophage activation studies

Resident peritoneal cells were collected by lavage and cultured in RPMI media (Gibco) containing 10% FBS (Cellgro), 10 mM HEPES (Gibco) and supplements (L-glutamine, pen/strep, 2β-mercaptoethanol) at 10^6 cells/mL and 10^5 cells/well in a 96-well plate. Cells were washed with phosphate-buffered saline (PBS, Gibco) to remove non adherent cells, primed with 1 μg/mL crude lipopolysaccharide derived from *Escherichia coli* (LPS, Sigma) and then stimulated as indicated. 250 μg/mL Aluminum hydroxide (Alum), 250 μg/mL silica, 100 μg/mL calcium pyrophosphate dihydrate (CPPD), 1mM ATP, 250 ng/mL each of lethal factor and protective antigen from *Bacillus anthracis* (LT), 10 ug/mL muramyl dipeptide (MDP), 5 ug/mL titanium dioxide (TiO2). For LT treatment, cells were cultured in DMEM without glutamine and containing 2 mM Glutamax (Invitrogen), 10% FBS, 2 mM HEPES, and 50 ug/mL gentamycin. For ATP stimulation, cells were incubated in BSSH buffer. Cell culture supernatants were collected and stored at -80°C. After collection of media, cells were lysed with and total protein was measured.

Real-time PCR Analysis

Total RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit. TaqMan universal PCR master mix and recommended probes were detected by ABI Prism 7900HT detection system (Applied Biosystems). Gene expression was calculated as the relative expression of *Mefv*, *Il1β*, or *Il6* normalized by *Gapdh* or *18s*.

Protein analyses

IL-1β and IL-6 ELISA (eBioscience), lactate dehydrogenase (LDH, Clontech) and total protein (BCA protein assay, Thermo Scientific Pierce) protocols were followed.

LPS/ATP treatment *in vivo*

Mice were treated with 1 mg/mouse of crude LPS at time 0 h, followed by 500 µL/mouse of 30 mM ATP at 2 h, and harvested at 2.5 h. The peritoneal cavity was lavaged with PBS. Peritoneal cell concentrations and viability were assessed using a hemocytometer and trypan blue. Samples were centrifuged to pellet cells and the supernatant was collected for IL-1β ELISA.

Animal Care and Use

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of the University of North Carolina at Chapel Hill.

Results

Pyrin-deficient mice displayed normal responses to LPS

The N-terminal cleaved fragment of Pyrin has been reported to interact with IκB-α and the p65 subunit of NF-κB, increasing the activity of this transcription factor (28). If this model is correct, it is reasonable to expect that the induction of NF-κB transcripts after treatment of cells with inflammatory mediators such as LPS would be attenuated in *Mefv*$^{-/-}$ mice. To test this hypothesis, we compared the mRNA levels of two NF-κB sensitive transcripts, *Il1β* and *Il6*, in macrophages before and after exposure to LPS. Basal levels of mRNA for both of these cytokines were similar in the *Mefv*$^{-/-}$ and control macrophages. As expected, treatment of the cells with LPS for 24 hours resulted in a robust increase in expression of these genes. However, the magnitude of the increase in expression of *Il1β* and *Il6* was not altered in cells lacking Pyrin (Figure 4.1*A*).

To address this point further, we examined the level of IL-6 and IL-1β in the supernatant of cultures of rpMΦ before and after LPS treatment. IL-6, similar to most cytokines, is released through a classical secretion pathway (4). Consistent with the increase in mRNA levels, a dramatic increase in the level of IL-6 was observed in the supernatants from the LPS-treated cells. No difference, however, was observed in IL-6 levels in samples from wild-type (WT) and Pyrin-deficient cell cultures (Figure 4.1*B*). Similar to IL-6, LPS results in an increase in expression of *Il1β* mRNA, but efficient release of this cytokine requires processing of the immature protein by Caspase-1 (7). Thus, not unexpectedly, the level of IL-1β in the supernatant collected from macrophage cultures is low, and while these levels increased significantly after LPS treatment, the magnitude of this increase was small. Loss of Pyrin did not augment or attenuate the release of IL-1β by rpMΦ either left untreated or following treatment with LPS (Figure 4.1*C*). Survival of the macrophages, either under control conditions or in the presence of LPS was not altered by the lack of Pyrin: levels of the cytoplasmic enzyme

LDH in the supernatant did not differ between samples collected from *Mefv*$^{-/-}$ and WT cultures (Figure 4.1*D*).

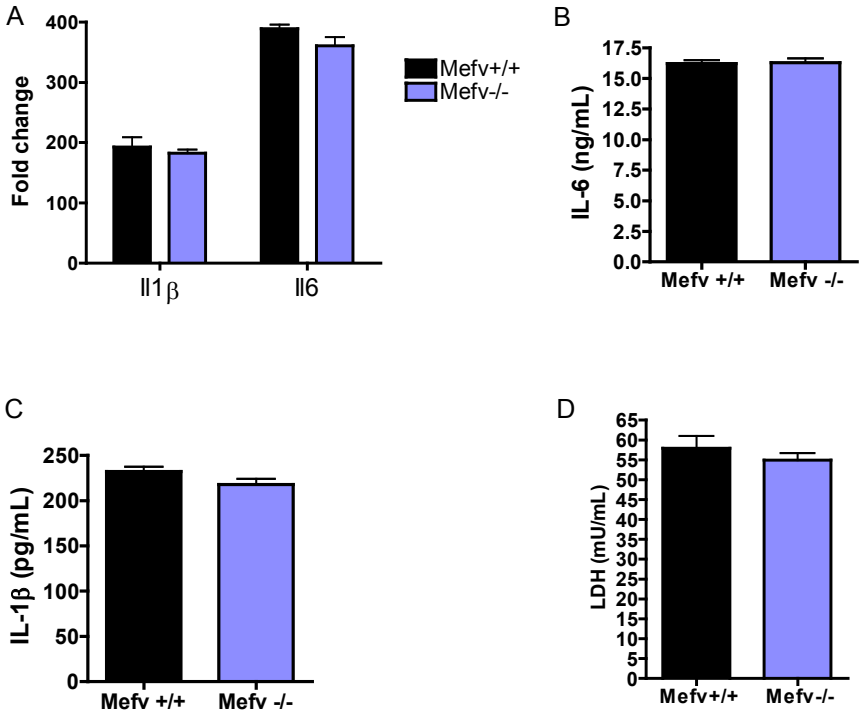


Figure 4.1. Loss of Mefv does not affect the response to LPS. rpMΦ from *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ littermate mice were untreated or treated with 1 μg/mL LPS for 24 h. *A*, Il1β and Il6 expression at the transcript level is expressed as fold induced by LPS treatment. n = 3 mice per genotype. *B* and *C*, Concentration of IL-6 and IL-1β in supernatants from LPS-treated rpMΦ cultures as determined by ELISA. n = 6 mice per genotype. *D*, The concentration of lactate dehydrogenase (LDH) released into cell supernatants was used as an indicator of cell death.

NLRP3 Inflammasome-dependent IL-1β production is increased in *Mefv*-deficient macrophages

To determine if a loss of Pyrin modulates inflammasome-mediated IL-1β production, WT and *Mefv*$^{-/-}$ rpMΦ were exposed to a variety of elicitors that induce NLRP3 inflammasome-dependent IL-1β production. Cells were pre-treated with LPS to induce IL-1β transcription and then stimulated with one of the following NLRP3 inflammasome elicitors: titanium dioxide (TiO2), aluminum hydroxide (alum), calcium pyrophosphate dehydrate crystals (CPPD), silica, or ATP (6, 29). IL-1β and IL-6 levels in the cell supernatants were determined by ELISA. As expected, treatment of cells with compounds that trigger assembly of the NLRP3 inflammasome resulted in substantial increase in the levels of extracellular IL-1β, compared to cultures treated with LPS alone. In addition, in all cases, higher IL-1β cytokine levels were measured in supernatants collected from *Mefv*$^{-/-}$ cultures compared to those collected from similarly treated WT cultures. The magnitude of the increase in the levels of IL-1β in *Mefv*$^{-/-}$ cultures compared to controls varied depending on the stimulus, ranging from an increase of 1.5-fold in cells exposed to silica to an increase of almost 5-fold in the response of *Mefv*$^{-/-}$ cells to Alum (Figure 4.2*A*). No difference was observed in the levels of IL-6 present in the samples from WT and *Mefv*$^{-/-}$ cultures (Figure 4.2*B*). Both mature and pro-IL-1β can be released into the supernatant after cell death, and most ELISAs do not bind exclusively to the mature protein. It is therefore possible that the increase in IL-1β in samples collected from *Mefv*$^{-/-}$ cultures reflects an increase in cell death. To address this question, we measured levels of LDH in the supernatant. Many of the agents used to stimulate NLRP3 assembly decrease cell viability (30), and the magnitude of this decrease was dependant on the agent used to elicit the inflammasome, but viability was not influenced by the absence of Pyrin: LDH levels did not differ significantly between *Mefv*$^{-/-}$ and WT macrophage cultures (Figure 4.2*C*).

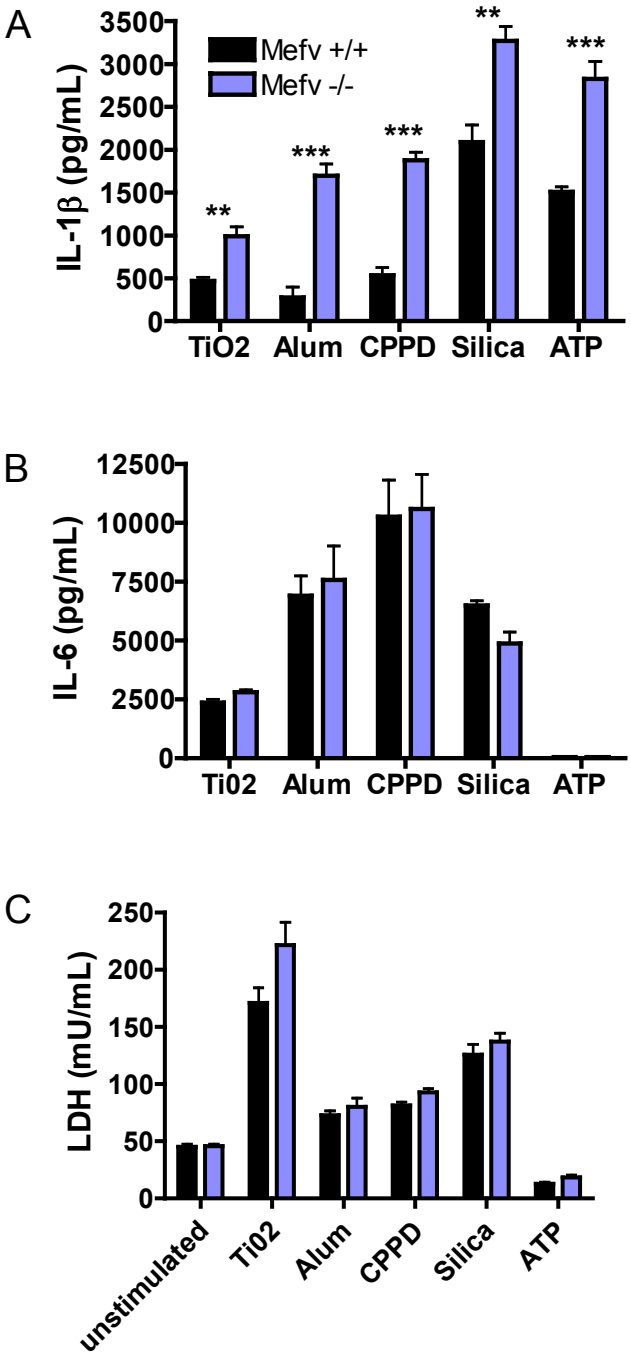


Figure 4.2. A loss of Pyrin causes increased IL-1β protein levels in response to NLRP3 inflammasome stimuli. rpMΦs from *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ littermate mice were exposed to LPS and the indicated stimuli. The concentration of IL-1β, *A*, and IL-6, *B*, cytokines in cell culture supernatants was determined by ELISA. *C*, LDH protein levels detected in cell supernatants. The total protein of cell lysates was measured to verify equivalent plating of cells. TiO2, titanium dioxide; Alum, aluminum hydroxide; CPPD, calcium pyrophosphate dihydrate; ATP, adenosine 5' triphosphate. n = 6 mice per genotype. Results are representative of at least 4 independent experiments. A student's t test was used to calculate p values for WT versus Mefv-/- cultures. **, $p \leq 0.001$; ***, $p < 0.0001$.

Negative regulation of IL-1β production is not limited to the NLRP3 inflammasome

Caspase-1 activation and IL-1β release is also observed after exposure of macrophages to agents that mediate the assembly of NLRC4 and NLPR1b -containing inflammasomes (7). We therefore asked whether a loss of Pyrin impacts only NLRP3-mediated IL-1β release or whether it also modulates IL-1β release secondary to assembly of these inflammasomes. WT and *Mefv*$^{-/-}$ rpMΦ were treated with LPS and exposed to agents known to elicit the NLRC4 and NLRP1b inflammasomes. The NLRC4 inflammasome is assembled in response to flagellin, while NLRP1b is required for IL-1β production in response to *Bacillus anthracis* (anthrax) lethal toxin (LT). Muramyl dipeptide (MDP) alone and MDP plus titanium dioxide (TiO_2) activate both NLRP1 and NLRP3 inflammasomes (7, 29). Again, significantly higher levels were observed in samples collected from cultures of *Mefv*$^{-/-}$ macrophages compared to similarly treated WT controls (Figure 4.3*A*). In contrast, IL-6 and LDH levels in supernatants collected from WT and *Mefv*$^{-/-}$ cultures were similar (Figure 4.3*B* and *C*).

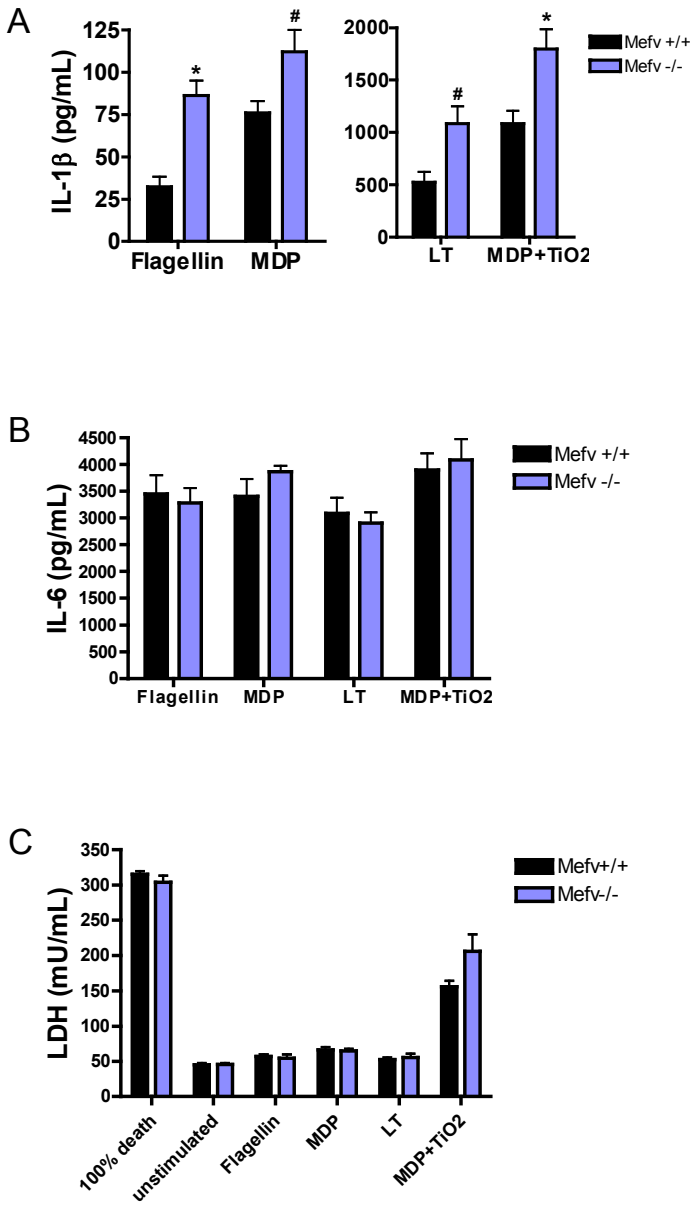


Figure 4.3. A loss of Pyrin causes increased IL-1β protein levels in response to NLRC4 and NLRP1b inflammasome stimuli. rpMΦs from *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ littermate mice were exposed to LPS and the indicated stimuli. The concentration of IL-1β, *A*, and IL-6, *B*, cytokines in cell culture supernatants was determined by ELISA. *C*, LDH protein levels detected in cell supernatants. The total protein of cell lysates was measured to verify equivalent plating of cells. MDP, muramyl dipeptide; LT, lethal toxin of *Bacillus anthracis*; TiO2, titanium dioxide. n = 6 mice per genotype. Results are representative of at least 4 independent experiments. A student's t test was used to calculate p values for WT versus Mefv-/- cultures. #, $p < 0.05$; *, $p \leq 0.01$.

IL-1β levels after LPS/ATP treatment *in vivo* were not affected by the loss of Pyrin

To determine if Pyrin affected inflammasome-mediated IL-1β production *in vivo*, we exposed mice to LPS and ATP. In contrast to the significant 2-fold increase in IL-1β levels in supernatants of macrophages treated with LPS/ATP *in vitro* ($p < 0.0001$), *Mefv*-/- and WT mice released similar amounts of IL-1β *in vivo* (Figure 4.4*A*). The concentration and viability of peritoneal cells was also unaffected by the loss of Pyrin (Figure 4.4*B* and *C*). Previous studies have demonstrated that systemic IL-1β levels are increased *in vivo* after LPS/ATP treatment by a NLRP3 inflammasome-dependent mechanism (31, 32), and we were able to verify these findings using our experimental system (Figure 4.4*D* and *E*). However, given the variability which is inherent to this experimental system, it is unlikely that a 2-fold change in IL-1β levels would be detected.

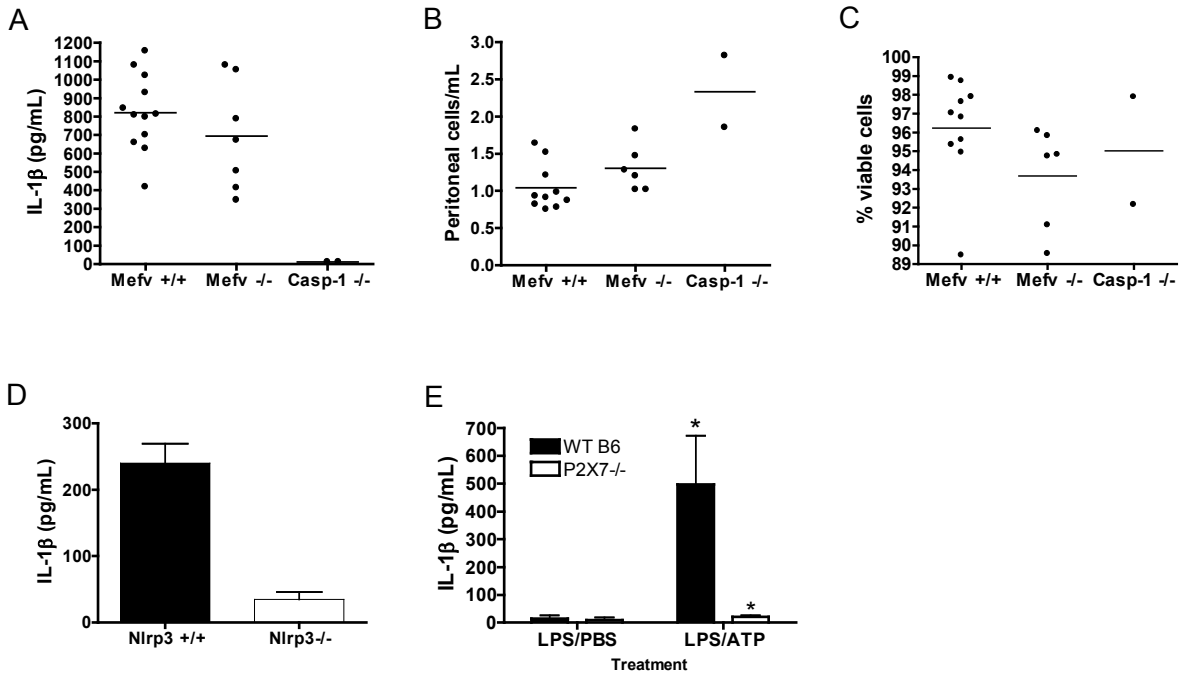


Figure 4.4. Loss of Pyrin does not affect the response to LPS/ATP treatment *in vivo*. *A – C*, *Mefv*$^{+/+}$, *Mefv*$^{-/-}$, and *Caspase-1*$^{-/-}$ mice were treated with 1 mg/mouse of crude LPS at time 0 h, followed by 500 µL/mouse of 30 mM ATP at 2 h, and harvested at 2.5 h. The peritoneal cavity was lavaged with 4 mL of PBS. *A*, IL-1β concentration in lavage fluid as detected by ELISA. *B*, Cell concentrations were determined using a hemocytometer and *C*, trypan blue to assess viability. Each dot represents one mouse in *A – C*. *D*, *Nlrp3*$^{+/+}$ and *Nlrp3*$^{-/-}$ mice were treated as in *A*. n = 3 mice per genotype. $p < 0.05$. *E,* WT B6 and *P2rX*$_7^{-/-}$ mice were treated with LPS followed by either PBS or ATP and harvested as in *A* using 3 mLs of PBS. n = 3 and 3 for LPS/PBS and 4 and 7 for LPS/ATP. *, $p < 0.05$.

Discussion

Using Pyrin null mice, we show a marked change in the response of primary macrophages to a number of inflammatory stimuli; specifically, a significant increase in the release of IL-1β observed in cultures derived from *Mefv*$^{-/-}$ mice in comparison to WT mice.

Since the discovery of the genetic lesions which cause FMF, numerous models have emerged that attempt to incorporate information gleaned from the genetic and clinical characteristics of the disease, from the structure of the protein and its pattern of expression, and from studies of the gene in model systems. One model suggests that Pyrin is a substrate for Caspase-1, and the N-terminal fragment of Pyrin potentiates inflammation by interacting with proteins of the NF-κB pathway. An increased ratio of cleaved to intact protein was detected in clinical samples from FMF patients (28). However, other investigators have not observed increased NF-κB-dependent luciferase activity following overexpression of full-length *MEFV* (16, 21). Our studies of mouse macrophages lacking Pyrin do not support a role for Pyrin in activity of the NF-κB pathway, although more extensive studies are required. An increase in expression of NF-κB sensitive transcripts was not observed in *Mefv*$^{-/-}$ cells in a naïve state or after exposure to LPS. As expected, *i.p.* injection of LPS resulted in an acute peritonitis characterized by a rapid drop in body temperature and accumulation of inflammatory cells in the peritoneal cavity. However, again, no difference was observed in the response of the mutant mice compared to control littermates.

The phenotype of the *Mefv* null mice differs in some aspects from those reported previously for mice carrying a disrupted *Mefv* locus. Macrophages from these mice were shown to release more IL-1β than wild type cells. In addition, these animals displayed an exaggerated cryogenic response to low-dose LPS, and an increase in morbidity upon exposure to high-dose LPS or LPS and D-Gal. An increase in the number of inflammatory cells recovered from peritoneal lavage fluid was observed in these mice after exposure to thioglycolate. Furthermore, apoptosis

was impaired in macrophages collected from animals homozygous for the targeted locus (14). In contrast, we did not observe an increase in the release of IL-1β from *Mefv*$^{-/-}$ cells treated with LPS alone, and as discussed above, the response of the *Mefv*$^{-/-}$ animals to LPS delivered *i.p.* could not be distinguished from that of the controls. We also failed to detect any change in the survival of *Mefv* null cells: no difference in the rate of cell death was observed in cultured macrophages, or in the number of cells surviving after stimulation of these cultures with inflammatory mediators. In addition, *ex vivo* survival of recruited peritoneal neutrophils lacking Pyrin was not altered (data not shown). One explanation for the differences between the mutant *Mefv* line characterized in this earlier report and the line described here is that in the former, a *Mefv* transcript and a truncated Pyrin protein were created by the targeted allele. In fact, the level of this truncated protein, based on western analysis, appeared to be higher than that observed for the native protein. It is possible that this truncated protein conferred unique functions not observed in mice carrying a null allele.

While we did not observe an increase in the IL-1β release by macrophages in response to LPS alone by cells lacking Pyrin, increased levels of IL-1β were detected in the culture media after stimulation of these mutant cells with agents that lead to inflammasome assembly. The increase in IL-1β levels was observed in supernatants collected from cells stimulated with agents known to trigger assembly of NLRP1b, NLRP3, and NLRC4 inflammasomes. Increased release of IL-1β from cells in response to all of the tested inflammatory stimuli is inconsistent with a model in which Pyrin modulates IL-1β production by regulating the assembly of a specific inflammasome, such as the NLRP3 inflammasome. It is unlikely that Pyrin acts to block ASC or Caspase-1 association with the inflammasome, because no alteration in Caspase-1 activity was observed between *Mefv*$^{-/-}$ and wild-type cells (data not shown). Our results differ from studies carried out with a human monocyte/macrophage cell line and PBMCs in which silencing of the *MEFV* transcript suppressed secretion of IL-1β (25, 26), and from transfection studies in which

overexpression of Pyrin increased Caspase-1 activity and IL-1β release (21). However, our results are consistent with models in which Pyrin acts as a negative regulator of innate immune pathways, and with a genetic model in which Pyrin mutations causing FMF represent loss-of-function mutations. In opposition to the studies mentioned above, silencing of *MEFV* has been shown to increase IL-1β release from a human monocyte/macrophage cell line (15, 17). This model is also supported by transfection studies in which wild-type Pyrin inhibited pro-IL-1β processing in macrophage cell lines, whereas the FMF-associated alleles proved less active in these assays (14, 15).

It is becoming increasingly apparent that production of IL-1β is regulated at many different levels, and that the relative importance of these mechanism(s) is likely dependant on many factors including the cell type, its local environment, and the nature of the stimuli (33). For example, recently, induction of autophagy by rapamycin was reported to result in degradation of pro-IL-1β and reduced secretion of IL-1β (34). Interestingly, in one study, examination of PMNs from FMF patients revealed a gene expression pattern consistent with impaired basal autophagy (35), raising the possibility that increased IL-1β levels in FMF patients and the increase in IL-1β release by the *Mefv*$^{-/-}$ macrophages reflect a defect in regulation of cellular levels of pro-IL-1β. Many other means by which Pyrin could modulate release of IL-1β are apparent. For example, alteration in uptake of particulate matter and/or decrease in lysosomal stability or transport could increase IL-1β maturation in response to many stimuli (7). It has been previously reported that mice with peritonitis induced by octacalcium phosphate (OCP) crystals had elevated IL-1β levels, and IL-1β release is was not impaired by the loss of the inflammasome components NLRP3 or ASC (36). In another study, crystal-induced IL-1β production was blocked by colchicine, a prophylactic drug for FMF patients (37). Furthermore, higher levels of reactive oxygen species have been reported in cells from FMF patients (38), and reactive oxygen species can increase IL-1β release (39-41). Pyrin may regulate the

release of mature IL-1β from the cytoplasm, a process that is poorly understood (5). Together, these data support a hypothesis that Pyrin regulates trafficking of vesicles containing IL-1β. Further studies will be required to identify at which of these many steps Pyrin acts to limit release of IL-1β.

Recently, an additional mouse line carrying a mutant *Mefv*$^{-/-}$ gene was reported. No difference in the release of IL-1β by macrophages after treatment with LPS or LPS and ATP, after infection with *Salmonella typhimurium*, or after tranfection of DNA was reported (24). A number of explanations for these differences are possible. First, while our studies use mature peritoneal macrophages which express high levels of Pyrin (Figure 2.2*B*), Chae, et al. examine IL-1β production in bone marrow-derived macrophages. As cultured bone marrow-derived macrophages express very low levels of Pyrin, the impact of the loss of this protein on IL-1β processing and/or release might not be as apparent with this cell type as on comparison of cells with higher levels of expression. In addition, the two studies use mice of differing genetic backgrounds: those reported here carried out entirely with co-isogenic 129S6 mouse lines, while those of Chae and collegues use N6 B6.129 animals. It is possible that the regulatory role of Pyrin might be more apparent on some genetic backgrounds. Correspondingly, the severity of FMF can differ dramatically between individuals carrying the same mutation (1).

The demonstration here that loss of Pyrin can result in increase in IL-1β release is consistent with the original designation of FMF as a recessive genetic disorder. However, perhaps for no other autoinflammatory disorder is the validity of extrapolating results from mouse studies more subject to scrutiny than for FMF. Pyrin encoded by the mouse gene lacks the C-terminal domain, termed B30.2, that is present in the human protein. This complicates comparison of gene function, not only because numerous functions including interaction with Caspase-1, NLRPs1-3, and IL-1β have been assigned to this domain (17), but also because the majority of the FMF mutations are located in this C-terminal region (14). In addition, major

differences in the regulation of the gene have been noted between human and mouse: while the mouse gene is induced by IL-4 (14), this cytokine inhibits expression of the human ortholog (42). These differences raise the possibility that one or more functions of Pyrin is unique to the human protein and is therefore difficult to model in the mouse. Thus, until novel methods and reagents are developed for addressing this limitation, we can only ascertain that Pyrin can act to limit the activity of proinflammatory pathways, namely the release of IL-1β by macrophages, in at least some species.

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References

1. Ben-Zvi, I., and A. Livneh. 2011. Chronic inflammation in FMF: markers, risk factors, outcomes and therapy. *Nat Rev Rheumatol* 7:105-112.

2. Meinzer, U., P. Quartier, J. F. Alexandra, V. Hentgen, F. Retornaz, and I. Kone-Paut. 2011. Interleukin-1 Targeting Drugs in Familial Mediterranean Fever: A Case Series and a Review of the Literature. *Semin Arthritis Rheum*.

3. Mariathasan, S. 2007. ASC, Ipaf and Cryopyrin/Nalp3: bona fide intracellular adapters of the Caspase-1 inflammasome. *Microbes Infect* 9:664-671.

4. Stanley, A. C., and P. Lacy. 2010. Pathways for cytokine secretion. *Physiology (Bethesda)* 25:218-229.

5. Andrei, C., C. Dazzi, L. Lotti, M. R. Torrisi, G. Chimini, and A. Rubartelli. 1999. The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol Biol Cell* 10:1463-1475.

6. Davis, B. K., H. Wen, and J. P. Ting. 2011. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol* 29:707-735.

7. Schroder, K., and J. Tschopp. 2010. The Inflammasomes. *Cell* 140:821-832.

8. Bruey, J. M., N. Bruey-Sedano, R. Newman, S. Chandler, C. Stehlik, and J. C. Reed. 2004. PAN1/NALP2/PYPAF2, an inducible inflammatory mediator that regulates NF-kappaB and Caspase-1 activation in macrophages. *J Biol Chem* 279:51897-51907.

9. Chen, G. Y., M. Liu, F. Wang, J. Bertin, and G. Nunez. 2011. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol* 186:7187-7194.

10. Elinav, E., T. Strowig, A. L. Kau, J. Henao-Mejia, C. A. Thaiss, C. J. Booth, D. R. Peaper, J. Bertin, S. C. Eisenbarth, J. I. Gordon, and R. A. Flavell. 2011. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 145:745-757.

11. Normand, S., A. Delanoye-Crespin, A. Bressenot, L. Huot, T. Grandjean, L. Peyrin-Biroulet, Y. Lemoine, D. Hot, and M. Chamaillard. 2011. Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci U S A* 108:9601-9606.

12. Rathinam, V. A., Z. Jiang, S. N. Waggoner, S. Sharma, L. E. Cole, L. Waggoner, S. K. Vanaja, B. G. Monks, S. Ganesan, E. Latz, V. Hornung, S. N. Vogel, E. Szomolanyi-Tsuda, and K. A. Fitzgerald. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11:395-402.

13. Stehlik, C., and A. Dorfleutner. 2007. COPs and POPs: modulators of inflammasome activity. *J Immunol* 179:7993-7998.

14. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* 11:591-604.

15. Chae, J. J., G. Wood, S. L. Masters, K. Richard, G. Park, B. J. Smith, and D. L. Kastner. 2006. The B30.2 domain of pyrin, the familial Mediterranean fever protein, interacts directly with Caspase-1 to modulate IL-1beta production. *Proc Natl Acad Sci U S A* 103:9982-9987.

16. Dowds, T. A., J. Masumoto, F. F. Chen, Y. Ogura, N. Inohara, and G. Nunez. 2003. Regulation of cryopyrin/Pypaf1 signaling by pyrin, the familial Mediterranean fever gene product. *Biochem Biophys Res Commun* 302:575-580.

17. Papin, S., S. Cuenin, L. Agostini, F. Martinon, S. Werner, H. D. Beer, C. Grutter, M. Grutter, and J. Tschopp. 2007. The SPRY domain of Pyrin, mutated in familial Mediterranean fever patients, interacts with inflammasome components and inhibits proIL-1beta processing. *Cell Death Differ* 14:1457-1466.

18. Consortium, T. I. F. 1997. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. *Cell* 90:797-807.

19. Consortium, F. F. 1997. A candidate gene for familial Mediterranean fever. *Nat Genet* 17:25-31.

20. Richards, N., P. Schaner, A. Diaz, J. Stuckey, E. Shelden, A. Wadhwa, and D. L. Gumucio. 2001. Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem* 276:39320-39329.

21. Yu, J. W., J. Wu, Z. Zhang, P. Datta, I. Ibrahimi, S. Taniguchi, J. Sagara, T. Fernandes-Alnemri, and E. S. Alnemri. 2006. Cryopyrin and pyrin activate Caspase-1, but not NF-kappaB, via ASC oligomerization. *Cell Death Differ* 13:236-249.

22. Booth, D. R., J. D. Gillmore, H. J. Lachmann, S. E. Booth, A. Bybee, M. Soyturk, S. Akar, M. B. Pepys, M. Tunca, and P. N. Hawkins. 2000. The genetic basis of autosomal dominant familial Mediterranean fever. *QJM* 93:217-221.

23. Dundar, M., E. F. Emirogullari, A. Kiraz, S. Taheri, and M. Baskol. 2010. Common Familial Mediterranean Fever gene mutations in a Turkish cohort. *Mol Biol Rep*.

24. Chae, J. J., Y. H. Cho, G. S. Lee, J. Cheng, P. P. Liu, L. Feigenbaum, S. I. Katz, and D. L. Kastner. 2011. Gain-of-Function Pyrin Mutations Induce NLRP3 Protein-Independent Interleukin-1beta Activation and Severe Autoinflammation in Mice. *Immunity*.

25. Gavrilin, M. A., S. Mitra, S. Seshadri, J. Nateri, F. Berhe, M. W. Hall, and M. D. Wewers. 2009. Pyrin critical to macrophage IL-1beta response to Francisella challenge. *J Immunol* 182:7982-7989.

26. Seshadri, S., M. D. Duncan, J. M. Hart, M. A. Gavrilin, and M. D. Wewers. 2007. Pyrin levels in human monocytes and monocyte-derived macrophages regulate IL-1beta processing and release. *J Immunol* 179:1274-1281.

27. Yu, J. W., T. Fernandes-Alnemri, P. Datta, J. Wu, C. Juliana, L. Solorzano, M. McCormick, Z. Zhang, and E. S. Alnemri. 2007. Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants. *Mol Cell* 28:214-227.

28. Chae, J. J., G. Wood, K. Richard, H. Jaffe, N. T. Colburn, S. L. Masters, D. L. Gumucio, N. G. Shoham, and D. L. Kastner. 2008. The familial Mediterranean fever protein, pyrin, is cleaved by Caspase-1 and activates NF-kappaB through its N-terminal fragment. *Blood* 112:1794-1803.

29. Yazdi, A. S., G. Guarda, N. Riteau, S. K. Drexler, A. Tardivel, I. Couillin, and J. Tschopp. 2010. Nanoparticles activate the NLR pyrin domain containing 3 (Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1alpha and IL-1beta. *Proc Natl Acad Sci U S A* 107:19449-19454.

30. Willingham, S. B., D. T. Bergstralh, W. O'Connor, A. C. Morrison, D. J. Taxman, J. A. Duncan, S. Barnoy, M. M. Venkatesan, R. A. Flavell, M. Deshmukh, H. M. Hoffman, and J. P. Ting. 2007. Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe* 2:147-159.

31. Solle, M., J. Labasi, D. G. Perregaux, E. Stam, N. Petrushova, B. H. Koller, R. J. Griffiths, and C. A. Gabel. 2001. Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 276:125-132.

32. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.

33. Herzyk, D. J., J. N. Allen, C. B. Marsh, and M. D. Wewers. 1992. Macrophage and monocyte IL-1 beta regulation differs at multiple sites. Messenger RNA expression, translation, and post-translational processing. *J Immunol* 149:3052-3058.

34. Harris, J., M. Hartman, C. Roche, S. G. Zeng, A. O'Shea, F. A. Sharp, E. M. Lambe, E. M. Creagh, D. T. Golenbock, J. Tschopp, H. Kornfeld, K. A. Fitzgerald, and E. C. Lavelle. 2011. Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. *J Biol Chem* 286:9587-9597.

35. Mitroulis, I., I. Kourtzelis, K. Kambas, A. Chrysanthopoulou, and K. Ritis. 2011. Evidence for the involvement of mTOR inhibition and basal autophagy in familial Mediterranean fever phenotype. *Hum Immunol* 72:135-138.

36. Narayan, S., B. Pazar, H. K. Ea, L. Kolly, N. Bagnoud, V. Chobaz, F. Liote, T. Vogl, D. Holzinger, A. Kai-Lik So, and N. Busso. 2011. Octacalcium phosphate crystals induce inflammation in vivo through interleukin-1 but independent of the NLRP3 inflammasome in mice. *Arthritis Rheum* 63:422-433.

37. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.

38. Davtyan, T. K., G. S. Hakopyan, S. A. Avetisyan, and N. R. Mkrtchyan. 2006. Impaired endotoxin tolerance induction in patients with familial Mediterranean fever. *Pathobiology* 73:26-39.

39. Carta, S., S. Tassi, I. Pettinati, L. Delfino, C. A. Dinarello, and A. Rubartelli. 2011. The rate of IL-1{beta} secretion in different myeloid cells varies with the extent of redox response to Toll-like receptor triggering. *J Biol Chem*.

40. Cruz, C. M., A. Rinna, H. J. Forman, A. L. Ventura, P. M. Persechini, and D. M. Ojcius. 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282:2871-2879.

41. Marques-da-Silva, C., M. Chaves, N. Castro, R. Coutinho-Silva, and M. Guimaraes. 2011. Colchicine inhibits cationic dye uptake induced by ATP in P2X2 and P2X7 receptor-expressing cells: implications for its therapeutic action. *Br J Pharmacol* 163:912-926.

42. Centola, M., G. Wood, D. M. Frucht, J. Galon, M. Aringer, C. Farrell, D. W. Kingma, M. E. Horwitz, E. Mansfield, S. M. Holland, J. J. O'Shea, H. F. Rosenberg, H. L. Malech, and D. L. Kastner. 2000. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood* 95:3223-3231.

CHAPTER 5

The role of Pyrin in neutrophils

In FMF patients, neutrophils accumulate at sites of inflammation, implying that these immune cells may play a role in disease pathophysiology. Neutrophils from patients with FMF are reported to have altered activity and survival in *ex vivo* assays. We hypothesize that genetic lesions within the *MEFV* gene alter a primary function of Pyrin in neutrophils and promote FMF pathology. To address this hypothesis, we created a mouse line lacking the *Mefv* gene and Pyrin protein. We characterized the development, recruitment, and survival of neutrophils. We did not detect any differences in neutrophils from *Mefv*$^{-/-}$ mice compared to neutrophils from wild-type mice using these criteria, despite high expression of *Mefv* in murine neutrophils. While more extensive characterization is necessary, these results do not support the hypothesis that neutrophil phenotypes seen in FMF patients result from mutations to the *MEFV* gene.

Introduction

Neutrophils are very dynamic cells and are critical for the host pathogen response. Neutrophils are the first line of defense against bacterial, yeast, and fungal infections. Their rapid recruitment to sites of tissue damage helps to prevent the early spread of infection and shape the overall immune response. In addition to infection, neutrophils also respond to mechanical injury (such as cuts or burns), oxidative stress (brought on by ischemia/reperfusion), and thermal stress (brought on by overheating or excessive exercise). Significant neutrophil deposition can be maintained in peripheral tissue during chronic inflammation.

Three pieces of correlative evidence support a model in which neutrophils play a role in FMF pathophysiology. First, during inflammatory attacks, neutrophils accumulate at sites of inflammation. Furthermore, the lifespan of neutrophils is 1–3 days (1), which correlates with the duration of symptoms and suggests that neutrophils mediate the onset and amelioration of inflammation. While these data suggest that FMF-associated inflammation engages neutrophils, they do not clarify if mutations in *Mefv* directly affect neutrophils or if neutrophil accumulation is an indirect result of perturbations to immune signaling upstream of neutrophil recruitment, activation, and/or survival. Second, colchicine can successfully ameliorate inflammatory episodes in many FMF patients. As colchicine is sequestered mainly by neutrophils (2), this suggests that altering neutrophil function can alter the outcome of FMF. Colchicine modulates the oxidative burst, phagocytosis, and chemotaxis functions of neutrophils (3-5). Still, the therapeutic effects of colchicine cannot distinguish between a direct reversal of the cause of FMF pathophysiology or an indirect bypass of exacerbated upstream signaling. Third, *Mefv* is expressed more highly in neutrophils than any other cell type tested. This argues that *Mefv* could play a direct role in neutrophil function.

Previous studies with neutrophils from FMF patients demonstrate altered neutrophil physiology. Oxidative burst and phagocytosis are increased in neutrophils from FMF patients

compared to controls (3-11). Interestingly, neutrophils demonstrates a biphasic phenotype in which activation of neutrophils collected from FMF patients between attacks is more intense than in neutrophils harvested from FMF patients during an attack period (4, 8, 9). Furthermore, previous work demonstrates that neutrophils from FMF patients are more susceptible to apoptosis. Following endotoxin (LPS) exposure, neutrophils from healthy human donors show a decrease in apoptosis, while neutrophils from FMF patients show an increase in apoptosis compared to untreated neutrophils (10).

To test the hypothesis that Pyrin functions in neutrophils to mediate acute episodic inflammation associated with FMF, *in vitro* and *in vivo* experimental systems were used to determine if a deficiency of the Pyrin protein affected neutrophils. In this chapter, studies designed to isolate and differentiate a role of *Mefv* in intrinsic neutrophil processes are presented. The following chapter describes *in vivo* models of peritonitis that were designed to detect differences in not only neutrophil function, but also macrophage function and the overall immune response in *Mefv*$^{-/-}$ mice compared to WT mice. The development, recruitment, and survival of neutrophils were not affected by the loss of Pyrin function.

Methods

Isolation of bone marrow neutrophils

The polymorphonuclear fraction that contained bone-marrow neutrophils was isolated from total bone marrow by centrifugation through a density gradient per manufacturer's instructions (density 1.083 g/mL and 1.119 g/mL; Histopaque, Sigma-Alrich). Greater than 90% of cells were classified as bone-marrow neutrophils based upon morphological analysis of cytospun cells stained with Hema-3 (Protocol, Fisher Scientific).

Peritoneal neutrophil recruitment

Wild-type (WT) and *Mefv*$^{-/-}$ mice were treated with 1 mL of 1 mg/mL zymosan-A (from *Saccharomyces cerevisiae*; Sigma-Aldrich) dissolved in PBS, by intraperitoneal (*i.p.)* injection using a 27G needle. Zymosan-A solution was aspirated through a 30G needle before injections to ensure the solution was homogeneous. Mice were sacrificed at 4 h after treatment, and the peritoneal cavity was lavaged with 4 mLs of Delbucco's phosphate-buffered saline (PBS; Gibco) to collect peritoneal cells. The majority of peritoneal cells were elicited neutrophils. Cell numbers within the peritoneal lavage fluid were determined using a hemocytometer.

In vitro neutrophil survival studies

WT and *Mefv*$^{-/-}$ thirteen-week-old female mice were used in these studies. Elicited peritoneal neutrophils were collected in PBS at 4 h following zymosan-A treatment, as stated above. Cells were kept on ice during harvest. Samples were diluted 2-fold with PBS and then washed once with 10 mLs of PBS containing 10% fetal bovine serum. Centrifuge steps were done at 230 x g for 10 minutes at 4°C. Cells were eluted in 13 mLs of DMEM containing 10% FBS, 150 U/mL of penicillin, 150 µg/mL of streptomycin, 0.438 mg/mL of L-glutamine (all from Invitrogen), gentamicin, and 57.2 µM 2-Mercaptoethanol (Sigma-Aldrich). Cell densities were determined using a hemocytometer, and cells were diluted to 1 x 10^6 cells/mL. Cells were kept

at room temperature in a laminar flow hood while counting. 400 µL (4 x 10^5 cells) were plated into each well of 48-well plates. Cell cultures were maintained in a constant environment (humidified 37°C, 5% CO_2) for the indicated times. When cultured in 10% serum, 80 – 90% of neutrophils are strongly or loosely -adherent to the culture dish. At harvest, wells were pipette-mixed in order to collect the majority of neutrophils in the well.

Trypan Blue

Viability was assessed by hemocytometer using cell samples containing 0.1x volume of trypan blue. Percent viability was calculated as the number of live cells excluding trypan blue divided by the total number of cells.

LDH Assays

Cell culture samples were collected from duplicate wells per mouse for each timepoint. Samples were centrifuged at 200 x g for 5 minutes at room temperature. The clarified supernatant was stored at -80°C until use. The amount of LDH released into the culture media was measured by a colorimetric assay following the manufacturer's protocol (Clontech). Results were standardized to the total LDH released from cells treated with 1% Triton-X (100% lysis control) in order to control for differences in cell plating densities. Absorbance values were corrected by values at 0h to control for differences in initial viability. The percentage of total LDH released was calculated using the formula: ((Absorbance at time X – Absorbance at 0h)/(Absorbance of 100% lysis control – Absorbance at 0h)) x 100%.

Flow cytometry

Fluorescently activated cell sorting (FACS) was used to detect Gr-1 receptor expression, phosphatidylserine translocation, and nuclear membrane permeability using the fluorescently labeled agents APC-conjugated anti-Gr-1 (Ly-6G and Ly-6C; 553129, BD Pharmingen), FITC-conjugated Annexin V (An V; BD Pharmigen), and propidium iodide (PI; Cell Technology).

Triplicate wells were pipette-mixed and 400 µL was collected and stained for either Gr-1 and An V or for PI. For samples stained with Gr-1 and An V, 4 mL of PBS was added as an initial wash step, and then cells were washed with Annexin V binding buffer. Cells were resuspended in 110 µL of binding buffer containing 5 µL of An V and 5 µL of mouse BD Fc Block (anti-CD16/CD32; 553142, BD Pharmigen), which was used to prevent background staining. Samples were incubated for 10 minutes and then 2 µL of Gr-1 or isotype antibody was added. Samples were incubated 10 minutes and then 400 µL of binding buffer was added. FACS staining was done at room temperature. Gr-1 positive/negative gate parameters were set using a sample stained with APC-conjugated IgG2b, κ isotype control (553991, BD Pharmigen). For samples stained with PI, cells were collected and stained directly in culture media to avoid unnecessary disturbances to the cells or removal of dead cells. PI was used at 0.01x volumes. Samples were scanned using a Beckman Coulter CyAn ADP Analyzer, and data were analyzed using Summit V5.2 software.

Animal Care and Use

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of UNC Chapel Hill.

Statistical analyses

A two-tailed unpaired t-test was used to determine statistical probability of differences between WT and *Mefv*$^{-/-}$ neutrophils, with a significance limit of $p \leq 0.05$.

Results

Development of neutrophils is not impaired by the loss of Pyrin

As discussed in Chapter 2 and visualized in Figures 2.1 and 2.2, *Mefv* is expressed in myeloid-lineage cells, and specifically, in neutrophils, at a higher level than any other cell type. It is expressed in bone marrow neutrophils, a relatively immature and inactive neutrophil subpopulation. Despite the already high expression level, *Mefv* expression was increased 23-fold at the transcript level in mature peritoneal neutrophils compared to bone marrow neutrophils. Since expression of *Mefv* was increased concurrently with cell maturation, we asked if Pyrin contributes to neutrophil differentiation. Since neutrophils develop from myeloid precursor cells in the bone marrow, we characterized the abundance and maturity of neutrophils within the bone marrow of wild-type and *Mefv*$^{-/-}$ mice. By visual analysis of cells stained with Hema-3, greater than 95% of cells fit the morphological criteria of neutrophils. The total number, viability, and relative abundance of neutrophils in the bone marrow was unaffected by the loss of Pyrin (Figure 5.1*A* and data not shown). Neutrophils are also commonly identified by Gr-1 surface receptor expression. The Gr-1 receptor is involved in neutrophil adhesion, and it expression is restricted to mature neutrophils. Using FACS analysis, Gr-1 expression was found to be similar ($p = 0.82$) between WT and *Mefv*$^{-/-}$ samples (Figure 5.2). These analyses suggest that *Mefv*-deficiency does not cause a defect in neutrophil development.

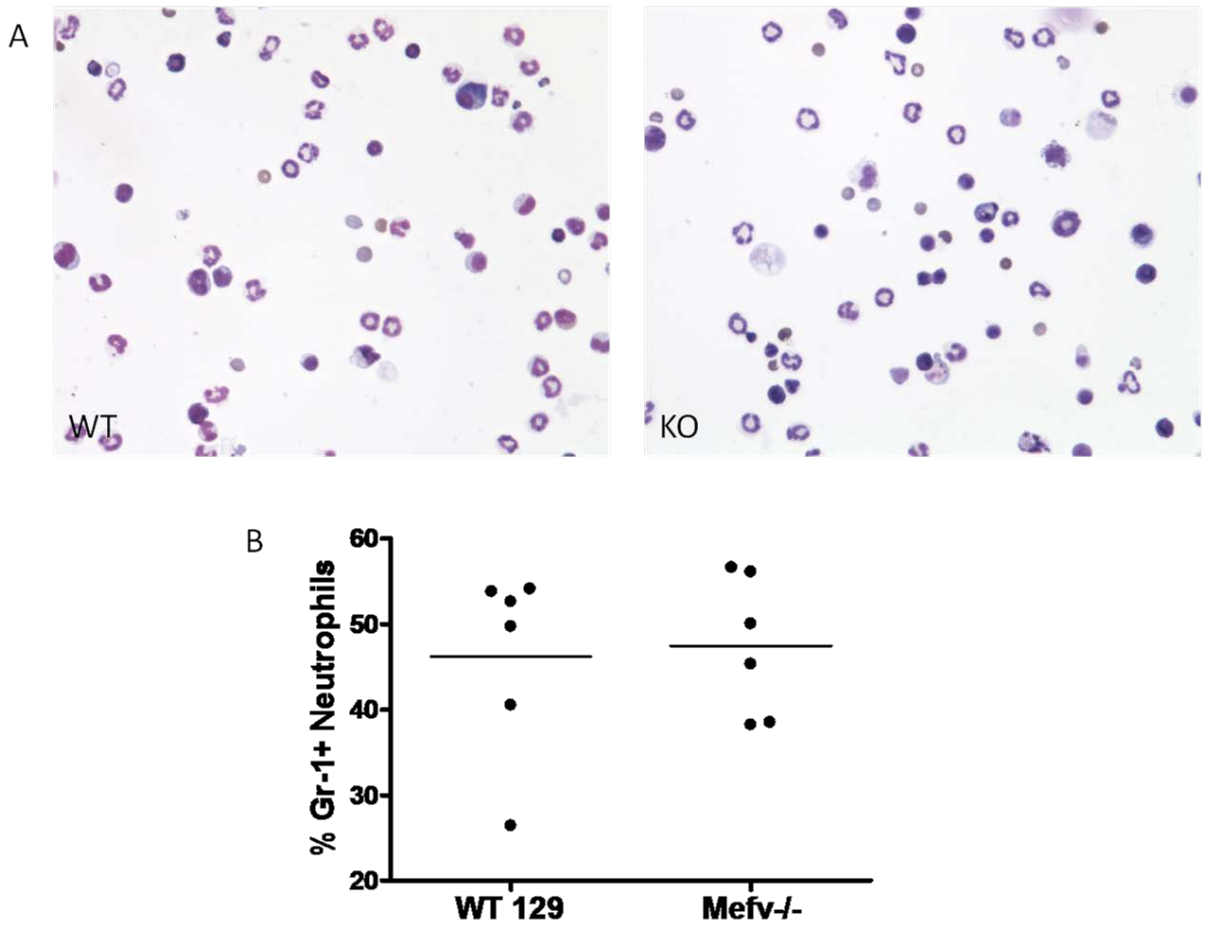


Figure 5.1. *Mefv*-deficiency does not affect neutrophil differentiation. The polymorphonuclear fraction was isolated from total bone marrow of wild-type (WT 129) and *Mefv*$^{-/-}$ (KO) mice using a density (Histopaque) gradient. *A*, Micrographs of cells from WT (left) and KO (right) mice. Cells were cytospun and stained with Hema-3. Neutrophils have an almost translucent cytoplasm and a darkly stained, multi-lobular, U-shaped nucleus. *B*, The percentage of cells expressing Gr-1 was detected by FACS analysis. Each dot represents one mouse. $p = 0.82$.

Neutrophil recruitment in *Mefv* null mice is normal

Since an large number of neutrophils are detected at inflamed tissues in FMF patients (12), and excessive neutrophil recruitment can cause uncontrolled inflammation, we postulated that

exacerbated neutrophil recruitment to peripheral tissues contributes to FMF disease pathology. Thus, we predicted that recruitment of neutrophils would be augmented in *Mefv*$^{-/-}$ animals compared to controls. To test this hypothesis, Zymosan-A, a component of yeast, was used to induce peritonitis and elicit neutrophils to the peritoneal cavity. Neutrophils were rapidly recruited to the peritoneal cavity, and by 4 h post-treatment, the peritoneal cavity contained 6-fold more cells compared to that of untreated mice, and 95% of cells were classified as neutrophils based upon morphological analysis, which is striking increase from untreated mice in which less than 5% of peritoneal cells were neutrophils. The total number of peritoneal cells and the percentage of cells expressing Gr-1 was similar between *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ animals (Figure 5.3*A* and *B*). These studies indicate that Pyrin deficiency does not augment, nor inhibit, neutrophil recruitment.

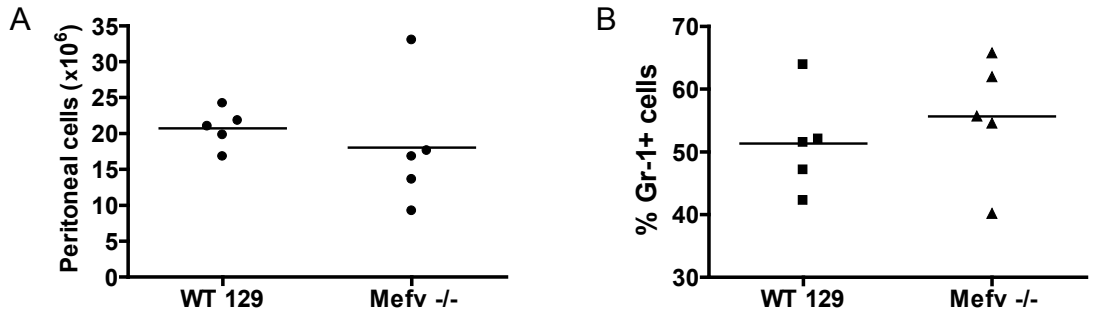


Figure 5.2. *Mefv*-deficiency does not affect neutrophil recruitment. *Mefv*$^{+/+}$ (WT 129) and *Mefv*$^{-/-}$ mice were treated by *i.p.* injection with 1 mL of 1 mg/mL Zymosan-A. At 4 h after treatment, peritoneal cells were collected. *A*, The total number of peritoneal cells was determined using a hemocytometer. Results are representative of several independent experiments. *B*, The percentage of peritoneal lavage cells expressing Gr-1, as determined by FACS analysis. Each dot represents one mouse, n = 5 mice per genotype.

Neutrophil survival

Since inflammatory episodes in FMF patients are characterized by neutrophilia, yet we were unable to detect a difference in neutrophil recruitment, we reasoned that Pyrin might affect neutrophil survival. To examine this, the constitutive cell death of recruited peritoneal neutrophils was monitored *ex vivo*. The percentage of cells retaining integrity of the cell membrane was determined by trypan blue exclusion and this number decreased at each timepoint tested: 0, 4, 6, 12, and 17 h. Additionally, the level of lactate dehydrogenase (LDH), an enzyme that is released as cells die, in the supernatant of cultured neutrophils was monitored over a 24 h timecourse. Both methods of testing showed that at least 90% of neutrophils died by 24 h in culture, and the rate of cell death was especially rapid between 6 and 16 h. However, the extent and the rate of neutrophil cell death was unchanged between *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ neutrophils (Figure 5.3*A* and *B*). FACS analyses was also used to determine if there were small changes in the survival of *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ neutrophils that could not be detected by trypan blue or LDH assays. At 0, 4, and 8 h, the percentage of wild-type and *Mefv*$^{-/-}$ neutrophils that were stained with Gr-1 and Annexin-V or Propidium Iodide was similar (Figure 5.3*C* and *D*). Consisent Gr-1 expression at all timepoints indicated that results were not skewed by a loss of receptor expression (Figure 5.3*E*). The rapid decrease in total cell counts made FACS analysis inconsistent at later timepoints, but LDH release remained a precise assessment of cell survival even at the later timepoints. These analyses indicate that a loss of Pyrin does not affect constitutive neutrophil cell death.

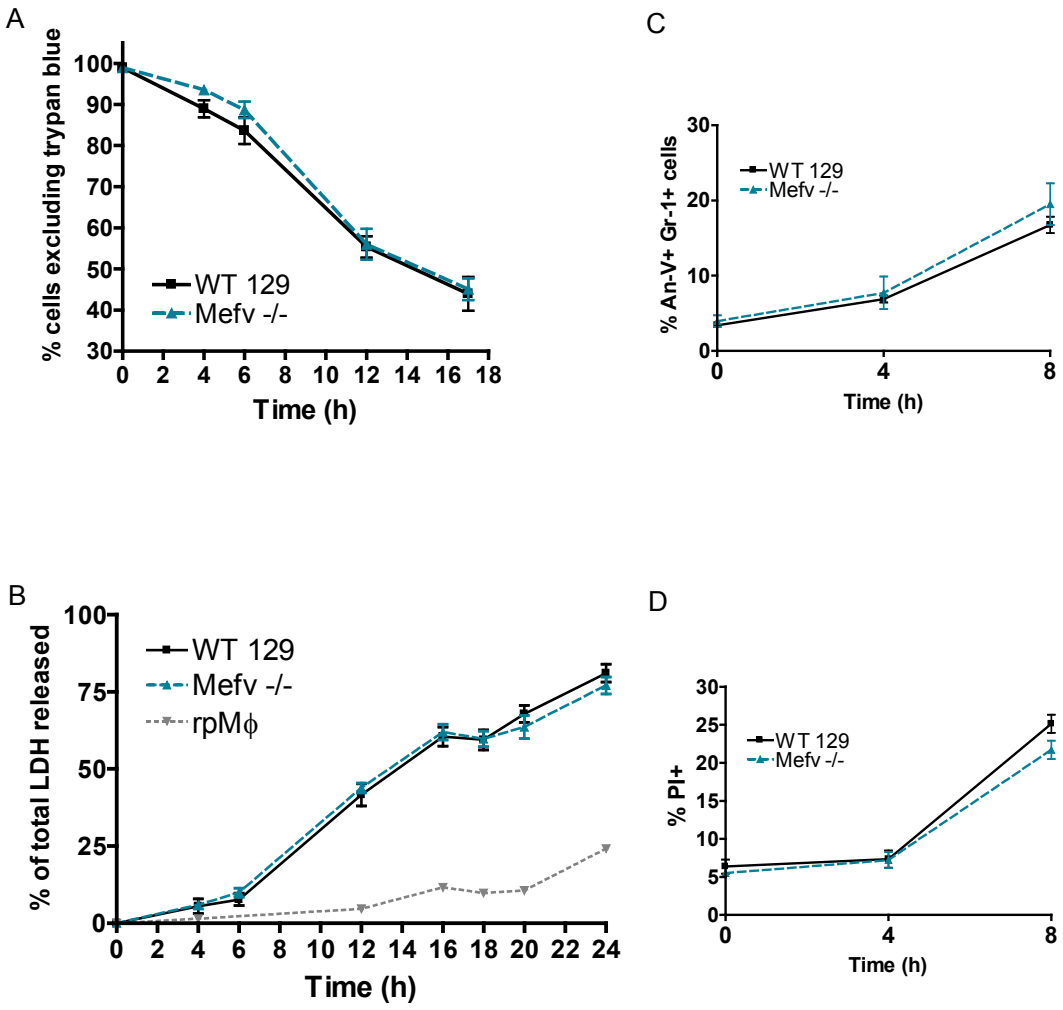


Figure 5.3. The loss of Pyrin does not affect neutrophil survival. Recruited peritoneal cells were collected from wild-type and *Mefv*$^{-/-}$ mice at 4 hours after treatment with Zymosan-A. Peritoneal lavage cells were placed in culture at a density of 10^6 cells/mL at 0 h. At least 90% of cells were classified as neutrophils by morphological assessment of cells stained with Hema-3 and visualized under a microscope. *A*, The percentage of cells excluding trypan blue was assessed at the indicated times using a hemocytometer. *B*, Neutrophil cell death is represented as the percent of total lactate dehydrogenase (LDH) released into the media at each timepoint compared to 100% lysis control samples. n = 4 wild-type and 5 *Mefv*$^{-/-}$ mice for *A* and *B*. Results of 3 replicate wells/mouse were averaged in *B*. *C* and *D*, The percentage of apoptotic (Annexin-V+) neutrophils (Gr-1+), in *C*, or dead (Propidium Iodide+) cells, in *D*, at 0, 4, and 8 h. The percentage of Gr-1+ cells was similar at each timepoint. n = 5 mice per genotype.

Discussion

The high expression of *Mefv* in neutrophils, alongside previous functional research using neutrophils from FMF patients, suggests that the Pyrin protein has an important function in neutrophils. However, we did not detect a change in neutrophil development, recruitment, or survival due to the loss of Pyrin expression.

Recruitment of neutrophils to the peritoneal cavity was similar between WT and *Mefv*$^{-/-}$ animals, despite elevated *Mefv* expression concurrent with recruitment (see Chapter 2). We used an established experimental system to elicit neutrophils; zymosan-A-induced peritonitis has been used in previous studies to detect genes important in neutrophil recruitment in mice, including LTB_4, TNF-α, and IL-8 (13, 14). Furthermore, colchicine impacts neutrophil chemotaxis (5), so it is logical to assume that FMF patients have altered neutrophil chemotaxis caused by mutations in Pyrin. However, our study is the first to compare WT neutrophils with neutrophils lacking Pyrin. The data presented here leaves open the possibility that Pyrin is involved in neutrophil migration in response to other stimuli. It is also important to keep in mind that most studies describing altered chemotaxis of neutrophils following colchicine treatment examine neutrophils in *ex vivo* assays. Previous studies have shown neutrophil phenotypes *in vitro* that cannot be detected *in vivo* (15). The difference between the *in vitro* and *in vivo* environment cannot be overlooked, especially since neutrophils dynamically respond to external cues from other cell types. Thus, it is also possible that a mechanism(s) prevents altered Pyrin function from having an effect *in vivo*.

We also saw no differences in the rate of spontaneous, or constitutive, apoptosis of neutrophils. In contrast, neutrophils from FMF patients display altered survival *ex vivo*. The spontaneous apoptosis of neutrophils from FMF patients was increased compared to healthy controls (17). Additionally, while exposure to LPS extends the lifespan of wild-type neutrophils, LPS treatment reduces the lifespan of neutrophils from FMF patients (9).

Pyrin protein binding partners ASC, Caspase-1, and Siva are expressed in neutrophils. Expression of ASC, a Pyrin protein binding partner, is absent or weak in non-activated human neutrophils, but is expressed by neutrophils at sites of peripheral tissue inflammation. Its expression is upregulated by LPS and by IL-1α, IL-1β, IFN-α, IFN-γ, and TNFα pro-inflammatory cytokines. These results suggest that ASC is a positive regulator of inflammation in neutrophils at the early stages of an immune response. However, caspase-dependent, Fas-mediated death signaling also increases human ASC expression concurrent with neutrophil apoptosis, suggesting that in later stages of the immune response, ASC promotes apoptosis of neutrophils and resolution of inflammation (18). Caspase-1 shows a similar pattern. Its expression is increased by LPS (Chapter 2 and (19)), but Caspase-1 deficient neutrophils show a delay in spontaneous neutrophil apoptosis *in vitro*, suggesting that Caspase-1 promotes apoptosis in neutrophils (15). Siva, which also interacts with Pyrin, is a proapoptotic protein (20). Whether or not Siva modulates apoptosis in neutrophils is unknown. Gene network mathematic predictions have failed to link Siva-mediated apoptosis to the pathogenesis of FMF (21).

It remains possible that the experimental systems used in these studies lack the resolution required to detect a phenotype in neutrophils from *Mefv* null animals. For example, the methods used to assess neutrophil development from myeloid precursor cells are not sensitive to potential variations within the Gr-1+ neutrophil subset. In humans, many neutrophil subsets are described, but these states are not well-defined for mouse neutrophils. Furthermore, neutrophil survival was examined *in vitro* because this method allows greater sensitivity; however, *in vivo*, non-activated neutrophils can migrate to the bone marrow, spleen, or liver where they apoptosis, and activated neutrophils can be phagocytosed by macrophages. Thus, it is possible that a difference in neutrophil survival is the result of coordinated processes which are only detectable via *in vivo* models. We cannot rule out the possibility that further studies may reveal a role of Pyrin in neutrophil physiology. However, our studies demonstrate that Pyrin-deficient

neutrophils respond normally under at least some external conditions. Therefore, the neutrophil deposition seen in FMF and the ability of colchicine to prevent FMF attacks may be mediated by altered production of extrinsic cytokines or chemokines that indirectly affect neutrophil function.

References

1. Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 6:173-182.

2. Ben-Chetrit, E., and M. Levy. 1998. Does the lack of the P-glycoprotein efflux pump in neutrophils explain the efficacy of colchicine in familial Mediterranean fever and other inflammatory diseases? *Med Hypotheses* 51:377-380.

3. Akopian, V. P., S. A. Avetisian, and T. K. Davtian. 2005. [The effect of colchicine on the periodicity of the spontaneous and induced respiratory burst of granulocytes and monocytes during familial Mediterranean fever]. *Eksp Klin Farmakol* 68:36-39.

4. Akopian, V. P., S. A. Avetisian, and T. K. Davtian. 2005. [The effect of colcichine and iodine-lithium-alpha-dextrin on the phagocytosis of granulocytes and monocytes in patients with familial Mediterranean fever]. *Eksp Klin Farmakol* 68:36-39.

5. Phelps, P. 1970. Polymorphonuclear leukocyte motility in vitro: IV. Colchicine inhibition of chemotactic activity formation after phagocytosis of urate crystals. *Arthritis Rheum* 13:S25-33.

6. Avetisian, S. A., G. S. Akopian, and T. K. Davtian. 2006. [Modulation of endotoxin-induced respiratory splash of granulocytes and monocytes in patients with Familial Mediterranean Fever by iodine-lithium-alpha-dextrin and sodium thiosulfate]. *Patol Fiziol Eksp Ter*:11-13.

7. Avetisian, S. A., T. K. Davtian, G. S. Akopian, A. M. Manukian, and D. A. Pogosian. 2006. [Spontaneous and inducible respiratory burst of monocytes and neutrophils in periodic autoinflammatory fever]. *Patol Fiziol Eksp Ter*:11-12.

8. Davtian, T. K., S. A. Avetisian, G. S. Akopian, and V. M. Arutiunian. 2005. [A respiratory splash of monocytes and neutrophils in familial Mediterranean fever]. *Ter Arkh* 77:39-43.

9. Davtyan, T. K., G. S. Hakopyan, S. A. Avetisyan, and N. R. Mkrtchyan. 2006. Impaired endotoxin tolerance induction in patients with familial Mediterranean fever. *Pathobiology* 73:26-39.

10. Davtyan, T. K., V. A. Harutyunyan, G. S. Hakobyan, and S. A. Avetisyan. 2008. Heightened endotoxin susceptibility of monocytes and neutrophils during familial Mediterranean fever. *FEMS Immunol Med Microbiol* 52:370-378.

11. Gogus, F., I. Fresko, Y. Elbir, E. Eksioglu-Demiralp, and H. Direskeneli. 2005. Oxidative burst response to monosodium urate crystals in patients with Behcet's syndrome. *Clin Exp Rheumatol* 23:S81-85.

12. Ben-Zvi, I., and A. Livneh. 2011. Chronic inflammation in FMF: markers, risk factors, outcomes and therapy. *Nat Rev Rheumatol* 7:105-112.

13. Monteiro, A. P., C. S. Pinheiro, T. Luna-Gomes, L. R. Alves, C. M. Maya-Monteiro, B. N. Porto, C. Barja-Fidalgo, C. F. Benjamim, M. Peters-Golden, C. Bandeira-Melo, M. T. Bozza, and C. Canetti. 2011. Leukotriene B4 mediates neutrophil migration induced by heme. *J Immunol* 186:6562-6567.

14. Ribeiro, R. A., C. A. Flores, F. Q. Cunha, and S. H. Ferreira. 1991. IL-8 causes in vivo neutrophil migration by a cell-dependent mechanism. *Immunology* 73:472-477.

15. Rowe, S. J., L. Allen, V. C. Ridger, P. G. Hellewell, and M. K. Whyte. 2002. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. *J Immunol* 169:6401-6407.

16. Molad, Y., A. Fridenberg, K. Bloch, P. Langevitz, M. Mukamel, J. Sulkes, M. Pras, and A. Livneh. 2004. Neutrophil adhesion molecule expression in familial Mediterranean fever: discordance between the intravascular regulation of beta2 integrin and L-selectin expression in acute attack. *J Investig Med* 52:58-61.

17. Ozen, S., D. Uckan, E. Baskin, N. Besbas, H. Okur, U. Saatci, and A. Bakkaloglu. 2001. Increased neutrophil apoptosis during attacks of familial Mediterranean fever. *Clin Exp Rheumatol* 19:S68-71.

18. Shiohara, M., S. Taniguchi, J. Masumoto, K. Yasui, K. Koike, A. Komiyama, and J. Sagara. 2002. ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils. *Biochem Biophys Res Commun* 293:1314-1318.

19. Watson, R. W., O. D. Rotstein, J. Parodo, R. Bitar, and J. C. Marshall. 1998. The IL-1 beta-converting enzyme (Caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1 beta. *J Immunol* 161:957-962.

20. Balci-Peynircioglu, B., A. L. Waite, C. Hu, N. Richards, A. Staubach-Grosse, E. Yilmaz, and D. L. Gumucio. 2008. Pyrin, product of the MEFV locus, interacts with the proapoptotic protein, Siva. *J Cell Physiol* 216:595-602.

21. Goulielmos, G. N., E. Petraki, D. Vassou, E. Eliopoulos, D. Iliopoulos, P. Sidiropoulos, I. Aksentijevich, D. Kardassis, and D. T. Boumpas. The role of the pro-apoptotic protein Siva in the pathogenesis of Familial Mediterranean fever: A structural and functional analysis. *Biochem Biophys Res Commun* 402:141-146.

CHAPTER 6

In vivo models of peritonitis

Inappropriate activation or resolution of an immune response can lead to inflammatory disorders such as Familial Mediterranean Fever (FMF). FMF is an autoinflammatory disorder caused by mutations in the gene *Mefv* which encodes the protein Pyrin. Previous studies presented in Chapter 4 indicate that Pyrin negatively regulates IL-1β production, but the contribution of Pyrin to innate immune responses *in vivo* remains unclear. To study the role of Pyrin in the initiation and resolution of innate immune responses *in vivo*, inflammation was induced in *Mefv* null mice via multiple peritonitis models. Using physiological parameters, cellular profiling, and molecular protein assays, we were able to detect active innate immune responses in wild-type and *Mefv*$^{-/-}$ animals. However, *Mefv* null mice did not display impaired or exacerbated inflammation compared to wild-type mice. Compensatory mechanisms may prevent Pyrin-deficiency from altering immune responses *in vivo*, in response to at least some innate immune stimuli.

Introduction

The acute inflammatory attacks that are characteristic of Familial Mediterranean Fever (FMF) are mediated by the innate immune system. An innate immune response is a very dynamic process that results from the interplay of resident immune cells and epithelial cells that perceive immune stimuli, recruited immune cells that migrate to the site of inflammation, endothelial cells that are involved in immune cell extravasation, and physiological processes including fever.

The kinetics of cellular efflux, cell activation, and cell death are intricately regulated. Cell recruitment is orchestrated by multiple cytokines and chemokines, including IL-1β, TNFα, IL-8, macrophage inflammatory protein-2 (MIP-2), and monocyte chemoattractant protein-1 (MCP-1) (1), the majority of which are released by macrophages. Since we previously showed that Pyrin regulates IL-1β levels (see Chapter 4), it is important to note that IL-1β enhances the recruitment of neutrophils to localized sites of inflammation (2). During pathogen clearance and resolution of the immune response, the kinetics of immune cell death are controlled by a balance of pro-apoptotic and anti-apoptotic, or survival, signals intrinsic and extrinsic to the cell and is affected by the presence of a pathogen or immune stimulant (3, 4). Cell death can be either an autonomous process or involve the interplay of multiple phagocytic immune cells. Phagocytosis of neutrophils by macrophages is an important mechanism for limiting tissue inflammation (5). Given the interplay of numerous processes and cell types involved in immune cell recruitment and cell death, it is important to examine these processes *in vivo*.

Fever accompanies the inflammatory episodes of FMF (6). Fever is a regulated physiological response that facilitates survival of the host during infection (7). In mice, the body temperature markedly declines before a subsequent, subtle fever response (8). The initial hypothermic response is often used to study pathogen-induced changes in temperature regulation. The magnitude and duration of hypothermia correlates with the endotoxin load in

LPS-induced peritonitis models (9). Since our previous work in Chapter 4 indicates that Pyrin negatively regulates IL-1β production, it is important to note that IL-1β mediates body temperature in both humans and mice (7, 10). In mice, IL-1β levels increase in a dose-dependent manner following LPS treatment *in vivo* (9), and IL-1β-deficient mice treated systemically with LPS have a shortened hypothermic phase compared to wild-type mice (10-12). Thus, by modulating IL-1β production, or through another uncharacterized function, Pyrin may modulate thermic responses.

Previous studies suggest that Pyrin is involved in cell recruitment and survival during inflammation (8). In FMF patients, extreme neutrophil deposition is apparent in inflamed tissues, and the *ex vivo* survival of neutrophils harvested from FMF patients is altered (3, 13). Also, previously described interactions between Pyrin and cytoskeletal elements (14-16) may influence cell shape, which is necessary for extravasation; cell motility, which is necessary for cell migration; and cell death, which is influenced by cytoskeletal integrity (17).

To assess the contribution of Pyrin in the activity, initiation, and resolution of the innate immune responses *in vivo*, we induced peritonitis in *Mefv*$^{-/-}$ and *Mefv*$^{+/+}$ (WT) mice. We drew upon knowledge of the most common symptoms for FMF—peritonitis, immune cell deposition in peripheral tissues, and fever—to guide testing of Pyrin-deficient mice. We exposed mice to thioglycolate, LPS, and *Psuedomonas aeruginosa*, and compared the physiologic, cellular, and molecular responses of *Mefv*$^{-/-}$ and WT animals. We found that *Mefv*$^{-/-}$ and WT mice had similar innate immune responses in these experimental systems.

Materials and Methods

Thioglycolate-induced peritonitis

Mice were treated with 2 or 3 mLs of 3% Brewer's thioglycolate medium by *i.p.* injection. The peritoneal cavity was lavaged with 4 mLs of PBS to collect peritoneal cells. Total peritoneal cells were counted using a hemocytometer. Lavage samples were cytospun and stained with Hema-3. Cell identity was based upon classification of morphology using microscopic visual analysis. FACS analysis was used to detect 7AAD, Gr-1+, and Mac-1+ staining as previously described in Chapter 5. Peritoneal lavage fluid was used to determine IL-1β concentration by ELISA (BD Biosciences). Lavage samples were processed to isolate myeloperoxidase (MPO) enzyme and its catalytic activity was evaluated by colorimetric assay.

In vivo LPS treatment

Mice were treated with 2.5 mg/kg or 0.25 mg/kg of LPS (serotypes 127:B8 and 055:B5, respectively) by *i.p.* injection. Body temperature was measured with a rectal thermometer probe. Mice were sacrificed at 20 h (2.5 mg/kg of LPS) or 24 h (0.25 mg/kg of LPS). The peritoneal cavity was lavaged with 4 mLs of PBS. Weight loss was calculated using body weights measured at 0 h and the time of harvest. Peritoneal lavage fluid was used to determine IL-1β and TNFα concentrations by ELISA (BD Biosciences). Peritoneal cell concentrations and MPO were measured as described above.

Pseudomonas aeruginosa infection

Cultures of WT PAK strain bacteria were started from glycerol stocks and grown overnight in Luria broth media. A 1:100 dilution was made in brain-heart infusion media and cultures were grown to OD 0.3. A 1:27 dilution in PBS was used for injections. The CFU/mouse was calculated by plaque assay using a dilution of the injected culture and determined to be 5.4 x 10^6. Mice were harvested at 19 h post-infection. The peritoneal cavity was lavaged with 4 mLs

of PBS. Blood was collected by cardiac puncture. Samples were lysed to release intercellular bacteria. Dilutions were spread on Luria broth plates and the number of bacterial plaques was counted following an overnight incubation of the plates at 37°C. Cellularity and IL-1β concentrations of peritoneal lavage fluid, body weight, and body temperature were measured as described above.

Animal Care and Use

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines of UNC Chapel Hill.

Statistical analyses

A two-tailed unpaired t-test was used to determine statistical probability of differences between wild-type and *Mefv*$^{-/-}$ cohorts, with a significance limit of $p \leq 0.05$.

Results

A loss of Pyrin does not affect cell recruitment or survival *in vivo*

Since autoinflammatory disorders such as FMF result from overactivation or a lack of resolution of the innate immune response, we reasoned that *Mefv* might play a role in either mobilization of cells to an inflamed tissue and/or retention of cells at the inflamed tissue. To evaluate the kinetics of cell recruitment, cell retention, and cell death in *Mefv* null mice, peritonitis was induced with the sterile immunostimulant, thioglycolate, which has been used in several previous studies to distinguish differences in neutrophil and/or macrophage recruitment in knock-out mice (18). Cellularity in fluid lavaged from the peritoneal cavity was evaluated at multiple times post-treatment. Both neutrophils and macrophages were recruited to the peritoneal cavity in this experimental system (Figure 6.1*A*), which is consistent with previously published results for WT mice (1). Neutrophil recruitment was rapid: neutrophils were detected in the lavage fluid at 2 h after the induction of peritonitis. The absolute number of neutrophils continued to increase through 5.5 h but was decreased at 24 h and more markedly decreased by 4 d (Figure 6.1*B*). Macrophages displayed slower kinetics, as there was not a significant increase in the total number of macrophages at 2 h or 4 h, but an increase was noted at 24 h and was more significant at 3 d and 4 d (Figure 6.1*A, C*, and data not shown).

We analyzed 4 h and 4 d timepoints more extensively and assessed total cellularity, the percentage of dead cells, and cellular composition in the peritoneal lavage fluid. Following treatment with 3 mLs of 3% thioglycolate, mice displayed a 6-fold increase in the total number of peritoneal lavage cells at both 4 h and 4 d post-treatment. Total cellularity was similar in $Mefv^{+/+}$ and $Mefv^{-/-}$ mice at both timepoints (Figure 6.1*D*). At 4 h, 10% of cells were dead, and 50% of cells at 4 d were considered dead by 7AAD staining. Since thioglycolate is a sterile immunostimulant, these values reflect endogenous mechanisms of regulating inflammation by controlled cell death. The percentage of dead cells was similar for $Mefv^{+/+}$ and $Mefv^{-/-}$ mice at

both timepoints (Figure 6.1*E*). Similar values of both total cell number and percentage of dead cells suggest that cell recruitment and cell death kinetics are not affected by the loss of Pyrin. Consistent with this, there was no difference in the cell composition of *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ animals at 4 h or 4 d (Figure 6.1*F* and *G*). Similar studies using Zymosan A, a component of *Saccharomyces cerevisiae* yeast, to induce peritonitis also showed that at 4 h post-treatment, the total number of cells and the percentage of Gr-1+ neutrophils in the peritoneal cavity were similar between WT and *Mefv*$^{-/-}$ animals (see Chapter 5 and Figure 5.2).

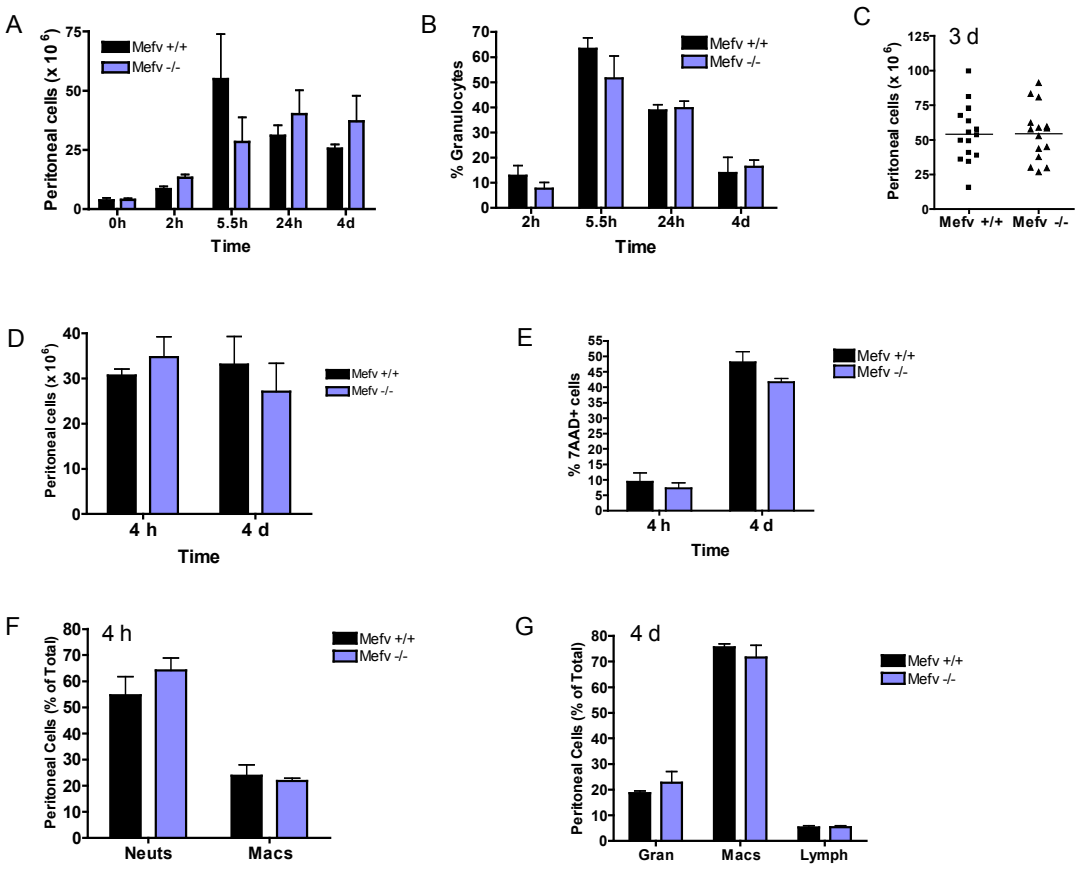


Figure 6.1. Loss of *Mefv* does not affect peritoneal cell recruitment or survival. *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ mice were treated with 3 mLs of 3% Brewer's thioglycolate medium by *i.p.* injection. At the indicated times, the peritoneal cavity was lavaged with 4 mLs of PBS to collect peritoneal cells. *A*, The total number of peritoneal lavage cells at the indicated times after the induction of peritonitis. *B*, The percentage of granulocytes was determined by visual analysis of cytospin samples stained with Hema-3. Samples correspond to *A*. n = 3 mice per genotype for *A* and *B*. *C*, Peritoneal cell concentration at 3 d after the induction of peritonitis, as determined by hemocytometer. n = 15 mice per genotype. *D*, The total number of peritoneal lavage cells at 4 h and 4 d after the induction of peritonitis. *E*, The percentage of peritoneal cells from *D* that were stained with 7AAD, a marker of dead cells. *F*, The relative abundance of neutrophils (Mac-1+/Gr-1+) and macrophages (Mac-1+/Gr-1-) in the peritoneal lavage fluid at 4h, as determined by FACS analysis. Samples correspond to the 4 h timepoint in *D*. *G*, The percentage of granulocytes, macrophages, and lymphocytes was determined by visual analysis

of cytospin samples stained with Hema-3. Samples correspond to the 4 d timepoint in *D*. n = 3 – 5 mice per genotype for *D* – *G*.

We also considered the possibility that a subtle difference in immune cell recruitment and/or clearance might be resolved by inducing a weaker immune response. Using a 1/3 lower dose of thioglycolate to induce peritonitis, we examined the 4 d timepoint. However, we found no difference between *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ cohorts in the total number of cells, the percentage of dead cells, or the relative number of neutrophils (Figures 6.2*A* – *C*). These results suggest that a loss of Pyrin function does not affect cell recruitment, cell retention, or cell death in the peritoneal cavity following treatment with thioglycolate.

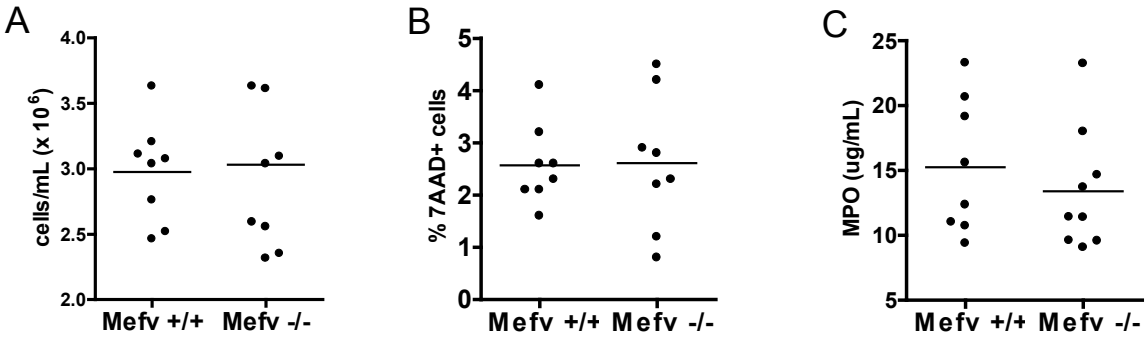
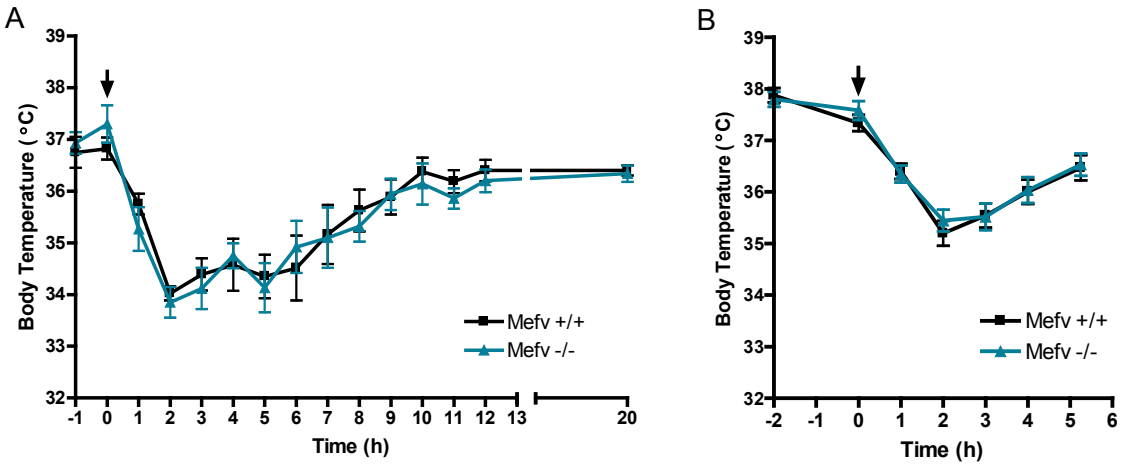


Figure 6.2. Loss of *Mefv* does not affect the cell profile at 4 d after the induction of mild peritonitis. *Mefv*$^{+/+}$ and *Mefv*$^{-/-}$ mice were treated with 2 mLs of 3% Brewer's thioglycolate medium by *i.p.* injection. At 4 d after the induction of peritonitis, the peritoneal cavity was lavaged with 4 mLs of PBS to collect peritoneal cells. *A*, Peritoneal cell concentration at 4 d, as determined by hemocytometer. *B*, The percentage of cells stained with 7AAD as detected by FACS analysis. *C*, The relative abundance of neutrophils as determined by myeloperoxidase (MPO) activity within peritoneal lavage samples. n = 8 *Mefv*$^{+/+}$ and 9 *Mefv*$^{-/-}$ mice.

A loss of Pyrin does not affect the response to LPS *in vivo*

Next, wild-type and *Mefv*$^{-/-}$ mice were evaluated following treatment with 2.5 or 0.25 mg LPS/kg body weight (serotypes 127:B8 and 055:B5, respectively). Mice were lethargic following LPS treatment in a dose-dependent manner that was independent of *Mefv* genotype. LPS induced a rapid drop in temperature, with core body temperature reaching a minimum at 2 hours after treatment. Both the magnitude of the drop in temperature, as well as the kinetics of the temperature change and subsequent recovery of the animals was similar in the *Mefv*$^{-/-}$ and control cohorts. No difference was observed in the cryogenic response of the *Mefv*$^{-/-}$ and wild-type control animals to *i.p.* LPS at both doses of endotoxin examined ($p > 0.8$; Figure 6.3*A* and *B*). In addition to hypothermia, LPS treatment results in a measurable drop in body weight. Weight loss was also similar between WT and Mefv$^{-/-}$ cohorts (Figure 6.3*C*). At 20 (2.5 mg/kg LPS) or 24 (0.25 mg/kg LPS) hours after treatment peritoneal lavage samples were collected. The total number of immune cells present in the peritoneal cavity increased with the dose of LPS, however neither the number or composition of cells recruited to the peritoneal cavity distinguished the *Mefv*$^{-/-}$ mice from control animals (Figure 6.3*C*). The concentration of IL-1β and TNF-α cytokines in the peritoneal lavage fluid was examined by ELISA. In WT and *Mefv*$^{-/-}$ animals, levels of both cytokines were below the limit of detection (data not shown).



C

LPS Dose	Genotype	% Wt loss	Peritoneal cells/mL	MPO (ng/mL)
2.50 mg/kg	WT	5.74 ± 0.50	$8.18 \pm 0.89 \times 10^6$	133.0 ± 6.07
	Mefv-/-	5.20 ± 0.61 (p = 0.51)	$7.95 \pm 0.94 \times 10^6$ (p = 0.88)	138.3 ± 10.54 (p = 0.65)
0.25 mg/kg	WT	5.54 ± 0.65	$9.32 \pm 0.87 \times 10^5$	
	Mefv-/-	5.02 ± 0.44 (p = 0.51)	$9.11 \pm 0.80 \times 10^5$ (p = 0.83)	

Figure 6.3. Loss of *Mefv* does not affect the response to LPS. WT and *Mefv*$^{-/-}$ mice were treated with 2.5 mg/kg (*A*, n = 8 WT and 5 *Mefv*$^{-/-}$ mice) or 0.25 mg/kg (*B*, n = 18 and 19) crude LPS by i.p. injection at time 0 h, as indicated by arrows. Rectal temperature at the indicated times is shown in *A* and *B*. The table shown in *C* lists other parameters of inflammation corresponding to the mice in *A* and *B*: percent of body weight lost between 0 h and the time of harvest, concentration of immune cells present in the peritoneal lavage fluid, and relative abundance of neutrophils in lavage samples as determined by myeloperoxidase (MPO) activity within peritoneal lavage cell samples.

A loss of Pyrin does not affect the outcome of *Pseudomonas aeruginosa* infection

To examine whether or not Pyrin shapes the overall immune response to live pathogens, mice were infected with *Pseudomonas aeruginosa*. *P. aeruginosa* is an opportunistic bacteria that can cause pneumonia, necrotising enterocolitis, urinary tract infection, skin infection, and septic shock in humans. In mice, depending upon the initial bacterial burden, wild-type mice either die or completely clear the bacteria by 48 hours post-infection (19). *P. aeruginosa* infection induces inflammasome-dependent IL-1β release (20), so it is reasonable to hypothesize that mutations in Pyrin may alter IL-1β production and/or the course of infection. The bacterial load was optimized in this study so that WT 129 mice would show signs of bacterial burden but no lethality at a relatively late time-point post infection, so that a role for Pyrin in either the early phase and/or the late phase of the response would be evident. 5.4 x 10^6 CFU/mouse were injected into the peritoneal cavity of mice, and animals were assessed at 19 h post-infection. While all mice had some bacteria remaining in the peritoneal cavity at 19 h, mice were split into two populations. One group showed signs of bacterial clearance. Bacterial burdens in these mice ranged from 10^2 – 10^3 CFU/mL, which is less than the initial burden of 1.35 x 10^6 CFU/mL. The other group showed signs of bacterial proliferation, having burdens of 10^7 – 10^9 CFU/mL, which is above the initial load. Importantly, the same population split was seen in WT and *Mefv*$^{-/-}$ groups, indicating that the response of individual mice within each genotype differs more than the response of mice between the two genotypes at this timepoint (Figure 6.4*A*). The bacterial load in the blood showed a similar trend. Mice that were clearing the bacterial infection in the peritoneal cavity had no bacteria in the blood, while some mice with high bacterial loads in the peritoneal cavity were also septic. Again, the population split was independent of the *Mefv* genotype. 50% of WT animals (2 of 4 tested) were septic, and 43% (3 of 7) *Mefv*$^{-/-}$ mice were septic (Figure 6.4*B*). The total number of peritoneal cells was increased in infected mice compared to PBS (vehicle) treated mice; however, the cell counts were similar between WT and *Mefv*$^{-/-}$ animals (Figure 6.4*C*). The IL-1β concentration in the peritoneal lavage

fluid correlated with bacterial burden; mice with the highest bacterial burdens also had the highest production of IL-1β; however, cytokine levels were not affected by the loss of Pyrin (Figure 6.4*D*). Corresponding to the cell and molecular indicators of inflammation, infection caused a 7% loss of body weight, and a decrease in body temperature, but again, results were independent of the *Mefv* genotype (Figure 6.4*E* and data not shown). Together, these results suggest that a loss of Pyrin does not affect the outcome of *P. aeruginosa* bacterial infection; however, a more extensive timecourse analysis is necessary.

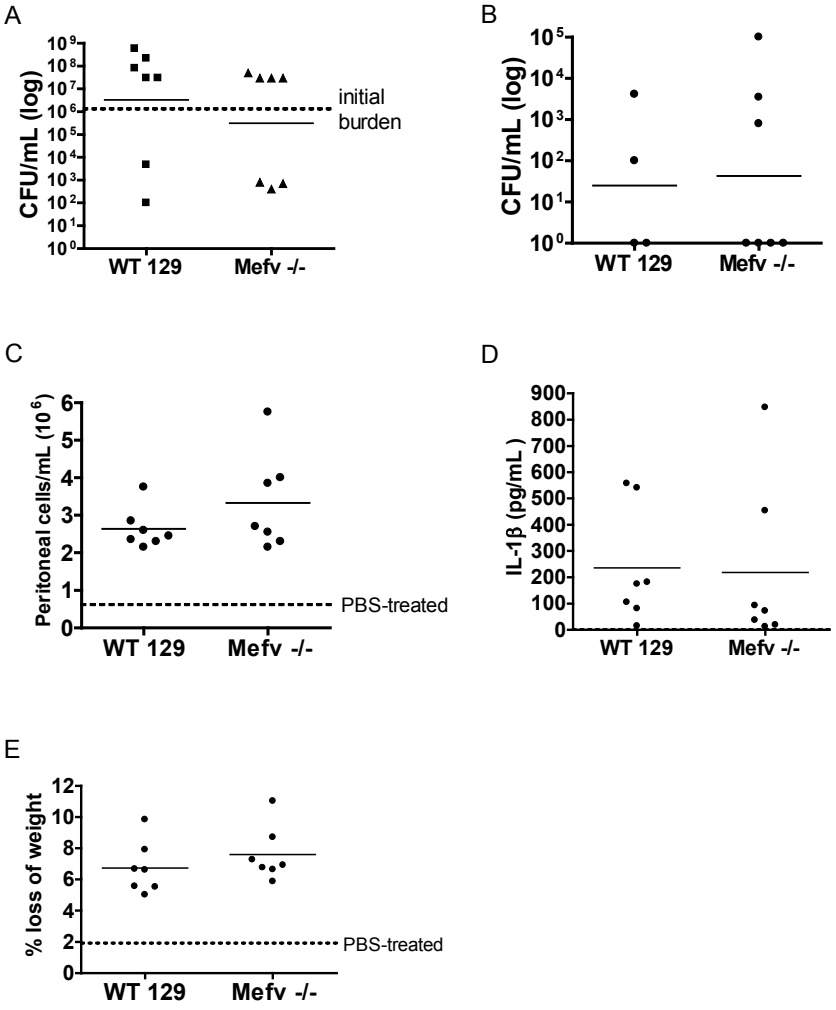


Figure 6.4. Loss of Pyrin does not affect the outcome of *Pseudomonas aeruginosa* infection. 5.4 x 10^6 CFU/mouse or PBS vehicle were injected into the peritoneal cavity of WT and *Mefv*$^{-/-}$ mice (n = 7 and 7), and animals were assessed at 19 h post-infection. The peritoneal cavity was lavaged with 4 mLs of PBS. *A* and *B*, Number of live bacteria (colony forming units (CFU) per mL of peritoneal lavage fluid, *A*, or mL of blood, *B*. The initial burden in the peritoneal cavity was determined by plating a sample of the injected solution. *C,* Peritoneal cell concentration, as determined by hemocytometer. Mice with greater bacterial loads in the peritoneum had slightly lower cell counts. *D*, IL-1β concentration in peritoneal lavage fluid was determined by ELISA. *E*, The percent of body weight lost during the course of infection (19 h). Each dot represents one mouse.

Discussion

This chapter describes studies to assess the *in vivo* innate immune response of *Mefv* null mice. These studies provide a functional complement to the work described in previous chapters in which we characterized the activation and survival of individual immune cell types isolated from the mouse. Following exposure to thioglycolate, LPS, and *P. aeruginosa*, WT and *Mefv*$^{-/-}$ mice showed similar physiological, cellular, and IL-1β responses. Peritoneal exposure to thioglycolate and LPS demonstrated that *Mefv*$^{-/-}$ mice recruit and retain inflammatory cells at sites of inflammation normally ($p = 0.83$ and 0.88, respectively). The hypothermic response induced by LPS was similar in *Mefv*$^{-/-}$ and WT mice. No differences in cytokine production or bacterial load were detected between *Mefv*$^{-/-}$ and WT mice following *P. aeruginosa* infection. While our results failed to support our hypothesis that Pyrin-deficiency causes exacerbated innate immune responses, they leave open the possibility that Pyrin may be involved in some, but not all, immune responses. It is also possible that compensatory mechanisms within the mouse prevent Pyrin-deficiency from altering the immune response *in vivo*.

Since we wanted to test the contribution of Pyrin to the general innate immune response, we used sterile peritonitis models, so that the interpretation of our results was not complicated by the pathogenicity of a microorganism. Furthermore, we chose LPS because *Mefv* expression is induced by LPS (Chapter 3 and previously published reports (8)). This affect on gene expression is not unique to murine *Mefv*. In rats, intratracheal instillation of LPS increases *Mefv* expression in bronchial aviolar lavage cells (21), and treatment with LPS also increases expression of human *MEFV* (22). Consistent with expression data, exacerbated hypothermia was observed after LPS treatment in mice carrying alleles encoding a truncated Pyrin protein. However, the possibility that Pyrin is involved in pathogen defense through a mechanism that is only apparent upon infection with live pathogens, such as bacterial clearance, was also addressed. *Pseudomonas aeruginosa* activates NF-κB-dependent *Il1β* transcription and

inflammasome-dependent IL-1β release (20, 23-26). Specifically, association of LPS with TLRs 2 and 4 and of flagellin with TLR5 results in MyD88 and NF-κB dependent *Il1β* transcription. Induction of *Il1β* expression is dose-dependent according to the bacterial burden (24-26). Release of IL-1β from macrophages exposed to *P. aeruginosa in vitro* is dependent upon *Caspase-1*, *Nlrc4*, and *Nlrp3*, but not *Pycard* (20, 23). Accordingly, Caspase-1 cleavage is absent in *Nlrc4*$^{-/-}$ macrophages, and *in vivo*, reduced IL-1β production in *Nlrc4*$^{-/-}$ mice was accompanied with increased bacterial burden (20). Given these previous findings, *P. aeruginosa* infection was a good model system to evaluate the contribution of Pyrin to bacterial defense.

Previous work has demonstrated that genetic perturbation of *Mefv* in mice causes exacerbated inflammatory responses. Chae, et al. created and characterized mice carrying a disrupted *Mefv* allele. The promoter and 5' end of the gene, which encodes the pyrin domain and a small portion of the linker region, were maintained, but the remaining 3' end, which encodes the B-box, coiled-coiled, and B30.2 domains, was replaced with DNA encoding green fluorescent protein (GFP). This allele, *Mefv*^{trunc}, encodes a truncated Pyrin protein that the authors describe as hypomorphic. Homozygous mutant mice exhibited phenotypes resembling FMF. An increased number of inflammatory cells was observed at 4 d after thioglycolate-induced peritonitis, perhaps as a result of impaired macrophage cell death in these animals (8). Relative to wild-type mice, homozygous mutant mice displayed exacerbated LPS-induced hypothermia and increased lethality following LPS and D-gal treatment. Peritoneal macrophages from these mice produced more IL-1β after immune stimulation *in vitro*. It is unclear why *Mefv*$^{trunc/trunc}$ mice with hypomorphic Pyrin function would have different immune responses compared to WT littermates, while *Mefv*$^{-/-}$ mice with no Pyrin function have responses similar to coisogenic WT mice. While effort was taken to match experimental variables between these studies, factors such as housing conditions and genetic background

could not be controlled. For example, the gut microbiome is influenced by external housing conditions, and it significantly alters the course of peritoneal inflammation (27). The genetic background of mice affects endotoxin-induced lethality and IL-1β production in WT mice (28). Furthermore, IL-1 receptor antagonist (IL-1ra) deficiency has varying degrees of severity in BALB/cA, C57BL/6, and 129 mice (reviewed in (29)). Interestingly, the cellularity of *Mefv*$^{-/-}$ and WT cohorts in this study was similar to that of *Mefv*$^{trunc/trunc}$ mice in the previous study, but differed from the wild-type mice. The dramatic difference between wild-type responses lends support to the idea that genetic and/or microbiome influences may contribute to the findings of these studies.

In chapter 3, we showed that the loss of Pyrin increases IL-1β levels, however, release of mature IL-1β requires a danger signal. Thus, it is not surprising that IL-1β levels were not dramatically increased *in vivo*. *Mefv*$^{trunc/trunc}$ mouse macrophages, on the other hand, released significant IL-1β in response to LPS. Therefore, it is not surprising that cell recruitment and thermic responses, which are regulated in part by IL-1β, were exacerbated in these mice. It is important to note that the mechanism of cellular recruitment induced by thioglycolate differs from that induced by IL-1β. In most cases, knock-out mice that display a difference in IL-1β-induced cell recruitment also display a difference in thioglycolate-induced cell recruitment (18). However, an example to the contrary is *Icam2*$^{-/-}$ mice, which have reduced neutrophil recruitment in response to IL-1β treatment but not thioglycolate treatment (30). It remains possible that changes in IL-1β production alter cellular recruitment in Pyrin-deficient mice.

It is possible that the experimental systems that we chose were not sensitive enough to resolve a functional contribution of Pyrin. We submit several arguments against this possibility. 1) We used well-established model systems that have been used in the past to detect functional changes in mouse models. 2) We reproduced experiments that were used previously to demonstrate inflammatory hyperresponsiveness in homozygous *Mefv* mutant mice. 3) We

further mitigated this risk by using multiple models of inflammation. We chose diverse models to stimulate robust (thioglycolate, Zymosan-A, and *P. aeruginosa*) and mild inflammation (0.25 mg/kg LPS), in order to entertain the possibility that Pyrin modulates only robust or only mild inflammation. 4) Large cohorts were used in these studies in order to resolve a more subtle phenotype. However, the variability that is inherent to *in vivo* studies does severely limit the ability to conclude that there is no phenotype in *Mefv* null animals. Nevertheless, our studies suggest that a complete loss of Pyrin function does not affect the sensitivity or magnitude of an innate immune response *in vivo* following treatment with at least some stimuli.

References

1. Henderson, R. B., J. A. Hobbs, M. Mathies, and N. Hogg. 2003. Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. *Blood* 102:328-335.

2. Rogers, H. W., C. S. Tripp, R. D. Schreiber, and E. R. Unanue. 1994. Endogenous IL-1 is required for neutrophil recruitment and macrophage activation during murine listeriosis. *J Immunol* 153:2093-2101.

3. Davtyan, T. K., G. S. Hakopyan, S. A. Avetisyan, and N. R. Mkrtchyan. 2006. Impaired endotoxin tolerance induction in patients with familial Mediterranean fever. *Pathobiology* 73:26-39.

4. Ledford, J. G., M. Kovarova, and B. H. Koller. 2007. Impaired host defense in mice lacking ONZIN. *J Immunol* 178:5132-5143.

5. Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 6:173-182.

6. Ben-Zvi, I., and A. Livneh. 2011. Chronic inflammation in FMF: markers, risk factors, outcomes and therapy. *Nat Rev Rheumatol* 7:105-112.

7. Leon, L. R. 2002. Invited review: cytokine regulation of fever: studies using gene knockout mice. *J Appl Physiol* 92:2648-2655.

8. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* 11:591-604.

9. Blanqué R, M. C., Millet S, Gardner CR. 1996. Hypothermia as an indicator of the acute effects of lipopolysaccharides: comparison with serum levels of IL1 beta, IL6 and TNF alpha. *General pharmacology* 27:973-977.

10. Alheim, K., Z. Chai, G. Fantuzzi, H. Hasanvan, D. Malinowsky, E. Di Santo, P. Ghezzi, C. A. Dinarello, and T. Bartfai. 1997. Hyperresponsive febrile reactions to interleukin (IL) 1alpha and IL-1beta, and altered brain cytokine mRNA and serum cytokine levels, in IL-1beta-deficient mice. *Proc Natl Acad Sci U S A* 94:2681-2686.

11. Kozak, W., M. J. Kluger, D. Soszynski, C. A. Conn, K. Rudolph, L. R. Leon, and H. Zheng. 1998. IL-6 and IL-1 beta in fever. Studies using cytokine-deficient (knockout) mice. *Ann N Y Acad Sci* 856:33-47.

12. Kozak, W., H. Zheng, C. A. Conn, D. Soszynski, L. H. van der Ploeg, and M. J. Kluger. 1995. Thermal and behavioral effects of lipopolysaccharide and influenza in interleukin-1 beta-deficient mice. *Am J Physiol* 269:R969-977.

13. Ozen, S., D. Uckan, E. Baskin, N. Besbas, H. Okur, U. Saatci, and A. Bakkaloglu. 2001. Increased neutrophil apoptosis during attacks of familial Mediterranean fever. *Clin Exp Rheumatol* 19:S68-71.

14. Mansfield, E., J. J. Chae, H. D. Komarow, T. M. Brotz, D. M. Frucht, I. Aksentijevich, and D. L. Kastner. 2001. The familial Mediterranean fever protein, pyrin, associates with microtubules and colocalizes with actin filaments. *Blood* 98:851-859.

15. Shoham, N. G., M. Centola, E. Mansfield, K. M. Hull, G. Wood, C. A. Wise, and D. L. Kastner. 2003. Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. *Proc Natl Acad Sci U S A* 100:13501-13506.

16. Yu, J. W., T. Fernandes-Alnemri, P. Datta, J. Wu, C. Juliana, L. Solorzano, M. McCormick, Z. Zhang, and E. S. Alnemri. 2007. Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants. *Mol Cell* 28:214-227.

17. Badour, K., J. Zhang, F. Shi, M. K. McGavin, V. Rampersad, L. A. Hardy, D. Field, and K. A. Siminovitch. 2003. The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. *Immunity* 18:141-154.

18. Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 7:678-689.

19. Kim, S. H., B. Y. Lee, G. W. Lau, and Y. H. Cho. 2009. IscR modulates catalase A (KatA) activity, peroxide resistance and full virulence of Pseudomonas aeruginosa PA14. *J Microbiol Biotechnol* 19:1520-1526.

20. Sutterwala, F. S., L. A. Mijares, L. Li, Y. Ogura, B. I. Kazmierczak, and R. A. Flavell. 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. *J Exp Med* 204:3235-3245.

21. Rao, K. M., and T. Meighan. 2006. Exposure in vivo to silica or lipopolysaccharide produces transient or sustained upregulation, respectively, of PYPAF7 and MEFV genes in bronchoalveolar lavage cells in rats. *J Toxicol Environ Health A* 69:481-490.

22. Chae, J. J., G. Wood, K. Richard, H. Jaffe, N. T. Colburn, S. L. Masters, D. L. Gumucio, N. G. Shoham, and D. L. Kastner. 2008. The familial Mediterranean fever protein, pyrin, is cleaved by Caspase-1 and activates NF-kappaB through its N-terminal fragment. *Blood* 112:1794-1803.

23. Kanneganti, T. D., M. Lamkanfi, Y. G. Kim, G. Chen, J. H. Park, L. Franchi, P. Vandenabeele, and G. Nunez. 2007. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 26:433-443.

24. Gadjeva, M., C. Paradis-Bleau, G. P. Priebe, R. Fichorova, and G. B. Pier. 2009. Caveolin-1 modifies the immunity to Pseudomonas aeruginosa. *J Immunol* 184:296-302.

25. Raoust, E., V. Balloy, I. Garcia-Verdugo, L. Touqui, R. Ramphal, and M. Chignard. 2009. Pseudomonas aeruginosa LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. *PLoS One* 4:e7259.

26. Tsay, T. B., C. J. Chang, P. H. Chen, C. M. Hsu, and L. W. Chen. 2009. Nod2 mutation enhances NF-kappa B activity and bacterial killing activity of macrophages. *Inflammation* \32:372-378.

27. Ivanov, II, K. Atarashi, N. Manel, E. L. Brodie, T. Shima, U. Karaoz, D. Wei, K. C. Goldfarb, C. A. Santee, S. V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda, and D. R. Littman. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485-498.

28. Yang, I. V., C. M. Wade, H. M. Kang, S. Alper, H. Rutledge, B. Lackford, E. Eskin, M. J. Daly, and D. A. Schwartz. 2009. Identification of novel genes that mediate innate immunity using inbred mice. *Genetics* 183:1535-1544.

29. Arend, W. P., and C. J. Guthridge. 2000. Biological role of interleukin 1 receptor antagonist isoforms. *Ann Rheum Dis* 59 Suppl 1:i60-64.

30. Huang, M. T., K. Y. Larbi, C. Scheiermann, A. Woodfin, N. Gerwin, D. O. Haskard, and S. Nourshargh. 2006. ICAM-2 mediates neutrophil transmigration in vivo: evidence for stimulus specificity and a role in PECAM-1-independent transmigration. *Blood* 107:4721-4727.

CHAPTER 7

Conclusions and Perspectives

Summary

This dissertation summarizes our work to elucidate the role of Pyrin in innate immune responses. Using a reverse genetics approach with Pyrin-deficient mice, we were able to examine the outcome of a complete loss of Pyrin function in an endogenous system. Building upon previous research addressing Pyrin function in systems with hypormorphic or hypermorphic Mefv expression, and guided by the pathology of FMF and *Mefv* expression, we chose to investigate the contribution of Pyrin in macrophage-dependent IL-1β production and in neutrophil processes. We identified a role of Pyrin in IL-1β production, as a negative regulator of inflammasome-mediated IL-1β levels. Pyrin null neutrophils, however, did not display altered phenotypes in an unstimulated state or in the context of innate immune responses, despite especially high expression of *Mefv* in wild-type neutrophils. Our results are consistent with a model in which mice in a steady-state have adequate immune function despite the loss of Pyrin, but during inflammation, the loss of Pyrin causes functional changes, and specifically, a loss in appropriate regulation of IL-1β levels.

Previous research is consistent with a functional contribution of Pyrin to the innate immune response and a model in which abnormal function, due to FMF-associated mutations, causes uncontrolled inflammation. Previous studies using in vitro human and mouse model systems

have shown that Pyrin contributes to macrophage-dependent IL-1β production (1-8); however, its specific contribution is unclear. Studies using neutrophils isolated from FMF patients indicate that neutrophils have altered chemotaxis, phagocytosis, oxidative burst, and survival (9-15), although the association of mutations in *Mefv* with these neutrophil phenotypes has not been determined. Thus, we addressed questions which remained as to how mutations in *Mefv* alter the innate immune response to cause FMF pathology.

We found that macrophages from wild-type and *Mefv*$^{-/-}$ mice release a similarly low amount of IL-1β after treatment with LPS; however, following the addition of a number of different agents that stimulate the inflammasomes, IL-1β levels in the supernatant of macrophages from *Mefv*$^{-/-}$ animals were elevated compared to those in the supernatant of macrophages from wild-type mice. While the concentration of IL-1β and the magnitude of the difference between *Mefv*$^{-/-}$ and wild-type macrophages changed depending on the treatment, the effect of Pyrin-deficiency remained the same. Exposure to NLRP1b, NLRP3, and NLRC4-dependent stimuli all led to increased levels of IL-1β in the supernatant of *Mefv*$^{-/-}$ macrophages compared to wild-type macrophages. Neutrophils, on the other hand, did not display any deficits due to a loss of Pyrin. No differences in development, recruitment, or survival were found. *In vivo*, the induction of peritonitis did not lead to detectable changes in the recruitment or survival of macrophages or neutrophils, or in the overall health of mice, as detected by parameters of body temperature, weight loss, and bacterial load. While the data from these studies suggest that there are mechanisms to protect against an alteration to the *in vivo* inflammatory response to at least some stimuli, further testing of Pyrin null mice is critical for making these conclusions. Certainly the importance of *Mefv* is clear, based upon its conservation in the genome and the association of mutations with an autoinflammatory phenotype.

This chapter discusses how our findings further previous research on Pyrin and FMF, primarily regarding the role of Pyrin in IL-1β production, and then hypothesizes where Pyrin fits

into the pathway for IL-1β production and release. Given that Pyrin negatively regulates IL-1β production, other mechanisms of endogenous regulation of inflammasome-dependent IL-1β production are discussed, in order to create a context for Pyrin's role in the complex regulation of IL-1β synthesis and release. Many questions remain, regarding not only Pyrin's regulation of IL-1β, but also mechanisms that regulate Pyrin's function. Finally, the genetics of FMF are revisited, given our findings that a loss of Pyrin function leads to an IL-1β phenotype that is characteristic of FMF.

Previous mouse models

Work published previous to our study also demonstrated that a genetic perturbation targeted to *Mefv* led to exacerbated pro-inflammatory responses. The targeted *Mefv* allele encodes a truncated Pyrin protein, in which the pyrin domain and a small portion of the linker region are fused to green fluorescent protein (GFP). Homozygous mutant mice ($Mefv^{trunc/trunc}$) displayed exacerbated LPS-induced hypothermia, increased inflammatory cell recruitment in response to thioglycolate, and increased lethality following LPS and D-gal treatment. Furthermore, peritoneal macrophages from $Mefv^{trunc/trunc}$ mice had excessive IL-1β production and decreased apoptosis *in vitro* (1). These findings certainly support an important role for Pyrin *in vivo* and suggest that disrupting *Mefv* significantly alters innate immune responses to induce and/or maintain exacerbated inflammatory responses. However, there are limitations which prevent a logical inference to the function of human Pyrin and the consequences of FMF-associated mutations based upon studies using $Mefv^{trunc/trunc}$ mice. Due to the presence of a partial protein, and furthermore, a GFP-fusion protein, it is difficult to define the disrupted allele as causing a partial or complete loss of wild-type Pyrin function or as producing a gain of wild-type function or even novel function. It is interesting that in Pyrin null mice, elevated IL-1β levels were also detected, but exacerbated hypothermia, cellularity, and sepsis-induced lethality phenotypes, and the decreased macrophage cell death phenotype, were not reproduced. It is

possible that these differences result from function(s) of the truncated, GFP-fusion, Pyrin protein that is present in *Mefv*$^{trunc/trunc}$ mice.

A major caveat to using the mouse to model human Pyrin function is the absence of the B30.2 domain from the mouse protein. Recently-published work addresses the function of the B30.2 domain by creating a hybrid *Mefv* gene in which the promoter, PYD, BB, and C-C domains of the mouse protein are intact, and the B30.2 domain of the human Pyrin protein is attached to the C-terminal end (16). Despite significant effort, a knock-in of the WT B30.2 domain could not be created, perhaps because it was incompatible with life. However, within this system, the effects of three missense mutations in the B30.2 domain were examined. All three mutations, collectively referred to here as mutB30.2, caused delayed growth, spontaneous inflammation, more circulating immune cells, and elevated IL-1β levels in homozygous mice. Heterozygous *Mefv*$^{mutB30.2/+}$ and *Mefv*$^{mutB30.2/-}$ were unaffected. The investigators concluded that mutations in the B30.2 region cause a gain-of-function and have a dosage effect. Similar to the *Mefv*$^{trunc/trunc}$ mice, it is difficult to define the chimeric Pyrin protein as causing a gain-of-function. To address this question, it was emphasized that Pyrin deficient mice, with a loss-of-function, had no disease phenotype in the assays used for testing. Specifically, the relative number of circulating CD11b^{+} cells was similar to *Mefv*$^{+/+}$ mice. *Mefv*$^{-/-}$ mice did not display increased IL-1β processing in response to NLRP3, NLRC4, or AIM2 stimuli. However, mice did secrete more IL-1β following treatment with LPS alone. In contrast, our results show that the loss of Pyrin causes significantly elevated IL-1β levels following treatment with NLRP3, NLRC4, and NLRP1b inflammasome stimuli, but not following treatment with LPS alone. Taking these IL-1β phenotypes into consideration, the conclusions that Pyrin null mice have no phenotype and that B30.2 missense mutations cause a gain-of-function are less clear. Another possible conclusion from studies using mutB30.2 mice is that the introduction of the wild-type B30.2 domain into the mouse protein causes a gain-of-function, either increased wild-type function or novel function,

and that missense mutations in the B30.2 domain cause a partial loss of the gained function. This later model in which missense mutations cause a loss-of-function is more consistent with autosomal recessive inheritance of FMF.

Where in the pathway?

IL-1β was undetected in peritoneal lavage samples from *Mefv*-/- and WT mice at 20 and 24 h following *in vivo* exposure to LPS, 4 h or 4 d following thioglycolate treatment, and untreated cells. Therefore, the removal of *Mefv* from the mouse does not cause spontaneous IL-1β release. Furthermore, in our studies, treatment with LPS alone did not alter IL-1β production from *Mefv* null macrophages in comparison to WT macrophages. Thus, the regulation of IL-1β production by Pyrin is independent of IL-1β transcription. Rather, inflammasome activation remains a requirement for the release of IL-1β from Pyrin-deficient macrophages. This is consistent with the episodic nature of inflammation in FMF—an unknown endogenous stimulus activates an inflammatory response, but severe inflammation is not chronic.

Pyrin can negatively regulate IL-1β production that is mediated by multiple inflammasomes. Therefore, Pyrin most likely contributes to a part of this pathway which is common to multiple modes of pathogen uptake and signaling through multiple inflammasome complexes. Since ASC and Caspase-1 are common between the inflammasomes, a likely possibility is that Pyrin interacts with ASC and/or Caspase-1, sequestering them away from inflammasome complexes. Previous studies detected protein interactions between Pyrin and both ASC and Caspase-1 (1, 2, 6-8, 17). In particular, one study demonstrated that Pyrin competed with NLRP3 for binding to ASC (3).

Mice deficient in inflammasome components ASC or Caspase-1 are more resistant to endotoxin-induced lethality than WT mice (18), while mice lacking IL-1β have unaltered

susceptibility compared to wild-type mice (19). These data should be reproduced using all three knock-out mice in parallel, but they provide preliminary *in vivo* evidence that inflammasome-dependent, IL-1β-independent signaling contributes to inflammation. Indeed, the formation of inflammasome complexes is associated with not only IL-1β production, but also inflammation-induced cell death (20). As expected, we observed an increase in macrophage cell death following exposure to stimuli which activate inflammasome complexes. However, inflammasome-associated cell death was not affected by the loss of Pyrin. This places Pyrin's role in inflammasome function as part of a divergent pathway that is limited to IL-1β production and not cell death.

Initial work suggested that inflammasome activation leads to both IL-1β production and cell death in a ASC-dependent, Caspase-1-dependent manner. Thus, it was perplexing to us that Pyrin affected IL-1β production but not cell death. We anticipated that if Pyrin affected inflammasome complex formation, or a theoretical common function upstream of inflammasome complex formation, we would observed changes in cell death as well as IL-1β production. However, more recent work has begun to tease apart two partially overlapping, but independent pathways for IL-1β production and cell death. IL-1β production in response to *Legionalla pneumophila* requires NLRC4, ASC, and Caspase-1, but cell death is dependent on only NLRC4 and Caspase-1 and not ASC (21). These findings elucidated the possibility that Pyrin could interact with ASC and affect IL-1β production without affecting cell death. Further research has shown that divergent pathways control IL-1β production and cell death in response to *Francisella novicida*, *Salmonella* typhimurium, and *Pseudomonas aeruginosa*, as well as *Legionella pneumophila*. Caspase-1 and ASC are both required for IL-1β production, but Caspase-1 is also absolutely required for cell death and ASC is not (22). These results are consistent with a model in which Pyrin sequesters ASC to prevent the formation of inflammasomes complexes that mediate IL-1β production. However, importantly, the "death

complex" is formed by CARD-CARD interactions between NLRC4, NLRP1b, or a CARD-Aim2 chimera and Caspase-1. Thus, there is still no model to explain how Pyrin can regulate NLRP3 inflammasome complex formation without affecting cell death as well as IL-1β production.

Our results are consistent with a model in which Pyrin negatively regulates IL-1β release from the cell. IL-1β, IL-18, IL-33, and high mobility group box protein 1 (HMGB1) are uniquely shuttled to the plasma membrane in endolysosomes, in contrast to most proteins which are sorted within the Golgi into vesicles released from the cell (23). The interplay between inflammasome complex formation, endolysosomes, and cytokine release is undefined. Immunofluorescence microscopy studies place the inflammasome complexes and ASC specks within the cytosol, however, Caspase-1 may cleave pro-IL-1β within the endolysosomes. Although not directly proven, sequestering Caspase-1 activity within the endolysosomes would explain how the cell might protect itself against off-target cleavage of other proteins by Caspase-1 (24). In support of this model, mature Caspase-1 release can be detected concurrent with IL-1β release (25). The mechanism by which IL-1β and Caspase-1 enter the endolysosomes is a mystery (24). Are active transporters required or do endolysosomes form around ASC specks? Another unanswered question is what cytoskeletal elements and associated proteins are necessary for shuttling of the endolysosomes through the cell. Since Pyrin has been shown to interact with cytoskeletal elements in addition to inflammasome complexes, it is reasonable to hypothesize that Pyrin may be a link between these elements that are necessary for IL-1β production. This link, called an immune synapse, was previously described.

There are several approaches to test the model that Pyrin is involved in IL-1β release from the cell. Cellular colocalization would be an ideal way to visualize an interaction between Pyrin, the cytoskeleton, and active Caspase-1, however, reagents necessary to facilitate image resolution to such detail are currently unavailable. A more amenable method to test the

hypothesis that Pyrin interacts with inflammasome proteins ASC and/or Caspase-1 in cells is to quantify the formation of ASC specks and the activation of Caspase-1. If Pyrin interacts with ASC to prevent inflammasome complex formation, then the percentage of ASC-speck positive cells should be increased in macrophages from Pyrin-deficient mice compared to macrophages from wt mice. Similarly, Caspase-1 activity should be increased. If Pyrin interacts with the catalytic subunits of pro-Caspase-1 or mature Caspase-1, then Caspase-1 activity will still be increased, however, ASC-speck formation should be similar between Pyrin-deficient and wt cells. This pattern of results is seen in cells treated with Caspase-1 inhibitors (26). It is important to recognize that Pyrin's affect on IL-1β production is subtle and a phenotypic difference in these assays is also likely to be subtle. Therefore, a detectable difference argues for a role of Pyrin in inflammasome complex formation and/or function, but results which are similar between WT and *Mefv* null cells is inconclusive. It could indicate that Pyrin does not affect inflammasome function, but alternatively, it could indicate that the phenotype could not be resolved by these assays.

An easy, albeit inconclusive, way to suggest that Pyrin's regulatory action is through endolysosome shuttling rather than inflammasome complex formation is to compare HMGB1 release from Pyrin deficient and sufficient macrophages following treatment with LPS. Since HMGB1 is released through endolysosomes, but does not require Caspase-1 cleavage, detection of HMGB1 levels provides a nice assay to separate Caspase-1 activation and endolysosome shuttling functions. Elevated levels of HMGB1 release from Pyrin-deficient macrophages suggest that Pyrin's effect is on a function common to HMGB1 and IL-1β release, i.e. endolysosome shuttling. Similar levels of HMGB1, on the other hand, argue that the regulatory role of Pyrin is limited to IL-1β and not HMGB1, and thus suggests that Pyrin regulates inflammasome function.

Together, these assays provide complementary methods to detect whether Pyrin affects inflammasome complex formation or IL-1β release. A question that could remain following this work is how Pyrin affects IL-1β release following inflammasome stimulation, but has no effect on IL-1β release in response to LPS alone. In mice, little IL-1β is released without additional danger stimuli to activate the inflammasome. Given the subtle regulatory effect of Pyrin, it is possible that a difference in IL-1β levels following LPS treatment could not be detected. It is also possible that a small amount of mature or immature IL-1β is released from macrophages through another mechanism, such as passive diffusion from dying cells (7). Pro-IL-1β can be cleaved following its release from the cell (27). Further experiments are necessary to address this possibility. Initially, it would be interesting to test IL-1β release following other stimuli that activate production of pro-IL-1β but do not activate the inflammasome. It would also be interesting to examine a mouse with Caspase-1 and Pyrin deficiencies. This would be another way to discern whether or not the regulatory effect of Pyrin is dependent upon the inflammasome complex, although given the small amount of IL-1β release independent of the inflammasome, the detection limit of reagents may prevent resolution of a phenotype.

Endogenous regulation of inflammasome-dependent IL-1β production

Considering the severe consequences which result from unregulated IL-1β signaling, it is not surprising that there are multiple stages at which IL-1β signaling is controlled. As previously mentioned, IL-1β transcription and the stability of pro-IL-1β protein is influenced by multiple factors (28-30). Additionally, regulation of inflammasome complexes influences IL-1β release. This section focuses on regulation of inflammasome signaling as a mechanism to fine-tune the timing and magnitude of IL-1β production.

Sequestration of inflammasome complex proteins

Two classes of negative regulators that prevent inflammasome protein interactions are CARD-only proteins (COPs) and PYD-only proteins (POPs). As their names suggest, these proteins have CARD or PYD protein binding domains, but lack the functional NACHT and LRR domains that are necessary to promote Caspase-1 activation. COPs and POPs bind to the CARD or PYD domains, respectively, of the NLRs, ASC, and/or Caspase-1 to prevent inflammasome complex interactions and Caspase-1 activation. Human COP, INCA (inhibitory CARD) and ICEBERG have been shown to block IL-1β production using *in vitro* overexpression systems (31-33). Similarly, human pyrin-only proteins POP1 and cPOP2 have been shown to regulate IL-1β production *in vitro*. POP1 was originally shown to increase IL-1β production, but a recent protein binding study suggests that POP1 may decrease NLR-dependent IL-1β production, similar to cPOP2 (34-37). The *in vivo* function of these genes has not been examined, because they are absent from the mouse genome. Pyrin, on the other hand, is suspected to act as a POP that negatively regulates IL-1β production in both humans and mice.

Additionally, Caspase-12 deficient mice display increased IL-1β cytokine production, but the significance of Caspase-12 in humans is questioned because the region that mediates the regulatory effect in mice is missing from the human gene (38). Bcl-2 and Bcl-X_L, on the other hand, bind NLRP1 and reduce Caspase-1 activation in human cell lines, and Bcl-2 deficient mice showed increased IL-1β production in response to muramyl dipeptide (MDP) treatment *in vivo* (39). p202, a HIN-family protein, inhibits AIM2-dependent inflammasome activity in response to dsDNA. p202 can bind dsDNA and also AIM2, so it may function to sequester one or both of these to prevent Caspase-1 activation (40). The serpin proteinase inhibitor 9 (PI-9) inhibits Caspase-1 and pro-IL-1β processing in human vascular smooth muscle cells (41).

Feedback loops

In addition to inflammasome complex disruption, a second mechanism of regulation occurs through feedback loops that converge upon NF-κB. NF-κb promotes transcription of *IL-1β, NLRP2, NLRP3, ICEBERG*, and *MEFV*. Increasing *IL-1β, NLRP2,* and *NLRP3* transcription should increase inflammasome-dependent IL-1β production and promote an inflammatory response, whereas ICEBERG and Pyrin inhibit IL-1β production. Activation of the IKK/NF-κB pathway, in turn, is regulated by IL-1β, TNFα, NLRP2-4, Pyrin, and ASC in a complex manner. Reconstitution of the pathway in HEK293T cells indicates that IL-1β or TNFα treatment alone induces NF-κB activity, and overexpression of NLRP3 or Pyrin also increases NF-κB activity above baseline (42). ASC alone dose-dependently reduces NF-κB activity (43) and abrogates the effects of IL-1β and TNFα (42). Interestingly, although NLRP3 and Pyrin increase NF-κB activity in untreated cells, they reduce the effect that TNF-α treatment has in promoting NF-κB activity. Moreover, while ASC alone reduces TNFα-dependent NF-κB activity, ASC increases NF-κB activity when NLRP3 or Pyrin is combined with TNFα. In this study, ASC affects IKKα and IKKβ activity through its PYD (42). To add further regulation, the expression of *Mefv* and IL-1 receptors 1 and 2 (IL-1r1 and IL-1r2) are also driven by NF-κB in a TNF-α dependent manner (44).

Other investigations have shown that, in untreated cells, *Pycard* (ASC) overexpression induces NF-κB activity (45). The NLRs PYNOD (NLRP10) and NLRP2 reduce NF-κB activity which is promoted by ASC. PYNOD binds to ASC (46) to inhibit ASC-mediated induction of NF-κB activity. NLRP2 inhibits NLRP3-mediated induction as well as ASC-mediated induction of NF-κB activity (45). NLRP2 expression is promoted by NF-κB in a TNFα-dependent manner. Since TNFα is itself regulated by NF-κB activity, NLRP2 mediates a feedback regulatory loop (47). Fas-associated factor 1 (FAF1) also binds to NLRP3 and inhibits NLRP3 and ASC-

mediated activation of NF-κB. Furthermore, immune stimulants can influence the expression of regulatory genes. For example, LPS increases NF-κB-mediated transcription of pro-IL-1β, ASC, NLRP3, Pyrin, and FAF1 (48). Thus, timing of NF-κB-driven gene expression and NF-κB regulation can shape the transcriptome and the immune response. As previously mentioned, in one study Pyrin increased NF-κB translocation (49) and in another study Pyrin increased NF-κB activity (42), but other investigators have seen no change in NF-κB activity following the overexpression of Pyrin (3, 8).

In addition to regulatory loops that affect NF-κB activity, NF-κb-mediated regulatory loops also affect inflammasome activity. In addition to *IL-1β, NLRP2, NLRP3, ICEBERG*, and *MEFV*, as previously mentioned, Serpin plasminogen activator inhibitor 2 (PAI-2) and Bcl-X_L are NF-κB regulated genes. Both negatively regulate Caspase-1 dependent IL-1β secretion. Therefore, NF-κb-dependent genes negatively regulate IL-1β release, whose transcription is positively regulated by NF-κB. Interestingly, PAI-2 is not expressed in THP-1 cells, which release IL-1β following stimulation with LPS only (50). PYNOD, mentioned above as an inhibitor of ASC-dependent NF-κB activity, also inhibits IL-1β release. Using both human and mouse proteins, PYNOD was shown to co-immunoprecipitate with IL-1β and Caspase-1, indicating that its affect on IL-1β release occurs through inflammasome complex interaction and not just NF-κB-regulated transcription of pro-IL-1β. In Jurkat cells, PYNOD expression slightly increased following LPS stimulation, thus PYNOD expression can also be regulated by inflammatory signaling in at least some hematopoietic cells (46).

A recent study in mice demonstrated another mechanism of inflammasome regulation: cell-mediatied suppression by adaptive immune cells. Activated effector and memory $CD4^+$ T-cells suppress inflammasome activity during the primary adaptive immune phase and during secondary immune challenge. T-cell ligands, including CD40, mediate this effect (40). These

findings provide a nice conceptual advancement as to how the innate immune response can be resolved, at least in part, by action of the adaptive immune response.

PRY-SPRY Domain

Another TRIM-family protein, TRIM30α, has been shown to regulate IL-1β production at both the transcriptional level and inflammasome-mediated processing level. Knockdown of TRIM30α in the immortalized mouse macrophage cell line J774 led to increased IL-1β production in response to multiple NLRP3-inflammasome elicitors (51). Overexpression of TRIM30α in transgenic mice cause reduced IL-1β production *in vivo* following treatment with MSU (51). Another SPRY-containing protein, estrogen-responsive B-box protein, was also shown to regulate inflammasome activity, albeit in a positive manner (52).

In summary, regulation of inflammasome-dependent IL-1β production is a complex process, mediated by NF-κB activation and regulatory feedback loops that converge upon Caspase-1 activity. Further research efforts are necessary to discern how all of these regulatory mechanisms, now including Pyrin, synergize to provide appropriate regulation of IL-1β-dependent immune responses.

Regulation of Pyrin's function

Splice variants

One interesting area for future research involves the study of *Mefv* splice variants. Several investigators have observed that *Mefv* has one primary transcript but several minor transcripts (53). To date, studies have characterized splice variants in PBMCs, granulocytes, and sonovial fibroblasts (54). So far, it appears that some splice variants may be unique to one or more cell types. However, no study has used the same methodology to detect splice variants of different

cell types in parallel. Thus, the apparent differences in the presence of splice variants between cell types could reflect the relative detection capabilities of the experimental systems used. Future studies should address whether or not splice variants vary in a qualitative or quantitative way between different cell types. A related question is whether or not splicing changes within a cell type as the cell matures or becomes activated. Indeed the abundance of minor splice variants increases in sonovial fibroblasts following activation with LPS (54). Is splicing also regulated by external signals from neighboring cells through soluble mediators or cell-cell interaction? Another important question is: What are the functional consequences of differential splicing? Do splice variants have increased, decreased, or novel function in caparison to the primary transcript? By potentially changing the overall structure of Pyrin, these transcripts could favor different protein-protein, protein-cytoskeletal, or intramolecular interactions. Or perhaps they change the protein stability of Pyrin. A third avenue for future research regarding *Mefv* splicing is addressing the hypothesis that mutations in *Mefv* affect splicing. So far, there is no evidence to directly support this hypothesis, and there are no mutations at splice donor or acceptor sites. However, since the abundance of minor transcripts increases concurrently with immune activation, and FMF disease is caused by aberrant immune activation, it seems possible that mutations could increase the abundance of splice variants and cause immune activation.

To address the first question, cells could be sorted based upon type, maturity, and activation by flow cytometry and assessed for the relative abundance of splice variants by quantitative real-time PCR. This experiment should be done using both human and mice tissues, since there is substantial evidence for splice variants of the human *Mefv* gene, but a broader range of tissues is available from the mouse. To assess the function of splice variants, two complementary experimental systems could be used. First, *Mefv* genes capable of expressing only one splice variant could be overexpressed in a human cell line that lacks

endogenous expression of Pyrin. This system allows for independent evaluation of the function of each splice variant. IL-1β production, NF-κB activity, ASC-speck formation, Caspase-1 activity, and cellular localization should be assessed. Second, antibodies specific to a splice variant of Pyrin could be used to determine protein binding capabilities and cell localization of endogenous Pyrin in primary human cells. This method is likely to produce results with better correlation to what occurs *in vivo*, but technical limitations could prevent a clear interpretation of results. Lastly, to assess the hypothesis that mutations affect splicing, *Mefv* genes with FMF-associated mutations could be overexpressed in a human cell line followed by quantification of the abundance of each splice variant by real-time PCR.

Cleavage of Pyrin

The human Pyrin protein was shown to be cleaved by Caspase-1. By, overexpressing human Pyrin and Caspase-1 proteins in murine PT67 cells, the Pyrin protein was observed at multiple molecular weights. The production of the lower molecular weight form of Pyrin was blocked by the Caspase-1 inhibitor z-VAD-fmk (49). It is important to note that *Pycard* is not expressed in PT67 cells. The expression of *Pycard* (ASC), as is typical in an endogenous system, could alter the interaction of Pyrin and Caspase-1, since Pyrin binds to both of these proteins. ASC may sequester Pyrin or Caspase-1 from interacting with the other or promote the formation of a trimeric protein complex. It is also interesting to note that these cells were unstimulated. In another study using THP-1 monocytes, two different weights of endogenous human Pyrin were detected by western blotting with an antibody that recognizes the N-terminal portion of Pyrin, similar to the aforementioned study. The estimated size of the smaller protein correlates nicely. Stimulation of cells with cold temperature (30°C) stimulated NLRP3 inflammasome-mediated Caspase-1 activity, and decreased the abundance of full-length Pyrin. However, the Caspase-1 inhibitor did not prevent the disappearance of the predominant 85 kDa Pyrin band. Thus, this study suggests that Caspase-1 does not mediate cleavage of Pyrin. The

authors instead suggest that Pyrin is degraded when the NLRP3 inflammasome is activated by cold temperature (5).

In FMF patient samples, there is a higher percentage of a low molecular weight form of Pyrin, compared to healthy controls (49). It is tempting to conclude that Caspase-1-mediated cleavage of Pyrin is increased as a result of FMF-associated mutations within the Pyrin protein and that the cleavage of Pyrin alters its function to produce the FMF disease phenotype. However, interpretation of data from human patient samples is complicated by a couple experimental limitations. First, the presence of a lower molecular weight band is not necessarily indicative of cleavage of Pyrin. Instead, it could be the natural protein product of a shorter transcript of the *MEFV* gene. Second, it is unclear if the samples from FMF patients and healthy controls have similar cell populations. Differential splicing of *MEFV* in neutrophils and macrophages (or perhaps other cell types) could explain the difference in the relative abundance of isoforms of the Pyrin protein.

Caspase-1 is expressed in most cells which express Pyrin: human macrophages (55), neutrophils (56, 57), dendritic cells (58), B and T lymphocyte cell lines (59, 60), and primary fibroblasts (61), and it can be detected by RT-PCR in synovial tissue of some, but not all patients with arthritis or healthy controls (62). Caspase-1 activity is increased in the psoriatic lesions of the skin (63), and in B-cells, Caspase-1 can mediate apoptosis (59, 60), thus Caspase-1 is functional in non-myeloid cells. The overlapping expression pattern of Caspase-1 with Pyrin is consistent with the idea that Caspase-1-mediated cleavage of Pyrin mediates the function of Pyrin.

However, the site of Caspase-1 cleavage of Pyrin is not conserved among all species. The cleavage site was mapped to amino acids 330 – 31 of the human protein, between aspartate and serine amino acids (49). The amino acid sequence is conserved in the chimpanzee,

monkey, and dog Pyrin proteins. In the mouse and rat protein, however, the corresponding amino acids are two serines. Thus, it is unlikely that the mouse and rat Pyrin protein can be cleaved by Caspase-1, although cleavage may be conserved in the chimpanzee, monkey, and dog.

The genetics of FMF

Inferences about the function of Pyrin have been made based upon dominant versus recessive inheritance: dominance suggests that mutants have a gain-of-function; recessivity suggests mutants have a loss of WT Pyrin function. Our data indicate that a loss of Pyrin function causes aberrant IL-1β production. We did not identify a phenotype in intrinsic neutrophil function, but the loss of Pyrin function may affect neutrophils in a manner that was beyond our detection by the assays we chose for testing. Together, our data support a model for FMF-associated mutations causing a loss-of-function, which in turn supports a recessive inheritance model. We cannot, however, rule out the possibility that some point mutants create a gain-of-function phenotype.

It is surprising that there is a large population of FMF patients with only one or no identified mutation in *MEFV*, although analysis is typically confined to examination of mutational hotspots. Two phenomena could explain this: 1) the recently established Tel Hashomer criteria and molecular genetic testing for mutations in *MEFV* facilitate diagnosis of FMF in milder cases, including asymptomatic individuals, and milder cases may be associated with unidentified mutations in *MEFV*, and 2) in response to changing selection pressures, new allelic variants are emerging that are beyond detection of current testing panels. Thus, caution should be used when concluding dominant or recessive inheritance patterns based upon an absence of molecularly-defined mutations.

Intramolecular interactions within Pyrin were reported by multiple investigators (5, 7), and could be an indication that either Pyrin interacts with itself on a monomeric level, or that Pyrin forms multimers with itself. Indeed, there is evidence to support a model in which Pyrin forms homotrimers (7). It is interesting to assimilate this idea with our knowledge of FMF inheritance. Our research indicates that the loss of function of one allele is not compensated through increased expression of the other allele of *Mefv*. Thus, if we assume that both wt and mutant alleles are expressed equally in heterozygous individuals, then it is reasonable to assume that each trimer of Pyrin could have 0, 33, 67 or 100% of the function of a trimer from homozygous WT individuals. These percentages represent the extreme where one allele has full function and the other allele has a complete loss-of-function. In actuality, since mutations are point mutations, and primarily missense mutations, they may cause only a partial loss-of-function. Additionally, most individuals with two mutant alleles are compound heterozygotes. Therefore, the range of functionality of Pyrin trimers varies greatly. On a phenotypic level, this broad functionality could explain the broad range in severity or apparent penetrance of FMF disorder. This does not rule out the possibility that there are genetic modifiers of the FMF phenotype.

The presence of the same *MEFV* genotype in patients with different FMF severity, even asymptomatic in some cases, indicates that there is really interesting biology that we don't yet understand. Unidentified modifiers of FMF pathology probably exist. Modifiers of disease penetrance and/or severity of symptoms can be either genetic or environmental. For example, in a large cohort of 2,067 patients, there was a female:male ratio of 1.38 (64). This bias may be due to the contribution of sex-specific genetic modifiers, but this conclusion warrants further investigation. An environmental influence of FMF pathology is clear. The country of residence affects the prevalence of *MEFV* mutations, the emergence of new mutations, and disease severity, specifically the risk of developing amyloidosis (65, 66). Populations within the countries of France, Turkey, Armenia, Spain, Lebanon, and Italy carry the largest number of

sequence variants (66-69), suggesting that environmental stressors within the Mediterranean region are selecting for *Mefv* allelic variants.

Mefv may protect against a pathogen that is most prevalent in the Mediterranean region. Functional protein analysis supports this hypothesis. The PRY-SPRY region within the B30.2 domain of Pyrin forms a 3D-structure with hypervariable loops, similar to the variable regions within immunoglobulin proteins. The PRY-SPRY domain is common to Tripartite motif (TRIM) family proteins. Among these are TRIM5α and TRIM21 (Ro52). A single amino acid substitution within TRIM5α has led to susceptibility for HIV infection in humans while chimpanzees that retain the evolutionary wild-type sequence remain resistant to HIV. Mutations within TRIM21 lead to systemic lupus erethymatosus susceptibility. Mutations in TRIM5α and TRIM21, along with the most severe disease-causing mutation (M694V) within Pyrin/TRIM20, map to a hyper-variable loop region of the PRY-SPRY domain. Thus, both the structural similarity between Pyrin and other TRIM-family proteins, and the association of PRY-SPRY mutations with disease, support a model in which the amino acids within the variable loop regions undergo frequent substitutions throughout evolution. These allelic variants may be maintained as a way of adapting new or better immune defenses to resist a pathogen(s). Consistent with this hypothesis, Pyrin expression is increased in response to treatment with LPS, retroviral infection, and treatment with some pro-inflammatory cytokines (7, 70). To provide a conceptual framework for this hypothesis, the phenotypes caused by mutations in *Mefv* may follow a model for mutations in the beta globin chain of hemoglobin, wherein heterozygosity provides protection against malaria, but homozygosity causes the sickle-cell trait.

References

1. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* 11:591-604.

2. Chae, J. J., G. Wood, S. L. Masters, K. Richard, G. Park, B. J. Smith, and D. L. Kastner. 2006. The B30.2 domain of pyrin, the familial Mediterranean fever protein, interacts directly with Caspase-1 to modulate IL-1beta production. *Proc Natl Acad Sci U S A* 103:9982-9987.

3. Dowds, T. A., J. Masumoto, F. F. Chen, Y. Ogura, N. Inohara, and G. Nunez. 2003. Regulation of cryopyrin/Pypaf1 signaling by pyrin, the familial Mediterranean fever gene product. *Biochem Biophys Res Commun* 302:575-580.

4. Gavrilin, M. A., S. Mitra, S. Seshadri, J. Nateri, F. Berhe, M. W. Hall, and M. D. Wewers. 2009. Pyrin critical to macrophage IL-1beta response to Francisella challenge. *J Immunol* 182:7982-7989.

5. Papin, S., S. Cuenin, L. Agostini, F. Martinon, S. Werner, H. D. Beer, C. Grutter, M. Grutter, and J. Tschopp. 2007. The SPRY domain of Pyrin, mutated in familial Mediterranean fever patients, interacts with inflammasome components and inhibits proIL-1beta processing. *Cell Death Differ* 14:1457-1466.

6. Seshadri, S., M. D. Duncan, J. M. Hart, M. A. Gavrilin, and M. D. Wewers. 2007. Pyrin levels in human monocytes and monocyte-derived macrophages regulate IL-1beta processing and release. *J Immunol* 179:1274-1281.

7. Yu, J. W., T. Fernandes-Alnemri, P. Datta, J. Wu, C. Juliana, L. Solorzano, M. McCormick, Z. Zhang, and E. S. Alnemri. 2007. Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants. *Mol Cell* 28:214-227.

8. Yu, J. W., J. Wu, Z. Zhang, P. Datta, I. Ibrahimi, S. Taniguchi, J. Sagara, T. Fernandes-Alnemri, and E. S. Alnemri. 2006. Cryopyrin and pyrin activate Caspase-1, but not NF-kappaB, via ASC oligomerization. *Cell Death Differ* 13:236-249.

9. Gogus, F., I. Fresko, Y. Elbir, E. Eksioglu-Demiralp, and H. Direskeneli. 2005. Oxidative burst response to monosodium urate crystals in patients with Behcet's syndrome. *Clin Exp Rheumatol* 23:S81-85.

10. Avetisian, S. A., T. K. Davtian, G. S. Akopian, A. M. Manukian, and D. A. Pogosian. 2006. [Spontaneous and inducible respiratory burst of monocytes and neutrophils in periodic autoinflammatory fever]. *Patol Fiziol Eksp Ter*:11-12.

11. Akopian, V. P., S. A. Avetisian, and T. K. Davtian. 2005. [The effect of colcichine and iodine-lithium-alpha-dextrin on the phagocytosis of granulocytes and monocytes in patients with familial Mediterranean fever]. *Eksp Klin Farmakol* 68:36-39.

12. Davtian, T. K., S. A. Avetisian, G. S. Akopian, and V. M. Arutiunian. 2005. [A respiratory splash of monocytes and neutrophils in familial Mediterranean fever]. *Ter Arkh* 77:39-43.

13. Davtyan, T. K., G. S. Hakopyan, S. A. Avetisyan, and N. R. Mkrtchyan. 2006. Impaired endotoxin tolerance induction in patients with familial Mediterranean fever. *Pathobiology* 73:26-39.

14. Davtyan, T. K., V. A. Harutyunyan, G. S. Hakobyan, and S. A. Avetisyan. 2008. Heightened endotoxin susceptibility of monocytes and neutrophils during familial Mediterranean fever. *FEMS Immunol Med Microbiol* 52:370-378.

15. Ledford, J. G., M. Kovarova, and B. H. Koller. 2007. Impaired host defense in mice lacking ONZIN. *J Immunol* 178:5132-5143.

16. Chae, J. J., Y. H. Cho, G. S. Lee, J. Cheng, P. P. Liu, L. Feigenbaum, S. I. Katz, and D. L. Kastner. 2011. Gain-of-Function Pyrin Mutations Induce NLRP3 Protein-Independent Interleukin-1beta Activation and Severe Autoinflammation in Mice. *Immunity*.

17. Richards, N., P. Schaner, A. Diaz, J. Stuckey, E. Shelden, A. Wadhwa, and D. L. Gumucio. 2001. Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem* 276:39320-39329.

18. Sutterwala, F. S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G. S. Lichtenberger, E. P. Grant, J. Bertin, A. J. Coyle, J. E. Galan, P. W. Askenase, and R. A. Flavell. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of Caspase-1. *Immunity* 24:317-327.

19. Joosten, L. A., F. L. Van De Veerdonk, A. G. Vonk, O. C. Boerman, M. Keuter, G. Fantuzzi, I. Verschueren, T. Van Der Poll, C. A. Dinarello, B. J. Kullberg, J. W. Van Der Meer, and M. G. Netea. 2010. Differential susceptibility to lethal endotoxaemia in mice deficient in IL-1alpha, IL-1beta or IL-1 receptor type I. *APMIS* 118:1000-1007.

20. Willingham, S. B., D. T. Bergstralh, W. O'Connor, A. C. Morrison, D. J. Taxman, J. A. Duncan, S. Barnoy, M. M. Venkatesan, R. A. Flavell, M. Deshmukh, H. M. Hoffman, and J. P. Ting. 2007. Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe* 2:147-159.

21. Case, C. L., S. Shin, and C. R. Roy. 2009. Asc and Ipaf Inflammasomes direct distinct pathways for Caspase-1 activation in response to Legionella pneumophila. *Infect Immun* 77:1981-1991.

22. Broz, P., J. von Moltke, J. W. Jones, R. E. Vance, and D. M. Monack. 2010. Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe* 8:471-483.

23. Stanley, A. C., and P. Lacy. 2010. Pathways for cytokine secretion. *Physiology (Bethesda)* 25:218-229.

24. Wewers, M. D. 2004. IL-1beta: an endosomal exit. *Proc Natl Acad Sci U S A* 101:10241-10242.

25. Miao, E. A., J. V. Rajan, and A. Aderem. 2011. Caspase-1-induced pyroptotic cell death. *Immunol Rev* 243:206-214.

26. Broz, P., K. Newton, M. Lamkanfi, S. Mariathasan, V. M. Dixit, and D. M. Monack. 2010. Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J Exp Med* 207:1745-1755.

27. Pham, C. T. 2006. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 6:541-550.

28. Herzyk, D. J., J. N. Allen, C. B. Marsh, and M. D. Wewers. 1992. Macrophage and monocyte IL-1 beta regulation differs at multiple sites. Messenger RNA expression, translation, and post-translational processing. *J Immunol* 149:3052-3058.

29. Harris, J., M. Hartman, C. Roche, S. G. Zeng, A. O'Shea, F. A. Sharp, E. M. Lambe, E. M. Creagh, D. T. Golenbock, J. Tschopp, H. Kornfeld, K. A. Fitzgerald, and E. C. Lavelle. 2011. Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. *J Biol Chem* 286:9587-9597.

30. Mitroulis, I., I. Kourtzelis, K. Kambas, A. Chrysanthopoulou, and K. Ritis. 2011. Evidence for the involvement of mTOR inhibition and basal autophagy in familial Mediterranean fever phenotype. *Hum Immunol* 72:135-138.

31. Druilhe, A., S. M. Srinivasula, M. Razmara, M. Ahmad, and E. S. Alnemri. 2001. Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. *Cell Death Differ* 8:649-657.

32. Lamkanfi, M., G. Denecker, M. Kalai, K. D'Hondt, A. Meeus, W. Declercq, X. Saelens, and P. Vandenabeele. 2004. INCA, a novel human caspase recruitment domain protein that inhibits interleukin-1beta generation. *J Biol Chem* 279:51729-51738.

33. Lee, S. H., C. Stehlik, and J. C. Reed. 2001. Cop, a caspase recruitment domain-containing protein and inhibitor of Caspase-1 activation processing. *J Biol Chem* 276:34495-34500.

34. Bedoya, F., L. L. Sandler, and J. A. Harton. 2007. Pyrin-only protein 2 modulates NF-kappaB and disrupts ASC:CLR interactions. *J Immunol* 178:3837-3845.

35. Dorfleutner, A., N. B. Bryan, S. J. Talbott, K. N. Funya, S. L. Rellick, J. C. Reed, X. Shi, Y. Rojanasakul, D. C. Flynn, and C. Stehlik. 2007. Cellular pyrin domain-only protein 2 is a candidate regulator of inflammasome activation. *Infect Immun* 75:1484-1492.

36. Srimathi, T., S. L. Robbins, R. L. Dubas, H. Chang, H. Cheng, H. Roder, and Y. C. Park. 2008. Mapping of POP1-binding site on pyrin domain of ASC. *J Biol Chem* 283:15390-15398.

37. Stehlik, C., M. Krajewska, K. Welsh, S. Krajewski, A. Godzik, and J. C. Reed. 2003. The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and pro-Caspase-1 regulation. *Biochem J* 373:101-113.

38. Saleh, M., J. C. Mathison, M. K. Wolinski, S. J. Bensinger, P. Fitzgerald, N. Droin, R. J. Ulevitch, D. R. Green, and D. W. Nicholson. 2006. Enhanced bacterial clearance and sepsis resistance in Caspase-12-deficient mice. *Nature* 440:1064-1068.

39. Bruey, J. M., N. Bruey-Sedano, F. Luciano, D. Zhai, R. Balpai, C. Xu, C. L. Kress, B. Bailly-Maitre, X. Li, A. Osterman, S. Matsuzawa, A. V. Terskikh, B. Faustin, and J. C. Reed.

2007. Bcl-2 and Bcl-XL regulate proinflammatory Caspase-1 activation by interaction with NALP1. *Cell* 129:45-56.

40. Guarda, G., and A. So. 2010. Regulation of inflammasome activity. *Immunology* 130:329-336.

41. Young, J. L., G. K. Sukhova, D. Foster, W. Kisiel, P. Libby, and U. Schonbeck. 2000. The serpin proteinase inhibitor 9 is an endogenous inhibitor of interleukin 1beta-converting enzyme (Caspase-1) activity in human vascular smooth muscle cells. *J Exp Med* 191:1535-1544.

42. Stehlik, C., L. Fiorentino, A. Dorfleutner, J. M. Bruey, E. M. Ariza, J. Sagara, and J. C. Reed. 2002. The PAAD/PYRIN-family protein ASC is a dual regulator of a conserved step in nuclear factor kappaB activation pathways. *J Exp Med* 196:1605-1615.

43. Sarkar, A., M. Duncan, J. Hart, E. Hertlein, D. C. Guttridge, and M. D. Wewers. 2006. ASC directs NF-kappaB activation by regulating receptor interacting protein-2 (RIP2) Caspase-1 interactions. *J Immunol* 176:4979-4986.

44. Dong, J., E. Jimi, C. Zeiss, M. S. Hayden, and S. Ghosh. 2010. Constitutively active NF-kappaB triggers systemic TNFalpha-dependent inflammation and localized TNFalpha-independent inflammatory disease. *Genes Dev* 24:1709-1717.

45. Kinoshita, T., Y. Wang, M. Hasegawa, R. Imamura, and T. Suda. 2005. PYPAF3, a PYRIN-containing APAF-1-like protein, is a feedback regulator of Caspase-1-dependent interleukin-1beta secretion. *J Biol Chem* 280:21720-21725.

46. Wang, Y., M. Hasegawa, R. Imamura, T. Kinoshita, C. Kondo, K. Konaka, and T. Suda. 2004. PYNOD, a novel Apaf-1/CED4-like protein is an inhibitor of ASC and Caspase-1. *Int Immunol* 16:777-786.

47. Fontalba, A., O. Gutierrez, and J. L. Fernandez-Luna. 2007. NLRP2, an inhibitor of the NF-kappaB pathway, is transcriptionally activated by NF-kappaB and exhibits a nonfunctional allelic variant. *J Immunol* 179:8519-8524.

48. Kinoshita, T., C. Kondoh, M. Hasegawa, R. Imamura, and T. Suda. 2006. Fas-associated factor 1 is a negative regulator of PYRIN-containing Apaf-1-like protein 1. *Int Immunol* 18:1701-1706.

49. Chae, J. J., G. Wood, K. Richard, H. Jaffe, N. T. Colburn, S. L. Masters, D. L. Gumucio, N. G. Shoham, and D. L. Kastner. 2008. The familial Mediterranean fever protein, pyrin, is cleaved by Caspase-1 and activates NF-kappaB through its N-terminal fragment. *Blood* 112:1794-1803.

50. Greten, F. R., M. C. Arkan, J. Bollrath, L. C. Hsu, J. Goode, C. Miething, S. I. Goktuna, M. Neuenhahn, J. Fierer, S. Paxian, N. Van Rooijen, Y. Xu, T. O'Cain, B. B. Jaffee, D. H. Busch, J. Duyster, R. M. Schmid, L. Eckmann, and M. Karin. 2007. NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell* 130:918-931.

51. Hu, Y., K. Mao, Y. Zeng, S. Chen, Z. Tao, C. Yang, S. Sun, X. Wu, G. Meng, and B. Sun. 2010. Tripartite-motif protein 30 negatively regulates NLRP3 inflammasome activation by modulating reactive oxygen species production. *J Immunol* 185:7699-7705.

52. Munding, C., M. Keller, G. Niklaus, S. Papin, J. Tschopp, S. Werner, and H. D. Beer. 2006. The estrogen-responsive B box protein: a novel enhancer of interleukin-1beta secretion. *Cell Death Differ* 13:1938-1949.

53. Medlej-Hashim, M., N. Nehme, E. Chouery, N. Jalkh, and A. Megarbane. 2010. Novel MEFV transcripts in Familial Mediterranean fever patients and controls. *BMC Med Genet* 11:87.

54. Diaz, A., C. Hu, D. L. Kastner, P. Schaner, A. M. Reginato, N. Richards, and D. L. Gumucio. 2004. Lipopolysaccharide-induced expression of multiple alternatively spliced MEFV transcripts in human synovial fibroblasts: a prominent splice isoform lacks the C-terminal domain that is highly mutated in familial Mediterranean fever. *Arthritis Rheum* 50:3679-3689.

55. Yazdi, A. S., S. K. Drexler, and J. Tschopp. 2010. The role of the inflammasome in nonmyeloid cells. *J Clin Immunol* 30:623-627.

56. Rowe, S. J., L. Allen, V. C. Ridger, P. G. Hellewell, and M. K. Whyte. 2002. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. *J Immunol* 169:6401-6407.

57. Watson, R. W., O. D. Rotstein, J. Parodo, R. Bitar, and J. C. Marshall. 1998. The IL-1 beta-converting enzyme (Caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1 beta. *J Immunol* 161:957-962.

58. Reich, K., A. Heine, S. Hugo, V. Blaschke, P. Middel, A. Kaser, H. Tilg, S. Blaschke, C. Gutgesell, and C. Neumann. 2001. Engagement of the Fc epsilon RI stimulates the production of IL-16 in Langerhans cell-like dendritic cells. *J Immunol* 167:6321-6329.

59. Schrantz, N., D. A. Blanchard, F. Mitenne, M. T. Auffredou, A. Vazquez, and G. Leca. 1999. Manganese induces apoptosis of human B cells: caspase-dependent cell death blocked by bcl-2. *Cell Death Differ* 6:445-453.

60. Hallan, E., H. K. Blomhoff, E. B. Smeland, and J. Lomo. 1997. Involvement of ICE (Caspase) family in gamma-radiation-induced apoptosis of normal B lymphocytes. *Scand J Immunol* 46:601-608.

61. Feldmeyer, L., M. Keller, G. Niklaus, D. Hohl, S. Werner, and H. D. Beer. 2007. The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. *Curr Biol* 17:1140-1145.

62. Kolly, L., N. Busso, G. Palmer, D. Talabot-Ayer, V. Chobaz, and A. So. 2010. Expression and function of the NALP3 inflammasome in rheumatoid synovium. *Immunology* 129:178-185.

63. Johansen, C., K. Moeller, K. Kragballe, and L. Iversen. 2007. The activity of Caspase-1 is increased in lesional psoriatic epidermis. *J Invest Dermatol* 127:2857-2864.

64. Dundar, M., E. F. Emirogullari, A. Kiraz, S. Taheri, and M. Baskol. 2010. Common Familial Mediterranean Fever gene mutations in a Turkish cohort. *Mol Biol Rep*.

65. Shohat, M., and G. J. Halpern. 2011. Familial Mediterranean fever-A review. *Genet Med* 13:487-498.

66. Milhavet, F., L. Cuisset, H. M. Hoffman, R. Slim, H. El-Shanti, I. Aksentijevich, S. Lesage, H. Waterham, C. Wise, C. Sarrauste de Menthiere, and I. Touitou. 2008. The infevers autoinflammatory mutation online registry: update with new genes and functions. *Hum Mutat* 29:803-808.

67. Touitou, I., S. Lesage, M. McDermott, L. Cuisset, H. Hoffman, C. Dode, N. Shoham, E. Aganna, J. P. Hugot, C. Wise, H. Waterham, D. Pugnere, J. Demaille, and C. Sarrauste de Menthiere. 2004. Infevers: an evolving mutation database for auto-inflammatory syndromes. *Hum Mutat* 24:194-198.

68. Sarrauste de Menthiere, C., S. Terriere, D. Pugnere, M. Ruiz, J. Demaille, and I. Touitou. 2003. INFEVERS: the Registry for FMF and hereditary inflammatory disorders mutations. *Nucleic Acids Res* 31:282-285.

69. 2011. Infevers: an online database for autoinflammatory mutations. In *Infevers.com*, 2008 ed.

70. Centola, M., G. Wood, D. M. Frucht, J. Galon, M. Aringer, C. Farrell, D. W. Kingma, M. E. Horwitz, E. Mansfield, S. M. Holland, J. J. O'Shea, H. F. Rosenberg, H. L. Malech, and D. L. Kastner. 2000. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood* 95:3223-3231.