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α-enolase: A new player in NLRP3 inflammasome activation

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1. Abbreviations

1,3-BPG	1,3-biphosphoglycerate
2-PG	2-phosphoglycerate
AD	Acidic transactivation domain
AIM2	Absent in melanoma 2
ALR	AIM2-like receptor
AP-1	Activator protein 1
APAF1	Apoptosis protease-activating factor 1
ASC	Apoptosis-associated speck-like protein containing CARD
BHB	β-hydroxybutyrate
BIR	Baculoviral inhibitory repeat
BMDM	Bone marrow-derived macrophage
BRCC3	BRCA1/BRAC2-containing complex subunit 3
CAPS	Cryopyrin-associated periodic fever syndromes (CAPS)
CARD	Caspase-recruitment domain
CD14	Cluster of differentiation 14
CE	Cholesterol esterase
CINCA	Chronic infantile neurological cutaneous and articular syndrome
CLIC	Chloride intracellular channel
CLR	C-type lectin receptor
COP	CARD-only protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DAG	Diacylglycerol
DAMP	Danger-associated molecular pattern
DDX3X	DEAD-box helicase 3 X-linked
DED	Death effector domain
DHAP	Dihydroxyacetone phosphate
dsDNA	Double stranded DNA
dTGN	Dispersed trans-Golgi network
ETC	Electron transport chain
EtOH	Ethanol
EV	Empty vector
FACS	Fluorescence-activated cell sorting
FCAS	Familial cold autoinflammatory syndrome
FIIND	Function-to-find domain
FMF	Familial mediterranean fever
G3P	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI3P	Glycerol-3-phosphate
GlcNAc	N-acetylglucosamine
GOI	Gene of interest
GPD2	Glycerol-3-phosphate dehydrogenase 2
GPS	Glycerol-3-phosphate shuttle
GSDMD	Gasdermin D
GSDME	Gasdermin E
hIPSC	Human induced pluripotent stem cell
HK1	Hexokinase 1
HMGB1	High mobility group box 1
HOIL-1L	IRP2 ubiquitin ligase 1L
HR	Homologous recombination
IAA	Iodoacetate
iBMDM	Immortalized BMDM

IFI16	Interferon-inducible 16
IKK	IκB kinase
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-18	Interleukin-18
IL1R1AcP	IL-1R accessory protein
iNOS	Inducible nitric oxide synthase
IP3R	IP3 receptor
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
KHSV	Kaposi's sarcoma-associated virus
KO	Knocked-out
LFC	Logarithmic fold change
LLOMe	Leu-Leu-O-methyl-esther
LMP	Lysosomal membrane permeabilization
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LUBAC	Linear ubiquitin assembly complex
MAM	ER mitochondria-associated membranes
MAPK	Mitogen-associated protein kinase
MARK4	Microtubule-affinity regulating kinase 4
MAVS	Mitochondrial antiviral signalling protein
MBP1	Myc-binding protein 1
MDP	Muramyl dipeptide
MD2	Myeloid differentiation 2
MKS	Muckle-Wells syndrome
MLKL	Mixed-lineage kinase domain-like pseudokinase
MOI	Multiplicity of infection
mtDNA	Mitochondrial DNA
mTORC1	Mammalian target of rapamycin complex 1
mtROS	Mitochondrial ROS
MyD88	Myeloid differentiation 88
NACHT	NAIP, CIITA, HET-E, and TEP-1 domain
NAD	Nicotinamide adenine dinucleotide
NAIPs	NLR family of apoptosis inhibitory proteins
NBD	Nuclear-binding domain
nCEH	Neutral cholesteryl ester hydrolase
NEK7	NIMA related kinase 7
NF-κB	Nuclear factor-κB
NHEJ	Non-homologous end joining
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOMID	Neonatal onset multisystem inflammatory disease
NOX4	NADPH oxidase 4
NPC1	Niemann-Pick C1
NQO2	NRH-quinone oxidoreductase 2
OAA	Oxalacetate
OXA	Oxalate
oxPAPC	Oxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcoline
P_2XR_7	P_2X purinoreceptor 7
PAMP	Pathogen-associated molecular pattern
PEP	Phosphoenolpyruvate
PKA	Protein kinase A
PKCδ	Protein kinase C-delta
PKD	Protein kinase D

PKK	PKC-associated kinase
PKM2	Pyruvate kinase isoform M2
PKN1/2	Protein kinases 1 and 2
PKR	RNA-dependent protein kinase
PLA	Plasmin
PLC	Phospholipase C
PLG	Plasminogen
PMA	Phorbol-12-myristol-13-acetate
POP	PYD-only protein
PRR	Pattern recognition receptor
PTPN22	Protein tyrosine phosphatase non-receptor type 22
PYD	Pyrin domain
RAGE	Receptor for advanced glycation end-products
RET	Reverse electron transport
RhoA	Ras homolog family member A
RIPK2	Receptor interacting serine/threonine kinase 2
RLR	RIG-1-like receptor
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SCAP	SREBP cleavage-activating protein
SCF^{FBXL2}	Skp-cullin-F box L2
SCFA	Short chain fatty acids
SERCA	Sarco/ER Ca^{2+}-ATPase
sgRNA	Small guide RNA
SLC4A1	Solute carrier family member 4 a 1
SRBEP2	Sterol regulatory element-binding protein 2
STARD3	StAR-related lipid transfer protein 3
SYK	Spleen tyrosine kinase
TAK1	Transforming growth factor β-activated kinase 1
TBK1	TANK-binding kinase 1
TCA	Tricarboxylic cycle
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
tPA	Tissue-type PLG activator
TRAF3	Tumour necrosis factor receptor-associated factor 3
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon β
TRIM20	Tripartite motif-containing protein 20
TRP	Transient receptor potential
TWIK2	Two-pore domain K^+ channel (K_{2P})
TX-100	Triton X-100
ULK1	Unc-51 like autophagy activating kinase 1
uPA	Urokinase-type PLG activator
USP50	Ubiquitin specific peptidase 50
VRAC	Volume regulated anion channel
WT	Wild type

2. Introduction

2.1 – Innate immunity

All throughout evolution, living organisms have been pushed to continuously adapt to their environment and to develop strategies to survive and pass on their genetic signatures to their offspring. Over time, this evolutionary pressure has led to the appearance of a plethora of most elegant and intricate mechanisms of self-preservation. Although complex functions are usually associated to multicellular organisms, prokaryotes have already been shown to display some specialized defence mechanisms to protect themselves against dangerous entities. One example of this is the widely studied immune response of bacteria to bacteriophages, which relies on the expression of a set of proteins that can target viral DNA to halt virus replication.

While bacterial defence to infection depends on single-cell driven action, most of the immune responses in multicellular organisms are based on the finely orchestrated collaboration between highly specialized cells with specific functions. Therefore, immunity in higher eukaryotes is understood in a scale comprising the cooperation of several parts of the organism. Accordingly, immunity has been divided in two types depending on the specificity of the response to the danger signal and the subsequent mobilization of immune cells: innate immunity is triggered first and constitutes the early host barrier of defence. Its role is to rapidly clear out the foreign particles and limit their spread. This is followed by the onset of adaptive immunity, which is based on the generation of memory against the specific pathogen that can be used to fight it more effectively if encountered in the future.

Unsurprisingly, there is no single molecule that allows immune cells to systematically distinguish between foreign hindrances and the material that belongs to the host. The fact that innate immune cells can distinguish between pathogenic bacteria and the host's own microbiome in the gut is a great example of how finely tuned responsiveness to foreign microorganisms is (Magalhaes et al., 2007). However, there are certain molecules or "patterns" which can be found repeatedly in several pathogenic bacteria and viruses, which the immune system has been trained to detect as noxious. These patterns are collectively known as pathogen-associated molecular patterns (PAMPs), and can be found conserved across bacteria, fungi and viruses. Immune cells are also capable of detecting patterns originated in the host, which must be cleared out to keep systemic homeostasis. These are known as danger-associated molecular patterns (DAMPs). Interestingly, albeit DAMP recognition is necessary for processes of such importance as the clearance of damaged cells, it is also responsible for sterile inflammation related to many diseases, and can aggravate their severity. I will explore this in more detail further below in this introduction.

Immune cells have in the course of evolution acquired a repertoire of germline-encoded sensors that are highly specialized to detect individual PAMPs and DAMPs. Upon recognition, they can trigger the onset of a signalling cascade culminating in pro-inflammatory events directed to clear out the infection. These sensors are known as pattern recognition receptors (PRRs), and constitute a key link in host defence among mammals.

2.2 – Pattern recognition receptors (PRRs)

PRRs are diverse in nature and structure, in accordance to the molecular pattern they interact with (Takeuchi and Akira 2010). However, they do not always bind directly to the PAMP or the DAMP itself, but can in some cases sense cellular perturbances promoted by the original insult. Collectively, PRRs are divided according to their localization in the cell and the domains contained in their sequence. PRR families localized in the plasma membrane and the endosomal membrane include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which serve to survey the entrance of pathogens from the extracellular space. On the other hand, the RIG-1-like receptors (RLRs), the AIM2-like receptors (ALRs) and the NOD-like receptors (NLRs) PRRs are located in the cytoplasm, and therefore respond to internal signals.

2.2.1 – Transmembrane PRRs: TLRs and CLRs

Toll like receptors were described as key components of the immune system for the recognition of pathogens by Bruce. A. Beutler and Jules. A. Hoffman. Their work in the study of these receptors lead to their distinction of receiving the prestigious Novel prize in Physiology in 2011. The Toll-like receptors (TLRs) are characterized by N-terminal Leucine rich repeats (LRRs), which are in charge of binding directly to their ligand, a central transmembrane region, and a cytoplasmic C-terminal Toll/IL-1R homology (TIR) domain. They are usually expressed in cells of the myeloid lineage, such as macrophages and dendritic cells. Ten TLRs have been identified in humans and twelve in mice (Osamu Takeuchi & Akira, 2010). TLRs show different specificity for different ligands and can form dimers. For example, TLR2 can form heterodimers with TLR1 or TLR6, which recognize triacyl and diacyl lipoproteins respectively in macrophages and dendritic cells (O. Takeuchi et al., 2001). Conversely, TLR4 requires the formation of complexes with proteins outside of the TLR family to transduce the signal upon interaction with its ligand. Specifically, TLR4 recognizes lipopolysaccharide (LPS) and forms a complex with myeloid differentiation 2 (MD2) together with the co-receptor Cluster of differentiation 14 (CD14), which drives the activation of transcriptional programs downstream of bacterial sensing (Hoshino et al., 1999). TLR3, 7, 8 and 9 on the other hand recognize bacterial- and viral-borne, as well as endogenous in some cases, nucleic acids (Shizuo Akira et al., 2006), which leads to the production of type I interferon and of other pro-inflammatory cytokines. While the TLR1, 2, 4 and 6 variants are found mostly in the plasma membrane, the latter are often found in the endoplasmic reticulum and in endolysosomes. TLR signalling converges into the activation of similar programs leading to the recruitment of Myeloid differentiation 88 primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon β (TRIF). This ultimately leads to the activation of the Mitogen-associated protein kinase (MAPK) cascade and IκB kinases (IKKs) leading to activator protein 1 (AP-1) and nuclear translocation of the Nuclear Factor-κB (NF-κB)-mediated transcription of several pro-inflammatory cytokines.

Similar to TLRs, CLRs contain transmembrane regions to mediate their localization in plasma membranes. Specificity to their ligand is given instead by a carbohydrate-binding domain in their C-terminal end, which can recognize carbohydrates of several origins, including bacteria, viruses and

fungi. I refer here to a comprehensive review for further details about this family of immune receptors (Geijtenbeek & Gringhuis, 2009).

2.2.2 – Cytosolic nucleic acid-binding PRRs: ALRs and RLRs

Absent in melanoma 2 (AIM2)-like receptors (ALRs) are found in tandem arrays in the same genomic loci in most mammals. Numbers of ALR genes can vary widely across mammals: the human genome contains four ALR genes, while the mouse loci is comprised of up to 13. In contrast to other PPRs, ALRs are characterized by the presence of a C-terminal HIN domain, which can directly interact with double stranded DNA (dsDNA), and an N-terminal pyrin domain. Albeit ALRs can bind nucleic acids, their target DNA is different from that of TLR3, 7, 8 and 9, as TLR can only bind to endocytosed nucleic acids, and ALRs solely bind to nucleic acids in the cytoplasm instead. Therefore, these receptors have been involved in the inflammatory response linked to cytosolic DNA. In most cases, the sensed dsDNA originates from phagocytosed bacteria or viruses. In particular, Interferon-inducible 16 (IFI16) has been shown to drive the response against Kaposi's sarcoma-associated virus (KHSV) (Kerur et al., 2011) and to be a crucial regulator of STING-dependent interferon production in some bacterial infections. The best studied PRR in this family is however AIM2, which has been shown to be activated in response to a myriad of pathogens. Interestingly, AIM2 protein levels have been linked to inflammation during senescence, a form of terminal cell cycle arrest associated to cellular aging. Old fibroblasts with higher expression of AIM2 have been shown to produce increased levels of senescence-associated secretory cytokines (Duan et al., 2011), suggesting that AIM2 might be involved in the sensing of extra-nuclear DNA in this cellular process.

RIG-I-like or RLRs are capable of sensing and binding nucleic acids in the cytoplasm as well. RLRs however are specialized in the detection of viral RNAs instead of DNA. This interaction with RNA is mediated by their central ATPase containing DExD/H box helicase domain. Additionally, two of the members of this family, RIG-I and MDA5, contain a C-terminal caspase-recruitment domain (CARD) that mediates downstream signalling upon activation of the receptor, and is not present in the third known member of the family, LGP2 (Yoneyama et al., 2005). Similarly to IFI16, RLRs can induce type I interferon secretion upon interaction with their ligand. Additionally, the protein levels of all these receptors are low in resting cells and similarly to ALRs, are highly induced by type I interferon signalling (Kang et al., 2004), creating a collective positive feedback loop for a potent antiviral response.

2.2.3 – The NOD-like receptor (NLR) family

NLR proteins are Nuclear-Binding domain (NBD) and Leucine Rich Repeat (LRR) containing receptors (NLR), also referred to as NOD-like receptors. This family of 22 members in humans, is evolutionarily related to the NB-LRR proteins in plants, where similarly to their mammalian paralogues, they can sense bacterial and viral PAMPs (Medzhitov, 2001). All NLR proteins contain a highly conserved central NOD domain, also known as NACHT or NBD domain, generally followed by a C-terminal LRR domain that confers specificity to their ligand, with the exception of NLRP10. N-terminal domains are heterogeneous across the family, and have been used as the basis for further categorization in subfamilies (Fig. A).

While all proteins from the NLRP subfamily are limited by a pyrin domain (PYD) as the N-terminal fragment, NLRA and NLRC proteins usually display a CARD domain typical of cysteine proteases. The only member of the NLRA subfamily additionally contains an acidic transactivation domain (AD), a transcription factor domain that facilitates its interaction with regulatory proteins involved in gene expression. In contrast, NLRB proteins have a baculoviral inhibitory repeat (BIR) domain, which is understood to have an anti-apoptotic activity, and has inspired the common name used to refer to this subfamily: NLR family of apoptosis inhibitory protein (NAIPs).

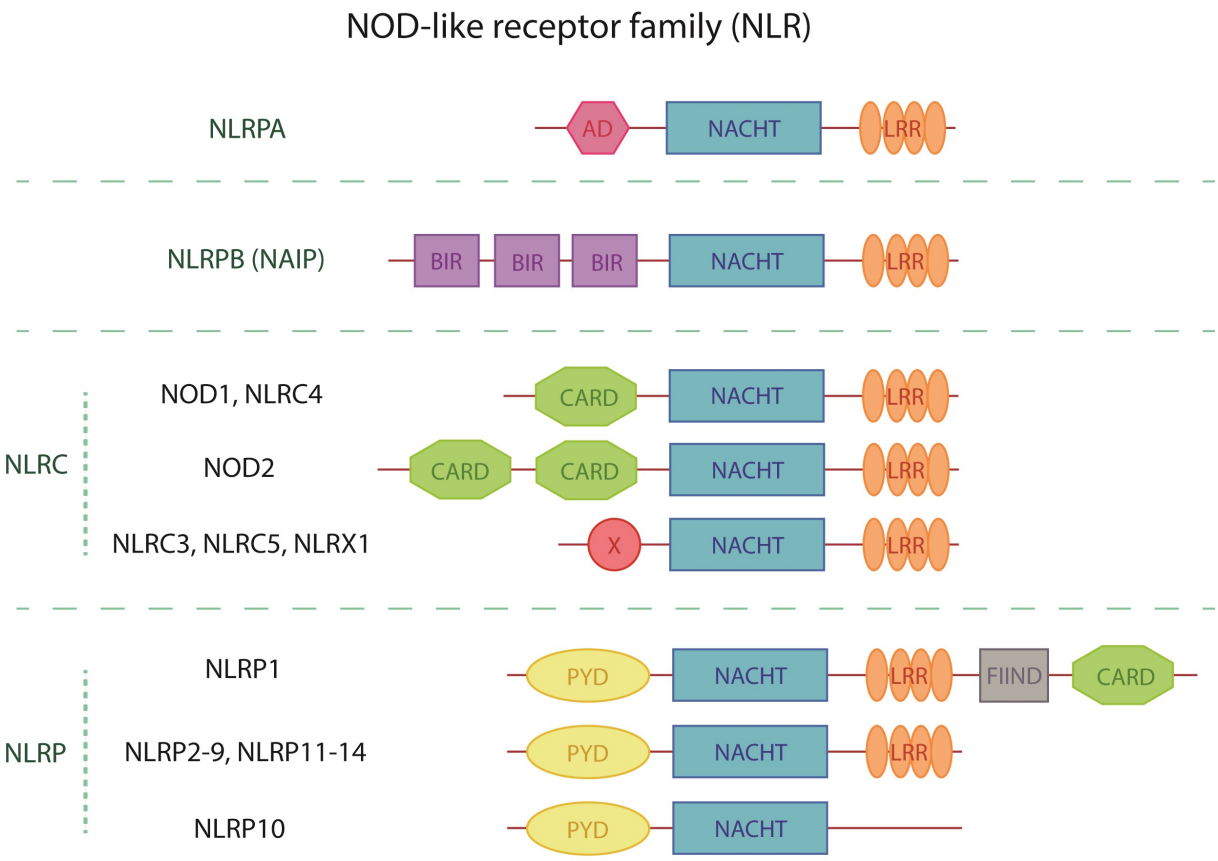


Fig. A. Classification of the members of the NLR family. The classification is done according to the presence of specific domains: acidic transactivation domain (AD), NAIP, CIITA, HET-E, and TEP-1 domain (NACHT) otherwise known as NOD domain, leucine reach repeats (LRR), baculoviral inhibitory repeat (BIR), caspase recruitment domain (CARD), pyrin domain (PYD) and function-to-find domain (FIIND). X represents so far unidentified domains.

Both CARD and PYD found in NLRA, NLRC and NLRP proteins belong to the death-fold structural family, characterised by a death-domain and death-effector domain, which can trigger cell death and inflammation. Indeed, it has been shown that activation of NLRs leads in many instances to the activation of caspase-1, a member of the cysteine protease family that is responsible for the maturation and secretion of several pro-inflammatory cytokines in immune cells. This can take place by direct interaction with the homotypic CARD domains in caspase-1 in the case of NLRA and NLRC. In contrast, NLRP proteins have been shown to be able to indirectly activate caspase-1. Interestingly, NLR proteins have a similar architecture as apoptosis protease-activating factor 1 (APAF1), the main orchestrator of the assembly and activation of the apoptosome, a central platform that coordinates the apoptotic cell death program in response to mitochondrial death signals. This similarity hinted to the discovery that

NLR proteins can actually assemble complexes in a similar fashion as APAF1 and thus play a key role in inflammation as central hubs controlling inflammatory events downstream of NLR activation.

This is not a unique characteristic to some NLR proteins. Albeit many PRRs orchestrate different mechanisms to cope with and limit infection, few of these receptors have been found to engage in a similar response, which is dependent on the formation of a big complex, whose activation requires caspase-1 recruitment and is responsible of inflammatory events including pro-inflammatory cytokine production and cell death. The complex was described for the first time by Martinon and colleagues as a caspase-activating complex that leads to Interleukin-1β (IL-1β) maturation and secretion (Martinon et al., 2002). The name they gave this newly-found structure was the inflammasome.

2.3 – The Inflammasomes are key platforms in immunity

The inflammasome is a multimeric protein complex that serves as an activation platform for caspase-related activation of pro-inflammatory cytokines in response to pathogenic and sterile molecular signals. Its size is at the scale of microns and it can already be observed in bright field light microscopy (Masumoto et al., 1999). Martinon and colleagues first described the inflammasome in the context of NLRP1 activation, but many other inflammasomes have been uncovered in the last two decades. To date, PRRs known to form this complex include several members of the NLRP family, including NLRP1, NLRP2, NLRP3 and NLRP6, NLRP7, NLRP9b, the NLRC4, the pyrin protein and AIM2 and IFI16 from the ALR family (Fig. B), albeit the amount of knowledge varies widely for each in terms of how they are formed and in which instances they are assembled. The main features of the inflammasome proposed by Martinon and colleagues stay true for most of them. Generally, the inflammasome contains a sensor molecule, the PRR, which confers the complex with its specificity to a particular ligand and gives it its name. Another indispensable member of the inflammasome is the effector protein, which is usually caspase-1, whose activation is directly responsible for cytokine maturation and cleavage of other substrates involved in the downstream inflammatory events. The other protein members of the inflammasome are highly variable depending on the PRR that drives the formation of the complex, which draws the guidelines for the assembly of the rest of its constituents and determines the time and the scale of the response to the original molecular pattern.

In order to understand the relevance of the inflammasome in the innate immune response in response to PAMPs and DAMPs, it is necessary to address independently every inflammasome. Thus, the inflammasome has become a major area of study in immunity, and deeper knowledge has been accumulated over the years about the inflammasomes that are mostly engaged to respond to noxious molecular signals.

2.3.1 – The AIM2 inflammasome

The AIM2 inflammasome was first identified upon the observation that caspase-1 activation in response to microbial cytosolic DNA was dependent on apoptosis-associated speck-like protein containing CARD (ASC) but independent of other PRRs such as TLR3, TLR7, TLR8, NLRP3 and of interferon regulatory

factors (Muruve et al., 2008). AIM2 inflammasome assembly requires the homotypic interaction of the AIM2 C-terminal PYD domain with the PYD domain of ASC. The ASC^{PYD} domain has a tendency to oligomerize into star-shaped branched filaments or “specks” that serve as platforms for caspase-1 clustering (Cai et al., 2014; Jones et al., 2010; A. Lu et al., 2014). Upon binding to AIM2 and oligomerization, ASC^{CARD} becomes exposed and can further interact with the CARD domain of caspase-1 (Sborgi et al., 2015). On the other hand, the N-terminal $AIM2^{HIN200}$ can bind directly to either viral (cytomegalovirus, vaccinia virus) or bacterial dsDNA like the bacterial DNA of *Francisella tularensis* (Alnemri, 2010). This requires the initial lysis of the bacteria, which then releases its DNA, facilitating its interaction with AIM2 in the cytosol (Jones et al., 2010; Sauer et al., 2010).

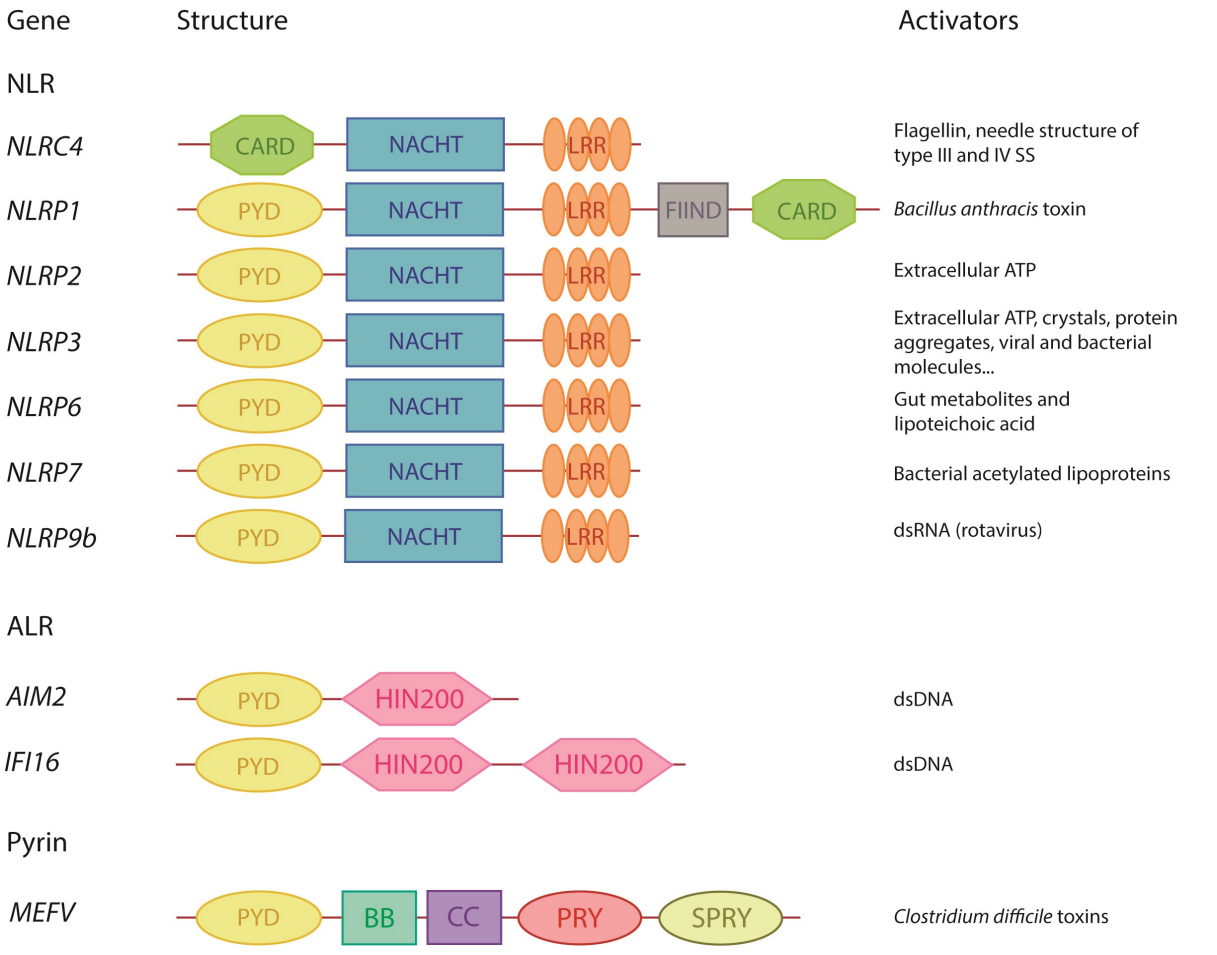


Fig. B Inflammasome-forming PRR domain structure and their activators. Mainly members of the NLRP subfamily of NLRs and NLRC4 from the NLRC subfamily can form this type of complex, and respond to different signals from yeast, bacterial and viral origin. AIM2 and IFI16 from the ALR family respond to dsDNA in the cytosol. Pyrin is the most recently reported protein outside of the conventional PRRs that is capable of forming an inflammasome.

Although polymerization of ASC is in big part responsible for the dimensions of the macromolecular complex, a germinal accumulation of AIM2 in close proximity is necessary to rapidly and efficiently nucleate ASC and to assemble the inflammasome. In comparison to NLR proteins, AIM2 lacks a NACHT domain for self-oligomerization, and therefore depends on an alternative mechanism to promote the growth of the complex at the initial stages of inflammasome assembly. Initial studies on the AIM2

inflammasome structure based on X-ray crystallography of the $AIM2^{HIN200}$ domain complexed with dsDNA, proposed the dsDNA braid itself serves as a scaffold for oligomerization (Jin et al., 2012). This model was later on confirmed by a couple of studies where $AIM2^{HIN200}$ was shown to interact with up to four base pairs of DNA, and to be in contact with six adjacent $AIM2^{HIN200}$ molecules (Jin et al., 2012; A. Lu et al., 2015). Unfortunately, crystallization of full-length AIM2 protein has not been achieved to date due to technical limitations. This limitation is mostly due to the tendency of $AIM2^{PYD}$ to form aggregates. However, the distribution of the domains in the AIM2 structure could hint at possible models. In fact, the HIND and PYD domains are separated by a rather long linker region, which could allow the PYD domain to rotate around the DNA core to find other $AIM2^{PYD}$ and oligomerize, which would be followed by higher recruitment of ASC and ASC filament formation.

Timely assembly of the AIM2 inflammasome is of utmost importance, and dysregulation of this process can result in disease. Upon loss of homeostasis, AIM2 can be sensitive to interact with the dsDNA of the host. Indeed, increased AIM2 expression is associated with psoriasis, and AIM2 has been shown to interact with cytosolic DNA that could originate from phagocytosis of damaged keratinocytes (Dombrowski et al., 2011). High expression of AIM2 could similarly explain severity of inflammation in abdominal aortic aneurysm and systemic lupus erythematosus (Dihlmann, Erhart, et al., 2014; Javierre et al., 2010). In contrast, low levels of AIM2 correlate with development of pancreatic and colorectal cancer (Dihlmann, Tao, et al., 2014; Man et al., 2015; Ponomareva et al., 2013). Actually, AIM2 stands for Absent in Melanoma 2, and was originally discovered as one of the genes suppressing tumourigenicity in malignant melanoma (DeYoung et al., 1997). Indeed, DNA release into the cytosol upon transient nuclear rupture has been reported for cancer cells, which could potentially activate AIM2. Therefore, AIM2 activation is required to allow efficient response to microbial infections and to control tumour progression, but must be finely regulated to avoid aberrant inflammation and autoinflammatory diseases.

2.3.2 – The Pyrin inflammasome

The pyrin inflammasome has been more recently added to the group of inflammasome-forming sensors. Its structure does not align with any of the pre-existing PRR families, and thus has not been included in any of the former groups. Pyrin, also known as Tripartite motif-containing protein 20 (TRIM20), is composed by a PYD domain and two B-boxes and a coiled-coil domain. In contrast to its murine orthologue, the human pyrin contains a C-terminal SPRY/PRY domain of unknown function (Papin et al., 2007).

Little is known in terms of structure or signalling pathway leading to the formation of this inflammasome. In fact, pyrin was initially thought to serve as a negative regulator for other inflammasomes, by competing with other PRRs to interact with the ASC^{PYD}. This hypothesis was based on the observation that mutations in the SPRY domain caused Familial Mediterranean Fever (FMF), an autoinflammatory disease. These mutations were initially thought to lead to a loss of function of the SPRY domain (Chae et al., 2006; Hesker et al., 2012). However, mice knocked-out for the gene coding for pyrin, *Mefv*, responded normally to inflammasome activators, and when knocked-in with human *MEFV* mutated in

the SPRY domain, were shown to activate caspase-1 in an ASC-dependent but NLRP3-independent manner (Chae et al., 2011). Accordingly, these authors proposed that mutations in SPRY observed in FMF patients were rather leading to gain-of-function of pyrin, resulting in the aberrant activation of pyrin, responsible for the formation of the inflammasome. This clarification was crucial to redirect efforts in the field into understanding the mechanisms underlying pyrin-induced ASC recruitment and pyrin inflammasome assembly. Recently, exciting developments in this direction have been made. One study showed that pyrin is phosphorylated by protein kinases 1 and 2 (PKN1/2), and this phosphorylation promotes pyrin interaction with 14-3-3 proteins to inhibit pyrin function (Y. H. Park et al., 2016). However, PKN1/2 can become inactivated by inactivation of Ras homolog family member A (RhoA), a member of the Rho family of GTPases. This stops phosphorylation of newly-synthesized pyrin and therefore halts its interaction with 14-3-3 proteins, rendering pyrin ready for inflammasome activation. All toxins described to date to activate the pyrin inflammasome do so by inhibiting RhoA. That is the case of *Clostriudium difficile* toxin B, *Clostridium botulinum* C3 toxin and effector proteins like VopS from *Vibrio parahaemolyticus* and IbpA from *Histophilus somni* (Xu et al., 2014). It has been proposed that pyrin could sense other events downstream of RhoA inactivation, such as changes in actin dynamics (Waite et al., 2009). All in all, the mechanism underlying pyrin inflammasome remains largely unexplored, but RhoA inactivation seems to be a common event downstream of its activators.

2.3.3 – The NLRC4 inflammasome

NLRC4 was the first protein of the NLR family identified as a potential inducer of cell death based on its similarity to APAF1 (Geddes et al., 2001). NLRC4, as a member of the NLRC subfamily, contains an N-terminal CARD domain and no PYD domain. Therefore, nucleation of ASC theoretically should not be required to target caspase-1 to NLRC4 and subsequent NLRC4 inflammasome activation. However, it has been shown that the presence of ASC in the complex might be crucial for caspase-1 activation and maturation of pro-inflammatory cytokines (Broz et al., 2010). Furthermore, the NLRC4 inflammasome requires the presence of yet other factors to be successfully assembled. Albeit NLRC4 is responsible for the recruitment and polymerization of ASC through $NLRC4^{CARD}$ against many facultative intracellular pathogens, NLRC4 fails to bind directly to any of the PAMPs that trigger NLRC4 activation. Instead, NLRBs, or NAIPs, have been shown to bind specifically certain bacterial molecules that activate the NLRC4 inflammasome. It was originally believed that only one gene coding for a NAIP in the human genome could sense the needle structure of the type III and IV secretion systems required for the injection of virulence factors in the infected cell (J. Yang et al., 2013; Y. Zhao et al., 2011). It was later shown that another isoform of NAIP exists in monocyte-derived human macrophages, which can bind flagellin of the bacterial locomotion machinery in the genus *Salmonella* (Kortmann et al., 2015). In contrast to human NAIP, a larger family of Naip codifying genes have been found in mouse, including *Naip1*, *Naip5* and *Naip6,* which can independently bind each ligand described for human NAIP (Amer et al., 2006; Franchi et al., 2006; Miao et al., 2006) and additionally *Naip2*, which can bind the basal rod component of the flagellar body (Kofoed & Vance, 2011; Y. Zhao et al., 2011). NAIP proteins bind to their cognate ligands through their NACHT domain, instead of LRR (Tenthorey et al., 2014), and can then interact with NLRC4. One possible requirement for binding of NAIP to NLRC4 could be the

phosphorylation of NLRC4^{NACHT} upon bacterial infection by Protein kinase C-delta (PKCδ) on Ser^{533} (Qu et al., 2012). This still remains unclear, as phosphorylation takes place in *Salmonella* infection but not *Shigella*-induced NLRC4 inflammasome activation, and therefore does not appear to be a universal mechanism for NLRC4 inflammasome activation (Qu et al., 2012; Suzuki et al., 2014).

Discovery of NAIPs as a founder member of the NLRC4 inflammasome was of key relevance to elucidate the structure of the NLRC4 inflammasome. So far, flagellin-Naip5 and PrgJ-Naip2 Nlrc4 inflammasomes have been purified and analysed by negative-stain and cryo-electron microscopy (Diebolder et al., 2015; Halff et al., 2012; Z. Hu et al., 2015). Altogether, these studies have unveiled key features of the NLRC4 inflammasome. One such example is the observation of the presence of 10 to 12 spokes corresponding to individual protomers of NLRC4. NLRC4 activation putatively led to higher flexibility of the protein and to an increase of the surface to interact with the close-by NLRC4 protomers, progressively facilitating oligomerization. They also showed that a single NAIP could be found in each complex, showing the great potential of a single molecule of this PRR. Hence, NAIP proteins are key factors in NLRC4 inflammasome assembly, and the diversity of the *Naip* locus has conferred NLRC4 a high versatility to a variety of signals, making it a major player in host defence against pathogens like *Salmonella typhimurium*, *Shigella flexneri* and *Pseudomonas aeruginosa*.

2.3.4 – The NLRP1 inflammasome

The NLRP1 inflammasome was first described from the observation that absence of NLRP1 lead to defective response to *B. anthracis* spores (Martinon et al., 2002; Moayeri et al., 2010; Terra et al., 2010). The NLRP1 inflammasome in humans is orchestrated by a unique NLRP1 protein coded by *NLRP1*. In contrast, mice have three different isoforms, *Nlrp1a*, *Nlrp1b* and *Nlrp1c*, albeit Nlrp1c is a pseudogene incapable of forming this complex. Furthermore, a crucial difference between NLRP1 in these two species is its domain composition. Human NLRP1 contains a PYD domain, a CARD domain, a NOD domain, LRR and a FIIND, while the murine orthologues lack the PYD domain at the C-terminal of the protein. In basal conditions, auto-proteolytic cleavage of the FIIND domain takes place, which has been shown to be necessary for later NLRP1 inflammasome function (Finger et al., 2012). NLRP1 can interact directly with caspase-1 through its CARD domain, but nucleation of ASC through NLRP1^{PYD} stabilizes the complex. Therefore, similarly to NLRC4, NLRP1 benefits from the presence of ASC in the complex. In one study it has been shown that ASC plays an indispensable role for caspase-1 autoproteolysis in murine Nlrp1b inflammasome activation by the *Bacillus anthracis* (Anthrax) lethal toxin (Van Opdenbosch et al., 2014). Mechanistically, this toxin has three distinct functions: first, its “protective antigen” forms a channel which facilitates the access of the lethal factor into the cytosol. Once in this cellular compartment, it inactivates immune signalling by cleaving MAPK kinases. Finally, it appears to cleave the first 4 kDa at the N terminus of Nlrp1b, although it is not well understood how this affects overall Nlrp1b function (Chavarría-Smith & Vance, 2013). This proteolytic cleavage has been proposed to relieve the protein from the auto-inhibition constraint by the N-terminal domain, which would increase the accessibility of Nlrp1b for self-oligomerization. Following cleavage, the N-terminal fragment is then ubiquitylated by the E3 ubiquitin ligase UBR2 (Xu et al., 2019) and sent for proteasomal degradation,

completely releasing the C-terminal fragment and rendering it ready for oligomerization (Chui et al., 2019; Sandstrom et al., 2019).

The Anthrax lethal toxin plays a very specific function in the activation of Nlrp1b, which has been unseen for any other toxin to date. No such mechanism has been described for human NLRP1 to date in response to this toxin. However, Chavarría-Smith and colleagues showed that truncation of the N-terminus of human NLRP1 leads to activation of NLRP1 inflammasome and IL-1β secretion (Chavarría-Smith & Vance, 2013). Furthermore, human NLRP1 has been found to form an inflammasome *in vitro* in response to a component of peptidoglycan, muramyl dipeptide (MDP) (Faustin et al., 2007), presumably through the binding of MDP to $NLRP1^{LRR}$ to induce NLRP1 oligomerization. Taken together, these observations suggest that both murine and human NLRP1 need to undergo conformational changes in order to engage the NLRP1 inflammasome.

2.3.5 – The NLRP3 inflammasome

Another member of the NLRP subfamily that can form an inflammasome is NLR Family Pyrin Domain Containing 3 (NLRP3). The NLRP3 is constituted by the NACHT self-oligomerization domain, a C-terminal LRR domain and a PYD N-terminal domain (Fig. B). This protein is not only present in mammals, but extends to other eukaryote classes and is able to form inflammasomes in distant species including zebrafish (J.-Y. Li et al., 2019). Its ubiquitous presence across eukaryotes already suggests a key role of NLRP3 inflammasome in the immune system. Indeed, the study of NLRP3 has been a great source of interest in the past couple of decades because of the intrinsically complex nature of its activation. NLRP3 has been shown to respond to a plethora of diverse stimuli, including bacterial, viral and fungal pathogens, crystals, aggregates and DAMPs, such as ATP or hyaluronan. It is generally agreed upon that given the diversity of these stimuli, NLRP3 activation cannot be activated by the direct binding of the receptor with the stimuli. Instead, NLRP3 is believed to sense a host-derived factor altered by the irruption of these agents. Several possible factors that could be responsible for NLRP3 activation have been proposed to date, including K^+ efflux: (Muñoz-Planillo et al., 2013), mitochondrial, ER and Golgi NLRP3 translocation (Misawa et al., 2013; N. Subramanian et al., 2013; Zhou et al., 2011), mitochondrial damage as defined by mitochondrial reactive oxygen species (mtROS) (Zhou et al., 2011), release of mitochondrial DNA (mtDNA) (Nakahira et al., 2011; Shimada et al., 2012) and the presence of mitochondrial lipid cardiolipin in the outer mitochondrial membrane (Iyer et al., 2013), cytosolic release of lysosomal cathepsins (Hornung et al., 2008) and changes in metabolism (Moon, Hisata, et al., 2015). Despite this volume of literature, a universal event upstream of NLRP3 has not yet been found, and understanding of this inflammasome still poses a great conundrum in the field. I will look at each of these potential players in NLRP3 inflammasome further below, and explain in more detail what is currently known in the activation of this inflammasome.

2.3.6 – The noncanonical inflammasome

Above described canonical inflammasomes involve caspase-1 activation through assembly of the inflammasome complex containing the original activated sensor. Strikingly, Gurung and colleagues

showed that caspase-1 was activated in murine immune cells upon infection with enteropathogens such as *Escherichia coli*, *Citrobacter rodentium* and *Vibrio cholera*, in a process that required caspase-11 for pro-inflammatory cytokine secretion (Gurung et al., 2012). They also showed that cell death associated to inflammasome activation was dependent on caspase-11 but not caspase-1, indicating that caspase-11 drives endotoxemia in this type of infection. This is further demonstrated by complete enterotoxic resistance in double *Casp1* -/- and *Casp11* -/- mice, but not in single knock-out alone (S. Wang et al., 1998). Entheropathogens are commonly classified as Gram negative, as determined by their capacity to retain crystal violet used in the Gram staining for bacterial characterization. Murine caspase-11 has been shown to be able to bind directly to lipopolysaccharide (LPS) of Gram negative, but not Gram positive bacteria. This is likely due to the presence of acylated lipid A in LPS from Gram negative pathogens. This activation is completely independent of TLR4, which usually binds to LPS in the extracellular space, but instead requires internalization of LPS for inflammasome activation (Hagar et al., 2013; Kayagaki et al., 2013). The orthologue of caspase-11 in human is caspase 4 and caspase 5. It has now been shown that all three proteins can bind directly to the lipid A in LPS, and that this interaction is sufficient to induce oligomerization and activation of these caspases (Shi et al., 2014). Shi and colleagues additionally showed that the CARD domain in caspase-11 is directly mediating the binding with the ligand, but the CARD domain of the closely related caspase-1 protein is incapable of mimicking this action.

However, although caspase-11 in mice and caspase 4 or 5 in human have been shown to activate inflammatory cell death, maturation of the traditional cytokines secreted during inflammasome activation absolutely requires the activation of caspase-1. In the recent years, a link between the canonical and noncanonical inflammasome has been established, which helps explain how the cross-talk between these two caspases occurs (W. He et al., 2015). He and colleagues demonstrated that activation of the noncanonical inflammasome by LPS leads to release of ATP and the activation of the P_2X purinoreceptor 7 (P_2XR_7). P_2XR_7 is a channel that promotes Ca^{2+} and Na^+ influx, two of the ions whose flux has been suggested to play a role in the NLRP3 inflammasome activation. Additionally, although it does not seem to be acting as the source of K+ efflux, P_2XR_7 can cooperate with the two-pore domain K^+ channel (K_{2P}) (TWIK2) to mediate K^+ efflux (Di et al., 2018), a widely accepted inducer of NLRP3 activation. Caspase-11-derived pannexin-1 activation could also participate to further exacerbate the K^+ efflux (D. Yang et al., 2015). Lastly, cell death activated by caspase-11 is lytic, and results in the formation of pores in the plasma membrane, which could also contribute to K^+ efflux (J. Shi et al., 2014). All these events could converge in the activation of the NLRP3 canonical inflammasome in a cell-intrinsic mechanism (Kayagaki et al., 2015). Therefore, the initial activation of caspase-11 would drive a stream of events stimulating the subsequent formation of NLRP3 inflammasome and activation of caspase-1. A similar process seems to be triggered in response to oxidized phospholipid 1-palmitoyl-2-arachidonoyl—sn-glycero-3-phosphorylcoline (oxPAPC). Like LPS, oxPAPC can directly bind to caspase-11 and activate the noncanonical pathway in murine macrophages (Zanoni et al., 2016). However, a more recent study proposed that oxPAPC inhibited rather than activated caspase-11, and therefore it is not yet clear what role these lipids play for inflammasome activation (Chu et al., 2018).

These findings have been shown in macrophages, but recent studies have proposed additional mechanisms underlying caspase-1 activation upon noncanonical inflammasome formation by other immune cells. This has been shown to be the case for neutrophils, where LPS triggers caspase-11 activation and cell death, which results in the release of neutrophil extracellular traps (NETs) in a process known as NETosis (K. W. Chen et al., 2018).

2.3.7 – Collaboration between inflammasomes

As indicated above, different inflammasomes can detect different molecular patterns depending on the PRR that constitutes the sensor element of the complex. Not only the triggering of the noncanonical inflammasome can lead to the activation of others, but induction of several inflammasomes can take place at the same time. Pathogens are complex molecular systems, and as such are composed of a wide variety of molecules which, if released in the cell, can simultaneously interact with different sensors. Indeed, several reports have shown the interplay between different types of inflammasomes in response to a sole pathogen. Unsurprisingly, the NLRP3 inflammasome in particular has been shown to be prevalent in these instances, in accordance with its capacity to sense a wide spectrum of stresses in the cell. For example, *Salmonella* contains NAIP-activating flagellin and can induce metabolic perturbations that have been proven to induce co-localization of NLRP3 and NLRC4 in the cytosol of murine macrophages (Man et al., 2014, p. 4). In this context, the two sensors were proposed to cooperate to generate a single inflammasome platform (Broz et al., 2010; Man et al., 2014). Nlrp3 can also be activated jointly with Aim2, and both have been shown to engage inflammasome activation upon the detection of *Aspergillus fumigatus*, a pathogen responsible for invasive pulmonary aspergillosis, a severe fungal infection in the lung (Karki et al., 2015). During malaria infection inflammation depends on phagocytosis of the *Plasmodium* species and the detection of their genomic DNA in the cytosol that can be sensed by Aim2, and of hemozoin sensed by Nlrp3 in murine macrophages (Kalantari et al., 2014). In both of these studies, the immune response was reduced when mice were defective for one of the sensors, and upon the ablation of the two, mice succumbed to infection. Even more surprisingly, some studies have pointed to the possibility of a Nlrc4, Nlrp3 and Aim2 triple-sided inflammasome in response to some bacteria. This is the case of *Listeria monocytogenes*, which causes one of the most common diseases resulting from bacterial food poisoning (Wu et al., 2010). *Listeria* species are phagocytosed and lysed in the internal compartments of phagocytes, where they can release several molecules that either directly activate Nlrc4 and Aim2, and indirectly the Nlrp3 inflammasome.

The ASC speck is a fundamental part of many inflammasomes, including NLRP3 and AIM2 inflammasomes, and contributes to a robust activation of caspase-1 in other inflammasomes, such as those formed by NLRP1 and NLRC4. It has now been observed that newly formed ASC specks from a cell undergoing inflammasome-induced cell death can be released into the extracellular space and phagocytosed in neighbouring cells, where they can continue to nucleate cytosolic ASC and activate caspase-1 to further propagate the inflammatory signal (Baroja-Mazo et al., 2014; Franklin et al., 2014). As ASC serves as a direct scaffold in this situation, this process appears to be PRR independent, but equally serves to amplify the same pro-inflammatory responses through caspase-1 activation.

2.3.8 – Caspase-1 activation

The final purpose of the assembly and growth of the inflammasome scaffold is the recruitment and activation of proteases that have the ability to mature pro-inflammatory cytokines and activate the molecular mechanisms leading to a lytic cell death. The aspartate-specific cysteine family of proteases, also known as caspases, have been tightly linked to the organization and execution of several death programs, including apoptosis or the pro-inflammatory lytic death programs: necroptosis and pyroptosis. This role on cell death was first proposed upon the discovery of ced-3 as a key factor in cell death during the development of *Caenorhabditis elegans* (Ellis & Horvitz, 1986). Several homologues for this protein were found to exist in mammals. CED-3, which was later termed Interleukin-1β-converting enzyme (ICE) and is currently known as caspase-1, was the first homologue of ced-3 described in mammals (Yuan et al., 1993). Newer members to the family have been added ever since, to make a total of 18 to date. The great variety of caspases in the mammal genome responds to the greater specialization of each of the members of the family to different functions. Accordingly, caspases have been subdivided in two different categories. The first subgroup is composed of the apoptotic caspases, which include caspase 2, 3, 7, 8, 9 and 10. This subfamily can be in turn divided into initiator (2, 8, 9, 10) and executioner or effector caspases (3, 6, 7). Initiator caspases comprise prodomains, Death effector domain (DED), Pyrin domain (PYD), death domain (DD) or Caspase Recruitment Domain (CARD), to mediate their dimerization or recruitment into larger protein complexes, respectively. Classically, apoptotic caspases were the proteins to have been associated to a programmed type of cell death, while lytic cell death was believed to be rather independent of a tightly controlled cellular pathway. However, caspase-1, 4, 5, and 11 have been shown to be major players in a tightly controlled pathway underlying inflammatory cell death. As indicated above, the orthologues of murine caspase-11 and bovine caspase-13 in human (caspase 4 and caspase 5) can sense LPS and trigger inflammasome-dependent cell death. In contrast, caspase-1, the first member of the family, was soon shown to respond to a variety of pathogens, and more recently to sterile signals in the host, and acts as the effector protein of inflammasome to trigger pro-inflammatory events downstream of infection.

Albeit they have different specificities and different targets, caspases have rather similar mechanisms of activation. Caspases are synthesized as zymogens, and therefore need to undergo proteolytic maturation in order to become fully active. Effector caspases are usually activated by proteolytic processing performed by mature initiator caspases. Other caspases however, including initiator caspases themselves such as caspase-8 or caspase-9, but also caspase-1, can concentrate locally, allowing the protomers to mature each other by auto-catalytic processing. For this proximity to occur, caspases own homotypic-interaction domains which typically consist of 6 or 7 antiparallel α helixes that allow them to form tightly packed structures with other caspase protomers. For example, both the apoptosome and the inflammasome have a double-ringed wheel structure with 7- or 8-fold symmetry (Duncan et al., 2007; Faustin et al., 2007), and recruitment of their cognate caspase, caspase-9 and caspase-1 respectively, is sufficient to mature the caspase and activate the complex.

Once the inflammasome complex becomes active, caspase-1 can perform different functions. As indicated above, caspase-1 was first described as the protease in charge of the maturation of IL-1β, a

pro-inflammatory cytokine (Yuan et al., 1993). Later on, it was also shown that caspase-1 can cleave yet another pro-inflammatory cytokine critical in innate immunity, Interleukin-18 (IL-18) (Fantuzzi & Dinarello, 1999). More recently, caspase-1 has been reported to be directly responsible for the cleavage of a protein from the pore-forming gasdermin family, gasdermin D (GSDMD), which is critical for inflammasome-induced cell death known as pyroptosis (J. Shi et al., 2015). All these events play an essential role in the propagation of inflammation to timely combat pathogens, and understanding how they work mechanistically is key to understand the importance of the inflammasome as a main platform in innate immunity (Fig. C).

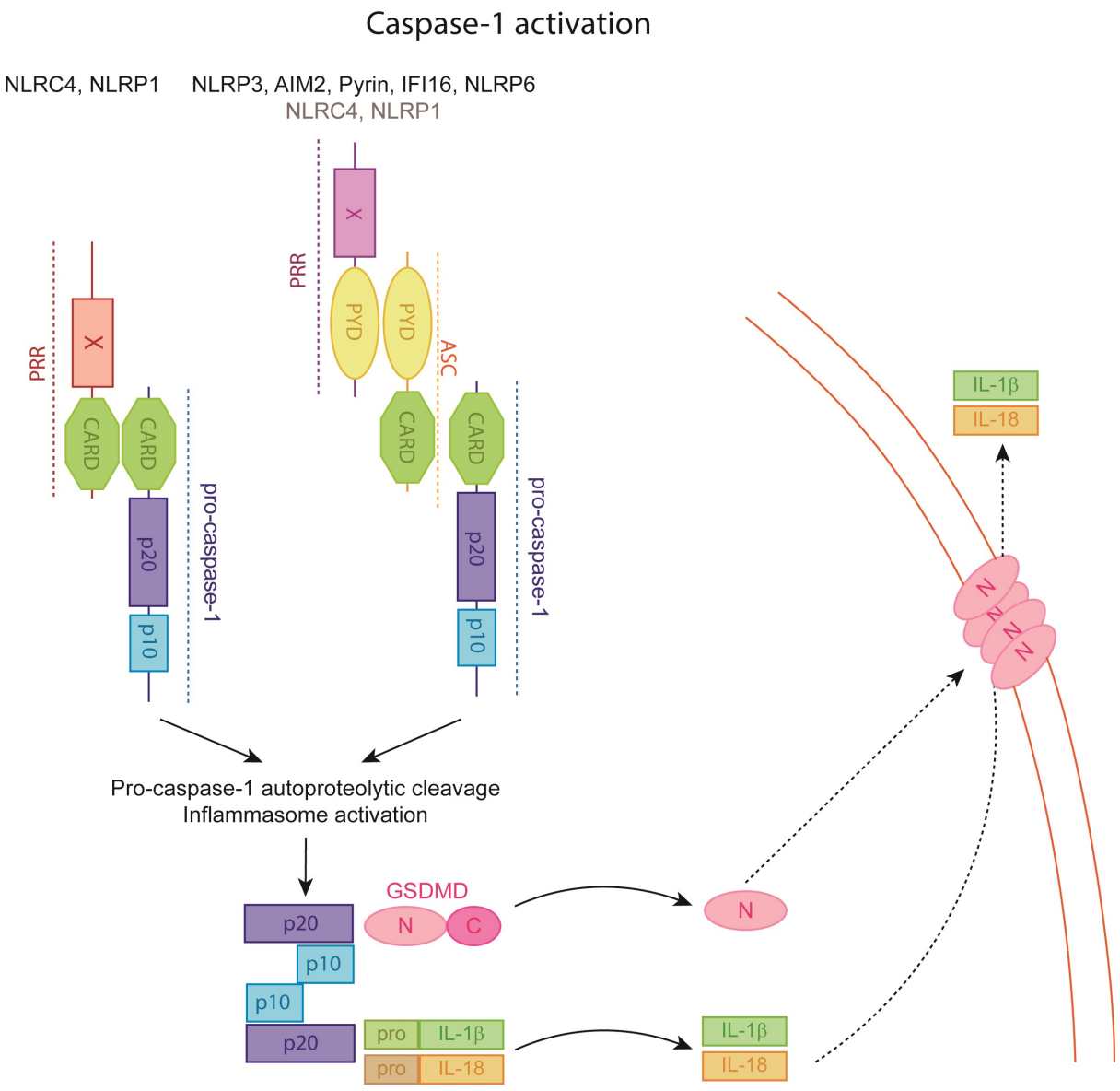


Fig. C. Recruitment of caspase-1 by CARD domains leads to pro-caspase-1 auto-processing and caspase-1 activation. PRRs such as NLRC4 and NLRP1 can recruit pro-caspapase-1 directly. The other members of the NLR and ALR rely on the recruitment of ASC through their PYD domain. NLRC4 and NLRP1 can recruit ASC first to make the activation of the inflammasome more robust. Recruitment of pro-caspase-1 leads to proximity-induced consecutive pro-caspase-1 auto-proteolytic cleavage of the p20 and p10 subunits. Caspase-1 heterotetramers cleave and activate the GSDMD and IL-1β and IL-18 precursors, which can then be secreted through oligomerized N-terminal GSDMD pores.

2.3.9 – GSDMD cleavage and induction of pyroptosis

Pyroptosis was first termed by Fink and Cookson to describe the type of inflammatory cell death induced by infections from bacteria such as *Salmonella* and *Shigella* (Fink & Cookson, 2005). Etymologically, the word is composed of two parts, the Greek word 'pyro' which means fire or fever, and 'ptosis' which can be translated to fall. In contrast to apoptosis it is non-homeostatic, and therefore it is intended to trigger a response in the surrounding environment of the dying cell, required to amplify inflammation in this site.

Morphologically, pyroptotic cells are clearly distinct to apoptotic cells, but show common features with the other type of programmed lytic cell death, necroptosis. Apoptotic cells have a condensed nucleus and cytoplasm, and display inner fragmentation into membrane-bound fragments, while keeping their plasma membrane intact. In contrast, pyroptotic cells and necroptotic cells are characterized by swelling, rupture of the plasma membrane and subsequent release of cytoplasmic contents in the extracellular space. This rupture is mediated by the formation of pores composed of mixed-lineage kinase domain-like pseudokinase (MLKL) oligomers in necroptosis, whilst pyroptosis relies in the oligomerization of GSDMD protomers (Kayagaki et al., 2015; J. Shi et al., 2015). These first studies on GSDMD showed that GSDMD in basal conditions is cytosolic, and remains inactive by the autoinhibitory function of its C-terminal fragment on the pore-forming N-terminal fragment (Ding et al., 2016). This is a common occurrence in the Gasdermin family, where at least Gasdermin A, B, C, D and E are known to date to follow a similar activation strategy by different proteases (Chao et al., 2017; Ding et al., 2016; J. Shi et al., 2015). GSDMD in particular contains a WEHD motif in the linker region separating its two fragments, which can be targeted by both caspase-1 and caspase-11. Upon activation of either caspase, the C-terminal constraint on the N-terminal fragment is released, thus rendering the oligomerizing surface of N-terminal accessible and allowing GSDMD to translocate to the plasma membrane and form pores. GSDMD pores have an inner diameter of 10 to 14 nm, which is sufficient to allow the passage of ions in an out of the membrane, facilitate cytokine secretion, and induce pyroptosis and autocrine NLRP3 inflammasome activation by noncanonical inflammasomes. In addition, GSDMD has a bactericidal function, as it can interact with cardiolipin in the bacterial membrane (X. Liu et al., 2016), and can help amplify the response by interacting with cardiolipin at the outer mitochondrial membrane, exacerbating mitochondrial damage.

While the requirement of GSDMD in inflammasome-induced pyroptosis upon strong stimulation of caspase-1 is widely accepted, the absence of GSDMD does not seem to be sufficient to block cell death upon longer periods of stimulation. Indeed, prolonged activation of caspase-1 but not caspase-11 in *Gsdmd* -/- murine macrophages remains lethal, indicating that there are other substrates of caspase-1 that can mediate cell death, namely other members of the gasdermin family (Kayagaki et al., 2015). Sustained activation of apoptotic caspases can trigger what is known as secondary necrosis in an ASC-dependent manner (W. He et al., 2015), which is consistent with the ability of ASC specks to activate caspase-8 in the absence of caspase-1 (Pierini et al., 2012). This type of cell death occurs by failure to clear apoptotic cells by phagocytes, which leads to lysis of the plasma membrane and release of cellular contents to the extracellular space. Although it is ASC-dependent, it can lead to secretion of mature type

1 interleukins and looks similar to pyroptosis morphologically. Recent studies have shown that this pathway is independent of GSDMD, and relies instead on the cleavage of another member of the Gasdermin family, GSDME (C. Rogers et al., 2017, p. 20; Schneider et al., 2017).

Albeit GSDMD pores seem to be essential for pyroptotic cell death specifically, the mechanism underlying their formation and translocation is not yet clear. In germinal studies on GSDMD it was shown that the N-terminal fragment of GSDMD can form pores in liposomes *in vitro*, and therefore it tends to assemble in this kind of structure irrespectively from other proteins (Ding et al., 2016). A cryo-EM structure of the GSDMA3 pore showed that interactions between GSDMA3 and acidic lipidic head groups in the membrane could be necessary for pore formation (Ruan et al., 2018). They also observed the formation of these pores outside of the membrane, which supports the possibility of Gasdermin pores being oligomerized in the cytosol and then inserted in the membrane, although it is not clear if the "soluble pores" they visualized were just an *in vitro* artefact. Other researchers tracked the dynamics of the formation of N-terminal GSDMD pores and observed that insertion of GSDMD protomers in the membrane preceded oligomerization (Mulvihill et al., 2018). These conflicting observations reveal the complexity of this process, which is likely to become yet more complex in intact cellular systems.

Faithful activation of caspase-1 and pyroptosis are requirements to ignite inflammatory events downstream of inflammasome activation to ultimately stop the replication of intracellular pathogens in infected immune cells, while at the same time promoting depletion of bacteria by exposing them to neighbouring immune cells. For this reason, release of DAMP trough pyroptosis is an important source of inflammatory cues, but production and secretion of more specialized molecules play a key role in the progression of inflammation. This is the case for type 1 interleukins IL-1α, IL-18 and IL-1β and High mobility group box 1 (HMGB1). HMGB1 can bind to the inflammatory receptor for advanced glycation end-products (RAGE) in the extracellular space and TLR2, 4 and 9 in a paracrine manner (Sims et al., 2010). IL-18 triggers expression of IL-17 in Th_{17} and can polarize T cells toward Th_1 or Th_2 profiles in combination with other cytokines (S. Akira, 2000). IL-1α and IL-1β are close relatives and promote similar functions in the organism binding to the ubiquitous IL-1 receptor (IL1R1): they promote adaptive Th_1 and Th_{17} T cells and humoral activity, although IL-1β has been associated to systemic activity, while IL-1α seems to have an effect more locally (Fantuzzi & Dinarello, 1999). Interestingly, all three members of the interleukin 1 family, IL-1α and IL-1β and IL-18 are leaderless cargoes produced as inactive precursors and lack a classical signal peptide to drive their secretion through the conventional ER-Golgi axis. A key difference between them is that IL-1α is cleaved independently of caspase-1 function. Albeit secretion of IL-1β and IL-18 is not dependent on their maturation state, caspase-1 is required not only for their maturation but also for their secretion. Some mechanisms that have been proposed to date to explain unconventional secretion of these cytokines include microvesicle shedding, exosomes, secretory autophagy and secretory lysosomes (C. Andrei et al., 1999; Cristina Andrei et al., 2004; MacKenzie et al., 2001; Qu et al., 2007). However, new insights on pyroptosis have raised the possibility that GSDMD pores, which have a diameter that is wide enough to allow the passage of these relatively small interleukins, are directly responsible for their secretion. Indeed, IL-1β and IL-18 fail to be secreted in *GSDMD* -/- cells, supporting this hypothesis of a passive leakage of IL-1β and IL-18 through the pores (W. He et al., 2015; J. Shi et al., 2015). Future studies will reveal whether pore formation is sufficient for

the rapid secretion of cytokines in cell models or whether additional active mechanisms are required to ensure their fast release into the extracellular space.

2.4 – The NLRP3 inflammasome

The panel of signals that activate the NLRP3 inflammasome has only grown over the years. However, a good understanding of the mechanisms underlying its activation has not followed at the same rate, and many claims on the nature of this pathway have been largely disputed leaving little common ground in the field. One of the less polarizing findings is that NLRP3 inflammasome activation is a biphasic process that requires two signals. The first signal initiates the priming step, and usually is a cytokine, a DAMP or a PAMP. This recognition can trigger different signalling cascades that culminate in the activation of transcriptional programs to upregulate core members of the inflammasome itself and its substrates. In addition, some post-translational modifications can take effect during this step. A second signal is then required to activate these proteins and for the assembly of the complex itself, which results in the enzymatic activation of the inflammasome and subsequent cytokine secretion during cell death.

In this section we will explore in more detail which events take place during this two-step process, and we will take a peek into the situations which do not fall into such a model.

2.4.1 – The priming step

The priming step takes place to effectively prime or “get the cell ready” for a robust response during the activation step. Priming can be induced by several molecules, commonly through their binding with a PRR receptor. The most well-studied case is the interaction of the rather ubiquitous bacterial LPS with the TLR4 receptor in the plasma membrane. Other molecules can interact with cytokine receptors such as RAGE or IL-1R, which can equally activate the priming cascade in response to HMGB1 and IL-1 interleukins respectively. There are also less physiological means of priming the cells like phorbol-12-myristol-13-acetate (PMA), which do not act at the level of the plasma membrane but instead can penetrate the cell and induce the activation of MAP kinases, PKCβI/II, NADPH oxidase and IKKs (Huan Qiao & May, 2009). Furthermore, the immune system can prime NLRP3 through the anaphylatoxin C3a and C5a receptors (Cao et al., 2016). All these stimuli signal through a signalling cascade that ultimately leads to nuclear translocation and activation of NF-κB and AP-1, respectively. In the case of TLR receptors, Interferon regulatory factors (IRFs) also shuttle to the nucleus to induce expression of interferon inducible genes. Nuclear NF-κB can bind to the promoter of several genes, and induce expression of several members of the inflammasome pathway. This process is known as transcriptional priming.

2.4.1.1 – Transcriptional priming

The pathway that leads to NF-κB import into the nucleus in response to TLR4 signalling induced by LPS is depicted below in Fig. D (F. Bauernfeind et al., 2011; Gurung et al., 2014). Briefly, the cascade is

typically initiated upon ligand sensing by membrane receptors. In the case of TLR4, this leads to the activation of the receptor complex to render active MyD88 and TRIF. TRIF promotes the expression of IFN inducible genes by IRF factors. TRIF can activate TANK-binding kinase 1 (TBK1), which phosphorylates IκB kinases i and e (IKKi/IKKe) that subsequently phosphorylate IRF3 to trigger its homodimerization or heterodimerization with IRF7. These dimers can translocate to the nucleus, where they induce expression of some genes. On the other hand, MyD88 can be recruited to the activated TLR4 complex and subsequently recruit Interleukin-1 receptor-associated kinase (IRAK) family members IRAK1 and IRAK4. This leads to their phosphorylation and ubiquitylation, which is required for activation of TNF receptor associated factor 6 (TRAF6). IRAK1, IRAK4 and TRAF6 together form a complex that drives activation of both the MAP kinase and the IKK pathway through MAP3K7. On one hand, the IKK complex is activated, which can subsequently phosphorylate IκB. In normal conditions IκB tethers NF-κB in the cytosol. Once IκB is phosphorylated it can be ubiquitylated and degraded in the proteasome. It is worth to mention that PI3K is at the same time activated by MAP3K7, and can induce Akt activation, which also contributes to release of NF-κB from IkB proteins. This enables NF-κB to translocate to the nucleus to activate inflammatory transcriptional programs. On the other hand, the complex formed by IRAK1/4 and TRAF6 leads to activation of the MAPK pathway. Firstly, MAP3K7 phosphorylates MEK family members (MKK), which can activate p38 and c-Jun N-terminal kinases (JNKs). Through these two signalling pathways, AP-1 will become active and will translocate to the nucleus.

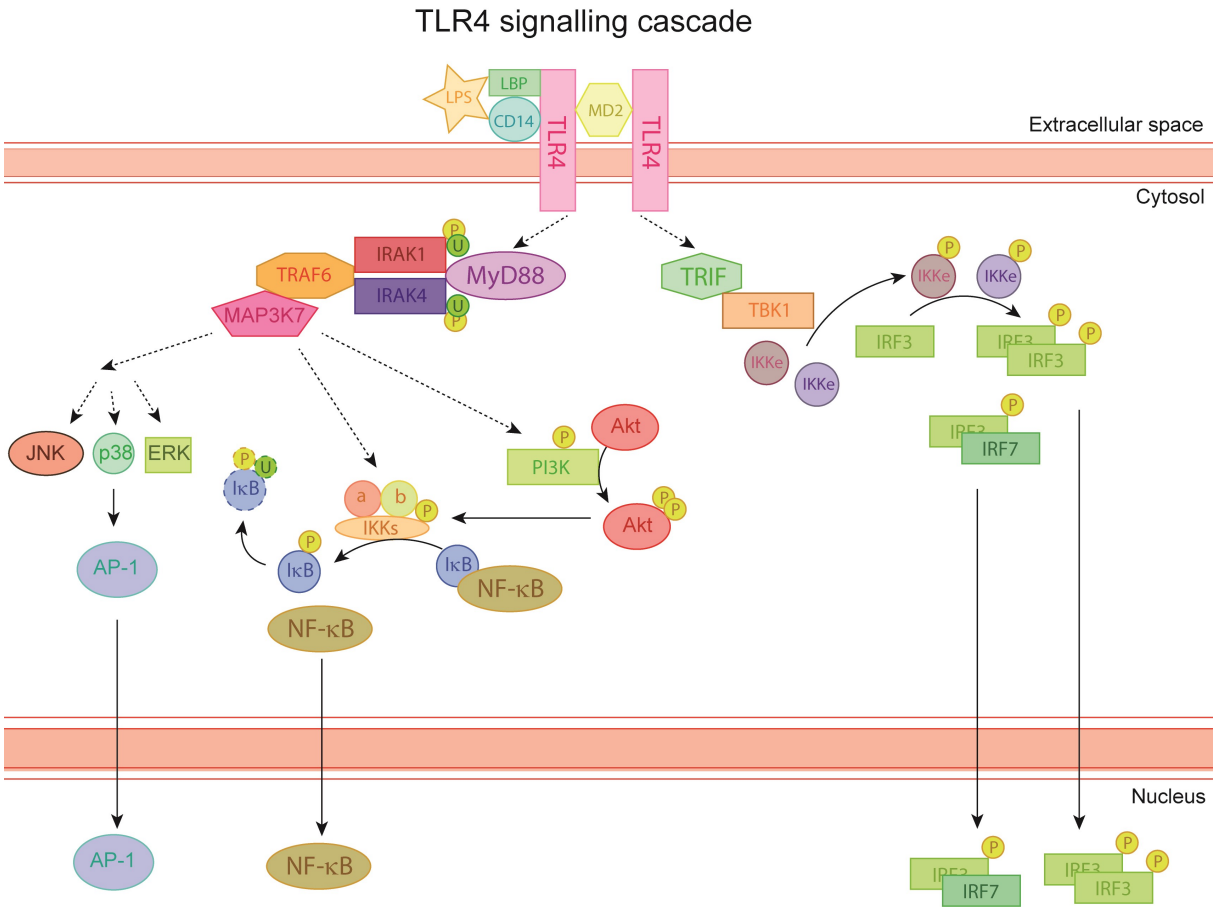


Fig. D. Transcriptional priming of NLRP3 by LPS. Signal transduction of LPS through the TLR4-CD14-LBP-MD2 complex. TLR4 activation activates the pathway leading to IFN inducible gene expression through TRIF activation and subsequent phosphorylation events. MyD88 phosphorylates IRAK1/4, which trigger activation of TRAF6 and MAP3K7. MAP3K7 activation initiates a cascade of phosphorylation events culminating in the nuclear translocation of AP-1 and NF-κB to activate the priming transcriptional programs.

Upon nuclear import of NF-κB, transcriptional priming will still require more than three hours to allow expression and translation of pro-inflammatory genes such as the precursor of IL-1β (pro-IL-1β) and NLRP3. In particular, exogenous expression of NLRP3 seems to alleviate the priming requirement for NLRP3 inflammasome activation, suggesting NLRP3 expression is the limiting factor in this step (F. Bauernfeind et al., 2011; F. G. Bauernfeind et al., 2009). It has been shown in previous studies that not only transcription of NLRP3 might be regulated, but also that translation of NLRP3 could be regulated by some miRNAs (F. Bauernfeind et al., 2012; Haneklaus et al., 2012). Once translated, some proteins may still require post-translational modifications to stabilize their abundance.

2.4.1.2 – Priming-related post-translational modifications

Proteins are in many occasions not synthesized in their functional form, but require additional modifications to promote a specific localization, conformation and activity. One of the main sources in the cell to produce these changes are other proteins that are capable of modifying specific residues in the peptide chain. This takes place in a time and space-dependent manner.

In the case of members of the inflammasome, many protein alterations have been shown to happen that stimulate progressive activation of the NLRP3 inflammasome. NLRP3 itself is highly modified during the priming step. NLRP3 ubiquitylation takes place soon after its synthesis. Indeed, the Skp-cullin-F box L2 (SCF^{FBXL2}) complex ubiquitylates the K691 residue (K689 in mouse Nlrp3) and targets it for proteasomal degradation, in order to avoid aberrant accumulation of NLRP3 (Han et al., 2015). Pellino2 has also been shown to ubiquitylate NLRP3, although it seems that this mechanism is rather indirect and therefore it remains unclear which residue in NLRP3 is affected (Humphries et al., 2018). A strong priming signal induces other modifications to alleviate the constraint of ubiquitin in NLRP3 function. A clear example of this is the phosphorylation of S198 (S194 in mouse) by JNK1 downstream of MyD88 activation and TRIF signalling, which has been suggested to be the key NLRP3 modification during the priming step (N. Song et al., 2017). S198 phosphorylation licenses NLRP3 for further modifications by deubiquitylation, and therefore is required for later events such as NLRP3 self-oligomerization. Additionally, transcriptional priming leads to the expression of SCFFBXO3 E3 ligase, which targets SCF^{FBXL2} for degradation and thus limits its ubiquitylation activity on K691 (Han et al., 2015). Therefore NLRP3 expressed in transcriptional priming will undergo ubiquitylation, and additional post-translational modifications are required to stabilize and render NLRP3 active.

Post-translational modifications during the priming step do not only occur on NLRP3. Ubiquitylation of K174 in ASC with K63 ubiquitin chains has also been shown. This process seems to take place downstream of tumour necrosis factor receptor-associated factor 3 (TRAF3) in response to RNA virus infections (Guan et al., 2015). However, this has not been shown in any other context of NLRP3 priming.

2.4.1.3 – Limiting priming: NLRP3 desensitization

Tight regulation of NLRP3 levels is required to avoid uncontrolled inflammation and tissue injury. Prolonged stimulation of TLR4 but not other TLRs leads to desensitization of NLRP3 to activation signals to limit host damage. One key feedback mechanism for this is the secretion of IFNβ. On one hand, it has been shown that IFNβ can activate inducible Nitric oxide synthase (iNOS) and TRIM30. iNOS inhibits NLRP3 but not other inflammasome-related PRRs by nitric oxide (NO) production (Hernandez-Cuellar et al., 2012). TRIM30 inhibits NLRP3 inflammasome by reducing the production of ROS (Y. Hu et al., 2010), although ROS requirement during the priming step remains debated. IFNβ can also inhibit expression of pro-IL-1α and pro-IL-1β via the STAT1 transcription factor, by inducing secretion of interleukin-10 (IL-10), which can play an autocrine function to down-regulate the expression of these cytokines (Guarda et al., 2011). Not only transcription for these cytokines can be down-regulated, but also maturation of pro-IL-1β by caspase-1 can be inhibited upon expression of a20 during priming (Duong et al., 2015).

There are other proteins induced by TLR4 activation that play a role in desensitization that are not dependent on IFN. TRIM31 ligase becomes activated in response to long-term priming, and can directly ubiquitylate the PYD domain in NLRP3 with K48 ubiquitin chains, which targets NLRP3 for proteasomal degradation (H. Song et al., 2016). Even NF-κB itself has been proposed to create a negative feedback loop to self-limit the production of pro-IL-1β by both upregulating the expression of negative regulators of the inflammasome and downregulating expression of members of the MyD88 cascade (Afonina et al., 2017). Another protein that has been reported to have a key role in NLRP3 desensitization and in particular to LPS tolerance is Glycerol-3-phosphate dehydrogenase 2 (GPD2), which can act as a switch from an inflammatory to a desensitized state in the cell (Langston et al., 2019, p. 2). Importantly autophagosome-dependent targeting of the inflammasome complex itself to lysosomal degradation can limit inflammation (C.-S. Shi et al., 2012). Therefore, the cell uses a whole variety of mechanisms to prevent tissue damage in the host.

2.4.1.4 – One-step inflammasome activation

Noteworthy, in human and porcine cells, but not in murine cells, strong LPS signalling can be sufficient not only to prime cells but also to induce NLRP3 inflammasome activation (Gaidt et al., 2016; Piccini et al., 2008). This pathway relies on TLR4 activation, and does not culminate in pyroptotic cell death in contrast to the canonical and the non-canonical NLRP3 inflammasomes. The activation of this so-called alternative inflammasome depends on the presence of NLRP3, ASC and caspase-1, and its assembly is independent on K^+ efflux and ASC nucleation. Instead, this pathway has been shown to rely on the activation of TRIF downstream of TLR4. TRIF orchestrates the signalling cascade leading to expression of IFN inducible genes, but is also a key protein in the activation of the ripoptosome, a protein complex constituted by RIPK1, FADD, RIPK3 and caspase-8. Upon binding of TLR4 to LPS and subsequent TRIF activation, the ripoptosome is assembled, and caspase-8 becomes active. Deficiency of any of the proteins in the complex blocks secretion of alternative inflammasome-associated IL-1β completely (Gaidt et al., 2016). It is not yet clear which role caspase-8 plays in the formation of this particular

inflammasome. None of the proteins that form the NLRP3 inflammasome complex seem to be cleaved in this situation, and therefore it is likely that caspase-8 could mature a still unknown factor involved in the activation of NLRP3 itself.

Until now, only priming had been reported to be sufficient to induce NLRP3-dependent caspase-1 actiation using the main components of the inflammasome response. However, a novel pathway of NLRP3 inflammasome activation independently of priming has recently been reported (Gritsenko et al., 2020). Gritsenko and colleagues showed that human monocytes but not monocyte-derived macrophages can form the NLRP3 inflammasome, undergo pyroptosis and secrete IL-18 but not IL-1β in the absence of a priming signal. This suggested that while priming is important for a robust NLRP3 inflammasome signalling, activating signals can be sufficient to trigger a lower grade of inflammation in some systems.

2.4.2 – The activation step

Under most circumstances, both a priming and an activating signal are required for NLRP3 inflammasome activation. As previously mentioned, the wide responsivity of NLRP3 suggests that activation of this sensor does not take place through direct interaction with the signal itself, but rather through a factor induced downstream of the signal. This also helps to explain why extracellular signals which do not pass the cell barrier can activate NLRP3. There has been extensive work to uncover the mechanisms involved in the activation of NLRP3, and so far there has been little consensus on a single universal mechanism linking all presently known activation signals. In this section, I will explain the processes which are based on abundant existing data. An overview of these mechanisms is provided in Fig. E.

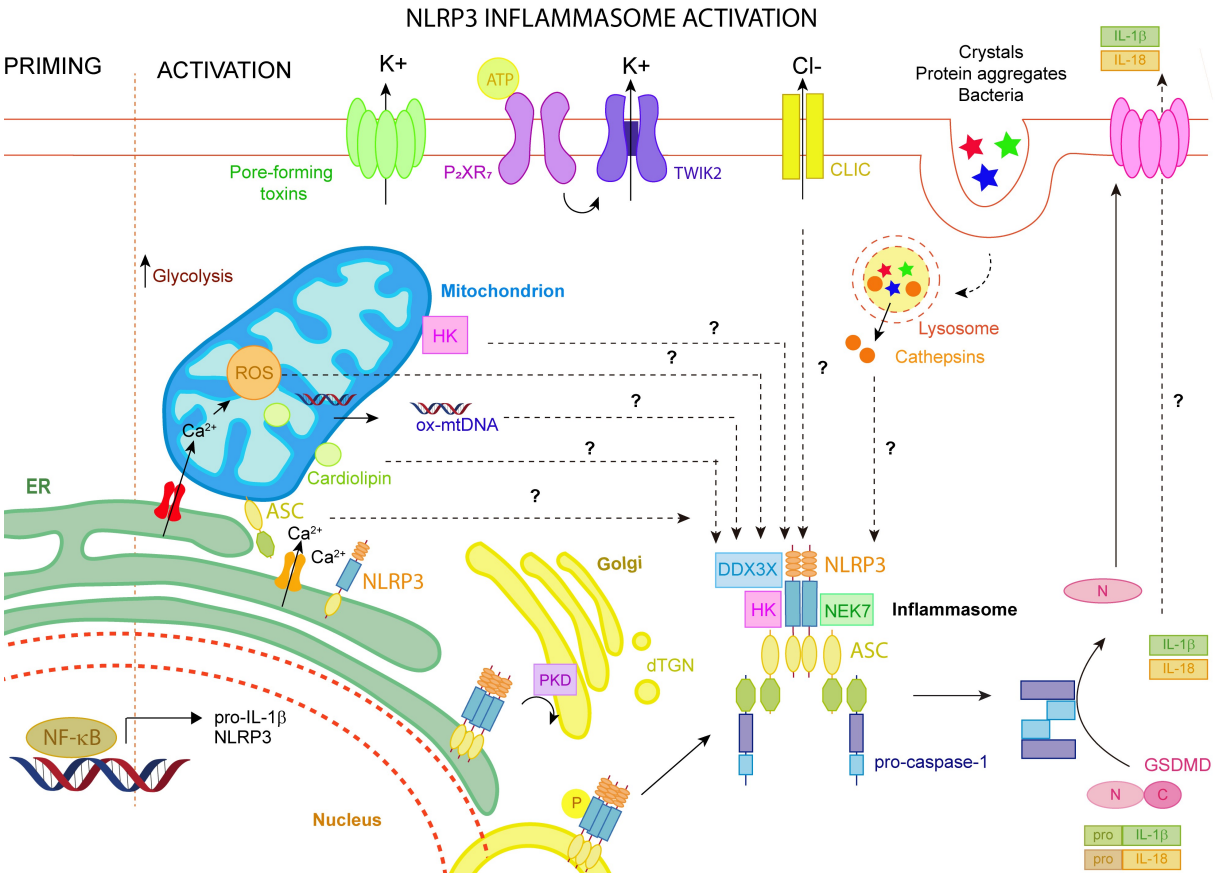


Fig. E. NLRP3 inflammasome activation is regulated by several cellular processes. The processes include K^+ efflux (through NLRP3 activators themselves or by channels such as TWIK2), release of Ca^{2+} from cellular stores and Ca^{2+} from the extracellular medium, mitochondrial damage through accumulation of mtROS and subsequent release of ox-mtDNA. Upon stimulation with crystals and/or particulate matter, release of lysosomal proteases upon lysosomal permeabilization can occur. Localization of NLRP3 and ASC is tightly regulated in response to these upstream mechanisms, but the exact steps leading to the final oligomerization of NLRP3 and assembly of the NLRP3 inflammasome complex are not yet fully understood. What links these mechanisms together is also poorly understood. These events converge into NLRP3 inflammasome assembly and activation of caspase-1 leading to cleavage of pro-inflammatory cytokines and of the pore-forming protein GSDMD, which translocates to the plasma membrane to form pores. More detailed description of mechanisms depicted can be found in the sections below.

2.4.2.1 – Ion fluxes

Ions play essential roles in many cellular functions. Functioning as cofactors, they can serve as accessory molecules to facilitate the activity of certain enzymes. They can also bind and inhibit proteins. They can act as secondary messengers to transduce a signal, and therefore constitute essential links in a signalling cascade. They have the potential to modify membrane polarity and their mobilization can help to cope with cellular stress. Hence, it is no surprise that changes in ion concentration were one of the first events proposed to be involved in NLRP3 inflammasome activation. So far, K^+, Na^+, Ca^{2+} and Cl^- ions have been proposed to be involved in this process, which I will further explore below. A summary of changes in ion mobilization can be found in Fig. F.

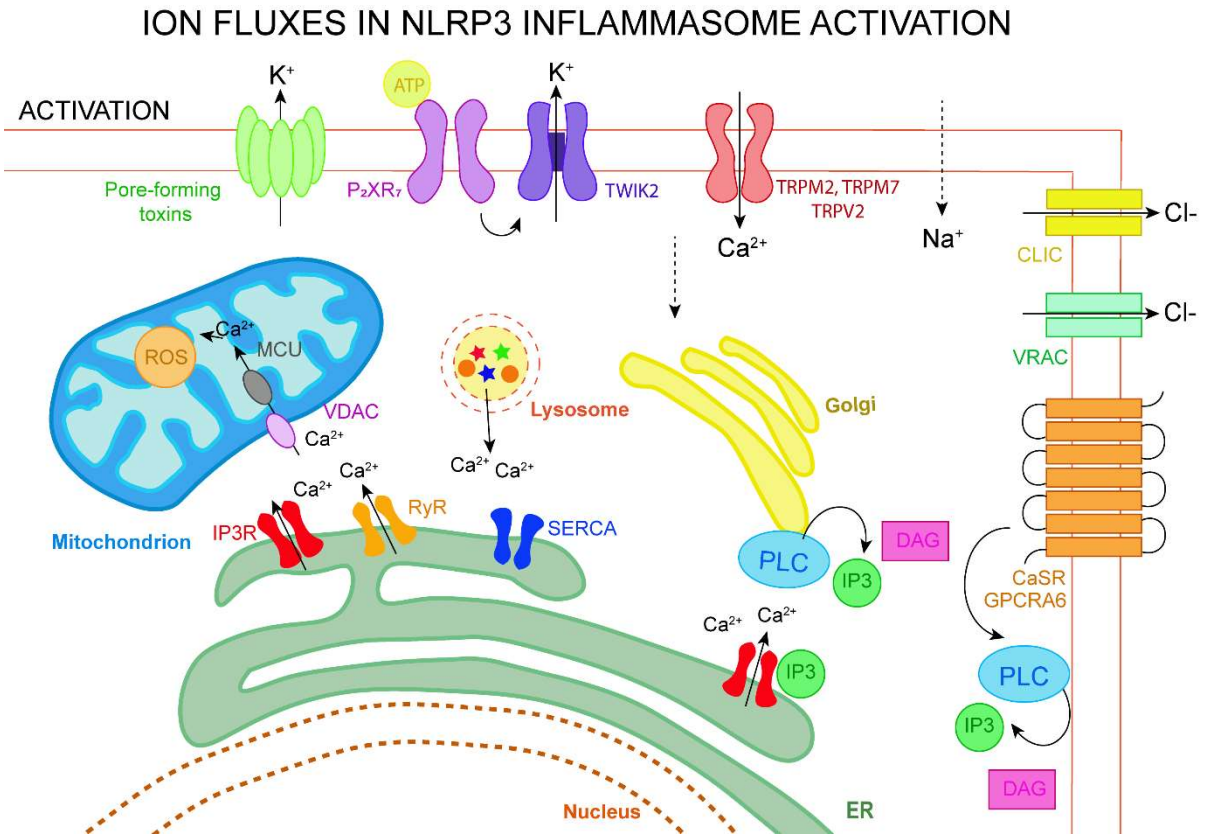


Fig. F. Ion fluxes during the NLRP3 inflammasome response. Induction of the NLRP3 inflammasome leads to changes in the concentration of K^+, Na^+, Cl^- and Ca^{2+}. K^+ efflux is common to most NLRP3 inflammasome activators. Extracellular ATP can activate the P_2XR_7 channel in the plasma membrane, which activates TWIK2 to mediate K^+ release. K^+ can also be released through NLRP3-activating pore-forming toxins, and can be induced by lysosomal permeabilization and mitochondrial damage. Typically release of K^+ leads to internalization of Na^+ from the extracellular medium. Cl^- efflux also serves to compensate the loss of positive charge and is released through VRAC and CLIC channels. Ca^{2+} accumulation in the cytosol can have several sources: activation of TRP channels (TRPM2/7 and TRPV2) in the plasma membrane or release of Ca^{2+} from organelles such as ER (through RyR and IP3R) or damaged compartments like the lysosomes. IP3 is required for IP3R activation, and is a product of PLC, which can be activated by GPCR such as CaSR and GPCRA6. Ca^{2+} can also be incorporated by mitochondria through the MCU channel, and leads to accumulation of mtROS and other consequences of mitochondrial damage.

K^+ efflux is to date the most consistent process in response to NLRP3 activators upstream of NLRP3 inflammasome assembly. In 2007, K^+ efflux was first proved to be a common trigger for NLRP3 inflammasome activation (Pétrilli et al., 2007). However, the mechanistic link between the loss of K^+ in the cytosol and the activation of NLRP3 is still unclear. In a study it was reported that K^+ efflux does not activate NLRP3 directly, but instead activates NIMA related kinase 7 (NEK7) to bind NLRP3 (Y. He et al., 2016, p. 7; H. Shi et al., 2016). Activation of the NLRP3 mutant R258W is not affected by high levels of extracellular K^+, suggesting that K^+ may affect NLRP3 inflammasome assembly inducing changes in NLRP3 conformation (Meng et al., 2009). With time, it has been proven that some new NLRP3 activators can robustly induce NLRP3 inflammasome activation independently of changes in K^+ flux. These are imiquimod and imiquimod derivatives such as CL097 (Groß et al., 2016). Although the mechanism of action of these molecules is still not completely understood, Groß and colleagues demonstrated that activation of NLRP3 in response to these molecules is highly dependent on production of mitochondrial

reactive oxygen species (mtROS). mtROS are generated in response to many stresses and are a typical sign of mitochondrial damage. These authors showed that upon imiquimod treatment mtROS were produced as a result of the inhibition of quinone oxidoreductases and of the mitochondrial complex I. However, a deeper understanding of how mtROS in this situation led to NLRP3 activation is still required. Additionally, K^+ efflux has been shown to play a role in the activation of Nlrp1b in response to anthrax lethal toxin of *Bacillus anthracis*, suggesting that even if K^+ is required, this mechanism might not be specific to NLRP3 (Pétrilli et al., 2007).

K^+ efflux is not an isolated event in the cells. Changes in a particular ion commonly result in the mobilization of other ions to keep the net charge and hence membrane potential constant. In their original study, Muñoz-Planillo and colleagues proposed that Na^+ influx is coupled to K^+ efflux, but higher concentrations of Na^+ are not the driving force for activation of the inflammasome (Muñoz-Planillo et al., 2013). However, one study in the same year proposed that changes in both Na^+ and K^+ are necessary to activate the NLRP3 inflammasome in response to alum and lysosomal destabilization (M. Katsnelson & Dubyak, 2013). The knowledge on Na^+ influx in inflammasome activation has not been expanded in recent years, and therefore the requirement of Na^+ influx in this process is still unclear.

Cations are not the only ions that have been proposed to be important for NLRP3 inflammasome activation. Cl^- efflux can also contribute to compensate the loss of a positive charge by K^+ efflux. Cl^- efflux was first shown to promote caspase-1 and IL-1β production in response to ATP even before the inflammasome was discovered (Verhoef et al., 2005). Later studies have supported a role for Cl^- efflux based on the observation that the NLRP3 inflammasome cannot become activated upon inhibition of Cl^- channels in the plasma membrane (Compan et al., 2012; Daniels et al., 2016; Tang et al., 2017). Originally the Volume regulated anion channel (VRAC) was reported to be required for Cl^- efflux and NLRP3 inflammasome activation (Daniels et al., 2016). Later it was proposed that VRAC is activated by chloride intracellular channels (CLICs) to induce NLRP3 inflammasome activation (Tang et al., 2017). Tang and colleagues additionally confirmed that Cl^- efflux was dependent on K^+ efflux, and proposed that its contribution to form the NLRP3 inflammasome activation consisted in promoting interaction of NLRP3 with NEK7. More recently, Green and colleagues have shown that LRRC8A, a core component of the VRAC Cl- channels is required for NLRP3 activation in the context of hypotonic shock, but not in response to DAMPs (Green et al., 2020). Altogether these reports indicate that Cl^- efflux may be an important event upstream of NLRP3 inflammasome activation.

Ca^{2+} is another cation that can shuttle in and out of the cell to keep charge homeostasis and maintain the membrane potential. In particular, Ca^{2+} influx has been highly debated in the field, and its significance in the pathway remains unclear. Ca^{2+} mobilization is more complex in comparison to Na^+ flow from the extracellular space. The concentration of Ca^{2+} outside of the cell is usually higher than in the cytosol, but not necessarily more elevated than in other compartments of the cell. Ca^{2+} can accumulate in several organelles such as lysosomes, the endoplasmic reticulum and mitochondria. There it can regulate the activity of some proteins or can function in responses to cope with different types of stress, such as the unfolded protein response in the ER. Therefore, a net increase of Ca^{2+} in the cytosol observed upon activation of the inflammasome (Murakami et al., 2012) can be difficult to analyse, as it cannot be traced back to the original source, or to a specific cellular compartment.

Additionally, when either Ca^{2+} channels in the plasma membrane or the ER open, the channels located in other compartments tend to follow to maximize the local concentration of Ca^{2+}. To date, several Ca^{2+} channels in the plasma membrane from the family of transient receptor potential (TRP) receptors, and in the endoplasmic reticulum such as the IP3 receptors (IP3Rs) and the ryanodine receptor (RyR) have been shown to participate in Ca^{2+} influx in this context (Compan et al., 2012; G.-S. Lee et al., 2012; Murakami et al., 2012; Triantafilou et al., 2013; Zhong et al., 2013). Mechanistically, activation of phospholipase C (PLC) by several NLRP3 inflammasome stimuli leads to production of IP3 which can activate the IP3Rs in the ER. PLC can also be activated by GPCRs in the plasma membrane such as the calcium-sensing receptor (CaSR) and GPCR6A to activate the inflammasome (G.-S. Lee et al., 2012). Currently it has been proposed that high Ca^{2+} in the cytosol could initially originate from the ER, as inhibition of Ca^{2+} mobilization by 2-aminoethoxy diphenylborinate (2-APB) strongly decreased NLRP3 inflammasome activation, while having no effect on NLRC4 and AIM2 inflammasomes (G.-S. Lee et al., 2012; Murakami et al., 2012). In line with these observations, it has been reported that K^+ promotes release of Ca^{2+} specifically from ER stores, which later on leads to further influx of Ca^{2+} by activating channels in the cell membrane (Yaron et al., 2015). One additional possible source of Ca^{2+} ions is the lysosome. Lysosomes could contribute to higher cytosolic Ca^{2+} levels upon lysosomal permeabilization, a common feature in silica- and alum-induced NLRP3 inflammasome activation (Murakami et al., 2012), Altogether, albeit based on observations from different cellular compartments, these studies coalesce in the understanding that Ca^{2+} influx is important for NLRP3 inflammasome activation. However, not all the literature points in this direction. In the aforementioned study from Muñoz-Planillo and colleagues, they showed that high Ca^{2+} in the cytosol is not sufficient to trigger NLRP3 inflammasome, and rather serves to balance the ionic charge of the lost K^+ (Muñoz-Planillo et al., 2013). Additionally, a more recent study showed that BAPTA inhibits the inflammasome independently of its function as a Ca^{2+} chelator, and instead proposed that Ca^{2+} influx is downstream of NLRP3 inflammasome activation (M. A. Katsnelson et al., 2015).

The participation of Ca^{2+} in NLRP3 inflammasome activation therefore remains highly debated. One of the main sources of this uncertainty is the lack of a tool to completely block Ca^{2+} mobilization, as in the current models, mobilization of Ca^{2+} is only partially dampened and NLRP3 activation is reduced but not blocked. Furthermore, the drugs used for Ca^{2+} studies can affect several channels upon longer incubation periods, thus complicating a clear understanding of which compartments indeed are play a role in NLRP3 inflammasome activation. Better tools should be used in the future to study Ca^{2+} influx in more depth. A second source of debate is what function Ca^{2+} influx could have in NLRP3 activation. Based on the observation that ASC-NLRP3 interaction *in vitro* is increased in the presence of higher concentrations of Ca^{2+}, one possibility is that Ca^{2+} could facilitate the recruitment of ASC to the NLRP3 oligomers (G.-S. Lee et al., 2012). It has also been postulated that instead of accumulation of Ca^{2+} in the cytosol, the key event for NLRP3 activation could be accumulation of Ca^{2+} in mitochondria derived from ER stores. This is based on the observation that excessive Ca^{2+} in the cytosol leads to a dramatic loading of Ca^{2+} in the ER, which channels this ion to mitochondria, resulting in mitochondrial Ca^{2+} overload and mtROS production (Camello-Almaraz et al., 2006; Lemasters et al., 2009). Therefore, Ca^{2+} could play an important role to induce mitochondrial damage, another rather controversial event upstream of NLRP3 inflammasome activation.

2.4.2.2 – Mitochondrial dysfunction

Mitochondrial dysfunction is defined as the loss of mitochondrial fitness and subsequent failure of mitochondrial processes. It can take place as a consequence of different events such as imbalances of membrane potential of the inner mitochondrial membrane, alteration of the activity at the level of the mitochondrial respiratory chain or issues in the transport of critical metabolites for normal mitochondrial activity. These problems usually result in two main deleterious consequences: a lower energetic output and the accumulation of toxic molecules released upon defects in the oxidative respiratory chain known as mtROS. Additionally, extensive mitochondrial damage can lead to the release of mtDNA into the cytoplasm and changes in the lipid composition of mitochondria, such as the flipping of cardiolipin from the inner to the outer mitochondrial membrane. These events are upstream of several cell stress responses, one of them being NLRP3 inflammasome activation.

Indeed, ROS production, especially from mitochondria, was one of the first triggers to be identified to activate the NLRP3 inflammasome (Heid et al., 2013; Zhou et al., 2011). Many activators of this inflammasome have been shown to trigger the generation of mtROS at non-homeostatic levels. What is less clear is what specific role mtROS play in the induction of NLRP3 activation. A study indicated that ROS are particularly important in the priming step, as NLRP3 expression appears to be dampened by ROS inhibitors (F. Bauernfeind et al., 2011). Indeed, the nuclear factor erythroid 2-related factor (NRF2) can inhibit NLRP3 inflammasome activation by regulating antioxidant gene expression, and thus modulating mtROS levels (G. Liu et al., 2017). Previous studies have shown that activation of NRF2 also lowers activation of the NF-κB-dependent transcriptional programs, which could explain why levels of NLRP3 during priming are low in the absence of ROS in the cell (Wenge Li et al., 2008). However, the relevance of NRF2 as a regulator of NLRP3 is not yet clear. *Nrf2*$^{-/-}$ murine macrophages do not engage a robust inflammasome assembly in response to various stimuli of Nlrp3 and Nlrc4, suggesting that Nrf2 participation in this pathway is not so direct (C. Zhao et al., 2014, p. 2). Other investigators have proposed that ROS has a particularly important role in early stages of the activation step by regulating activation of NEK7, to allow its interaction with NLRP3 (Y. He et al., 2016, p. 7; H. Shi et al., 2016). As previously mentioned, imiquimod-derived NLRP3 inflammasome activation seems to also strongly rely on the production of mtROS (Groß et al., 2016). Following these data, it is to be expected that inhibition of mitochondrial ROS production should block to some extent NLRP3 activation. Additionally, activation of the NLRP3 inflammasome in a ROS-independent manner has also been reported (Jabaut et al., 2013), thus indicating that ROS accumulation is not the universal event responsible for NLRP3 inflammasome activation.

Release of mtDNA is also a common event linked to mitochondrial dysfunction. Noteworthy, NLRP3^{NACHT} can bind nucleotides, albeit its ability to interact with DNA does not seem to be essential for NLRP3 inflammasome assembly. Excitingly, oxydized mtDNA and NLRP3 have been shown to co-localize in the cytosol in response to NLRP3 inflammasome activators (Shimada et al., 2012). This observation supports the possibility that mtDNA released by damaged mitochondria downstream of activating signals could directly bind and activate NLRP3. Soon after this novel discovery, the first concerns were raised on the specificity of the inflammasome response to mtDNA. There are other

sensors that can bind to dsDNA, and therefore it was to be expected that this molecule could be an activator for a wider range of inflammasomes. Interestingly, recent studies have shown that mtDNA in its native state can interact directly with the dsDNA-sensing Aim2 PRR to form the Aim2 inflammasome, while oxidated mtDNA (ox-mtDNA) is particularly important for the onset of Nlrp3 inflammasome activation (Zhong et al., 2018). Supporting the important role of mtDNA, Zhong and colleagues also showed that LPS can induce the replication of mtDNA in murine bone marrow-derived macrophages (BMDMs), along with mtROS production, which could serve to create an environment for a more robust activation of Nlrp3 downstream of the activating signal.

Cardiolipin has also been proposed to play an important role for NLRP3 inflammasome activation (Iyer et al., 2013). Cardiolipin in a basal state resides in the inner mitochondrial membrane, and in response to mROS accumulation flips to the organelle's outer membrane. When the inflammasome is induced, cardiolipin appears to co-localize with Nlrp3 and partial inhibition of cardiolipin synthesis using saturated fatty acids affects the ability of macrophages to assemble the complex. More recently it has been reported that both Nlrp3 and caspase-1 can directly bind cardiolipin in the outer mitochondrial membrane, suggesting that cardiolipin might have a role in the spatial localization of the NLRP3 inflammasome components (Elliott et al., 2018). Finally, cardiolipin has been shown to have high affinity to the N-terminal fragment of GSDMD (X. Liu et al., 2016). The presence of cardiolipin in the outer mitochondrial membrane could thus serve to exacerbate mitochondrial damage by the formation of pores at this location. This becomes even more interesting when one takes into account that bacterial membranes also contain cardiolipin, consistent with the origin of mitochondria from symbiotic bacteria. Therefore, cardiolipin in the bacterial membrane could potentially recruit GSDMD and lead to formation of pores in this structure, which in turn would contribute to the lysis and the digestion of the pathogen.

The exhaustion of mitochondrial units in a healthy cell usually triggers the activation of mitophagy, a form of macroautophagy that consists in the engulfment and digestion of mitochondria that are no longer functional. In line with the previous observations, inhibition of mitophagy in phagocytes leads to the accumulation of defective mitochondria, which is sufficient to activate the NLRP3 inflammasome (Nakahira et al., 2011). The great extent of damaged mitochondria in the context of the inflammasome could render the mitophagy machinery incapable of clearing completely all the dysfunctional organelles, thus compromising the possible efforts to halt the inflammasome.

2.4.2.3 – Lysosomal permeabilization

Lysosomal membrane permeabilization (LMP) is the process by which lysosome membranes become unstable and porous, which typically leads to leakage of protons to the cytosol, the subsequent increase of the lysosomal pH, and even the release of lysosomal enzymes when the lysosomal membrane is ruptured. Lysosomes have been termed as the bins of the cell due to their ability to capture toxic substances in the cell and to drive their degradation. Consequently, it is of high importance to swiftly repair breaks in the lysosomal membrane in order to keep the cell healthy. Upon mild permeabilization of the lysosomal membrane, the endosomal sorting complex required for transport (ESCRT) machinery is recruited, which binds to the site of the break and sews the membrane back together (Radulovic et

al., 2018). When damage is too great to repair, lysosomes are instead targeted for a selective form of macroautophagy known as lysophagy (Hung et al., 2013). Both processes are intended to maintain homeostasis and avoid the release of hazardous components to the cytosol.

LMP takes place in response to some activators of the NLRP3 inflammasome, and lysosomes in these conditions seem not to be repaired properly, and cannot be cleared out by lysophagy. Actually, it has been proposed that LMP is induced by some activating signals that are endocytosed and accumulated in these acidic compartments, and the subsequent rupture leads to the release of lysosomal components to the cytosol that activate NLRP3. These activators are quite distant in terms of their composition, but are often big and organized in crystalline structures. The first case of such activation of NLRP3 inflammasome was described for phagocytosis of monosodium urate (MSU) crystals, the causative agent of gout (Martinon et al., 2006). Other structures have since been shown to activate the inflammasome in a similar fashion, including asbestos and silica (Dostert et al., 2008), the protein PrPSc in prion disease (F. Shi et al., 2012), extracellular cholesterol, and particulate matter (Hornung et al., 2008), a term that encompasses solid particles that trigger inflammation in the lung such as silica or asbestos. In addition, phagocytosis of some pathogens like bacteria from the group B of the genus *Streptococcus* can likewise trigger LMP (Gupta et al., 2014).

The mechanism underlying LMP-derived NLRP3 activation is not yet well understood. In their study using particulate matter, Hornung and colleagues reported that NLRP3 is activated as a result of the leakage of the lysosomal enzyme cathepsin B (Hornung et al., 2008). In their study they showed that inhibition of cathepsin B dampened NLRP3 inflammasome activation. Currently, other cathepsins have been proposed to play a role in NLRP3 activation dependent on their release into the cytosol, including Cathepsin B, X, L and S. However, if not blocked simultaneously, the NLRP3 inflammasome can still assemble (Orlowski et al., 2016), thus suggesting that cathepsins have rather redundant roles. This redundancy is supported by an earlier study that showed murine cathepsin B-depleted BMDMs responded normally to hemozoin and crystal treatments and were able to mature caspase-1 and secrete IL-1β (Dostert et al., 2009). Noteworthy, low grade LMP by low concentrations of Leu-Leu-O-methyl-esther (LLOMe) induces K^+ efflux in the plasma membrane, which has been already shown to be a strong inducer of NLRP3 inflammasome activation (M. A. Katsnelson et al., 2016). Conversely, higher concentrations of LLOMe do not induce any changes in cytosolic K^+ and do not activate the NLRP3 inflammasome, while still triggering cell death. This suggests that NLRP3 inflammasome formation requires an organized sequence of events in response to LMP, which likely do not get orderly activated in the event of a rapid and dramatic lysosomal rupture.

2.4.2.4 – Metabolic shift and glycolysis

Inflammation and metabolism are known to be linked to each other. A clear example of dependency of inflammation on metabolism is the pathogenesis of the metabolic syndrome. The metabolic syndrome is a constellation of coinciding disturbances including glucose intolerance, obesity, hypertension and dyslipidemia, which prime an individual to the development of metabolic diseases such as cardiovascular disease, type 2 diabetes or fatty liver disease. This syndrome is often accompanied by

low-grade inflammation, which plays an essential role in its pathophysiological consequences. In the context of the NLRP3 inflammasome, extracellular cholesterol accumulates in the arteries that can be phagocytosed by macrophages and activate the NLRP3 inflammasome, and this is in part responsible for the formation of atherosclerotic lesions (Duewell et al., 2010). Similarly, NLRP3 can also be activated by circulating free fatty acids, which are more abundant in type 2 diabetes patients (Zhou et al., 2010). Therefore, aberrant accumulation of certain metabolites can drive inflammatory processes by activating the inflammasome. Reciprocally, induction of inflammatory programs can produce drastic changes in the cellular metabolism, which often can serve to prime the cell to maximize the inflammatory potential. In this section, we will explore some of the main metabolic changes the cell undergoes for this purpose.

As indicated above, one of the better studied metabolic adaptations in inflammasome-primed cells is the mitochondrial damage. The latter results in loss of function of mitochondria, rendering the respiratory chain non-functional and leading to accumulation of mtROS in the cell. One obvious consequence of ineffective mitochondria is the shift from the tricarboxylic acid (TCA) cycle to the glycolytic pathway to maintain the necessary energy production to sustain the cell in the short term. Indeed, priming of macrophages with both LPS and PMA has been shown to favour the transcriptional upregulation of genes codifying glycolytic enzymes and a concomitant increase of the glycolytic flux, although mitochondrial state was not addressed in these studies (Mehrotra et al., 2014; Tannahill et al., 2013). This shift was shown to be dependent on the activation of hypoxia-inducible factor 1α (HIF-1α) leading to an in increase in IL-1β transcription. Additionally, TCA block leads to accumulation of the metabolite succinate, which further stabilizes HIF-1α. Interestingly, a recent study by Humphries and colleagues showed that fumarate, another intermediate produced in the TCA has an inhibitory function on pyroptosis (Humphries et al., 2020). Fumarate is required for succination of GSDMD, which leads to a defect in its processing and oligomerization, and effectively blocks cell death. Therefore, favouring the glycolysis over TCA can also serve to minimize the production of inhibitors of the NLRP3 inflammasome inflammatory pathway. Glycolytic flux is not only increased during priming, but also during the activation step through activation of mammalian target of rapamycin complex 1 (mTORC1) (Moon, Hisata, et al., 2015). In this study, the authors showed that silencing of hexokinase 1 (HK1) leads to inhibition of NLRP3 inflammasome activation. It is however not clear whether the contribution of HK1 to inflammasome activation is due to a globally increased glycolytic flux or to a function independent of glycolytic regulation. In another study, pyruvate kinase isoform M2 (PKM2) was shown to be required to activate the NLRP3 and AIM2 inflammasomes, but not the NLRC4 inflammasome, and pharmacological inhibition of PKM2 dampened inflammasome activity (Xie et al., 2016). However, PKM2 did not seem to be directly activating the inflammasome, but rather acted by increasing glycolytic flux itself.

Not all studies support the vision that glycolysis stimulates NLRP3 inflammasome activation. One of these studies has further supported a possible role of HK in inflammasome activation independently of its metabolic function (Wolf et al., 2016). This study showed that N-acetylglucosamine (GlcNAc) from digested bacterial walls can bind to HK at the mitochondrial surface and promote its re-localization into the cytosol. This process was proposed to promote NLRP3 inflammasome activation independently of K^+ efflux and mitochondrial damage. In contrast, Sanman and colleagues reported that the inflammasome is induced not by an increase of glycolytic flux or glycolytic enzyme function, but by the

exact opposite (Sanman et al., 2016). They inhibited enzymes from early and late glycolysis and showed that this lead to higher levels of caspase-1 cleavage and inflammasome activation. They proposed that this was due to an increase in the nicotinamide adenine dinucleotide (NAD) NAD+/NADH ratio, which they speculated resulted in production of mtROS. However, some of the figures representing the results in this article do not appear very convincing to me. Furthermore, it is generally understood that it is the high NADH/NAD+ ratio, and not the opposite, which drives the generation of mtROS (Murphy, 2009). Therefore these results should be handled with precaution when interrogating the role of glycolysis in inflammasome activation.

Alongside with sugar metabolism, other metabolic programs can contribute to NLRP3 inflammasome activation. However, they appear to be activating signals per se, rather than mediators of events upstream of NLRP3 inflammasome activation. As mentioned above, synthesis or diet-derived free fatty acids can activate the NLRP3 inflammasome (Moon et al., 2016; Moon, Lee, et al., 2015; Wen et al., 2011). Saturated fatty acids can suppress AMPK activation and in turn increase ROS production by increasing levels of mitochondrial NADPH oxidase 4 (NOX4) (Wen et al., 2011). Activation of AMPK through its agonist metformin has been showed to dampen NLRP3 inflammasome in a model of diabetes in mouse, supporting a role of AMPK in down-tuning NLRP3 activation, (F. Yang et al., 2019). Therefore, AMPK activation negatively regulates NLRP3 inflammasome activation, probably through its involvement in the induction of autophagy. The reactivity of the NLRP3 inflammasome does not seem to be limited to the host's own fatty acids, but appears to be activated also with microbial-derived metabolites known as short chain fatty acids (SCFA). SCFA do not seem to act through an increase in ROS production, but instead can act as agonists to the GPR43 and GPR109A coupled to Gi/o proteins. This interaction leads to changes in ion fluxes and membrane polarization (Covington et al., 2006) , which would be responsible for NLRP3 activation in this context.

Another lipid that has been shown to play a role in NLRP3 activation is cholesterol. It is known that cholesterol crystals can by phagocytosed by macrophages, and their accumulation in the lysosome can result in lysosomal permeabilization and subsequent NLRP3 inflammasome activation (Duewell et al., 2010). However, it has been recently shown that resident cholesterol in the cellular membranes is also involved in the activation of the NLRP3 inflammasome. In 2018 two articles were published discussing the role of this small molecule in this pathway. De la Roche and colleagues showed that blocking cholesterol trafficking in the endocytic axis partially inhibits NLRP3 inflammasome activation by modifying the cholesterol levels at the ER (de la Roche et al., 2018). In contrast, Guo and colleagues proposed that cholesterol depletion in the ER drives NLRP3 inflammasome activation by inducing the translocation of a complex formed by the sterol regulatory element-binding protein 2 (SREBP2), the SREBP cleavage-activating protein (SCAP) and NLRP3 to the Golgi apparatus (Guo et al., 2018). While the two findings hint at different mechanisms, it is clear that lipid composition of cellular organelles has a non-negligible effect on NLRP3 inflammasome activation.

In line with an inflammatory role of circulating glucose, fatty acids and cholesterol, caloric restriction is known to reduce inflammation. Caloric restriction causes a decrease in blood glucose levels and drives the switch of metabolism towards β-oxidation of lipids to obtain energy. Metabolites accumulated as a consequence of this shift include ketone bodies such as β-hydroxybutyrate (BHB), which have been

shown to inhibit the NLRP3 inflammasome specifically in response to several stimuli by blocking K^+ efflux and ASC oligomerization (Youm et al., 2015).

Clearly, there is compelling evidence that changes in metabolism have key functions in the NLRP3 inflammasome response, albeit the mechanisms underlying these changes are incompletely understood.

2.4.2.5 – Accessory proteins in the NLRP3 inflammasome

The NLRP3 inflammasome has traditionally been understood to be composed by NLRP3, caspase-1 and ASC, which is required to bring the sensor and the effector proteins together in space. However, in the recent years more molecules have been shown to be localized to this structure, possibly to aid in the formation of the complex or its enzymatic activity. A couple of these proteins have been mentioned in previous sections. HK1 has been proposed to aid in the localization of NLRP3, albeit it is not yet clear whether it stays in the complex in the longer term. There have not been recent developments on HK1 function. One of the recent advances in the field has been the discovery of NEK7 in the NLRP3 inflammasome complex. NEK7 is a mitotic kinase required for the formation of the mitotic spindle and the separation of centrosomes (Souza et al., 2015). Nek7 was first found in the context of Nlrp3 inflammasome activation in a genetic screen of murine immortalized BMDMs (iBMDMs) based on cell death resistance upon nigericin treatment (Schmid-Burgk et al., 2016). Reports in the same year helped to clarify the function of NEK7 (Y. He et al., 2016; H. Shi et al., 2016). These studies showed that NEK7 is required for canonical and noncanonical NLRP3 inflammasome activation downstream of K^+ efflux. They showed that NEK7 can bind directly to the LRR domain of NLRP3, which triggers NLRP3 self-oligomerization. This interaction is dependent on the catalytic domain of NEK7, although so far NLRP3 has not been shown to be phosphorylated by this kinase. Additionally, co-localization of NLRP3 and NEK7 has been observed in a cryo-electron microscopy structure of the inflammasome (Sharif et al., 2019), further supporting an important role of NEK7 in NLRP3 inflammasome formation. However, it has been recently shown that in some cases NEK7 is not absolutely required for NLRP3 inflammasome activation. Indeed, transforming growth factor β-activated kinase 1 (TAK1)-dependent priming has been shown to bypass the absence of NEK7 in both murine and human systems (Schmacke et al., 2019). Thus, NEK7 is not an essential member of the inflammasome complex.

Another protein that has been recently shown to localize in the NLRP3 inflammasome is the DEAD-Box Helicase 3 X-Linked (DDX3X) (Samir et al., 2019). DDX3X is a well-known factor involved in the formation of stress granules, a type of aggregates composed of RNA and other molecules that form in the cytoplasm during cellular stress. In this study the authors revealed that DDX3X found in the NLRP3 inflammasome, and its sequestration in stress granules blocks NLRP3 inflammasome assembly. Importantly, *Ddx3x*$^{-/-}$ myeloid murine BMDMs secreted less IL-1β in response to LPS, but this reduction was only partial, suggesting that DDX3X is not completely essential for Nlrp3 inflammasome activation either.

All in all, DDX3X localization in the inflammasome seems to serve not only the purpose of activating this platform but as a checkpoint to lead the cell into cell death or into a coping mechanism such as stress

granule formation. Demonstrating how the cells direct this protein to perform one or another function will be important to have a good insight into how inflammation is prioritized over other mechanisms intended to keep cell homeostasis. Likewise, NEK7 is required during mitosis, and its targeting to the inflammasome might be essential to stop the cell from dividing in inflammatory conditions.

2.4.2.6 – Regulation of NLRP3 localization in time and space

It is increasingly evident that post-translational modifications and localization of NLRP3 play a key role to permit the formation of the inflammasome. Indeed, while NLRP3 localizes mostly in the cytosol and the ER compartment upon cell priming, activation signals lead to changes of its localization over time.

As mentioned previously, one of the most common means to modify the function of a protein is through post-translational changes. To the phosphorylation and ubiquitylation events described in the priming section, new modifications can be added to the NLRP3 protein to regulate its capacity to oligomerize and its localization in the cell. For instance, one of the crucial deubiquitylation events is induced by the K63-specific deubiquitylase BRCA1/BRAC2-containing complex subunit 3 (BRCC3) (Ren et al., 2019). BRCC3 removes K63 ubiquitin chains in the C-terminal of NLRP3 to block its proteasomal degradation, albeit the specific residues affected remain unknown (Py et al., 2013). Initially it was shown that early in the activation step, NLRP3 can become phosphorylated by different kinases. One of the first enzymes proposed to do so is the double stranded RNA-dependent protein kinase (PKR), also known as EIF2AK2 (B. Lu et al., 2012) but the role of this protein has been debated in later studies (Y. He et al., 2013). Another protein kinase reported to be involved in NLRP3 function was protein kinase A (PKA) by phosphorylating NLRP3 at the S295 (S298 in mouse) residue, which results in the blockade of inflammasome (Guo et al., 2016). However, my host laboratory has shown that this phosphorylation is key to define localization of NLRP3 in the cell compartments, and both premature phosphorylation and no phosphorylation at all fully blocked the potential of NLRP3 to assemble the inflammasome (Zhang et al., 2017).

One of the earliest events observed in response to DAMPs and PAMPs is the relocation of mitochondria close to the ER at the perinuclear region by transport along acetylated microtubules (Misawa et al., 2013). This already happens during the priming step, which could be a consequence not only of mitochondrial damage, but suggests that changes in the mitochondrial network might be relevant for the activation of the inflammasome. Interestingly, NLRP3 recruitment to mitochondria has been observed to happen already during the priming step (Elliott et al., 2018). NLRP3 has been proposed to translocate from the ER membrane to mitochondria upon stimulation by priming and activating signals. It has been reported early on that assembly of the inflammasome takes place upon mitochondrial outer membrane permeabilization (MOMP), one of the main events resulting from mitochondrial damage. Indeed, inhibition of voltage dependent channels and destabilization of the mitochondrial membrane reduced NLRP3 inflammasome activation (Zhou et al., 2011). This finding is supported by the fact that overexpression of Bcl-2, which blocks mitochondrial outer membrane permeabilization, can inhibit the inflammasome. It is possible that the link for NLRP3 trafficking to the mitochondrial membrane is newly exposed cardiolipin in the outer mitochondrial membrane resulting from mitochondrial damage (Iyer et

al., 2013), but other possible mediators such as mitochondrial antiviral signalling proteins (MAVS) have been proposed (N. Subramanian et al., 2013). However, an article contending the relevance of MAVS was published short after this first finding, (S. Park et al., 2013) and therefore the relevance of MAVS should be reassessed. Alternatively, one of the first mechanisms proposed for NLRP3 translocation was based in the observation that upon incubation with microtubule destabilizing drugs, NLRP3 and microtubule-affinity regulating kinase 4 (MARK4) can interact and migrate along acetylated microtubules to mitochondria (Franklin et al., 2014, p. 4; X. Li et al., 2017). Furthermore, we have demonstrated that NLRP3 localizes in the ER mitochondria-associated membranes (MAMs) in close proximity to the Golgi apparatus (Zhang et al., 2017). We showed that oligomerized NLRP3 can become phosphorylated in this compartment, thereby promoting its release from the membrane to nucleate ASC and form the inflammasome. However, most studies where NLRP3 localization was studied have an important technical limitation: the signal of NLRP3 antibodies does not appear to be very specific. Alternatively the approach to overexpress proteins tagged with fluorescent proteins has been taken, raising the concern that some of these results could be not representative of the physiologic event.

Recently, a new mechanism has been proposed that leads to NLRP3 inflammasome assembly. Disruption of the trans-Golgi network in what is termed dispersed trans-Golgi network (dTGN) has been described as a cellular event downstream of both K^+ efflux-dependent and independent activators (J. Chen & Chen, 2018). NLRP3 contains a polybasic stretch of 4 lysine residues which interacts with the negatively charged PI4P, a highly abundant inositol in the TGN. These authors showed that this interaction leads to the clustering of NLRP3 and subsequent assembly of the ASC filaments at the dTGN. We had previously observed the close proximity of NLRP3 to the Golgi, albeit we did not report particular Golgi structures (Zhang et al., 2017). According to our observations, diacylglycerol (DAG) in the Golgi recruits protein kinase D (PKD), which then can phosphorylate NLRP3 in the S295 residue, and allows release of oligomerized NLRP3. In light of the data from Chen and colleagues, it is possible that NLRP3 recruitment could take place initially at the MAMs, and proximity to the Golgi in the GMAM compartment could facilitate NLRP3 translocation to the dTGN. However, further experiments will be required to determine what the exact sequence of events is in this context. Following the new dTGN paradigm, it has been proposed that NLRP3 translocation to the Golgi takes place as a complex with SREBP2 and SCAP (Guo et al., 2018). Undoubtedly, more studies will soon arise to define what mechanism underlies NLRP3 translocation to the Golgi.

Modifications on NLRP3 such as S295 phosphorylation are essential not only during inflammasome activation, but can also be key to dampen inflammasome activity, and could serve as a mechanism to self-limit inflammation. Such is the phosphorylation of Y861 (Y859 in mouse), which reduces inflammasome activity by targeting NLRP3 to the autophagosome (Spalinger et al., 2016, 2017). This event takes place in an ASC-dependent manner, and thus this phosphorylation is intended to limit inflammasome activity later on. Conversely, protein tyrosine phosphatase non-receptor type 22 (PTPN22) can be recruited in an equally ASC-dependent manner to NLRP3 and dephosphorylates Y861 in response to activating stimuli to sustain the already active inflammasome and inflammasome-dependent secretion of IL-1β (Spalinger et al., 2016). Possibly, each specific situation will determine which the best strategy for the cell to follow is.

2.4.2.7 – Post-translational modifications on ASC

NLRP3 is not the only protein that needs to be modified for its proper sorting in the cell. One similar example is the ASC protein which, on its native state, cannot be recruited to NLRP3.

To date, two main modifications on ASC are known to be important for inflammasome assembly. First, ASC is linearly ubiquitylated by by iron responsive element binding protein 2 (IRP2) ubiquitin ligase 1L (HOIL-1L) in the linear ubiquitin assembly complex (LUBAC). This ubiquitylation is required for the interaction between ASC and NLRP3, although it is not yet clear whether this happens upstream or downstream of the activating signal (Rodgers et al., 2014). ASC can also be ubiquitylated with K63 chains upon LPS priming and needs to be later deubiquitylated by ubiquitin specific peptidase 50 (USP50) downstream of the activating signal (J. Y. Lee et al., 2017). The other key event necessary for ASC function is its phosphorylation on ASC^{CARD} (Hara et al., 2013). This phosphorylation is required for ASC interaction with immature pro-caspase-1. There are two tyrosine kinases that have been shown to be involved in this post-translational modification: Spleen tyrosine kinase (SYK) and JNK (Hara et al., 2013). Both kinases can phosphorylate among others Y146 (Y144 in mouse), a critical residue for NLRP3 inflammasome assembly. Activation of SYK or JNK depends on the upstream events leading to the formation of the complex. While SYK becomes activated upon infection with *Candida albicans* (Gross et al., 2009), the MAP3K7-JNK axis is rather activated downstream of lysosomal damage (Okada et al., 2014). It is not yet clear whether *Candida* species can likewise accumulate in phagolysosomes and trigger LMP, and therefore the interplay between these two kinases needs to be looked at in more detail in the future. Altogether, the outcome of this particular phosphorylation is the translocation of ASC to the site where NLRP3 resides. In turn, although pro-caspase-1 seems not to be sensitive to post-translational modifications, its localization in the cytosol changes upon nucleation of ASC, attracted by the available nucleated ASC^{CARD}. Additionally, pro-caspase-1 has been shown to interact with cardiolipin in the outer mitochondrial membrane from damaged mitochondria, supporting the notion of mitochondria serving as a docking site for inflammasome assembly (Elliott et al., 2018; Iyer et al., 2013). However, given the new evidence suggesting that dTGN is instead leading the formation of the inflammasome, more experiments to clearly distinguish where this process takes place will be necessary.

2.5 – The NLRP3 inflammasome in disease

As one of the main mechanisms for the first line of defence from dangerous entities, the activation of the NLRP3 inflammasome response needs to be tightly coordinated. Timely and robust activation are key to limit the spread of pathogens, and to neutralize the threat in the host as soon as possible to minimize damage and severity of disease. Therefore, detailed studies of the inflammasome could be useful to find new ways to support the activation of this complex in order to amplify its activity in case of need. In practice, however, it is aberrant activation of NLRP3 that has been found to be particularly prevalent in a wide spectrum of diseases. Therefore, a better understanding of how the NLRP3 inflammasome is activated can give rise to possible targets to tune down NLRP3 in the event of excessive inflammation. In this section we will explore what some of the self-regulatory mechanisms involved in NLRP3 inflammasome function are, how loss of proper control of this process can be

responsible for the appearance of some diseases, and finally which are some venues that are being currently used to limit the harm derived from an overactive NLRP3 inflammasome.

2.5.1 – Regulation of NLRP3 inflammasome function

Our organism has developed strategies aimed at the reduction of the collateral damage caused in the host by a sustained activation of the NLRP3 inflammasome. These mechanisms are specific to this complex, but similar processes have been found to regulate other inflammasomes. We will describe some of the main negative regulators known to modulate NLRP3 function, and negative feedback mechanisms aimed at terminating inflammasome signalling.

Negative regulation can be done at different levels of the pathway leading to inflammasome activation: before NLRP3 becomes activated, on NLRP3 oligomerization itself, at the step of ASC and pro-caspase-1 recruitment to the complex, and also downstream of the activation of the complex to dampen the propagation of inflammation. Here we will explore what are some of the main mechanisms involved at each step (Fig. G).

Translocation of proteins to compartments where they cannot exercise their function is an efficient approach to prevent inflammasome formation upstream of the activation signal. For example, ASC is captured in the nucleus by IκB kinase-α (IKKα) in the priming step, thereby making it unavailable for other proteins in the cytosol (Martin et al., 2014). Consistently, loss of IKKα leads to hyper-activation of the inflammasome. Additionally, another regulatory mechanism that can be initiated during the priming step is the interferon-mediated repression of pro-IL-1β and pro-IL-18 transcription. Interferons are cytokines that play key functions to activate immune cells and induce defence responses. Type I interferons in particular have been shown to reduce expression of the precursors of IL-1β and IL-18 in monocytes, macrophages and dendritic cells (Guarda et al., 2011; Mayer-Barber et al., 2011), through a STAT-dependent mechanism. Type II interferons have similarly been shown to be able to repress production of pro-IL-1β, albeit only in monocytes, and the mechanism underlying this inhibition remains obscure (Mayer-Barber et al., 2011). Furthermore, type II interferons can play additional roles to dampen inflammasome activation through the production of inducible nitric oxide synthase iNOS and subsequent NO-mediated NLRP3 nitrosylation (Mishra et al., 2013). In this study they showed that NO generated by iNOS can inhibit the inflammasome assembly via thiol nitrosylation of NLRP3. These are all functions that had already been described previously, as they serve as a mechanism of desensitization of NLRP3, and represents one of the earliest tools activated to self-limit the activity of the inflammasome.

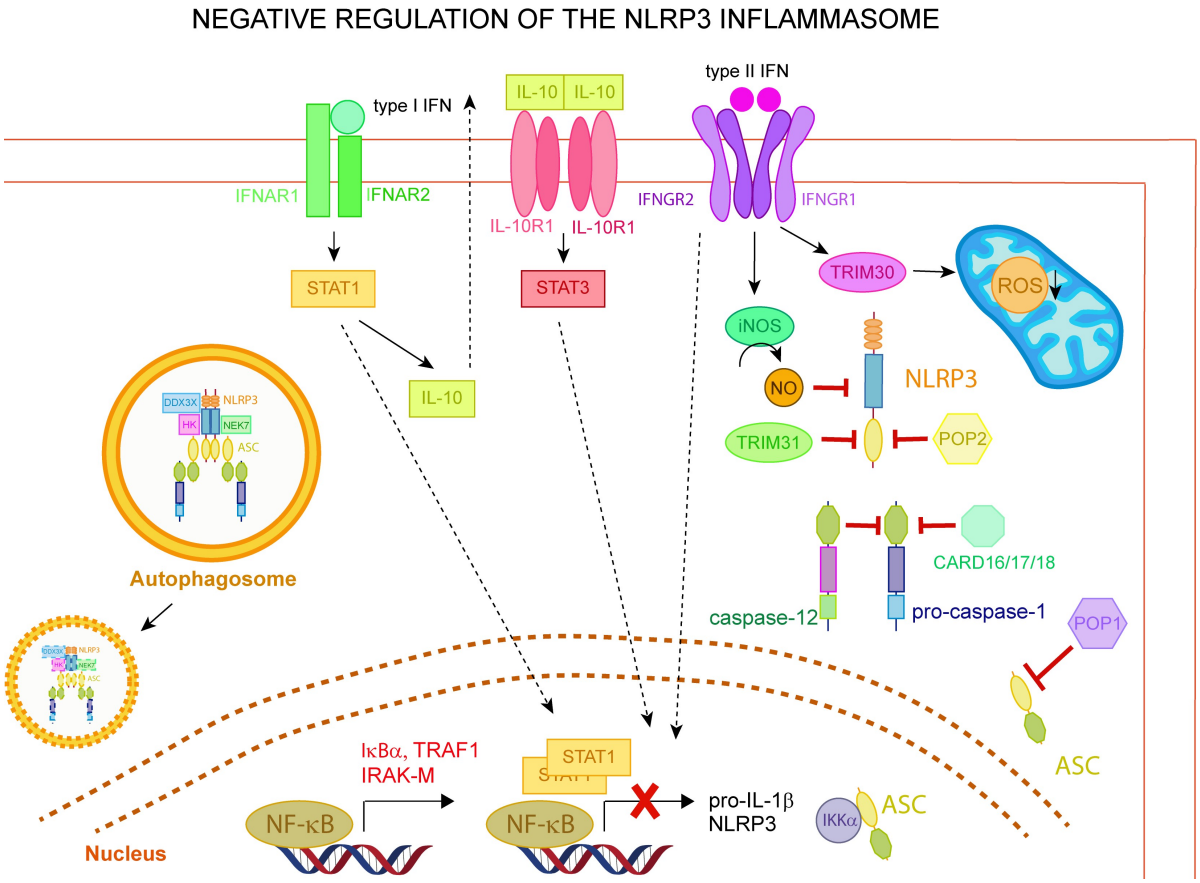


Fig. G. Mechanisms of negative regulation of the NLRP3 inflammasome. The NLRP3 inflammasome response is tuned down by mechanisms that become activated at different points of the signalling cascade. During the priming step through TLR4, mechanisms of desensitization of NLRP3 are activated, directly by expression of negative regulators through NF-κB, or by their activation downstream of pro-inflammatory cytokines secreted in this pathway like type I and II interferons. Essentially, type I interferons signal through STAT proteins to block expression of Pro-IL-1β and NLRP3. This can take place directly by translocation of STAT1 to the nucleus, or by the paracrine effect of IL-10 secreted in the process. Type II interferons lead to this effect in monocytes, while they can also inhibit NLRP3 directly through NO production. Simultaneously TRIM30 is activated, which leads to lower production of mtROS. A family of decoy proteins with similar sequences to PYD of NLRP3 and ASC or CARD of caspase-1 can inhibit these proteins by direct homotypic interactions. Caspase-12 has a similar effect in inhibiting caspase-1. Finally, activated inflammasome can be engulfed and degraded by through autophagy to finalize the signal.

A particularly interesting subset of proteins that are capable of repressing the NLRP3 inflammasome, specifically in higher primates, is the family of Pyrin-domain (PYD)-only proteins (POPs). POPs are small cytoplasmic decoy proteins that are constituted by a sole PYD domain, which can bind to PRR^{PYD} or ASC^{PYD} by direct homotypic interaction. There are four POPs identified to date, and all of them share homology to PYD domains found in different proteins, making them highly specialized for distinct proteins. Initially, pyrin was included as one of the members of this family, but later was shown to be capable of orchestrating a whole inflammasome by itself, effectively excluding it from the POP family (Papin et al., 2007). The first member of the family, POP1, can interact with ASC^{PYD} (de Almeida et al., 2015). In accordance, POP1 deficiency leads to higher NLRP3 inflammasome activation in response to activating signals. Interestingly, POP1 expression is induced by IL-1R1 activation and TLRs, thus suggesting it can play a function to terminate the inflammatory signal downstream of inflammasome

activation. Since POP1 directly interacts with ASC, it is also likely that, if expressed, might be inhibiting other inflammasomes that depend on ASC recruitment to form the complex, such as the AIM2 or the pyrin inflammasome. POP3 on the other hand, has been shown to have a sequence similarity to $AIM2^{PYD}$ and can bind to AIM2 and other proteins that contain the HIN200 domain (Khare et al., 2014), thus down-regulating the activation of AIM2 inflammasome specifically. POP2 has sequence similarity to $NLRP3^{PYD}$ instead, and its overexpression can block NLRP3 inflammasome formation (Bedoya et al., 2007; Dorfleutner et al., 2007). Similarly to POP1, POP2 is expressed downstream of TLR activation and NF-κB translocation to the nucleus (Ratsimandresey et al., 2017). Together, this evidence suggests that POP2 could also play a role to self-limit inflammasome-induced inflammation. However, studies where POP2 was exogenously expressed in mice showed that in this model overexpression conferred additional resistance to bacterial infection (Periasamy et al., 2017). Therefore more efforts need to be put in this direction to help to clarify the exact function of POPs in the modulation of the NLRP3 inflammasome.

Similarly to POPs, CARD-only proteins (COPs) are constituted by a single domain that can homotypically interact with CARD domains from caspases. Like POPs, COPs are only expressed in higher primates. So far three members in this family have been described: CARD16 (PSEUDO-ICE), CARD17 (INCA) and CARD18 (ICEBERG) (Druilhe et al., 2001; Humke et al., 2000; Lamkanfi et al., 2004). All three COPs present highly homologous sequences to the $CASP1^{CARD}$, and probably were originated from the duplication of this genomic region. They repress the inflammasome by directly binding to pro-caspase-1 and blocking its recruitment by ASC. Therefore, similarly to POP1, COPs might not only inhibit NLRP3 inflammasome activation but also other inflammasomes. Additionally, PSEUDO-ICE and ICEBERG can bind and inhibit receptor interacting serine/threonine kinase 2 (RIPK2), a potent activator of NF-κB, thus blocking the NLRP3 inflammasome at two different levels (Druilhe et al., 2001; Humke et al., 2000; Lamkanfi et al., 2004). More recently, a protein that could be playing a similar role to COPs has been identified in mouse. Caspase-12, a poorly studied member of the caspase family has been found to directly interact with pro-caspase-1 through homotypic CARD-CARD interactions, and overexpression of caspase-12 leads to inhibition of the activity of the Nlrp3 inflammasome in murine cells (Saleh et al., 2006). A more recent study however suggested that this phenotype in mice was rather due to the lack of caspase-11 expression in the strain of mice used (Vande Walle et al., 2016). In humans, it has been suggested that it is a truncated form of caspase-12 originated from a premature STOP codon that is responsible for the inhibitory function on pro-caspase-1 (Saleh et al., 2004). All in all, COP expression could be a promising strategy to dampen the pathogenicity of aberrant inflammasome activation, not only in the context of NLRP3 inflammasome but of other inflammasomes as well.

It has been hinted throughout this introduction that cells possess several mechanisms downstream of inflammasome activation that become activated specifically upon inflammasome induction and serve to terminate inflammation at later stages of the pathway. This is essential to restore homeostasis and limit damage from excessive inflammation. As previously mentioned, some of these pathways are related to the activation of cytokines or other proteins during the priming step that are capable of inhibiting transcription of some of the main players of the inflammasome pathway, or on the modification of

members of the inflammasome in order to limit their function. Finally, activation of autophagy for NLRP3 inflammasome degradation seems to be one of the final steps to completely terminate NLRP3 function. These mechanisms altogether are essential and their impaired regulation can result in disease.

2.5.2 – NLRP3 activity in inflammatory diseases

Inflammation is a very common event across human diseases, and usually responds to the presence of danger signals. Interestingly, several diseases can induce similar cellular responses that converge into the accumulation of danger and stress molecules that can be sensed by PRRs. Indeed, NLRP3 responds to many of these stresses, and NLRP3 activation is one of the main initiators of a cascade of inflammatory processes which, if not finely-tuned, can lead to severe damage in the organism. Additionally, mutations in members of the inflammasome, and more specifically in NLRP3, are responsible for pathogenicity of some autoinflammatory diseases, in which inflammation can be triggered even in the absence of any hazard. In this section we will look at how *NLRP3* polymorphisms can directly induce disease and how NLRP3 plays an essential role in the induction of inflammation associated to other diseases.

2.5.2.1 – Mutations of NLRP3 in autoinflammatory diseases

Inherited autoinflammatory diseases are characterised by recurrent episodes of inflammation independently of self-antibodies or antigen-specific T cells. This differentiates them from auto-immune diseases driven by autoreactive antibodies, in which lymphocytes play a central role in the pathogenesis. The underlying cause of inherited autoinflammatory diseases are polymorphisms contained in genes that codify for proteins taking part in the inflammatory pathway. Such is the case for a family of diseases known as Cryopyrin-associated periodic fever syndromes (CAPS), which are characterised by gain-of-function mutations in NLRP3 that lead to aberrant NLRP3 inflammasome activation. From milder to more severe, the group of CAPS diseases include Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells syndrome (MKS) and Chronic infantile neurological cutaneous and articular syndrome/neonatal onset multisystem inflammatory disease (CINCA/NOMID) (Feldmann et al., 2002; H. M. Hoffman et al., 2001). These diseases produce urticarial skin rashes and prolonged episodes of fever in response to a higher secretion of pro-inflammatory cytokines. Mutations in *NLRP3* in these patients usually are concentrated in the NACHT domain, but can also be found in other parts of the sequence. Overall, these mutations cause NLRP3 auto-oligomerization, which is sufficient to assemble the rest of the components of the inflammasome (Aksentijevich et al., 2007; Brydges et al., 2009; Y. Nakamura et al., 2012). Unfortunately, a single copy of these gain-of-function mutations is sufficient to drive NLRP3 inflammasome activation, increasing the likelihood of the apparition of these diseases. Surprisingly some CAPS patients do not have any mutation in the sequence that codifies NLRP3 itself, suggesting that in some cases aberrant NLRP3 activation might not stem from changes in NLRP3 itself, but from hyper-activation of the promoter or the dampening of events that limit NLRP3 activation. Albeit there is some knowledge of key features of CAPS, the rather low prevalence in the population (one or

two cases per million), could account for the current lack of a finer treatment for patients suffering from these diseases.

2.5.2.2 – NLRP3 as a driving force of inflammation

Considering the great spectrum of danger molecules and stresses that NLRP3 can sense, it is not surprising that NLRP3 inflammasome activation arises as one of the main inflammatory events triggered by tissue damage, which becomes progressively prevalent as a result of ageing. Indeed, the pathogenicity of many diseases that appear later in life present chronic inflammation, for which NLRP3 sensing of danger signals is in great part responsible. Hence, albeit the molecules that drive inflammation in these diseases can be quite diverse, NLRP3 inflammasome activation is the unified consequence of degeneration of most tissues.

In many instances this is due to the fact that many of these pathologies lead to the abnormal accumulation of structures that can be phagocytosed by myeloid cells and accumulated in the lysosomes. As indicated in previous sections, conditions included in the metabolic syndrome and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease lead to such events, which result in the permeabilization of lysosomes and the leakage from these structures, although the mechanisms leading to NLRP3 inflammasome activation downstream of lysosomal permeabilization are not yet well defined. Additionally, some respiratory diseases based in the internalization of crystalline substances from the outer environment likewise follow this same mechanism. This is the case of interstitial lung diseases such as silicosis and asbestosis, which are caused by the accumulation in the lung of inhaled substances like silica and asbestos, respectively (Cassel et al., 2008; Hornung et al., 2008). Exposure to silica is common in many areas, and is known to be one of the leading causes of occupational disease, particularly in mine workers. Furthermore, if sustained for longer periods, the inflammation caused by these diseases can derive into more severe pathologies such as lung cancer (Brown, 2009).

Altogether, the severity and particularly the high incidence of these diseases in the population reveals the importance to find therapies that can block NLRP3 inflammasome efficiently in order to avoid the great extent of damage derived from inflammation in these diseases. Promising advances have been put forward in the recent years, which I will discuss further below.

2.5.3 – Current therapies based on inhibition of the NLRP3 inflammasome pathway

Therapies to regulate the effects of the NLRP3 inflammasome can be designed targeting different proteins involved in the NLRP3 inflammasome pathway. It is however important to note that proteins commonly serve more than one function, and thus when targeted with drugs, secondary effects could arise which in some instances might be difficult to predict at early stages of the drug characterization. Additionally, the NLRP3 inflammasome has essential functions in the eradication of pathogenic infections, and if blocked in excess, bacterial and viral pathogens could be incompletely cleared, leading to further damage of the host. Fine tuning the stage at which the NLRP3 inflammasome is targeted can

be useful to reduce the functions that are blocked in the process, and thus limit the effects of the drug. Conversely, if wider effects on inflammation are wanted, this can be achieved by targeting the function of molecules downstream of different inflammatory processes. In this section we will explore current therapies directed to the dampening of IL-1 function, and I will introduce the drugs designed to inhibit events upstream, downstream and directly on NLRP3 function, which could become go-to therapies for inflammatory diseases in a not so distant future.

2.5.3.1 – IL-1-directed therapies to reduce inflammation

Anti-IL-1 therapies have been present for some time as a general mean to block excessive inflammation. Members of the IL-1 family of cytokines are the main drivers of inflammation in the surrounding cells downstream of not only NLRP3 inflammation, but also all the other canonical inflammasomes described to date. Therefore, anti-IL-1 therapies can reduce inflammation in a more global manner, not targeting specifically the NLRP3 inflammasome.

These therapies have been shown to operate with great success against some of the aforementioned pathologies, including type 2 diabetes (Larsen et al., 2007) and juvenile rheumatoid arthritis (Dinarello et al., 2012). Inhibition of IL-1 has proven particularly beneficial in CAPS patients (Ter Haar et al., 2013), and is now a standard to treat these diseases. However, there is no unique way to target IL-1-induced inflammation. To date, three different drugs have been approved by the US food and Drug Administration and the European Medicines Agency for this purpose: anakinra, rilonacept and canakinumab. They function by targeting different molecules in IL-1 signalling. The first and the most commonly used drug is anakinra, which is a non-glycolsylated human IL-1R1 antagonist (Goldbach-Mansky et al., 2006). It competes with IL-1 to bind to the IL-1R1 thereby limiting the activation of the IL-1R1 cascade. Anakinra is currently used to treat rheumatoid arthritis in CAPS patients and in patients suffering from Still's disease, a rare disease with similar symptomatology to CAPS. Rilonacept contains ligand-binding domains from the human IL-1R1 and the IL-1R accessory protein (IL1R1AcP) fused to the Fc portion of a human immunoglobulin receptor (Hal M. Hoffman et al., 2012). In contrast to anakinra, rilonacept serves as a decoy protein of IL-1R1 that can bind directly and sequester IL-1β and IL-1α, thereby stopping these cytokines from reacting with its receptor. Rilonacept is less frequently used and is mostly given to treat patients with CAPS. Finally, canakinumab is a drug based on an antibody that can also target IL-1 to avoid its interaction with IL-1R1, but it is specific to IL-1β and does not interfere with IL-1α signalling (Kuemmerle-Deschner et al., 2011). Similarly to anakinra, canakinumab is used to treat CAPS and Still's disease patients, along with patients suffering from gouty arthritis.

These therapies have been shown to have positive effects on patients of CAPS and arthritis, but not all the symptoms can be resolved using this treatment only, as not all downstream effects of NLRP3 are blocked (Neven et al., 2010). For example, the other main pro-inflammatory cytokine produced downstream of NLRP3 inflammasome activation, IL-18, does not signal through IL-1R1 but IL-18R, and cannot interact with either canakinumab or rilonacept. Therapies with monoclonal antibodies against IL-18 have been proposed to complement these drugs, and so far are showing promising results (Ozaki et al., 2015). However, a joint IL-1R1 and IL-18 therapy would still be insufficient to address other

consequences of NLRP3 inflammasome such as HMGB1 secretion or release of DAMPs resulting from the pyroptotic cell death. Therefore, therapies targeting the inflammasome at earlier stages of the inflammatory process could be of high interest to simultaneously tackle all the inflammatory events taking place downstream of NLRP3 activation. Not only this, but directly targeting the NLRP3 inflammasome would make this process less invasive, as the drugs would not perturb other sources of inflammation signalling through IL-1.

2.5.3.2 – Targeting NLRP3 activation for an early blockade of inflammation

NLRP3 inhibition has been an attractive therapeutic strategy since the NLRP3 inflammasome was first discovered to be responsible for caspase-1 activation and IL-1 secretion. However, this is a rather recent discovery, and albeit few molecules are now known to block NLRP3, their effects in the human organism are still poorly understood. Here I will explain which drugs have been shown to have promising results in cells and mouse models, and thus might be interesting for clinical testing in the future. So far, molecules in this category are MCC950, CY-09, OLT1177, tranilast and oridonin, which directly target NLRP3, and Bay 11-7082 or β-hydroxibutyrate (BHB), which interact with targets upstream of NLRP3 activation.

MCC950 is a diarylsulfonylurea compound that prevents the nucleation of ASC initiated by NLRP3 both in human and mouse macrophages (Coll et al., 2015). Coll and colleagues showed that MCC950 does not compromise events occurring during the priming step, and its activity is independent of changes in both K^+ and Ca^{2+}. A more recent article by Coll and colleagues has revealed that MCC950 can directly interact with the NACHT domain of NLRP3, inhibit ATP hydrolysis and thus NLRP3 activation (Coll et al., 2019). The specificity of MCC950 to this domain in NLRP3 along with other studies suggest that only the NLRP3 but no other inflammasomes are sensitive to MCC950 (Coll et al., 2015; Daniels et al., 2016; Dempsey et al., 2017). Albeit its high specificity and robust inhibition of the inflammasome are very attractive features of MCC950, so far this drug has not been approved for therapies. Similarly to MCC950, CY-09, a cystic fibrosis transmembrane conductance regulator (CFTR) channel inhibitor C-172 can inhibit the NLRP3 inflammasome (H. Jiang et al., 2017) by directly binding the same NLRP3 motif in the NACHT domain, but not that of other NLR proteins. This results in the blockade of the ATPase activity of the receptor, and therefore inhibition entails the same outcome as the previous drug. However, no clinical trials have been commenced with this drug to my knowledge. OLT1177 is a β-sulfonyl nitrile compound, and a very promising drug (Marchetti, Swartzwelter, Koenders, et al., 2018). It is currently in phase II clinical trials to treat acute gouty arthritis (Toldo & Abbate, 2018). It has been shown to lower oxidative stress in cells extracted from mice treated with this drug (Marchetti, Swartzwelter, Gamboni, et al., 2018) and it can interact with NLRP3 to induce a reduction of NLRP3's ATP hydrolase activity, suggesting it might cause NLRP3 inflammasome inhibition in a similar manner to the two previous drugs. Interestingly, tranilast, an anti-allergy drug used to treat several inflammatory diseases has been shown to interact with the NACHT domain of NLRP3 albeit NLRP3 inhibition does not seem to be due to a blockade of its ATPase activity (Yi Huang et al., 2018). Oridonin, another anti-inflammatory drug has similarly been shown to bind to the NACHT domain on the residue C279 by a covalent bond, and in this case this interaction seems not to limit ATPase function neither, but

interestingly enough appears to stop NLRP3 from interacting with NEK7 (H. He et al., 2018), albeit NEK7 is supposed to bind to $NLRP3^{LRR}$, not the NACHT domain. In summary, the NACHT domain seems to be the key domain to modulate NLRP3 function. This does not come as a surprise given its importance for self-oligomerization of NLRP3 and the high number of gain-of-function polymorphisms found in CAPS patients in this region of the *NLRP3* gene.

Other inhibitors of the NLRP3 inflammasome that do not target directly NLRP3 have also been reported. Bay 11-7082, a molecule that can target and inhibit IκB kinase-β, hence blocking NF-kB from translocating to the nucleus (Juliana et al., 2010), shows inhibition of the NLRP3 inflammasome and NLRP3-dependent caspase-1 activation. However, due the fact that this molecule targets NF-κB in a broader way, it could affect many other pathways which require transcriptional expression regulated by this transcription factor. Hence, Bay 11-7082 may have too wide effects with potentially disadvantageous outcomes in the organism. A drug that acts during the activating step but upstream of NLRP3 is BHB, which has been shown to specifically block this inflammasome but not others by preventing K^+ efflux (Youm et al., 2015). Most activators of NLRP3 require K^+ efflux early on during the activation step to activate the signalling pathway that leads to NLRP3 activation, but there are some exceptions to this rule (Groß et al., 2016). Therefore, this drug would not be effective for this kind of situations, and cannot be used as a universal NLRP3 inflammasome inhibitor.

3. Aims of the project

There are currently several molecules that have potential to be used for therapies to treat CAPS and inflammatory conditions resulting in NLRP3 over-activation. However, it is on the best interest for patients to limit the amount of unwanted secondary effects in these therapies. These kind of effects can arise in response to strategies targeting a wider group of biological processes, as it is the case for IL-1 inhibition and strategies limiting priming. Unexpectedly, drugs directly targeting NLRP3 can also have hazardous effects independent of their function on NLRP3, such as liver toxicity in patients treated with MCC950 (Mangan et al., 2018). For this reason, it is of great interest to find new proteins that could be targeted to block inflammasome activation. Not only co-treatment with drugs targeting the NLRP3 at different levels of inflammasome could be a highly robust tool to tune down NLRP3-dependent inflammation. This would also allow to reduce drug concentration, hence putting forward molecules that have not been presently used for clinical trials due to solubility problems or cytotoxic effects at higher doses.

However, for this approach to be effective, new bona-fide partners of NLRP3 need to be found. As shown at length in this introduction, no protein has yet been shown to be essential for NLRP3 inflammasome activation other than the members of the NLRP3 inflammasome itself. Thus, my main aim during this thesis was to develop an experimental approach that would allow to find new key factors in NLRP3 inflammasome activation in an unbiased manner. To this end, I established a CRISPR/Cas9 genome-wide screen in the THP-1 human monocyte-like cell line and later investigated how one the top hits found in this screen, *ENO1*, could be involved in NLRP3 inflammasome activation.

4. MATERIALS AND METHODS

4.1 Reagents

PMA (Ref. P1585), nigericin sodium salt (Ref. N7143), sodium iodoacetate (Ref. I2512) sodium oxalate (Ref. 379735), ionomycin calcium salt from *Streptomyces* (Ref. IO634), thapsigargin (Ref. T9033), phosphor(enol)pyruvic acid monopotassium salt (Ref. 860077), D(+)-2-phosphoglyceric acid sodium salt hydrate (Ref. 79470), Hoechst 33342 (Ref. 14533), PolyBrene (Ref. TR1003), proteinase K from *Tritirachium album* (Ref. P2308), TRIzol reagent (Ref. 93289), Triton X-100 (Ref. T9284), NP-40 IGEPAL (Ref. I3021), saponin (Ref. 47036), digitonin (Ref. D-141),Tween 20 (Ref. P2287) and Oil red O (Ref. O0625) were from Sigma-Aldrich. Immobilon-P membranes (Ref. IPVH00010) were from Merck. Z-VAD-FMK was from Tocris (Ref. 2163). Sytox Green (S7020), Lipofectamine 2000 (Ref. 11668-019), PrestoBlue (Ref. A13261) and SuperScript IV cDNA synthesis kit (Ref. 18090050) were from Invitrogen. Phenol (Ref. 51252) and Phenol-chloroform (Ref. EU0302) were from Euromedex. FastStart Essential DNA Green Master (Ref. 31498300) and X-tremeGENE 9 DNA Transfection Reagent (Ref. 6366244001) were from Roche. Herculase II polymerase kit (Ref. 600679) was from Agilent. AMPure XP beads (Ref. A63880) were from Beckman Coulter. The ENO1 Human Activity Assay Kit (Ref. ab117994) was from Abcam. Paraformaldehyde 16% (Ref. 15710) was from Electron Microscopy Sciences. The Amplex Red cholesterol kit (Ref. A12216) was from Thermo Fisher Scientific. MCC950 (inh-mcc) and CL097 (tlrl-c97) were from InvivoGen.

4.2 Plasmids

pMX26 Cas9-mCherry for stable Cas9 cell line generation was kindly provided by Bernardo Reina at the IGBMC. Human CRISPR Knockout Pooled Library and the LentiGuide-Puro backbone were deposited by Zhang's lab. The pBOB plasmid was a kind gift from Professor Jiahuai Han's lab in Xiamen University, China, and was used for lentiviral integration of α-enolase mutants for rescue experiments. The pX330-Cas9-P2A-EGFP plasmid was generated by inserting a P2A-RGFP sequence into pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid deposited in Addgene, and it was used for cloning of sgRNA and transient Cas9-gRNA expression.

4.3 Antibodies

Several commercial antibodies were used for immunoblot and immunostaining experiments: N-terminal ENO1 antibody was from Abcam (Ref. ab155102), the antibody targeting the C-terminal of ENO1 was from Interchim SA (Ref. NBP1-31324), the Flag M2 antibody was from Sigma-Aldrich (Ref. F1804), the p20 caspase-1 antibody was from Coger reactifs (Ref. AG-20B-0048-C100), the IL-1β antibody was from R&D Systems (Ref. AF-201), the GSDMD antibody was from Novus biologicals (Ref. NBP2-33422), the NLRP3 antibody was from Coger reactifs (Ref. AG-20B-0014-C100), the ATP5B antibody was purchased from Abcam (Ref. ab14730), antibody to target β-actin was from Cell Signaling (Ref. 07/2018), ASC antibody was from Santa Cruz (Ref. sc-22514-R) and TOM20 used for immunostainings was from Santa Cruz (Ref. SC-11415).

4.4 Cell culture

All cell lines were purchased from ATCC and maintained at 37ºC with 5% CO_2 in a humidified incubator. All THP-1 cells except *ENO1* KO THP-1 cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 10 mM HEPES, 1 mM of sodium pyruvate, gentamycin and 50 µM β-mercaptoethanol. *ENO1* KO cells in particular were grown with 15% instead of 10% foetal calf serum. HEK 293T cells were grown in DMEM with low glucose 1 g/L, 10% foetal calf serum and 1% penicillin and streptomycin. Phoenix A cells were grown in DMEM with 4.5 g/L glucose with 10% of serum in the presence of penicillin and streptomycin. For iBMDM culture, cells were maintained in DMEM with 4.5 g/L glucose, 10% of heat-inactivated foetal calf serum supplemented with penicillin and streptomycin.

4.5 THP-1 *ENO1* knock-out cell generation by CRISPR Cas9 editting

For transient pX330-P2A-EGFP expression in THP-1 cells the X-tremeGENE 9 DNA Transfection Reagent was used. Briefly, 3 µL of reagent were mixed with 100 µL OptiMEM medium. 1 µg of pX330-Cas9-P2A-EGFP-containing *ENO1*-gRNA1 and *ENO1*-gRNA2 were added to the solution, and incubated for 15 minutes. At the same time, $1.5x10^6$ THP-1 cells were counted and spinned down at 120 g during 5 minutes. At the end of incubation, pelleted cells were carefully mixed with the transfection solution containing the reagent and the plasmids, and 2.5 mL of THP-1 culture medium were added to the mix. Once properly homogenized, the cell solution was seeded in a 35 mm dish, and further incubated for 48 hours. At the end of incubation, the peak of expression of pX330-P2A-EGFP plasmids was reached and cells were then prepared for FACS sorting as indicated below. Sorted cells were cultured for 48 hours in filtered medium to facilitate cell recovery after sorting. Finally, cells were counted and seeded in 96 well plates at a density of 70 cells/plate to ensure single cell growth. Medium was routinely changed and cells splitted until cell number reached the yield to perform experiments. Clones were screened by immunoblotting with the N-terminal ENO1 antibody, and clones were the band was completely absent were used for experiments.

4.6 Cell treatments for NLRP3 inflammasome activation

To prime cells for NLRP3 inflammasome activation, THP-1 cells were incubated with PMA 100 nM during three hours for acute experiments or for three days to optimize cell visualization by immunostaining. In the case of primary iBMDMs, cells were incubated with LPS at a concentration of 100 ng/mL for three hours, followed by the treatment of interest. Activation of NLRP3 inflammasome activators was done incubating THP-1 cells or iBMDMs 15 µM nigericin and 30 µM CL097 during one hour. Inhibitors used to block NLRP3 inflammasome were 10 µM MCC950 and 10 µM Z-VAD-FMK. Inhibitors to block glycolytic enzymes were sodium iodoacetate and sodium oxalate at different concentrations. When inhibitors were used, cells were pre-incubated for one hour with inhibitor or vehicle, then for an additional hour with both inhibitor and NLRP3 inflammasome activator. To induce Ca^{2+} release from cell stores cells were pre-treated for one hour with 10 µM ionomycin or 10 µM thapsigargin, and then co-treated with this reagent and the NLRP3 inflammasome activator. To rescue

of α-enolase function up concentrations ranging from 50 μM to 2 mM of phospho(enol)pyruvic acid monopotassium salt were used.

4.7 Flow cytometry

For cell sorting experiments, THP-1 cells were spinned down at 120 g for 5 minutes, and pellets were re-suspended in cold PBS supplemented with 10% of foetal calf serum. Cells were filtered using 0.45 μm filters to avoid aggregates and to optimize cell sorting. Cells were sorted at 4ºC using a 100 μm nozzle and collected in fresh medium for further culture. For cell analysis, medium was collected from PMA-primed and treated THP-1 cells, and cells were detached from the plate using PBS supplemented with 5 mM EDTA followed by repeated pipetting. Medium and cells were pooled together and centrifuged at 120 g for 5 minutes. Pelleted cells were then re-suspended in cold PBS and 1 μM of Sytox green was added to the suspension. Cells were kept in ice until analysis. $3x10^5$ events were recorded per sample in each experiment.

4.8 Cloning

Primers used for sgRNA cloning in the LentiGuide-Puro backbone are the following:

Primer	Sequence
LentiGuide-Puro-CASP1-sgRNA1 F	5’ CACCGTAATGAGAGCAAGACGTGTG 3’
LentiGuide-Puro-CASP1-sgRNA1 R	5’ AAACGTTCTTCTAGGAATACTGTCC 3’
LentiGuide-Puro-CASP1-sgRNA2 F	5’ CACCGACAGTATTCCTAGAAGAAC 3’
LentiGuide-Puro-CASP1-sgRNA2 R	5’ AAACCACACGTCTTGCTCTCATTAC 3’
LentiGuide-Puro-ENO1-sgRNA1 F	5’ CACCGGTCATCGGCATGGACGTAG 3’
LentiGuide-Puro-ENO1-sgRNA1 R	5’ AAACCTACGTCCATGCCGATGACC 3’
LentiGuide-Puro-ENO1-sgRNA2 F	5’ CACCGCAGCAGGTACATCTCGCCTG 3’
LentiGuide-Puro-ENO1-sgRNA2 R	5’ AAACCAGGCGAGATGTACCTGCTGC 3’
LentiGuide-Puro-ATP5B-sgRNA1 F	5’ CACCGATTTGCTCCCATTCATGCTG 3’
LentiGuide-Puro-ATP5B-sgRNA1 R	5’ AAACCAGCATGAATGGGAGCAAATC 3’
LentiGuide-Puro-ATP5B-sgRNA2 F	5’ CACCGCTAGCTCCCTATGCCAAGGG 3’
LentiGuide-Puro-ATP5B-sgRNA2 R	5’ AAACCCCTTGGCATAGGGAGCTAGC 3’
LentiGuide-Puro-NKX2-6-sgRNA1 F	5’ CACCGCGCGGCTTCGCCACATCCGC 3’
LentiGuide-Puro-NKX2-6-sgRNA1 F	5’ AAACGCGGATGTGGCGAAGCCGCGC 3’
LentiGuide-Puro-NKX2-6-sgRNA2 F	5’ CACCGATGGTTCGGAGCCTCCTGG 3’
LentiGuide-Puro-NKX2-6-sgRNA2 R	5’ AAACCCAGGAGGCTCCGAACCATC 3’
LentiGuide-Puro-CCNH-sgRNA1 F	5’ CACCGCAATCCTTACAGACCATTTG 3’
LentiGuide-Puro-CCNH-sgRNA1 R	5’ AAACCAAATGGTCTGTAAGGATTGC 3’
LentiGuide-Puro-CCNH-sgRNA2 F	5’ CACCGCCGGGAGAGTCCTCTTGGAC 3’
LentiGuide-Puro-CCNH-sgRNA2 R	5’ AAACGTCCAAGAGGACTCTCCCGGC 3’
LentiGuide-Puro-RIBC2-sgRNA1 F	5’ CACCGCCTAGCCCTTAAGAAAGATC 3’
LentiGuide-Puro-RIBC2-sgRNA1 R	5’ AAACGATCTTTCTTAAGGGCTAGGC 3’
LentiGuide-Puro-RIBC2-sgRNA2 F	5’ CACCGAGGATTTAAACTTCCATGAG 3’
LentiGuide-Puro-RIBC2-sgRNA2 R	5’ AAACCTCATGGAAGTTTAAATCCTC 3’
LentiGuide-Puro-DZIP1L-sgRNA1 F	5’ CACCGAAGCAGAGCTCATTCATCAG 3’

LentiGuide-Puro-DZIP1L-sgRNA1 R	5’ AAACCTGATGAATGAGCTCTGCTTC 3’
LentiGuide-Puro-DZIP1L-sgRNA2 F	5’ CACCGAGAGTGGACCAAACTTTATG 3’
LentiGuide-Puro-DZIP1L-sgRNA2 R	5’ AAACCATAAAGTTTGGTCCACTCTC 3’
LentiGuide-Puro-Eno1-sgRNA1 F	5’ CACCGTGGCTGTCTGCAAAGCTGGT 3’
LentiGuide-Puro-Eno1-sgRNA1 R	5’ AAACACCAGCTTTGCAGACAGCCAC 3’
LentiGuide-Puro-Eno1-sgRNA2 F	5’ CACCGCATTGCTGACTTGGCCGGCA 3’
LentiGuide-Puro-Eno1-sgRNA2 R	5’ AAACTGCCGGCCAAGTCAGCAATGC 3’
LentiGuide-Puro-Eno1-sgRNA3 F	5’ CACCGTGTCTGCAAAGCTGGTGCCG 3’
LentiGuide-Puro-Eno1-sgRNA3 R	5’ AAACCGGCACCAGCTTTGCAGACAC 3’

SgRNAs were first annealed together at 95ºC and cloned into the plasmid’s BsmBI site by T4 DNA ligation. *E. coli* competent cells were subsequently transformed with the ligation product and seeded in plates in the presence of 100 µg/mL Ampicilin, single clones cultured in liquid medium and three clones for each sgRNA were sequenced using the primers described below. For lentiviral expression of full proteins and EGFP fusion proteins, human α-enolase and MBP1 transcripts codified by *ENO1* were amplified from cDNA from THP-1 cells and cloned into the donor plasmid. E167Q and E211Q mutants were generated from the original α-enolase transcript using primers localized at the region of the mutation of interest. EGFP was fused into these constructs by PCR ligation of both sequences. The primers used were the following:

Primer	**Sequence**
pBOB-flENO1 F	5’ TCTAGAGAATTCGGATCCGCCACCATGTCTATTCTCAAGATCCATGCC 3’
pBOB-flENO1 R	5’ TTCCATGGCTCGAGCCCGGGTTACTTGGCCAAGGGGTTTCTGAAGTTC 3’
pBOB-MBP1 F	5’ TCTAGAGAATTCGGATCCGCCACCATGGAACAGAAAATAAATCT 3’
pBOB-C/term-EGFP F2	5’ ACTTCAGAAACCCCTTGGCCAAGGTGAGCAAGGGCGAGGAGCTGTT3’
pBOB-C/term-EGFP R1	5’ AACAGCTCCTCGCCCTTGCTCACCTTGGCCAAGGGGTTTCTGAAG
pBOB-C/term-EGFP R2	5’ TTCCATGGCTCGAGCCCGGGTTACTTGTACAGCTCGTCCATGCCG 3’
pBOB-E167Q F	5’ TGGCAACAAGCTGGCCATGCAG**CAG**TTCATGATCCTCCCAGTCGG 3’
pBOB-E167Q R	5’ CCGACTGGGAGGATCATGAACTG**CTG**CATGGCCAGCTTGTTGCCA 3’
pBOB-E211Q F	5’ AGATGCCACCAATGTGGGGGAT**CAA**GGCGGGTTTGCTCCCAACA 3’
pBOB-E211Q R	5’ TGTTGGGAGCAAACCCGCC**TTG**ATCCCCCACATTGGTGGCATCT 3’

To clone these constructs in the lentiviral pBOB backbone, Exonuclease III (Takara) was used to digest and ligate constructs into the vector following manufacturer’s instructions. *E. coli* competent cells were transformed with the ligation product, and clones were tested using colony PCR (following the same set up as the PCR used to obtain the constructs) to find colonies containing the construct of interest. Then, culture was amplified and plasmid digested BamHI and SmaI restriction enzymes to visualize the construct size. Finally, sequencing was done to confirm the presence of the insert in the plasmid and the successful mutation in the E167 and E211 sites respectively.

4.9 Transfection and lentiviral transduction

Lentiviral transduction was used for constitutive expression of LentiGuide-Puro-gRNA and mutant and recombinant proteins cloned in the pBOB backbone. Transfection was performed using Lipofectamine 2000, following manufacturer's instructions. Briefly, HEK293T cells obtained from ATCC were used to package lentivirus harbouring the construct of interest. Cells were seeded 12h prior to transfection at a 40% cell density in a 35 mm plate. The transfection mix was composed of 8 µL of Lipofectamine 2000 equilibrated at room temperature for 5 min with OptiMEM, 2 µg of Lenti-mix containing plasmids encoding for Gag, Env and Pol lentiviral proteins and 2 µg of the plasmid of interest. Lipofectamin complexes were then incubated during 20 minutes, and added drop by drop to cells with freshly changed medium. Medium was renewed upon 8 hours of culture. Virus-containing supernatant was collected 48 hours after transfection and filtered using a 0.22 µm strainer. For THP-1 transduction, the filtered supernatant was mixed with $2x10^5$ THP-1 cells and THP-1 culture medium at a 1:1 ratio to reach 1.2 mL volume. 1.2 µL of PolyBrene was added to the mix to increase transduction efficiency. Cells were spin-fected at 2500 rpm during 30 minutes. Cells were then incubated at 37ºC for four hours to allow recovery, followed by a change of medium to remove excess virus. Upon 48 hours of incubation green fluorescence was assessed in the inverted microscope for constructs containing EGFP-recombinant proteins or alternatively puromycin treatment at 1 µg/mL concentration was initiated for a total length of three days. At the end of the treatment cells were split twice before being used for experiments. In the case of iBMDM transduction, cells were seeded the day before transduction to reach 10% cell confluency at the moment of the infection. Cell medium was removed, and supernatants from HEK 293T transfected cells were filtered and added to the cells together with PolyBrene. Cells were spinned during one hour at 2500 rpm. No iBMDM medium was kept during spin-fection. Cells were left to recover at 37ºC for 4 hours, at the end of which they were washed and fresh medium was added. To select successfully transduced cells, puromycin selection was performed during four days using 2 µg/mL puromycin, due to a higher resistance to this antibiotic in the control non-transduced iBMDM population.

4.10 Cas9-mCherry cell line generation

Phoenix A cells were used for retroviral packaging of pMX26, which codifies for a Cas9-mCherry-Flag fusion protein. Transfection of Phoenix A cells was done using Lipofectamine 2000 as described in the previous section, with the according retroviral-specific plasmid mix. THP-1 transduction was also performed following the same protocol described in the previous section. iBMDM were transduced with the same virus solution twice in order to maximize transduction efficiency. Red fluorescence was observed in an inverted epifluorescence microscope 48 hours after transduction. Cells were prepared for FACS as described previously, mCherry+ cells selected and cultured for 48 hours in filtered medium to facilitate cell recovery after sorting. Finally, cells were counted and seeded in 96 well plates at a density of 70 cells/plate to ensure single cell growth. Medium was routinely changed and cells split until cell number was sufficient to perform Western Blot. Most competent clones were chosen in the basis of Cas9-mCherry-Flag expression as determined by intensity of bands observed by Flag immunoblotting, cell sensitivity to nigericin) treatment and *CASP1* knock-out efficiency when lentivirally co-transduced with LentiGuide-Puro-*CASP1*-gRNA1-Puro and LentiGuide-Puro-*CASP1*-gRNA2-Puro.

4.11 Dynamic pyroptosis curves

To follow cell death in real time, THP-1 cells were seeded in a clear-bottom 96 well plates in the presence of PMA 100 nM. The medium was changed after three hours, and fresh full medium was added. The next day, cells were incubated for one hour with Hoechst 33342 in full medium. Then, a solution of nigericin 15 µM and Sytox green was added to the cells, and immediately after, the plate was introduced in a chamber at 37ºC and 5% for fluorescence imaging in an inverted microscope. More than 200 cells per condition were followed over the course of 2 hours and 20 minutes, and pictures were taken every 10 minutes.

4.12 sgRNA cell library generation

Viral particles were produced by the Molecular Biology platform in the IGBMC, following HEK 293T packaging cell transfection as described previously. Virus titre necessary to obtain 20% transduction efficiency in the selected THP-1 Cas9+ clone #22 was determined by cell viability following virus transduction and puromycin treatment following Broad protocols. Briefly, cells were spin-fected as previously described using serial dilutions of human CRISPR knock-out pooled virus library. Medium was changed upon 4 hours of recovery at 37ºC, and cells were incubated for further 48 hours to express the virus. Cells were then incubated with 1 µg/mL puromycin for three days, which lead to complete cell death of THP-1 Cas9-mCherry mock transduced cells. At the end of the treatment, cells were collected and viability was assessed by PrestoBlue assay. PrestoBlue shows higher absorbance to 570nm wavelengths in the presence of reductive environments, typical of living cell medium. To test the viability for each of the aforementioned transduction conditions, puromycin-treated cells were seeded in 96 well plates in the presence of 90 µL of medium, and the day after 10 µL of PrestoBlue reagent were added. Cells were incubated for 10 minutes at 37ºC, after which absorbance was immediately measured using an absorbance microplate reader at 570 nm. Following the plotting of the resulting data, the condition of 0.53376 titre units of virus in HEK293T was defined to obtain a Multiplicity of Infection (MOI) of 0.2 in the Cas9+ expressing THP-1 cells.

4.13 Pooled genome-wide CRISPR Cas9 cell library production and treatment with nigericin

$200x10^6$ THP1 Cas9-mCherry cells were cultured, spinned down at 120 g during four minutes and re-suspended in THP-1 medium containing 0.53376 MOI^{HEK}/cell, as defined during the set-up of the screen. Cells were seeded in 10 cm dishes and spin-fected at 2500 rpm during 30 minutes. Cells were allowed to recover, and after four hours were seeded in 15 cm plates. Upon two days of incubation, cells were treated with 1 µg/mL of puromycin and further incubated for three days to kill all non-successfully transduced cells from the culture. Cells were then seeded in new medium, and further cultured for two weeks to allow recovery and obtain enough cell numbers to freeze cells from the library and to perform the cell death experiment. A minimum of $80x10^6$ cells were kept at all times in culture to ensure enough coverage for each gRNA in the cell library pool. Upon two weeks, $80x10^6$ cells were seeded with PMA

100 µg/mL as previously described. After three hours, the medium was changed, and the following day $40x10^6$ cells were treated with nigericin 15 µM or ethanol vehicle for 2 hours and 15 minutes. Cells were then prepared as previously shown and surviving cells sorted following the increase of Sytox Green permeability and the resulting green fluorescence. A maximum of 11% surviving cells was attained in each biological replicate. Surviving cells were collected for further sample processing.

4.14 Bulk genomic DNA extraction

Surviving cells were pelleted by centrifugation at 120 g for 4 minutes. Cells were re-suspended in a lysis buffer containing 100 mM Tris at pH = 8, 5mM EDTA, 200 mM NaCl, 0.2% SDS and freshly added proteinase K for a final concentration of 200 µg/mL at a ratio of $1.25x10^6$ cells/mL. Digestion was done at 55ºC overnight. For genomic DNA extraction, first this solution was mixed at 1:1 ratio with phenol, vortexed for 15 seconds and then separated in two phases by centrifugation at 16000 g for 15 minutes. The upper phase was collected and mixed at even volumes with a mix of phenol, chloroform and isoamyl alcohol at a 25:24:1 ratio. The lower phase was mixed with lysis buffer in order to completely separate the residual genomic DNA at the interphase between phenol and the aqueous phase. The following steps for this later phase were performed as indicated for the aqueous phase. All tubes were vortexed thoroughly and centrifuged at 16000 g for 15 minutes. Aqueous phases were collected and mixed with chloroform or phenol chloroform solution respectively. Upon mixing, centrifugation was repeated at 16000 g and final aqueous phases were mixed with ethanol absolute, centrifuged with the previous settings and mixed with ethanol 70% to complete DNA precipitation by centrifugation at 16000 g for 30 minutes. The final pellet was re-suspended in a small volume of water, and the DNA concentration was quantified using the Nanodrop (Life Technologies).

4.15 Bulk sgRNA locus amplification from genomic DNA

Locus containing the sgRNA were amplified from the genomic DNA using two types of PCR. Herculase II polymerase was used following the manufacturer's protocol for the PCR reaction. Two types of PCR programs were conducted depending on each sample in order to ensure successful amplification of the regions of interest.

Standard PCR protocol	
t (s)	**T (ºC)**
180	95
30	95
30	61
30	72
300	72
∞	14

34 Cycles

Touch-Down PCR		
t (s)	T (ºC)	
180	95	
30	95	
45	67 → 52	15 Cycles
60	72	
30	95	
45	52	25 Cycles
60	72	
300	72	
900	4	
∞	23	

In order to keep primer diversity during Illumina sequencing the forward primer (Argon) was a mix of eight different primers with common 5' first nucleotides (5' FORW = AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT… 3') and different nucleotides at the 3' end. To allow simultaneous sequencing of all samples in the same sequencing chip, the reverse primer (Kermit) was unique to each independent sample for few nucleotides, but shared homologous 5' starting nucleotides (START = CAAGCAGAAGACGGCATACGAGAT) and 3' end nucleotides:

(END=GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCA*C*T*G*T)

Primer (forward)	Sequence
Argon P5_P1	5' FORW-TTGTGGAAAGGACGAAAC*A*C*C*G 3'
Argon P5_P2	5' FORW-CTTGTGGAAAGGACGAAAC*A*C*C*G 3'
Argon P5_P3	5' FORW-GCTTGTGGAAAGGACGAAAC*A*C*C*G 3'
Argon P5_P4	5' FORW-AGCTTGTGGAAAGGACGAAAC*A*C*C*G 3'
Argon P5_P5	5' FORW-CAACTTGTGGAAAGGACGAAAC*A*C*C*G 3'
Argon P5_P6	5' FORW-TGCACCTTGTGGAAAGGACGAAAC*A*C*C*G 3'
Argon P5_P7	5' FORW-ACGCAACTTGTGGAAAGGACGAAAC*A*C*C*G 3'
Argon P5_P8	5' FORW-GAAGACCCTTGTGGAAAGGACGAAAC*A*C*C*G 3'

Sample	**Primer (reverse)**	**Sequence**
EXP1 – EtOH (MTPZ2)	Kermit P7_P1	5' START-TTGAGTAT-END 3'
EXP1 – Nigericin (MTPZ4)	Kermit P7_P5	5' START-TTGAACCG-END 3'
EXP2 – EtOH (MTPZ9)	Kermit P7_P3	5' START-AATCCAGC-END 3'
EXP2 – Nigericin (MTPZ10)	Kermit P7_P6	5' START-TTCTCAGC-END 3'
EXP3 – EtOH (MTPZ11)	Kermit P7_P7	5' START-CCTCCAAT-END 3'
EXP3 – Nigericin (MTPZ12)	Kermit P7_P8	5' START-TTAGACTA-END 3'
Plasmid library (MTPZ6)	Kermit P7_P4	5' START-AACTGTTA-END 3'

The first Biological replicate was amplified using the standard PCR protocol. The second and third replicates were amplified using the Touch-Down PCR due to inefficient product amplification with the standard protocol. For each method one single reaction was run initially, in which samples of 5 µL of reaction were taken to determine the optimal cycle number for product amplification and minimal unspecific product formation. 5 µL of each reaction were then run in an agarose gel to confirm successful product amplification.

4.16 PCR product purification and sequencing

The PCR product at the expected size of 350 bp was purified from the PCR reaction mix using AMPure beads following the manufacturer's protocol. Briefly, well homogenized 100 µL of PCR reaction for each sample were placed in a tube, mixed softly with the same volume of AMPure beads and placed in a magnetic rack for bead separation. Medium was cleared up, and beads were rinsed with 70% ethanol twice. Finally, DNA was eluted using 50 µL ultra-pure water and the resulting DNA solution was quantified using the Nanodrop. Upon DNA purification of each sample, samples were provided to the Sequencing Platform at the IGBMC for sequencing. Samples were first analysed to determine their purity using a BioAnalyzer QC (Agilent). Upon selection of DNA peak of about 350 bp, samples were mixed together along with 30% Phix to introduce variability and optimize Illumina sequencing efficiency. Each product was amplified up to 50 bp, and a total of $250x10^6$ reads were attained for the whole sequencing chip. Output data was obtained in FASTQ files, which were then used for statistical analysis.

4.17 Genome-wide screen statistical analysis

Sequencing reads were first trimmed using Cutadapt and sgRNA counted using Bowtie2 matching threshold = 35 (Langmead & Salzberg, 2012) directly from the sequencing FASTQ files. Data was analyzed using MAGeCK 0.5.6 (Wei Li et al., 2014). The method used to identify gene hits was MAGeCK RRA with the default parameters and using counts per million (cpm) as parameter for the normalization method. MAGeCK RRA allows to compare two experimental conditions with several biological replicates. MAGeCK RRA ranked sgRNAs based on their P-values calculated from the negative-binomial model and used a modified RRA algorithm named α-RRA to identify positively or negatively selected genes. Genes that were significantly enriched upon nigericin treatment as compared to the vehicle ethanol were tailored down to genes with 3 ≤ gRNA enriched, 0.01 ≥ p-value and LFC ≥ 1.14, corresponding to LFC of *GSDMD*.

4.18 RT-qPCR Analysis

Cells were homogenized using TRIzol reagent. RNA was phase separated upon mixing of cell-containing TRIzol and chloroform. Aqueous phase containing RNA was then cleaned-up using 70% ethanol. The supernatant was discarded and the RNA re-suspended in ultra-pure water. The concentration of RNA was then measured using Nanodrop. All isolated RNA was retro-transcribed to

cDNA using SuperTranscriptase IV cDNA synthesis kit using Oligo-dT primers for full RNA-wide retrotranscription following manufacturer's instructions. Real Time quantitative PCR (RT-qPCR) was then set up using five times diluted cDNA in reaction with Fast Start or Sybr Green RT-qPCR mix along with primers targeting the cDNA of interest. RT-qPCR was conducted in a LightCycler 480 (Roche). Melting curves were assessed and expression values for each gene of interest normalized to human β-Actin values. The following primers were used:

Gene	Primer Forward	Primer Reverse
Cas9	5' CCACCTCGTCCACGATGTTGCC 3'	5' AGAAGAACCTGATCGGCGCCCT 3'
IL1B	5' AATCTGTACCTGTCCTGCGTGTT 3'	5' TGGGTAATTTTTGGGATCTACACTCT 3'
CCNH	5' GCTCACTTGTGCATTTTTGGCCTGC 3'	5' GCGGGTCTTTAAGTCGATGAGGAAGC 3'
RIBC2	5' AGAAGCCAGAAACTCGCCGTGA 3'	5' TGTGTAGAGGGCCTCTGCGCAT 3'
ACTB	5' CACCAACTGGGACGACAT 3'	5' ACAGCCTGGATAGCAACG 3'

4.19 ENO1 human activity assay

The ENO1 Human Activity Assay Kit was used to determine the level of α-enolase activity in WT and *ENO1* KO THP-1 cells. To summarize, this kit allows to quantitatively determine the amount of enzymatically active α-enolase in a given sample by capturing the enzyme in a microplate using high affinity antibodies. Once bound, α-enolase activity is quantified by consumption of NADH in the buffer supplied by the company, which also contains pyruvate kinase and lactate dehydrogenase. α-enolase's product phosphoenolpyruvate serves as a substrate for pyruvate kinase, and the product of this enzyme, pyruvate, as a substrate for lactate dehydrogenase, which oxidizes NADH to NAD+ to produce lactate. NADH consumption is monitored by the decrease of NADH absorbance at 340nm. For this purpose, $2x10^6$ WT and *ENO1* KO cells were treated with PMA and collected. Briefly, cells were washed twice with cold PBS and re-suspended in Extraction Buffer at a $2x10^7$ cell/mL concentration. To obtain complete cell lysis, cells were then incubated on ice for 20 minutes, and protein-containing supernatant collected upon centrifugation at 16000 g for 20 minutes. In a preliminar setup of the experiment using serial dilutions, the protein concentration chosen for next experiments was defined as 50 µg/mL. 50 µL of each dilution used to test in the microplate. Samples were incubated for 2 hours to ensure total enzyme binding to the plate, and then washed out twice with Wash Buffer. Finally, Activity solution was added to each well, and kinetic absorbance read was started immediately at 340nm for 60 minutes. Every well was read once per minute.

4.20 Immunofluorescence and live imaging

For fixed cell visualization, THP-1 cells were seeded in UV-treated coverslips and incubated during 3 days in PMA to induce attachment of cells to the plate and cell priming. Cells were then treated and washed with PBS twice. All the steps involved in sample preparation were performed at room temperature. Fixation was performed with 4% paraformaldehyde in PBS for 15 minutes. Coverslips were subsequently washed three times with PBS, followed by blocking with 0.1% saponin 3% Bovine Serum Albumine during one hour. The cognate primary antibody was diluted in the blocking solution and

coverslips were further incubated for one hour. Coverslips were then washed three times in 0.1% saponin PBS buffer, followed by an incubation with the secondary antibody diluted in the blocking buffer for 45 minutes in a wet chamber protected from light. The secondary antibody solution was removed and coverslips newly washed with PBS. To mount the cells, DAPI-containing mowiol solution was used and coverslips were mounted in slides. Slide visualization was done 12 hours after mounting in an epifluorescence microscope.

4.21 Immunboblotting

To detect of secreted proteins, medium from treated cells was collected. Protein content was isolated using the methanol-chloroform method. Briefly, the medium was mixed by vortexing with methanol, chloroform and H_2O sequentially. Then, samples were centrifuged at 14000 g during one minute. After, the pellet was resuspended in methanol, followed by a centrifugation at 14000 g this time for two minutes. After protein was precipitated, the pellet was mixed with the loading solution for Western Blot analysis, and run in a tricine gel. To detect intracellular protein levels, cells were lysed using 1×SDS sample buffer or 1× RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM Sodium pyrophosphate, 1 mM NaVO4 and 1 M NaF) supplemented with protease inhibitor cocktail (Roche diagnostics). Protein was quantified using Bradford, and same amount of protein was mixed with loading buffer and loaded in a glycine gel. Electrophoresis was performed for the medium and the lysate samples, and then proteins were transferred into an Immobilon-P membrane using semi-dry transfer. Upon completion of this step, membranes were blocked in Tris Saline Buffer with Tween 20 supplemented with 3% of bovine serum albumin for one hour. Membranes were then incubated overnight in the presence of the primary antibody of interest. The next day, membranes were washed with TBS Tween three times, and then incubated with the secondary antibody during one hour. Cells were after this washed three more times with the same washing buffer, and incubated with the ECL reagent.

4.22 Detergent permeabilization experiments

THP-1 cells were seeded in the presence of PMA 100 nM and incubated during three hours. Then, cells were washed with pre-warmed PBS and incubated in a solution of PBS with the detergent of choice: 0.2 or 0.3 pmol/cell concentrations were used for Triton X-100, and cells were incubated during 10 minutes. The same concentrations were used for NP-40, but cells were only incubated during 5 minutes. For digitonin treatments, 20 µM, 30 µM or 50 µM concentrations were used, and cells were incubated with the detergent only during one minute. In the case of saponin, solutions of 0.016% or 0.03% in PBS were used in experiments, and cells were incubated during 5 minutes. All solutions were prepared freshly before the experiment. At the end of treatments, cells were newly washed with pre-warmed PBS, and then left to recover in complete medium. The next day, cells were recovered as indicated in section 3.7, incubated in the presence of Sytox green, and cell survival was quantified by FACS.

4.23 Lipid droplet visualization

To determine lipid droplet content in THP-1 cells, cells were seeded in coverslips in the presence of PMA 100 nM for days, after which they were challenged with nigericin for one hour. Following this, cells were fixed in 4% PFA during 15 minutes. Then, the coverslips were washed with PBS three times, and then incubated in 60% isopropanol for 5 minutes. This was followed by an incubation with 0.25g/mL Oil Red O in 60% isopropanol for 10 minutes. In order to wash out excess Oil Red O from cells, coverslips were washed three times in PBS during 10 minutes. Then coverslips were mounted in slides using Mowiol containing DAPI. Image acquisition was performed using an epifluorescence microscope.

4.24 Cholesterol measurement assays

To measure total cholesterol levels in the cell, the Amplex Red cholesterol kit was used. This kit can measure cholesterol content by using a cholesterol oxidase enzyme, which oxidizes cholesterol and produces H_2O_2, which can be then detected using the Amplex Red reagent, a highly stable probe of H_2O_2. For Amplex Red and H_2O_2 to react horseradish peroxidase (HRP) is added to the reaction. In this setup only free cholesterol levels are assessed. The kit also provides cholesteryl esterase, which can hydrolyse cholesteryl esters into cholesterol to measure total cholesterol content in the sample of interest. Experiments were performed following the protocol indicated by the manufacturer, with some changes indicated below. Briefly, $1x10^6$ THP-1 cells were counted and divided into two groups: $0.5x10^6$ were lysed using RIPA buffer described above to determine protein content using Braford reagent. The other $0.5x10^6$ cells were re-suspended in 500 µL of modified RIPA buffer that does not contain protease or phosphatase inhibitors, which can alter the reads of fluorescence in the experiment. In parallel, all the components of the reaction buffer were equilibrated at room temperature and mixed in the amounts indicated by the manufacturer. Additionally, a reaction buffer lacking cholesteryl esterase was prepared to quantify free cholesterol content only. 100 µL of cell suspension and 100 µL of reaction buffer with or without cholesteryl esterase were pipetted in a chimney well F-bottom 96 well plate (Greiner bio-one). Every condition was pipetted in duplicates. The plate was then incubated at 37ºC during 30 minutes, and fluorescence was detected at 570 nm at the end of the incubation. Content of cholesterol in the samples was interpolated from a standard curve of a cholesterol solution provided in the kit. Cholesterol content was then normalized by the amount of protein content in each sample. This was expressed as ng cholesterol/µg protein.

4.25 Statistical analysis

For statistics in the CRISPR/Cas9 screen see section 3.17. For the rest of experiments, and provided the distribution was normal, unpaired Student t-test was used to compare two groups independently. For FACS experiments, the percentage of Sytox green-negative cells was calculated in comparison to all events, otherwise the opposite was indicated. For immunofluorescence experiments, a minimum of 100 cells were counted per condition, and percentage of levels containing ASC specks were used to compare different biological replicates. In all cases, the data was expressed as mean ± SD. Differences

were considered significant when P-value < 0.05. Degree of significance was indicated using the following symbols: * represented P-value < 0.05, ** represented P-value < 0.005 and *** represented P-value < 0.0005. ns was used when P-value > 0.05, and therefore differences were not considered significant.

5. RESULTS

5.1 CRISPR/Cas9 genome-wide screen setup in THP-1 cells

The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-associated Cas system technology for genome editing is based on the naturally occurring pathway triggered in prokaryotes in response to bacteriophage infection. The great versatility of this system has led to a revolution in genome editing. Indeed, the contributions of two of its pioneers, Emmanuelle Charpentier and Jennifer Doudna, have been awarded the Nobel Prize in Chemistry of 2020.

The current tools used for CRISPR/Cas9 genome edition make use of the Cas9 protein itself commonly engineered from *Streptococcus pyogenes*, and a small guide RNA (sgRNA) containing both crRNA and tracrRNA targeting the gene of interest (GOI) upstream of a PAM sequence. Once expressed in cells or organisms of interest, Cas9 interacts with the sgRNA and can be recruited to the GOI, where it generates double strand breaks (DSBs). This leads to the activation of the DNA damage repair (DDR) pathways in the cell: homologous recombination (HR) or non-homologous end joining (NHEJ). If HR is activated, the DNA is repaired with high-fidelity using the sister chromatid as a template, thus limiting the activation of HR to the S and G2 phases of the cell cycle (X. Li & Heyer, 2008). Alternatively, if NHEJ is activated, NHEJ proteins set to stitch the two ends together, meaning NHEJ is not restricted to a specific phase of the cell cycle. The possibilities of CRISPR/Cas9 technology are almost endless, and depending on the type of the DDR pathway, it can serve multiple purposes. If, for example, two sgRNAs are provided spanning a region of interest, the whole region can be deleted out of the genome, which can be useful in studies of motif or domain functions of a protein. Additionally, if a partially homologous artificial template is provided, point mutations of interest can be introduced, or knock-in cell lines can be produced with the sequence of interest. Conversely, NHEJ has proven a very simple tool to introduce small modifications in the sequence of the GOI. These modifications can likely disrupt the GOI reading frame completely, leading to transcripts with premature STOP codons and therefore idly-folded peptide chains, which are rapidly targeted for proteasomal degradation upon translation, factually knocking-out the function of this gene.

The development of CRISPR/Cas9 sgRNA programmable gene-engineering has started a whole new paradigm in genome-wide functional studies. Soon after the first articles on CRISPR/Cas9 gene editing, efforts to scale up sgRNA-directed gene editing of the whole genome started, and already in 2014 the first CRISPR/Cas9 genome-wide knock-out screens were published (Shalem et al., 2014; T. Wang et al., 2014). They were only conducted as proof-of-concept studies to show the reliability of CRISPR/Cas9 for genome-wide screens, but showed the exciting potential of this technique. It is no surprise that this kind of screens have since become a common practice for unbiased genome-wide studies, especially given their simplicity and versatility in comparison to other tools like RNAi-based screens. In line with the increase of tools available for Cas9 technologies, the set up for screens has improved dramatically. The first small libraries of sgRNAs used for screens were constituted by cherry-picked sgRNAs. Now genome-wide libraries are designed using bioinformatic tools considering different criteria, such as RNA structure or the specific locus targeted in the gene sequence. Recent advances have gone the extra mile by including more refined on-target and off-target assessment studies to increase specificity and

efficacy of sgRNAs. Following these improvements, new tools like the Brie for the mouse genome and Brunello for the human genome sgRNA libraries have been developed and have shown to be superior to their previous counterparts (Doench et al., 2016). In fact, they have now been successfully used in an increasing number of genome-wide screens (Sanson et al., 2018; Shifrut et al., 2018).

To our knowledge, the CRISPR/Cas9 genome-wide technology has been used in four instances to uncover new key proteins in the inflammasome pathway at the date of the publication of this thesis. The first screen by Shi and colleagues (J. Shi et al., 2015) set off to describe new proteins involved in the induction of pyroptosis by activation of caspase-11 upon LPS electroporation, in immortalized BMDMs (iBMDMs). In this screen they found Gsdmd as a key protein directly involved in caspase-11-dependent pyroptosis, and further experiments also showed the key role of Gsdmd in caspase-1-dependent pyroptosis. Additionally, in the context of caspase-1-dependent pyroptosis, Schmid-Burgk and colleagues unveiled Nek7 as a key protein upstream of Nlrp3 function required for iBMDM cell death in response to nigericin (Schmid-Burgk et al., 2016). More recently, Chui and colleagues have performed a CRISPR/Cas9 genome-wide screen to describe new proteins required for Nlrp1b-mediated pyroptosis in response to lethal anthrax toxin in the mouse RAW 264.7 murine macrophage cell line (Chui et al., 2019). They showed that pyroptosis downstream of Nlrp1b is dependent on the activation of the N-end proteasomal degradation pathway, which is required to freeing the C-terminal domain of Nlrp1b and engage into Nlrp1b inflammasome activation. Finally, in the course of this thesis, I have collaborated with Benaoudia and colleagues to explore the mechanism underlying noncanonical inflammasome activation using a CRISPR/Cas9 genome-wide screening approach. This has resulted in the identification of IRF1 and IRF2 as indispensable elements for caspase-4, -5 and -11 expression and hence in inflammasome formation, and we confirmed the role of IRF2 in caspase-4 expression in human induced pluripotent stem cell (hIPSC)-derived macrophages (Benaoudia et al., 2019).

However, other than the NEK7 and GSDMD mentioned above, not many novel proteins were found in the main hits of these screens to be important for pyroptosis downstream of NLRP3 inflammasome activation in these screens. Importantly, both of these screens were performed in the context of a murine model, which has already been shown to differ in some instances from human cells. Furthermore, after their publication, new more optimized libraries for CRISPR/Cas9 genome-wide screens have been published and made available to the public. Therefore, we set up to perform a novel CRISPR/Cas9 genome-wide screen to investigate the mechanism leading to NLRP3 inflammasome-dependent cell death using a highly-optimized screening approach in a human system. For this purpose, we set to conduct a novel screen using the human genome pooled CRISPR sgRNA library Brunello in an acute monocyte-like leukaemia cell line, THP-1 cells.

5.1.1 Generation of Cas9-expressing THP-1 cells

THP-1 was chosen as a model to induce NLRP3-derived inflammation due to several reasons: first, its capability to be cultured and amplified; secondly, the possibility of differentiation to terminal macrophage-like cells; third, their high sensitivity to lentiviral transduction and four, their robust response to NLRP3-dependent cell death. THP-1 cells are not responsive to traditional signals that induce the

priming step in the cell due to their lower expression of CD14 in comparison to primary cells or mouse cell lines. They also fail to adhere in response to LPS (Bosshart & Heinzelmann, 2004). CD14 is a key component in the highly sensitive signalling complex that mediates responses to LPS, along with TLR4 and myeloid differentiation factor MD2 (J.-I. Kim et al., 2005; B. S. Park et al., 2009). THP-1 can however be primed with phorbol-12-myristate-13-acetate (PMA), which likewise induces transcriptional priming and post-translational modifications. Mechanistically, PMA activates protein kinase C (PKC), which leads to the activation of PKC-associated kinase (PKK), and this protein can in turn activate IKKα and IKKβ to engage NF-κB function (S.-W. Kim et al., 2014) triggering cell adherence to the extracellular space mediated by AMPK and Syk signalling (Chang et al., 2012). Three hours of incubation with PMA at 100 nM is sufficient for the induction of these programs, followed by 24 hours of culture in the absence of PMA to facilitate marker expression and adaptation (Chanput et al., 2013). Upon priming, THP-1 show high sensitivity to nigericin-induced NLRP3 inflammasome activation and subsequent cell death. PMA-primed THP-1 cells were therefore treated with nigericin to induce robust NLRP3 inflammasome activation.

Initially, a Cas9-stably expressing THP-1 cell line was generated to produce the cell library. For this purpose, cells were transduced with retrovirus generated from the plasmid pMX26, carrying a Cas9-mCherry-Flag expression cassette that allows FACS sorting of transduced cells by red fluorescence. Single cell colonies were selected. Best initial candidates were determined on the basis of Cas9 protein expression levels and sensitivity to nigericin-induced IL-1β secretion and cell death. This was determined by cleavage and secretion levels of pro-inflammatory IL-1β and by permeation of Sytox green, a poly-charged nucleic acid dye which is repelled by the charged lipids in the plasma membrane in normal conditions, and can only penetrate cells undergoing a lytic type of cell death.

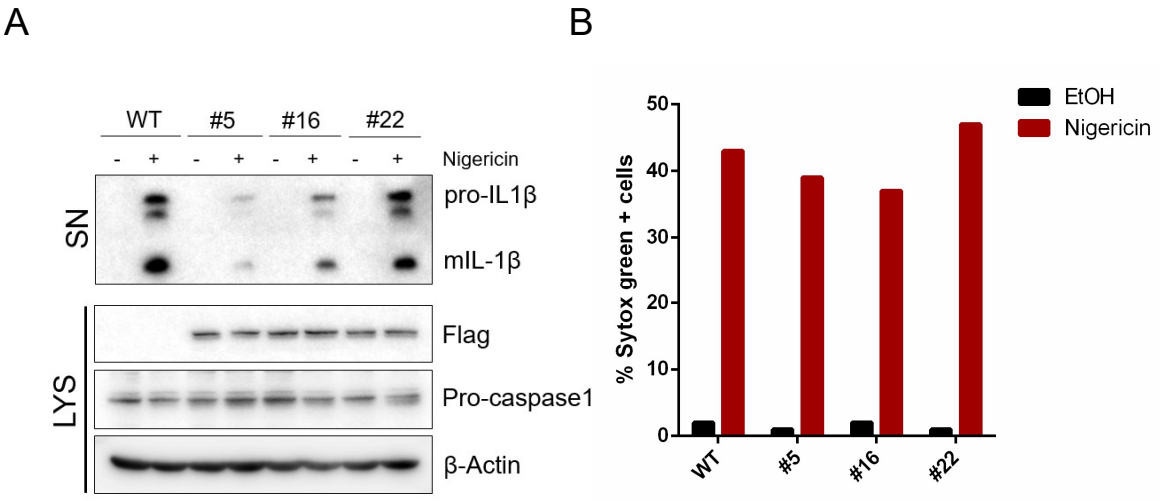


Fig. 1. Cas9 expression and nigericin sensitivity in pMX-26-transduced THP-1 clones. Cells were primed with PMA for three hours and incubated in the presence of nigericin for one hour to activate the NLRP3 inflammasome. (A) Immunoblotting of selected clones expressing high levels of Cas9. Protein levels of Cas9 are determined by Flag protein detection. Secretion of mature and pro-IL-1β in the supernatant was used as an indication of NLRP3 inflammasome-dependent caspase-1 activation and pyroptosis. (B) Permeation of Sytox green as measured by green fluorescence detection in an epifluorescence microscope. Cells were treated with Sytox Green after Nigericin treatment and immediately taken images of for quantification.

The three selected Cas9-expressing clones showed a degree of sensitivity to nigericin comparable to the parental cell line (Fig. 1). To further narrow down the number of possible candidates, Cas9 activity in these clones was determined testing knock-out efficiency upon lentiviral transduction with a *CASP1*-targeting sgRNA, which has been previously used in the laboratory to generate *CASP1* human knockout cell lines. All clones showed high efficiency to knock-out *CASP1* (Fig. 2). Clone #22 was chosen as it was mostly sensitive to nigericin, deletion of pro-caspase-1 was most efficient and it was proliferating in culture most efficiently.

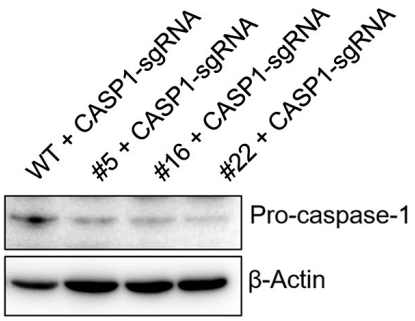


Fig 2. Cas9-expressing clones have lower caspase-1 levels upon transduction with a *CASP1*-targeting sgRNA. Cells were transduced with *CASP1*-sgRNA, followed by selection with puromycin 1 µg/mL for three days to remove cells without sgRNA. Cells were allowed to recover in antibiotic-free medium, and two days after cells were lysed for protein detection by Western Blot.

5.1.2 Determination of virus titre for CRISPR library generation

The success of a screen is dependent on the quality of its pooled knock-out library, whereby following principles should be considered. First, sgRNAs should have highest on-target efficacy and lowest off-target scores, and these scores should be similar from one sgRNA to another, to ensure faithful distribution of knock-outs in the library. For this first purpose, I decided to choose the highly optimized Brunello library for our screen. Secondly, sgRNAs should be represented equally in the library to ensure all genes are targeted to the same extent. Finally, only one sgRNA should be expressed per cell, in order to avoid the presence of false positives in the final result. For both equal distribution of sgRNAs and limitation to one disturbance per cell, determination of the amount of library virus to be used in the transduction of the Cas9-expressing cells requires special attention. Lentiviral transduction per se confers a great advantage over plasmid libraries counterparts, as it simplifies the control of the amount of sgRNAs that can be accepted by each cell. Previous analysis have already explored which are the best conditions for viral transduction to ensure a good representation of the library (Doench, 2018), which suggest that the optimal compromise between a high coverage – the amount of cells successfully transduced with a particular sgRNA – and limited cell numbers is a coverage of 500, using a virus transduction efficiency of 20%. This is based on the Poisson distribution of the number of sgRNA that are integrated per cell, which defines 20% as the best balance between cell numbers required to get full representation of the library while keeping the maximum disturbances per cell at 1 (Fig. 3A). 20% of transduction efficiency correlates with MOI = 0.2 for that specific cell line, as MOI represents the amount of viral particles required to transduce one cell. Cells that fail to be transduced by the virus can be easily

cleared out by antibiotic selection. For this reason, we used the virus titre calculated in the HEK 293T cell line originally used for the virus packaging (MOI^{HEK}) to test which would be the amount of virus required to obtain a MOI of 0.2 in THP-1 cells (MOI^{THP-1}). Transduction with the virus was followed by puromycin selection and a cell viability assay (Fig. 3C and 3D).

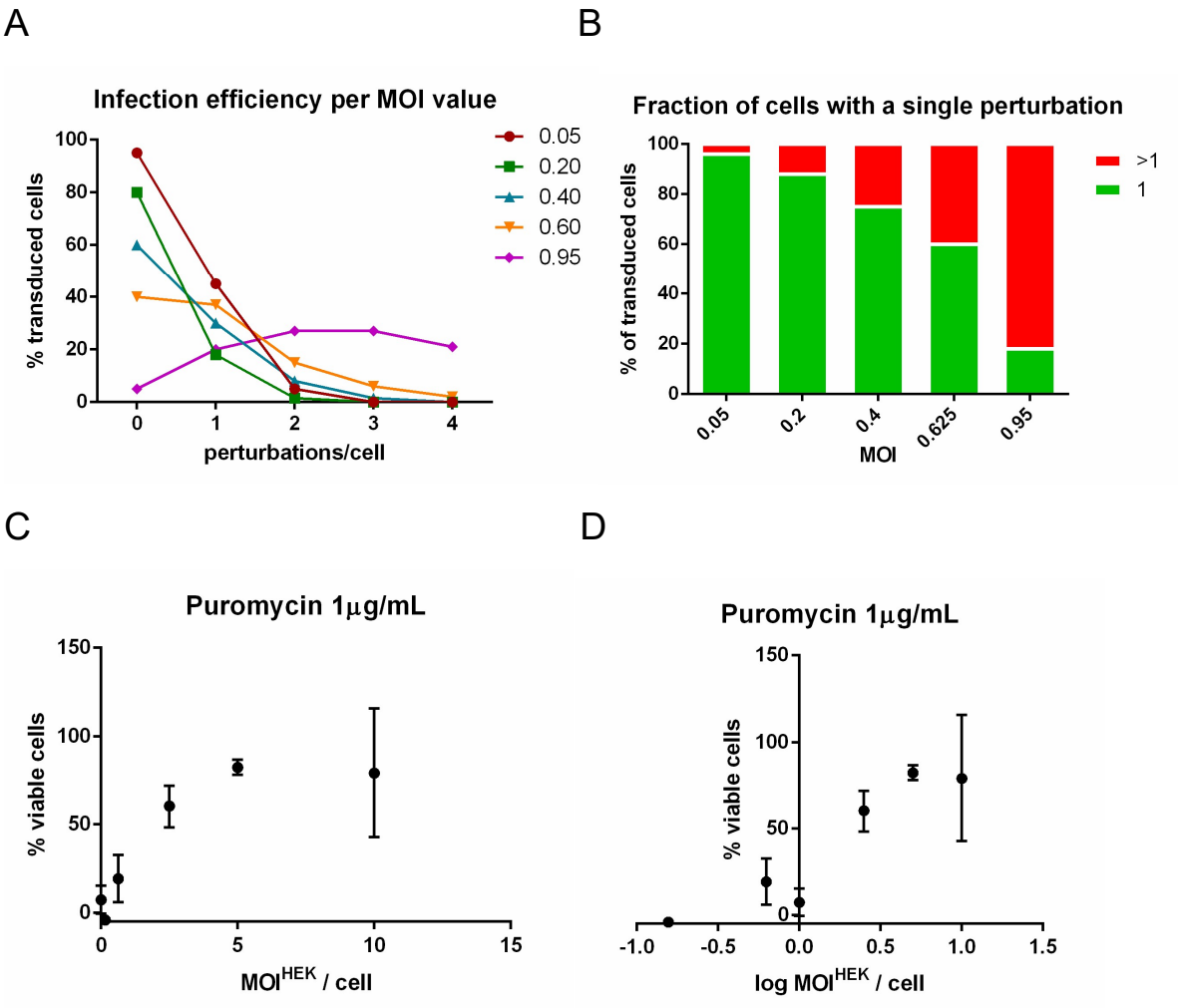


Fig 3. The optimal MOI^{THP-1} is 0.2. Following the Poisson distribution (A and B), the optimal MOI^{THP-1}= 0.2, in order to limit the amount of cells used in the experiment but maximize the number of cells containing a unique sgRNA after transduction, while minimizing the possibility to get more than two sgRNA integrated in the same cell. At MOI = 0.2 theoretically the % of cells with a single perturbation/cell is 20%. Cells were transduced with different MOI^{HEK}/cell, followed with a treatment with puromycin for three days and the PrestoBlue viability assay. Cell viability was plotted in relation to MOI^{HEK} calculated per each THP-1 cell (C). Log MOI^{HEK}/cell was used to linearize the function and calculation of MOI^{THP-1} (D).

The linearized function of Fig. 3D where the Y axis is of % viable THP-1 cells and the X axis the log of MOI^{HEK}per cell was used to calculate the number of MOI^{HEK}required to obtain 20% of successfully transduced and thus viable THP-1 cells in the PrestoBlue assay. The result was 0.53376 MOI^{HEK} per each THP-1 cell.

5.1.3 Description of the nigericin treatment for positive selection

Genome-wide screens can be divided in two different types depending on the outcome of the experiment: positive selection or negative selection screens. While negative selection screens are

based on the observation of which perturbations deplete the presence of cells over time, positive screens are based on the survival of only those perturbations that confer resistance to the treatment tested, and therefore to an enrichment of the population of interest. In particular for positive selection, it is of interest to use a setup where the treatment will lead to very few survivors and will minimize the amount of false positives in the final result.

Based on this premise, we chose nigericin as the activating stimulus to induce the NLRP3 inflammasome in our screen. Nigericin is a pore-forming toxin derived from *Streptomyces hygroscopicus* which acts as a strong K^+ ionophore and can activate the NLRP3 inflammasome in a variety of immune cells (Schroder & Tschopp, 2010). THP-1 cells are highly responsive to this drug and can undergo high levels of cell death at relatively low concentrations of the toxin, making it a fast and rather low-costing option to treat big amounts of cells in the screen.

To visualize the survival curve for THP-1 in response to nigericin over time, THP-1 cells were treated with PMA and the day after treated for one hour with Hoechst, which can migrate across the plasma membrane independently of the cell's viability, and stain the nucleus by intercalating the DNA. Cells were then treated with nigericin in parallel of Sytox Green, which in contrast to Hoechst can only stain the nucleus of cells when their membranes become porous as a consequence of the formation of GSDMD pores. At the end of two hours of treatment the cell death level was over 90% and the curve was slowly approaching a plateau (Fig. 4).

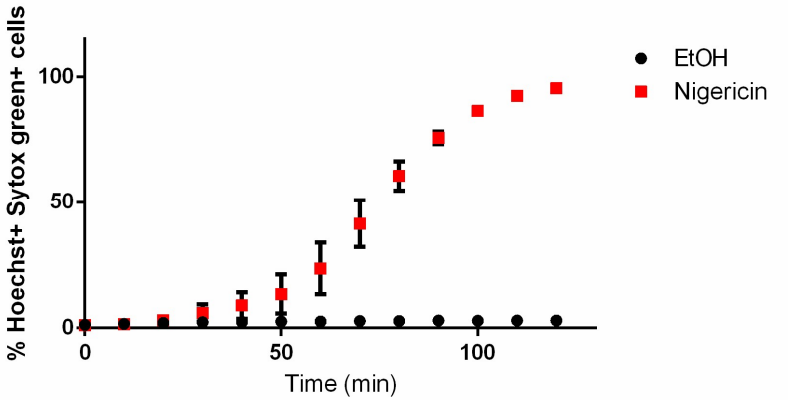


Fig. 4. Dynamic cell death curve of THP-1 wild type cells in response to nigericin. THP-1 cells were incubated for two hours in the presence of 15 µM nigericin at 37ºC and 5% CO_2. Hoechst was used as a general marker of the nucleus to allow visualization of all cells in the microscope. Sytox green co-localization with Hoechst was used as a marker of lytic cell death. Pictures were taken every 10 minutes in 10 regions of the well to quantify a minimum of 200 cells per condition. The experiment was repeated twice.

In sight of this result, we chose to treat the cells in the screen for 2 hours and 15 minutes with nigericin at the commonly used concentration of 15 µM to get a maximum of 10% living cells at the end of the treatment. However, this experiment was performed using WT THP-1 cells. Therefore we could not base our predictions for the Cas9 #22 library on the assumption that these cells would display the same behaviour upon Cas9 expression and lentiviral transduction. Therefore, nigericin-induced cell death was compared between WT cells and the pooled Brunello sgRNA cell library (Fig 5).

Of note, at this point of my work I decided to use a different readout to determine the yield of cell death after the induction of pyroptosis. This decision stemmed from the observation that upon activation of the pyroptotic cell death program, cells detach from the surface and are out of focus, rendering them not visible in the microscope. The consequence of this technical limitation is that the amount of Sytox green-positive cells remain underestimated throughout experiments. From this point on, we thus decided to use fluorescence-associated cell sorting (FACS) for further experiments instead, which is far more sensitive and additionally provides a clear profile of the distribution of the fluorophore in the population. In this new setup we quantified Sytox green-negative cells as opposed to positive green signal, as lytic cells could be counted more than once by the analyser on the basis of the fluorescence distributed in multiple fragments originating from one dying cell.

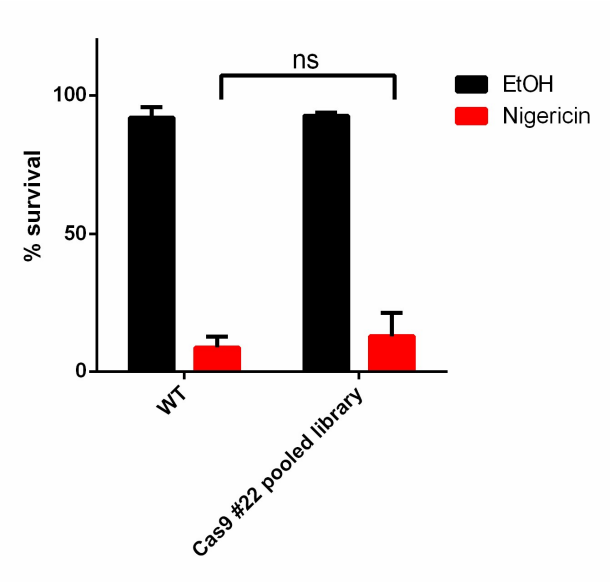


Fig. 5. The knock-out pooled library has similar sensitivity to nigericin as compared to WT cells. Cells were primed with PMA for three hours and incubated in the presence of 15 µM nigericin for further 2 hours and 15 minutes. permeation% survival was determined by % of Syotx green-negative cells. Cells were analysed by FACS to distinguish Sytox green positive and negative cells. Sytox green-negative cells are indicated as surviving cells in graph. Experiments were performed three times. ns indicates non-significant differences.

The results did not vary widely between the two populations upon nigericin treatment after two hours, and therefore the same condition could be used to obtain over 90% cell death in the experiment with the pooled knock-out library. Slightly higher survival was observed in this population in comparison to the WT cells, but this difference was not significant. This could be a consequence of partial loss of sensitivity to nigericin upon stress in response to viral transduction, or of the presence of pyroptosis-unresponsive cells through the knock-out of a gene necessary for this pathway. Still, less than 10% survival was observed in both populations, and therefore 2 hours and 15 minutes of treatment were kept as the condition of choice for the screen.

5.1.4 Final CRISPR/Cas9 genome-wide screen setup

Cas9 #22 cells were transduced with the packaged lentiviral Brunello library following the scheme in Fig. 6. Transduction was repeated three independent times with newly thawed cells using the $MOI^{THP\text{-}1}$ calculated previously.

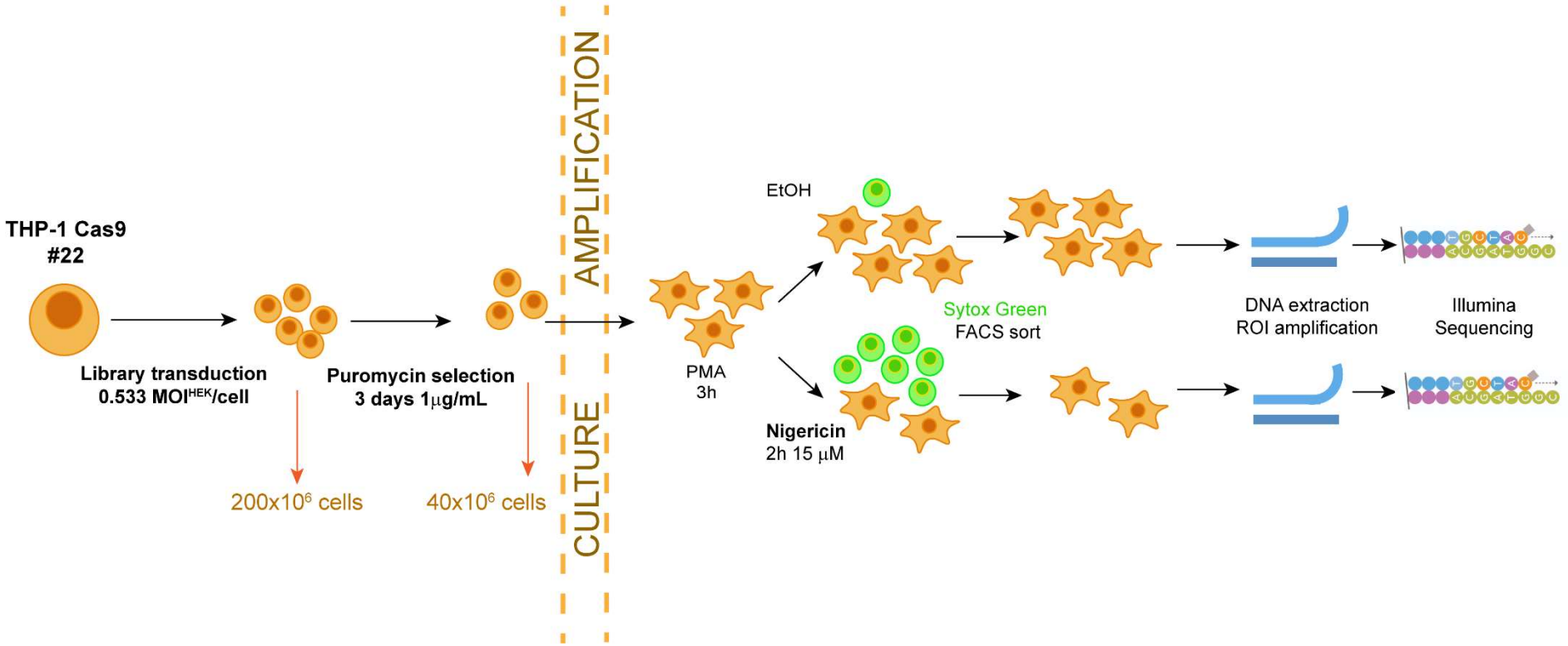


Fig. 6 CRISPR/Cas9 pooled genome-wide screen setup. Briefly, $200x10^6$ Cas9-expressing THP-1 cells were transduced with a total of well homogenised $106.7x10^6$ MOI^{HEK} to reach 20% transduction efficiency. Cells were then selected with puromycin for three days at 1 µg/mL. Then, cells were amplified in normal medium, and two weeks after $80x10^6$ cells were treated with 100 ng/mL PMA for three hours. Cells were allowed to recover in normal medium overnight, and the day after half of the cells were treated with 15 µM nigericin during 2 hours and 15 minutes, and the other half with ethanol vehicle. Cells were then incubated with Sytox Green, and surviving knock-outs were identified as Sytox green-negative cells and were sorted out of the population using FACS. For both treatments, surviving cells were digested overnight with Proteinase K, and their genome was extracted the day after using the phenol chloroform extraction method. Integrated sgRNA were amplified from the genome using previously described PCR protocols. Amplicons were purified using AMPure beads, and subsequently sequenced using Illumina sequencing.

Nigericin treatment showed to provide highly similar results in cell death for all three independently transduced libraries, and error bars for the same treatment were low (Fig. 7). This was important to allow comparison of results between the three replicates and to determine statistical significance.

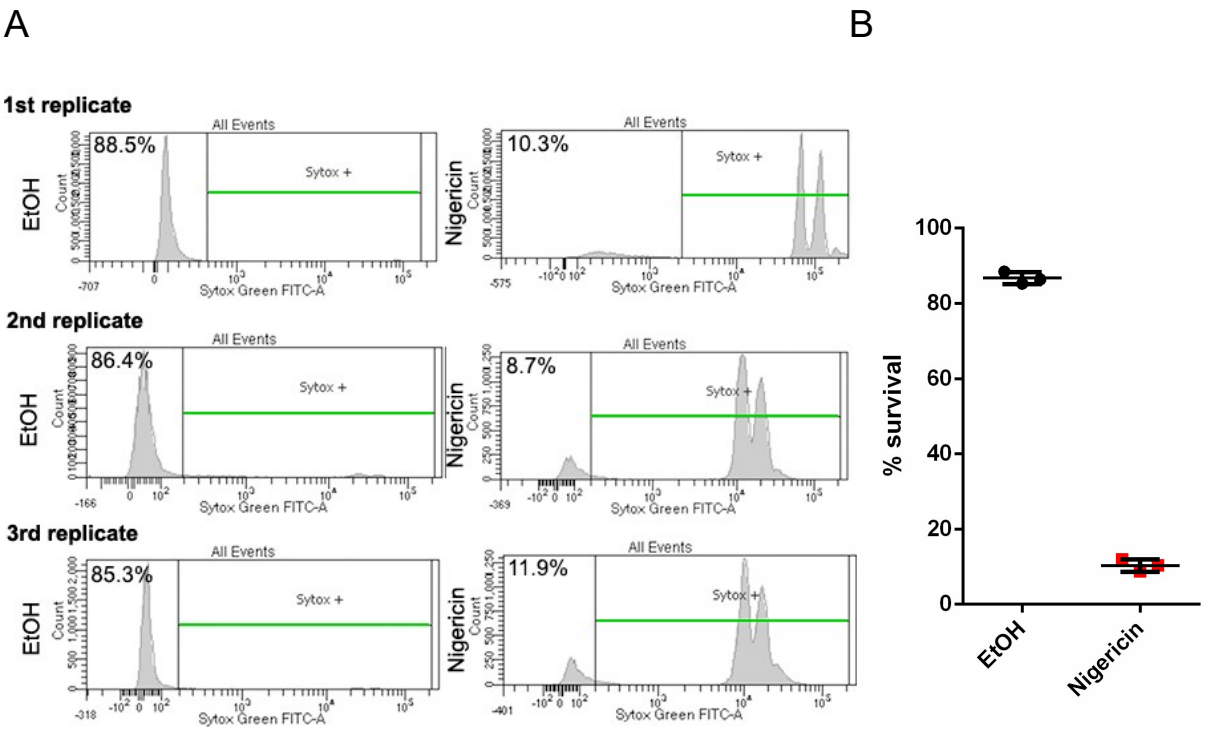


Fig. 7 Cell survival across replicates upon nigericin treatment was about 10%. (A) FACS sort results of control ethanol (EtOH) and nigericin treatment for 2 hours and 15 minutes for all biological replicates, which originate from three independent lentiviral transductions. Percentages indicate the proportion of the population that was considered to be Sytox green negative. (B) Plot of survival across all replicates. Error bars indicate variance between the three replicates.

Sytox green-negative cells were recovered and digested with Proteinase K-containing digestion buffer and DNA was extracted following the steps indicated in Materials and Methods.

5.1.5 sgRNA loci amplification by PCR

In order to uncover the genetic signature of surviving cells in the control and the nigericin treatment, the lentiviral sgRNA library provides yet another great advantage. Integrated sgRNAs remain in the genome, and therefore can be traced back at any step of the screen, in comparison to short-lived plasmids that are rapidly digested in the cytosol. Furthermore, the architecture of the sgRNAs in the Brunello library is such that the flanking regions around the sgRNA are common for the whole sgRNA pool. Therefore, universal amplification of sgRNAs can theoretically be done using simply one primer forward and one primer reverse for the whole library. In practical terms, sgRNAs can be amplified in bulk using the same PCR reaction (Fig. 8). All six samples were amplified using a mix of forward staggered primers, which provide additional variability across the flow-cell, to help maximize the number of reads for each sample in a setup of low diversity of primers. The reverse primer used for each sample was, on the contrary,

different for each replicate and condition. This allows for all samples to be amplified together in the flow-cell and to be able to be compared to each other directly.

Each independent sample might accumulate PCR inhibitors, which can be co-purified during DNA extraction, and be detrimental for amplification of the sgRNA amplicons. The amount of PCR inhibitors can largely vary from one sample to another, and therefore PCR conditions need to be set up independently for each replicate. Optimal settings for sgRNA loci amplification were determined using a small sample of the ethanol treatment for each replicate sample. Nigericin samples, however, started from a significantly lower amount of DNA than their vehicle counterparts, and therefore this optimization step would result in a great loss of DNA in comparison. For this reason, and given EtOH and nigericin samples for the same biological replicate were processed in parallel during every step of sample preparation, nigericin samples were amplified with the same conditions chosen for their respective control. Simple PCR for the first biological replicate (R1-EtOH and R1-Ni) was performed following the program specified in the protocol section. The optimal number of cycles of PCR was chosen as a balance between visualization of the amplicon of sgRNA at the right size, 350 bp, and before the apparition of additional bands from possible unspecific or denatured sequences (Fig. 8). Then, the rest of the DNA was used following these conditions to amplify the rest of the sample. Unfortunately, the second and third replicates (R2 and R3) did not display any band at 350 bp using this reaction setup. In this case sgRNAs were amplified instead using Touch-Down PCR (TD-PCR), which is characterized by a starting annealing temperature much higher than melting temperature (T_m) of the primers, which with every cycle progressively goes down to the T_m. This approach serves to increase specificity of the reaction (Korbie & Mattick, 2008). In this case amplicons at 350 bp were obtained, but additional bands could be observed at a higher molecular weight in the gel. Given the big increase of the cycles necessary for these protocol, it is likely these bands belong to denatured DNA upon exhaustion of primers (Korbie & Mattick, 2008).

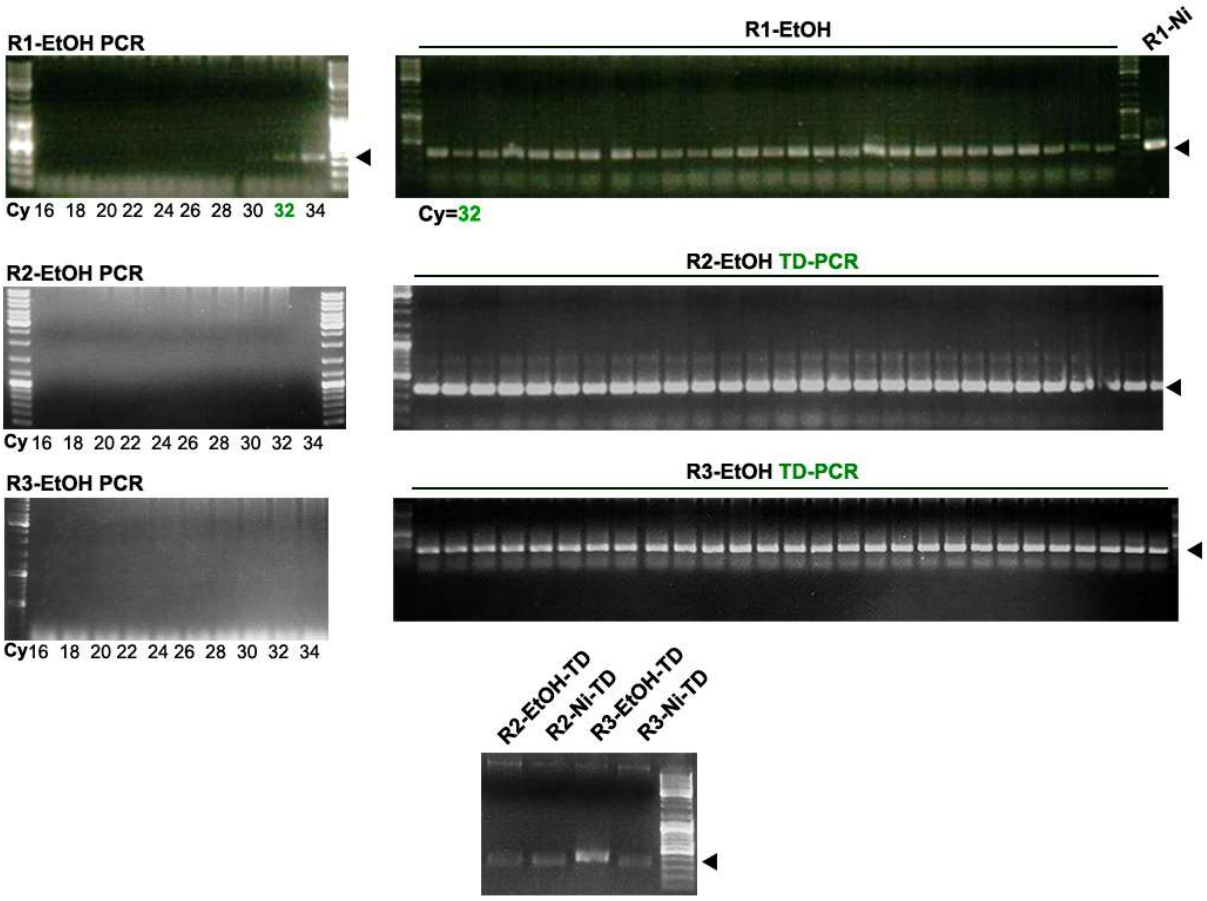


Fig. 8. sgRNA loci from all three replicates were successfully amplified from genomic DNA samples. Sample R1-EtOH was tested with standard PCR settings to define the cycles of amplification. Cycle 32 was chosen, and used for amplification of all R1-EtOH DNA and R1-Ni. A standard PCR set up for sample R2-EtOH and R3-EtOH was unsuccessful. The same program of Touch down PCR (TD-PCR) was used for amplification of all DNA in R2 and R3 samples and for the Nigericin treatments in these replicates. R2 and R3 samples were run together in an agarose gel.

After all the genomic DNA template was used, the PCR reaction mixes were pooled together and well homogenised for DNA purification following the protocol described in the Materials and Methods. Then, purified DNA was sent to be sequenced by Illumina next-generation sequencing.

5.2 The CRISPR/Cas9 genome-wide screen confirmed the requirement of currently known proteins involved in NLRP3 inflammasome activation and uncovered new potential players

5.2.1 Quality control of the CRISPR/Cas9 genome-wide screen

Several indicators of sequencing quality were assessed to determine the reliability of the sequencing result. Read depth serves as an indicator of how evenly samples have been amplified in the flow-cell of the sequencer and how well the extracted sequences can be traced back to the original Brunello library (Fig. 9A). Counts for a specific sequence or sgRNA are not compared between all samples directly. Instead, they are normalized to the total number of counts in that sample and then compared to the normalized counts in the control replicate for that specific treatment. Therefore, high count reads are to

be desired across samples, but small changes from one sample to another do not interfere greatly with the interpretation of the final result. Importantly, no striking differences in total reads or read-depth were observed between samples amplified using standard PCR or TD-PCR. Of note, the EtOH control for replicate 1 showed more failed alignments to the library than any other sample, although it had been prepared with the standard PCR protocol. This suggests that additional bands observed in the agarose gels after TD-PCR do not correspond to unspecific sequence amplification, but rather to denatured DNA that can then be still used as template during the sequencing step. Over all, aligned reads across the board went from 20 to 45 million, indicating that minimum average coverage in each sample was of 250 counts per sgRNA. This coverage has been used successfully in previous positive selection screens (Hart et al., 2015; T. Wang et al., 2014). Overall, this amount of reads, albeit not ideal, was sufficient to process sgRNA counts and find statistically significant differences between the nigericin treatment and the control.

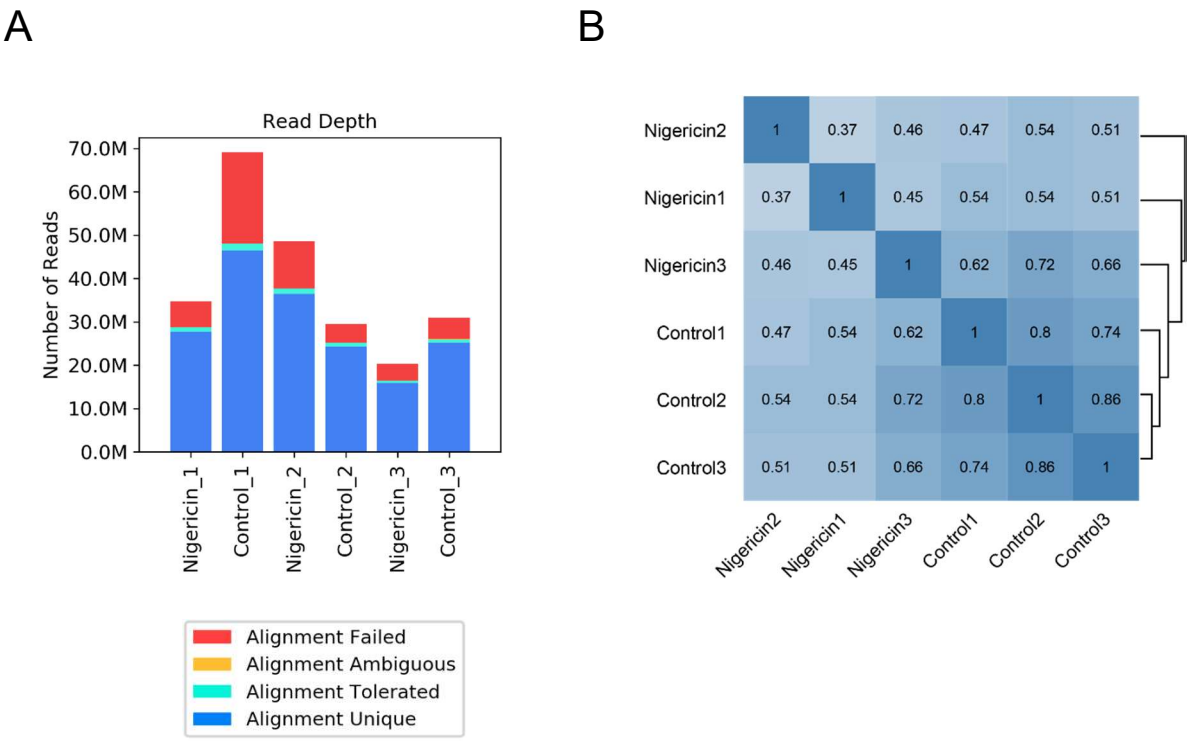


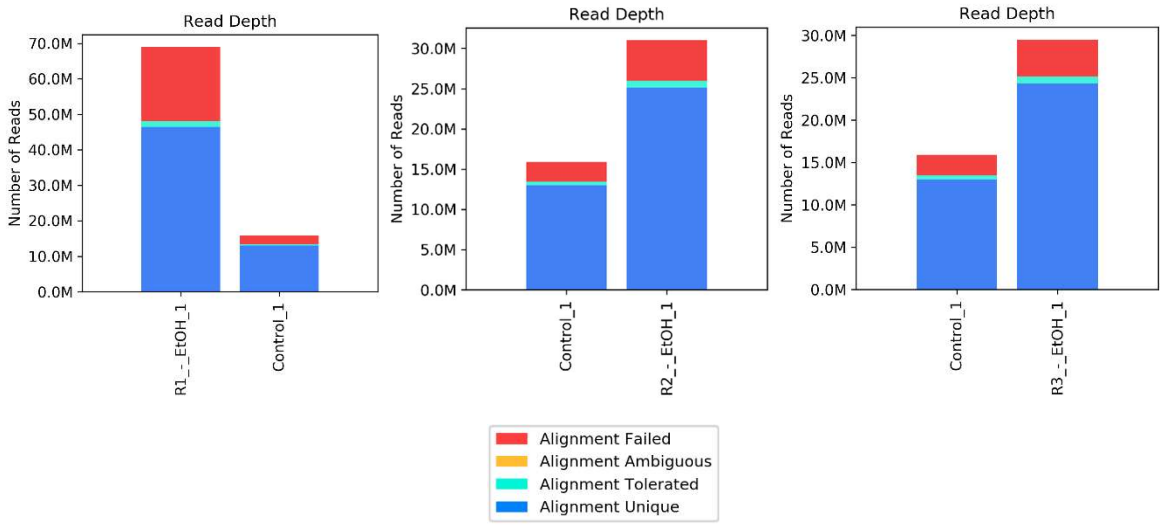
Fig. 9. Quality control of the CRISPR/Cas9 screen. (A) Read depth across the flow-cell in Illumina sequencing. Reads correspond to number of sequences sequenced per each sample. Reads were divided in failed, ambiguous, tolerated or unique alignments depending on the traceability of the sequence to the original Brunello sgRNA library. Unique, ambiguous and tolerated alignments were used to determine the number of counts for each sgRNA. (B) Similarity between different samples by sgRNA count, graph created using the CRISPR-VISPR tool.

An even distribution of the gene knock-outs across the pooled cell library is key to assess gene function without technical bias. In order to determine how conserved the distribution of sgRNA between samples was in our screen, sgRNA counts were compared across each sample. Control 2 and 3 were the most similar. Control 1 had lower similarity to the other two control replicates, probably owing to a longer period of culture, in the grounds of characterization of nigericin sensitivity. Altogether the three control replicates were arguably similar. For Nigericin samples, the sgRNA distribution was not as conserved. This could be due to accumulation of certain knock-out populations overtime, or could simply be a result of stochastic differences in cell fitness at the moment of the experiment. Indeed, the knock-outs of

interest at the end of the experiment are the ones that were most conserved across the three nigericin replicates which are expected to be the least subjected to these kind of variations. These genes will be selected below using specific statistical tests.

Not only similarity between libraries is required for faithful results at the end of the screen, but also knock-out libraries should have a similar distribution of sgRNAs in comparison to the original plasmid library, and this should be consistent in all biological replicates. To evaluate this, a sample of the original plasmid pool was included in the flow-cell, sequenced, and compared to each independent replicate control sample (Fig. 10A).

A



B

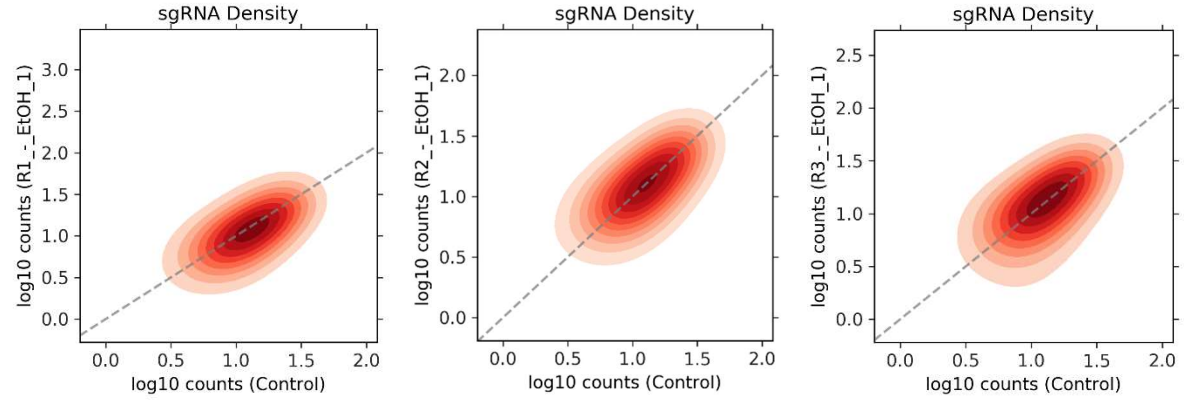


Fig. 10. sgRNA density is similar across replicates and in comparison to the plasmid library. (A) Quality of reads for each sgRNA was divided in categories according to its traceability to the Brunello sgRNA library. (B) Plot of log counts per sgRNA comparing each ethanol sample and the original plasmid library.

Read depth of the plasmid library was lower than that of ethanol samples, but that did not pose a problem as coverage of sgRNA in the original sgRNA pool is not subject of the efficiency of Cas9. Noteworthy was the fact that sgRNAs seemed to be distributed similarly between the plasmid library and each replicate (Fig. 10B). Differences between the original library and the replicates can be due to uneven virus packaging, loss of essential gene representation in the library or accumulation of knocked-out cells

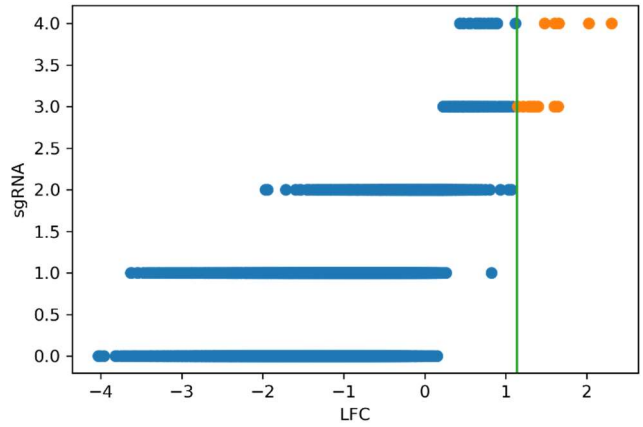
for genes whose absence provides a survival or proliferation advantage. Noteworthy, the results in R1-EtOH/Plasmid did not appear to be more skewed than in the other two replicates, indicating that long periods of culture do not seem to affect dramatically the composition of knock-outs in the library.

5.2.2 Statistical analysis of the CRISPR/Cas9 screen and final Hit List

The outcome of a positive selection CRISPR/Cas9 genome-wide screen needs to reflect the genes with the highest likeliness to be required in a specific cellular process based on the pooled enrichment of each of their targeting sgRNAs. Comparison of big data is not simple, and specific packages of functions have been designed to do this. Among other functions, these packages contain several possibilities for statistical analysis. To extract the data enclosed in our screen we have chosen the Model-based Analysis of Genome-wide CRISPR/Cas9 Knockout (MAGeCK) (Wei Li et al., 2014). This model allows the bulk processing, alignment and comparison of enrichment of sgRNA and genes between an experimental group and the control population. MAGeCK prioritizes sgRNAs, genes and pathways to provide a robust list of genes enriched in the group treated with the drug of interest. We chose the modified Robust Ranking Aggregation (α-RRA) algorithm available in MAGeCK for statistical analysis. This algorithm assumes that if a gene has no effect in selection, all its sgRNAs should be distributed uniformly along the ranked list of all sgRNAs. Therefore, it assigns gene priority based on whether the consistent placement of its targeting sgRNAs in the top tier of enriched sgRNAs.

Upon launching MAGeCK, a comprehensive list ranking all genes according to their P-value and base 2 logarithmic fold change (LFC) was generated. One of the critical steps in screen analysis is to define where the cut-off determining the genes of interest should be set. Usual considerations include setting significance as a maximum P-value = 0.01 and selecting genes with more than half sgRNAs enriched in the treatment group. In this case, the Brunello library harbours 4 different sgRNA targeting every gene, and therefore this number was set at 3. Genes were organized by decreasing number of logarithmic fold change, from the highest to lowest enriched genes. Still, determining which genes made the cut was not straight forward: several genes followed the previous criteria, with slight variations in LFC. It is rarely obvious where to set the line that defines the outlier genes, and it is important to tailor the genes to a stringent number that makes its analysis feasible. We finally decided to set the threshold at GSDMD, the main effector of pyroptosis downstream of NLRP3 inflammasome activation (Fig. 11), thus simplifying the original list to a stringent list of 18 final genes.

A



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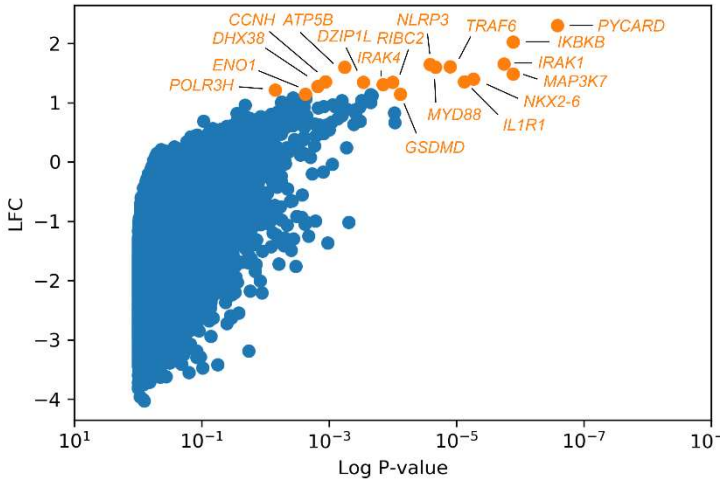


C

Rank	Gene	Num	LFC	P-value	Rank	Gene	Num	LFC	P-value
1	*PYCARD*	4	2.30	2,59E-07	10	*IL1R1*	3	1.35	7,51E-06
2	*IKBKB*	4	2.02	1,29E-06	11	*CCNH*	3	1.34	1,14E-03
3	*IRAK1*	4	1.65	1,81E-06	12	*RIBC2*	3	1.34	1,00E-04
4	*NLRP3*	3	1.64	2,62E-05	13	*DZIP1L*	3	1.34	2,92E-04
5	*TRAF6*	4	1.60	1,27E-05	14	*IRAK4*	3	1.30	1,43E-04
6	*MYD88*	3	1.59	2,15E-05	15	*DHX38*	3	1.27	1,50E-03
7	*ATP5B*	3	1.59	5,66E-04	16	*POLR3H*	4	1.21	7,01E-03
8	*MAP3K7*	4	1.48	1,29E-06	17	*ENO1*	3	1.14	2,35E-03
9	*NKX2-6*	3	1.39	5,44E-06	18	*GSDMD*	3	1.14	7,59E-05

Fig. 11. Top hits enriched upon nigericin treatment in the CRISPR/Cas9 genome-wide knock-out screen. (A) Genes plotted according to LFC and number of enriched sgRNAs. Threshold (green line) is set at LFD= 1.14, genes LFD> 1.14 are represented in orange. (B) Plot of genes according to their LFC and P-values. Genes that have a LFD>1.14 are represented in orange and are named by their ID. (C) List of hits obtained by MAGeCK. Num is

number of sgRNA significantly enriched in the nigericin treatment compared to the control. LFC is log fold change. The list contains only genes from the original result that followed 3 limiting criteria: Num ≥ 3, P-value ≤ 0,01 and LFC ≥ LFC^{GSDMD}. LFC was the parameter used to order the hits in the list. Genes already known to participate in priming or NLRP3 inflammasome activation are marked in green.

Excitingly, two members of the NLRP3 inflammasome complex (NLRP3 and ASC, codified by *PYCARD*) and several genes involved in cell priming (*IKBKB, IRAK1, TRAF6, MYD88, MAP3K7* and *IRAK4*), were found in the top positions of the final hit list. There were some priming genes of slightly lower LFC that did not make the cut into this refined selection, such as the NLRP3-activating phosphatase codified by *PPP2R2A* (LFC = 1.13). It was initially surprising that *CASP1* was not included in the top hits. This could however be explained by the low efficacy of 2 out of 4 sgRNA targeting this gene. Therefore, it is to be expected that some of the genes left out of the list might be false negatives. Nonetheless, the absence of *CASP1* due to 2 low-performance sgRNAs rather suggests the hits obtained in this list are true hits, and the presence of an abundant number of expected hits in the highest ranks clearly demonstrates the robustness of this screen.

New genes that could be potentially required for nigericin-induced cell death were identified: *ATP5B*, *NKX2-6*, *CCNH*, *RIBC2*, *DZIP1L*, *DHX38*, *POLR3H* and *ENO1*. None of these genes have been documented to date to play a role in the NLRP3 inflammasome cascade leading to pyroptosis except for *ENO1*. Interestingly, inhibition of the *ENO1* product α-enolase has been proposed to induce the NLRP3 inflammasome by downregulating glycolytic flux, leading to perturbed NAD+/NADH ratios and to a build-up of mitochondrial ROS species (Sanman et al., 2016). It should hence be expected for ENO1 knock-out cells to be depleted upon nigericin treatment, instead of enriched, as observed in my screen. However, results in this study on *ENO1* were not solid, and contribution of downregulating glycolytic flux as an event activating the NLRP3 inflammasome has already been introduced as a subject of discussion elsewhere (Próchnicki & Latz, 2017). Therefore, I decided to study the requirement of this gene in the pathway along with the rest of hits.

One useful tool to get a hint of which candidate genes could indeed play a function in the NLRP3 inflammasome signalling cascade is to search for predicted enriched pathways. However, there did not seem to be specific functions unifying the genes selected in our list outside of NF-κB signalling cascade and immune responses. Additionally, the function of some of these genes remains largely unknown. Therefore there were no hints to suggest which genes were the most faithful hits. This uncertainty raised the need for an unbiased approach to answer this question. To this end, a secondary screen limited to these genes can be a useful tool to pave the next step forward and unravel which hits this list hold true interest for the understanding of the NLRP3 inflammasome pathway.

5.2.3 Confirmation mini-screen

Inspired by the experimental workflow of the genome-wide screen itself, we decided to conduct a secondary mini-screen for the shortlisted genes making use of the same gene editing technology. *DHX38* and *POLR3H* were not included in the confirmation screen. DHX38 has essential functions in most cells, rendering *DHX38* knock-out cells unviable (Y. Wang & Guthrie, 1998). This is thus very likely a false-positive hit. POLR3H, as a subunit of the RNA polymerase III, has already been shown to play

a role in the activation of NF-κB upon LPS priming and its deletion leads to lower pro-inflammatory cytokine secretion (Graczyk et al., 2015). For the rest of the hits, I designed two sgRNA targeting each gene that did not overlap with the ones designed in the Brunello library. sgRNAs were cloned into LentiGuide vector, which is the same backbone of Brunello library. Cas9 #22 cells were then co-transduced with two sgRNA-expressing lentivirus. Following selection with puromycin, cells were primed with PMA and treated with nigericin and analysed as indicated previously using FACS (Fig. 12).

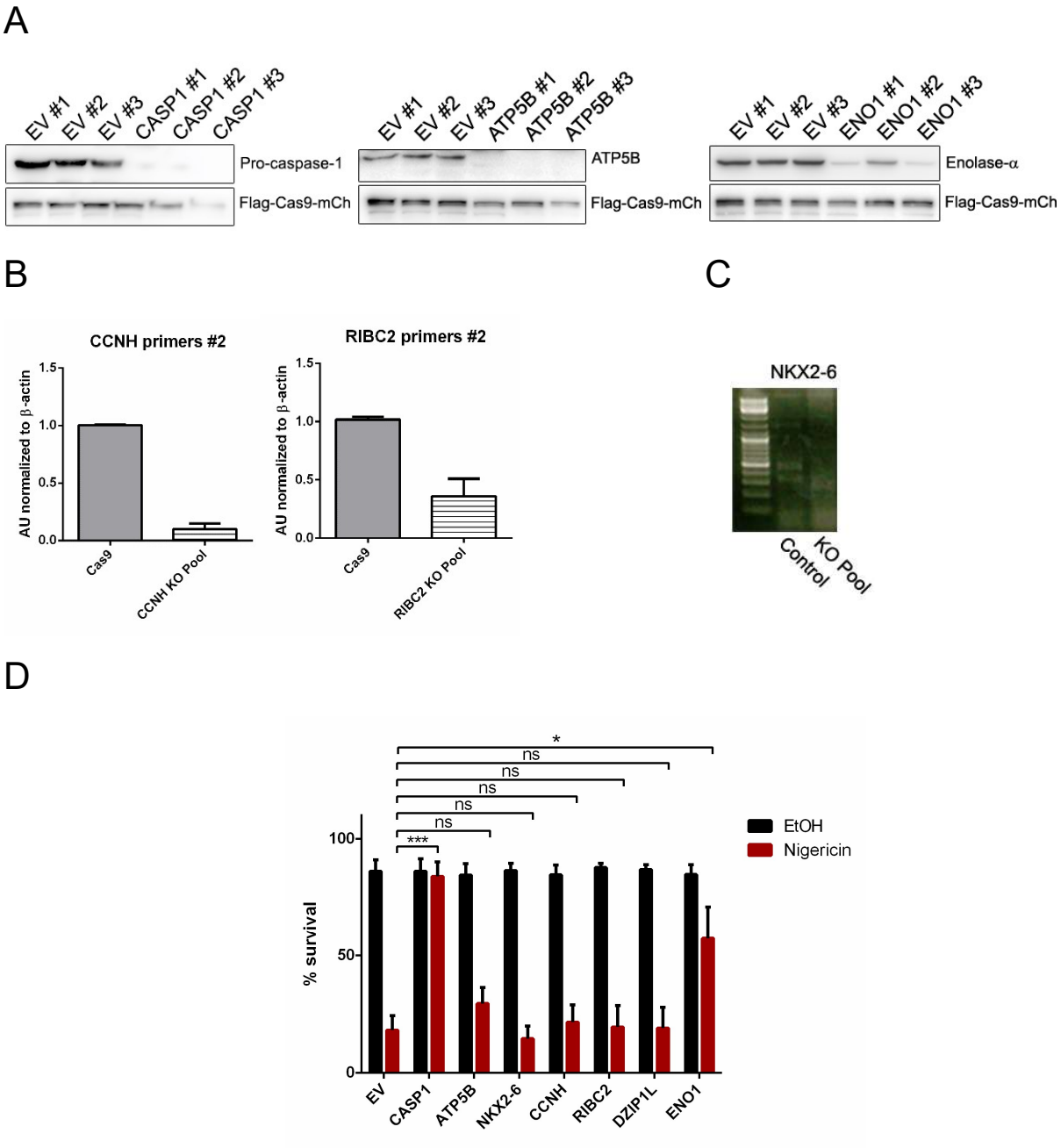


Fig. 12. *ENO1* knock-out pools are partially protected of nigericin-induced pyroptosis. Three independent transductions were performed in order to obtain three independent knock-out pools for each gene. (A) Immunobloting for indicated proteins using extracts from pooled knock-out cells and empty vector (EV) controls. (B) Quantification of mRNA levels of CCNH and RIBC2 in knock-out pools. (C) PCR amplification of the region flanking the NKX2-6 sgRNA 1 and 2 in the *NKX2-6* KO pool. Images show representative example out of three experiments. (D) Percentage of Sytox green- cells in each knock-out pool was used as the parameter to define non-pyroptotic cells. Cells were primed with PMA for three hours, and the next day for one hour in the presence of nigericin,

followed by Sytox green incubation and FACS. Experiments were repeated three independent times and significance was determined by P-value < 0.05. * represents P-value < 0.05 and *** represents P-value < 0.0005, ns represents non-significant differences.

Out of all the genes tested, the *ENO1* knock-out pool stood out as the only population with a protective effect comparable to the control *CASP1* knock-out pool. Some cells still underwent pyroptosis, but this could be due to the incomplete knock-out in the pool, as confirmed in the immunoblotting for this protein (Fig. 12A). *ATP5B* KOs showed a weak tendency to protect cells from cell death. The other knock-out pools did not show any substantial effect in this setup, while knock-out efficiency appeared to be comparable to *CASP1* as assessed by protein levels in their respective pools, with the exception of *DZIP1L*, which could not be characterized. It is still possible that the remaining protein levels in the knock-out pools could be sufficient to drive cell death in response to nigericin. However, the striking result observed in the *ENO1* KO pool indicated that *ENO1* held great promise as a potential component of the pathway leading to NLRP3 inflammasome activation and subsequent pyroptosis. Although the effects in the other genes were low like *ATP5B* or unnoticeable, it could be of interest to design new sgRNAs to optimize knock-out efficiency, in which case stronger effects that go unnoticed in this setup could emerge.

5.3 *ENO1* plays an essential role in NLRP3 inflammasome assembly

5.3.1 Generation of *ENO1* KO single-cell clones in THP-1 cells

Phosphopyruvate hydratase, otherwise known as enolase, is the 9th enzyme of the glycolytic pathway, and it is responsible for the penultimate step of glucose catabolism to pyruvate. It was first described by Lohmann and Meyerhof in 1934 in muscle extracts as a metalloenzyme active in the presence of Mg^{2+}. The ubiquitous nature of glycolysis as a basic process to produce energy from glucose makes enolase widely present in most organisms, and the gene codifying for this enzyme is highly conserved across species. In all prokaryotes, enolase is codified by a single gene, while in vertebrates the enzyme can be transcribed from three independent genes. *ENO1* is expressed in all tissues and codifies for α-enolase, whilst *ENO2* is expressed specifically in neurons and neuroendocrine tissues to form γ-enolase and *ENO3* is the form transcribed in muscle, and codifies for β-enolase. More recently, a putative *ENO4* gene has been described, which appears to be expressed mostly in testis and shares the same enzymatic activity as the other members of this family (N. Nakamura et al., 2013). All these isoforms share the feature that in order to become enzymatically active they require Mg^{2+} and engage in the formation of dimers, in which two enolase subunits organize in an antiparallel manner (Fletcher et al., 1976). Depending on the tissue, enolase can form homodimers or heterodimers, which display a similar enzymatic activity.

As aforementioned, α-enolase inhibition has been previously shown to lead to pro-caspase-1 proteolysis and maturation of pro-IL-1β in LPS-primed BMDMs. This observation has been related to a general effect of down-regulation of glycolysis in Nlrp3 inflammasome activation (Sanman et al., 2016). However, the approach used in this paper to inhibit α-enolase was to use a putative enolase inhibitor known as ENOblock or AP-III-a4. It has been later shown that ENOblock does inhibit the enzymatic activity of α-enolase (Satani et al., 2016). For this reason, we decided to take a genetic approach to

deplete *ENO1* in THP-1 cells in order to confirm the result observed in the genome-wide screen and in the confirmation mini-screen. To generate a stable cell line lacking α-enolase function, the sgRNA1 and sgRNA2 targeting exon 8 in the human *ENO1* gene used for the mini-screen (Fig. 13A) were sub-cloned in a plasmid containing Cas9 and EGFP used for transient expression in THP-1 cells. Successfully transfected cells were selected on the basis of EGFP fluorescence, and grown as single cell colonies until clones were sufficiently amplified to obtain protein lysates for Western blot (Fig. 13B). Clones lacking α-enolase expression were selected by Western Blot.

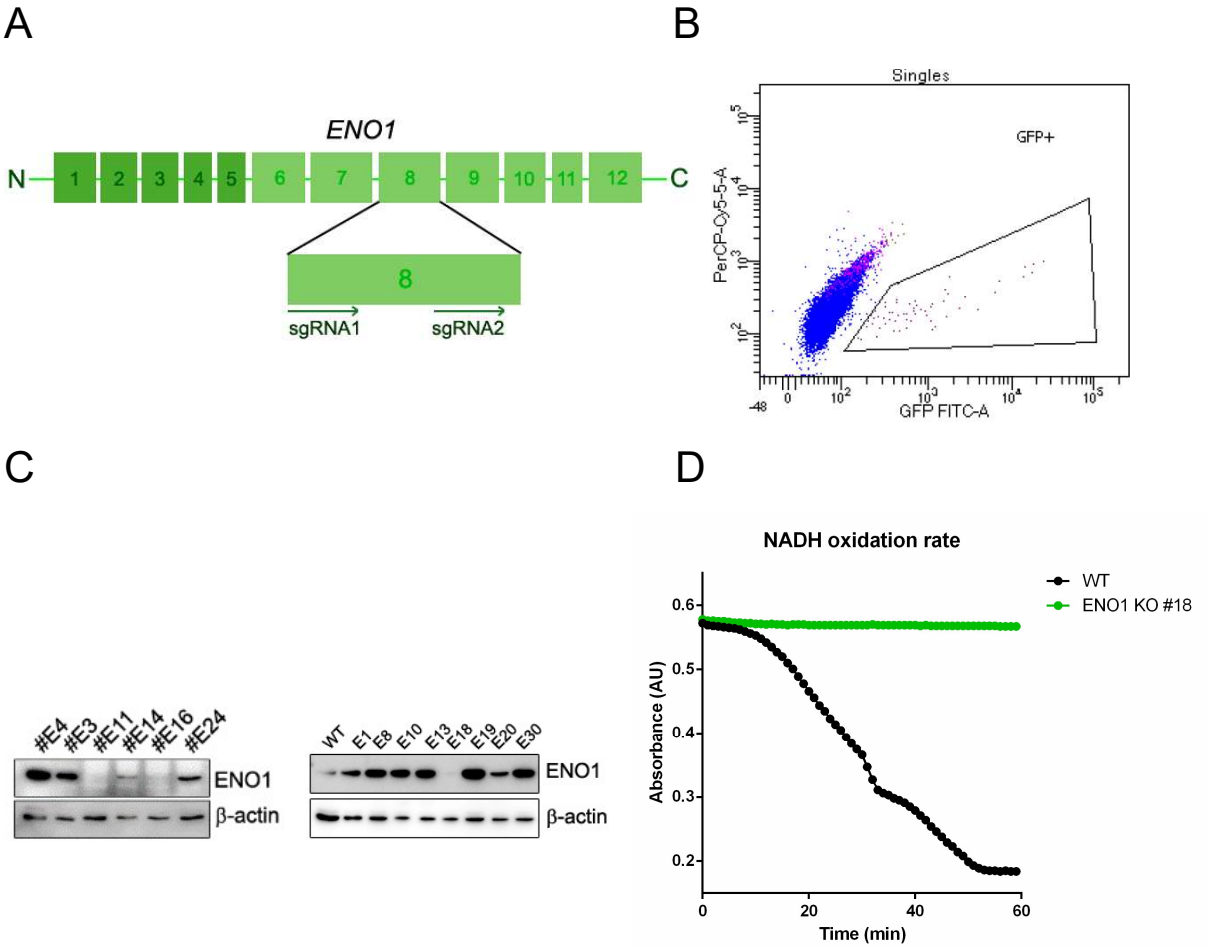


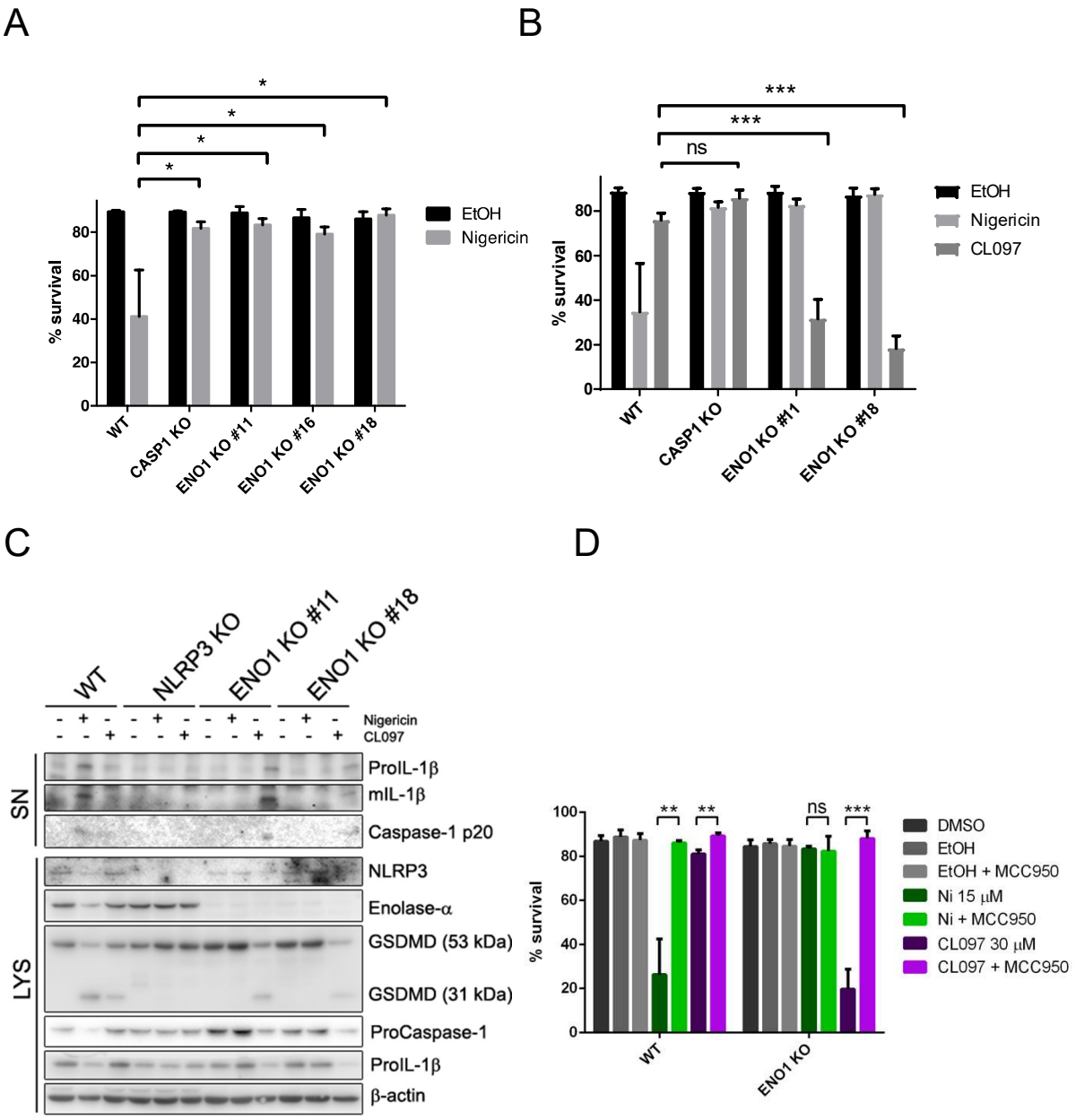
Fig. 13. Generation of *ENO1* KO THP-1 cells. (A) sgRNA1 and sgRNA2 were designed targeting the exon 8 of the human *ENO1* gene sequence for CRISPR/Cas9 gene editing. (B) EGFP+ cells upon co-transfection with sgRNA1-Cas9-EGFP and sgRNA2-Cas9-EGFP were sorted out using FACS and further cultured. (C) EGFP+ cells grown in single cell cultures were amplified, and protein lysates were produced to assess protein levels of α-enolase by Western blot. (D) NADH oxidation rate using a kit of enolase enzymatic activity. WT and *ENO1* KO cells were primed for three hours. Protein extracts were isolated and serially diluted to obtain different protein amounts. α-enolase in protein extracts was bound to a surface coated with α-enolase antibodies, then incubated in the presence of pyruvate kinase, ADP, LDH and NADH. Plates were read during one hour, and measurements were taken every minute for each sample.

Clones #11, #16 and #18 were selected for further experiments to characterize the function of the *ENO1* gene based on their α-enolase protein levels (Fig. 13C). Additionally, α-enolase activity was confirmed to be completely absent in clone #18 (Fid. 13D). Of note, a high proportion of clones showed problems to cope with growth from single cells, and apoptosis could be detected in many of the clones before Western blot analysis. This is not surprising, as it has already been previously reported that silencing of

ENO1 with shRNA leads to higher apoptosis rates (Hui Qiao et al., 2018). Furthermore, all *ENO1* KO clones showed lower cell division rates in comparison to their wild type counterparts, which has already been reported when α-enolase is targeted with drugs or genetically silenced in cell lines (Capello et al., 2015; Dai et al., 2018).

5.3.2 *ENO1* KO THP-1 cells are resistant to cell death induced by nigericin

Next I asked whether these cells were indeed resistant to cell death induced by nigericin. For this purpose, clones #11, #16 and #18 were primed with PMA, treated with nigericin and further incubated with Sytox green to analyse cell death on the basis of the permeation of this dye.



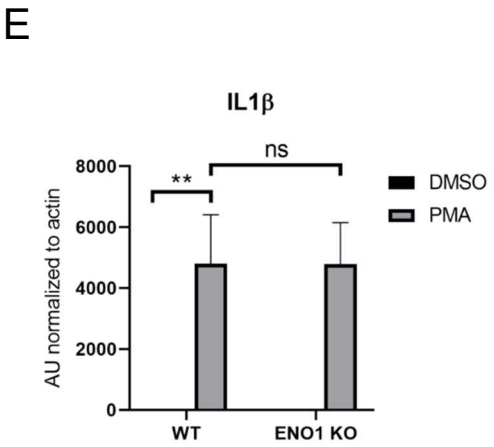


Fig. 14. *ENO1* KO cells are protected against nigericin-induced cell death but display high levels of lethality in response to CL097. (A) *ENO1* KO #11, #16 and #18, *CASP1* KO and WT cells were primed as indicated above and treated for one hour with 15 µM nigericin (A) or 30 µM CL097 (B). (C) Western blot of WT, *NLRP3* KO and *ENO1* KO clones in the previous conditions. *ENO1* KO cells did not secrete IL-1β or caspase-1 and did not cleave GSDMD in response to nigericin, but did so in response to CL097. (D) Cell death by nigericin and CL097 was blocked by pre-incubation with the NLRP3 inhibitor MCC950 at a 10 µM concentration in *ENO1* KO and WT cells. Experiments were repeated three independent times and significance was determined by P-value < 0.05. * represents P-value < 0.05, ** represents P-value < 0.005 and *** represents P-value < 0.0005, ns represents non-signficant differences.

Although cell survival varied across replicates in WT cells, probably due to different time frames cells were kept in culture, it was strikingly evident that *ENO1* KO were completely protected from cell death triggered by nigericin in these conditions (Fig. 14A). All three clones behaved in a similar manner, and conferred a protection comparable to that of *CASP1* KO. This result suggests that the protein encoded by the *ENO1* gene is essential in response to this toxin, and therefore suggests a role of *ENO1* in NLRP3 inflammasome activation. I next asked whether this resistance to cell death was unique to K^+-dependent NLRP3 activators such as nigericin, or it was universal to all NLRP3 inflammasome activators. Imiquimod has been shown to activate the canonical inflammasome but independent of K^+ efflux, rather relying on the accumulation of mtROS species by targeting mitochondrial reductases and the oxidative phosphorylation chain Complex I (Groß et al., 2016). Cells were thus treated with a potent imidazoquinoline derived from imiquimod (CL097), which is more efficient to activate the NLRP3 inflammasome than imiquimod itself.

Unexpectedly, *ENO1* KO cells became permeable to Sytox green when treated with CL097 (Fig. 14B). Interestingly, cell death in response to CL097 was markedly more pronounced and occurred more rapidly than seen in WT counterparts. The *ENO1* KO clone #16 was not used in this or any subsequent experiments due to its low rate proliferation, but this phenotype was observed in both other clones. Immunoblotting of cell lysates from these cells demonstrated that inflammasome activation occurred in the *ENO1* KO clones in response to CL097, while there was no activity visible in lysates derived from nigericin-stimulated cells (Fig. 14C). Typical markers of inflammasome activation observed in *ENO1* KO cells in response to CL097 but not nigericin included cleavage of GSDMD responsible for pore-forming

activity, and pro-IL-1β and pro-caspase-1 maturation and secretion. This result suggested that the activation of the inflammasome was triggered by CL097 but not nigericin in *ENO1* KO cells. It still could be that NLRP3 inflammasome activation is not the cause of cell death. To answer whether the observed cell death is NLRP3 inflammasome dependent, cells were treated with MCC950, an inhibitor of NLRP3. MCC950 specifically binds to the NACHT domain of NLRP3 and inhibits its self-oligomerization capacity (Coll et al., 2019). Strikingly, CL097-derived cell death in both WT and *ENO1* KO cells was completely blocked when pre-treated with MCC950 (Fig. 14D). Together, these results indicate that α-enolase is required for NLRP3 inflammasome activation and pyroptosis in response to nigericin, but not Imiquimod.

I next wanted to understand at which step of NLRP3 inflammasome activation α-enolase is playing a role. α-enolase owns a nucleotide binding domain and can bind to DNA in some cases, although this has so far only been observed *in vitro* (Feo et al., 2000; W. Wang et al., 2005). I therefore asked whether ENO1 is involved in the transcriptional expression of some of the members of the inflammasome. Albeit the protein levels of pro-IL-1β in the protein lysates varied across experiments, the RT-qPCR result clearly showed that pro-IL-1β is expressed in primed *ENO1* KO to a similar extent as primed WT cells (Fig. 14E). NLRP3 protein levels were abundant in *ENO1* KO cells as seen in WT but not NLRP3 knockout cells. My results indicate that α-enolase does not have a function at the level of the regulation of the NF-κB pathway activated during the priming step. Additionally, protein levels of other members of the inflammasome like pro-caspase-1 and the downstream effector of cell death GSDMD were also assessed by Western blot, but no striking differences could be observed in any of these proteins when compared with those in WT cells, thus suggesting protection from nigericin-induced pyroptosis in *ENO1* KO cells is not caused by altered expression of currently known inflammasome-related proteins. These results do not rule out the possibility that it might have a role in the activation of events leading to post-translational modifications required for NLRP3 activation. I have shown that *ENO1* KO cells can form an active NLRP3 inflammasome in response to CL097. As already described above, the mechanism leading to inflammasome activation in response to CL097 is different to the one reported for nigericin. Even though imiquimod induces inflammasome activity independently of K^+ efflux, it may still engage a mechanism that is downstream of K^+ efflux. α-enolase may thus be upstream of this mechanism.

Interestingly, α-enolase protein levels appeared to be reduced in lysates of nigericin-treated cells. No additional bands of α-enolase were observed in response to nigericin, suggesting that cleavage of α-enolase is not responsible for reduced levels of total α-enolase. Interestingly, it has been previously shown that α-enolase can be engulfed in exosomes and be secreted outside of the cell, which could help propagate and activate pro-inflammatory programs in a paracrine manner (Bae et al., 2012). It will be interesting to explore in the future how reduction in α-enolase protein abundance occurs in response to nigericin.

At this point, it is clear that α-enolase acts upstream of pro-caspase-1 maturation, but is not involved in the initial steps of cell priming. It was therefore important to characterize at which level of the activating step this enzyme acts. Although the activating step is not well understood mechanistically, there are few key events that allow the cell to engage NLRP3 inflammasome assembly, processes that can be tested experimentally. ASC nucleation, for example, can easily be assessed using immunostaining with ASC-specific antibodies to understand whether the inflammasome complex is assembled in *ENO1* KO cells. The branched ASC forms the scaffold for the macromolecular structure of the inflammasome, and

therefore can be easily observed as a great puncta or speck in the cytosol of cells in which ASC has been successfully nucleated by NLRP3. Hence, we used this technique to determine the capacity of WT and *ENO1* KO cells to form these specks (Fig. 15).

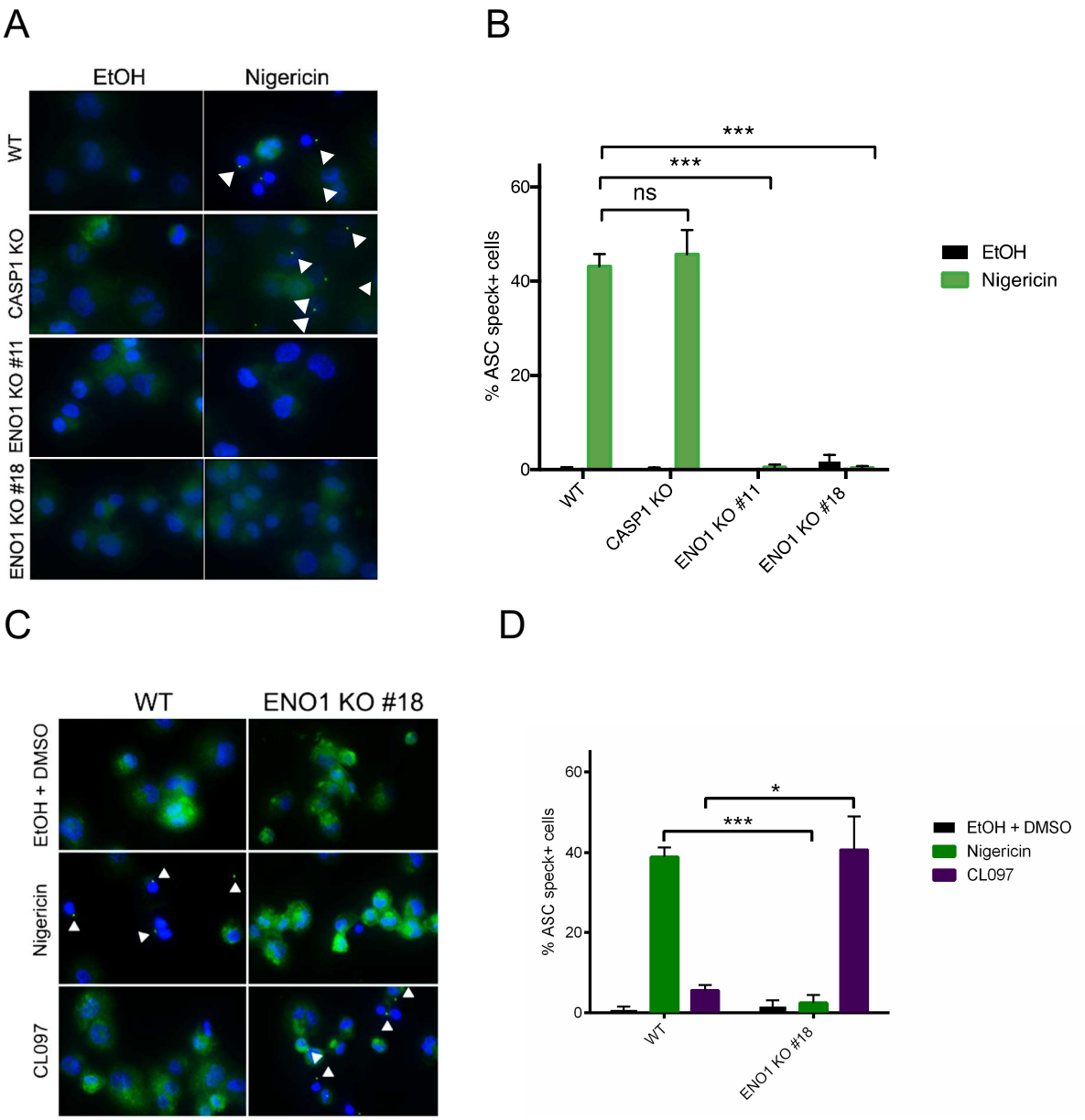


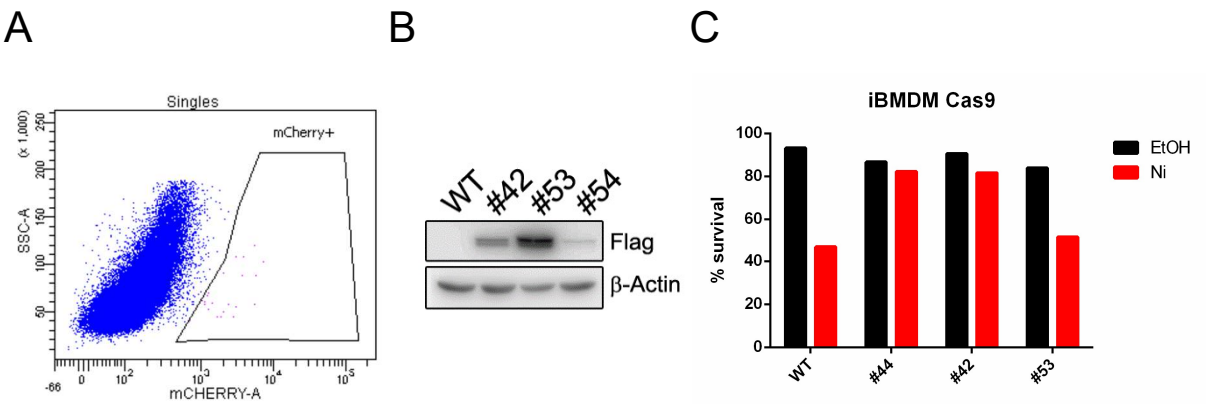
Fig. 15. *ENO1* KO cells do not form ASC specks in response to nigericin, but can form them in response to CL097. Cells were primed and incubated with nigericin and CL097 as described previously. (A) Arrows point at ASC specks (green), which are found close to the nucleus (blue) in response to nigericin (A) or CL097 (C) and were quantified as number of cells containing a speck in WT, *CASP1* KO and two *ENO1* KO clones after incubation with nigericin (B) or CL097 (D). Experiments were repeated three independent times and significance was determined by P-value < 0.05. * represents P-value < 0.05 and *** represents P-value < 0.0005, ns represents non-significant differences.

While both WT and *CASP1* KO cells formed ASC specks, none of the *ENO1* KO clones tested showed these structures in response to nigericin (Fig. 15A, B). ASC nucleation is a rather late step in the pathway leading to NLRP3 inflammasome activation. Once the speck is formed, pro-caspase-1 can be rapidly recruited to the complex to complete NLRP3 inflammasome assembly. This result thus indicates that in nigericin treatments, α-enolase has a function prior to formation of this complex. Additionally, the

absence of ASC specks in *ENO1* KOs supports the hypothesis that α-enolase's role is fundamental in this pathway. Conversely, *ENO1* KO were capable to form ASC specks in response to CL097 (Fig. 15C, D). Therefore, α-enolase has an essential function upstream of NLRP3 inflammasome assembly in response to nigericin but not in response to K^+-independent NLRP3 inflammasome activator CL097.

5.3.3 iBMDM *ENO1* KO cells also show reduced responsiveness to nigericin-mediated NLRP3 inflammasome activation

The striking phenotype of *ENO1* KO cells in response to nigericin lead me to ask whether α-enolase function was conserved among species. To answer this question, I sought to study this aspect in murine macrophages. *Eno1* depletion in mice has a developmentally lethal phenotype (Dai et al., 2018). To bypass this limitation, I set to generate *Eno1* KO immortalized bone marrow-derived macrophages (iBMDM). I first generated Cas9-stably expressing iBMDM cell lines using similar strategy for THP-1 Cas9 #22 cells. iBMDMs show certain difficulties in genetic manipulation. This is likely because these cells sense and respond to foreign DNA. This was indeed confirmed by the low efficiency of transduction with retrovirus carrying the Cas9-mCherry-Flag expression cassette in comparison to THP-1 cells (Fig. 16A). The few successfully transduced mCherry+ iBMDMs were sorted out and grown independently until protein levels of Flag-Cas9-mCherry could be assessed. Out of all Flag+ clones, clone #42 and #53 had the most similar morphology to WT cells, and were further tested (Fig. 16B). Clone #53 was chosen due its greater levels of cell death in response to nigericin in LPS-primed cells (Fig. 16C). To generate the *Eno1* KO iBMDMs, three sgRNAs targeting the exon 6 of the Eno1 gene were designed (Fig. 16D). Through the combination of two designed sgRNAs, three conditions were applied. After puromycin selection, α-enolase protein levels were assessed by immunoblotting (Fig. 16E). Cells were primed with LPS, then treated with Nigericin (Fig. 16F).



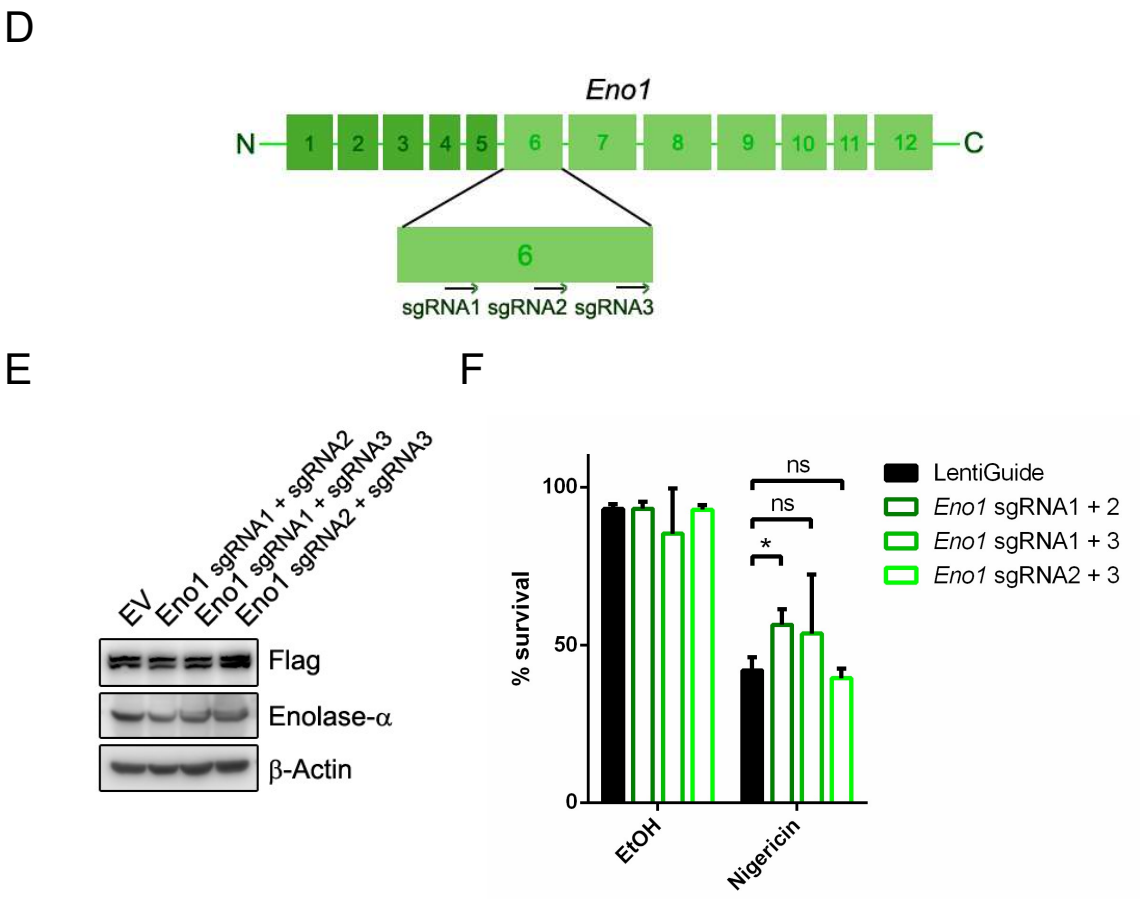


Fig. 16. Expression of *Eno1*-targeting sgRNA in Cas9-expressing iBMDMs partially protects iBMDM from nigericin-induced cell death. (A) mCherry+ cells upon lentiviral transduction with lentiviral particles for Cas9-mCherry-Flag genomic integration were sorted using FACS and further cultured. (B) Selection of single cell-derived clones expressing the mCherry-Cas9-Flag protein by Western blot. (C) Nigericin sensitivity was assessed in Flag- (clone #44) and Flag+ (clone #42 and #53) cells in comparison to WT iBMDMs upon priming with LPS. Cell survival was assessed using Sytox green staining. (D) sgRNA1, sgRNA2 and sgRNA3 were designed targeting the exon 6 of the murine *Eno*1 gene sequence for CRISPR/Cas9 gene editing. (E) Clone 53 was transduced with coupled sets of lentivirus for *Eno1*+sgRNA integration. Transduced cells were selected with 2 ug/mL puromycin, and then lysed to test *Eno1* protein levels by immunoblotting. (F) Transduced cells were primed with LPS for three hours, then treated with 15 μM nigericin or vehicle. This experiments was repeated three independent times and significance was determined by P-value < 0.05 in the form of *, ns represents non-signficant differences.

The protein levels of α-enolase upon lentiviral transduction with *Eno1*-targeting sgRNAs appeared to be slightly reduced. There are several factors that can have contributed to this low knock-out efficiency. First, as stated above, iBMDMs are difficult to transduce. Secondly, these experiments were performed in pools after puromycin selection, and therefore cells were not genetically homogeneous. Additionally, iBMDMs appear to be more resistant than THP-1 cells to this antibiotic. Hence, a high concentration of puromycin needed to be used to induce sufficient cell death in the control non-transduced cells. After puromycin treatment there were not many survivors in any of the pools generated. For this reason, cells needed to be cultured for a longer period of time prior to experiments. It has been previously observed in THP-1 that *ENO1* KO grow slower than WT cells, possibly due to an energetic deficiency. Therefore, WT cells could overgrow *Eno1* KO cells during the extended period of culture. All in all, the loss of α-enolase in the transduced pools was rather mild. However, a protective effect could be observed in the

sgRNA1+sgRNA2 population in comparison to the control when primed with LPS and challenged with nigericin, and this difference was statistically significant. This was followed by a milder effect in the sgRNA1+sgRNA3 pool, clear albeit not statistically significant, and no changes could be observed in the last sgRNA2+sgRNA3 pool. These changes seem to correlate with the differences in protein levels of α-enolase by Western blot.

Two important pieces of information can be drawn from this data: first, α-enolase loss can to an extent protect cell lines of human and murine origin from nigericin-derived cell death. Secondly, this protective effect is not solely relevant to PMA-differentiated monocyte-like cells, but is also present in LPS-primed immortalized macrophages, a more physiologically relevant model. This suggests that the requirement of α-enolase for nigericin-induction of the inflammasome is rather universal.

5.4 Enzymatic activity of α-enolase is required for the NLRP3 inflammasome activation

5.4.1 The longer transcript of *ENO1*, α-enolase, is dominantly expressed in THP-1 cells

As I have shown above that *ENO1* gene is required for nigericin-induced NLRP3 inflammasome activation, the next step was to define which protein product of the *ENO1* gene was responsible for this function. Interestingly, the *ENO1* gene not only encodes the full-length α-enolase, but also encodes a truncated protein, Myc-binding protein-1 (MBP1), lacking the first 96 amino acids of α-enolase by using an alternative start codon (indicated in lighter green in Fig. 13A, 16D) (A. Subramanian & Miller, n.d.). MBP1 is found in the nucleus and has been shown to bind and repress the promoter of c-Myc, a proto-oncogene that controls the expression of several genes involved in cell proliferation and the development of cancer. In line with its function, expression of MBP1 has been shown to be low both in tumour and non-tumourogenic cell lines (Presti et al., 2010). Conversely, α-enolase has been reported to be overexpressed in highly proliferative cells such as cancer cells from breast, pancreatic and colon tumours (Altenberg & Greulich, 2004; Sedoris et al., 2010), but also has an important role in energy production in stem cells (Simsek et al., 2010). This does not come as a surprise, as one of the better studied changes in cancer cells is the tendency to favour aerobic glycolysis, a switch traditionally termed the Warburg Effect (Warburg et al., 1927).

I thus set to ask which isoform could be involved in the activation of NLRP3 inflammasome in THP-1 cells. So far, loss of a band of approximately 50 kDa by immunoblotting using an α-enolase N-terminal antibody which cannot detect MBP1 has been used to select *ENO1* KO in THP-1 and iBMDMs (Fig. 13C, 16D). To assess both α-enolase and MBP1 expression, THP-1 WT samples were immunoblotted using two different types of antibodies: the N-terminal antibody, which can solely bind to the full length form of α-enolase, and a C-terminal antibody, which recognizes an epitope present in both isoforms.

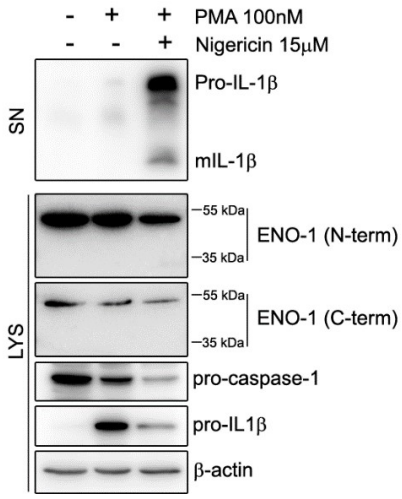
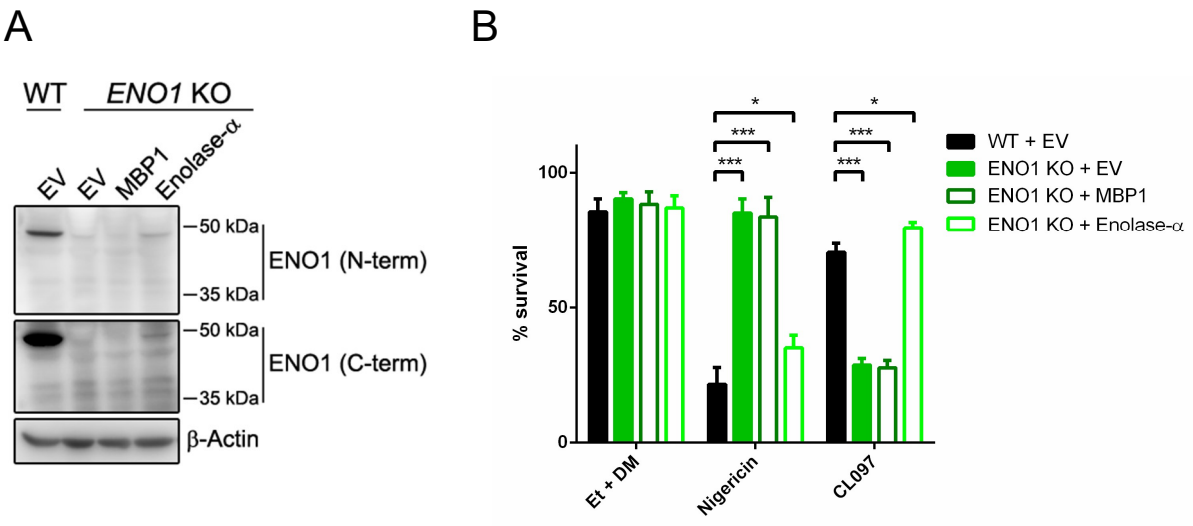


Fig. 17 Protein levels of MBP1 cannot be detected in THP-1 cells by Western blot. THP-1 cells were treated with PMA for three hours, incubated in normal medium overnight and nigericin 15 μM was used the next day to activate the NLRP3 inflammasome. Whole cell lysates were used for immunoblotting.

As expected, the N-terminal antibody bound to a protein of roughly 50 kDa in the Western blot (Fig. 17). This was the case for the C-terminal antibody too, corresponding to the expected size of the full length α-enolase form. Therefore, this result suggests that α-enolase is the most abundant product of *ENO1* in THP-1 cells.

In order to confirm that α-enolase and not MBP1 is playing a role in NLRP3 inflammasome activation by nigericin, I generated MBP1- and α-enolase-coding lentiviral particles to express the two proteins separately and monitored their function in *ENO1* KO cells in the context of NLRP3 inflammasome activation.



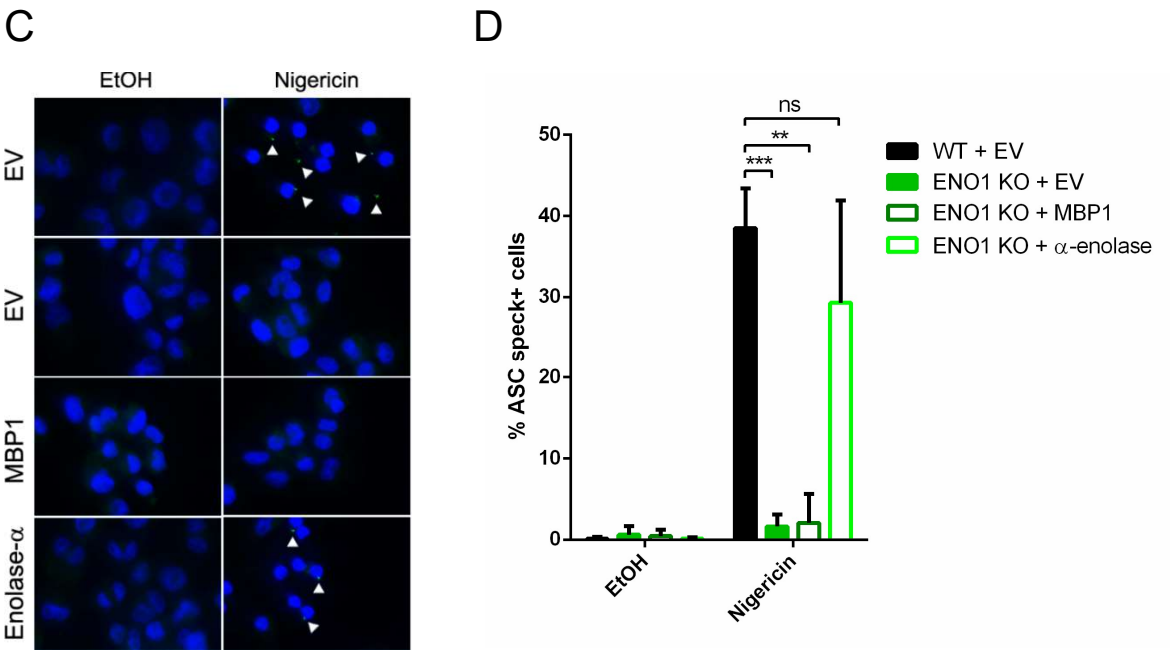


Fig. 18. α-enolase expression restores NLRP3 inflammasome activation in *ENO1* KO THP-1 cells in response to nigericin. Cells were lentivirally transduced to express α-enolase or MBP1. (A) Protein levels were assessed by Western blot. (B) Survival to nigericin and CL097 treatments was determined by Sytox green premeation levels. (C) Cells were immunostained with an ASC antibody to quantify ASC speck formation in treated cells (D). All experiments were repeated three times. Experiments were repeated three independent times and significance was determined by P-value < 0.05. * represents P-value < 0.05, ** represents P-value < 0.005 *** represents P-value < 0.0005, ns represents non-signficant differences.

No signal could be detected for MBP1 in cells transduced with this construct (Fig. 18A). c-Myc drives the proliferation program in several cancer cell lines, and has been shown to be highly expressed in resting THP-1 cells (J. Lee et al., 1987). Therefore, MBP1 expression might block c-Myc expression, leading to cell division arrest and cell death, as previously observed in breast cancer cells (Ghosh et al., 2006). Indeed, MBP1 transduced cells showed high levels of cell death soon after lentiviral transduction, and then recovered over time. A different approach should be taken in the future to address MBP1 function of NLRP3 inflammasome.

On the other hand, expression levels of α-enolase were detectable by Western Blot but were very low in comparison to endogenous α-enolase in WT cells. Stunningly, albeit α-enolase expression was low, it rescued almost entirely the cell death phenotype in response to nigericin in *ENO1* KO cells (Fig. 18B). Excitingly, these protein levels were also sufficient to restore responsiveness to CL097 more similar to WT cells. This was further proved by a similar extent of ASC speck formation in the α-enolase expressing cells in comparison to their WT counterparts (Fig. 18C, D). Altogether, these results suggest that α-enolase is the product of *ENO1* expression required for NLRP3 inflammasome activation in response to nigericin. Importantly, these results show that the phenotype observed in *ENO1* KO cells is resulting from the depletion of this gene specifically, and not due to an off-target effect of the sgRNAs.

5.4.2 Expression of wild type, but not enzymatically-inactive α-enolase rescues cell death in *ENO1* KO cells

Albeit its role in glycolysis is the most well-known function of α-enolase, this protein has been associated with other functions in the recent years. In mammals, it has been reported that α-enolase can interact with tubulin and may have an effect on the microtubule skeleton (Keller et al., 2007). All mammalian enolase isoforms have also been found to be involved in the transport of tRNA in mitochondria (Baleva et al., 2015). Noteworthy, α-enolase has already been proposed to be an important inducer of inflammation due to its ability to bind plasminogen (PLG) in the plasma membrane (Plow & Das, 2009). PLG, similarly to pro-caspase-1, is a zymogen that can be converted to the serine protease plasmin (PLA) upon its binding to α-enolase, which triggers the activation of its proteases urokinase-type PLG activator (uPA) or tissue-type PLG activator (tPA) (Didiasova et al., 2014). Concomitant to its activation, PLA degrades proteins of the extracellular matrix such as fibrin, which leads to cell mobility and mediates immune processes including monocyte recruitment (Wygrecka et al., 2009). In sight of the diversity of α-enolase functions, it is challenging to determine what particular function is needed for inflammasome activation. We first sought to investigate whether the enzymatic activity of α-enolase was important for its function upstream of the NLRP3 inflammasome formation in response to nigericin.

For this purpose, *ENO1* KO THP-1 cells were transduced with a wild type form of α-enolase along with two mutants carrying mutations on key residues essential for the conversion of 2-phosphoglycerate (2-PG) into phosphoenolpyruvate (PEP): E167 and E210. E167 is involved in an early event of the reaction and along with K398 mediates the interaction of the enolase subunit with Mg^{2+} (Brewer et al., 1993). Mg^{2+} is important to place the negative charge of the carboxyl group of 2-PG in the catalytic site of the enzyme. Once the substrate is at this site, E210 plays a fundamental role in the final dehydration of the substrate to PEP (Poyner et al., 1996). For this experiment, the glutamate residues E167 and E210 were mutated to glutamine, which lacks the negative charge critical for glutamate function. Cells were challenged with nigericin to determine whether the cell death phenotype was restored.

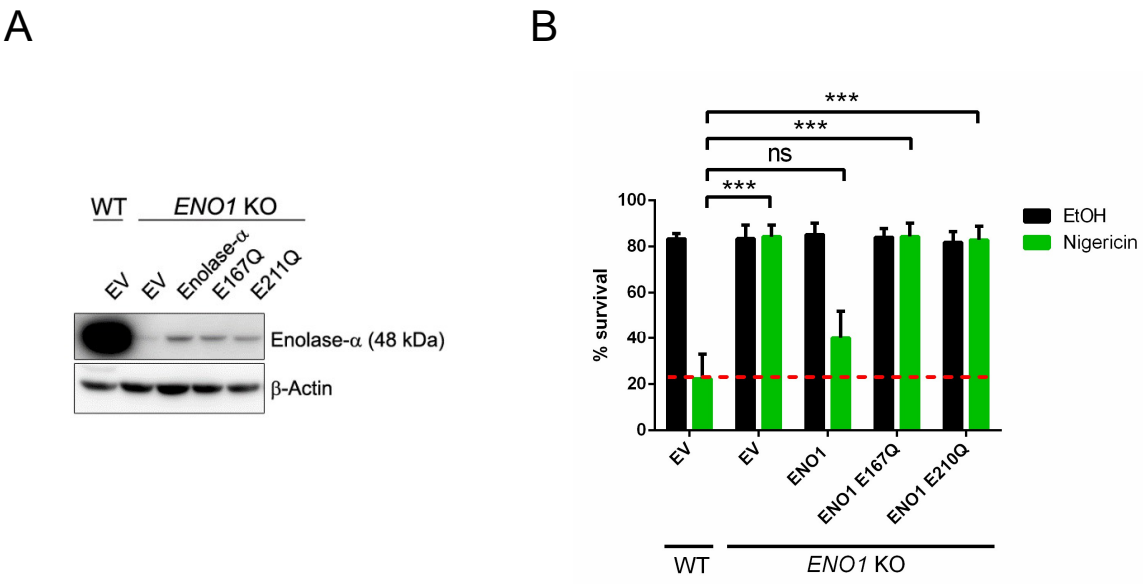
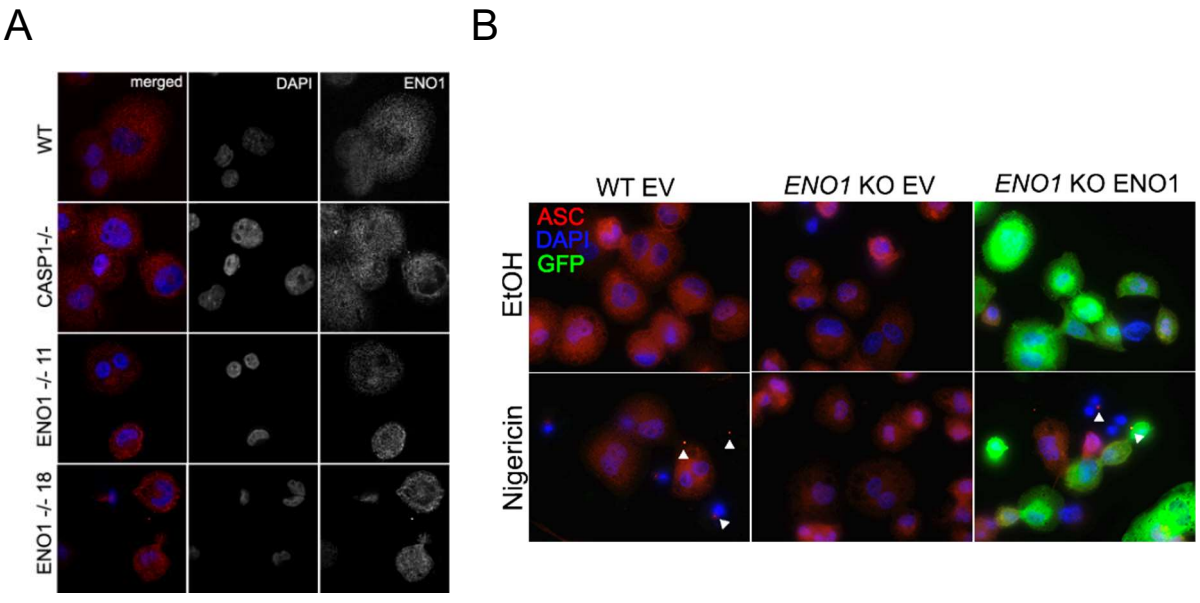


Fig. 19. Expression of enzymatically active α-enolase reinstates nigericin-induced cell death. Cells were lentivirally transduced with constructs encoding for wild type α-enolase (ENO1) or enzymatically-dead mutants (E167Q and E210Q). (A) Immunoblotting of all rescue cell lines for the C-terminal antibody of ENO1. (B) % survival

for each rescue cell line upon priming and PMA and nigericin treatment as indicated above. Levels of cell death in WT cells treated with nigericin is indicated as a red dashed line. Cell survival is measured by lack of Sytox green signal determined by FACS. Statistics are done using results from three independent experiments. Experiments were repeated three independent times and significance was determined by P-value < 0.05. *** represents P-value < 0.0005, ns represents non-signficant differences.

As previously shown, expression of α-enolase even at very low levels was sufficient to restore cell death in response to nigericin (Fig. 19A). E167Q and E210Q did not restore cell death levels as seen in WT cells (Fig. 19B). This strongly indicates that the glycolytic function of α-enolase is critical for its role in this pathway. Therefore, reconstitution of enzymatic activity of α-enolase is sufficient to restore the cell death phenotype in response to nigericin.

In parallel, *ENO1* KO cells were transduced with α-enolase fused to an EGFP coding sequence on its C-terminal end to allow visualization of α-enolase by microscopy. The current antibody targeting α-enolase used for immunofluorescence does not appear to be specific in immunofluorescence experiments, as it shows similar patterns of staining in WT and *ENO1* KO cells (Fig. 20A). Therefore, expression of EGFP-tagged α-enolase can serve as a tool to track changes in the pattern of localization of α-enolase upon induction of the inflammasome in living cells. Exogenous expression of proteins can lead to undesirable accumulation of proteins in other compartments in the cell and partly mask the original function of the protein (Prelich, 2012), but can still be informative. Furthermore, the relatively low expression levels of our α-enolase constructs suggests that the visualized EGFP signal of α-enolase is likely not artefactual (Fig. 20B,C). Therefore, we used this tool to assess two features in THP-1 cells: the cellular localization of α-enolase and percentage of successfully transduced cells.



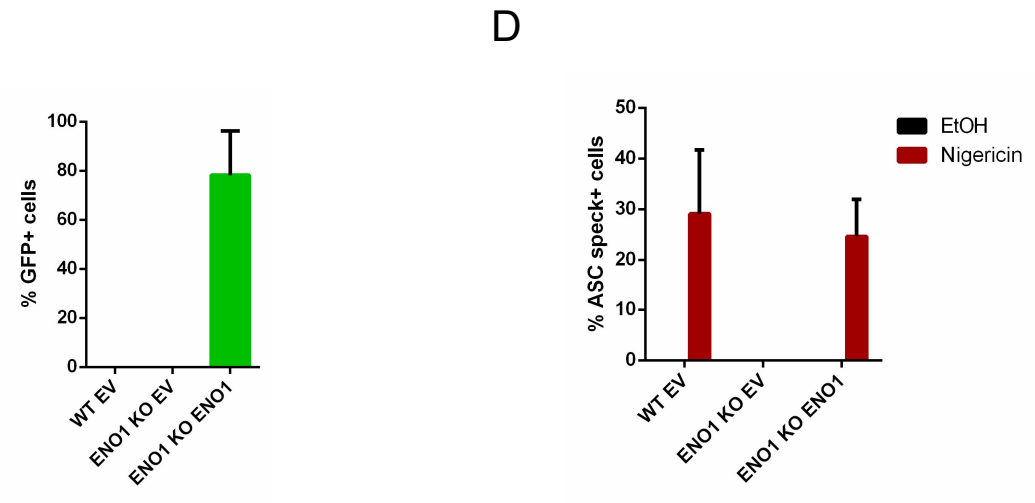


Fig. 20. α-enolase localizes to the cytoplasmic compartment. (A) THP-1 WT, *CASP1* KO and two *ENO1* KO clones were fixed and stained with the C-terminal α-enolase antibody. (B) Cells were transduced with EV or a wild type α-enolase-EGFP construct, followed by immunostaining with an ASC specific antibody. ASC specks are indicated by white arrows (C) Quantification of EGFP+ cells. (D) Quantification of cells that contained an ASC speck upon incubation with PMA followed by nigericin or vehicle treatment. Quantifications were made using data from two experiments from two independent lentiviral transductions.

The basal localization of α-enolase is mostly cytoplasmic, where it executes its glycolytic function. Upon nigericin incubation, the staining in the cytoplasm decreased, but no other structures or clear patterns of α-enolase localization were observed (Fig. 20B). EGFP did not appear to disturb α-enolase activity, as α-enolase-EGFP expressing cells could form ASC specks to a similar extent as WT cells (Fig. 20D). I have shown that α-enolase protein levels decrease substantially upon NLRP3 inflammasome activation in WT cells, but not in *NLRP3* KO cells (Fig. 14C). Therefore, the loss of α-enolase-EGFP signal could be due to the leakiness of pyroptotic cells, which become increasingly porous as cell death progresses. Together, these results support a key role of the enzymatic activity of α-enolase in the formation of the NLRP3 inflammasome.

5.4.3 Inhibition of glycolysis upstream of α-enolase blocks NLRP3 inflammasome activation in response to nigericin

To further confirm that the catalytic function of α-enolase is important for NLRP3 inflammasome activation, it would be important to use pharmacological means to block this enzyme. AP-III-a4 also known as ENOblock is currently widely used for this purpose (Jung et al., 2013). However, a more recent assessment of the activity of this reagent indicated that ENOblock does not act directly on α-enolase, and *in vitro* incubation with this molecule does not result in a decrease in 2-PG conversion to PEP (Satani et al., 2016). Therefore it is not a reliable tool to decrease α-enolase activity. However, following the observation that α-enolase enzymatic activity is required for NLRP3 inflammasome activation in response to nigericin, it should be expected that the inhibition of early steps in the glycolytic pathway could similarly block NLRP3 inflammasome activation. To this end, I set to challenge glycolysis following a similar set-up as Ho and colleagues (Ho et al., 2015). Iodoacetate (IAA) was used to inhibit

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which catalyses the conversion of glyceraldehyde-3-phosphate (G3P) into 1,3-biphoshpglycerate (1,3-BPG) in the sixth step of glycolysis. Albeit it did not score in the top places of the list, GAPDH was among the glycolytic enzymes whose sgRNAs showed a higher enrichment in the screen. Next, I targeted pyruvate kinase (PK), the last enzyme in the glycolytic pathway, which catalyses the conversion of PEP to pyruvate using oxalate (OXA).

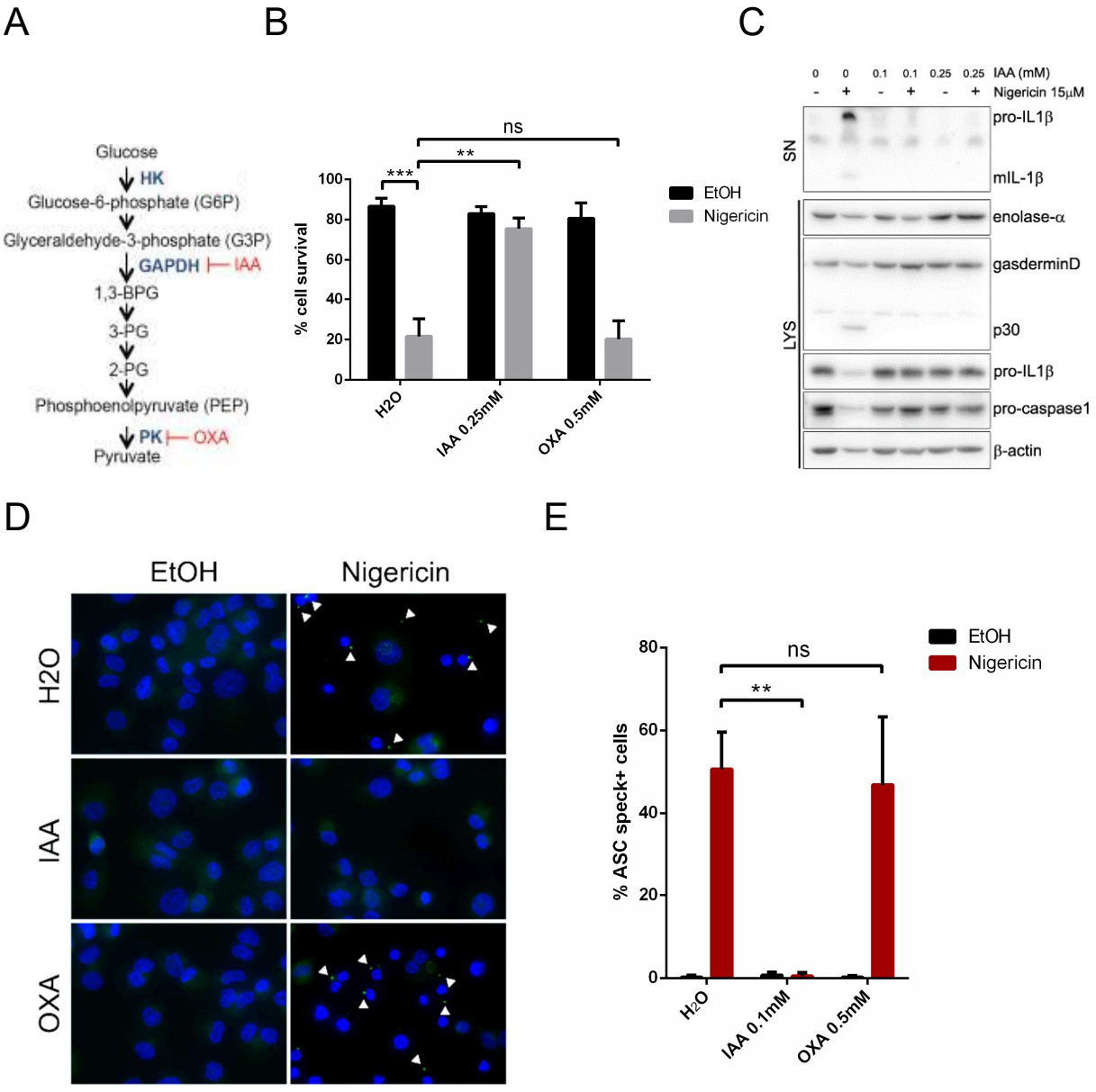


Fig. 21. Inhibition of GAPDH with IAA protects THP-1 cells from nigericin-induced NLRP3 inflammasome activation. WT THP-1 cells were primed with PMA for three hours, incubated with increasing doses of IAA and sub-lethal doses of OXA for one hour, followed by a treatment with nigericin. (A) The glycolytic pathway. IAA inhibits GAPDH and OXA inhibits PKM2 at the last levels of glycolysis. (B) % of cell death was determined by resistance to sytox green permeation and therefore lack of green signal measured by FACS. (C) Immunoblotting of main members of the inflammasome at different concentrations of IAA. (D) ASC speck formation in fixed cells at different concentrations of IAA, quantified in (E). Experiments were repeated three independent times and significance was determined by P-value < 0.05. ** represents P-value < 0.005 and *** represents P-value < 0.0005, ns represents non-significant differences.

IAA appeared to block the inflammasome completely, as shown by the lack of Sytox green permeation (Fig. 21B), the undetectable band of mature GSDMD and pro-IL-1β in the Western Blot (Fig. 21C), and the complete absence of ASC speck formation upon treatment with nigericin (Fig. 21D and E). IAA is a thiol alkylating agent that can target cysteine residues in the peptide sequence of GAPDH. However, IAA has also been proposed to target caspase-1 and potentially other caspases by a similar mechanism. Indeed, incubation with high concentrations of IAA has previously been shown to induce apoptosis, which is likely due to the activation of caspase-3 (L. Jiang et al., 2013). The absence of ASC specks in cells treated with this drug suggests that in this context IAA also inhibits NLRP3 inflammasome through a mechanism upstream of caspase-1 auto-activation. GAPDH catalyses a reaction three steps upstream of α-enolase in the glycolytic pathway. Therefore, IAA may alter the production of a downstream metabolite required for NLRP3 inflammasome formation.

Importantly, pre-treatment with OXA did not result in a clear perturbation in the induction of pyroptosis in response to nigericin. PK inhibition should not perturb the enzymatic activity of α-enolase, but should result in an increase in PEP levels. However, inhibition of the pyruvate kinase muscle isoform 2 (PKM2) leads to generalized reduction of glycolytic flux and lower production of glycolysis intermediary metabolites such as PEP itself (Xie et al., 2016). Therefore, the net result of inhibition of PK with OXA would be the balance between these two events, and this could explain the lack of a striking difference in cell death when OXA was used.

In summary, these results show that ablation of α-enolase or inhibition of glycolysis upstream of this enzyme have a protective effect against NLRP3 inflammasome activation. Inhibition of GAPDH with IAA will slow down 1,3-BPG production, thus decreasing 2-PG production. Therefore, these results also indicate that protection against nigericin-induced inflammasome activation in *ENO1* KO cells is not due to an accumulation of 2-PG.

5.4.4. α-enolase does not activate the inflammasome by facilitating cytosolic Ca^{2+} mobilization

The substrate of α-enolase, 2-PG, does not have an inhibitory role in NLRP3 inflammasome activation. Could the product of α-enolase activity, PEP, be required in this pathway instead? PEP has been reported in several instances to play a role in Ca^{2+} mobilization. PEP was first found to inhibit Ca^{2+} uptake in mitochondria in rat heart and liver several decades ago (Chudapongse & Haugaard, 1973). More recent studies have shown that it rather acts to block the flux of Ca^{2+} to the ER instead of mitochondria and this leads to the accumulation of Ca^{2+} in the cytosol. It has now been shown that PEP is a natural inhibitor of sarco/ER Ca^{2+}-ATPase (SERCA), which is the main cation pump promoting Ca^{2+} accumulation in ER stores (Ho et al., 2015). T cells in the tumour microenvironment are exposed to hypoxic conditions, which leads to induction of anaerobic glycolysis. PEP can accumulate and block SERCA in the ER, and subsequent Ca^{2+} mobilization in the cytosol triggers a boost of tumour-specific CD4 and CD8 T responses. Thus, it has been proposed that PEP serves as a metabolic checkpoint to activate anti-tumour T cell responses (Ho et al., 2015).

Ca^{2+} has been a subject of study for years also in the context of the NLRP3 inflammasome pathway but as outlined above it remains rather debated. In sight of the requirement of the enzymatic activity of α-enolase for mounting a strong nigericin response, we asked whether mimicking the effect of PEP in Ca^{2+}

stores could rescue cell death in *ENO1* KO cells. To do so, we used two compounds known to increase Ca^{2+} in the cytosol: thapsigargin and ionomycin.

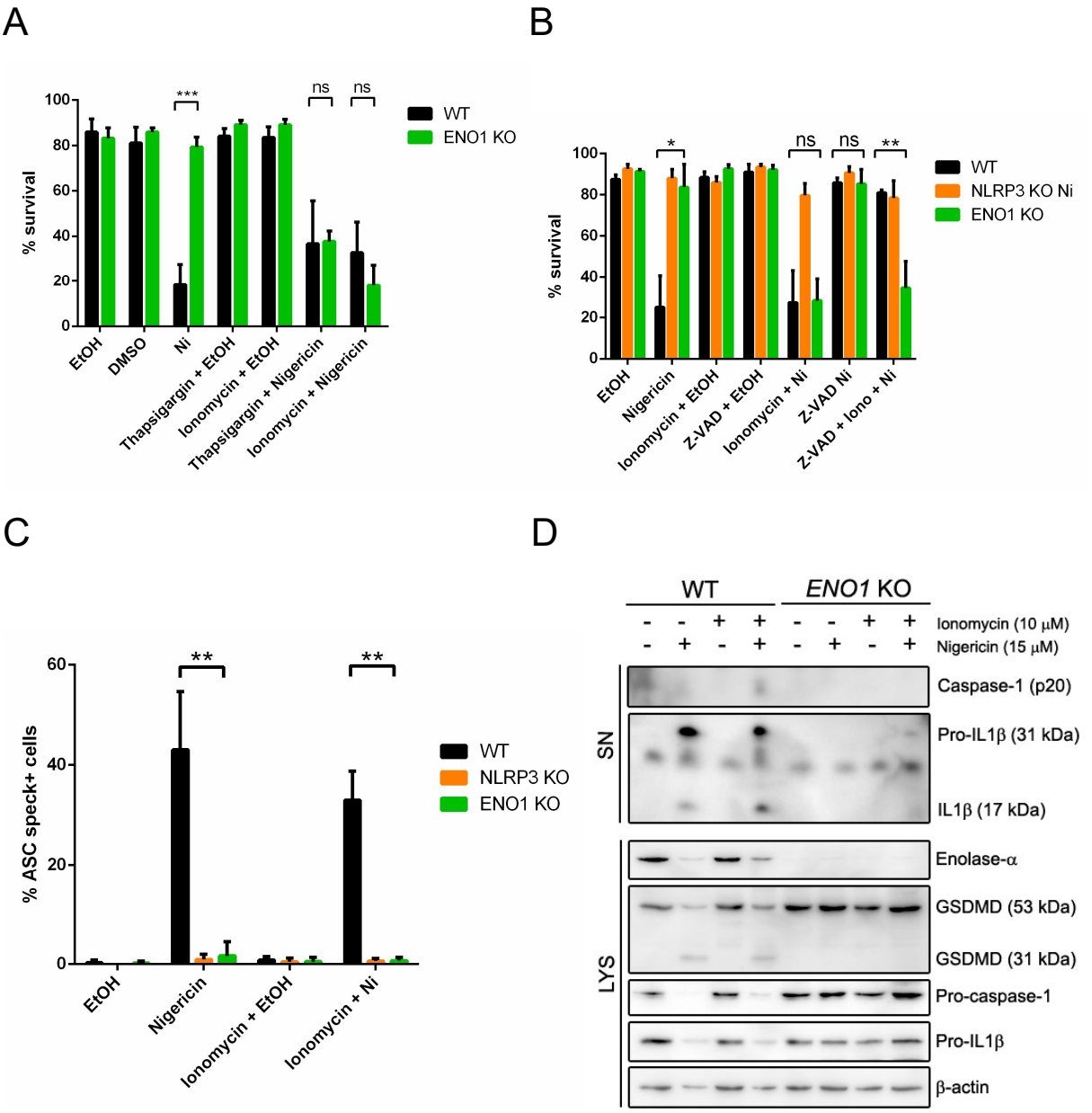


Fig. 22. Increasing cytosolic Ca^{2+} renders cells sensitive to nigericin-induced cell death but does not restore NLRP3 inflammasome activity in *ENO1* KO cells. Cells were primed with PMA, pre-incubated for one hour with 10 μM ionomycin or 10 μM thapsigargin and then incubated with nigericin. Cell survival was determined by FACS upon incubation with Sytox green. (A) Cell death in response to nigericin in WT and *ENO1* KO cells pre-treated with ionomycin or thapsigargin. (B) Cell death in WT, *NLRP3* KO and *ENO1* KO cells pre-treated with Z-VAD or ionomycin and then challenged with nigericin. (C) ASC speck formation was determined by ASC immunostaining in cells treated with nigericin in the presence or absence of ionomycin. (D) Protein levels of the main NLRP3 inflammasome markers were analysed by Western blot. Experiments were repeated three independent times and significance was determined by P-value < 0.05. ** represents P-value < 0.005 and *** represents P-value < 0.0005 ns represents non-significant differences.

As shown in Fig.22A, no effect in cell survival could be observed when cells were incubated with thapsigargin or ionomycin and the vehicle ethanol, in line with previous findings showing that influx of

Ca^{2+} is not sufficient to activate the inflammasome in the short term. However, I observed that treatment with either thapsigargin or ionomycin rendered *ENO1* KO cells sensitive to nigericin-induced lytic cell death. On one hand, thapsigargin is a synthetic drug that, similarly to PEP, can inhibit SERCA in the ER and block Ca^{2+} storage in this compartment and acts on other Ca^{2+} channels upon longer incubation (T. B. Rogers et al., 1995). Interestingly, long incubation of thapsigargin has been shown to induce NLRP3 inflammasome activation, which probably depends on ER stress (Menu et al., 2012). However, the acute effect observed in my experiments upon treatment with nigericin suggests that, in this setup, thapsigargin rather functions to increase the mobility of cytosolic Ca^{2+}. In fact, I used conditions precluding cell death in WT cells in response to thapsigargin or ionomycin alone. This is further supported by the dramatic effect of ionomycin in nigericin-induced cell death in *ENO1* KO cells. Mechanistically, ionomycin is an ionophore that allows passage of Ca^{2+} from highly concentrated cell compartments to the cytosol. As the main reservoir of Ca^{2+} in the cell, ER is particularly sensitive to ionomycin treatment, and therefore represents the main source of newly accumulated Ca^{2+} in the cytosol. Albeit both thapsigargin and ionomycin mainly target the ER, ionomycin has been shown to increase cytosolic Ca^{2+} particularly quickly and strongly (Y. Huang & Putney, 1998). Indeed, ionomycin induced a strong cell death upon nigericin treatment. Thus, so far my results strongly supported that Ca^{2+} mobilization to the cytosol restores lytic cell death in response to nigericin in *ENO1* KO cells.

Importantly, as mentioned in the introduction chapter of this thesis, there are other forms of lytic cell death unrelated to inflammasome activation. In order to investigate whether the observed cell death in ionomycin and thapsigargin-treated *ENO1* KO corresponded to pyroptosis, cells were pre-treated with Z-VAD-FMK (Z-VAD), a pan-caspase inhibitor (Fig. 22B). This molecule blocked the cell death induced by nigericin in wild type cells, both in the presence or absence of ionomycin. Ionomycin did not trigger any form of cell death in *NLRP3* KO cells treated with nigericin, and cell viability in this cell type remained unaltered throughout treatments. Strikingly, survival levels in *ENO1* KO cells treated with ionomycin and nigericin remained low in the presence of Z-VAD, suggesting that lytic cell death-induced by ionomycin and nigericin treatment in *ENO1* KO cells is a caspases-independent cell death. It was further shown that *ENO1* KO cells did not form ASC specks when treated with ionomycin and nigericin, in contrast to their WT counterparts (Fig. 22C). Finally, cells were lysed and the protein levels of the main markers of inflammasome activity were assessed by immunoblotting (Fig. 22D). *ENO1* KO cells co-treated with ionomycin and nigericin did not display the typical features of NLRP3 inflammasome activation and pyroptosis: no cleavage of GSDMD, pro-caspase-1 or pro-IL-1β could be observed. Interestingly, pro-IL-1β could be detected in the medium of *ENO1* KO cells upon treatment with nigericin and ionomycin, although this was not consistent across all 3 replicates. This could be explained by the passive leakage of this protein across the highly permeable membrane. Altogether, these results suggest that α-enolase activity and possibly PEP production prevents induction of an alternative lytic cell pathway by de-repressing Ca^{2+} mobilization. However, de-repression of Ca^{2+} mobilization downstream of α-enolase is not sufficient to restore NLRP3 inflammasome activation and pyroptosis.

If PEP is indeed responsible for NLRP3 inflammasome activation, the presence of PEP should be sufficient to restore cell responsiveness to nigericin and pyroptosis. To test this, *ENO1* KO cells were incubated with PEP using different strategies in order to replenish the levels of this metabolite in the cell.

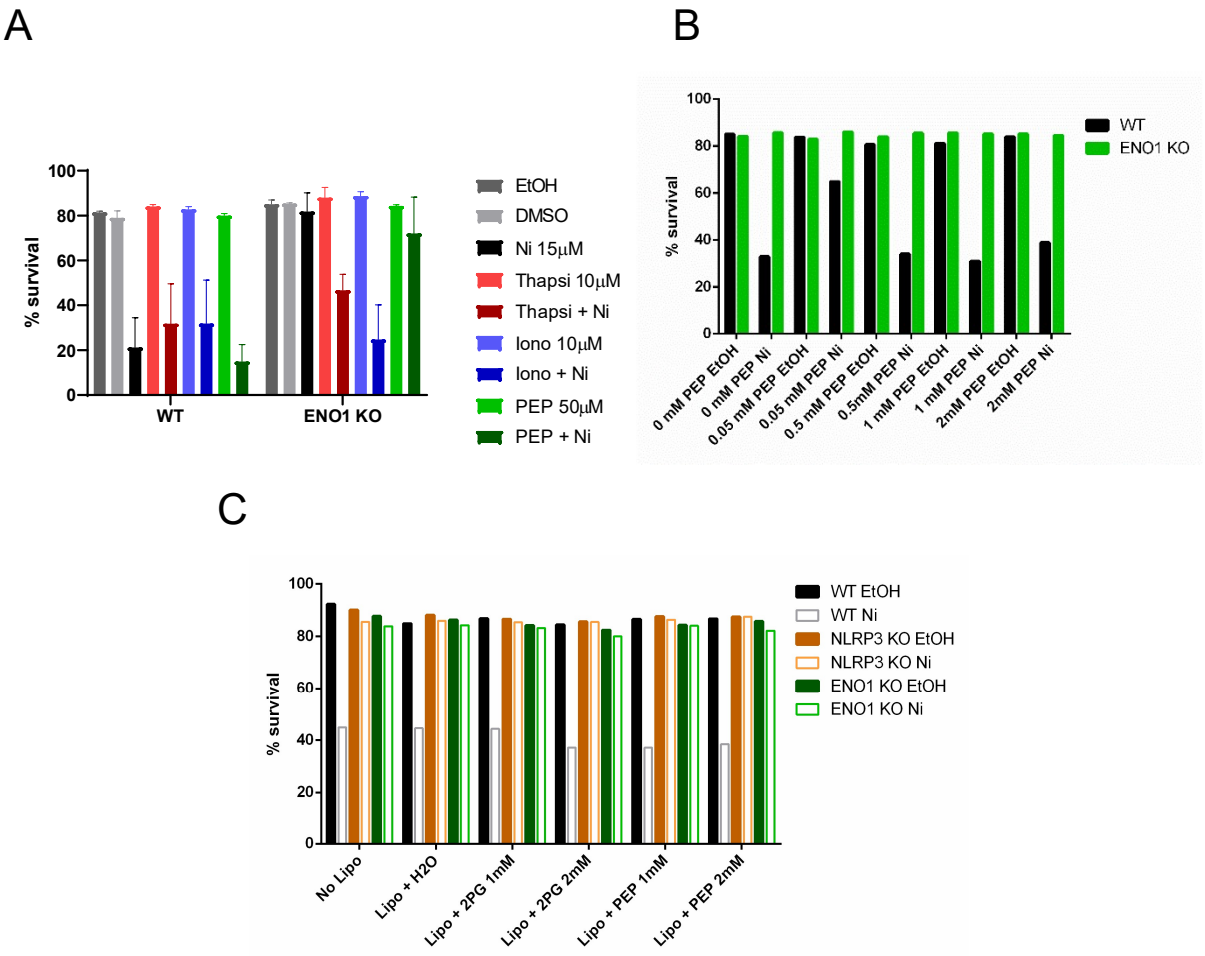


Fig. 23. High concentration and lipofectamine-based delivery of PEP did not lead to the rescue of nigericin-induced cell death in *ENO1* KO cells. Cells were incubated during one hour with a low PEP concentration of 50 µM (A) and growing high concentrations from 0 to 2mM of PEP (B), then challenged with a nigericin treatment. (C) Cells were transfected using lipofectamine in the presence of 1mM or 2mM of 2-PG or PEP in the medium. After four hours cells were challenged with nigericin. Cell survival was determined by Sytox green staining using FACS. Experiments were performed once (A, C) or two times (B).

As shown in Fig. 23A, although a slight rescue of the death phenotype could be observed by FACS, PEP efficiency was far lower than thapsigargin and ionomycin. Noteworthy, PEP transport across the plasma membrane is dependent on Band 3, otherwise known as Solute carrier family member 4 a 1 (SLC4A1) (Hamasaki et al., 1983). However, Band 3 is not expressed ubiquitously, and a search in expression databases quickly revealed that this gene does not appear to be translated in cells of the myeloid lineage, according to data from The Human Protein Atlas. Consequently, the comparatively lower effect of PEP in rescuing the cell death phenotype could be due to its inefficient internalization into THP-1 cells. To bypass this limitation, several strategies were tested to facilitate uptake of PEP in THP-1 cells, including treatment with higher concentrations of PEP (Fig. 23B) or Lipofectamine 2000-mediated transfection (Fig. 23C). PEP addition did not restore lytic cell death activity in *ENO1* KO cells nor did it amplify the latter in WT cells upon activation with nigericin. A similar approach was used with 2-PG. However, no differences were found in this case neither. There are two possible ways to interpret

these results. First, it is possible that none of this approaches led to uptake of 2-PG or PEP by these cells. In that case, we cannot confirm or exclude a role of these metabolites in nigericin-induced pyroptotic cell death. Alternatively, it is possible that permeation to 2-PG and PEP was achieved, but did not yield any quantifiable change in cell death. In order to elucidate which of the two scenarios is taking place, in the future it will be necessary to determine PEP levels in these samples using a quantitative method, such as mass spectrometry.

As none of the previous approaches successfully demonstrated the product of α-enolase leading to altered sensitivity to nigericin-induced cell death in *ENO1* KO cells, a more potent strategy to permeate the cell membrane for PEP entry was used. To this end, cells were treated with a low concentration of a nonionic surfactant, Triton X-100 (TX-100). This detergent is commonly used to permeate cells to antibodies in immunostaining experiments, and at higher concentrations to lysate cells for protein extraction. It has also been used for transient permeabilization of living cells at low concentrations (van de Ven et al., 2009). For this purpose, WT, *NLRP3* KO and *ENO1* KO cells were treated with TX-100 at different concentrations. Cells can also be permeabilized using digitonin, which is a detergent commonly used to permeabilize the plasma membrane without affecting the nuclear membrane. Similarly to TX-100, it has been reported to allow permeabilization without compromising viability at very low concentrations (Miyamoto et al., 2008). Therefore, we tested both detergents in an attempt to render the plasma membrane more permeable.

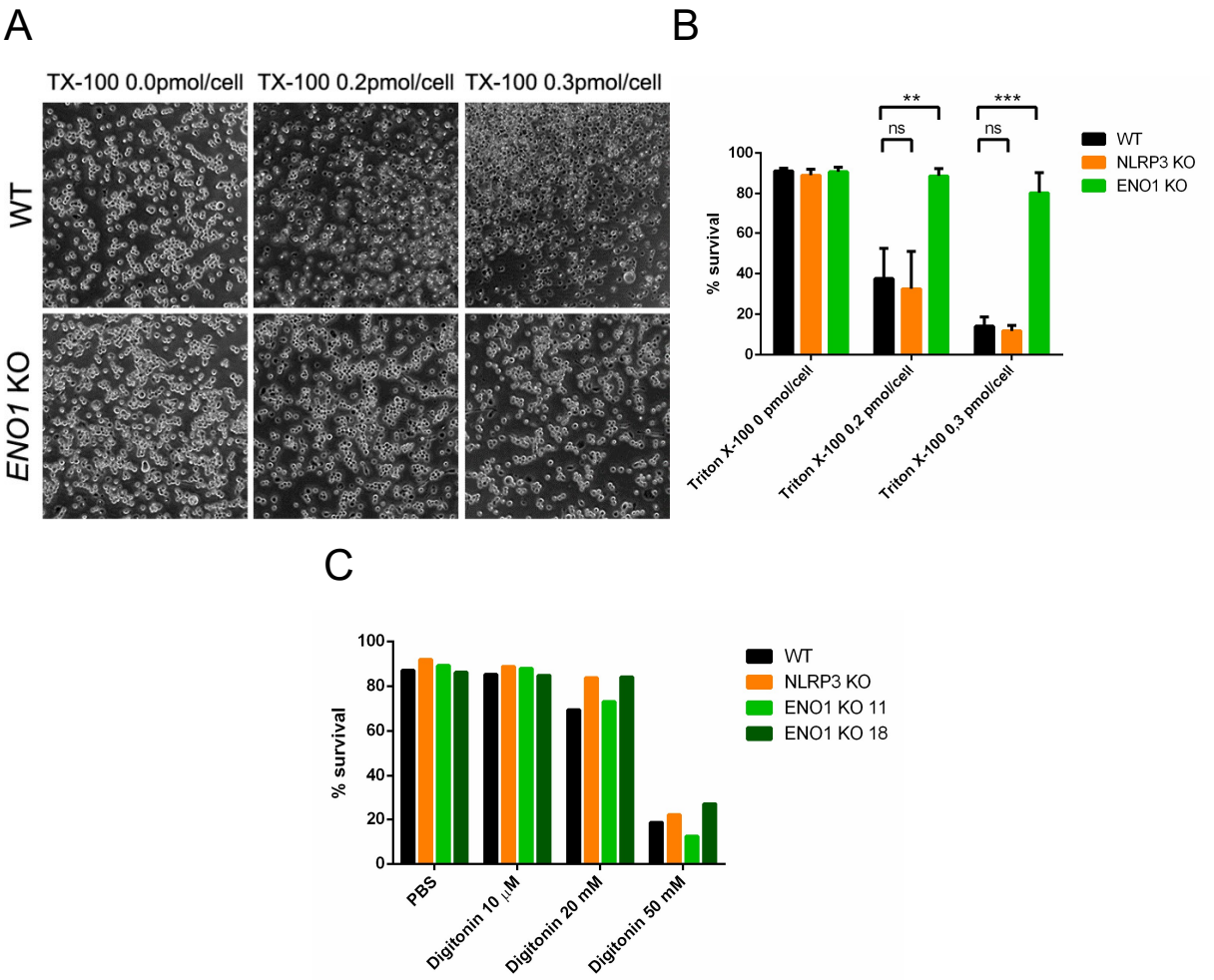


Fig. 24 *ENO1* KO cells are resistant to cell death induced by Triton X-100 but not digitonin. Cells were treated with PMA, followed by different concentrations of TX-100 or digitonin, then left to recover in fresh medium overnight before cell viability was assessed. (A) Brightfield image of WT and *ENO1* KO cells 24h after treatment with 0.2 pmol and 0.3 pmol/cell Triton X-100. (B) Quantification of Sytox Green-negative cells 24h after treatment with Triton X-100. (C) Quantification of Sytox Green-negative cells 24h after treatment with digitonin at concentrations ranging from 10 to 50 mM. Significance in cell death survival upon Triton X-100 treatment was drawn from three independent experiments. Triton X-100 experiments were repeated three independent times and significance was determined by P-value < 0.05. ** represents P-value < 0.005 and *** represents P-value < 0.0005 ns represents non-significant differences.

Unexpectedly, brightfield microscope images revealed the onset of cell death in WT cells even at the lowest concentration of TX-100 (Fig. 24A). Dying cells looked largely necrotic, and granulation could be observed in the medium. Macrophage function is highly sensitive to ion fluxes, and particularly to K^+ efflux, which can drive the activation of the inflammasome (Muñoz-Planillo et al., 2013). The formation of pores in the membrane is likely to facilitate the ionic exchange with the external medium, which could drive the egress of K^+ from the cell and thus trigger NLRP3 inflammasome activation and subsequent cell death. However, cell death was also present in *NLRP3* KO to a similar extent as WT cells, albeit they lack the key protein for the formation of this complex. A possible explanation to this phenomenon could be that other organelle membranes may be equally permeabilized by this detergent. For example, mitochondrial outer membrane permeabilization (MOMP) can on one hand release DAMPs which can activate the NLRP3 inflammasome, but also leads to the leakage of cytochrome c and other mitochondrial proteins that can activate apoptosis. Lysosomal fitness is likewise important for cell viability, and LMP is known to trigger necrotic-like cell death. Therefore, it is possible that in cells that become permeable by TX-100, this detergent could access the intracellular space and trigger one or more of these processes culminating in cell death. Strikingly, *ENO1* KO were completely resistant to cell death induced by this detergent (Fig. 24B). Therefore, this result hinted that depletion of α-enolase rendered these cells resistant to TX-100 permeabilization per se, instead of blocking a specific pathway of cell death. This was further supported by the observation that *ENO1* KO do undergo cell death upon permeabilization with a different detergent, digitonin (Fig. 24C). In this instance, cell death levels were similar to *NLRP3* KO and WT cells, and particularly efficient when higher concentrations of this detergent were used. In light of this unexpected results, I next sought to investigate the reason underlying insensitivity of *ENO1* KO cells to TX-100. In fact, different detergents are known to have distinct affinities to membranes with different lipid compositions. Given the fact that NLRP3 binds to endomembranes with distinct lipid compositions, these seemingly unrelated findings may provide a link between α-enolase activity and NLRP3 inflammasome activation.

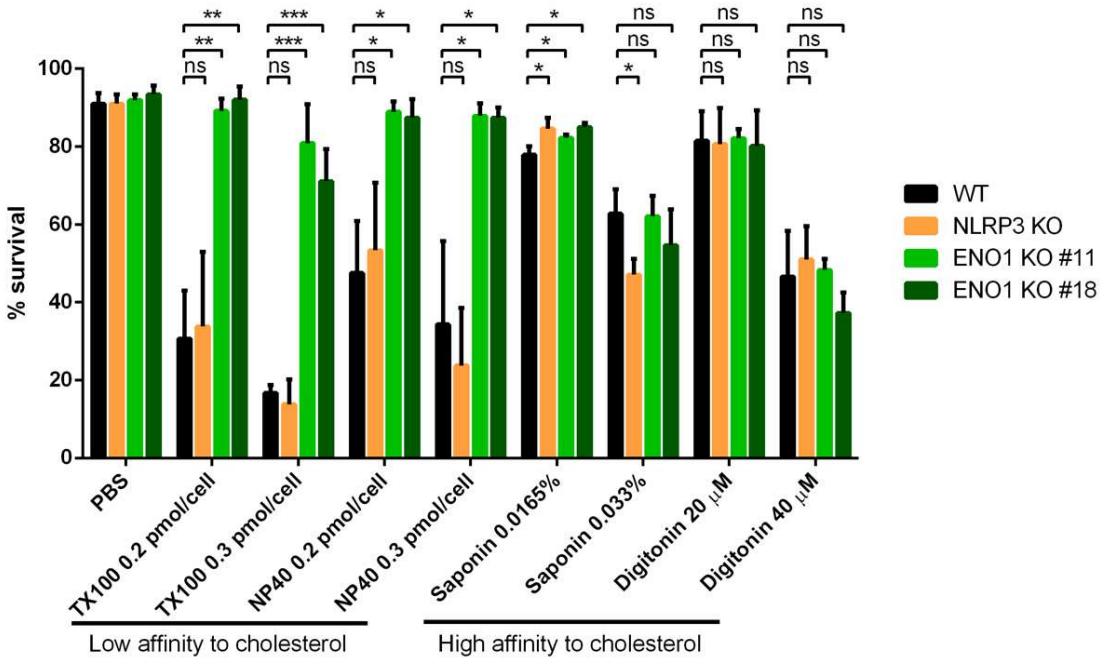
5.5 *ENO1* KO cells are less sensitive to detergents with low affinity to cholesterol

Understanding the chemistry behind detergent-mediated membrane permeabilization is key in order to elucidate how these two detergents can have different capacities to permeabilize *ENO1* KO cell membranes. Both TX-100 and digitonin are surfactants, a type of amphiphilic compounds that spontaneously form structures to minimize surface tension with the surrounding solvent. This leads to the organization of these molecules in spherical micelles or in layers, depending on the size of the

molecule. Similarly, long-tailed amphiphilic lipids in the cell such as phospholipids form lipidic membranes in order to isolate their hydrophobic tails from the governing aqueous environment. Due to their highly similar nature, surfactants can solubilize lipids and in some instances proteins of the cellular membrane. At low surfactant concentrations this leads to the transient formation of pores, which can be used to permeabilize the membrane for a limited time. However, depending on their structure, surfactants will be more likely to solubilize some lipids more than others. Indeed, insolubility of membranes by detergents can be used to determine the structure of biological membranes. For instance, TX-100 is a nonionic surfactant that has been shown to be inefficient to solubilize rafts, regions in the lipid bilayer rich in sphingolipids and cholesterol (London & Brown, 2000). Conversely, digitonin is a steroidal surfactant that interacts selectively with cholesterol (Nishikawa et al., 1984). Therefore, digitonin is particularly effective to solubilize membranes rich in this type of lipid, such as the plasma membrane, and cannot permeabilize low-cholesterol membranes like the nuclear membrane (Colbeau et al., 1971).

Based on my previous results, lack of TX-100-induced death in *ENO1* KO in comparison to WT and *NLRP3* KO cells might reveal a change in the composition of the plasma membrane. I next asked whether this result could be reproduced with other detergents owing similar affinities to cholesterol as TX-100 and digitonin. For this purpose, the low cholesterol affinity Triton family member NP40 was used as a detergent with low affinity to cholesterol, and the steroidal surfactant saponin was used due to its high affinity to cholesterol.

A



B C

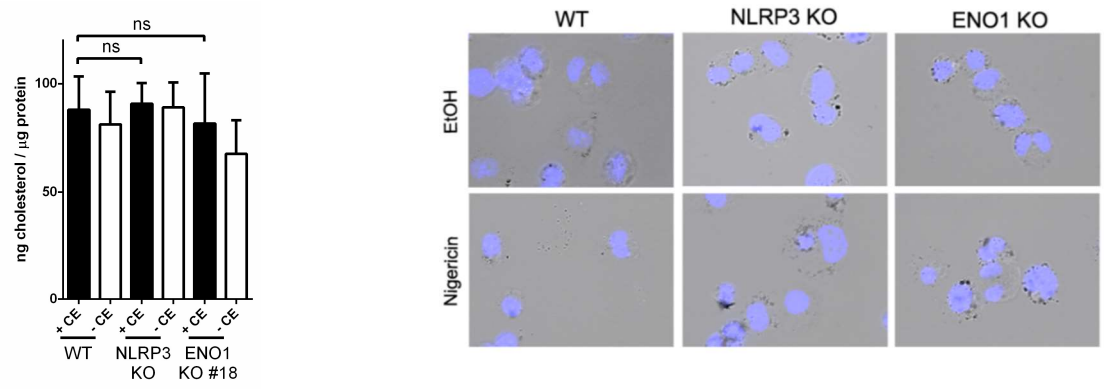


Fig. 25. Cholesterol-solubilizing surfactants can trigger cell death by permeabilizing *ENO1* KO cell membranes. (A) Cells were treated with the indicated concentrations of TX-100, NP40, saponin or digitonin. Cells were left to recover in fresh medium, and after 24h cell viability was assessed according to permeation to Sytox Green by FACS. (B) Cells were collected to analyse protein and cholesterol levels. Cholesterol content was determined using the Amplex Red cholesterol kit in the presence or absence of cholesterol esterase (CE). (C) Lipid droplets were visualized using Oil Red O staining (black). Nucleus were stained with DAPI (blue) for cell quantification. All experiments were repeated three times. Experiments were repeated three independent times and significance was determined by P-value < 0.05. * represents P-value < 0.05, ** represents P-value < 0.005 and *** represents P-value < 0.0005, ns represents non-significant differences.

All detergents were efficient to induce cell death in WT and *NLRP3* KO cells in a dose-dependent manner (Fig. 25A). However, *ENO1* KO clones 11 and 18 showed a strong resistance to NP40-induced cell death similar to TX-100. Conversely, both digitonin and saponin were capable to induce cell death in *ENO1* KO to a similar extent as in WT and *NLRP3* KO cells. This result strongly indicated that the cell death phenotype was tightly linked to a difference in the lipid composition of the membranes of *ENO1* KO cells, particularly in the cholesterol content in the plasma membrane. Interestingly Shand and West proposed two decades ago that enolase from different eukaryotic species could play an inhibitory role on the conversion the hydrolysis of cholesteryl esters to free cholesterol (Shand & West, 1995). Cholesterol is found in its free form in cellular membranes, but can be stored in lipid droplets reservoirs in the form of esters when the cholesterol requirements of the cell are satisfied. Macrophages resident in fatty deposits in blood vessels constantly ingest LDL proteins and differentiate into foam cells. These cells become overloaded with cholesterol in the form of cholesteryl esters in lipid droplets. In this article the authors showed both α-enolase and cholesteryl esters accumulate in foam cells, and proposed that α-enolase plays an inhibitory function on the neutral cholesteryl ester hydrolase (nCEH), a key enzyme to generate free cholesterol from these esterified lipids. According to my results using different surfactants, it is possible that *ENO1* KO cells might have higher levels of cholesterol in the membrane. Therefore, lack of α-enolase in these cells could be responsible for the sustained activity of nCEH leading to the conversion of all esterified cholesterol into free cholesterol that could relocate in cellular membranes. In order to test this hypothesis, free and total cholesterol levels were assessed in the cells, and lipid stores were visualized using oil red staining in PMA-primed THP-1 cells. However, no obvious changes in free or total cholesterol content were detected in *ENO1* KO cells in comparison to WT (Fig. 25B). This result excludes any major effects of α-enolase on the control of free cholesterol production from esterified reservoirs. However, α-enolase activity may still be required for proper sorting of

cholesterol in cellular compartments. This hypothesis is further supported by the lack of changes in size or quantity of lipid droplets in the cells by Oil Red O staining (Fig. 25C). All in all, these results indicate that a role of α-enolase to inhibit free cholesterol formation is not likely, but provide first hints supporting a possible role of α-enolase in compartmentalization of cholesterol. In fact, cholesterol has been recently linked to NLRP3 inflammasome activation. Thus, future experiments linking α-enolase activity and cholesterol trafficking in the context of NLRP3 inflammasome activation will be necessary.

5.6 Mitochondria constitutively cluster close to the Golgi apparatus in *ENO1* KO cells

Mitochondria are a key compartment in the cell for energy production. Their performance is tightly linked to the fitness of mitochondria per se, but is also regulated by the size and the localization of these organelles in space. Mitochondria can undergo processes of fission, or fragmentation into shorter entities, and fusion, in which the outer and inner mitochondrial membranes of independent mitochondria fuse to form a sole unit. The balance between these two mitochondrial processes is determined in many instances by the metabolic state of the cell, and if not properly regulated can be the cause of diseases such as Charcot-Marie-Tooth Neuropathy and Dominant Optic Atrophy (Alexander et al., 2000; Züchner et al., 2004). Positioning of mitochondria can change over time. Mitochondria can travel along microtubules with the help of motor proteins (Ball & Singer, 1982; Heggeness et al., 1978). This transport can be mediated by kinesins towards the + end of the microtubules, and dyneins are required to drive this transport towards the – end, where the microtubule organizing centre (MTOC) is located. As a matter of fact, mitochondrial function, size and localization are closely related to each other, and are all highly sensitive to metabolic changes in the cell. For instance, low glucose levels induce the formation of mtROS (Kajihara et al., 2017), a rise of ATP and an accumulation of defective mitochondria in the cell accompanied by the induction of mitochondrial fusion in the cytosol (S. B. Song & Hwang, 2019). Importantly, mtROS and mtDNA, two of the main indicators of mitochondrial injury, have been proposed to play an important role in NLRP3 inflammasome activation upstream of NLRP3 activation, and this function seems to be particularly relevant in the context of imiquimod and imiquimod derivatives. Here, I sought to investigate whether α-enolase depletion could have induced changes in the mitochondrial network using the mitochondrial membrane marker TOM20 (Fig. 26).

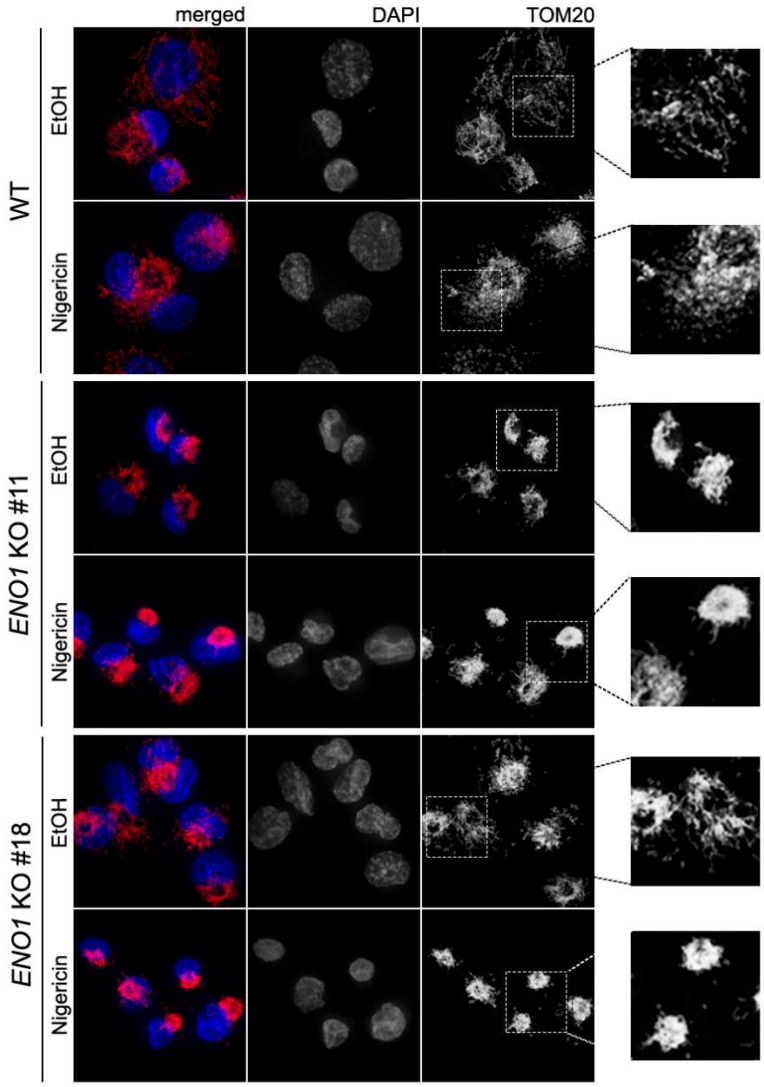


Fig. 26. Mitochondria of *ENO1* KO THP-1 cells are clustered in the perinuclear region. Immunofluorescence images of TOM20 mitochondrial staining from WT and *ENO1* KO cells primed with PMA for 72 hours, then treated with nigericin or EtOH for one hour.

One of the first changes in macrophages upon nigericin incubation has already been described to be mitochondrial fragmentation (Z. Wang et al., 2012). It has also been previously reported that priming of the inflammasome induces translocation of mitochondria to the – end of microtubules, and therefore to the MTOC, which is situated in the vicinity to the nucleus, the perinuclear ER and the Golgi apparatus (Misawa et al., 2013). Misawa and colleagues proposed that the newly rearranged mitochondria in the close proximity to the ER could be a key platform to facilitate the interaction between NLRP3 and ASC. Furthermore, it is possible that mitochondrial fragmentation could increase the available surface of mitochondria in order to maximize the formation of MAMs in the cell. According to my host lab study, NLRP3 is located in MAMs and needs to be close to the Golgi to be activated (Zhang et al., 2017). Taking all these observations together, mitochondrial fragmentation and reorganization do seem to facilitate NLRP3 inflammasome activation by nigericin.

In fact, a striking mitochondrial phenotype was observed in the two *ENO1* KO clones tested in comparison to the WT cells. Mitochondria clustered in *ENO1* KO clones in the perinuclear region even under non-stimulated conditions. Treatment with nigericin, albeit not triggering cell death, further

enhanced mitochondrial clustering leading to a more tightly packed mitochondrial staining in the perinuclear region as compared to WT controls. Such a mitochondrial phenotype has been also observed upstream of cytochrome c release in apoptotic cells (Haga et al., 2003) and as a result of HIF-1α inhibition (D. E. Lee et al., 2018). In both cases, mitochondrial clustering appeared to be concomitant with mitochondrial damage. Indeed, low glucose and thus impaired glycolysis can lead to an overdrive of mitochondrial oxidative phosphorylation, which over time develops into mitochondrial burnout and mitochondrial damage (Kajihara et al., 2017). Therefore, the accumulation of dysfunctional mitochondria at the MTOC could drive the local and sustained production of mtROS, which further perpetuates the damage. When taken together, these observations point to the possibility the depletion of *ENO1* and therefore lower glycolytic flux is responsible for the overburdened status of mitochondria, which leads to the exhaustion of this organelle and the accumulation of damaged mitochondria. This may also provide an explanation why *ENO1* KO cells are more sensitive to CL097-induced pyroptosis. However, further experiments need to be conducted to demonstrate that mitochondrial function is indeed compromised in *ENO1* knockout cells. Most importantly however, as *ENO1* KO cells are highly protected against nigericin-induced pyroptosis, mitochondrial damage might indeed be redundant for NLRP3 inflammasome activation under these conditions as previously suggested by other authors in the field.

In conclusion, even though my results did not pin down a specific mechanism as to how α-enolase promotes NLRP3 inflammasome activation, they pave the way for more targeted experiments to be conducted in the near future. Based on my results, the most promising avenue to pursue for the moment is the analysis of a role of α-enolase activity in cholesterol trafficking. Moreover, it is also tempting to address how restoration of Ca^{2+} mobilization in the absence of α-enolasecan trigger an alternative lytic cell death in response to nigericin stimulation, which may give us further mechanistic insights.

6. DISCUSSION

After the seminal discovery of the NLRP1 inflammasome (Martinon et al., 2002), there has been a strong interest to understand mechanisms as to how different inflammasomes are activated. Studies have unceasingly emerged to answer these questions, bringing new nuances to the field and drawing a growingly complex picture of this fundamental innate immune pathway.

As the most ubiquitous inflammasome, the NLRP3 inflammasome has been in the centre of efforts, also in the context of different diseases. Many mechanisms have been proposed to date to underlie the formation of the NLRP3 inflammasome complex, but still little consensus exists on the importance of and links among them. This stimulated my interest to find novel factors involved in this process, avoiding a bias from previously published data. To this end, I established a CRISPR/Cas9 genome-wide screen in THP-1 cells to identify new genes required upstream of pyroptosis induced by the NLRP3-activating nigericin toxin. The successful completion of this screen did not only result in the identification of a new exciting player in this pathway; it also provided a very exciting tool that can be used for the study of other NLRP3 inflammasome activators. Even more, such a screen can be adapted to resolve other scientific questions. For example, it can help unveiling activation of inflammasomes, which to date are still very poorly characterized.

In this discussion, I will lay out the main discoveries made during my thesis and my contribution to the field of the NLRP3 inflammasome. I will also propose possible ways to explain my findings considering what is currently known in the literature, and propose future directions to address the questions that I could not yet answer during the time I carried out my thesis work.

6.1 The CRISPR/Cas9 genome-wide screen is a robust tool to identify players in NLRP3 inflammasome activation.

Positive selection genome-scale CRISPR knockout screens are based on the enrichment of sgRNA targeting specific genes in a particular condition. Therefore, a good indicator of a positive screen performance is the presence of genes known to participate in the process of interest at the top tier of enriched genes.

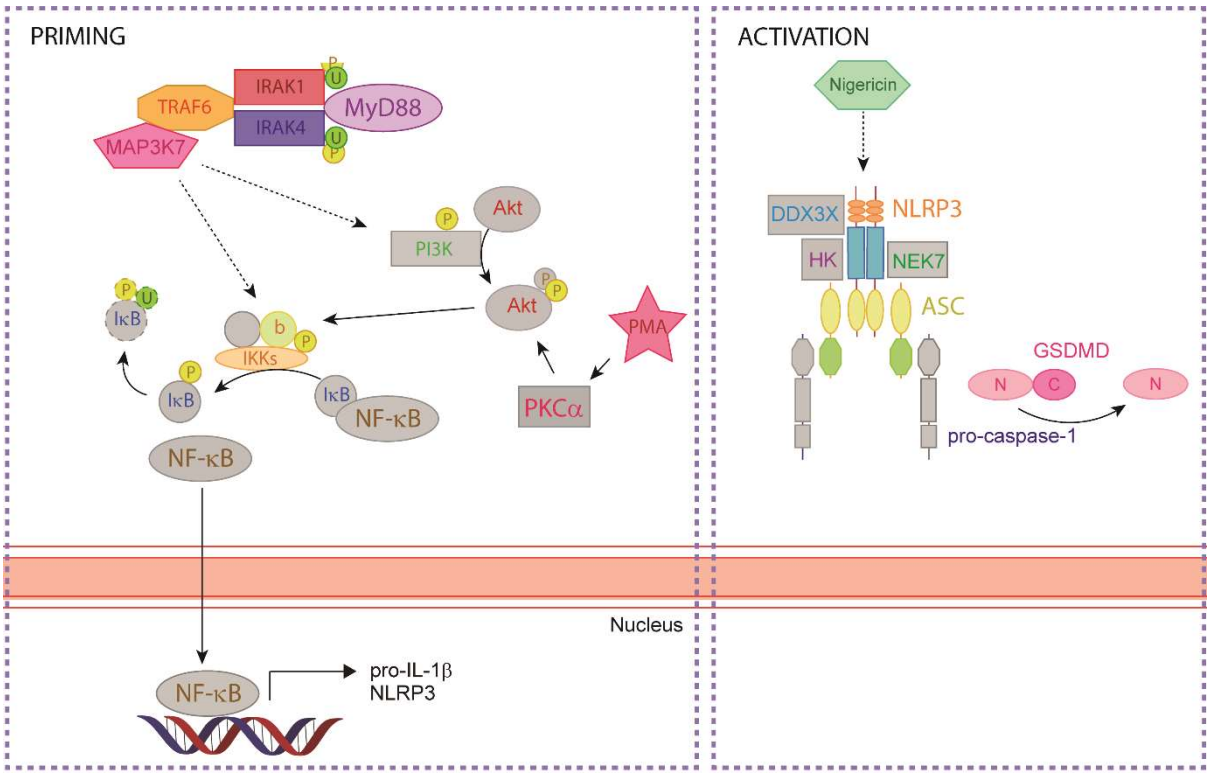


Fig. I. Proteins coded by genes ranking in the higher positions of the hit list from the CRIPSR/Cas9 knockout genome-wide screen. The proteins highlighted in colour were found in the top 18 hits in the list. The proteins that have been described to participate in the priming and activation steps but not found in the list are coloured grey.

In my screen, most of the genes with the highest score were previously known to encode for proteins either participating in the priming step of NLRP3 activation, members of the inflammasome itself, or encoding for the pore-forming GSDMD required for pyroptosis (Fig. I). This strengthens the quality of my screen. Interestingly, I also found genes encoding for the proteins participating in the early events of the priming step such as MYD88, IRAK1/4, TRAF6 and MAP3K7. The molecule I used to prime cells was PMA. PMA activates PKCα, which can phosphorylate Akt and trigger activation of the IKK complex. Thus, direct activation of the IKK complex would be sufficient to allow translocation of NF-κB to the nucleus and induce transcription of the inflammatory genes. Yet, activation of the NF-κB pathway may trigger release of cytokines that then in turn lead to activation of the canonical signalling events upstream of NF-κB. Moreover, it has been recently reported that human monocytes can activate the NLRP3 inflammasome without priming (Gritsenko et al., 2020). Indeed THP-1 is a cell line derived from an acute monocytic leukaemia sample in which signalling induced by priming might be constitutively switched on (Guzova et al., 2019). Yet, if switched off, it is expected to affect inflammasome activation in line with the results obtained in my screen.

Interestingly, none of the proteins reported to interact with the NLRP3 inflammasome core complex have been found to be enriched in my screen. The sgRNAs targeting *DDX3X* and all the *HK* isoforms had rather low LFC, suggesting that HK and DDX3X proteins are rather redundant. In the case of *NEK7*, two of the sgRNAs were clearly not enriched in all three cell libraries. However, a notable enrichment of the remaining two sgRNAs may indicate that *NEK7*, as reported, was indeed a hit in my screen.

There were no apparent processes or pathways linking the other genes in the top tier of the screen. A confirmation mini-screen succeeded to confirm that out of these hits, *ENO1* did have a role in nigericin-induced cell death. Moreover, *ATP5B* was at least partially required in this pathway, and this should be investigated further in the future. However, it could be interesting in the future to use less stringent criteria to set the cut off of positive hits in order to include genes that did not rank higher due to inefficiency of sgRNAs or changes in their coverage across biological replicates. It will also be important to assess the function of the other top hits in more depth; it is possible that knock-outs generated in the mini-screen were not efficient, and remaining protein levels could be sufficient to allow normal maturation of the NLRP3 inflammasome.

6.2 The glycolytic enzyme α-enolase is a novel key factor in NLRP3 inflammasome activation

According to my data, α-enolase, the penultimate enzyme in the glycolytic pathway, is the preferentially expressed form of the *ENO1* gene in THP-1 cells. This enzyme is up-regulated in hypoxic conditions in tumours to help the cell to cope with its energetic requirements. However, THP-1 cells in culture have accessibility to O_2 and do not express high levels of HIF-1α. Additionally, THP-1 cells have been described to rely primarily on β-oxidation and oxidative phosphorylation to meet their energetic requirements (Suganuma et al., 2010). Hence, α-enolase activity should not be expected to be particularly high in the steady state in THP-1 cells. Interestingly, both PMA and another form of priming, LPS, have been shown to transiently enhance glycolytic flux and up-regulate the expression of some glycolytic enzymes (Rodríguez□Espinosa et al., 2015; Wygrecka et al., 2009). Therefore, one of the important events during the priming step could be the acute up-regulation of α-enolase activation. Additionally, activation of the P_2XR_7 channel in the plasma membrane by extracellular ATP has been shown to induce the expression of glycolytic enzymes associated with an increase of the glycolytic flux (Amoroso et al., 2012). In my experimental setup, α-enolase protein levels did not change upon PMA priming in THP- cells. Noteworthy, activity of α-enolase and other glycolytic enzymes can directly be down-regulated by Unc-51 like autophagy activating kinase 1 (ULK1)-mediated phosphorylation (T. Y. Li et al., 2016). ULK1 is inhibited during cell priming by LPS, and this event is necessary for a fully active immune response (She et al., 2018). Therefore, enzymatic activity of α-enolase may become exacerbated during cell priming due to the loss of its ULK1-mediated inactivating phosphorylation. It will be interesting to study levels of α-enolase phosphorylation to better understand changes in its activity in the near future.

While it remains to be investigated whether α-enolase has a direct role in this pathway, in this thesis I have shown that α-enolase plays a key role in NLRP3 inflammasome assembly in response to nigericin. I have provided data suggesting that this role is conserved in murine iBMDMs, where a partial knock-out of *Eno1* led to a slight but significant decrease in cell death. This result suggested that α-enolase function in NLRP3 inflammasome activation may be conserved in human and in mice. However, *Eno1* knock-outs derived from a single cell will need to be generated in the future to confirm this finding. I have also shown that in response to the imidazoquinoline CL097, *ENO1* KO THP-1 cells are perfectly capable of forming the NLRP3 inflammasome scaffold and present maturation of key executor proteins in cell

death such as caspase-1 and GSDMD. Nigericin is a K^+ ionophore supporting H^+/K^+ antiport transport across cellular membranes. It thus triggers NLRP3 by inducing K^+ flux from the highly concentrated intracellular medium to the extracellular environment. The response of *ENO1* KO cells to this activator could thus signify a failure to mount the NLRP3 inflammasome to K^+ efflux, one of the most universal events upstream of most NLRP3 activators. Indeed, imiquimod and its derivatives are so far the only stimuli reported to activate the inflammasome independently of K^+ efflux (Groß et al., 2016). Considering this, *ENO1* KO may be resistant only to K^+-dependent NLRP3 inflammasome activators. It will be important to expand my findings using other NLRP3 inflammasome activators that induce K^+ efflux to confirm this hypothesis.

My current findings are in direct opposition to what has been published in regards to α-enolase function in a previous study (Sanman et al., 2016). Sanman and colleagues concluded that interruption of the glycolytic flux promotes NLRP3 inflammasome activation. They identified a new small molecule able to interact and inhibit both α-enolase and GAPDH and thus trigger NLRP3 inflammasome activation in BMDMs. They also showed that inhibition of these enzymes is sufficient to increase caspase-1 cleavage, but failed to detect any induction of IL-1β release. The authors concluded that activation of the NLRP3 inflammasome arises from the formation of ROS as a result of NADH depletion. It is commonly understood that it is rather accumulation, not loss of NADH that is responsible for ROS production (Murphy, 2009). Additionally, the α-enolase inhibitor they used to confirm their findings has been later proven not to block α-enolase enzymatic function (Satani et al., 2016). Additionally, the idea that inhibition of glycolysis induces NLRP3 activation contradicts the big body of evidence supporting glycolytic induction by LPS and PMA priming.

6.3 The glycolytic function of α-enolase is required for nigericin-induced NLRP3 inflammasome activation

The exogenous expression of different proteins codified by the *ENO1* gene in *ENO1* KO THP-1 cells was conducted in order to elucidate which protein, MBP1 or α-enolase, is required for NLRP3 inflammasome activation by nigericin. The role of MBP1 in this process could not be properly assessed due to high levels of cell death upon its expression. To circumvent this issue, in the future cells lacking the alternative start codon of MBP1 should be generated; this will allow to determine whether the NLRP3 inflammasome can be activated in the absence of MBP1. Importantly, expression of α-enolase could largely rescue the cell death phenotype in *ENO1* KO cells, suggesting that α-enolase is the main actor in *ENO1*'s role in this inflammatory pathway. The expression of catalytically dead mutants of α-enolase E167Q and E211Q failed to re-establish nigericin-induced cell death, thus indicating that the enzymatic function of α-enolase is important for NLRP3 inflammasome activation. The glycolytic activity of α-enolase takes place in the cytosol. The cytosolic localization of exogenously expressed α-enolase-EGFP in *ENO1* KO cells further supported this hypothesis.

Using inhibitors of enzymes upstream and downstream of α-enolase in glycolysis was a first approach to determine whether α-enolase function in particular is important for NLRP3 activation, or whether rather glycolytic flux was determinant. Inhibition of GAPDH with IAA in WT THP-1 cells completely blocked the inflammasome response to nigericin. Conversely, inhibition of PK with OXA led to no

observable changes in cell death in WT cells. Indicatively, it has been reported that loss of PK in BMDMs leads to a generalized decay of glycolytic activity, and to the defective activation of AIM2 and NLRP3 inflammasomes (Xie et al., 2016). The absence of a clear phenotype in my experiments could be explained by the dual effect of PK inhibition on PEP levels: as a limiting enzyme of the glycolytic flux as shown by Xie and colleagues, but also as the PEP-consuming enzyme at the rearmost step of glycolysis. Interestingly, cytosolic K^+ is required for the proper PK conformation to catalyse PEP conversion to pyruvate (Oria-Hernández et al., 2005). Therefore, K^+ efflux triggered by nigericin and other NLRP3 activators may contribute to the build-up of PEP levels during the activating step by partly inhibiting PK activation. Loss of NLRP3 inflammasome activation upon inhibition of GAPDH suggested that 2-PG does not have an inhibitory effect in this pathway. However, it is still unclear whether PEP depletion underlies the protective effect observed in *ENO1* KO cells. Altogether, this experiment suggests that the formation of a metabolite between GAPDH and PK is necessary for NLRP3 inflammasome activation. However, these results must be confirmed in a genetic model, as inhibitor studies always present the caveat of possible unspecific effects.

It will also be of great importance to confirm a reduction in PEP in the *ENO1* KO cells. There are two currently known physiological reactions leading to PEP formation: 2-PG dehydration and decarboxylation and phosphorylation of oxaloacetate (OAA). The reversible conversion of OAA to PEP is catalysed by PEP carboxykinase (PEPCK), which is mostly expressed in liver, where it plays a key role in gluconeogenesis, but can also be detected at considerable levels in kidney and adipose tissues (Zimmer & Magnuson, 1990). It seems unlikely, but it could be interesting to study whether *ENO1* KO cells could develop a compensatory mechanism for the lack of PEP production by activating PEPCK. Directly assessing the content of 2-PG and PEP in *ENO1* KO cells and in different steps of NLRP3 inflammasome activation in WT cells can also provide valuable information to better understand fluctuations of α-enolase activity throughout this inflammatory pathway.

6.4 α-enolase represses a different form of lytic cell death in favour of pyroptosis

In contrast to 2-PG, several functions of PEP outside of glycolysis have been reported, especially in bacteria. In these organisms, it is known to be the precursor to several essential metabolites in conditions of low glucose availability, and can constitute a source of energy in the cell (Walsh et al., 1996). However, there is limited knowledge on the role of PEP in animal cells. One of the more studied functions of this metabolite in eukaryotes is the regulation of cytosolic Ca^{2+} levels by modulating Ca^{2+} entry and release from cellular organelles.

Ca^{2+} concentration varies widely from a cell compartment to another. Cytosolic Ca^{2+} concentration in the steady state oscillates around 100 nM, but can dynamically increase up to 10-100 fold to activate distinct cellular functions. Changes in ionic Ca^{2+} concentrations can respond to an influx from the Ca^{2+}-rich extracellular environment or Ca^{2+} release from intracellular stores. In particular, mitochondria and the ER own ion channels that can rapidly release Ca^{2+} to enable its mobilization in the cytosol. Initially it was discovered that PEP can induce influx of Ca^{2+} from mitochondria (Chudapongse & Haugaard, 1973). More recently, it has been shown that this metabolite has a strong effect on ER stores, where it

inhibits the main entry channel of Ca^{2+}, SERCA, to increase cytosolic Ca^{2+} in T cells (Ho et al., 2015). Changes in enolase activity can thus dramatically alter Ca^{2+} levels in cell compartments.

Priming-mediated up-regulation of glycolysis should be expected to increase Ca^{2+} cytosolic levels. Strikingly, while *ENO1* KO were unresponsive to nigericin in normal conditions, pre-treatment with thapsigargin and ionomycin, which are known to increase Ca^{2+} levels in the cytosol, restored cell death in these cells. Importantly, imiquimod has been reported to regulate Ca^{2+} channels in the ER to induce Ca^{2+} release to the cytosol (A. Y. Huang & Wu, 2016; Nyberg & Espinosa, 2016). Thus, in the absence of PEP, CL097 may compensate for the lack of modulation of SERCA in the ER by prompting Ca^{2+} exit from this compartment through other Ca^{2+} channels. Ca^{2+} release would thus be the necessary event to mount K^+-dependent and -independent NLRP3 inflammatory responses. However, the contribution of changes in Ca^{2+} fluxes are still controversially discussed. In fact, my additional experiments clearly demonstrated that triggering Ca^{2+} influx in *ENO1* KO cells induced cell death independent of the NLRP3 inflammasome. Cell death was not accompanied by markers of inflammasome activation and it could not be blocked by the pan-caspase inhibitor Z-VAD-FMK. Hence, my results suggest that in a normal situation, when cells are treated with nigericin and possibly a threshold of cytosolic Ca^{2+} is reached α-enolase represses a caspase-independent lytic cell death. *ENO1* KO cells cannot modulate this mechanism, and thus die through this alternative pathway when thapsigargin or ionomycin are used. It is important to note that albeit *ENO1* KO cells did not cleave pro-caspase-1 and therefore did not mature pro-inflammatory cytokines in this condition, pro-IL1β was still secreted, which could also trigger inflammatory responses (Fantuzzi et al., 1997). Additionally, lytic cell death is intrinsically inflammatory, as it can prompt the release of DAMPs and contribute to amplify inflammation to neighbouring cells. This feature seemed to be unique to *ENO1* KO cells, as *NLRP3* KO survived the co-treatment with ionomycin and nigericin. It remains to be explored what type of lytic cell death cells undergo. The release of cellular contents in the medium and the presence of smaller cell fragments in culture were reminiscent of necroptosis (Vandenabeele et al., 2010). Indeed, the interplay between pyroptosis and necroptosis has been reported elsewhere (Fritsch et al., 2019). However, it will be important to dissect this pathway in the future. This can be done, for example, by challenging components of the currently known lytic cell death pathways in *ENO1* KO cells co-treated with nigericin and Ca^{2+} mobilizing drugs.

The question remains why can the cell activate two different mechanisms of cell death and actively represses one by α-enolase activity? It is comprehensible that in a normal situation pyroptosis should be prioritized over the alternative cell death pathway due to its higher inflammatory capacity. This active repression would be particularly relevant if key proteins required for the execution of cell death were common to both pathways. However, if α-enolase is absent and pyroptosis cannot be activated, the cell may make use of this alternative pathway to still send an alert to other immune cells to become activated and amplify inflammation. It remains unclear why α-enolase specifically represses this alternative cell death pathway, and there is so far no evidence on whether enzymatic activity in this case is also required. These questions will need to be clarified in the future.

6.5 Could α-enolase play a function during the priming step?

The failure to restore nigericin-induced NLRP3 inflammasome activation by Ca^{2+} influx in *ENO1* KO cells was a strong indicator that Ca^{2+}-related PEP activity was not the main function of α-enolase in the NLRP3 inflammasome pathway. A deeper search in the literature revealed a less known function for PEP. Four decades ago, Świerczyński and colleagues reported that incubation of human placental mitochondria with PEP *in vitro* lead to a dose-dependent oxidation and inactivation of Glycerol-3-phosphate dehydrogenase 2 (GPD2) (Świerczyński et al., 1976). They showed that this function was specific to PEP, as it could not be reproduced by other glycolytic metabolites. GPD2 is one of two enzymes present in the cell for the reversible interconversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (Gl3P). While the other isoform, GPD1, is located in the cytosol and catalyses mostly DHAP conversion to Gl3P, GPD2 is located in the inner mitochondrial membrane and catalyses the reverse reaction. Together, they form the glycerol-3-phosphate shuttle (GPS). GPS is a basic mechanism to regenerate NAD+ from glycolytic NADH and to transport reducing power to the electron transport chain (ETC) in mitochondria. Interestingly, GPD2 has recently been reported to be a key factor in LPS tolerance in mouse cells (Langston et al., 2019). This study reported that GPD2 is required to switch from a predominantly inflammatory state in early LPS priming to the loss of sensitivity to LPS-induced inflammation in longer incubation periods, known as LPS tolerance. They reported that GPD2 works as a switch from a moderate oxidative metabolism accompanied by Acetyl CoA production and subsequent histone acetylation for cytokine expression in early priming, to the complete shutdown of the ETC, loss of Acetyl CoA and thus cytokine expression in the long term. They concluded that this acute change of metabolism was due to the reverse electron transport (RET) phenomena. RET takes place upon excessive flux of electrons into the ETC, which can turn the flow of electrons backwards. Therefore, over-activation of GPD2 in longer incubations with LPS is likely responsible for this unique thermodynamic process by transferring too many electrons to the ETC. Interestingly, under longer incubations with LPS the authors showed an increase in GPD2 protein levels and activity, which may explain the turning point from priming and mild oxidative metabolism to RET and LPS tolerance. It could be speculated that premature activation of GPD2 could be responsible for the untimely onset of LPS tolerance by an early induction of RET.

In accordance with the study by Świerczyński and colleagues, LPS-mediated increase in glycolysis could lead to higher PEP levels, which may be necessary to slow down GPD2 at early stages of LPS priming. In that case, absence of PEP would result in the exacerbated activation of GPD2 in response to LPS, and prematurely desensitize cells to upcoming inflammatory stimuli. Most of my experiments have been performed with PMA. Langston and colleagues propose that LPS induces an increase of glycolytic output and NADH production, subsequent GPD1 activation and GPD2-mediated transfer of electrons to the ETC. Therefore, their findings in LPS are likely to be applicable in the context of PMA, as PMA has also been shown to activate glycolysis (Rodríguez□Espinosa et al., 2015). However, according to my data *ENO1* KO cells express IL-1β normally, based on IL-1β mRNA and pro-IL-1β protein levels upon treatment with PMA. This is not in line with the study by Langston and colleagues, as one of the main markers of LPS tolerance is reduced IL-6 and IL-1β expression. However, this could be explained if the time before enhanced GPD2 activation triggers RET is still sufficient to induce interleukin expression. If this hypothesis is correct, many other processes could be affected by NLRP3

activators as a result of the early onset of RET in *ENO1* KO cells. Acetyl CoA has several functions, and can be the source of acetylation not only for histones, but also for other proteins. Importantly, NLRP3 itself is acetylated in macrophages and this modification has been recently proposed to facilitate the assembly of the NLRP3 inflammasome (M. He et al., 2020; K. Zhao et al., 2019), providing a direct link to loss of Acetyl CoA levels with lack of NLRP3 inflammasome activation in *ENO1* KO cells. In parallel, RET is a strong source of ROS production in mitochondria, particularly of superoxide (Pryde & Hirst, 2011). However, ROS are generally understood to activate rather than dampen NLRP3 inflammasome activation (Heid et al., 2013; Sorbara & Girardin, 2011; Zhou et al., 2011). These events should be analysed in the future to determine whether they underlie the failure to activate the NLRP3 inflammasome in *ENO1*-deficient cells in response to Nigericin.

6.6 Modulation of GPD2 by α-enolase could explain changes in lipid homeostasis

GPD2 is a master regulator of glucose metabolism. The GPS system has in addition a key role in the modulation of lipid homeostasis. Langston and colleagues showed that *Gpd2*$^{-/-}$ BMDMs have high levels of Gl3P. Conversely, uncontrolled GPD2 activation could lead to an elevated consumption of Gl3P. Gl3P is a key metabolite for the biosynthesis of glycerolipids like triacylglycerols, diacylglycerols and phospholipids. Therefore, dysregulated activation of GPD2 may hinder this process and predictably result in changes in lipid composition in cellular membranes.

Interestingly, α-enolase has been reported to decrease levels of a different lipid, free cholesterol, by inhibiting the neutral Cholesteryl Ester Hydrolase (nCEH), albeit it is not yet clear how this works mechanistically (Shand & West, 1995). Cholesterol has been linked to immunity in the context of atherosclerosis, where cholesterol accumulates in the atherosclerotic plaques (Fessler, 2016). In these plaques, cholesterol can form structures that once phagocytosed permeabilize the lysosomal membrane and trigger the onset of NLRP3 inflammasome activation by lysosomal rupture (Hornung et al., 2008). Additionally pathogens can use the host's cell cholesterol to enter and proliferate within this cell (Coppens et al., 2000; Kaul et al., 2004). Therefore, changes in cholesterol levels and cholesterol organization in cell membranes are prone to affect the induction of a robust NLRP3 inflammasome response. Strikingly, *ENO1* KO cells were not responsive to cell death induced by membrane permeabilization with non-ionic surfactants such as Triton X-100 and NP40, while they responded normally to cholesterol-solubilizing detergents like digitonin and saponin. This result suggests that there is a disturbance in lipid composition in the plasma membrane of α-enolase-depleted cells. This observation would correlate with a putative increase of free cholesterol resulting from a highly active nCEH in *ENO1* KO cells. However, it has been reported that nCEH is expressed particularly in foam cells, and is not present in monocytes (Okazaki et al., 2008). Therefore, it will be important to determine whether nCEH is present in THP-1 cells. Furthermore, measurements of cholesterol and lipid droplet size did not exhibit striking differences between *ENO1* KO and WT cells. These results suggest that cumulative production of free cholesterol from *de novo* biosynthesis and cholesteryl ester hydrolysis is not altered in the absence of α-enolase. In that case, I see two scenarios that could explain insensitivity to Triton X-100 and NP40 in α-enolase deficient cells. On one hand, cholesterol trafficking could be

affected in *ENO1* KO cells, leading to the abnormal accumulation of cholesterol in the plasma membrane and deficiency of this lipid in other cellular membranes. It has been shown that perturbed cholesterol trafficking upon Niemann-Pick C1 (Npc1) depletion reduces cholesterol trafficking to the ER, and this dramatically reduces Nlrp3 inflammasome activation in murine macrophages (de la Roche et al., 2018). In a different study published the same year the authors concluded that it is low cholesterol levels in the ER that activate the NLRP3 inflammasome by inducing the translocation of a ternary complex formed by SREBP2, SCAP and recruitment of NLRP3 to the Golgi apparatus (Guo et al., 2018). These two apparently contradicting studies denote that a delicate balance of cholesterol levels in cellular organelles is required for the timely activation of the NLRP3 inflammasome. In my experiments, I also observed a dramatic difference in mitochondrial morphology between *ENO1* KO and their WT cells, which can be in part explained by the loss of glycolytic output and subsequent mitochondrial dysfunction. Cholesterol levels in the mitochondrial membranes are low, and small fluctuations may affect membrane potential and other thermodynamic properties, which could contribute to this phenotype. It could be interesting to study whether this could have an effect in NLRP3 inflammasome assembly. Interestingly, a study has shown that StAR-related lipid transfer protein 3 (Stard3)-deficient murine cells, which exhibit lower levels of cholesterol in mitochondria, are less sensitive to lethal anthrax toxin-induced cell death (Ha et al., 2012). In this study they showed clumping and aggregation of mitochondria in response to this toxin. It will therefore also be important to study activation of other inflammasomes with their cognate stimuli to determine whether the protective effect of *ENO1* KO cells is specific to NLRP3 only.

The second scenario is based on the enhanced production of DHAP from Gl3P in cells deficient of PEP. Deregulated activity of GPD2 leads to exacerbated conversion of Gl3P into DHAP. As previously mentioned, GL3P is a key metabolite for the generation of many phospholipids. Phospholipids are structural constituents of all cellular membranes. If phospholipids are not properly biosynthesized, this could affect the lipid composition of cellular membranes, including the plasma membrane. Therefore, it is possible that it is not the enrichment of cholesterol but rather the decrease of structural phospholipids that determines the sensitivity of these membranes to detergents with low affinity to cholesterol. In order to distinguish between these two options, it would be of high interest to assess the lipid composition in cell fractions of *ENO1* KO cells, and to stain cells with specific lipid dies to track lipid trafficking between cellular compartments.

Altogether, it appears that α-enolase activity is involved in the dynamic modification of the lipid composition of membranes in the cell. It is also important to keep in mind that as an ionophore, nigericin diffuses through the plasma membrane to transport cations in favour of the ionic gradient. So far, there has not been studies on this subject, but it cannot rule out that changes in membrane composition and fluidity could affect the capacity for this ionophore to diffuse across the membrane. It will thus be important to measure K^+ fluxes in response to nigericin in cells depleted for *ENO1* as compared to WT cells. It should also be considered that lipid composition in the plasma membrane is important for receptor trafficking, and if a constitutive defect exists in *ENO1* KO cells, this might affect translocation of proteins involved in NLRP3 inflammasome activation. Importantly, during the priming step (Elliott et al., 2018) and later in response to activating signals NLRP3 is localized to cellular membranes (J. Chen & Chen, 2018; Franklin et al., 2014; Zhang et al., 2017). In these studies, lipids have been directly linked to NLRP3 recruitment. Therefore, changes in lipid homeostasis may hinder proper localization of

NLRP3. In the future, NLRP3 should be tracked in *ENO1* KO cells in response to activating signals to determine whether its trafficking to a particular cellular compartment is affected.

6.7 *ENO1* KO cells respond differently in response to nigericin and imiquimod

Another unique feature of *ENO1* KO cells is that they are particularly sensitive to imiquimod-induced NLRP3 inflammasome activation. A better understanding of how CL097 and other imidazoquinolines may affect this pathway could give a hint why α-enolase is redundant under these conditions. Groß and colleagues showed that imiquimod inhibits the Complex I in the ECT and NRH-quinone oxidoreductase 2 (NQO2), which together yield a strong production of mtROS (Groß et al., 2016). Considering all putative functions of PEP described above, PEP depletion is most likely to result in oxidative stress; early RET leads to increased superoxide formation and mitochondrial damage through blockade of glycolysis, impaired glycolysis causes mitochondrial dysfunction and mtROS production (Kajihara et al., 2017) and profound changes in mitochondrial cholesterol may also affect mitochondrial function and fitness. Therefore, higher levels of mtROS species in *ENO1* KO cells could explain the enhanced inflammatory response in comparison to WT cells. However, it has been demonstrated that mtROS production does not seem to be sufficient to induce NLRP3 inflammasome (Jabaut et al., 2013), and it is not sufficient in our model to induce the NLRP3 inflammasome in response to nigericin. Together, these observations suggest that there is a unique mechanism underlying imiquimod-driven NLRP3 inflammasome activation. Even though CL097 induces inflammasome activity independent of K^+ efflux, CL097 could still act downstream of K^+ efflux and α-enolase activity. Alternatively, CL097 may trigger NLRP3 inflammasome activation through a completely independent mechanism. Understanding the mechanism as to how α-enolase controls NLRP3 inflammasome activity may thus also provide insights on possible CL097-dependent mechanisms.

6.8 α-enolase is an essential mediator of several inflammatory responses

My thesis work has revealed a new unanticipated role of α-enolase in one of the key pathways of the host defence system, the NLRP3 inflammasome pathway. The mechanism underlying this effect is not yet clear, but my work offers some exciting mechanistic scenarios to be further corroborated. I summarized them in a potential schematic model (Fig. II). Interestingly, α-enolase has been previously linked to other processes of innate immunity. It has been long known that α-enolase can bind to plasminogen in the plasma membrane surface of eukaryotic cells in cancer and facilitates cell migration (Felez et al., 1990; Nakajima et al., 1994), also in the case of monocytic migration (Wygrecka et al., 2009). Bacteria can take advantage of this system by exposing their own enolase, which can interact with the host's plasminogen and trigger proteolytic degradation of extracellular matrix to assist invasion of host cells (Bhattacharya et al., 2012). More recently localization of α-enolase to the monocytes' and macrophages' plasma membrane has been observed in the context of disease, namely in rheumatoid arthritis (Bae et al., 2012). Soluble α-enolase can also be found in synovial fluid of rheumatoid patients, and it can activate inflammatory programs in myeloid cells by inducing the TLR4-CD14 axis (Guillou et al., 2016). Additionally, α-enolase autoantibodies have been identified in several pathologies, and are

now regarded as markers of microbial infection and of autoimmune disease (Terrier et al., 2007). Interestingly, α-enolase also participates in adaptation to several types of stresses, including hypoxic stress, glucose deprivation and high temperature in mammal cells (Graven et al., 1993; Young & Elliott, 1989).

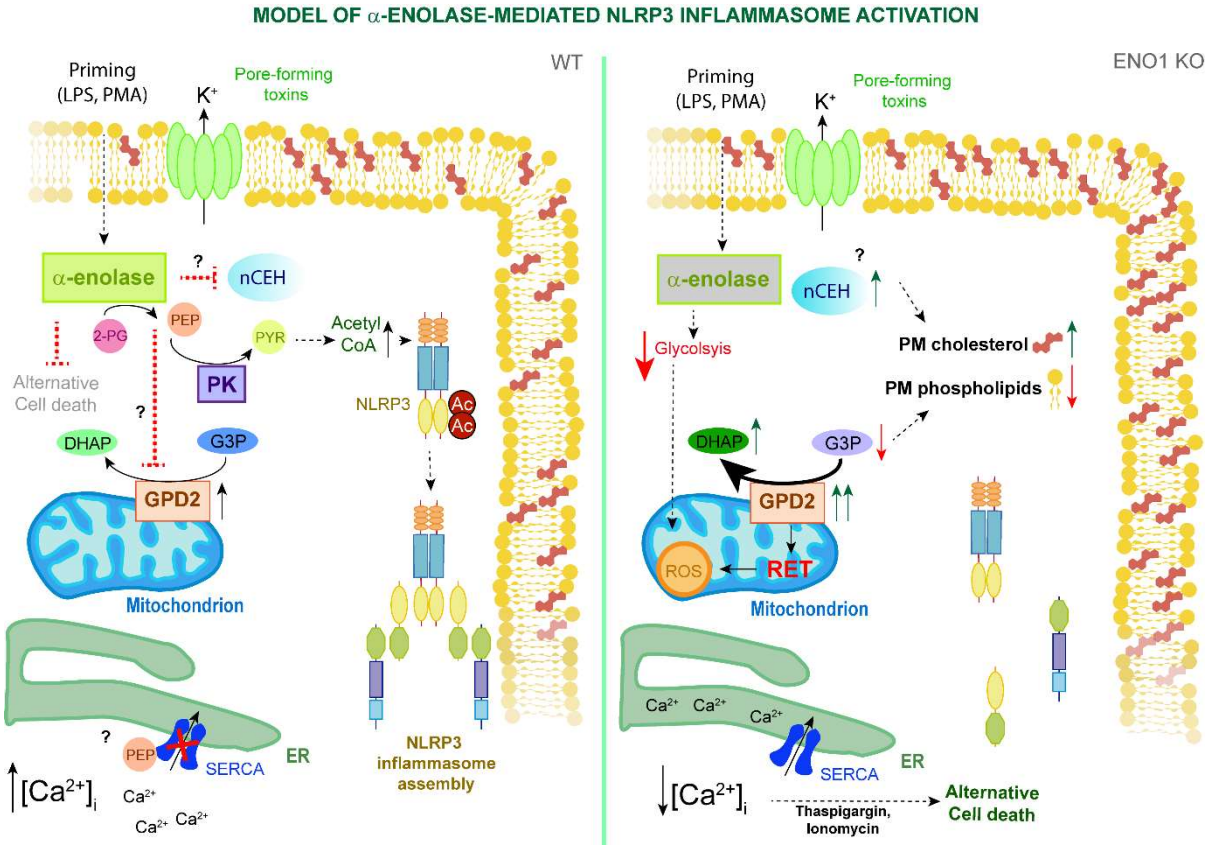


Fig. II. α-enolase deficiency may affect NLRP3 inflammasome activation at several levels. α-enolase expression and activation is induced in primed cells. Active α-enolase catalyses the conversion of 2-PG to PEP, which can play several functions in the cell: it can inhibit SERCA and inducing Ca^{2+} efflux from ER, it can block GPD2 activity in mitochondria and it is a precursor for Acetyl CoA synthesis, which is required for acetylation of several proteins, including histones and NLRP3. α-enolase has also been proposed to block nCEH and dampen formation of free cholesterol from cholesteryl ester reservoirs. Loss of α-enolase leads to lower glycolytic output, which can result in enhanced mitochondrial activity and on a long term can lead to mitochondrial damage ROS production. GPD2 activity in the absence of PEP may be exacerbated, leading to RET and oxidative stress in mitochondria, along with the depletion of the metabolite Gl3P. Changes in Gl3P and possibly cholesterol may modify membrane lipid composition in all organelles, and particularly lead to an increase of the cholesterol/phospholipid ratio in the plasma membrane. This could render these cells less sensitive to detergents that cannot solubilize cholesterol. Additionally, lack of PEP may lead to higher flux of Ca^{2+} to the ER, thereby lowering cytosolic Ca^{2+} levels. In *ENO1* KO cells, if drugs that increase Ca^{2+} mobilization are present, a Ca^{2+} threshold could be reached and trigger the onset of an alternative cell death pathway.

Developing new strategies to specifically target α-enolase could thus be an invaluable tool to alleviate exacerbated inflammatory processes. This is particularly interesting in the context of NLRP3 inflammasome activation. Further work in this direction may thus pave the way for interesting therapeutic approaches in combination with current anti-inflammatory therapies.

7. Conclusions

During my Thesis I set up a CRISPR/Cas9 genome-wide screen which revealed genes with a potential involvement in the pathway leading to NLRP3 inflammasome and subsequent pyroptosis. One of the top genes obtained in the screen, *ENO1*, has been successfully shown to be essential for NLRP3 inflammasome in response to nigericin, especifically upstream of NLRP3 inflammasome assembly. That is not the case for the K^+-independent NLRP3 activator CL097, in response of which *ENO1* KO cells can mount a strong NLRP3 inflammasome.

The larger protein encoded by *ENO1*, α-enolase, is responsible for its function in NLRP3 inflammasome activation. Mechanistically, enzymatic activity of α-enolase is required to reinstate cell death in response to nigericin in *ENO1* KO cells. Interestingly, depletion of α-enolase unblocks a caspase-independent alternative lytic cell death pathway when cells are co-treated with nigericin and Ca^{2+} mobilizing drugs. This cell death does not entail maturation of pro-IL-β, suggesting the presence of α-enolase is required for full-blown inflammatory responses. Additionally, depletion of α-enolase affects dramatically membrane solubility to some detergents, and leads to a striking changes in mitochondria morphology and localization. It is still unclear whether these phenotypes are directly linked to the lack of responsiveness to nigericin in these cells, and whether α-enolase plays an acute role in NLRP3 inflammasome activation. This shall be investigated in the future.

Taken together, these results indicate a key role of α-enolase in the NLRP3 pathway, and suggest α-enolase plays an important function to modulate different cell death programs in response to NLRP3 activators.

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Résumé de thèse

Titre : L'enzyme glycolytique α-enolase joue un rôle essentiel dans l'activation de l'inflammasome NLRP3

De tous les acteurs que l'immunité innée peut déployer pour combattre l'infection, les récepteurs de reconnaissance de formes (« PRR ») sont parmi les premiers à jouer un rôle prédominant. Ils sont chargés de détecter des entités nocives en détectant des motifs moléculaires spécifiques, et peuvent ensuite déclencher une réaction inflammatoire. Notre intérêt s'est porté sur la protéine « NOD-, LRR- and pyrin domain-containing protein 3 » (NLRP3), un PRR intracellulaire qui orchestre les processus inflammatoires en réponse à un large éventail de molécules microbiennes et aux signaux de danger endogènes. L'activation de ce senseur conduit à la formation d'un complexe protéique multimérique appelé inflammasome NLRP3. Ce complexe macromoléculaire est une plateforme inflammatoire responsable de la sécrétion de cytokines pro-inflammatoires. En outre, son activation entraîne généralement la pyroptose, un type de mort cellulaire lytique qui limite la propagation des bactéries phagocytées, facilite la libération de cytokines pro-inflammatoires et de signaux de danger pour alerter le reste de l'organisme afin de lutter efficacement contre les infections[1]. L'existence d'un facteur unique en aval de tous les stimuli activant NLRP3 semble probable. De nombreux événements communs en amont de l'activation de l'inflammasome NLRP3 ont été proposés ; Parmi lesquels y figurent l'efflux de K^{+} [2], l'influx de Ca^{2+} [3], la perméabilisation lysosomale[4], les modifications du métabolisme cellulaire[5] et les lésions mitochondriales[6]. Toutefois, aucun de ces facteurs ne s'est jusqu'à présent révélé nécessaires pour l'activation de l'inflammasome en réponse à tous les stimuli connus, et ce facteur universel présumé reste donc inconnu. Bien que des efforts aient été faits dans ce sens, les données et les conclusions opposées ralentissent l'identification de ce facteur universel. Il est donc intéressant de choisir une approche plus large afin d'éviter un biais indésirable pour aborder cette question.

La technologie CRISPR/Cas9 est apparue ces dernières années comme un outil essentiel pour l'étude des sciences du vivant. La polyvalence de cette technique permettant d'éditer le génome de manière rapide et évolutive a permis d'ouvrir la porte à de nombreuses possibilités. L'une d'entre elles a particulièrement éveillé mon intérêt : le criblage CRISPR/Cas9 à l'échelle du génome, permet d'évaluer le caractère essentiel de tous les gènes du génome pour une fonction donnée. En particulier dans la recherche sur le cancer, les criblages génétiques ont été largement utilisés pour découvrir l'implication des gènes particuliers[7,8]. Comme mentionné avant, l'activation de l'inflammasome entraîne dans la plupart des cas la mort pyroptotique des cellules.

Au cours de ma thèse, j'ai réalisé un criblage CRIPSR/Cas9 à l'échelle du génome pour identifier les gènes clés impliqués dans la mort cellulaire induite par l'activation de l'inflammasome NLRP3. Le criblage a été validé par l'identification d'une première série de gènes connus pour être impliqués dans l'amorçage cellulaire. Ce processus correspond à une première cascade d'événements nécessaires pour rendre la cellule compétente pour activer l'inflammasome. D'autres gènes codant pour certains membres de l'inflammasome NLRP3 lui-même, tels que NLRP3 et PYCARD, et un gène codant pour la protéine de formation des pores responsable de la pyroptose, GSDMD ont été mis en évidence. L'identification de ces gènes ont permis de confirmer le succès de mon criblage. De manière

intéressante, ce criblage m'a surtout permis de découvrir un gène qui semble essentiel dans l'activation de l'inflammasome NLRP3 et qui était jusqu'alors inconnu : *ENO1*.

J'ai pu confirmer le rôle de ce gène dans l'activation de l'inflammasome NLRP3 dans les cellules THP-1, une lignée cellulaire monocytique humaine. En effet, l'inactivation du gène codant pour α-enolase dans ces cellules (*ENO1* KO), ne permet pas d'assembler le complexe NLRP3. Par conséquent, elles étaient résistantes à la mort cellulaire induite par l'activation de NLRP3. Le gène *ENO1* encode pour deux formes de protéines, l'α-enolase et la protéine de liaison à Myc-1 (MBP1). La protéine MBP1 est la forme la plus courte, c'est un répresseur de tumeur bien connu qui agit sur le promoteur oncogène c-Myc[9]. En revanche, l'α-enolase est une enzyme clé dans la glycolyse, un processus catabolique hautement conservé pour décomposer le glucose et produire de l'énergie. En outre, il a été constaté que l'α-enolase a d'autres fonctions indépendantes de son activité enzymatique, en particulier chez les levures et les bactéries[10].

J'ai montré que l'α-enolase est la forme nécessaire à la formation de l'inflammasome NLRP3. L'expression de l'α-enolase dans les cellules *ENO1* KO est suffisante pour rétablir la pyroptose en réponse à l'activation de l'inflammasome. Il est intéressant de noter que la mutation des résidus essentiels pour l'activité catalytique de cette enzyme, E167 et E211, entraîne la perte complète de cet effet. Ce résultat suggère que l'activité enzymatique de l'α-enolase est nécessaire pour son rôle dans la formation de l'inflammasome NLRP3. Il a été démontré que le produit de l'activité catalytique de l'α-enolase, le phosphoénolpyruvate (PEP), est un puissant inhibiteur de la Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)[11]. Cela suggère que le PEP joue un rôle important pour bloquer l'accumulation du Ca^{2+} provenant réservoir du réticulum endoplasmique[11,12]. L'influx de Ca^{2+} a été précédemment proposé comme l'une des étapes clés pour induire l'activation de l'inflammasome[3]. Par conséquent, le PEP pourrait faciliter l'accumulation de Ca^{2+} dans le cytosol en bloquant son entrée dans le réservoir principal de Ca^{2+}. De manière intéressante, l'induction pharmacologique de l'influx de Ca^{2+} à partir des réserves de Ca^{2+}, déclenche la mort cellulaire des cellules *ENO1* KO, mais ne reproduit pas les principales caractéristiques de l'activation de l'inflammasome NLRP3 telles que la sécrétion d'interleukine et la maturation de la protéine GSDMD. Ces données suggèrent que malgré son rôle dans l'influx du Ca^{2+} cytosolique, l'α-enolase pourrait aussi exercer une fonction plus essentielle pour permettre le début de la formation de l'inflammasome NLRP3. Une observation clé de mon travail de thèse a mis en évidence que les cellules *ENO1* KO sont uniquement protégées des stimuli qui dépendent de l'efflux de K^+ pour activer l'inflammasome. L'imiquimod et l'imidazoquinoline CL097, qui ne dépendent pas de cet événement pour activer l'inflammasome mais plutôt de la formation d'espèces réactives de l'oxygène (ROS)[13], ont fortement activé l'inflammasome NLRP3 dans les cellules ENO1 KO. Il est donc probable que l'α-enolase joue une fonction de transduction de l'efflux de K^+ en un signal d'activation de NLRP3. Bien que l'on sache que l'imiquimod cible les TLR7/8 et entraîne la production de ROS et l'accumulation de calcium dans le cytosol[13-15], les cibles exactes de l'imiquimod restent encore mal connues. Il reste donc possible que l'un des effets de cette molécule reproduise en partie l'effet physiologique de l'α-enolase. Il sera intéressant d'aborder cette question à l'avenir.

Bien que de nouvelles protéines telles que l'enolase-α aient été proposées pour jouer des fonctions importantes dans l'activation de l'inflammasome NLRP3, l'absence de modèles cellulaires humains rend difficile la confirmation de ces résultats. À ce jour, les modèles les plus couramment utilisés sont soit

des lignées cellulaires dérivées de patients leucémiques, soit des cellules isolées de la moelle osseuse murine et différenciées en macrophages in vitro. Cependant, ces dernières années, l'usage de cellules souches pluripotentes humaines induites (hIPSC) permet de mimer d'une manière plus précise la réponse des macrophages primaires humains. Au cours de ma thèse, j'ai établi un protocole pour différencier les hIPSC en macrophages en adaptant le protocole décrit par Van Wilgenburg et al[16]. J'ai également mis en place un nouveau protocole d'inactivation de la fonction des gènes dans les hIPSC en utilisant la technologie CRISPR/Cas9. Ainsi, j'ai pu générer des macrophages dérivés des hIPSC, dont les gènes codant pour les protéines caspase-1 et IRF2 ont été invalidés. Les données générées avec ces cellules ont fait l'objet d'une publication dans le cadre d'une collaboration[17].

Dans l'ensemble, mes travaux ont permis de découvrir un nouvel acteur clé dans la formation de l'inflammasome NLRP3. L'α-enolase présente un grand potentiel en tant que nouvelle cible pour les thérapies visant à traiter les conditions caractérisées par une activation excessive de l'inflammasome NLRP3, telles que les syndromes auto-inflammatoires associés à la cryopyrine (CAPS). La synthèse d'inhibiteurs plus spécifiques de l'α-enolase dans le futur permettra d'évaluer sa fonction dans les hIPSC. Ils permettront également de valider la pertinence de cibler l'α-enolase dans les maladies inflammatoires dépendantes de l'inflammasome NLRP3.

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Marta PUIG GÁMEZ

α-enolase: A new player in NLRP3 inflammasome activation

Résumé

La protéine NLRP3 (protéine 3 contenant les domaines NOD, LRR et pyrine) est un senseur intracellulaire qui conduit à la formation d'un complexe protéique oligomérique inflammatoire connu sous le nom d'inflammasome NLRP3. Afin de comprendre ce mécanisme, j'ai réalisé un criblage à l'échelle du génome à l'aide de la technique CRIPSR/Cas9. Pour ce faire, j'ai activé NLRP3 dans la lignée cellulaire monocytaire de la leucémie humaine (THP-1) avec la Nigéricine, un puissant activateur de l'inflammasome. Ce criblage a mis en évidence l'α-enolase comme un nouveau régulateur de l'activation du NLRP3. Une étude plus approfondie de cette protéine a suggéré que son produit de réaction phosphoénolpyruvate (PEP) est nécessaire à l'activation de l'inflammasome. Ainsi, mes travaux de recherche ont permis de découvrir un acteur clé dans la formation de l'inflammasome. L'α-enolase devrait être considéré comme une nouvelle cible thérapeutique potentielle. Elle permettrait de limiter l'activation excessive de l'inflammasome NLRP3 qui caractérise certains syndromes auto-inflammatoires tels que ceux associés à la cryopirine (CAPS).

Résumé en anglais

NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) is an intracellular sensor that leads to the formation of an inflammatory multimeric protein complex known as the NLRP3 inflammasome. The promiscuous nature of NLRP3 suggests that sensing of NLRP3 does not occur directly. In this study, I have taken an unbiased approach to shed light on this question by performing a CRIPSR/Cas9 genome-wide study using a strong activator of the NLRP3 inflammasome in the human leukaemia monocytic cell line, THP-1. This screen unveiled α-enolase as a novel key regulator of NLRP3 activation. Further study of this protein suggested that the enzymatic function of this glycolytic enzyme is necessary for activation of the inflammasome, possible through its product phosphoenolpyruvate (PEP). Altogether, my work has uncovered a key player in the formation of the inflammasome, and introduce a new potential target to treat conditions characterized by an excessive NLRP3 inflammasome activation, such as the Cryopyrin-Associated Autoinflammatory Syndromes (CAPS).