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Characterization of TLR7-independent effects of Imidazoquinolines

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For my Dad

If an idea presents itself to us, we must not reject it simply because it does not agree with the logical deductions of a reigning theory.

Claude Bernard
An Introduction to the Study of Experimental Medicine, 1813

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Abbreviations

°C	degrees Celsius
2DG	2-deoxy-D-glucose
AIM2	absent in melanoma 2
AP-1	activator protein 1
APAF1	apoptotic protease activating factor 1
APC	antigen-presenting cell
APDC	ammonium pyrrolidinedithiocarbamate
ASC	apoptosis-associated speck-like protein containing a CARD
ATG16L1	autophagy-related protein 16-1
ATP	adenosine triphosphate
Bcl2	B-cell lymphoma 2 protein
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
BRCC3	BRCA1/BRCA2-Containing Complex Subunit 3
BSA	bovine serum albumin
C. albicans	*Candida albicans*
CARD	caspase-recruitment domain
CASR	calcium-sensing receptor
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazine
CD	cluster of differentiation
cGAS	cyclic GMP-AMP synthase
CIITA	major histocompatibility class II transactivator
cmk	chloromethylketone
CpG	unmethylated CpG-containing oligodeoxynucleotide
DAMP	danger/damage-associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double-stranded
DSS	disuccinimidyl suberate
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECAR	extracellular acidification rate
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
$FAD/FADH_2$	flavin adenine dinucleotide
FCS	fetal calf serum
FeS	iron-sulfur center

FMN	flavin mononucleotide
g	grams
g	gravity
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hours
HaCaT	human, adult, low calcium, high temperature keratinocytes
HBSS	Hanks-balanced salt solution
HEK293T	human embryonic kidney 293T
HEPA	high-efficiency particulate air filter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hPK	human primary keratinocytes
ICE	IL-1 converting enzyme
IFI16	interferon gamma inducible protein 16
IFN	interferon
IKK	inhibitor of κB kinase complex
IL	interleukin
INFAR	IFNα/β receptor
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
ISE	ion-selective electrode
IκB	inhibitor of κB
l	litres
LC3B	light chain 3B
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LRR	leucine rich repeat
M	molar
M-CSF	macrophage colony-stimulating factor
MAPK	mitogen-activated protein kinase
MAS	mannitol and sucrose buffer
MAVS	mitochondrial antiviral-signalling protein
MDA5	melanoma differentiation-associated gene 5
MDP	muramyl dipeptide
MHC	major histocompatibility
min	minutes
MOI	multiplicity of infection
MPO	myeloperoxidase
MSU	monosodium urate crystals
mtDNA	mitochondrial DNA
MyD88	myeloid differentiation factor 88
NAD^+/NADH	nicotinamide adenine dinucleotide
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate
NAIP	NLR family (formerly neuronal) apoptosis inhibitory protein
Ndi1	*S. cerevisiae* NADH dehydrogenase, internal 1
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NHDF	normal human dermal fibroblasts

NLR	NOD-like receptor
NLRC	NACHT, LRR, and CARD-containing protein
NLRP	NACHT, LRR, and PYD-containing protein
NOD	nucleotide-binding and oligomerization domain
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NP-40	nonident P-40
NQO	NAD(P)H:quinone oxidoreductase
OCR	oxygen consumption rate
P2rX7	P2X purinoceptor 7
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGN	peptidoglycan
poly(dA:dT)	poly(deoxyadenylic-deoxythymidylic) acid
PRR	pattern recognition receptor
Pycard	(gene name for ASC protein)
PYD	pyrin N-terminal homology domain
RBC	red blood cell
RET	reverse electron transport
RIG-I	retinoic acid-inducible gene I
RIP	receptor-interacting serine/threonine-protein kinase
RLR	RIG-I-like receptors
rm	recombinant murine
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOPF	specific opportunistic pathogen-free
SPF	specific pathogen-free
ss	single-stranded
TAK1	transforming growth factor β-activated kinase 1
TCA	tricarboxylic acid
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMPD	*N,N,N',N'*-tetramethyl-*p*-phenylenediamine
TNF	tumour necrosis factor
TRIF	TIR-domain-containing adaptor protein inducing IFNβ
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TXNIP	thioredoxin-interacting protein
U	units
Unc93b1	Unc-93 homolog B1
V	volts

Summary

The imidazoquinoline imiquimod is a topical immunomodulator that is used to treat genital warts and basal cell carcinoma. Its ability to activate Toll-like receptor 7 (TLR7) for the production of type I interferon and pro-inflammatory cytokines is presumed to underlie its efficacy. However, TLR7-independent effects of imiquimod, including apoptosis in keratinocytes and NLRP3 inflammasome activation in myeloid cells, may contribute to its efficacy or adverse effects. The purpose of this study was to investigate the mechanism of NLRP3 inflammasome activation by imiquimod. The imidazoquinolines imiquimod and CL097 induce conventional NLRP3 inflammasome activation, in that they trigger NLRP3-dependent ASC oligomerization, interleukin-1 secretion, and pyroptosis. Activation of NLRP3 by imiquimod and CL097 does not require endolysosomal TLRs, but it may involve leakage of endolysosomal components such as cathepsins into the cytoplasm. Potassium (K^+) efflux is accepted as a universal requirement for NLRP3 inflammasome activation. Surprisingly, imiquimod and CL097 do not induce or require K^+ efflux for NLRP3 activation. This is the first demonstration that NLRP3 activation can in principle occur independently of K^+ efflux. In addition, imiquimod and CL097 are identified as specific inhibitors of mitochondrial Complex I activity. In contrast to imiquimod and CL097, imidazoquinolines that lack the ability to inhibit Complex I do not activate NLRP3 and or trigger proliferation arrest in keratinocytes, the primary cellular target of imiquimod *in vivo*. NLRP3 activation by imiquimod and CL097 requires ROS, which is consistent with ROS production as a well-established outcome of Complex I inhibition. Since Complex I has a critical function in supporting tumour cell proliferation and survival, the data presented here suggest that Complex I inhibition by imiquimod may be a determinant of its clinical anti-tumour effects.

Zusammenfassung

Imiquimod ist ein Arzneistoff aus der Gruppe der Imidazoquinoline, der in Salbenform zur Behandlung genitaler Warzen und des Basalzellkarzinoms eingesetzt wird. Es wird allgemein angenommen, dass seine Fähigkeit, den Toll-*like* Rezeptor 7 (TLR7) zu aktivieren und dadurch die Produktion von Typ-I Interferonen und pro-inflammatorischen Zytokinen zu induzieren, seiner Wirksamkeit zu Grunde liegt. Es ist jedoch seit längerem bekannt, dass auch TLR7-unabhänige Effekte wie in Induktion von Zelltod in Keratinozyten und die Aktivierung des NLRP3 Inflammasoms in myeloiden Immunzellen zu den Wirkungen und Nebenwirkungen von Imiquimod beitragen können. Ziel dieser Studie war es, die Mechanismen der NLRP3 Aktivierung durch Imiquimod zu untersuchen. Es konnte gezeigt werden, dass die Imidazoquinoline Imiquimod und CL097 das konventionelle NLRP3 Inflammasom aktivieren, indem sie NLRP3-abhängige Oligomerisation des Proteins ASC, Ausschüttung von Interleukin-1 und pyroptotischen Zelltod induzieren. Während TLR7 und andere endosomale TLRs für diese Aktivität entbehrlich sind, scheint die Freisetzung von endosomalen Komponenten wie z.B. Cathepsinen in das Zytoplama beteiligt zu sein. Kalium (K^+) Ausstrom aus der Zelle ist als universeller, auslösender Faktor in der NLRP3 Aktivierung allgemein akzeptiert. Überraschenderweise ist K^+ Ausstrom weder für die NLRP3 Aktvierung durch Imiquimod beteiligt, noch induziert Imiquimod K^+ Ausstrom aus Zellen. Diese Ergebnisse belegen erstmalig, dass NLRP3 Aktivierung im Prinzip ohne K^+ Ausstrom möglich ist. Darüber hinaus konnte gezeigt werden, das Imiquimod und CL097 spezifische Inhibitoren von Komplex I der mitochondrialen Atmungskette sind. Im Gegensatz zu Imiquimod und CL097 sind andere Imidazoquinoline, die nicht das NLRP3 Inflammasom aktivieren auch keine Inhibitoren von Komplex I. Gleichermaßen führt nur die Behandlung von

Keratinozyten, den primären pharmakologischen Zielzellen, mit Imiquimod und CL097 aber nicht mit anderen Imidazoquinolinen, zum Stillstand des Zellwachstums. In Übereinstimmung mit der Fähigkeit von Komplex I Inhibitoren, die Produktion von Sauerstoffradikalen (ROS) zu induzieren, ist die Aktivierung von NLRP3 durch Imiquimod ROS-abhängig. Nachdem Komplex I eine wesentliche Funktion in Überleben und Proliferation von Tumorzellen hat, legen die hier vorgestellten Daten nahe, dass die klinische anti-Tumor Aktivität von Imiquimod mit seiner inhibierenden Wirkung auf Komplex I in Zusammenhang steht.

Introduction

The early view of the immune system was that it serves as the first line of defence against infection by distinguishing 'self' from 'non-self'. Over the past two decades, however, it has become appreciated that such a view is overly simplistic, and a more accurate view may be that the immune system functions as an elaborate and evolutionarily conserved means of detecting and protecting the host against 'danger' or infectious non-self (Janeway, 1989; Matzinger, 1994). Charles A. Janeway, Jr. hypothesized the existence of receptors for pathogen molecules on antigen-presenting cells (APCs) that would induce on these cells the expression of costimulatory molecules required for T cell activation (Janeway, 1989). Thus, an adaptive immune response would not be triggered unless two criteria were satisfied: a microbial stimulus and a non-self antigen. He termed these receptors for pathogen molecules on APCs pattern recognition receptors. Soon after, the CD14 component of the lipopolysaccharide (LPS) receptor complex, several microbial carbohydrate-binding lectin receptors, and the Toll-like receptor family were identified. In 2011, the Nobel Prize in Medicine was awarded to Ralph Steinman for his discovery of dendritic cells and their antigen presentation capacity, and to Jules Hoffmann and Bruce Beutler for their contributions that helped prove Janeway's pattern recognition theory.

It is now apparent that the innate immune system employs a wide variety of germline-encoded pattern recognition receptors (PRRs) for the detection of conserved structural motifs or genomic material of invading pathogens (pathogen-associated molecular patterns, PAMPs) and endogenous danger signals indicating host injury or stress (danger/damage-associated molecular patterns, DAMPs) (Medzhitov, 2009). Such receptors include transmembrane proteins that survey the extracellular space and

endosome (*e.g.* Toll-like receptors, TLRs; and C-type lectin receptors, CLRs), cytoplasmic nucleic acid-sensing receptors (*e.g.* DNA sensors such as absent in melanoma 2 (AIM2) and cyclic GMP-AMP synthase (cGAS)), and RNA sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5)) and the intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Upon signal detection, PRRs initiate cellular signalling cascades that culminate in the activation of transcription factors, cytokine production, altered cellular programming, and expression of costimulatory molecules. Some PRRs also directly activate microbicidal mechanisms, such as phagocytosis, production of reactive oxygen species (ROS) and release of toxic cytoplasmic granules. This elegant receptor system allows the resulting immune response to be exquisitely tailored to not only the class of pathogen (bacteria, protozoa, viruses, and fungi), but also to its location (within the cytoplasm, internal compartments, or outside of the cell).

Toll-like Receptors

TLRs are a family of pattern recognition receptors sharing homology with the *Drosophila* protein Toll. In *Drosophila*, Toll is required for induction of antimicrobial peptides, and Toll mutants succumb to lethal fungal infections (Lemaitre et al., 1996). Soon after, TLR4 was discovered by a database search for human homologs of Toll (Medzhitov et al., 1997), and by mapping of the genomic locus in mice responsible to resistance to LPS-induced shock (Poltorak et al., 1998). Since then, characterization of the ligands and functions of the dozen other TLRs has demonstrated an essential role for pattern recognition receptors in immunity (Akira et al., 2006).

TLRs are expressed on the cell surface and on endolysosomal membranes of myeloid cells. They primarily recognize evolutionarily conserved PAMPs derived from bacteria, fungi and viruses, but also some endogenous molecules (DAMPs). All TLRs have an extracellular (or lumenal) leucine-rich repeat domain (LRR) for ligand binding, and an intracellular Toll/interleukin-1R/Resistance (TIR) domain for engagement of innate immune signalling pathways. Ligand binding of TLRs triggers their dimerization and the recruitment of TIR domain-containing adaptor proteins. TLR signalling results in the activation of transcription factors including nuclear factor κB (NF-κB), activator protein-1 (AP-1) and interferon regulatory factor (IRF) family members which control the expression of pro-inflammatory cytokines, type I interferons (*e.g.* IFN-α and IFN-β), and genes that control the microbicidal activity and phenotypic maturation of myeloid cells (Akira and Takeda, 2004). Most cytokines induced by TLRs bear a signal peptide, which directs their co-translational translocation into the lumen of the endoplasmic reticulum (Murray and Stow, 2014). These cytokines are secreted conventionally, via secretory vesicles departing from the Golgi apparatus. The regulation of these cytokines occurs primarily at the level of gene expression. Expression of cytokines of the interleukin-1 (IL-1) family is also induced by TLR signalling, in a process termed priming that is described in detail later in this text. However, these cytokines do not have signal peptides and are secreted by an unconventional pathway that is tightly controlled by other PRR complexes called inflammasomes (Monteleone et al., 2015).

The adaptor protein MyD88 is essential for signalling downstream of most TLRs (except TLR3), and also of the IL-1 receptor (Kawai et al., 1999; Wesche et al., 1997). MyD88 recruits and activates members of the IL-1R-associated kinase (IRAK) family. IRAK1 and IRAK4 then activate a ubiquitin ligase complex containing the protein tumour

necrosis factor receptor-associated factor 6 (TRAF6), which activates the kinase TAK1 by ubiquitination (Akira and Takeda, 2004). In resting cells, NF-κB transcription factors are held inactive in the cytoplasm by inhibitor of κB (IκB). TAK1 phosphorylates and activates the IκB kinase (IKK) complex, which in turn phosphorylates IκB. This targets IκB for ubiquitination and proteasomal degradation (Karin and Ben-Neriah, 2000). Liberated NF-κB enters the nucleus and activates target genes. TAK1 also activates the AP-1 transcription factor, which in concert with NF-κB promotes the expression of pro-inflammatory target genes (Akira and Takeda, 2004).

TRIF is a TIR-containing adaptor protein that controls production of IFN-β production in response to double-stranded RNA recognition by TLR3, or LPS recognition by TLR4. In the TLR4 signalling pathway, it is also necessary for the late, MyD88-independent activation of NF-κB (Hoebe et al., 2003; Yamamoto et al., 2003). Recruitment of TRIF to endosomal TLR3 and also to internalized TLR4 leads to the phosphorylation of IRF3, and its nuclear translocation. IRF3 activation and subsequent IFN-β production occur independently of MyD88. Activation of other endosomal TLRs 7 and 8 (by single stranded RNA or imidazoquinolines), and 9 (by unmethylated CpG DNA) triggers IRF7-dependent production of IFN-α (Kawai et al., 2004). This pathway is especially important in plasmacytoid DCs, which express high levels of IRF7 and the nucleic acid sensing TLRs. In contrast to type I IFN production downstream of TLR3 and TLR4, this pathway is MyD88-dependent and TRIF-independent (Kawai et al., 2004). The IFN-α/β receptor (IFNAR) is present on nearly all somatic cells, and induces an antiviral response after IFN binding (Ivashkiv and Donlin, 2014).

Nod-like Receptors

NLRs are cytoplasmic proteins that are homologous to the NB-LRR disease resistance genes in plants. The functions of most mammalian NLRs remain uncharacterized. NLRs typically have a tripartite structure, consisting of a central and defining nucleotide-binding and oligomerization (NACHT) domain, C-terminal LRRs and an N-terminal protein interaction domain. Two major subfamilies of NLRs exist: NLRP proteins, which have a pyrin (PYD) effector domain, and NLRC proteins, which have a caspase-activation and recruitment (CARD) domain (Ting et al., 2008).

NOD1 and NOD2 are of the NLRC family were the first intracellular pattern recognition receptors to be described (Girardin et al., 2003a; 2003b; Inohara et al., 2003). They recognize, respectively, mesodiaminopimelic acid and muramyl dipeptide (MDP) substructures of the bacterial cell wall component peptidoglycan. Upon activation, they engage in homotypic CARD domain interactions with receptor-interacting serine/threonine-protein kinase 2 (RIP2), thereby activating pro-inflammatory genes, including various cytokines and chemokines under the transcriptional control of NF-κB and MAPK pathways (Strober et al., 2005). The NLRs CIITA and NLRC5 have been shown to act as transcriptional co-activators that control the expression of major histocompatibility (MHC)/human leukocyte antigen (HLA) class II and class I, respectively (Neerincx et al., 2013). Other NLRs, including NLRP1, NLRP3, NLRC4, and NAIP form complexes termed inflammasomes, which control the cleavage and secretion of IL-1β and IL-18 (Table 1).

Inflammasomes

Inflammasomes are cytoplasmic multiprotein complexes that serve as platforms for activation of the protease caspase-1, and for caspase-1-dependent proteolytic maturation and secretion of IL-1β and IL-18. Minimally, inflammasomes contain an oligomerized sensor/receptor protein connected to caspase-1, usually via the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD). Aside from the aforementioned NLRs, other PRRs such as the cytoplasmic nucleic acid receptors AIM2, RIG-I, and interferon gamma-inducible protein 16 (IFI16) have been shown to form inflammasomes (Table 1). Collectively, these PRRs couple the detection of a vast array of PAMPs and DAMPs to the caspase-1-dependent bioactivation of IL-1β and IL-18. Most of these inflammasomes have a defined set of structurally related activators that are often pathogen components, and that usually directly bind and activate the sensor protein. As described below, NLRP3 is the exception.

Table 1: *Inflammasomes and their ligands or activators*

PRR	**Activator/Ligand (*source*)**	**References**
NLRP1	anthrax lethal toxin (*B. anthracis*) MDP (*bacteria*)	(Boyden and Dietrich, 2006) (Faustin et al., 2007)
NLRP3	many diverse activators, ligand unknown	(Schroder and Tschopp, 2010)
NLRP6	unknown (*intestinal microbiota?)*	(Elinav et al., 2011)
NLRP7	lipopeptide (*bacteria*)	(Khare et al., 2012)
NLRP12	unknown (*Yersina pestis*)	(Vladimer et al., 2012)
NAIP/NLRC4	flagellin and Type III/IV Secretion System components (*bacteria*)	(Kofoed and Vance, 2011; Mariathasan et al., 2004; Zhao et al., 2011)
AIM2	dsDNA (*DNA viruses and bacteria*)	(Fernandes-Alnemri et al., 2009; Hornung et al., 2009)
RIG-I	5’ triphosphate RNA (*viruses and bacteria*)	(Poeck et al., 2010)
IFI-16	dsDNA (*DNA viruses*)	(Kerur et al., 2011; Monroe et al., 2014)
caspase-11	LPS (*Gram negative bacteria*)	(Shi et al., 2014)

NLRP3 Inflammasome

The NLRP3 inflammasome was among the first inflammasomes discovered, and is arguably the most important inflammasome in human disease (Agostini et al., 2004; Schroder and Tschopp, 2010). NLRP3 can be activated by a myriad of structurally diverse exogenous and endogenous factors, but none of these are known to be ligands. The ability of NLRP3 to respond to endogenous dangers signals, such as extracellular ATP, monosodium urate (MSU) crystals, cholesterol crystals, amyloid deposits and sterile environmental irritants such as asbestos and silica explains why it is implicated in inflammatory diseases such as gout (Martinon et al., 2006; So et al., 2007), atherosclerosis (Duewell et al., 2010), metabolic syndromes (Henao-Mejia et al., 2012; Vandanmagsar et al., 2011), Alzheimer's (Halle et al., 2008; Heneka et al., 2013), multiple sclerosis (Gris et al., 2010), asbestosis, silicosis (Dostert et al., 2008; Hornung et al., 2008) and contact hypersensitivity (Sutterwala et al., 2006; Watanabe et al., 2007). Furthermore, gain-of-function mutations in NLRP3 cause debilitating periodic fever syndromes such as familial cold auto-inflammatory syndrome (FCAS) and Muckle-Wells syndrome (MWS) (Aksentijevich et al., 2002; Hoffman et al., 2001; 2004). Despite over a decade of research and overwhelming disease relevance, the mechanism of NLRP3 inflammasome activation remains unresolved.

Priming

A first signal, termed priming, is necessary but not sufficient for robust NLRP3 inflammasome activation and IL-1β secretion (Figure 1) (Bauernfeind et al., 2009). The priming signal triggers the NF-κB-dependent upregulation of NLRP3 and proIL-1β, which are poorly expressed in resting myeloid cells (Guarda et al., 2011; O'Connor et al., 2003).

The expression of ASC, caspase-1, and other inflammasome sensor proteins (*e.g.* AIM2, NLRC4, *etc*) is largely unaffected by the priming signal, so the actual activation of most other inflammasomes is not as reliant on priming as the NLRP3 inflammasome. The most efficient and most commonly used priming agent is LPS, though ligands of other PRRs and cytokine receptors that activate NF-κB can also provide a priming signal (Franchi et al., 2009). In addition to its effect on expression of NLRP3 and proIL-1β, LPS-induced priming has rapid, poorly understood non-transcriptional effects that lower the threshold for NLRP3 activation (Juliana et al., 2012; Schroder et al., 2012). These non-transcriptional signals also enhance activation of caspase-1 in response to activators of other inflammasomes, suggesting they may also regulate components common to all inflammasomes such as ASC or caspase-1 (Schroder et al., 2012).

NLRP3 Activation

In the absence of an activator, NLRP3 is maintained in an inactive state in the cytoplasm. The LRRs of NLRP3 are proposed to sense the putative ligand, but also to mediate intramolecular autoinhibition of the NACHT domain in the absence of an activator (Figure 1). This inhibitory interaction may be sustained by chaperones such as Hsp90 and SGT1, which participate in autoinhibition of the NAIP/NLRC4 inflammasome (Mayor et al., 2007; Poyet et al., 2001). Under resting conditions NLRP3 is heavily ubiquitinated, which may divert it for proteosomal degradation and/or sterically hinder its interaction with putative ligands (Juliana et al., 2012; Py et al., 2013). Priming and activation signals both promote NLRP3 deubiquitination (Juliana et al., 2012). A signal for NLRP3 activation triggers its oligomerization by homotypic NACHT domain interactions. The clustered PYD domains of NLRP3 molecules (or other PRRs) act as a seed for prion-like polymerization

of the PYD- and CARD- domain containing protein ASC (Cai et al., 2014; Lu et al., 2014). A hallmark of inflammasome activation is the formation of large (1 μm), detergent insoluble ASC specks (Fernandes-Alnemri et al., 2007; Masumoto et al., 1999). The CARD domain of polymerized ASC recruits caspase-1, and caspase-1 clustering leads to its activation via autoprocessing. Active caspase-1 dissociates from the inflammasome and cleaves a variety of cellular targets including proIL-1β, proIL-18, and others (Denes et al., 2012). Caspase-1 activity triggers unconventional secretion of IL-1α, IL-1β, and IL-18, and also a form of cell death called pyroptosis (Figure 1).

Activation of NLRP3 can be triggered by many factors, including extracellular ATP, potassium (K^+) ionophores, crystalline and non-crystalline particles, and pathogens including viruses and the fungus *Candida albicans*. Rather than activating NLRP3 directly, these factors induce reactive oxygen species (ROS), K^+ efflux, endolysosomal leakage, and potentially other pathways (Figure 1). By unknown mechanism, these cellular perturbations are sensed by NLRP3 or unknown factors immediately upstream of NLRP3. Though the role of ROS in NLRP3 activation has been debated (Bauernfeind et al., 2011; Muñoz-Planillo et al., 2013), and endolysosomal leakage is implicated only in NLRP3 activation by particles and crystals (Hornung et al., 2008), it is accepted that K^+ efflux is a universal requirement for NLRP3 activation (Franchi et al., 2014; Muñoz-Planillo et al., 2013; Rivers-Auty and Brough, 2015).

Potassium Efflux

Along with Na^+ ions, K^+ ions are a primary determinant of membrane potential. K^+ concentrations are high within the cell (120-150 mM) and low (5 mM) outside the cell, whereas the opposite is true for Na^+. Healthy cells maintain a membrane polarity of

-50mV to -80mV, meaning that the inside of the cell has a net negative charge. Maintenance of membrane potential is important for many essential cellular processes. Since the discovery more than 20 years ago that the K^+/H^+ ionophore nigericin triggers the proteolytic activation and secretion of IL-1β, a role for K^+ efflux in this process has been postulated (Perregaux et al., 1992). This was supported by the finding that other inducers of K^+ efflux such as extracellular ATP (later found to be a P2rx7 cation channel agonist) and several additional K^+-permissive ionophores trigger processing and secretion of IL-1β (Perregaux and Gabel, 1994). A requirement for K^+ efflux in processing and secretion of IL-1β in response to ionophores was demonstrated by preventing K^+ efflux by incubating cells in medium containing high K^+ concentrations (Walev et al., 1995).

Shortly after these pioneering studies, caspase-1 was identified as the long-sought 'IL-1 converting enzyme' (ICE) (Kuida et al., 1995). In 2002, the inflammasome was discovered as the complex that controls caspase-1 activation and therefore the bioactivity of IL-1 (Martinon et al., 2002). It was then appreciated that PRRs such and NLRP3, NLRP1, and NLRC4 engage the inflammasome in response to distinct activators, and that inducers of K^+ efflux were activators of the NLRP3 inflammasome (Mariathasan et al., 2006). It was then published that high extracellular K^+ inhibited NLRP3 activation in response to ATP, nigericin, and other newly discovered NLRP3 activators (Pétrilli et al., 2007). The finding that particles indeed induce K^+ efflux and that K^+ efflux induced by K^+-free medium is 'sufficient' for NLRP3 activation, led to the proposal that K^+ efflux is the common trigger of the NLRP3 inflammasome (Muñoz-Planillo et al., 2013). Recently, it was also shown that 'non-canonical' NLRP3 activation in response to caspase-11 activation by cytoplasmic LPS (Hagar et al., 2013; Kayagaki et al., 2011; 2013) also requires K^+ efflux (Baker et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015).

Collectively these findings have led to the consensus that K^+ efflux is a universal requirement for Nlrp3 activation (Rivers-Auty and Brough, 2015).

Endolysosomal Leakage

The NLRP3 inflammasome drives inflammation in response to gout-associated MSU crystals, pseudogout-associated calcium pyrophosphate crystals (Martinon et al., 2006), the adjuvant alum (Eisenbarth et al., 2008), environmental irritants such as asbestos and silica (Dostert et al., 2008), cholesterol crystals (Duewell et al., 2010), and Alzheimer's disease-associated amyloid-β fibrils (Halle et al., 2008). Upon phagocytic uptake, these particles trigger leakage or rupture of the endolysosome (Halle et al., 2008; Hornung et al., 2008). Concomitant spilling of cathepsin B and potentially other molecules into the cytoplasm has been linked to NLRP3 activation by these particles, since cathepsin inhibitors and cathepsin B deficiency impaired NLRP3 activation by particles and crystals (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008). While the inhibitory effect of the cathepsin B/L inhibitor CA074Me have been reproduced by many laboratories, there are a number of reports of normal particle/crystal-induced NLRP3 activation in cathepsin B-deficient cells (Dostert et al., 2009; Muñoz-Planillo et al., 2013). Interpretation of these findings is also confounded by the observation that NLRP3 activation can itself trigger endolysosomal leakage (Fujisawa et al., 2007; Heid et al., 2013). Therefore, while endolysosomal leakage by particles and crystals coincides with NLRP3 activation, an open question is whether this is a cause or consequence of NLRP3 activation.

Reactive Oxygen Species

One-electron reduction of O_2 generates the short-lived radical superoxide (O_2-). Superoxide is the proximal reactive oxygen species (ROS), but is rapidly converted to other species. ROS are evolutionarily conserved signalling and microbicidal molecules, but excessive levels of ROS, resulting from an imbalance of ROS production and antioxidative mechanisms, can lead to oxidative stress, where cellular macromolecules including lipids, enzymes and DNA are irreversibly damaged (Dröge, 2002; Schieber and Chandel, 2014). Induction of ROS is observed in response to NLRP3 activators such and ATP, nigericin, particles, crystals and fungal pathogens, and antioxidants suppress NLRP3 activation by these agents (Cruz et al., 2007; Dostert et al., 2008; Gross et al., 2009; 2012; Pétrilli et al., 2007; Zhou et al., 2010b). Thus, ROS may be signals for NLRP3 inflammasome activation, but the mechanism by which ROS influence NLRP3 and the molecular source of this ROS remain unknown. In myeloid cells, the major sources of ROS are the phagosomal NADPH oxidase and mitochondria, and both have been investigated for their role in NLRP3 activation.

Respiratory burst is the rapid, non-mitochondrial generation of ROS in myeloid cells. The critical role of ROS in host defence is highlighted by patient mutations in NADPH oxidase components that lead to repeated, life-threatening bacterial and fungal infections in chronic granulomatous disease (Good et al., 1968). The activity of NADPH oxidase is regulated by assembly of inactive complexes upon myeloid cell activation (DeLeo and Quinn, 1996). Once assembled at the phagosome, this enzyme converts O_2 to superoxide, which is rapidly converted to hydrogen peroxide (Babior, 1984). These species are only mildly microbicidal. However, their killing potential is greatly increased by the action of myeloperoxidase (MPO), which converts hydrogen peroxide to

hypochlorous acid, the reactive agent in bleach. The ability of hypochlorous acid to destroy the activity of a wide array of biomolecules accounts largely for the microbicidal consequences of respiratory burst (Klebanoff, 1968).

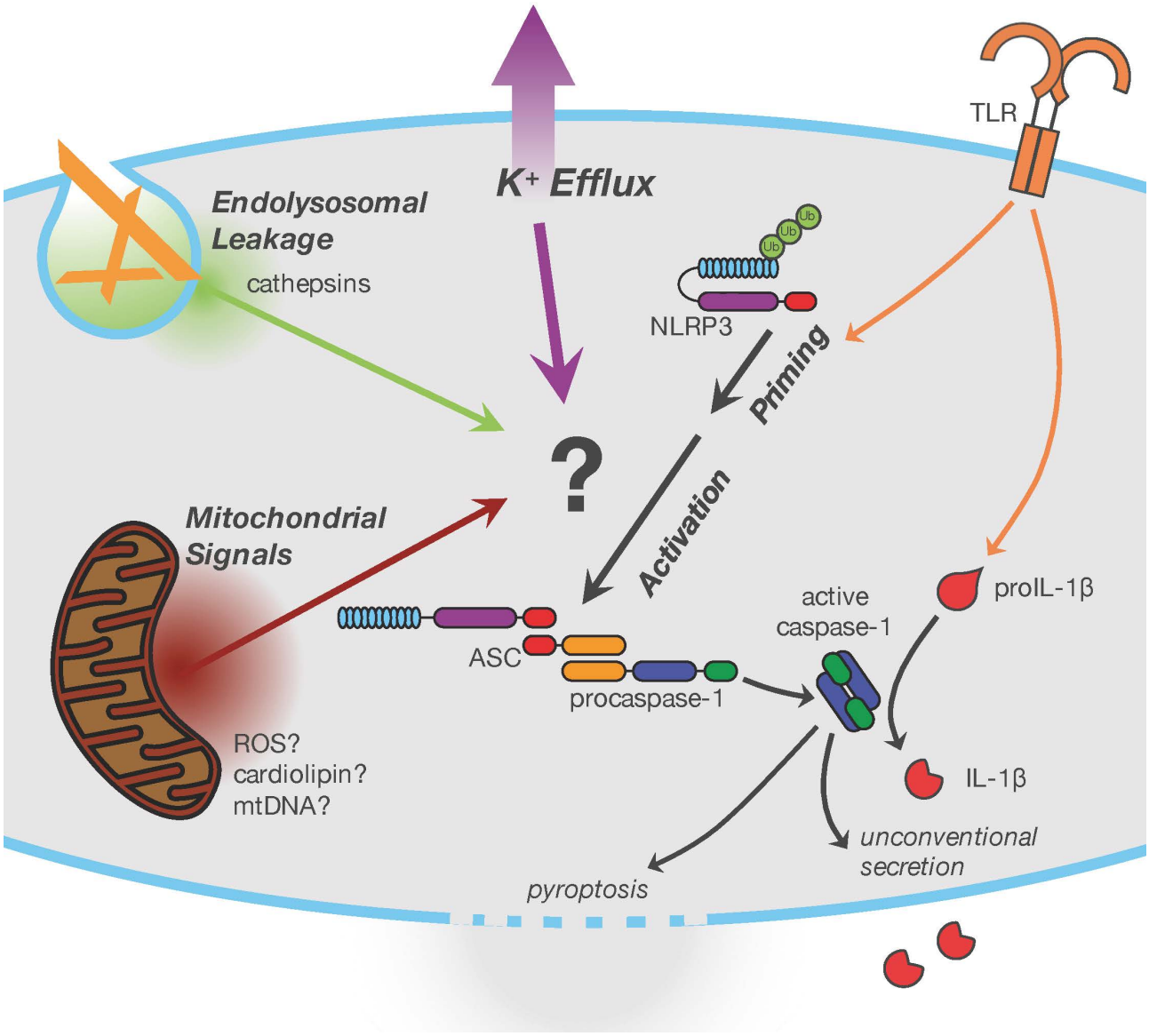


Figure 1: Mechanisms and cellular consequences of Nlrp3 inflammasome activation.

A priming signal provided by an NF-κB-activating receptor (*e.g.* a TLR) is necessary for upregulation of proIL-1β and NLRP3. Priming also has poorly understood non-transcriptional effects, including but not limited to deubiquitination of NLRP3, that promote but are not sufficient for NLRP3 activation. Activators such as inflammatory particles or crystals, ATP, and nigericin all trigger K^+ Efflux and ROS production, which are signals for NLRP3 activation. Other mitochondrial signals, such as the cardiolipin and mtDNA may be involved in NLRP3 activation. Inflammatory particles and crystals, such as MSU, silica, alum, and amyloid-β also trigger endolysosomal leakage of cathepsins and other molecules. It is currently not known whether NLRP3 senses K^+ Efflux, ROS, and endolysosomal leakage directly, or whether these signals are

Figure 1, continued relayed to NLRP3 by putative upstream factors which may integrate these signals. Activation of NLRP3 results in its oligomerization and the recruitment and polymerization of ASC to form a caspase-1-activating complex called the inflammasome. For simplicity, the inflammasome is represented as single molecules. Active caspase-1 processes proIL-1β to generate biologically active IL-1β. Caspase-1 also orchestrates unconventional secretion of IL-1β, and also a form of lytic cell death termed pyroptosis.

Mitochondria

Mitochondria are organelles that are commonly referred to as the 'powerhouse' of eukaryotic cells on account of their ability to produce ATP. They have an outer membrane that is permeable to molecules smaller than 5000 Da, and an inner membrane that is impermeable to most molecules, including protons. The space between the outer membrane and the inner membrane is called the intermembrane space. Within the inner membrane is the mitochondrial matrix, which harbours the enzymes for fatty acid oxidation and the tricarboxylic acid (TCA) cycle, as well as the maternally-inherited mitochondrial genome and protein synthesis machinery. The undulations of the inner membrane are known as cristae, and they provide an increased surface area for reactions that occur at the inner membrane, such as oxidative phosphorylation. In addition to there role as the metabolic hub of the cell, mitochondria have important functions in calcium signalling, redox signalling and cell death (Dröge, 2002; Galluzzi et al., 2012; McBride et al., 2006; Rizzuto et al., 2012; West et al., 2011b). Genetic and acquired mitochondrial dysfunction is implicated in aging and in many pathological conditions including cancer, inflammation, and neurodegeneration (Green et al., 2011; Lin and Beal, 2006; Nunnari and Suomalainen, 2012; Wallace, 2005; 2012).

Oxidative Phosphorylation

The TCA cycle in the mitochondrial matrix oxidizes carbon substrates to CO_2, and couples this to the reduction of electron carriers nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). In addition, intermediates of the TCA cycle can be funnelled into anabolic pathways. The electron transport chain consists of five multiprotein complexes that couple the energy generated from electron transfer from reduced carriers to oxygen to the generation of ATP (Figure 2). Complex I is an NADH:ubiquinone oxidoreductase. It transfers electron from NADH to ubiquinone via a flavin mononucleotide (FMN) and a series of iron-sulfur (FeS) centers distributed amongst its 44 subunits. Complex II is succinate dehydrogenase: it converts succinate to fumarate, and generates $FADH_2$ from FAD. Like Complex I, Complex II transfers electrons to ubiquinone. Ubiquinone is a lipophilic electron carrier in the inner mitochondrial membrane that shuttles electron from Complex I or Complex II to Complex III. Complex III accepts ubiquinone-derived electrons and transfers them to cytochrome *c* (cyt *c*), a heme-containing protein in the intermembrane space. Cytochrome *c* transfers electrons from Complex III to Complex IV, where they are finally transferred to the terminal electron acceptor O_2 to generate H_2O. Electron transfer reactions at Complex I, Complex III and Complex IV results in the pumping of protons out of the matrix into the intermembrane space. This generates a proton gradient across the inner mitochondrial membrane. The energy stored in this gradient is used by ATP Synthase (Complex V) to generate ATP from ADP and inorganic phosphate (Lehninger and Kennedy, 1948; Mitchell, 1961). Thus, in healthy mitochondria electron transport reactions are tightly coupled to ATP synthesis.

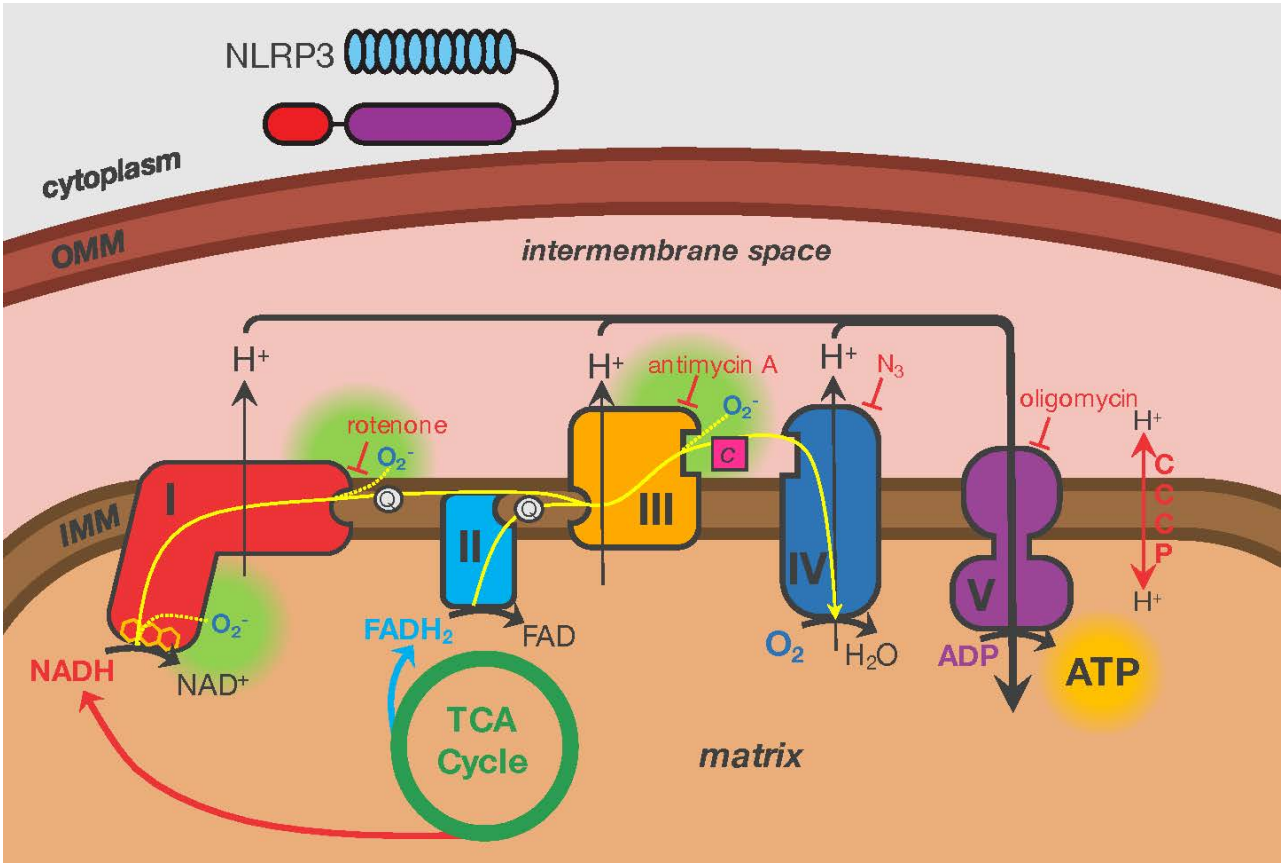


Figure 2: Oxidative Phosphorylation.

The electron transport chain at the inner mitochondrial membrane (IMM) consists of fives complexes. Transport of electron through these complexes (depicted by the solid yellow line) is coupled to the production of ATP. Within the mitochondrial matrix, the TCA cycle generates NADH, which donates electrons to Complex I. Complex II (succinate dehydrogenase) is part of the TCA cycle. It converts succinate and FAD to fumarate and $FADH_2$, and then accepts electrons from $FADH_2$. Complex I and Complex II transfer electrons to ubiquinone (Q) to generate ubiquinol. Complex III accepts electrons from ubiquinol and transfers them to cytochrome *c* (*c*), a protein that is associated with the IMM. Cytochrome *c* shuttles electrons to Complex IV, where they are transferred to O_2, the terminal electron acceptor. Electron transport at Complexes I, III, and IV is coupled to the pumping of protons (H^+) from the matrix into the intermembrane space. The energy of the resulting proton gradient is harvested by Complex V (ATP Synthase), which generates ATP from ADP and phosphate. Various inhibitors of the electron transport chain are shown. CCCP is an uncoupler: it allows respiration to continue, but prevents ATP production because it dissipates the proton gradient. Leakage of single electrons to O_2 at Complex I or Complex III generates superoxide (O_2^-), and this is enhanced in the presence of inhibitors of these complexes (I: rotenone, III: antimycin A). NLRP3 in the cytoplasm may sense (directly or indirectly) mitochondrial ROS.

Mitochondria in Innate Immune Signalling

Mitochondria are intimately involved in immunity. In addition to fulfilling the energetic and biosynthetic demands of mobile immune cells, they also act as an innate immune signalling nexus and contribute to antimicrobial effector mechanisms (West et al., 2011b; 2011a). Furthermore, recent studies have demonstrated that changes in immune cell phenotype are tightly coupled to mitochondrial metabolic pathways (Buck et al., 2015; Pearce and Everts, 2015).

One of the first indications for a role of mitochondria in innate immune signalling was the discovery mitochondrial antiviral-signalling protein (MAVS) (Meylan et al., 2005; Seth et al., 2005). Localization of MAVS to the outer mitochondrial membrane is required for type I IFN production in response to cytoplasmic RNA recognition by RIG-I and MDA5 (Kato et al., 2006; Seth et al., 2005). The NOD-like receptor NLRX1 is also mitochondrially located, and been implicated in ROS signalling and negative regulation of MAVS signalling (Moore et al., 2008; Tattoli et al., 2008).

Mitochondria are an important source of danger signals that can engage the innate immune response. Extracellular ATP released by dying cells triggers P2rX7-dependent NLRP3 activation (Mariathasan et al., 2006; Perregaux and Gabel, 1994; Solle et al., 2001). Mitochondria and their bacterial ancestors produce formylated peptides, which are among the most potent chemoattractants for immune cells (Witko-Sarsat et al., 2000; Zhang et al., 2010). Release of mitochondrial genomic DNA (mtDNA) into the cytoplasm or into the extracellular space can activate DNA-sensing PRRs in the cytoplasm (Allam et al., 2011; Nakahira et al., 2011; West et al., 2015) or TLR9 in the endosome, respectively (Zhang et al., 2010). As described below, mitochondrial ROS is emerging as another important regulator of innate immune signalling.

Sources of Mitochondrial ROS, and their Implication in NLRP3 Activation

Mitochondria are a significant source of ROS, even in cells that have NADPH oxidases. Superoxide is the proximal ROS produced by mitochondria, but it is rapidly converted to hydrogen peroxide by superoxide dismutase. Superoxide is produced by the one electron reduction of O_2. Direct electron donors for this reaction are the redox groups of proteins, such as FMN, FAD, and FeS centers, and ubiquinone. Mitochondrial proteins that can produce superoxide and other ROS are cytochrome *b5* reductase, monoamine oxidase, dihydroorotate dehydrogenase, α-glycerophosphate dehydrogenase, aconitase, dihydrolipoamide dehydrogenase subunit of α-ketoglutarate dehydrogenase, Complex I, Complex II, and Complex III (Andreyev et al., 2005). However, mitochondria also act as a ROS sink because they express peroxiredoxins, glutathione peroxidases and other enzymes that can detoxify ROS (Brown and Borutaite, 2012).

The rate of superoxide production by a given protein depends on three factors: 1) the concentration of O_2, 2) the concentration of the protein with its electron carrier in the reduced form, and 3) the second-order rate constant for the reaction between these factors (Murphy, 2009). Changes in oxygen concentration can occur as a result of external factors such as tissue oxygen concentration, but also internal factors such as the mitochondrial respiratory rate. The second-order rate constant for superoxide production by a given protein can be changed by post-translational modifications or conformational changes. However, the factor that is likely most important for determining the rate of superoxide production by a given protein is the proportion of that protein containing the reduced electron donor, which is in turn determined by the local redox state (*i.e.* the ratio of the oxidized and reduced forms of NAD, ubiquinone and other free electron carriers) (Murphy, 2009).

Complex I is thought to the most important mitochondrial source of ROS *in vivo* (Murphy, 2009). However, in healthy mitochondria Complex I produces very little ROS. Oxidative damage to Complex I itself, mitochondrial DNA encoding components of Compex I, and inhibitors of Complex I are well established triggers of mitochondrial ROS production (Ishikawa et al., 2008; St-Pierre et al., 2002; Verkaart et al., 2007). Defects in autophagic degradation of mitochondria (mitophagy) leads to an accumulation of dysfunctional mitochondria that produce excessive amounts of ROS. Autophagy-related protein 16-1 (ATG16L1) is a component of the autophagic machinery, and mutations in ATG16L1 have been Crohn's disease (Hampe et al., 2006). Macrophages deficient for ATG16L1 display elevated ROS levels and secrete IL-1β in response to LPS alone, suggesting that they have spontaneous inflammasome activation (Saitoh et al., 2008). A role for mitochondrial ROS in NLRP3 inflammasome activation was proposed in 2010 (Nakahira et al., 2011; Zhou et al., 2010b), after genetic evidence suggested the NADPH oxidases were not the source of NLRP3-activating ROS. Zhou *et al.* showed the the NLRP3 inflammasome is recruited to mitochondria and is activated by mitochondrial ROS that is produced in response to conventional NLRP3 activators or inhibitors of the respiratory chain such as rotenone (Complex I) and antimycin A (Complex III). Nakahira *et al.* followed up on the autophagy study described above by showing that macrophages deficient in other critical autophagy components (LC3B and Beclin-1) also produce more ROS and display enhanced IL-1β secretion in response to NLRP3 activators. Furthermore, they showed that NLRP3 activation in response to conventional activators or rotenone was impaired in ρ^0 macrophages, which are deficient for the mitochondrial genome (King and Attardi, 1989), and in macrophages treated with the mitochondrially targeted antioxidants (Nakahira et al., 2011). While there is still great interest in the role of

ROS in NLRP3 activation, there are many contradictory reports. For instance, many groups have reported failure to reproduce the finding that rotenone triggers inflammasome activation (Iyer et al., 2013; Juliana et al., 2012; Muñoz-Planillo et al., 2013; Youm et al., 2015). Other studies have suggested that ROS induced by LPS or rotenone promote priming, but are not sufficient to trigger NLRP3 activation (Bauernfeind et al., 2011; Juliana et al., 2012). Others suggest that ROS are dispensable for NLRP3 activation in response to some (Iyer et al., 2013) or all (Muñoz-Planillo et al., 2013) activators. Thus, the importance of ROS in NLRP3 activation is unresolved.

Imiquimod

Imiquimod (also known as R837 or S26308) is the most prominent member of a family of small nucleoside-analogues termed imidazoquinolines. It was discovered as an interferon-inducer with antiviral and anti-tumour activity. FDA approval for imiquimod was given in 1997 for the topical treatment of genital warts. Since 2004, it is also licensed for the local treatment of actinic keratosis and basal cell carcinoma. Today, imiquimod is available worldwide as a generic, and represents a standard therapy for the treatment of non-melanoma superficial skin cancers. It is also widely used as an inducer of T_H17-dependent psoriasis-like inflammation in mice (van der Fits et al., 2009).

It was not until 2002 that imiquimod and R848 (resiquimod) were determined to be ligands for TLR7 (Hemmi et al., 2002). Activation of TLR7 by imidazoquinolines or by its natural ligand single-stranded RNA (Diebold et al., 2004; Heil et al., 2004) triggers signalling via the MyD88 adaptor molecule. Both TLR7 and MyD88 are required for the production of type I IFNs and NF-κB-dependent pro-inflammatory cytokines (e.g. TNF, IL-6, proIL-1β) by these compounds. TLR7-dependent production of type I IFNs and pro-

inflammatory cytokines are important for the efficacy of imiquimod. For instance, in xenograft tumour models in mice, genetic deficiency for TLR7, MyD88, or IFNAR, or blocking antibodies against IFN-α/β reduced the inhibitory effect of imiquimod on tumour growth (Drobits et al., 2012; Sidky et al., 1992).

These studies and others lead to the assumption that the efficacy of imiquimod was due entirely to cytokine induction (Miller et al., 1999; Testerman et al., 1995; Tomai et al., 1995). However, it was also demonstrated that imiquimod can directly induce growth arrest or even apoptosis of tumour cells *in vitro* (Chen et al., 1988; Meyer et al., 2003; Schön et al., 2003). R848 is a more potent activator of TLR7 (Tomai et al., 1995), but lacks apoptotic activity (Schön et al., 2003). Imiquimod can also activate the NLRP3 inflammasome, for which its activation of TLR7 is dispensable (Kanneganti et al., 2006). The ability of imiquimod to trigger NLRP3 activation and direct growth arrest or apoptosis in tumour cells may contribute to its anti-tumour efficacy, but also to its adverse side effects, including itch, pain, and psoriasis-like inflammation. However, the mechanism by which imiquimod activates NLRP3 and triggers growth arrest or apoptosis are unknown.

Purpose of the study

Despite over a decade of research and overwhelming disease relevance, the mechanism of NLRP3 inflammasome activation remains unresolved. Determining the mechanism of NLRP3 activation would reveal potential targets for therapeutic intervention in the many auto-inflammatory diseases that have been linked to NLRP3 activation. It is presumed that K^+ efflux is a universal requirement for NLRP3 activation. Most NLRP3 activators such as the P2rX7 receptor agonist ATP, K^+ ionophores, whole pathogens, and inflammatory particles are known to trigger NLRP3 activation at least in part by their ability to induce K^+ efflux. The objective of this study was to investigate the mechanism of NLRP3 activation by the small molecule imiquimod, which is more amenable to molecular studies than other NLRP3 activators. Identification of imiquimod targets may also provide insight into how it induces apoptosis in tumour cells, which may be an important determinant of its anti-tumour efficacy. To this end, the contribution of endosomal TLRs, endolysosomal leakage, cell death, K^+ efflux, and mitochondrial pathways to NLRP3 activation by imiquimod were examined. Chemical proteomics and functional studies of mitochondria were used to determine novel molecular targets of imiquimod.

Materials and Methods

Reagents

If not stated otherwise, reagents were from the following suppliers. Buffers, salts, and detergents were from either Sigma-Aldrich or Carl Roth. Fine chemicals were from Sigma-Aldrich or Enzo. Tissue culture reagents including fetal calf serum (FCS) were from Thermo Scientific (formerly Invitrogen/Gibco). Tissue culture plasticware was from TPP or Sarstedt. Imidazoquinolines and all other TLR ligands were from Invivogen.

Mice

Nlrp3$^{-/-}$ (Martinon et al., 2006), *Pycard*$^{-/-}$ (Mariathasan et al., 2004), ICE$^{-/-}$ (*Casp1*$^{-/-}$/*11*$^{-/-}$) (Kuida et al., 1995), *Il1b*$^{-/-}$, *Il1a*$^{-/-}$ (Horai et al., 1998), and *Tlr7*$^{-/-}$ (Hemmi et al., 2002) mice on C57BL/6 were housed under SOPF or SPF conditions at the Zentrum für Präklinische Forschung (Munich, Germany), Institut für Medizinische Mikrobiologie, Immunologie und Hygiene (Munich, Germany), Centre for Infection and Immunology Lausanne (Switzerland), or Charles River Laboratories (Italy) in accordance with local and European guidelines. Colonies of inflammasome-deficient mice were maintained by interbreeding of heterozygous mice, and offspring were genotyped by PCR of DNA prepared from tail biopsies. *Unc93b1*$^{3d/3d}$ mice (Tabeta et al., 2006) were provided by Dr. Philipp Yu (Philipps-Universität Marburg). *NQO2*-/- mice on a C3H.129 background (Mailliet et al., 2004) along with age-and sex-matched C3H/HeOuJIco controls were housed at Charles River Facilities (Lyon, France), and were provided by Dr. Jean A. Boutin (Institut de Recherche Servier, France)

Mammalian Cell Culture

Cells were handled aseptically under a HEPA filter cell culture cabinet, and were cultured at 37°C/5% CO_2 in a humidified incubator, unless indicated otherwise. All centrifugation steps were at 400 *g* for 5 min at 4°C. Cells were enumerated using a hemocytometer and an inverted microscope. HEK293T human embryonic kidney cells (293T), HeLa human epithelial cells, 3T3 murine embryonic fibroblasts, NSC34 murine motorneuron-like cells, and normal human dermal fibroblasts (NHDF, provided by Prof. Holger Prokisch) were maintained in DMEM (25 mM glucose, 1 mM pyruvate, 4 mM glutamine) supplemented with 10% FCS and 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. HaCaT human keratinocytes were maintained in RPMI-1640 (11.1mM glucose, with GlutaMAX) supplemented with 10% FCS, and 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. To passage adherent cells, the cells were washed with phosphate-buffered saline (PBS) and treated for 5-10 min at 37°C with Trypsin and 0.05% EDTA. Dislodged cells were mixed with FCS-containing growth medium, centrifuged and resuspended in culture medium. Cells were passaged every 2-3 days as needed.

Isolation and Cultivation of Human Primary Keratinocytes

Human primary keratinocytes (hPK) were isolated from the roof of suction blisters raised on normal skin of the forearms of healthy donors. Single-cell suspensions were prepared by trypsinization of the blister roof and seeded on a feeder layer of 3T3/J2 fibroblasts treated with 10 µg ml^{-1} mitomycin C for 2 h. First-passage keratinocytes were cryopreserved in liquid nitrogen. hPK were grown in DermaLife keratinocyte growth medium (LifeLine CellTech) to 80-90% confluence, and were provided by Daniela Dittlein.

Generation of Murine BMDMs and BMDCs

Bone marrow from age- and sex- matched mice was obtained by flushing femurs and tibias with cold RPMI with 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin using a 26-gauge needle and syringe. Red blood cell (RBC) lysis was performed on single-cell suspensions of bone marrow by incubation with G-DEX II RBC Lysis Buffer (Intron Biotechnology) on ice for 5 min. RBC-depleted bone marrow was either frozen gradually to -80°C in FCS with 10% DMSO using a Mr. Frosty (Nalgene), or taken into culture immediately as previously described in detail (Schneider et al., 2013). To generate bone marrow-derived macrophages (BMDMs), bone marrow (10 x 10^6 cells in non-tissue culture treated Petri dishes) was cultured in DMEM containing 10% FCS, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 20 ng ml^{-1} recombinant murine macrophage colony stimulating factor (rmM-CSF, Immunotools) for 6-8 days. 10 mL of additional medium was given on day 2. Old medium and non-adherent cells were discarded on day 4 and replaced with 10 mL fresh medium on day 4 and every second day thereafter. To generate bone marrow-derived dendritic cells (BMDCs), bone marrow (5 x 10^6 cells in non-tissue culture treated Petri dishes) was cultured in RPMI containing 10% FCS, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, and 20 ng ml^{-1} recombinant murine granulocyte-macrophage colony stimulating factor (rmGM-CSF, Immunotools) for 6-8 days. 10 mL of additional medium was given on day 2. A complete medium change of 20 mL was performed on day 4 or day 5 and every 2 days thereafter.

Stimulation of BMDCs and BMDMs

After 6-8 days of differentiation adherent BMDMs and all cells from BMDCs cultures were lifted using Hank's balanced salt solution (HBSS) containing 0.05% EDTA,

and then plated in 96-well plates at a density of 0.12-0.15x10^6 cells per well. Before addition of inflammasome activators, cells were primed with 20 ng ml^{-1} *E. coli* K12 ultra-pure LPS for 3 h and treated with inflammasome activators for 0.5-6 h. All stimulations were performed in triplicate. Typical stimulus concentrations for TLR ligands used for priming or TNF production (2.5-4 h stimulation) were: 20-50 ng ml^{-1} LPS, 2 µg ml^{-1} R848, 10 µg ml^{-1} CpG DNA, 5 µg ml^{-1} PGN, 2 µg ml^{-1} Pam_3CSK_4. Typical inflammasome activator concentrations and times were as follows: 5 mM ATP 30 min, 5 µM nigericin 30-45 min, MOI 5 *Candida albicans* SC5314 4 h, 300 µg ml^{-1} MSU/alum/silica for 4 h, 2 µg ml^{-1} poly(dA:dT) 2-3 h (transfected with Lipofectamine 2000, Invitrogen), MOI 20 *Salmonella enterica* serovar Typhimurium *ΔaroA* (X3625) 1-2 h. Imidazoquinolines were used at 15-25 µg ml^{-1} or 70 µM for 1.5-2.5 h for inflammasome activation, though inflammasome activation can be detected as early as 10 min after stimulation with these compounds. Inhibitors were added after 2.5-3 h of priming, and 20-30 min before stimulation with inflammasome activators. Inhibitor concentrations were as follows, unless indicated otherwise: 20 µM zVAD-fmk (Enzo), 20 µM Ac-YVAD-cmk (Enzo), 20 µM CA074Me (Enzo), 20 µM PR-619, 30 µM ebselen (Enzo), 40 µM ammonium pyrrolidinedithiocarbamate (APDC), 5 µM MCC950. To minimize off-target effects of extracellular KCl, it was added and mixed well by careful pipetting immediately before addition of inflammasome activators. All inflammasome activators were carefully titrated and used at the lowest dose and the shortest time required to cause significant IL-1 secretion. Inhibitors were also titrated, and the lowest effective dose was used. MCC950 was provided by Prof. Matthew A. Cooper (University of Queensland) (Coll et al., 2015). MSU crystals were prepared as previously described (Martinon et al., 2006). Imject alum was from Pierce.

Cytokine Quantification

For cytokine quantification of cell-free supernatants, enzyme-linked immunosorbent assay (ELISA) kits for murine IL-1α, IL-1β, and TNF (Ready-Set-Go from eBioscience) were used according to manufacturer's instructions, except that half of the recommended volume was used. ELISA data is depicted as mean ± SEM of technical triplicates. Supernatants were diluted 1/3 -1/6 (all the same dilution factor within a single experiment) when it was expected that the highest value would be beyond the detection range of the kit (5 ng ml^{-1}). Absorbance at 450 nm was measured using a Tecan Sunrise instrument, and concentrations were interpolated from the standard curve using the accompanying Magellan software.

Determination of Cell Death

Cell death was quantified using trypan blue staining or by the CytoTox LDH assay kit from Promega, according to manufacturers instructions. 100% lytic cell death was determined in the LDH assay by lysing the cells by repeated freeze-thaw cycles.

Potassium Measurement

Intracellular K^+ measurements were performed using an ion-selective electrode (ISE) (Cobas analyzer, Roche). Cells were dislodged and 10^7 cells per condition were stimulated in suspension. After incubation with inflammasome activators, cells were immediately transferred to ice and pelleted by centrifugation at 400 *g* for 5 min at 4°C in 15 ml conical tubes. Medium was carefully and completely removed and subjected to IL-1β measurement by ELISA. The cell pellet was suspended in 150 µl of ultrapure water and cellular contend was released by repeated freeze-thaw cycles in liquid nitrogen. Debris

was pelleted by centrifugation at 14000 *g* for 10 min at 4°C and 100 µl of the clarified lysate was subjected to K^+ measurement.

Immunoblotting

Cell-free supernatant and cell lysates prepared in sodium dodecyl sulfate (SDS)- and dithiothreitol (DTT)-containing sample buffer, and triplicate samples were pooled. To analyse inflammasome formation, the NP-40-insoluble fraction of cells was left untreated or cross-linked with disuccinimidyl suberate (Thermo Scientific) before immunoblot analysis, as previously described (Fernandes-Alnemri et al., 2007). Samples were loaded onto SDS-polyacrylamide gels (prepared by Susanne Weiss, acrylamide content 8-15%) and proteins separated by application of 100-150 V for 1-2 h. Proteins were transferred from gels to nitrocellulose membranes (GE Healthcare) by application of 110 V for 1.5 h as previously described in detail (Gross, 2012). Membranes were rinsed in PBS-Tween (PBST) and protein loading checked by staining for 5 min in Ponceau S stain. After blocking the membranes in PBST + 5% skim milk powder for 1 h at room temperature, primary antibody was applied (diluted in PBST + 2.5% skim milk, 0.05% sodium azide) overnight at 4°C. The membranes were washed for at least 3 X 10 min in PBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted in PBST + 2% skim milk) for 0.5 – 1.5 h at room temperature. The membranes were then washed at least fives times in PBST for a total time of 45 min – 2 h. Enhanced chemiluminescence substrate (Western Lightning Plus from PerkinElmer or ECL Ultra from Lumigen) was applied to the membranes before they were imaged using a cooled charge-coupled device (CCD) camera (ChemoCam from Intas). Depending on the signal intensity, exposures of 3-20 min were taken such that the detection limit of the camera

(64k grayscale) was not exceeded. Images were inverted, so that the signal was black instead of white. To visualize fainter bands, black was set to progressively lower grayscales. White was always set to the 0 on the grayscale, so that all background signal was observed in the final image and that there were no changes to image contrast.

Primary antibodies were as follows: goat anti-mouse IL-1β (AF-401, R&D Systems), mouse anti-mouse caspase-1 p20 (Casper-1, Adipogen), hamster anti-mouse IL-1α (ALF-161, eBioscience), mouse anti-mouse NLRP3 (Cryo-2, Adipogen), rabbit anti-ASC (AL177, Adipogen), rabbit anti-Vimentin (5741 Cell Signaling), and rabbit anti-caspase-3 (9662 Cell Signaling).

Flow Cytometric Analysis of Endolysosomal Leakage

ASC-deficient BMDMs were incubated for 30 min with acridine orange (1 μg ml^{-1}), washed three times in phenol red-free HBSS with 5mM EDTA and 3% FCS. After a constant baseline was obtained by flow cytometry, the stimulation of cells with imiquimod, R848, CL097 and gardiquimod (100 μM) was followed as a time course for 1 h at 37°C. Maximum endolysosomal rupture was confirmed by addition of LLOMe (1 μM) in the last 10 min. Endolysosomal leakage was assessed by a ratiometric measurement of the change in distribution of the dye in the acidic endolysosomal compartment (red, on PerCP channel) and the cytoplasmic/nuclear compartment (green, on FITC channel). A FACS Aria III (BD Biosciences) flow cytometrer was used. Data were acquired with DIVA (BD Biosciences) and were analysed with FlowJo software.

Metabolic Analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was measured using XF96 Extracellular Flux Analyzer (Seahorse Bioscience). BMDMs or BMDCs (7-8x10^4/well in quadruplicates) were seeded the evening before the experiment in 96-well plates. The morning of the experiment the cells were primed with 50 ng ml^{-1} LPS for 2-3 h before the medium was changed to bicarbonate- and phenol red-free DMEM 5030 (Sigma) containing 20 ng ml^{-1} recombinant murine M-CSF or GM-CSF, 10 mM glucose, and 2 mM glutamine. The cells were then incubated for at least 1 h at 37°C in a non-CO_2 incubator. To avoid respiratory chain inhibition that occurs after 8 h of stimulation with TLR ligands, the time between LPS addition and the beginning of the experiment did not exceed 4 h (Everts et al., 2012). For analysis of intact cells, mix-wait-measure times 1 min – 2 min – 3 min were used, and stimuli were injected via ports. Imidazoquinolines were used at 70 μM (=20 μg ml^{-1} for imiquimod) unless indicated otherwise. Respiratory chain inhibitors were carefully titrated in initial experiments and then used at the following concentrations in subsequent experiments: 0.5 μM carbonyl cyanide *m*-chlorophenyl hydrazine CCCP, 3.5 μM oligomycin A, 2 μM antimycin A. The protocol for OCR measurements of HEK293T, 3T3, HeLa, NSC34, HaCaT, human primary keratinocytes, and NHDF fibroblast was the same except assay medium did not contain growth factors and cells were not LPS-treated. Cell lines were cultivated in DMEM with 10% FCS, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin, 2 mM glutamine, and 10 mM glucose using standard protocols.

To analyse the activity of individual respiratory chain complexes, BMDMs were permeabilized for 5 min in MAS buffer (220 mM mannitol, 70 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA, 0.4% fatty acid-free BSA pH 7.2)

containing 40 μM digitonin. Digitonin-containing medium was removed and replaced with MAS buffer containing 2 mM ADP along with combinations of TCA cycle intermediates and electron donors at the following concentrations: 10 mM succinate, 2 mM malate, 5 mM pyruvate, 5 mM glutamate, 2 μM rotenone, 0.5 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), 2 mM ascorbate (Salabei et al., 2014). Stimuli were added directly to the assay medium before measurement began. For analysis of permeabilized cells, mix-wait-measure times of 1 min – 10 s – 2.5 min were used, without initial equilibration. Intracellular ATP was quantified using a luciferase-based assay (CellTiterGlo Assay from Promega) and a Mithras microplate reader according to the manufacturers instructions. NAD^+/NADH ratios were measured by enzymatic cycling using a colorimeter NAD^+/NADH Quantification kit (Biovision).

Statistics

For technical replicates mean and standard error of the mean were calculated. For biological replicates, mean and standard deviation were calculated. These descriptive statistics were calculated using Excel (Microsoft). Data were analysed by a Student's t-test in Prism 6 (GraphPad). $p<0.05$ was considered significant. The results from all experiments were verified on at least two or three separate occasions.

Results

Imiquimod and CL097 activate the NLRP3 inflammasome

The TLR7 agonist imiquimod is known to activate the NLRP3 inflammasome (Kanneganti et al., 2006). In generating data for a previous study (Gross et al., 2012), it was confirmed that deficiency in NLRP3, ASC (*Pycard*$^{-/-}$), or caspase-1/11 (ICE$^{-/-}$) prevented IL-1β cleavage and secretion in response to imiquimod (Appendix 1A). To determine whether this activity is unique to imiquimod or shared by other TLR agonists, bone marrow-derived dendritic cells (BMDCs) were primed with LPS for 3 h and then treated with agonists of various TLRs, and with control inflammasome activators. In these previous experiments, all TLR agonists triggered TNF secretion from unprimed cells, but only imiquimod caused cleavage and secretion of IL-1β, IL-1α and caspase-1 in LPS-primed cells (Appendix 1B-1D). As previously shown, the TLR7 agonist R848 is a more potent inducer of NF-κB-dependent cytokine production than imiquimod (Appendix 1C) (Hemmi et al., 2002; Tomai et al., 1995). However, R848 from various commercial sources did not activate the inflammasome in LPS-primed BMDCs or bone marrow-derived macrophages (BMDMs) (Figures 3A-3C; Appendix 1B-1D), which is in contrast to a previous publication (Kanneganti et al., 2006). TLR7 binds imidazoquinolines and ssRNA (Heil et al., 2004; Hemmi et al., 2002). Other ligands of TLR7/8 were tested for their ability to trigger IL-1β secretion, but CL097 was the only additional inflammasome activator identified (Figure 3B, 3C). This suggests that TLR7 activation is insufficient for inflammasome activation by imidazoquinolines.

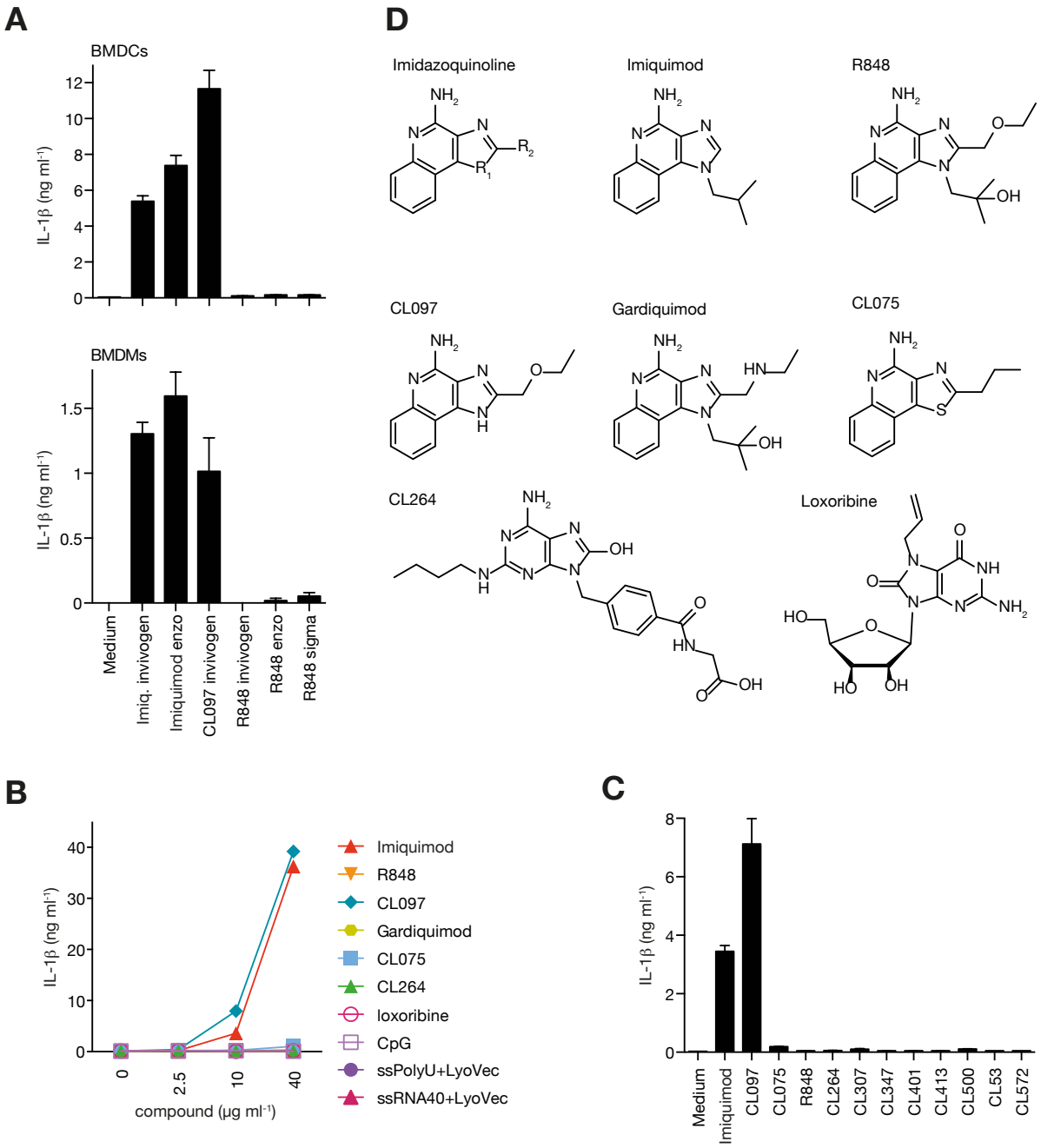


Figure 3: Imiquimod and CL097 are unique among imidazoquinolines and other TLR7 ligands in their capacity to induce induce IL-1β secretion.

(A) LPS-primed wild-type BMDCs (top) or BMDMs (bottom) were stimulated with 15 µg ml^{-1} imiquimod, CL097, or R848 from different commercial sources. IL-1β release was quantified from cell-free supernatants by ELISA.

(B, C) LPS-primed BMDCs were treated with increasing concentrations of imidazoquinoline or ssRNA ligands of TLR7 (B) or 16 µg ml^{-1} of the indicated TLR7 ligands from Invivogen (C). IL-1β secretion was quantified from cell-free supernatants by ELISA.

(D) Structures of selected imidazoquinolines and other TLR7/8 ligands used in this study.

ELISA data are depicted as mean ± SEM of technical triplicates.

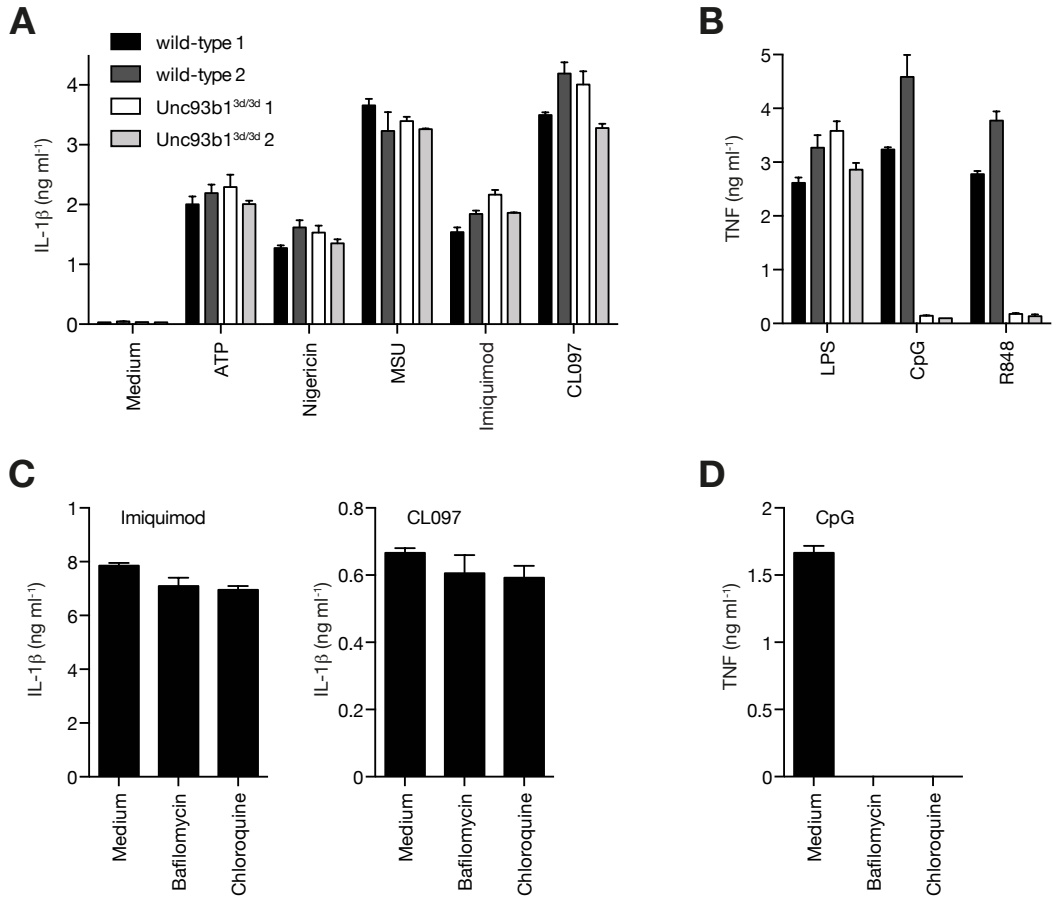


Figure 4: NLRP3 activation by imiquimod and CL097 does not require Unc93b1 endolysosomal acidification

(A, B) BMDCs from wild-type and Unc93b1$^{3d/3d}$ mice were primed with LPS (A) or left unprimed (B) and stimulated as indicated. Concentrations of and duration of stimulation with inflammasome activators is provided in the Materials and Methods. IL-1β (A) and TNF (B) were measured from cell-free supernatants by ELISA.

(C, D) BMDCs that were LPS-primed (C) or left unprimed (D) were treated for 30 min with bafilomycin A1 or chloroquine, and subsequently stimulated with imiquimod or CL097 (C) or CpG DNA (D). IL-1β (C) and TNF (D) secretion was quantified from cell-free supernatants by ELISA.

Data are depicted as mean ± SEM of technical triplicates.

Endosomal TLRs and acidification are dispensable for NLRP3 activation by imiquimod and CL097

It was previously demonstrated that TLR7 is dispensable for imiquimod-induced NLRP3 activation (Kanneganti et al., 2006). However, a recent report showed that NLRP3 inflammasome activation in response to endosomal bacterial RNA was dependent on

Unc93b1, but not TLRs 3, 7, or 9 (Eigenbrod et al., 2012). Unc93b1 is required for signalling of TLRs 3, 7, 8, 9, and 13 because it regulates the trafficking of these TLRs and other proteins from the ER to the endolysosome (Kim et al., 2008; Tabeta et al., 2006). Imidazoquinolines are known to accumulate in the endosome (Russo et al., 2011) and to signal via TLR7/8, but it is possible that other Unc93b1-dependent endosomal receptors for these compounds may exist. However, BMDCs from mice carrying an inactivating mutation in Unc93b1 (Tabeta et al., 2006) secreted normal amounts of IL-1β secretion when treated with imiquimod, CL097, ATP or nigericin (Figure 4A), but as expected failed to secrete TNF in response to CpG DNA or R848 (Figure 4B). Endolysosomal acidification is required for TLR signalling in this compartment, and can be blocked by bafilomycin A1 or chloroquine (Tabeta et al., 2006). Inhibition of endolysosomal acidification did not affect IL-1β secretion in response to imiquimod, CL097, or other NLRP3 activators (Figure 4C), but inhibited CpG DNA-induced TNF secretion as expected (Figure 4D). Thus, imiquimod-induced inflammasome activation does not require endolysosomal acidification, TLR7, or any other Unc93b1-dependent endolysosomal TLR.

P2rX7 is dispensable for NLRP3 activation by imiquimod and CL097

As adenine analogs, it is conceivable that imiquimod and CL097 could activate the P2rx7 purinoreceptor, which is required for NLRP3 activation by extracellular ATP (Solle et al., 2001). However, while P2rx7 deficiency prevented ATP-dependent IL-1β secretion from LPS-primed cells, it did not affect IL-1β secretion induced by imiquimod or CL097 (Figure 5). In contrast to these results, it was reported that P2rX7-deficient mast cells have a defect in imiquimod-induced IL-1β secretion (Nakamura et al., 2009). Differences in cell type may account for these divergent responses. For instance, it is possible that

the amount of ATP released after imiquimod-induced pyroptotic cell death may be greater in mast cells as compared to BMDCs.

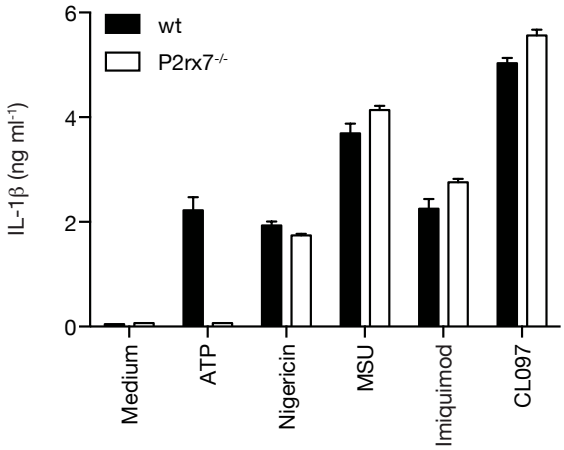


Figure 5: P2rX7 is dispensable for NLRP3 activation by imiquimod and CL097

LPS-primed BMDCs from wild-type or P2rx7-deficient mice were left untreated or stimulated as indicated. IL-1β was quantified from cell-free supernatants by ELISA. Data are depicted as mean ± SEM of technical triplicates.

Apoptosis and necroptosis are dispensable for NLRP3 activation by imiquimod and CL097

Imiquimod can trigger growth arrest and apoptosis in non-myeloid cells (Schön et al., 2003; Zagon et al., 2008). In myeloid cells, NLRP3 activation by certain triggers may involve apoptotic or necroptotic cell death pathways (Shimada et al., 2012; Vince et al., 2012). Therefore, it was important to examine whether cell death induction may contribute to imiquimod-induced NLRP3 activation. However, imiquimod induces negligible IL-1α and LDH release in NLRP3-deficient cells and no caspase-3 activation in myeloid cells treated with imiquimod (Figure 6A, 6B; Appendix 1A) (Allam et al., 2014). Thus, imiquimod-induced myeloid cell death is inflammasome-dependent. RIP1 and RIP3 are critical mediators of necroptosis, and inhibition of RIP1 kinase activity by necrostatin-1 blocks necroptosis (Cho et al., 2009; Degterev et al., 2008; Holler et al., 2000). IL-1β secretion in response imiquimod and other inflammasome activators was intact in

BMDCs treated with necrostatin-1, suggesting that necroptotic pathways are dispensable for NLRP3 activation (Figure 6C). Furthermore, RIP3-deficient and Bax/Bak-deficient cells show no defect in imiquimod-induced inflammasome activation, indicating that neither necroptosis nor mitochondrial Bax/Bak-dependent apoptosis contribute to NLRP3 activation by imiquimod (Allam et al., 2014; Vince et al., 2012).

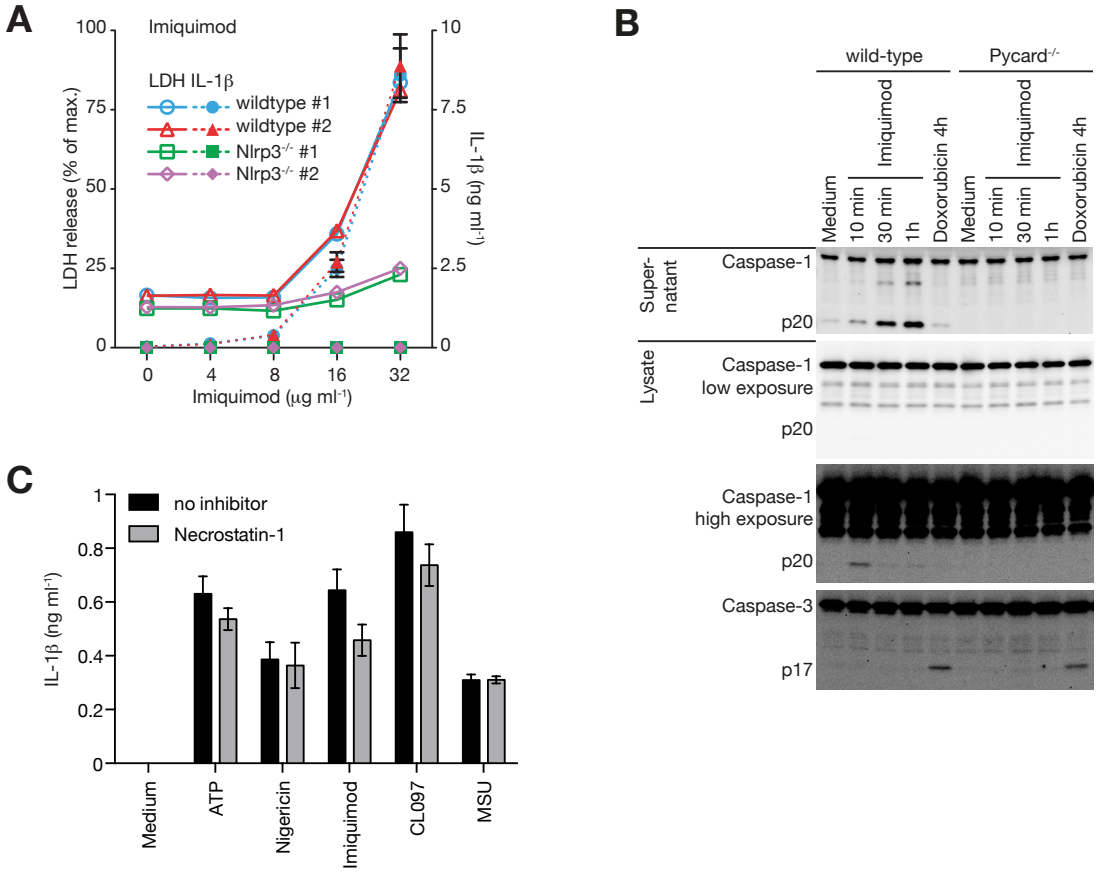


Figure 6: Imiquimod-induced NLRP3 activation results in pyroptosis, but not caspase-3 cleavage, and does not require RIP1 kinase activity.

(A) LPS-primed BMDCs from two wild-type and two NLRP3-deficient mice were treated with increasing amounts of imiquimod for 2 h. IL-1β (right Y axis, dotted lines) and LDH (left Y axis, solid lines) release were quantified from cell-free supernatants by ELISA and a colorimetric assay, respectively.

(B) LPS-primed BMDCs were left untreated or pretreated the RIP1 inhibitor necrostatin-1 (50 μM) and subsequently stimulated with different NLRP3 activators. IL-1β secretion was quantified from cell-free supernatants by ELISA. ELISA and LDH data are depicted as mean ± SEM of technical triplicates.

(C) LPS-primed BMDCs from wild-type and ASC-deficient (*Pycard*$^{-/-}$) mice were treated with imiquimod or doxorubicin (1 μM, positive control for apoptosis) for the indicated times. Caspase-1 release and cleavage from cell-free supernatants (top) and caspase-1 and caspase-3 cleavage in cell lysates were determined by immunoblotting.

Cathepsin B/L activity contributes to NLRP3 activation by imiquimod and CL097

Disruption of the endolysosomal compartment and subsequent leakage of cathepsins into the cytoplasm has been implicated in NLRP3 activation by particles, insoluble protein aggregates and crystals such as alum, silica, amyloid β, cholesterol crystals, and MSU (Halle et al., 2008; Hornung et al., 2008). Imidazoquinolines and other endosomal TLR ligands strongly accumulate in the endolysosome (Russo et al., 2011). These compounds may trigger NLRP3 activation by compromising the integrity of the endolysosome and leading to cathepsin release into the cytoplasm. In support of this hypothesis, the cathepsin B/L inhibitor CA074Me inhibited IL-1β secretion in response to not only MSU, but also imiquimod and CL097 (Figure 7A). MSU crystals require phagocytosis in order to disrupt the endolysosome and activate NLRP3 (Martinon et al., 2006). In contrast, imiquimod-induced IL-1β secretion was not inhibited by cytochalasin D, demonstrating that this small molecule does not rely on phagocytic uptake for NLRP3 activation (Figure 7A). However, neither the cathepsin B inhibitor II nor the pan-cathepsin inhibitor E64D inhibited imiquimod- or MSU-induced IL-1β secretion (Figure 7A). This is in line with previous genetic evidence that cathepsin B is not required for NLRP3 activation by MSU or imiquimod (Dostert et al., 2009). Cathepsin and phagocytosis inhibitors did not impair IL-1β secretion in response to NLRP3 activators like ATP and nigericin that do not trigger the endolysosomal leakage. LPS-induced TNF secretion was measured as a control for potential off-target effects of the inhibitors, but TNF secretion was intact in cells pretreated with these inhibitors (Figure 7B). Given the potent suppressive effect of CA074Me on imiquimod- and CL097-induced inflammasome activation, it was surprising to observe only a partial reduction in IL-1β secretion in cathepsin B/L double knockout cells (Figure 7C). Furthermore, the inhibitory effect of the cathepsin B/L inhibitor

CA074Me was intact in the double knockout cells, demonstrating that the inhibitor has effects on other proteases or cellular processes. Recent reports have suggested a high level of functional redundancy between endolysosomal cathepsins for the activation of NLRP3 by particles and crystals (Orlowski et al., 2015). Therefore, imiquimod-induced NLRP3 activation may involve multiple redundant cathepsins, and perhaps additional non-cathepsin targets of CA074Me.

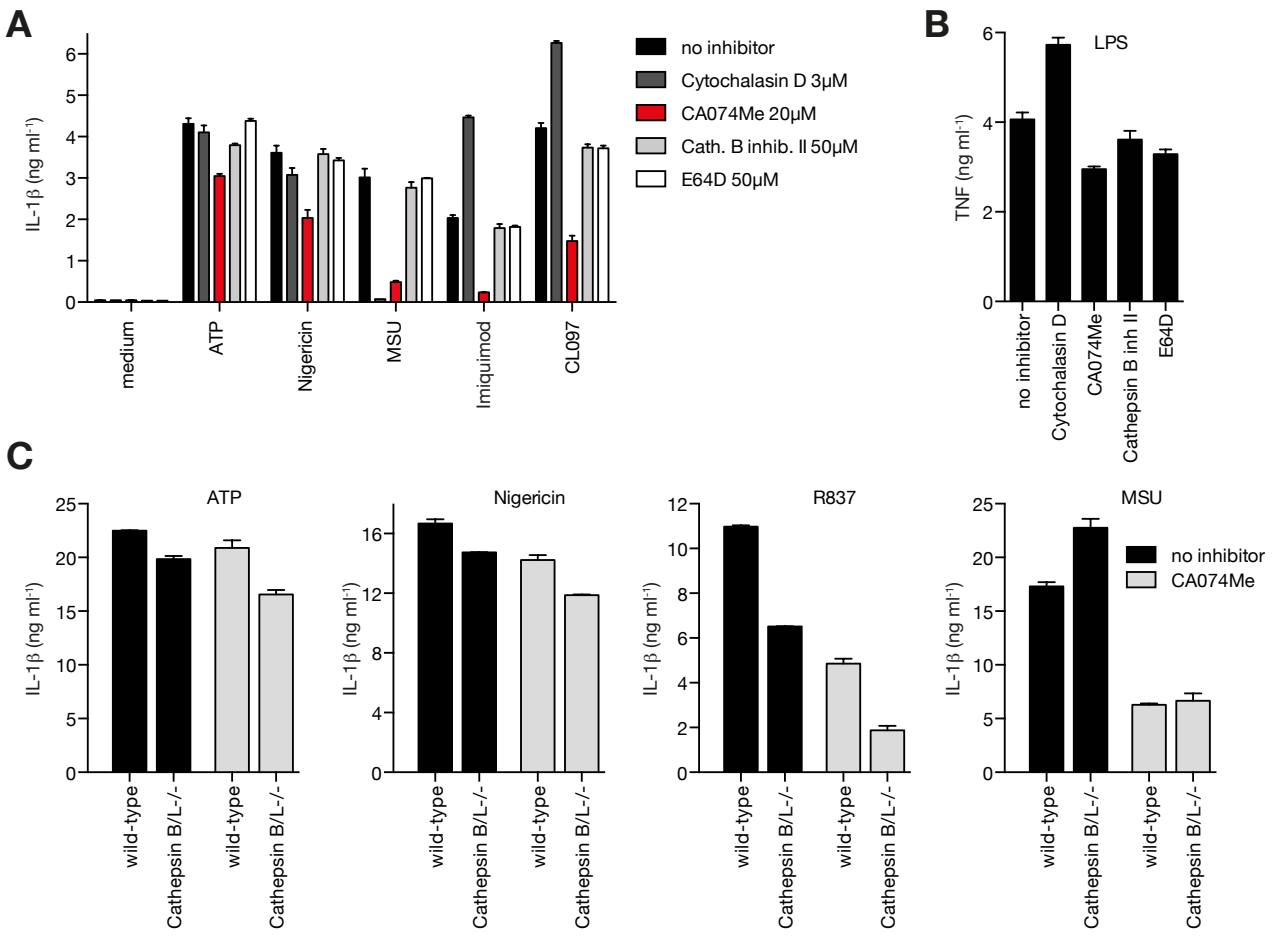


Figure 7: Cathepsin B/L activity contributes to NLRP3 activation by imiquimod and CL097

(A) LPS-primed wild-type BMDCs were treated with the inhibitors 30 min prior to addition of the indicated inflammasome activators. IL-1β release was quantified from cell-free supernatants by ELISA.

(B) Unprimed wild-type BMDCs were treated with various inhibitors 30 min prior to addition of 20 ng ml^{-1} LPS. TNF secretion was quantified from cell-free supernatants by ELISA.

(C) LPS-primed wild-type or cathepsin B and cathepsin L double-knockout BMDCs were left untreated or treated with the cathepsin inhibitor CA074Me 30 min prior to addition of the indicated inflammasome activators. IL-1β release was quantified from cell-free supernatants by ELISA.

ELISA data are depicted as mean ± SEM of technical triplicates.

Imidazoquinolines trigger endolysosomal leakage

To determine whether imiquimod or CL097 induce endolysosomal leakage, acridine orange staining was performed as previously described for NLRP3-activating particles (Hornung et al., 2008). Acridine orange accumulates in the acidic endolysosomal compartment, where it fluoresces red; cytoplasmic/nuclear acridine orange fluoresces green. The rupture of lysosomes leads to a redistribution of the dye, resulting in reduction in red fluorescence and increase in green fluorescence. Since inflammasome-dependent cell death pathways disrupt endolysosomal integrity (Fujisawa et al., 2007; Heid et al., 2013), ASC-deficient cells were used to determine whether imidazoquinolines trigger endolysosomal leakage upstream of NLRP3. Imiquimod, CL097 and gardiquimod, but not R848, caused endolysosomal leakage, as indicated by the loss of red fluorescence (Appendix 1E). The finding that gardiquimod triggers endolysosomal leakage, but not NLRP3 activation, suggests that endolysosomal leakage of cathepsins or other molecules is alone insufficient for NLRP3 activation by these compounds.

Extracellular calcium activates NLRP3 by forming a calcium phosphate precipitate

Some studies have suggested a role for calcium in NLRP3 activation (Lee et al., 2012a; Rimessi et al., 2015). During a previous study, the involvement of calcium signalling in NLRP3 activation was investigated by using the cell permeable calcium chelator BAPTA-AM (Gross et al., 2012). At concentrations of BAPTA-AM that were effective in inhibiting IL-1β secretion, impaired viability and secretion of non-inflammasome cytokines such as TNF was observed. Furthermore, the calcium ionophore ionomycin caused IL-1α release, but did not activate the NLRP3 inflammasome or trigger IL-1β secretion (Gross et al., 2012). Extracellular calcium (1 mM)

was reported in a recent *Nature* paper to trigger NLRP3 activation via the calcium sensing receptor (CASR) (Lee et al., 2012a). CASR activation leads to phospholipase C activation and subsequent inositol triphosphate-dependent release of ER calcium stores into the cytoplasm. CASR also inhibits adenylate cyclase activity, leading to a decrease in cytoplasmic cyclic AMP. Increased cytoplasmic calcium and decreased cyclic AMP concentrations both were proposed to lead to NLRP3 activation downstream of CASR. Therefore, it was important to reinvestigate the role of calcium in NLRP3 activation by repeating some of the critical experiments in this paper. In this paper, cells were stimulated with 1 mM $CaCl_2$ in RPMI without serum (Lee et al., 2012a). While IL-1β secretion was observed using these stimulation conditions, it also caused a fine precipitate to form on the cells (Figure 8A, 8B). Furthermore, we did not observe IL-1β secretion when the cells were stimulated with 1 mM calcium in serum-free DMEM or OptiMEM, which contain less phosphate than RPMI medium. This indicates that the calcium-induced precipitate rather than the soluble calcium ions were responsible for NLRP3 activation. Dr. Dominik Höhne (Lehrstuhl für Anorganische Chemie, TUM) analysed the amount of calcium and phosphorus in a dried sample of the precipitate that formed under these conditions. By weight, it was composed of 33.0% calcium and 17.8% phosphorous, giving a Ca:P ratio of 1.85:1. Pure $Ca_3(PO_4)_2$ is 38.7% calcium, 20.0% phosphorous 41.3% oxygen by weight, and has a Ca:P ratio of 1.94:1. Therefore, the precipitate formed in $CaCl_2$-supplemented RPMI is most likely a species of calcium phosphate. While the elemental analysis is consistent with $Ca_3(PO_4)_2$, it cannot be ruled out that the precipitate is a mixture of different calcium phosphate species. Others have also suggested that inflammasome activation in RPMI+$CaCl_2$ is a result of precipitate formation (Muñoz-Planillo et al., 2013). Pseudogout-associated calcium pyrophosphate

crystals were one of the first NLRP3 inflammasome activators described (Martinon et al., 2006), and other species of calcium phosphate also activate NLRP3 (Pazár et al., 2011). Our results demonstrate that neither ionomycin-induced increases in intracellular calcium (Gross et al., 2012), nor extracellular calcium ions trigger IL-1β secretion.

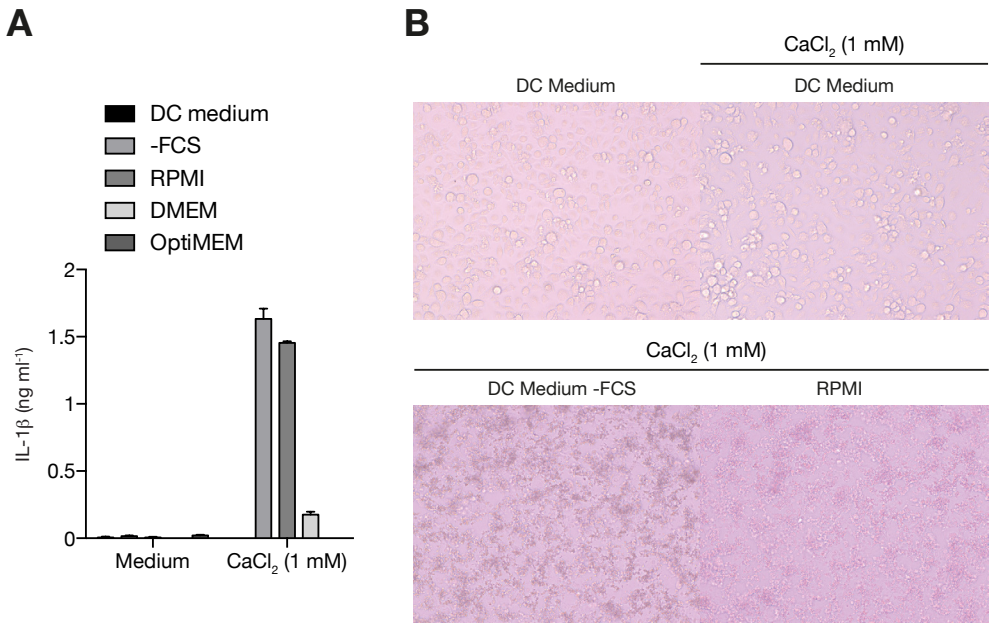


Figure 8: Only in serum-free but not serum-containing RPMI or serum-free DMEM or OptiMEM does $CaCl_2$ induce IL-1β secretion.

(A) Wild-type BMDCs were LPS-primed in DC Medium for 3 h, washed once with PBS and cultivated in different media as indicated. $CaCl_2$ (1 mM) was added, or cells were left untreated and cultivated for 3 h. IL-1β release was quantified from cell-free supernatants by ELISA. Data are depicted as mean ± SEM of technical triplicates.

(B) Images of the cells in A.

Deubiquitination is necessary for NLRP3 activation by imiquimod and CL097

Ubiquitination of NLRP3 and caspase-1 has been proposed to negatively regulate activation of the NLRP3 inflammasome (Juliana et al., 2012; Lopez-Castejon et al., 2013; Py et al., 2013). BRCC3 was recently proposed to be the deubiquitinase regulating NLRP3 activity (Py et al., 2013). Deubiquitinase inhibitors suppress NLRP3 activation, suggesting that ubiquitination of NLRP3 suppresses its activity and the deubiquitination may license NLRP3 activation. In accordance with what has been published for other NLRP3 activators, imiquimod-induced IL-1β secretion was inhibited by the deubiquitinase inhibitor PR-619 (Figure 9A). As expected, the caspase-1 inhibitor Ac-YVAD-cmk suppressed secretion of IL-1β (Figure 9A). LPS-induced TNF secretion was intact in cells treated with PR-619 and Ac-YVAD-cmk (Figure 9B).

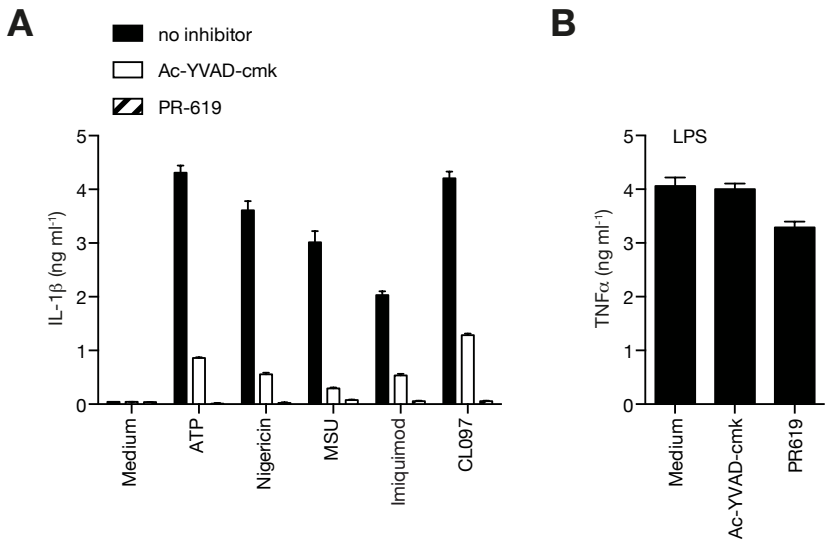


Figure 9: Imiquimod and CL097-induced inflammasome activation is sensitive to inhibition of deubiquitinating enzymes and of caspase-1.

(A, B) LPS-primed (A) or unprimed (B) BMDCs were treated for 30 min with 10 μM Ac-YVAD-cmk or 30 μM PR-619, and then stimulated with NLRP3 inflammasome activators (A) or LPS (B). IL-1β and TNF secretion was quantified from cell-free supernatants by ELISA.

Data are depicted as mean ± SEM of technical triplicates.

MAVS and microtubule polymerization are dispensable for NLRP3 activation

MAVS is an innate immune adaptor protein localized to the mitochondrial outer membrane. A role for mitochondria in NLRP3 inflammasome activation as been proposed (Zhou et al., 2010b). Mitochondria-derived factors such as ROS (Zhou et al., 2010b), calcium (Rimessi et al., 2015), mtDNA (Shimada et al., 2012), and cardiolipin (Iyer et al., 2013) may be involved in NLRP3 activation. NLRP3 would be best poised to sense these factors if it was localized to the mitochondria. Indeed, NLRP3 does localize to mitochondria and ER-mitochondria contact sites (Zhou et al., 2010b). Recently, it was suggested that MAVS is required for efficient NLRP3 inflammasome activation, suggesting that MAVS may serve as a mitochondrial docking site for NLRP3 (Subramanian et al., 2013). Consistent with other reports, wild-type and MAVS-deficient BMDCs secreted similar amounts of IL-1β (Figure 10A), suggesting MAVS is not universally required for NLRP3 activation (Allam et al., 2014; Muñoz-Planillo et al., 2013; Park et al., 2013).

The results of a compound library screen for inhibitors of nigericin-induced IL-1β secretion suggested that microtubule-dependent relocalization of NLRP3 to mitochondria is necessary for efficient NLRP3 activation (Misawa et al., 2013). The inhibitors used in this study, namely colchicine, podophyllotoxin and nocodazole, were tested for their ability to inhibit IL-1β secretion by activators of the NLRP3 and AIM2 inflammasomes (Figure 10B). While microtubule inhibitors did slightly reduce IL-1β secretion in response to nigericin, TNF secretion was inhibited to a comparable or greater extent, suggesting the effect of these inhibitors on NLRP3 activation is not specific.

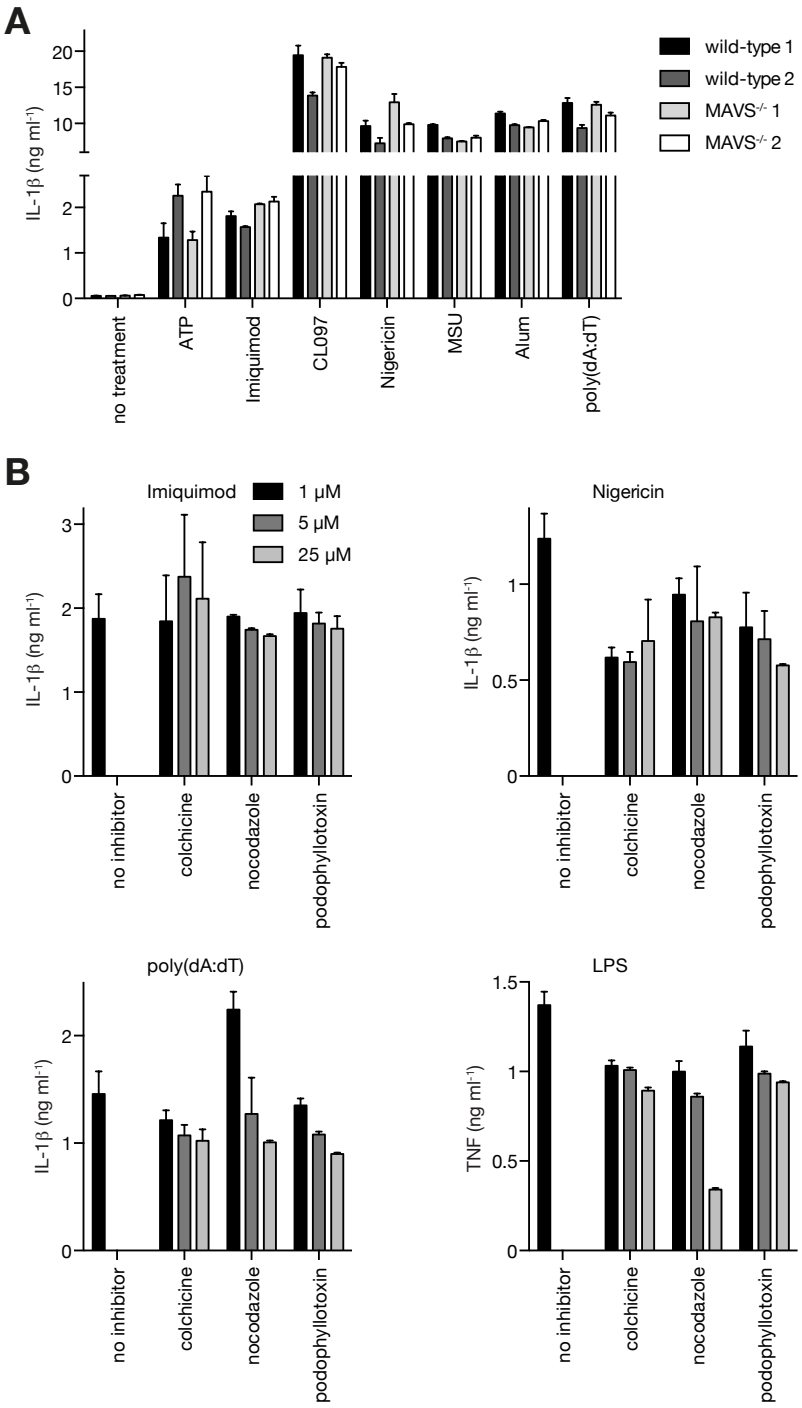


Figure 10: Imiquimod and CL097-induced inflammasome activation does not involve MAVS signalling and is sensitive to inhibition of microtubule dynamics.

(A) LPS-primed BMDCs from two wild-type and two MAVS-deficient mice were treated with different inflammasome activators as indicated. IL-1β secretion was quantified from cell-free supernatants by ELISA. (B) LPS-primed wild-type BMDCs were treated for 30 min with 1-25 µM colchicine, nocodazole, or podophyllotoxin and then stimulated with NLRP3 inflammasome activators as indicated. IL-1β secretion was quantified from cell-free supernatants by ELISA.

Data are depicted as mean ± SEM of technical triplicates.

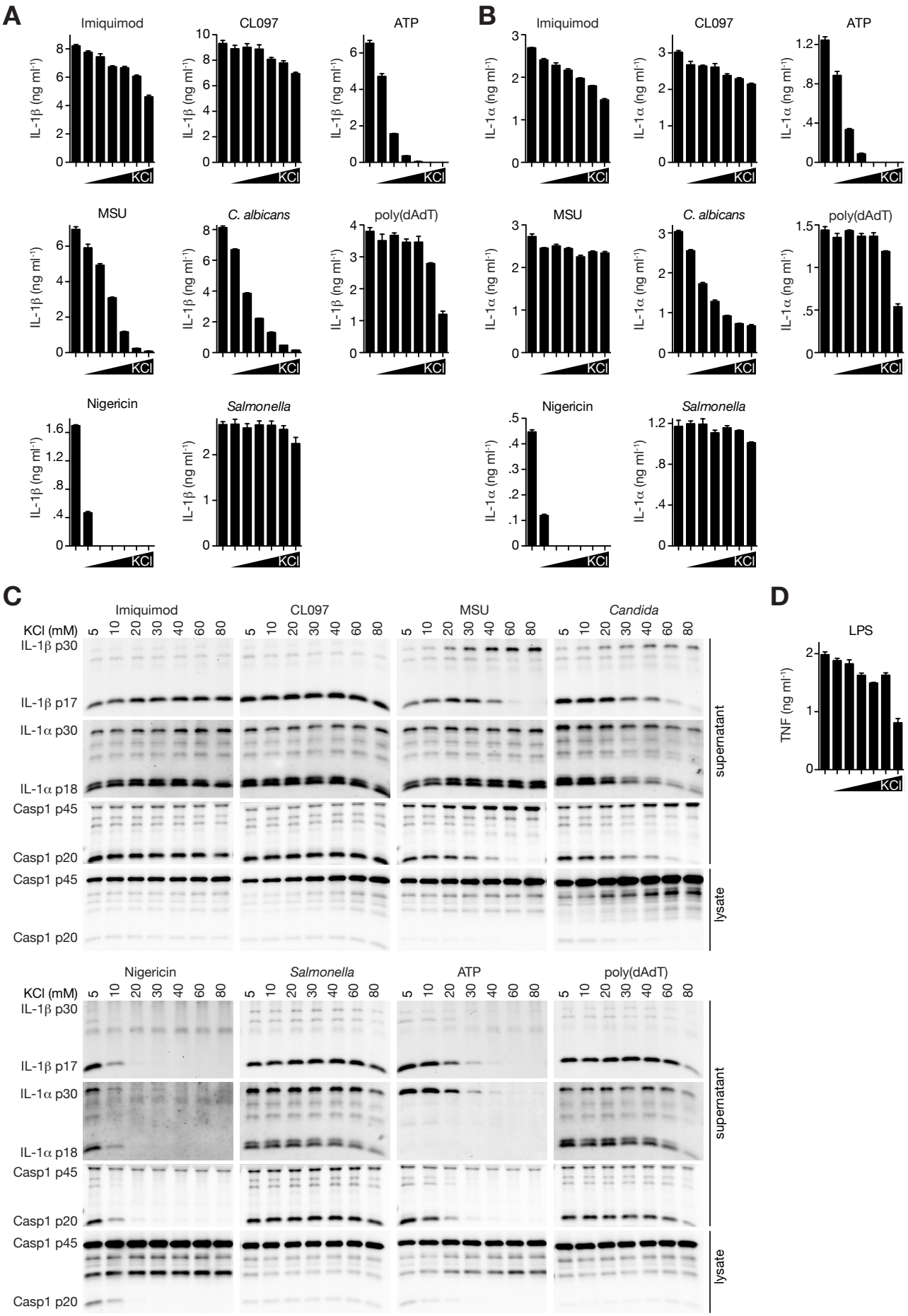
K^+ efflux is dispensable for NLRP3 activation by imiquimod and CL097

The mechanism of NLRP3 activation remains unknown, but it is accepted that K^+ efflux is universally required (Muñoz-Planillo et al., 2013; Rivers-Auty and Brough, 2015). K^+ efflux is triggered by all NLRP3 activators investigated thus far, including extracellular ATP, K^+-permissive pore-forming toxins, and particles/crystals (Muñoz-Planillo et al., 2013; Pétrilli et al., 2007). K^+-free medium, which is sufficient to trigger K^+ efflux, is activates NLRP3 (Muñoz-Planillo et al., 2013). The non-canonical caspase-11 inflammasome, which senses intracellular LPS, triggers NLRP3-dependent IL-1β secretion via caspase-11-dependent K^+ efflux (Baker et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015). Preventing K^+ efflux, albeit rather crudely by the use of high extracellular concentrations of KCl, inhibits NLRP3 activation by canonical and non-canonical activators.

Surprisingly, caspase-1 and IL-1 cleavage and secretion induced by imiquimod and CL097 was not blocked by extracellular KCl in LPS-primed BMDCs (Figure 11A-11D). ASC oligomerization in response to imiquimod was similarly intact in the presence of extracellular KCl (Figure 12A). NLRP3 Inflammasome activation by extracellular ATP, nigericin, MSU, and *Candida* was, as previously reported, inhibited with increasing concentrations of extracellular KCl (Figure 11A-11C). In contrast, the NAIP/NLRC4 inflammasome activator *Salmonella* and the AIM2 inflammasome activator, intracellular poly(dA:dT), behaved similarly to imiquimod and CL097 in that they triggered cleavage and secretion of caspase-1 and IL-1β even in the presence of higher extracellular K^+ levels (Figure 11A-11C), as expected.

However, at concentrations exceeding 60 mM, KCl suppressed not only NLRP3 activation by conventional activators, but also caused a slight reduction in dsDNA-

induced AIM2 inflammasome activation and LPS-induced TNF secretion, which are not dependent on K^+ efflux (Figure 11A-11D). Poly(dA:dT) and imiquimod activate ASC-dependent inflammasomes, which unlike the NAIP/NLRC4 inflammasome cannot directly recruit caspase-1 (Schroder and Tschopp, 2010). The slight reduction in poly(dA:dT)- or imiquimod-induced inflammasome activation at high extracellular concentrations of KCl may reflect the observed direct inhibition of K^+ on ASC oligomerization *in vitro* (Fernandes-Alnemri et al., 2007). However, non-physiological K^+ and Cl^- concentrations in the extracellular milieu most certainly has cellular effects beyond inhibiting K^+ efflux. Therefore, we examined whether imiquimod induces K^+ efflux. Wild-type or NLRP3-deficient BMDCs were treated with imiquimod or nigericin and intracellular K^+ concentrations and IL-1β secretion were measured from the same samples. Imiquimod triggered IL-1β secretion in wild-type BMDCs without triggering a loss of cellular K^+ in NLRP3-deficient cells (Figure 12B). These results were confirmed in independent experiments by several others in the lab, and the pooled results are shown in Appendix 1F. A minor decrease in cellular K^+ concentrations was observed only at later time points in wild-type cells (Figure 12B), which probably reflects pyroptotic release of K^+, as was observed in response to caspase-11 activation (Rühl and Broz, 2015). In contrast, nigericin-induced IL-1β secretion coincided with a large drop in cellular K^+ concentration (Figure 12B and Appendix 1F). Therefore, while it is conceivable that K^+ efflux may contribute to imiquimod-induced NLRP3 activation once pyroptosis is initiated, initial activation of NLRP3 by imiquimod does not induce and therefore cannot require K^+ efflux.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Imiquimod
CL097
ATP
MSU
C. albicans
poly(dAdT)
Nigericin
Salmonella
IL-1β (ng ml-1)
KCl
B
IL-1α (ng ml-1)
C
KCl (mM)
5 10 20 30 40 60 80
IL-1β p30
IL-1β p17
IL-1α p30
IL-1α p18
Casp1 p45
Casp1 p20
supernatant
lysate
Candida
D
LPS
TNF (ng ml-1)

← Figure 11: K^+ efflux-independent NLRP3 inflammasome activation by imiquimod and CL097.

(A, B) LPS-primed wild-type BMDCs were treated with increasing amounts of KCl, ranging from an additional 0 to 75 mM, resulting in final concentrations of 5, 10, 20, 30, 40, 60, and 80 mM directly prior to stimulation with the indicated inflammasome activators. IL-1β (A) and IL-1α (B) secretion was quantified from cell-free supernatants by ELISA.

(C) Supernatants from (A, B) were subjected to immunoblotting for the presence of the cleaved and uncleaved forms of IL-1β, IL-1α, and caspase-1. Cell lysates were subjected to immunoblotting for the presence of the cleaved and uncleaved forms of caspase-1.

(D) Unprimed BMDCs were treated with increasing amounts of KCl in parallel to (A, B) directly prior to stimulation with 20 ng ml^{-1} LPS for 3 h. TNF secretion was quantified from cell-free supernatants by ELISA. ELISA data are depicted as mean ± SEM of technical triplicates.

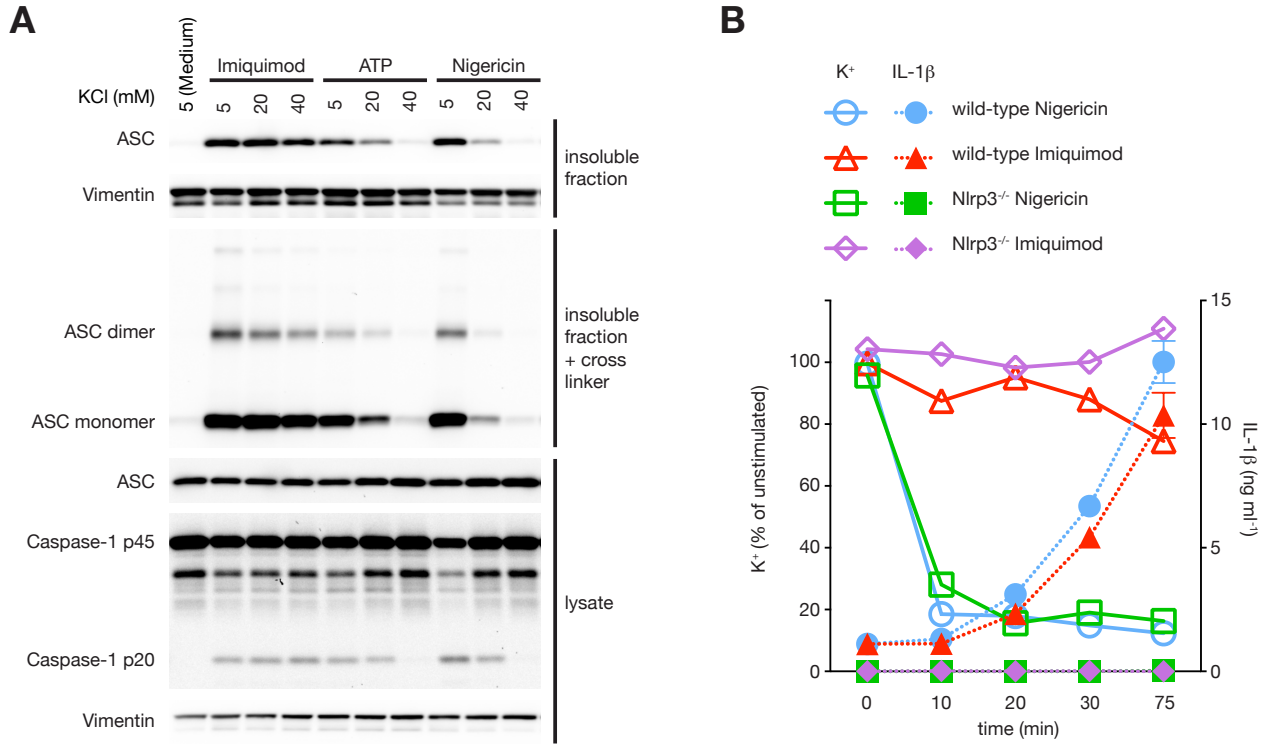


Figure 12: K^+ efflux-independent NLRP3 inflammasome activation by Imiquimod and CL097.

(A) LPS-primed BMDCs were pretreated with different concentrations of KCl or left uninhibited (medium = 5 mM K^+) as indicated and were left unstimulated or treated with imiquimod, ATP, or nigericin. ASC complexes in the NP40-insoluble fraction of these cells were enriched by centrifugation, cross-linked with DSS, and analysed by immunoblotting. Vimentin is a loading control for the lysate and the insoluble fraction

(B) Primed wild-type or NLRP3-deficient BMDCs were treated with nigericin (5 µM) or imiquimod (20 µg ml^{-1}) for the indicated time. IL-1β secretion was quantified from cell-free supernatants by ELISA (right Y axis, dotted lines). Intracellular K^+ concentrations from the same cells were determined by ISE, depicted as % of unstimulated cells (right Y axis, solid lines). ELISA data are depicted as mean ± SEM of technical triplicates.

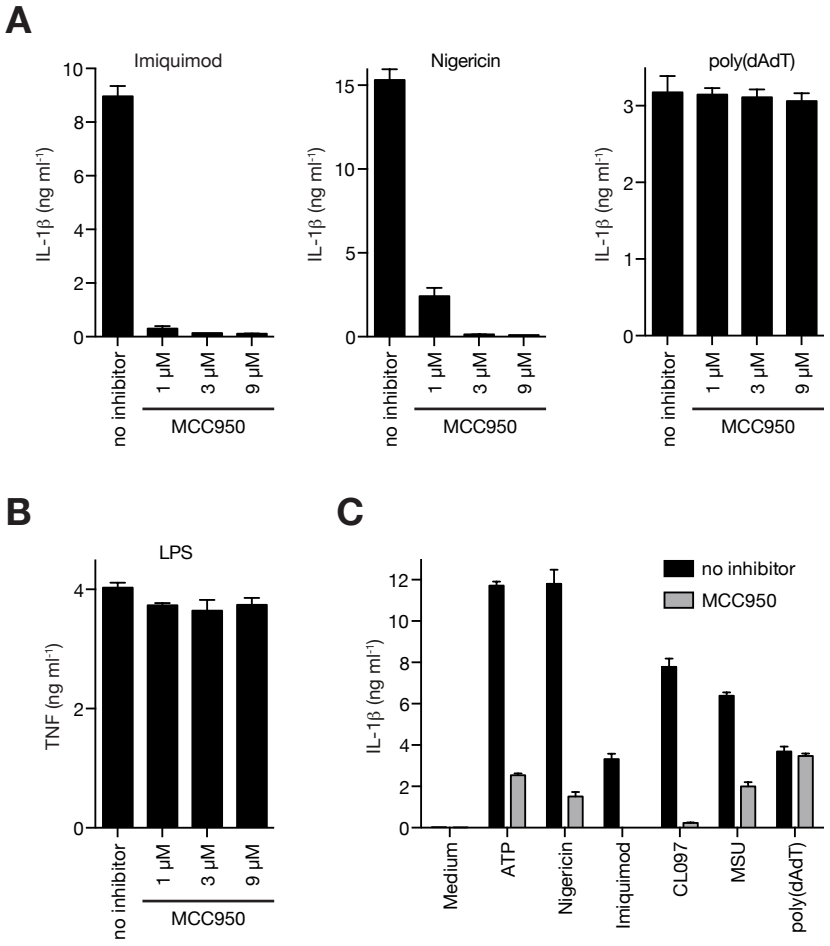


Figure 13: NLRP3 inflammasome activation by Imiquimod and CL097 is inhibited by MCC950

(A) LPS-primed wild-type BMDCs were treated with the indicated concentrations of MCC950 30 min prior to stimulation with imiquimod, nigericin, or poly(dA:dT). IL-1β secretion was quantified from cell-free supernatants by ELISA.

(B) BMDCs were treated with the indicated concentrations of MCC950 30 min prior to stimulation with LPS (20 ng ml^{-1}). TNF secretion was quantified from cell-free supernatants by ELISA.

(C) LPS-primed wild-type BMDCs were treated with 1 μM MCC950 30 min prior to stimulation with various inflammasome activators as indicated. IL-1β secretion was quantified from cell-free supernatants by ELISA. Data are depicted as mean ± SEM of technical triplicates.

MCC950 inhibits NLRP3 activation by imiquimod and CL097

The small molecule MCC950 (also known as CRID3) inhibits activation of the NLRP3 inflammasome by activators such as ATP, nigericin, particles, and intracellular LPS (Coll et al., 2015). It is not known how MCC950 prevents NLRP3 activation, but it does not affect K^+ efflux (Coll et al., 2015). Inflammasome activation by imiquimod,

CL097, and other NLRP3 activators was blocked by MCC950, while poly(dA:dT)-induced AIM2 inflammasome activation and LPS-induced TNF secretion were not inhibited by MCC950 (Figure 13). This suggests that imiquimod, CL097, and conventional (*i.e.* K^+-efflux dependent) NLRP3 activators trigger a common MCC950-sensitive pathway for NLRP3 activation.

Chemical proteomics identifies NQO2 as a target of imiquimod and CL097

In order to identify cellular targets of imiquimod and CL097 that may be determinants of their ability to activate NLRP3, Prof. Bernhard Küster and Dr. Guillaume Médard (Chair of Proteomics and Bioanalytics, TUM) performed chemical proteomics with an imiquimod/CL097 analogue that they synthesized (GM019, Appendix 2A). Though many proteins from lysates of LPS-primed BMDCs bound to GM019-coupled beads, only the interaction of NQO2 was specific in that it could be prevented by pre-incubation of BMDC lysates with free imiquimod (Appendix 2B, 2C). Dr. Sabine Schneider (Chair of Biochemistry, TUM), co-crystalized imiquimod and CL097 with NQO2. Within the active site of NQO2, imiquimod and CL097 formed an aromatic stack with the isoalloxazine rings of the FAD cofactor (Appendix 2D, 2E). These results identify NQO2 as a direct target of imiquimod and CL097.

NQO2 is dispensable for NLRP3 activation

To determine the functional significance of this interaction, NLRP3 activation in BMDCs from NQO2-deficient mice was analysed. While NQO2 was not required for NLRP3 activation (Figure 14A), NQO2 knockouts did display some differences in the kinetics of IL-1β secretion in response to imiquimod (Figure 14B). It is possible that

modulation of NQO2 activity by imiquimod, or modification of imiquimod by NQO2 may explain these subtle differences. NQO2 is a promiscuous quinone oxidoreductase that catalyzes the two-electron detoxification of quinones to form hydroquinones. Resveratrol, quercetin, melatonin (Buryanovskyy et al., 2004; Calamini et al., 2008), and the Bcr-Abl tyrosine kinase inhibitors imatinib and nilotinib also bind and inhibit NQO2 (Bantscheff et al., 2007; Rix et al., 2007; Winger et al., 2009). None of these compounds inhibited IL-1β secretion except at concentrations where they also inhibited LPS-induced TNF secretion (data not shown). Surprisingly, imatinib alone triggered substantial IL-1β secretion (Figure 15A). Like imatinib, nilotinib inhibits both Bcr-Abl and NQO2, but did not trigger IL-1β secretion (Figure 15A). Bosutinib, which inhibits Bcr-Abl but not NQO2, also caused IL-1β secretion (Figure 15A). IL-1β secretion in response to imatinib and bosutinib was NQO2-independent (Figure 15B), but ASC-dependent (Figure 15C, 15D) suggesting that these compounds activate an inflammasome in an NQO2-independent manner.

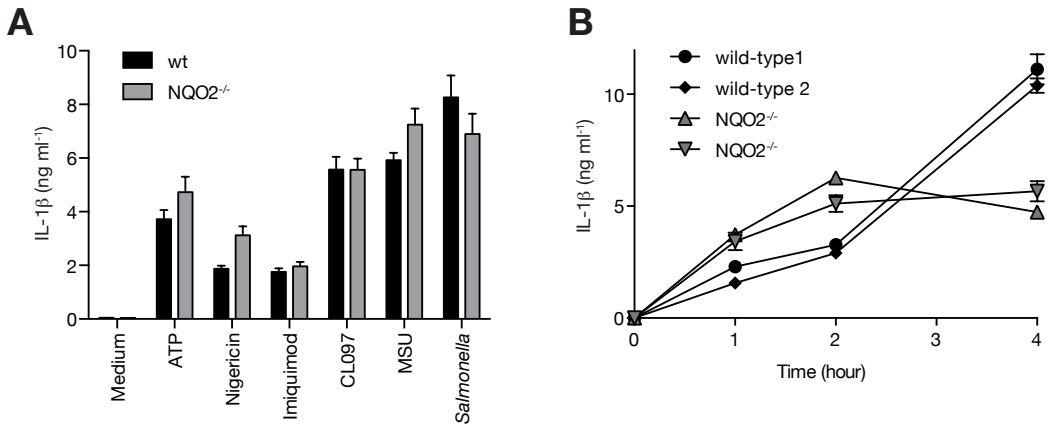


Figure 14: NQO2 is dispensable for NLRP3 inflammasome activation by imiquimod and CL097

(A) LPS-primed BMDCs from wild-type or NQO2-deficient mice were stimulated with different inflammasome activators as indicated. IL-1β secretion was quantified from cell-free supernatants by ELISA. (B) LPS-primed BMDCs from two wild-type and two NQO2-deficient mice were treated for the indicated periods with 15 μg ml^{-1} of Imiquimod. IL-1β secretion was quantified from cell-free supernatants by ELISA. Data are depicted as mean ± SEM of technical triplicates.

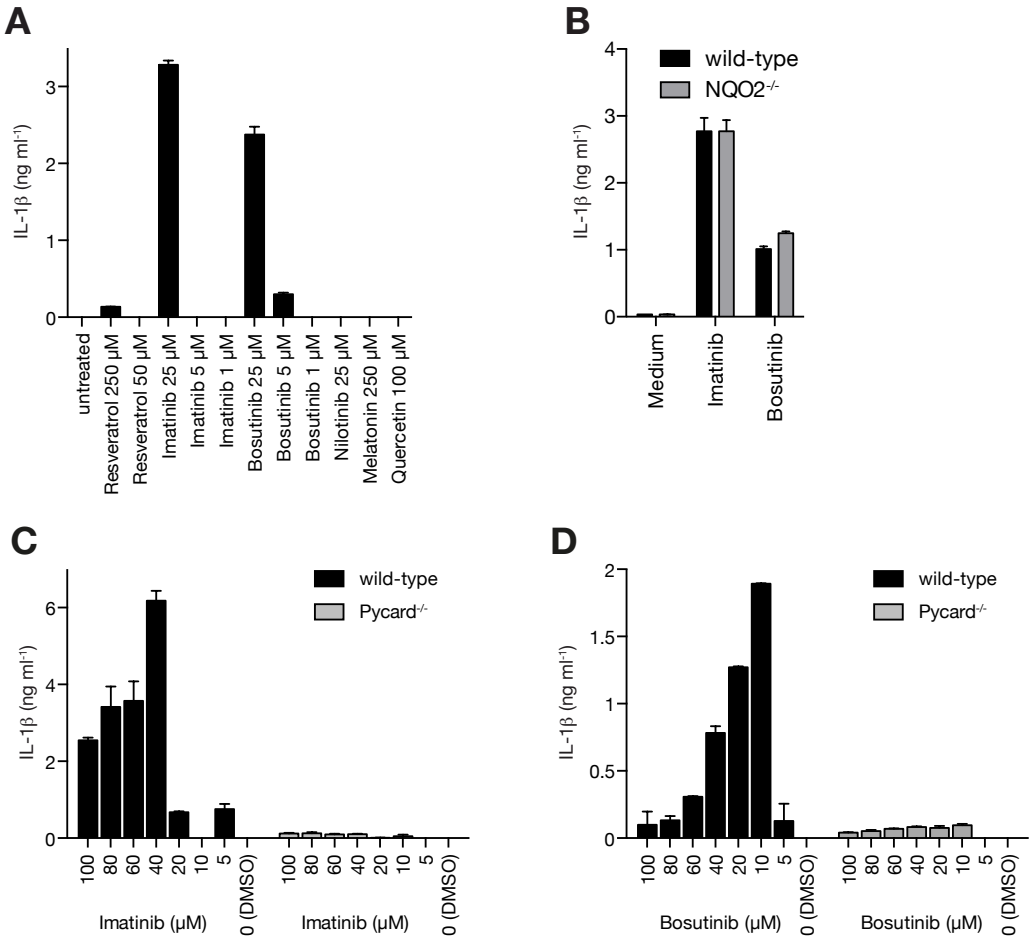


Figure 15: Certain tyrosine kinase inhibitors activate the inflammasome in an NQO2-independent manner.

(A) LPS-primed BMDCs were stimulated with different NQO2-binding (Imatinib and Nilotinib) or non-binding (Bosutinib) tyrosine kinase inhibitors as well as other reported NQO2-binding molecules at different doses as indicated. IL-1β secretion was quantified from cell-free supernatants by ELISA.

(B) LPS-primed wild-type and NQO2-deficient BMDCs were treated with Imatinib and Bosutinib. IL-1β secretion was quantified from cell-free supernatants by ELISA.

(C, D) LPS-primed wild-type and ASC-deficient (*Pycard*$^{-/-}$) BMDCs were treated with different doses of Imatinib (C) and Bosutinib (D) as indicated. IL-1β secretion was quantified from cell-free supernatants by ELISA.

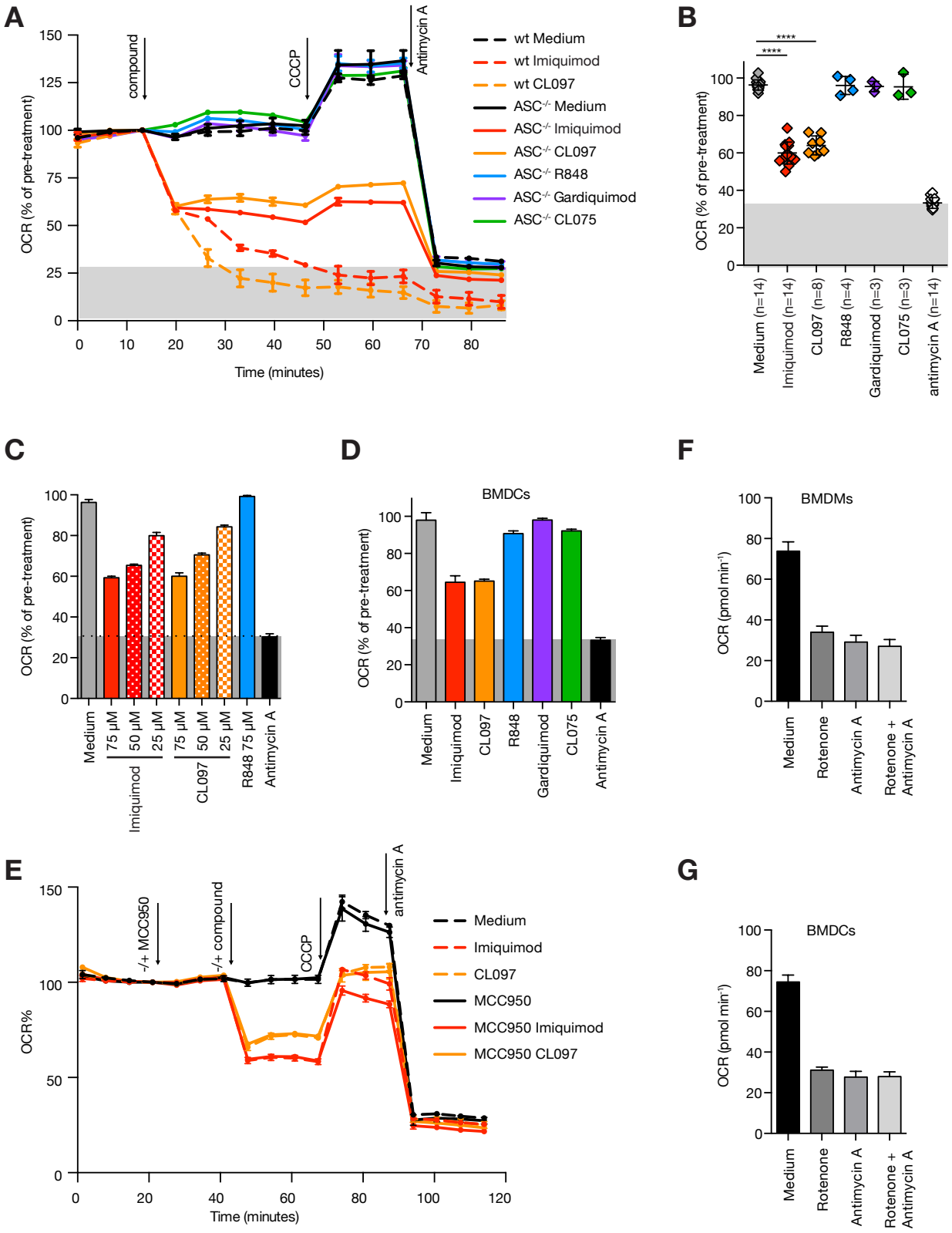
Data are depicted as mean ± SEM of technical triplicates.

Imiquimod and CL097 inhibit respiration

The human genome encodes many dehydrogenases that use FAD or FMN as a redox cofactor. Given that many of these flavoproteins function in mitochondrial metabolic processes, and that imiquimod binds the flavoprotein NQO2, it is possible that imiquimod might modulate mitochondrial function. Sequelae of mitochondrial dysfunction, such as ROS production, release of mitochondrial DNA, changes in the lipid composition of the outer membrane, and mitochondrial calcium fluxes have been linked to NLRP3 activation (Gurung et al., 2015). To determine whether imiquimod directly influenced (*i.e.* not indirectly via NLRP3 inflammasome activation) respiration as a key mitochondrial function, we measured the O_2 consumption rate (OCR) in ASC-deficient BMDMs. NLRP3-activating concentrations of imiquimod caused an immediate and dose-dependent decrease in the respiratory rate (Figure 16A-16C). The respiratory rate of wild-type cells continued to drop even below the rate of antimycin A-treated cells in which mitochondrial respiration is completely inhibited at the level of Complex III (Figure 16A), suggesting that in addition to the direct effect of imiquimod on mitochondrial respiration, inflammasome activation or pyroptosis also secondarily interferes with respiration. Interestingly, CL097 also suppressed respiration in ASC-deficient cells, but imidazoquinolines that did not activate NLRP3 also failed to suppress respiration (Figure 16A, 16B). This was also observed in BMDCs (Figure 16D). Furthermore, the NLRP3 inflammasome inhibitor MCC950 did not influence respiration (Figure 16E)

In analysing the respiratory profile of BMDMs and BMDCs, it was observed that the Complex I inhibitor rotenone inhibited respiration to the same extent as antimycin A, which inhibits Complex III (Figure 16F, 16G). Complex III accepts electrons from Complex I and Complex II via ubiquinone. The finding that rotenone inhibits respiration to the same

extents as antimycin A suggests that myeloid cells rely primarily on Complex I rather than Complex II as the site of entry of electrons into the respiratory chain.



←Figure 16: Imiquimod and CL097 inhibit mitochondrial respiration.

(A) Oxygen consumption rate (OCR) of wild-type (wt, dotted lines) and ASC-deficient ($ASC^{-/-}$, solid lines) BMDMs stimulated with imidazoquinolines (75 µM = 21 µg ml^{-1} for imiquimod) and subsequently with CCCP (0.5 µM) and antimycin A (2 µM). In (A), the grey area of the graph below the level of antimycin A-treated samples indicates the contribution of non-mitochondrial respiration (*i.e.* NADPH oxidase or other enzymes)

(B) OCR of BMDCs and BMDMs treated with 70-75 µM imidazoquinolines or 2 µM antimycin A for 7 min on six separate occasions. Each dot represents a reading from cells derived from an independent mouse. The grey area of the graph below the level of antimycin A-treated samples is the level of non-mitochondrial respiration. Mean ± SD of biological replicates is shown. A Student's t-test was performed to determine significant differences between imiquimod- or CL097-treated cells and their untreated counterpart from the same experiment. ****$p<0.0001$

(C) Oxygen consumption rate (OCR) of ASC-deficient BMDMs stimulated with increasing doses of imidazoquinolines or antimycin A (2 µM) for 7 min. The grey area of the graph below the level of antimycin A-treated samples is the level of non-mitochondrial respiration.

(D) OCR of ASC-deficient BMDCs, left untreated or stimulated with 75 µM of different imidazoquinolines as indicated or with antimycin A (2 µM) for 7 min. The grey area of the graph below the level of antimycin A-treated samples is the level of non-mitochondrial respiration.

(E) OCR of ASC-deficient BMDMs first treated with the NLRP3 inhibitor MCC950, 1 µM and subsequently stimulated with imidazoquinolines (75 µM) and finally with CCCP (0.5 µM) and antimycin A (2 µM).

(F, G) OCR of ASC-deficient BMDMs (F) or BMDCs (G), left untreated or treated with the Complex I inhibitor rotenone or the Complex III inhibitor antimycin A or with both inhibitors together for 7 min.

Data are depicted as mean ± SEM of technical quadruplicates.

Imiquimod and CL097 enhance glycolysis, but this fails to rescue ATP production

In the mitochondrial electron transport chain, electrons are transferred from reduced donors to O_2 via a series of complexes. Electron transport is coupled to the pumping of protons into the intermembrane space by Complexes I, III, and IV. ATP synthase (Complex V) uses the energy stored in the proton gradient to produce ATP. Mitochondrial ATP production can be suppressed by inhibitors of electron transport or by agents that disperse the proton gradient and therefore uncouple respiration from ATP production. In cells where mitochondrial ATP production is not sufficient to satisfy cellular demands, glycolysis is upregulated. Imiquimod and CL097 also enhanced the glycolytic

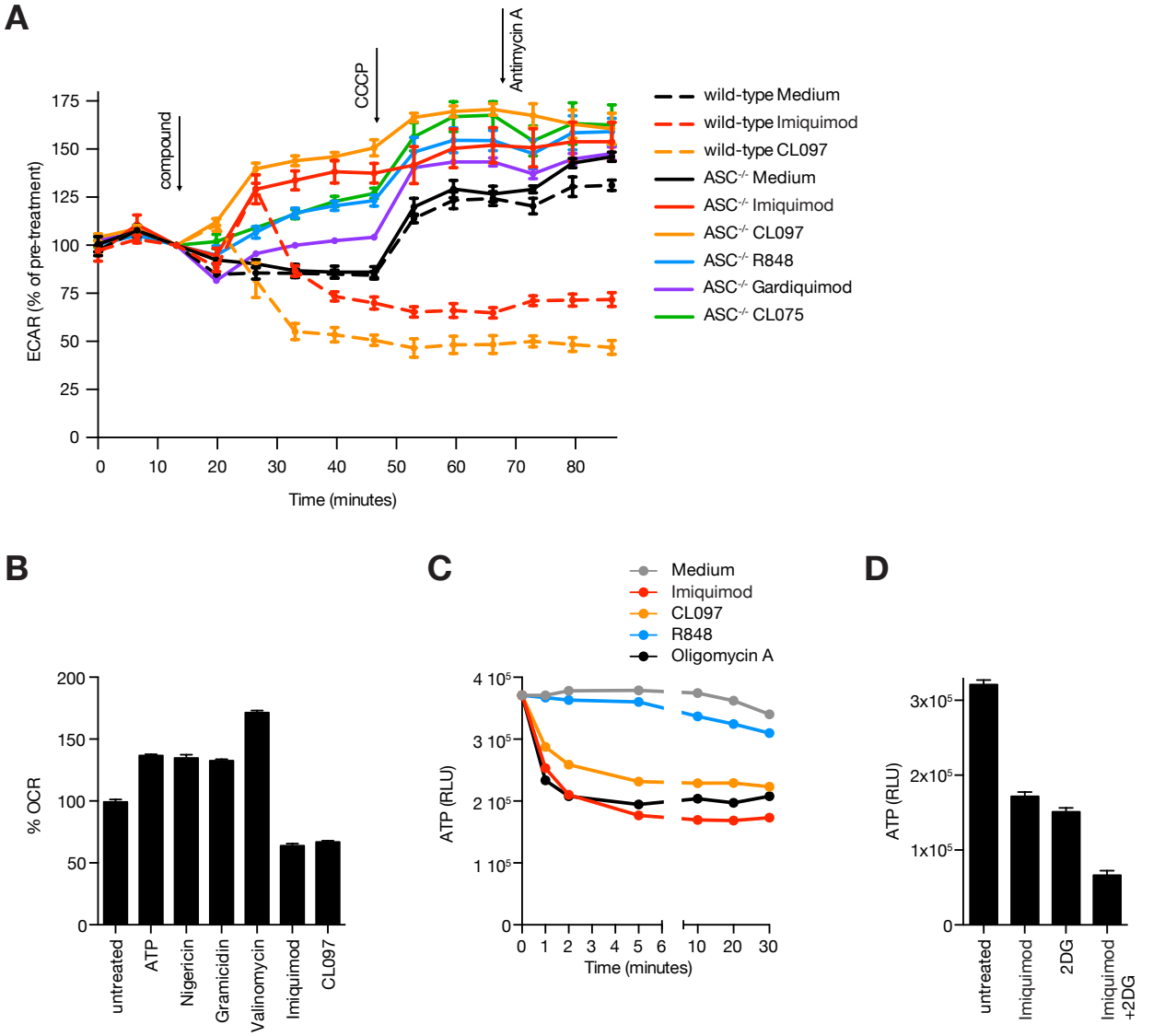


Figure 17: Imiquimod and CL097 enhance glycolysis, but this fails to rescue ATP production

(A) Extracellular acidification rate (ECAR) of wild-type (wt, dotted lines) and ASC-deficient (ASC$^{-/-}$, solid lines) BMDMs stimulated with imidazoquinolines (75 µM = 21 µg ml^{-1} for imiquimod) and subsequently with CCCP (0.5 µM) and antimycin A (2 µM).

(B) OCR of ASC-deficient BMDMs, left untreated or stimulated with different inflammasome activators and uncoupling agents for 7 min.

(C) Cellular ATP concentration of LPS-primed ASC-deficient BMDMs treated with imidazoquinolines (70 µM) or oligomycin A (3.5 µM) for the indicated times. Relative ATP concentrations were determined by luciferase signal intensity.

(D) Cellular ATP concentration of ASC-deficient BMDMs treated with Imiquimod, 2DG or a combination of the two. Relative ATP concentrations were determined by luciferase signal intensity.

Data are depicted as mean ± SEM of technical quadruplicates.

activity of BMDMs, as indicated by an increase in the extracellular acidification rate (ECAR, Figure 17A). R848, Gardiquimod, and CL075 also increased extracellular acidification rate, but not to the same extent as imiquimod or CL097. TLR ligands have been shown to increase glycolysis in myeloid cells (Everts et al., 2012; 2014; Krawczyk et al., 2010). The further increase in glycolysis in response to imiquimod or CL097 is probably a combined result of inhibition or respiration, and activation of TLR7-induced glycolysis. As expected, other respiratory chain inhibitors (*e.g.* rotenone, antimycin) and uncoupling agents (CCCP) also increased the extracellular acidification rate (Figure 17A). Other NLRP3 activators such as ATP, nigericin, gramicidin, and valinomycin enhanced respiration (Figure 17B), which is consistent with their described uncoupling activity (Ferguson et al., 1971). Imiquimod and CL097, but not R848, also caused a rapid reduction in cellular ATP levels in ASC-deficient cells, comparable to the reduction observed with the ATP synthase inhibitor oligomycin A (Figure 17C). ATP levels could be further suppressed in imiquimod treated cells by treatment with the glycolytic inhibitor 2-deoxy-D-glucose (2DG; Figure 17D). This suggests that the induction of glycolysis by imiquimod and CL097 does not rescue the defects in mitochondrial ATP production by these compounds.

Imiquimod and CL097 suppress respiration in human keratinocytes

Suppression of respiration by imiquimod was observed in a variety of human and murine non-myeloid cell types, indicating that this effect on respiration is not species- or cell type-specific (Figure 18A). Imiquimod and CL097 also suppressed respiration in human primary keratinocytes and the HaCaT human keratinocyte cell line (Figure 18B), which is important because keratinocytes are the primary cellular target of imiquimod *in*

vivo. Imiquimod and CL097 did not reduce ATP levels in HaCaT or human primary keratinocytes (Figure 18C), which is consistent with the fact that proliferating cell such as cancer cells rely more heavily on glycolysis than oxidative phosphorylation for ATP production.

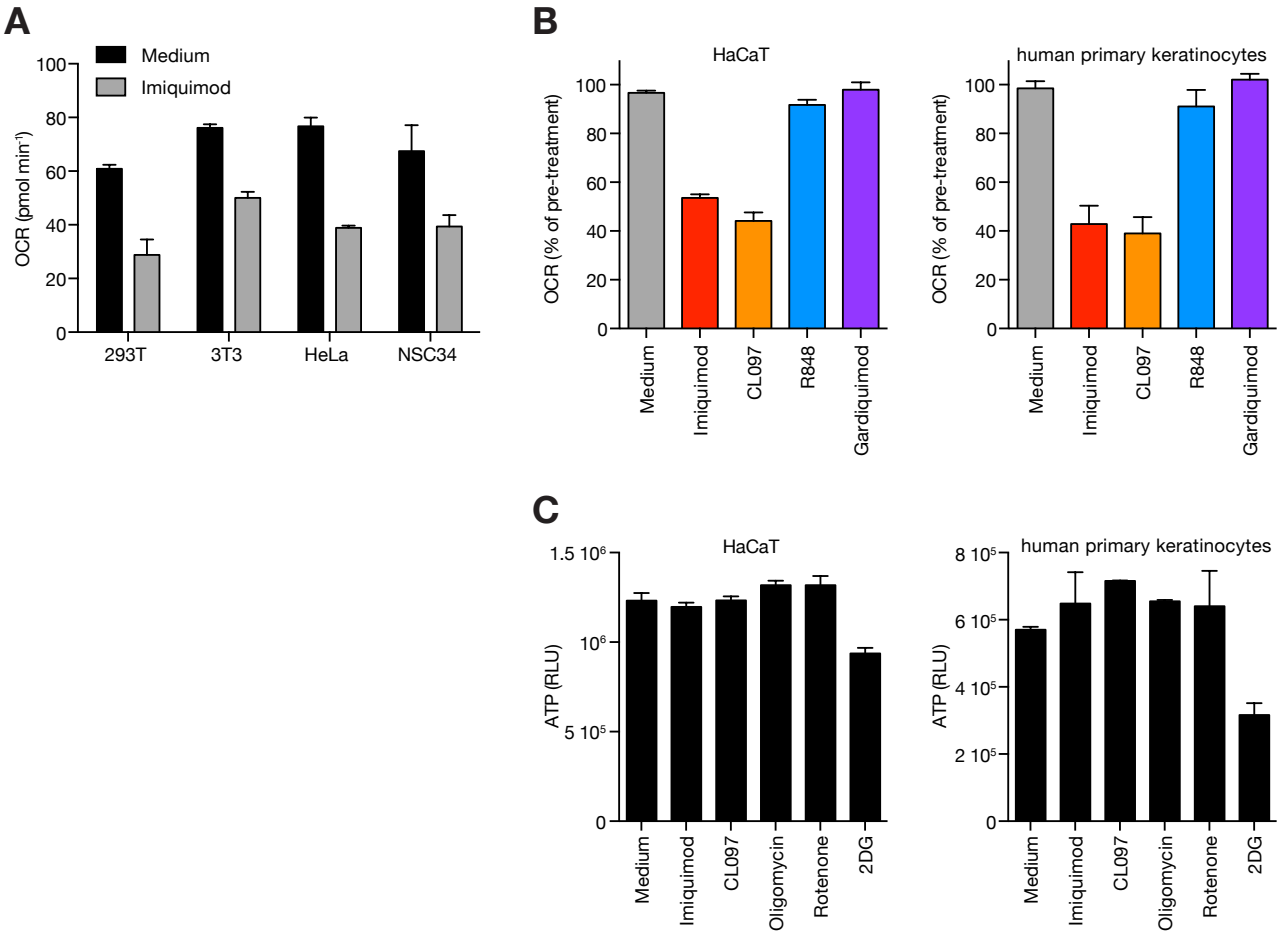


Figure 18: Imiquimod and CL097 inhibit respiration in diverse cell types but do not cause a decrease cellular ATP content human keratinocytes.

(A) Oxygen consumption rate (OCR) of the indicated human and murine cell lines, left untreated or stimulated with 75 µM imiquimod for 7 min.

(B) Oxygen consumption rate (OCR) of HaCaT keratinocytes and human primary keratinocytes, left untreated or stimulated with 70 µM of different imidazoquinolines for 7 min.

(C) Cellular ATP concentration of HaCaT keratinocytes and human primary keratinocytes treated with different inhibitors of the respiratory chain or with 2DG. Relative ATP concentrations were determined by luciferase signal intensity.

Data are depicted as mean ± SEM of technical quadruplicates

Imiquimod and CL097 inhibit mitochondrial Complex I activity

Imiquimod could suppress respiration by a variety of mechanisms including direct block of the electron transport chain, inhibition of the tricarboxylic acid (TCA) cycle, or by causing general defects in cellular or mitochondrial fitness. To examine the mechanism of imiquimod-induced impairment of respiration, we measured mitochondrial respiration in digitonin-permeabilized BMDMs. Digitonin is a cholesterol-binding detergent that selectively permeabilizes the plasma membrane without disrupting internal membranes with low cholesterol content such as the mitochondrial membranes. In permeabilized cells, the activity of individual respiratory complexes can be assessed by supplying exogenous TCA cycle intermediates or other electron donors (Salabei et al., 2014). Digitonin-permeabilized BMDMs were incubated with substrate combinations to feed Complex I (malate and pyruvate or glutamate), Complex II (succinate with rotenone) and cytochrome *c*/Complex IV (TMPD with ascorbate). These were combined with ADP in a phosphate-containing buffer, which is required for flux through the respiratory chain because ATP synthesis serves as a terminal acceptor for the potential energy generated by electron transport. Imiquimod and CL097 selectively blocked Complex I-mediated respiration, without affecting respiration via Complex II or cytochrome *c*/Complex IV (Figure 19A). As observed in intact cells, imidazoquinolines such as R848 and gardiquimod that do not activate NLRP3 also did not influence respiration in permeabilized cells (Figure 19B). Collectively, these data suggest that imiquimod and CL097 are specific inhibitors of Complex I activity. Imiquimod also decreased the NAD^+/NADH ratio in NLRP3-deficient BMDMs, which is consistent with Complex I inhibition (Figure 19C).

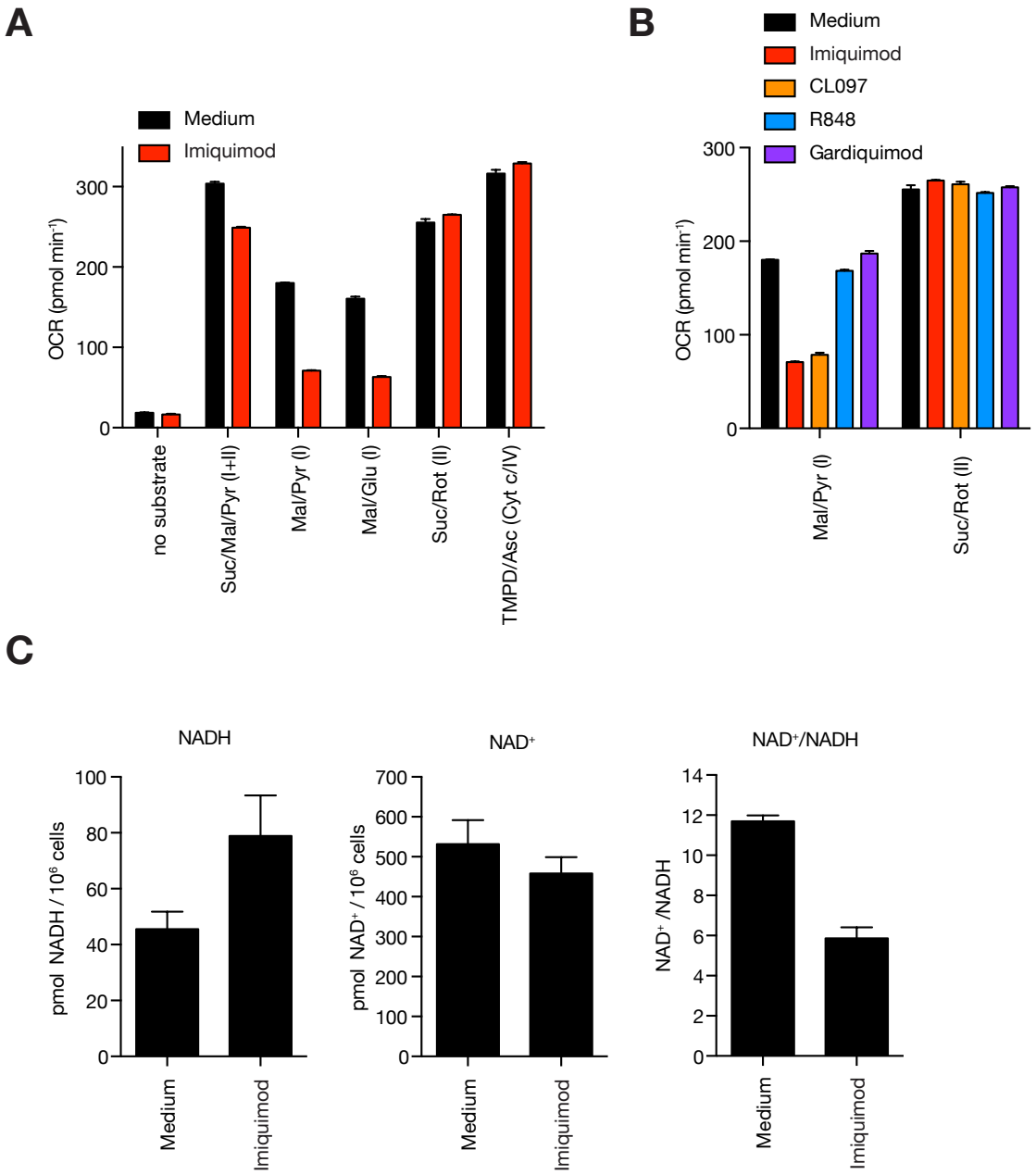


Figure 19: Imiquimod and CL097 inhibit mitochondrial Complex I activity.

(A) OCR of digitonin-permeabilized BMDMs that were given, in the presence or absence of imiquimod (70 µM), ADP and substrates of the TCA cycle/mitochondrial electron transport chain: succinate+malate+pyruvate (Complex I and II), malate+pyruvate (Complex I), malate+glutamate (Complex I), succinate+rotenone (Complex II), or TMPD+ascorbate (cytochrome *c*/Complex IV).

(B) OCR of digitonin-permeabilized BMDMs that were given, in the presence or absence of imidazoquinolines (70 µM), ADP and substrates of the TCA cycle/mitochondrial electron transport chain.

(C) Quantification of NAD^+ and NADH from NLRP3-deficient BMDMs treated with imiquimod or left untreated. Mean and standard deviation from two experiments performed on day 6 and day 8 of the same BMDM culture are depicted.

Complex I transfers electrons from NADH to ubiquinone with the concomitant pumping of protons into the intermembrane space. Some organisms express alternative, rotenone-insensitive NADH:quinone oxidoreductases that catalyze electron transfer from NADH to ubiquinone, but do not pump protons. Ndi1 from *Saccharomyces cerevisiae* is one such protein (de Vries and Grivell, 1988). Expression of Ndi1 rescues respiration in cells with genetic or pharmacological inhibition of Complex I (Bai et al., 2001; Seo et al., 1998; 1999; 2000). Indeed, Ndi1 expression in normal human dermal fibroblasts (NHDF) prevented the decrease in respiration observed with imiquimod and CL097 (Figure 20A, 20B). As expected, respiration in Ndi1-expressing fibroblasts was insensitive to the Complex I inhibitor rotenone, but sensitive to the Complex III inhibitor antimycin A. These results further suggest that imiquimod and CL097 are inhibitors of Complex I.

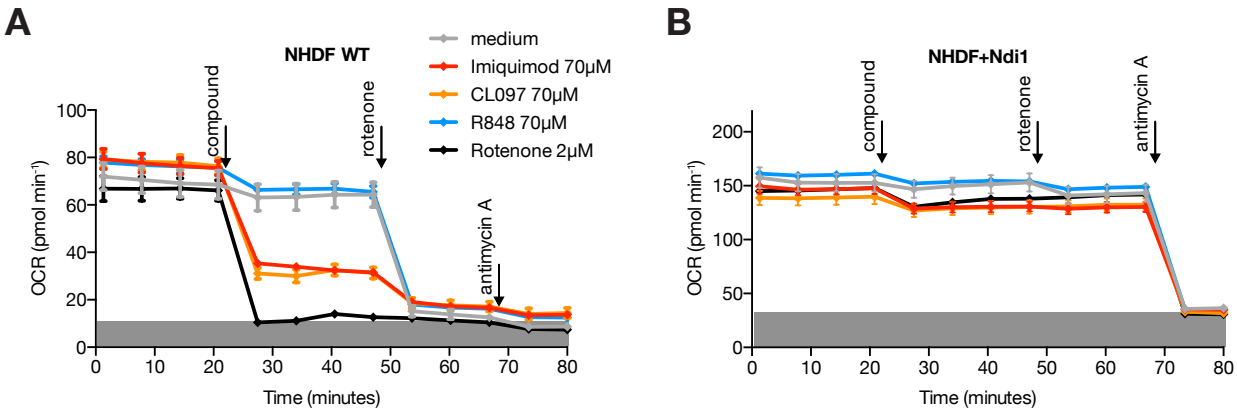


Figure 20: Ndi1 expression rescues Complex I inhibition by Imiquimod and CL097.

(A, B) Oxygen consumption rate (OCR) of NHDF (A) or NHDF stably expressing Ndi1 (B) treated with the indicated compounds and subsequently with rotenone (2 µM) and antimycin A (2 µM). The grey area of the graph below the level of antimycin A-treated samples indicates the contribution of non-mitochondrial respiration.

NLRP3 activation by imiquimod and CL097 requires ROS, but Complex I inhibition is not sufficient for NLRP3 activation

Complex I dysfunction is thought to be the major source of ROS *in vivo* (Murphy, 2009). Given that imiquimod inhibits Complex I activity, that Complex I inhibition by other agents triggers ROS, and that ROS are implicated in activating NLRP3 (Schroder and Tschopp, 2010), we hypothesized that antioxidants may block NLRP3 activation by imiquimod and CL097. LPS-primed BMDCs were pre-treated with the antioxidants ebselen and ammonium pyrrolidinedithiocarbamate (APDC) for 30 min before addition of NLRP3 activators. The glutathione peroxidase mimetic ebselen, and the ROS scavenger APDC both inhibited NLRP3 activation by nigericin, imiquimod, and CL097 without inhibiting LPS-induced TNF production, or dsDNA-induced activation of the AIM2 inflammasome (Figure 21A-21C). Therefore, ROS are required for NLRP3 activation by the Complex I inhibitors imiquimod and CL097. It was previously reported that rotenone and antimycin A trigger NLRP3 activation by promoting generation of mitochondrial ROS at Complex I and Complex III, respectively (Nakahira et al., 2011; Zhou et al., 2010b). However, some groups have reported failure to reproduce these findings (Iyer et al., 2013; Juliana et al., 2012; Muñoz-Planillo et al., 2013; Youm et al., 2015), and neither rotenone nor another Complex I inhibitor piericidin A triggered IL-1β secretion in our experiments (Figure 21D, 21E). This suggests that Complex I-derived ROS may be necessary but is not sufficient for NLRP3 activation.

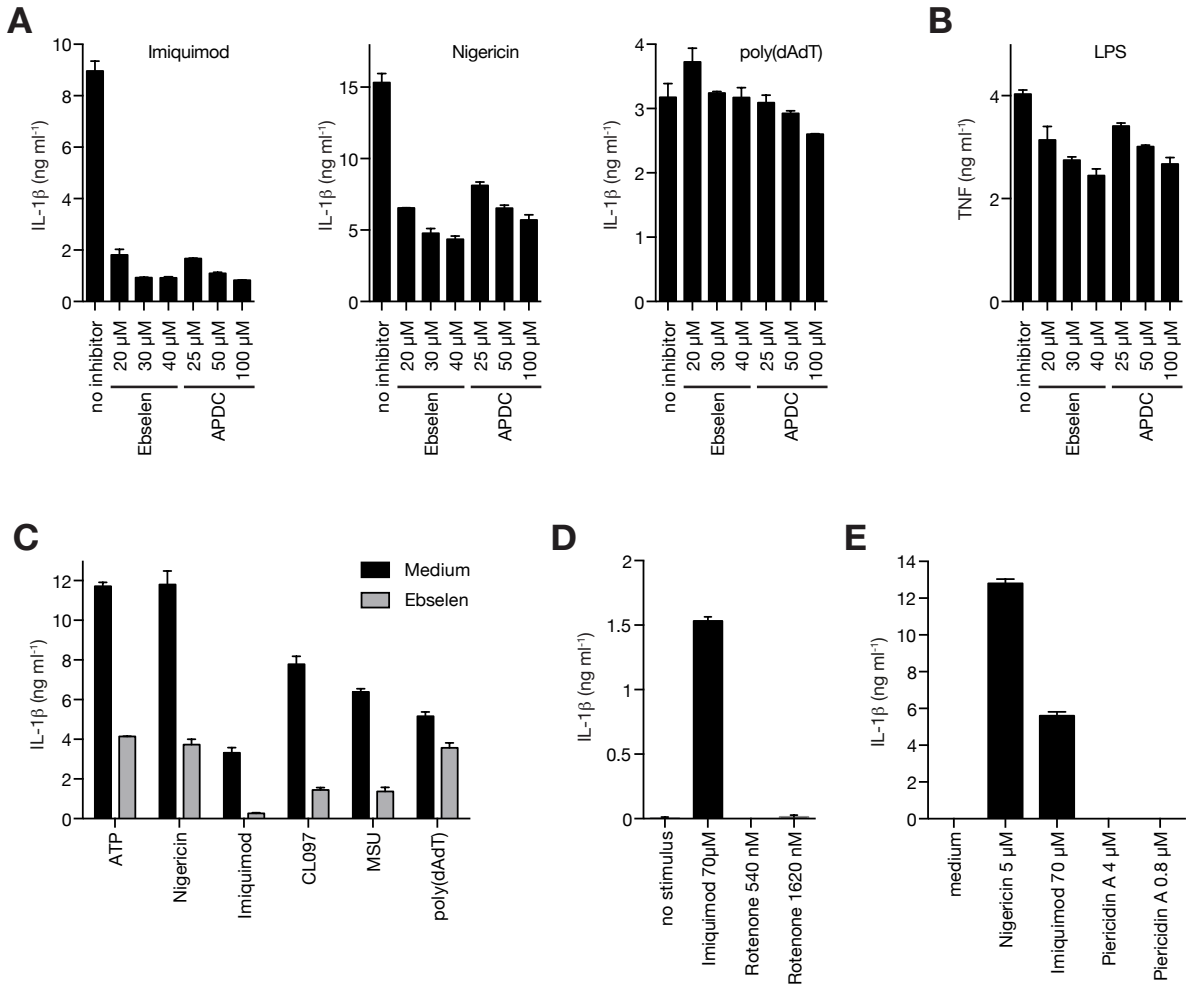


Figure 21: Imiquimod-induced inflammasome activation is sensitive to ROS-inhibitor treatment.

(A, B) LPS-primed (A) or unprimed (B) BMDCs were treated for 30 min with ebselen or APDC, and then stimulated with inflammasome activators (A) or LPS (B). IL-1β (A) or TNF (B) secretion was quantified from cell-free supernatants by ELISA

(C) LPS-primed BMDCs were treated for 30 min with ebselen, and then stimulated with different inflammasome activators as indicated. IL-1β secretion was quantified from cell-free supernatants by ELISA.

(D) LPS-primed BMDCs were stimulated with the indicated compounds for 3 h. IL-1β secretion was quantified from cell-free supernatants by ELISA.

(E) LPS-primed BMDCs were stimulated with the indicated compounds for 3 h. IL-1β secretion was quantified from cell-free supernatants by ELISA.

ELISA data are depicted as mean ± SEM of technical triplicates or quadruplicates.

Discussion

Imatinib activates the inflammasome in an NQO2-independent manner

Quinones are ubiquitous xenobiotics and environmental hydrocarbon combustion products. They are derivatives from aromatic compounds that contain an even number of –C(=O)- groups in place of -CH=. One-electron reduction of these compounds by cytochrome P450 reductases, cytochrome *b5* reductase, xanthine oxidoreductase, ubiquinone oxidoreductase, or other related enzymes leads to the formation of dangerous semiquinone radicals. In the presences of molecular oxygen, semiquinone radicals can trigger production of large amounts of ROS by redox cycling reactions (Kappus and Sies, 1981), which additionally leads to glutathione depletion (Di Monte et al., 1984; Thor et al., 1982), and formation of semiquinone adducts of DNA and other molecules (Joseph and Jaiswal, 1994). Menadione is a prototypical toxic quinone that undergoes redox cycling and is used as an experimental positive control for ROS production. The oxidative stress and damage to cellular macromolecules induced by these compounds acutely leads to cell death and tissue degeneration, but can also cause mutations and promote carcinogenesis (Schieber and Chandel, 2014).

In contrast to one-electron reduction of quinones, two-electron reduction produces hydroquinones, which can be innocuously excreted after conjugation to glucuronic acid or other molecules by phase II detoxification enzymes (Lind, 1985; Lind et al., 1982). This detoxification of quinones is performed by NAD(P)H:quinone oxidoreductase 1 (NQO1) and ribosyldihydronicotinamide:quinone oxidoreductase (NQO2). These homodimeric enzymes catalyse the obligatory two-electron reduction of quinones by a ping-pong mechanism. The nicotinamide-containing cofactor transfers a hydride ion to a tightly bound FAD to generate $FADH_2$. The nicotinamide-containing cofactor then vacates the

active site, allow the substrate to enter and accept the hydride from $FADH_2$ (Foster et al., 1999; Li et al., 1995).

NQO1 has been long known to protect mammals against the toxic effects of many quinone xenobiotics (Benson et al., 1980; Radjendirane et al., 1998). Antioxidant- and xenobiotic-response elements in the promoter of NQO1 allow for robust NQO1 upregulation upon sensing of various chemicals, which allows it to outcompete cytochrome P450 reductase for common quinone substrates (Nioi and Hayes, 2004; Talalay et al., 1988). For instance, the induction of NQO1 is thought to account for the anti-carcinogenic effects of isothiocyanates from broccoli (Zhang et al., 1992). However, NQO1 can also reductively activate certain quinone anticancer drugs such as mitomycin C and β-lapachone (Pink et al., 2000; Siegel et al., 1990). Like NQO1, NQO2 also protects against the carcinogenic effects of certain quinones, and reductively activates certain anticancer drugs such as CB1954 and apaziquone (Celli et al., 2006; Iskander et al., 2004). Despite their homology, there are differences in the regulation and co-factor, substrate, and inhibitor specificities of NQO1 and NQO2. While NQO1 uses NADH or NADPH as hydride donors, these are inefficient co-factors for NQO2. Instead, NQO2 uses rare, non-phosphorylated nicotinamide mononucleotides such as ribosyldihydronicotinamide or alkyldihydronicotinamides (Foster et al., 1999; Zhao et al., 1997). NQO2 also contains a Zn^{2+} or Cu^{2+} that promotes its stability but is not thought to be involved in catalysis (Foster et al., 1999). Furthermore, expression of NQO2 is poorly inducible by xenobiotics (Zhao et al., 1997).

To determine molecular targets of imiquimod/CL097 that might be required for their ability to activate the NLRP3 inflammasome, chemical proteomics was performed by Dr. Guillaume Médard and Prof. Bernhard Küster. NQO2 was identified as a binding

partner of imiquimod and CL097. To determine the involvement of NQO2 in NLRP3 inflammasome activation, NQO2-deficient mice and several NQO2 inhibitors were tested. NQO2 is inhibited by several drugs and dietary supplements, including nilotinib, imatinib (Bantscheff et al., 2007; Rix et al., 2007), chloroquine (Graves et al., 2002), resveratrol (Buryanovskyy et al., 2004), quercetin (Wu et al., 1997), and melatonin (Calamini et al., 2008). NLRP3 inflammasome activation in response to imiquimod and other activators was not impaired in the NQO2-deficient cells, or in cells treated with NQO2 inhibitors. However, we were surprised to observe that Bcr-Abl/NQO2 inhibitor imatinib triggered robust IL-1β secretion. Bosutinib, which inhibits Bcr-Abl but not NQO2, also caused IL-1β secretion. Like imatinib, nilotinib inhibits both Bcr-Abl and NQO2 (Rix et al., 2007; Winger et al., 2009), but did not trigger IL-1β secretion. Thus, the ability of tyrosine kinase inhibitors to inhibit NQO2 did not correlate with the ability to trigger IL-1β secretion. Furthermore, imatinib and bosutinib required ASC but not NQO2 for IL-1β secretion, demonstrating that NQO2 is dispensable of inflammasome activation by these compounds.

The tyrosine kinase inhibitor imatinib (Glivec) was the first molecularly-targeted cancer therapy. Imatinib was designed to target the fusion protein Bcr-Abl, which is derived from the t(9;22) chromosomal translocation (known as the Philadelphia chromosome) that causes up to 90% chronic myeloid leukemias and 15-25% of acute lymphoblastic leukemias (Heisterkamp et al., 1985). This fusion protein displays constitutive Abl kinase activity, and activates signalling pathways that lead to transformation of hematopoietic stem cells. Cancer cells, including those driven by Bcr-Abl, often display ‘oncogene addiction’, where their survival depends on the expression and activity of their dominant oncogene (Weinstein and Joe, 2008). Therapies such as

imatinib target this addiction, and trigger cancer cell death. Imatinib induces caspase-dependent apoptotic death in Bcr-Abl positive cancer cells (Dan et al., 1998; Gambacorti-Passerini et al., 1997), but several studies suggest that imatinib can also trigger other 'caspase-independent' cell death pathways (Kamitsuji et al., 2008; Lavallard et al., 2009; Okada et al., 2004; Yu et al., 2002). 'Caspase-independence' was determined in these studies using the pan caspase inhibitor zVAD-fmk, which can in fact induce necroptosis by preventing RIP1 and RIP3 inactivation by caspase-8 cleavage (Holler et al., 2000). Components of the necroptosis machinery can, under certain circumstances, promote NLRP3 activation (Kang et al., 2015; 2013; Wang et al., 2014; Yabal et al., 2014). Furthermore, we have found that zVAD-fmk fails to inhibit caspase-1-dependent cell death (pyroptosis), though it efficiently inhibits cytokine processing (Katharina Schneider, unpublished observations). Therefore, it will be interesting to investigate whether imatinib-induced cell death involves the NLRP3 inflammasome and/or necroptosis, and whether inflammasome activation by imatinib is relevant for its efficacy or adverse effects.

NLRP3 activation by imidazoquinolines is not related to their ability to activate TLR7/8

Though it was previously published that the established receptor for imiquimod, TLR7, is not required for NLRP3 activation by imiquimod (Kanneganti et al., 2006), it is shown here that Unc93b1 is also dispensable for imiquimod-induced NLRP3 activation. Since TLR3, 7, 8, 9 and 13 require Unc93b1 for signalling, this suggests that these TLRs and other putative Unc93b1-dependent molecules are also dispensable for imiquimod-induced NLRP3 activation. This was an important possibility to rule out because bacterial RNA-induced NLRP3 activation requires MyD88 and Unc93b1 but not TLR3, 7, or 9 (Eigenbrod et al., 2012). However, the authors of this paper also showed that the defect

observed in the Unc93b1 knockout could be rescued by providing an Unc93b1-independent priming agent, suggesting that they observed a priming defect, rather than a defect in the actual activation of NLRP3. In the experiments presented in this thesis, this potential confusion was avoided by always using LPS as an Unc93b1-independent priming agent.

In addition to imiquimod, the TLR7 agonist CL097 was identified in this study as a novel imidazoquinoline activator of the NLRP3 inflammasome. Most of the other imidazoquinoline TLR7/8 agonists tested did not trigger IL-1β secretion. The initial report in which imiquimod induced NLRP3 activation was described also found that R848 was an activator of NLRP3. The source of R848 in this paper is Coley Pharmaceuticals (Prof. Stefan Bauer, personal communication). We tested R848 from multiple commercial sources, but never observed IL-1β secretion in response to this compound. It is possible that the lot of R848 used by Kannaganti *et al.* was contaminated with NLRP3-activating imidazoquionlines. Recently, a library of imidazoquinolines was synthesized and tested for ability to activate TLR7/8 and ability to trigger secretion of IL-1β and other cytokines from unprimed cells (Schiaffo et al., 2014; Shi et al., 2012). Since they used unprimed cells, compounds that trigger IL-1β secretion in their experimental system are likely dual activators of TLR7 (to induce proIL-1β production) and NLRP3 (to trigger cleavage and secretion of IL-1β). Nonetheless, these studies reveal that there are likely many other imidazoquinolines that can activate the NLRP3 inflammasome. Furthermore, the results presented here and the cited studies strongly suggest that the structural determinants for TLR7/8 activation and NLRP3 activation by imidazoquinolines are distinct. We have established a collaboration with the authors of these studies, and will determine the structure-activity relationship of these compounds for NLRP3 activation in LPS-primed

cells. Given the structural characteristics of limited number of imidazoquinolines analysed so far, a low overall charge of the molecule may be a primary determinant of cellular/mitochondrial permeability, and by extension its ability to inhibit Complex I or activate NLRP3. We predict these studies will lead to identification of imidazoquinolines that can activate NLRP3, but are not ligands for TLR7/8. Furthermore, it will be interesting to determine whether an overlapping structure-activity relationship exists for Complex I inhibition and NLRP3 activation by imidazoquinolines.

Role of endolysosomal leakage and cathepsins in NLRP3 activation by imidazoquinolines

Uptake of inflammatory crystals and particles triggers leakage or even rupture of the endolysosome and NLRP3 activation. The finding that the lysomotropic peptide LeuLeuOMe triggers NLRP3 activation suggests that endolysosomal rupture is sufficient for NLRP3 activation. However, we have observed that LeuLeuOMe activates NLRP3 only at concentrations exceeding 0.5 mM, while 10 μM LeuLeuOMe is sufficient for complete endolysosomal rupture (data not shown, and unpublished findings of Dr. Ritu Mishra), suggesting that endolysosomal rupture is not sufficient. The evidence suggesting that concomitant liberation of cathepsins into the cytoplasm is necessary for NLRP3 activation is that the cathepsin B/L inhibitor CA074Me or genetic deficiency of cathepsin B blocks NLRP3 activation by particles and crystals (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008). However, cathepsin B-deficient cells displayed normal particle/crystal-induced NLRP3 activation in several papers, suggesting that other cathepsins or non-cathepsin targets of CA074Me may be involved in NLRP3 activation in response to particles (Dostert et al., 2009; Hari et al., 2014; Lima et al., 2013; Muñoz-

Planillo et al., 2013). Furthermore, a mechanistic explanation for how cathepsins activate NLRP3 is lacking.

It was surprising to observe that inflammasome activation by imiquimod or CL097 was inhibited by CA074Me. Unlike NLRP3 activating particles (Hornung et al., 2008), these compounds are soluble and do not require phagocytosis or endolysosomal acidification for NLRP3 activation, so it is not immediately obvious how they would disrupt the endolysosome to allow for cathepsin release. Inflammasome activation in response to imiquimod was partially reduced in cells deficient for cathepsins B and L, the major targets of CA074Me (Gewies and Grimm, 2003; Montaser et al., 2002; Steverding, 2011). The further reduction of inflammasome activation in cathepsin B/L double knockout cells treated with CA074Me demonstrates that this inhibitor has additional targets that may even contribute more to NLRP3 activation than cathepsin B and L together, which is supported by a recent publication (Orlowski et al., 2015).

Dr. Ritu Mishra and Dr. Oliver Gorka provided results (included in Appendix 1) from acridine orange-stained inflammasome-deficient cells that suggest that imiquimod, CL097 and gardiquimod (but not R848) induce endolysosome leakage. This might explain our finding that imiquimod, CL097, and gardiquimod are effective at inducing TLR7-dependent TNF production at low doses, but fail to trigger TNF production at high doses (>10 μg ml^{-1}) that trigger endolysosomal leakage (data not shown, and unpublished observations of Tamara Cikovic). This occurs even in NLRP3-deficient cells, which rules out that it is a consequence of pyroptosis. Gardiquimod is not a Complex I inhibitor, and high concentrations of gardiquimod also fail to induce TNF, so the inhibition of TNF secretion at high doses of imiquomod or CL097 is probably not a consequence of Complex I inhibition. Imiquimod can suppress CpG DNA-induced TLR9 signalling in a

TLR7-independent manner (Butchi et al., 2010), which may be related to the phenomenon that we observe. Furthermore, gardiquimod induces endolysosomal leakage but does not activate NLRP3. Together with our LeuLeuOMe findings described above, this suggests that endolysosomal leakage is not sufficient for NLRP3 activation. Another possibility that would be worth addressing experimentally is whether these compounds might inhibit endolysosomal acidification, as an inhibitor of endolysosomal acidification would be expected to have the same effect on the relative intensities of the red (acidic, lysosomal) and green (neutral pH, cytoplasm/nucleus) acridine orange signals, and on TNF secretion as the effects that we observed in response to imiquimod, CL097, and gardiquimod.

There have been dozens of follow-up studies since a role for endolysosomal leakage and cathepsins in NLRP3 activation by particles and crystals was initially proposed. Unfortunately, these studies have not resulted in substantial progress in our understanding of how NLRP3 is activated by endolysosomal leakage. Several experimental barriers and misconceptions may be responsible for these difficuties. Experiments, including our own, that implicate cathepsins B and L in NLRP3 activation by use of CA074Me should be interpreted carefully. CA074Me causes near complete inhibition of cathepsin B at 1-5 μM but causes only minor inhibition of NLRP3 activation at these doses (Mihalik et al., 2004; Orlowski et al., 2015). In contrast, many inflammasome studies use this inhibitor at concentrations exceeding 50 μM. At these concentrations, CA074Me has effects on many additional proteases and cellular processes; it stabilizes the endolysosome, inhibits apoptotic and necrotic cell death induced by particles, and can even inhibit NLRP3 activation by nigericin (Lima et al., 2013; Mihalik et al., 2004). The novel pan-cathepsin inhibitor K777 (Orlowski et al., 2015),

which lacks many of these off-target effects but still inhibits NLRP3 activation by particles, could be used instead of CA074Me but it is not yet commercially available.

There are also several difficulties in assessing the role of cathepsins using cathepsin-deficient mice. On the one hand, the role of a given cathepsin can be difficult to ascertain because of functional compensation by other cathepsins (Orlowski et al., 2015). Conversely, loss of multiple cathepsins can impair homeostatic signalling in immune cells, or is even neonatal lethal in the case of cathepsin B/L double knockout (Felbor et al., 2002; Orlowski et al., 2015; Tholen et al., 2014).

Another issue that confounds many studies is the use of inflammasome-proficient (wild-type) cells for the study of pathways upstream of NLRP3. Activation of caspase-1 in response to NLRP3 activation destabilizes the endolysosome (Figure 22), perhaps as part of the pyroptotic death program (Fujisawa et al., 2007; Heid et al., 2013; Lima et al., 2013). While NLRP3-induced endolysosomal destabilization may further promote activation of NLRP3, examination of endolysosomal integrity should also be performed in NLRP3/ASC/caspase-1-deficient cells or in the presence of the NLRP3 inhibitor MCC950 to determine conclusively whether activator itself triggers endolysosomal leakage. We find that imiquimod, CL097, and gardiquimod directly (*i.e.* not indirectly via inflammasome activation) trigger changes in the acidic compartment of the cell. However, we are currently examining whether this change results from endolysosomal leakage, from impairment of endolysosomal acidification, or a direct effect of these compounds on the acridine orange dye. In summary, the results presented here suggest that endolysosomal leakage and/or cathepsins may be involved in NLRP3 activation by imidazoquinolines. A mechanistic explanation for how endolysosomal leakage and/or cathepsins activate

NLRP3 will be required to conclusively demonstrate the importance of these pathways in NLRP3 activation.

K^+ Efflux is not a universal requirement for NLRP3 activation

K^+ efflux is accepted as a universal requirement for NLRP3 inflammasome activation (Muñoz-Planillo et al., 2013; Rivers-Auty and Brough, 2015). Indeed most NLRP3 activators, including ATP, nigericin, and particles require K^+ efflux for NLRP3 activation (Muñoz-Planillo et al., 2013). It was published recently that non-canonical activation of NLRP3 downstream of caspase-11 also requires K^+ efflux (Baker et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015). However, spontaneously active, disease-associated mutations in NLRP3 trigger IL-1β secretion that is not inhibited by extracellular K^+ (Muñoz-Planillo et al., 2013). Here, it is found that imiquimod and CL097 do not induce K^+ efflux, and do not require K^+ efflux for NLRP3 activation. This is the first demonstration that K^+ efflux is not a universal requirement for NLRP3 activation.

At high (>80 mM) concentrations of extracellular KCl, a slight reduction in IL-1β secretion in response to imiquimod was observed. Indeed, two previous publications concluded that imiquimod-induced NLRP3 activation was K^+ efflux-dependent by using extracellular KCl concentrations exceeding 100 mM (Nakamura et al., 2012; Pétrilli et al., 2007). There are several possible reasons why we arrive at a different conclusion. Non-physiological extracellular concentrations of KCl may have unspecific effects on cells that result in suppression of many pathways, including NLRP3 activation. In agreement with this, suppression of TNF production and AIM2 inflammasome activation was observed in response to extracellular KCl. Furthermore, changes in cytoplasmic K^+ concentrations may have a direct effect on ASC oligomerization downstream of NLRP3 activation.

Oligomerization of recombinant ASC *in vitro* is suppressed by high K^+ concentrations (Fernandes-Alnemri et al., 2007). This would explain the observation that IL-1β secretion induced by the ASC-dependent dsDNA-binding AIM2 inflammasome is slightly reduced in the presence of high extracellular KCl, while activation of the NAIP/NLRC4 inflammasome, which does not strictly require ASC (Broz et al., 2010; Gross et al., 2012; Mariathasan et al., 2004) was not affected by extracellular KCl.

However, the strongest argument in favour of the conclusion that K^+ efflux is not required for imiquimod-induced NLRP3 activation is that imiquimod does not trigger K^+ efflux. The only situation where we observed K^+ efflux after imiquimod stimulation was in inflammasome-proficient cells, where cellular K^+ content dropped by only 10%. In inflammasome-proficient cells, pyroptosis impairs plasma membrane integrity, leading to leakage of small molecules and ions such as K^+ (Fink and Cookson, 2006). K^+ efflux induced by K^+-free medium or by the K^+-selective ionophore valinomycin (nigericin is a K^+/H^+ ionophore) is sufficient to activate NLRP3 (Muñoz-Planillo et al., 2013; Perregaux and Gabel, 1994). Furthermore, it was recently demonstrated that caspase-11-dependent pyroptosis in response to intracellular LPS activates NLRP3 by inducing K^+ efflux (Baker et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015). Collectively, these findings would support a model where pyroptosis-induced impairment of plasma membrane integrity can provide a positive feedback loop for NLRP3 activation, even in response to activators like imiquimod and CL097 that can trigger NLRP3 activation without directly inducing K^+ efflux (Figure 22).

A major unanswered question is how K^+ efflux triggers activation of NLRP3 in response to conventional activators. Imiquimod and CL097 may bypass the requirement for K^+ efflux by triggering a signalling pathway that converges with the pathway that is

activated downstream of K^+ efflux. Alternatively, imiquimod and CL097 may activate pathways unrelated to K^+ efflux to trigger NLRP3 inflammasome activation through a distinct mechanism. For instance, imiquimod/CL097-induced ROS production by Complex I inhibition may provide a strong enough signal for NLRP3 activation that K^+ efflux is not necessary. However, this K^+ efflux-independent pathway for NLRP3 activation by imiquimod or CL097 is still inhibited by MCC950, a potent, specific, and universal inhibitor of NLRP3 activation that does not interfere with K^+ efflux (Coll et al., 2015). Further study of the molecular targets of imiquimod and MCC950 may reveal the mechanism of NLRP3 activation.

During the preparation of this thesis, another study has come to light suggesting that K^+ efflux is not a universal requirement for NLRP3 activation. Prof. V. Hornung described at the TOLL 2015 meeting in Marbella, Spain that NLRP3 activation in human monocytes is unconventional in that: LPS is sufficient for activation (Netea et al., 2009), ASC specks do not form, and it is not inhibited by extracellular KCl. This unconventional activation of NLRP3 is also inhibited by MCC950.

Imiquimod and CL097 are Complex I inhibitors

Here it is demonstrated that imiquimod and CL097 suppress respiration in a variety of human and murine cells. Importantly, the suppression of respiration in myeloid cells is not inflammasome-dependent, indicating that it is not simply a consequence of pyroptosis. However, imidazoquinolines that do not activate NLRP3 (R848, gardiquimod, and CL075) also failed to suppress respiration. This suggested that the ability of imidazoquinolines to suppress respiration may be related, perhaps even causally, to the ability to activate NLRP3. Experiments in permeabilized cells demonstrated that

imiquimod and CL097, but not imidazoquinolines incapable of NLRP3 activation, specifically inhibit Complex I activity, without influencing Complex II or downstream components of the electron transport chain. In line with the ability of these compound to inhibit Complex I, an increase in the NAD^+/NADH ratio and a marked decrease in ATP levels were observed in BMDMs.

Perhaps the most convincing evidence that imiquimod and CL097 inhibit Complex I comes from experiments in fibroblasts expressing Ndi1. Ndi1 is a homodimeric, rotenone-insensitive NADH:quinone oxidoreductase from *Saccharomyces cerevisiae* (de Vries and Grivell, 1988). It is localized peripherally to the matrix side of the inner mitochondrial membrane, and like Complex I catalyses the transfer of two electrons from NADH to ubiquinone. In contrast to Complex I, Ndi1 does not pump protons, and does not produce ROS (Seo et al., 1998). Because Ndi1 does not pump protons, its contributes to ATP production only by reducing ubiquinone (Bai et al., 2001). Expression of Ndi1 rescues the detrimental effects of genetic or biochemical Complex I-deficiency *in vitro* (Bai et al., 2001; Seo et al., 1998; 1999; 2000) and *in vivo* (Barber-Singh et al., 2011; Perry et al., 2011). Ndi1 expression is used to demonstrate the specificity of respiratory chain inhibitors for Complex I (Wheaton et al., 2014). Ndi1 expression in fibroblasts rescues the decrease in respiration observed in response to imiquimod and CL097, confirming that they are inhibitors of Complex I activity. In future studies, it will determined whether imiquimod binds directly to Complex I, for instance by in gel activity assay (Wittig et al., 2007). The potential relevance of Complex I inhibition in the anti-tumour efficacy is addressed later in the Discussion.

Role of ROS in NLRP3 activation by imiquimod

The purpose of this study was to determine the mechanism of NLRP3 activation by imiquimod, which was found to be a Complex I inhibitor. Complex I is thought to the most important mitochondrial source of ROS *in vivo* (Murphy, 2009). There are two modes of ROS production by Complex I. When NAD^+/NADH ratio is low, the flavin site of Complex I becomes fully reduced by excess NADH and reacts with O_2 to generate superoxide. This occurs when electron transport is operating in the forward (*i.e.* normal) direction, but is then blocked by an inhibitor (classically by rotenone) or defective components of the respiratory chain (Ishikawa et al., 2008; Murphy, 2009; St-Pierre et al., 2002). Interestingly, we observed a dramatic decrease in the NAD^+/NADH ratio in response to imiquimod, which suggests that imiquimod might induce this 'forward' mode of ROS production by Complex I. The second mode of superoxide production occurs during reverse electron transport (RET), where high succinate concentrations force Complex II to transfer electrons to ubiquinone to generate ubiquinol, which then forces electrons back to Complex I. ROS production during RET is blocked by rotenone, a Complex I inhibitor that binds to the ubiquinone site. Both the flavin site and the ubiquinone-binding site of Complex I can produce ROS during RET. ROS production via RET drives oxidative damage during ischemia-reperfusion injury (Chouchani et al., 2014).

Given that imiquimod and CL097 inhibit Complex I activity, that Complex I inhibition by other agents triggers ROS (Murphy, 2009; St-Pierre et al., 2002), and that NLRP3 activation by imiquimod is inhibited by antioxidants, one hypothesis is that imiquimod causes ROS production from Complex I and this ROS activates NLRP3 (Figure 22). Indeed, imiquimod and CL097, but not R848, cause an elevation in mitochondrial ROS levels as measured by MitoSOX (Dr. Ritu Mishra, unpublished observations). A

problem with this theory is that under the same experimental conditions where we see NLRP3 activation by the Complex I inhibitor imiquimod, we do not observe NLRP3 activation by the classical Complex I inhibitors rotenone or piercidin A. This is in contrast to the original reports implicating ROS in NLRP3 activation (Nakahira et al., 2011; Zhou et al., 2010b), but consistent with subsequent studies where rotenone did not trigger inflammasome activation (Iyer et al., 2013; Juliana et al., 2012; Muñoz-Planillo et al., 2013; Youm et al., 2015). This suggests that Complex I-derived ROS may be necessary but is not sufficient for NLRP3 activation in response to rotenone.

Another possible explanation for the ability of imiquimod but not rotenone to activate NLRP3 is that imiquimod may not trigger ROS by the same mechanism as rotenone. Imiquimod forms an aromatic stack with the isoalloxazine moiety of the flavin cofactor in the active site of NQO2. It may also bind the flavin site of Complex I, in contrast to rotenone and other established Complex I inhibitors which binds the ubiquinone site (Degli Esposti, 1998; Okun et al., 1999). Its binding of the flavin site may alter the quantity or species of ROS produced at that site when the NAD^+/NADH ratio is low. Alternatively, imiquimod may trigger ROS production at the ubiquinone site, where it could more easily diffuse through the outer mitochondrial membrane to trigger NLRP3 activation.

Imiquimod may even prevent NADH oxidation and ROS production at Complex I and cause excess NADH to trigger ROS production by other flavin-containing oxidoreductases instead. Though we established the ability of imiquimod to bind to the flavin-containing oxidoreductase NQO2, NQO2 was dispensable for NLRP3 activation by imiquimod, CL097, imatinib, and other conventional activators of NLRP3. The chemical proteomics screen for molecular targets of imiquimod did identify NQO2, but failed to

detect other targets of imiquimod such as TLR7 and Complex I. This suggests that imiquimod may have additional cellular/mitochondrial targets that were not detected by the screen. Given that Complex I and NQO2 are flavoproteins, and that imiquimod binds to the flavin site of NQO2, a reasonable conjecture is that imiquimod binds to other flavoprotein(s). ROS production or other consequences of imiquimod action on these proteins may be important for its ability to activate NLRP3. Recently, the flavoprotein xanthine oxidase has been implicated in NLRP3 activation (Ives et al., 2015). ROS production and IL-1β secretion triggered by particles (and to a lesser extent by ATP or nigericin) was suppressed by the xanthine oxidase inhibitor febuxostat or by xanthine oxidase knockdown. This suggests that xanthine oxidase may be a source of NLRP3-activating ROS. It will be interesting to determine whether xanthine oxidase activity is involved in NLRP3 activation by imiquimod, CL097 and imatinib.

The molecular basis of the effect of ROS on NLRP3 is not known. Physiological levels of ROS cause reversible oxidation of the thiol group of cysteine residues, which is a primary mechanism of redox signalling (Schieber and Chandel, 2014). Oxidized thiols can react with other thiols (to form disulfide bonds), amides, or glutathione (Finkel, 2012). Similarities can be drawn between the NLRP3 inflammasome and the apoptosome in that both involve oligomerized receptors (NLRP3 and APAF1, respectively) and activate caspases. ROS directly modify APAF1, and inhibition of APAF1 oxidation prevents apoptosome formation (Sato et al., 2004). The LRRs of NLRP3 are particularly enriched with cysteine residues that may be prone to oxidation. This might trigger conformational changes in NLRP3 that could promote its activation. Alternatively, NLRP3 may not sense ROS directly but instead may be activated by a redox-sensitive molecule/protein. Previous studies have suggested a role for the ROS-sensitive protein thioredoxin-

interacting protein (TXNIP) in NLRP3 activation. Inflammasome activators induced ROS-dependent association of TXNIP and NLRP3, and TXNIP deficiency impaired NLRP3 inflammasome activation (Zhou et al., 2010a). However, other reports were unable to reproduce these findings (Masters et al., 2010) suggesting that TXNIP may modulate NLRP3 activation but is not absolutely required.

If mitochondria are indeed the source of ROS necessary for NLRP3 activation, then an important issue is how NLRP3 comes in sufficient proximity to this source, since some types of ROS are poorly diffusible. It has been proposed that NLRP3 relocalization from the cytoplasm to the mitochondria is necessary for its efficient activation (Misawa et al., 2013; Zhou et al., 2010b). A role for the mitochondrial innate immune adaptor MAVS in the mitochondrial localization of NLRP3 has been suggested (Subramanian et al., 2013). The data presented here and by several others suggests that MAVS is not required for NLRP3 activation by most activators, but may be required for NLRP3 activation by poly(I:C) (Allam et al., 2014; Franchi et al., 2014; Park et al., 2013). In a compound library screen, microtubule polymerization inhibitors reduced IL-1β secretion in response to nigericin (Misawa et al., 2013). Though we observed a slight reduction in nigericin-induced IL-1β secretion with these inhibitors they did not influence imiquimod-induced IL-1β secretion. Therefore, our results do not support the conclusion that microtubule polymerization is necessary for NLRP3 activation.

In summary, the mechanism by which ROS activates NLRP3, and the molecular source of this ROS produced by most NLRP3 activators remains unknown. However, since we and others do not observe inflammasome activation in response to rotenone, a classic ROS-inducing inhibitor of Complex I, we speculate that Complex I-derived ROS may be necessary but is not sufficient for NLRP3 activation (Iyer et al., 2013; Juliana et

al., 2012; Muñoz-Planillo et al., 2013; Youm et al., 2015). Our data suggest that a flavoprotein target of imiquimod may provide a source of ROS necessary for NLRP3 activation. This protein may be Complex I, but it is unlikely to be NQO2 because NQO2-deficient mice show intact NLRP3 activation in response to imiquimod and other activators. In future experiments, it will be important to determine whether Complex I is indeed the source of ROS induced by imiquimod. We are currently generating the tools that will allow us to overexpress Ndi1 in myeloid cells. This should rescue the drop in mitochondrial respiration by imiquimod and CL097, as it does in fibroblasts treated with these compounds. If Complex I is the source of ROS induced by imiquimod, Ndi1 expression should prevent imiquimod-induced ROS production. However, other sequelae of Complex I inhibition, such as insufficiency of NAD^+ or ATP, may contribute to NLRP3 activation by these compounds. Indeed, NLRP3 activation by other factors coincides with a drop in cellular NAD^+ and ATP, though it uncertain whether this is a cause or effect of NLRP3 activation in those studies (Misawa et al., 2013; Nomura et al., 2015). If either ROS or NAD^+ insufficiency are necessary for NLRP3 activation by imiquimod, Ndi1 expression should prevent NLRP3 activation by imiquimod (Figure 22). These studies should provide a conclusive answer as to whether Complex I and ROS are necessary for NLRP3 activation by imiquimod. This could also be investigated by testing whether Complex I-deficient patients have alteration in NLRP3 activation by imiquimod or other NLRP3 activators.

Is imiquimod-induced NLRP3 activation relevant *in vivo*?

NLRP3 activation is a TLR7-independent effect of imiquimod that is not shared with R848, and which could be involved in its *in vivo* effects. Imiquimod is applied to the

skin, and within the skin, the inflammasome can be activated in not only myeloid cells, but also keratinocytes. The NLRP3 inflammasome in dermal myeloid cells could be activated by imiquimod to trigger secretion of IL-1 in the skin. Human keratinocytes express most components and substrates of the inflammasome, including NLRP3, ASC, caspase-1, proIL-1α and proIL-1β. In contrast, murine keratinocytes are not thought to express NLRP3 or proIL-1β (Drexler et al., 2012; Guarda et al., 2011; Yazdi et al., 2010), though there are contradictory reports (Feldmeyer et al., 2007). Imiquimod triggers the secretion of IL-1α but not IL-1β from human and murine keratinocytes (Barland et al., 2004; Uribe-Herranz et al., 2013). However, IL-1α is a danger signal that can be secreted by dying cells in the absence of inflammasome activation (Chen et al., 2007; Gross et al., 2012). The fact that murine keratinocytes do not express NLRP3 but still secrete IL-1α in response to imiquimod speaks against a role for the inflammasome in this response. In other words, IL-1α secretion by keratinocytes may be a passive consequence of imiquimod/Aldara-induced non-pyroptotic death in these cells. In theory, the imiquimod-induced release of ATP by dying cells could trigger a positive feedback loop for IL-1 secretion by activating NLRP3 in other resident and recruited myeloid cells.

The IL-1 receptor is ubiquitously expressed, and can trigger local and systemic reactions in response to its ligands (Sims and Smith, 2010). IL-1 is particularly important in the development of T_H17 responses (Acosta-Rodriguez et al., 2007). T_H17 responses drive psoriasis in humans (Di Cesare et al., 2009) and imiquimod/Aldara-induced psoriasis-like inflammation in mice (van der Fits et al., 2009). However, in addition to imiquimod itself, the isostearic acid carrier in the topical formulation of imiquimod (Aldara) also has TLR7-independent effects that include inflammasome activation (Walter et al., 2013). The finding that Aldara-induced psoriasis-like inflammation in mice is IL-1-

dependent but NLRP3 inflammasome-independent (Rabeony et al., 2015) and TLR7-independent (Walter et al., 2013) suggests that TLR7- and NLRP3-independent secretion of IL-1 in response to both imiquimod and isostearic acid drive the IL-1-dependent inflammation in this model.

The NLRP3 inflammasome may also play a role in the anti-tumour effects of imiquimod. In a mouse xenograft tumour model, the efficacy of oxaliplatin as a chemotherapeutic agent requires the NLRP3 inflammasome (Ghiringhelli et al., 2009). Oxaliplatin-induced cell death was accompanied by ATP release, which activates the NLRP3 inflammasome in infiltrating DCs. NLRP3-dependent IL-1 secretion was required to trigger an adaptive immune response against the tumour cells (Ghiringhelli et al., 2009). NLRP3 is reported to have anti-tumour function in other mouse models, including colitis-associated cancer (Allen et al., 2010; Zaki et al., 2010). The NLRP3 inflammasome can also have a tumour-promoting role. For instance, the inflammasome in myeloid cells promotes tumour development in inflammation-induced skin cancer in mice (Drexler et al., 2012). However, this is counteracted by a non-inflammasome, tumour suppressor role of ASC in keratinocytes (Drexler et al., 2012). Therefore, the role of the NLRP3 inflammasome and IL-1 in tumourigenesis and anti-tumour therapy is highly context-dependent (Menu and Vince, 2011). It is possible that imiquimod-induced NLRP3 activation and IL-1 secretion may influence the anti-tumour efficacy of imiquimod, but this has yet to be investigated.

A potential role for Complex I in the clinical efficacy of imiquimod

Early studies of imiquimod argued that it had no direct antiviral or anti-tumour activity (Miller et al., 1999; Testerman et al., 1995), and assumed that its efficacy was due

to induction of type I IFN (Miller et al., 1999; Testerman et al., 1995; Tomai et al., 1995). TLR7-dependent effects of imiquimod are indeed important for its efficacy (Drobits et al., 2012; Sidky et al., 1992). However, it has been known for many years that in terms of type I IFN production and NF-κB activation, R848 is effective at a 100-fold lower dose than imiquimod, both *in vitro* and *in vivo* (Tomai et al., 1995). Clinical studies of R848 for cutaneous viral infections, skin cancer, and vaccine adjuvant activity are ongoing. Despite its inferior ability to activate TLR7, imiquimod is the only FDA-approved imidazoquinoline. This raises the question whether imiquimod might have TLR7/IFN independent effects that contribute to it clinical success in the treatment of viral and malignant skin conditions.

Aside from NLRP3 activation, imiquimod has several other cellular effects that are thought to be independent of TLR7 activation and IFN production, and not shared with R848. It can modulate the activity of adenosine receptors (Kan et al., 2012; Schön et al., 2006; Wolff et al., 2013), trigger action potentials and release of ER calcium stores in dorsal root ganglia neurons (Hwang et al., 2014; Kim et al., 2011; Lee et al., 2012b), induce autophagy (Huang et al., 2010), and inhibit prion propagation (Oumata et al., 2013). Modulation of adenosine receptor signalling by imiquimod represses activation of oncogenic GLI transcription factors downstream of Hedgehog signalling (Wolff et al., 2013). Given the tumour-promoting role of the Hedgehog/GLI axis in basal cell carcinoma and other tumours, inhibition of Hedgehog signalling may contribute to the anti-tumour effect of imiquimod (Gruber et al., 2014).

Imiquimod can inhibit proliferation and induce cell death (Chen et al., 1988; Huang et al., 2010; 2012; Meyer et al., 2003; Schön et al., 2003; 2004; Smith et al., 2007; Sohn et al., 2014; Zagon et al., 2008). R848 lacks this activity (Huang et al., 2010; 2012; Schön et

al., 2003; 2004). 5-10 μg ml^{-1} of imiquimod is sufficient to induce death, and cytotoxicity increases up to doses of 200 μg ml^{-1}. These concentrations of imiquimod are 1000 fold lower than what is applied topically (5% Aldara cream = 50 mg g^{-1}) (Schön et al., 2003). Indeed, apoptotic cells are observed in human skin treated with Aldara (Schön et al., 2003). Imiquimod-induced cell death has the features of apoptosis, including TUNEL positivity, DNA fragmentation, Annexin V positivity, caspase-3 cleavage, and sensitivity to caspase inhibitors (Huang et al., 2010; Meyer et al., 2003; Schön et al., 2003). Cytochrome *c* release and cell death in response to imiquimod are inhibited by Bcl2 overexpression, whereas death receptor inhibition does not prevent cell death in response to imiquimod (Schön et al., 2003; 2004). Therefore, imiquimod triggers growth arrest and mitochondrial apoptosis, and it is possible if not likely that this contributes to its anti-tumour effects. However, a mechanistic explanation for these effects is so far lacking.

In most cells glucose is converted to pyruvate via glycolysis, and in the presence of sufficient O_2, pyruvate is further oxidized by mitochondria for the efficient generation of ATP by oxidative phosphorylation. Under hypoxic conditions, or as a result of active metabolic reprogramming, pyruvate is instead converted to lactate with the concomitant regeneration of NAD^+ that is necessary for further glycolytic ATP production (Fantin et al., 2006). The active shift to aerobic glycolysis is also known as the Warburg effect, which was first observed in cancer cells but which also takes place in certain immune cells and presumably other cell types (O'Neill and Hardie, 2013; Warburg et al., 1927). Thus, it was long presumed that within tumours, mitochondria were essentially asleep on the job: not contributing to ATP production, and not killing the tumour cells from within unless coerced by drugs.

Presumably on account of high glucose consumption and poor perfusion, concentrations of many nutrients but especially of glucose are limiting in the tumour microenvironment (Gullino et al., 1967; Hirayama et al., 2009; Urasaki et al., 2012). In a screen for genes required for cancer cell proliferation in low glucose conditions, subunits of Complex I were the most significant hits ($p<9x10^{-49}$), followed by other components of the electron chain (Birsoy et al., 2014). This demonstrates that the electron transport chain, especially Complex I activity, is a critical determinant of the ability of cancer cells to survive and continue proliferating when glucose is limiting (Birsoy et al., 2014). This is supported by numerous previous studies showing growth arrest in cells and tumours upon genetic or pharmacological inhibition of Complex I or other electron transport chain components (Cunningham et al., 1995; Fendt et al., 2013; Löffer and Schneider, 1982; Wheaton et al., 2014). ATP insufficiency and enhanced ROS production have been proposed as an explanation for why Complex I/electron transport chain inhibition results in growth arrest (Wallace, 1999). In normal cells, oxidative phosphorylation is, as compared to glycolysis, an efficient and preferred means of generating ATP. But the reliance of glycolytic cancer cells on mitochondrial respiration is counterintuitive from an energy/ATP-centric viewpoint. Indeed, recent studies demonstrate that a critical role of Complex I activity and the electron transport chain in proliferating cells is NAD^+ regeneration (Birsoy et al., 2015; Sullivan et al., 2015). While NAD^+ is an electron acceptor in many biosynthetic reactions, its critical function in proliferating cells is to support aspartate synthesis (Birsoy et al., 2015; Sullivan et al., 2015). Aspartate is needed as a building block for proteins, but also serves as an intermediate in purine nucleotide synthesis. These findings explain the phenomenon of pyruvate auxotrophy in cells with

genetic defects in the electron transport chain, since pyruvate allows for lactate dehydrogenase-mediated regeneration of NAD^+ (King and Attardi, 1989).

The data presented here demonstrate that imiquimod and CL097 are specific inhibitors Complex I. In myeloid cells, imiquimod and CL097 impair respiration, NAD^+ regeneration, and ATP synthesis, and trigger ROS production and ROS-dependent NLRP3 inflammasome activation. As already discussed, a priority is to determine whether Complex I inhibition is a determinant of inflammasome activation by imiquimod and CL097 by performing rescue experiments with Ndi1. However, the most clinically significant implication of the discovery that imiquimod is a Complex I inhibitor is that it provides a potential mechanistic explanation for its direct anti-proliferative and pro-apoptotic effects. The clinical efficacy of imiquimod may therefore reflect a Complex I-dependent effect on keratinocytes and a local induction of TLR7-dependent immune responses (Drobits et al., 2012) (Figure 22).

The anti-cancer effect of Complex I inhibitors in humans is not without precedence. The anti-diabetic drug metformin, whose mechanism of action was unknown for many years after it entered the clinic, was shown in retrospective studies to reduce the risk of cancer (Bowker et al., 2006; Evans et al., 2005). It is now known that metformin is a Complex I inhibitor (El-Mir et al., 2000; Owen et al., 2000), and that this underlies its anti-tumour effect (Wheaton et al., 2014).

While the finding that imiquimod inhibits the electron transport chain could explain an anti-proliferative effect resulting from NAD^+/aspartate-insufficiency, it does not fully account for its ability to induce apoptosis. In addition to the metabolic consequences of Complex I inhibition, imiquimod-induced ROS production may be necessary to push cells from growth arrest to apoptosis. Indeed, the Complex I inhibitor rotenone also inhibits cell

proliferation and spontaneous carcinogenesis in mice (Cunningham et al., 1995), and its ability to induce apoptosis is ROS-dependent (Li, 2002). The ability of rotenone to inhibit Complex I and trigger ROS production has been implicated in dopaminergic cell death and Parkinsonism that result from exposure to rotenone, a common piscicide (Barber-Singh et al., 2009; Betarbet et al., 2000; Marella et al., 2007; Sherer et al., 2003). In contrast, metformin inhibits Complex I but does not trigger ROS (Algire et al., 2012; Kelly et al., 2015; Ouslimani et al., 2005) and protects against Parkinsonism in mice and humans (Patil et al., 2014; Wahlqvist et al., 2012). Thus, the ability of Complex I inhibitors to trigger ROS production may be a determinant of their ability to kill tumour cells and dopaminergic neurons. Though imiquimod triggers ROS production, there is no evidence that it increases the risk of Parkinson's disease. However, an effect of imiquimod on dermal nociceptive neurons has been implicated in itch, pain, and psoriasis-like inflammation that are common adverse effects of topical imiquimod (Hwang et al., 2014; Kim et al., 2011; Riol-Blanco et al., 2014).

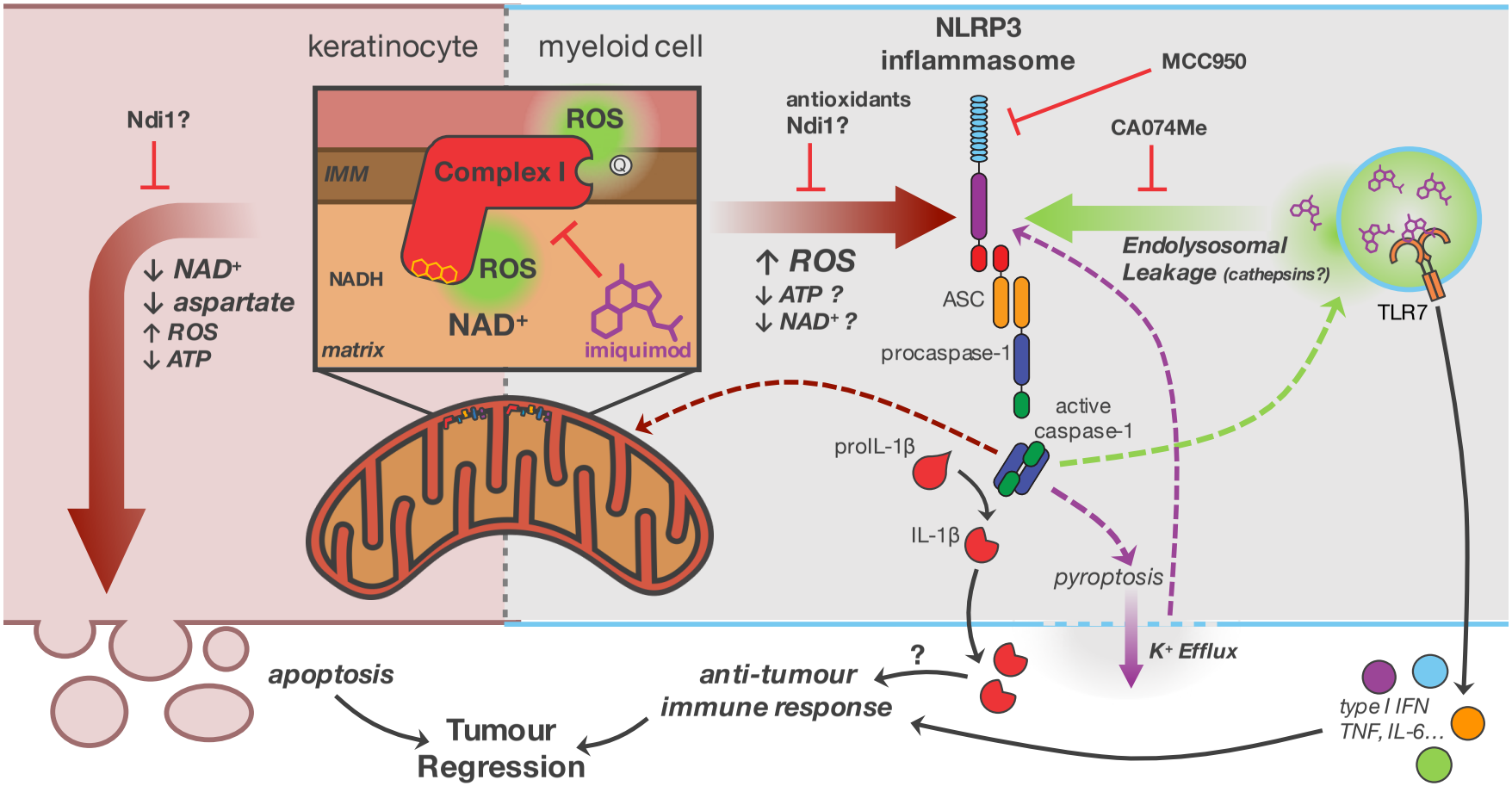


Figure 22: Proposed mechanism of the anti-tumour effects of imiquimod. In myeloid cells, imiquimod activates the NLRP3 inflammasome for the cleavage and secretion of IL-1β. Imiquimod induces endolysosomal leakage, and genetic or pharmacological (*i.e.* with CA074Me) inhibition of cathepsins B and L suppresses NLRP3 activation. Therefore, NLRP3 activation by imiquimod may require endolysosomal leakage. K^+ efflux is dispensable for NLRP3 activation by imiquimod. Imiquimod does not induce K^+ efflux, and NLRP3 activation by imiquimod is not inhibited by elevated extracellular K^+. Imiquimod inhibits mitochondrial Complex I, resulting in an increase in mitochondrial ROS production, and a decrease in cellular NAD^+ and ATP levels. Antioxidants suppress imiquimod-induced inflammasome activation, suggesting that Complex I-derived ROS are necessary for NLRP3 activation by imiquimod, and this may be suppressed by expression of Ndi1. Caspase-1 triggers pyroptosis, which allows for efflux of many molecules including K^+. Caspase-1 dependent ROS, endolysosomal leakage and K^+ efflux may provide positive feedback loops for NLRP3 activation by imiquimod and other NLRP3 activators. In tumour cells including transformed keratinocytes, Complex I is necessary for proliferation because it supports the regeneration of NAD^+, which is required for the synthesis of aspartate and nucleotides. Complex I inhibition by imiquimod may be responsible for the ability of imiquimod to induce growth arrest and apoptosis in these cells. The clinical efficacy of imiquimod as a topical anti-tumour therapy likely reflects a Complex I-dependent effect on keratinocytes, and a local induction of anti-tumour immune responses by that is promoted by TLR7 and NLRP3-dependent cytokine production.

Outlook

Imiquimod is a TLR7 ligand that is used to treat genital warts and basal cell carcinoma. TLR7-independent effects of imiquimod, including apoptosis in keratinocytes and NLRP3 inflammasome activation in myeloid cells, may contribute to its efficacy or adverse effects. Here it was demonstrated imiquimod and CL097 are specific inhibitors of mitochondrial Complex I activity. Since Complex I has a critical function in supporting tumour cell proliferation and survival, this study raises the possibility that Complex I inhibition by imiquimod may be important for its clinical anti-tumour effects.

The precise mechanism of NLRP3 activation by imiquimod and other activators remains unknown. However, a major conceptual advance of this study is that K^+ efflux is not a universal requirement for NLRP3 inflammasome activation. Identification of the mechanism of NLRP3 activation would reveal potential targets for therapeutic intervention in NLRP3-driven auto-inflammatory diseases. IL-1β-blocking antibodies and IL-1 receptor antagonists are currently used to treat NLRP3-dependent auto-inflammatory diseases. In contrast to these therapeutic proteins, a small molecule inhibitor of NLRP3 would be relatively inexpensive to produce and administer and would be less likely to block protective IL-1 from other inflammasomes. Ideally, such an agent would not only block acute symptoms, but also halt progression of inflammatory diseases.

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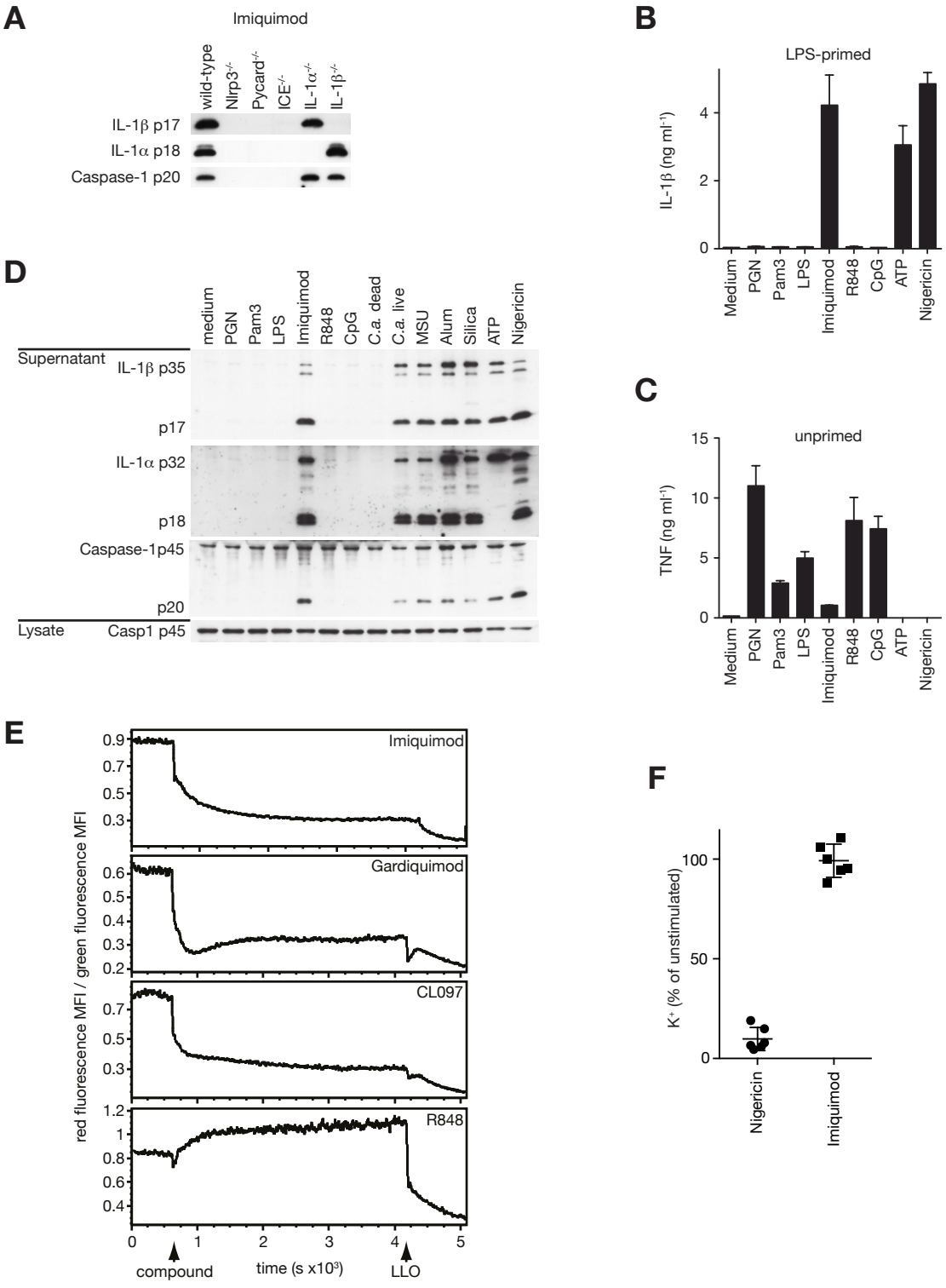
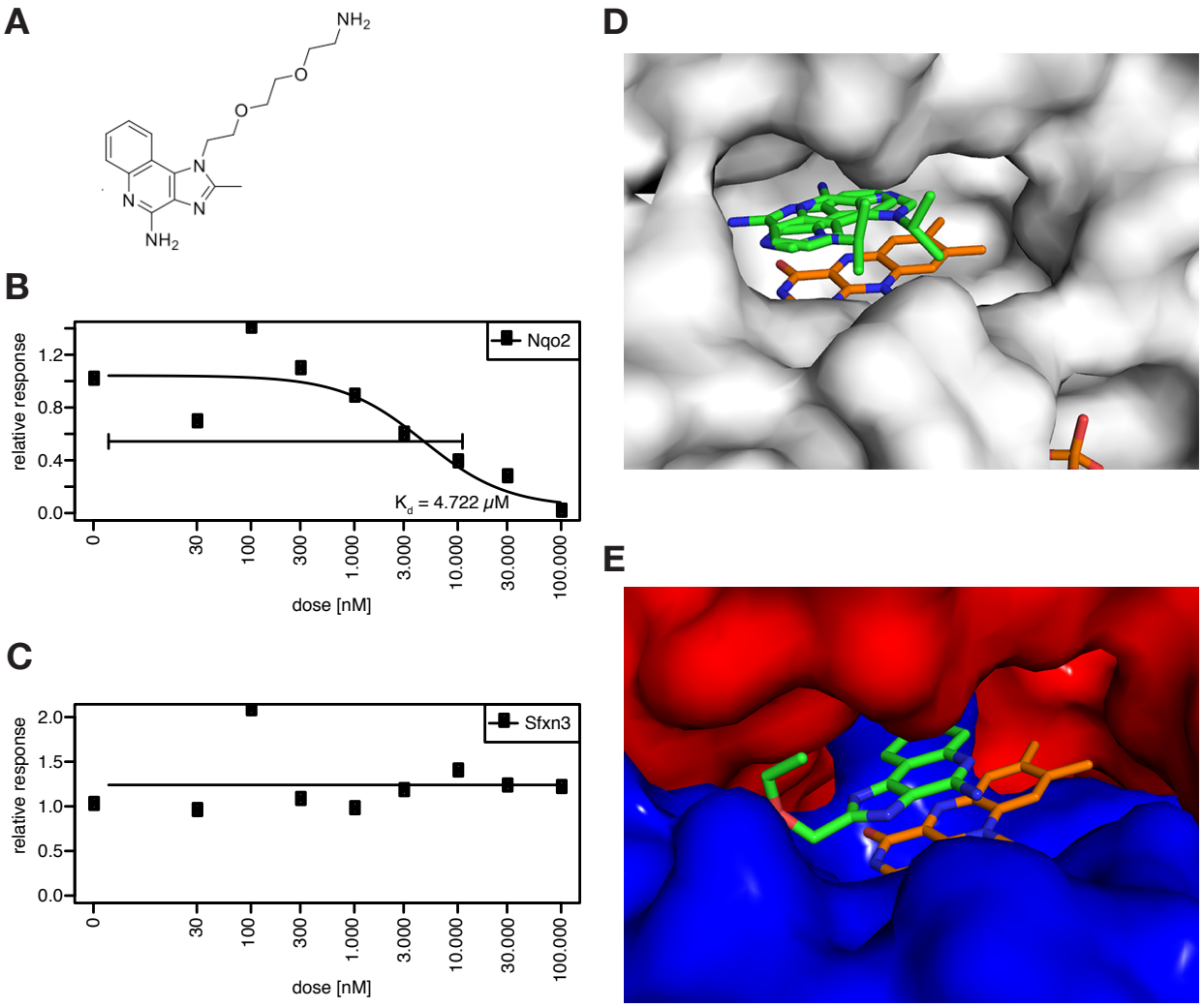
Appendix 1



Characterization of imiquimod-induced NLRP3 inflammasome activation. *Data from Dr. R. Mishra, Dr. O. Gorka, Dr. O. Groß, and G. Magnani*. Figure legend is on the next page

(A) LPS-primed BMDCs from wild-type and the indicated knockout strains were treated with 15 µg ml^{-1} imiquimod for 3 h. Cell-free supernatants samples were analysed for the presence of the cleaved forms of IL-1β, IL-1α, and caspase-1 by immunoblotting.
(B, C) BMDCs from wild-type mice were primed for 3 h with 20 ng ml^{-1} LPS (B) or left unprimed (C), and then stimulated as indicated. IL-1β (B) and TNF (C) were quantified from cell-free supernatants by ELISA.
(D) Cell-free supernatants and cell lysates from (B) were analysed for caspase-1 and the cleaved forms of IL-1β, IL-1α, and caspase-1 by immunoblotting.
(E) Assessment of endolysosomal leakage following stimulation with imidazoquinolines. ASC-deficient BMDMs labelled with acridine orange were analysed by flow cytometry for a time-lapse ratiometric measurement of the dye in cells upon stimulation with imiquimod, R848, CL097 or gardiquimod. Endolysosomal leakage is assessed by the change in the distribution of the dye in the acidic endolysosomal (PerCP channel) and cytoplasmic/nuclear (FITC channel) compartments of the cells.
(F) BMDCs from 6 different mice were stimulated on separate occasions with nigericin or imiquimod (15-20 µg ml^{-1}) for 30 min and intracellular K^+ concentrations were determined by ISE. Data is depicted as % K^+ content of unstimulated cells. Mean ± SD are shown.
ELISA data are depicted as mean ± SEM of technical triplicates.

Appendix 2



Imiquimod binds NQO2

Data from Dr. Guillaume Médard, Prof. Bernhard Küster, and Dr. Sabine Schneider

(A) Structure of GM019, synthesized by Dr. Guillaume Médard

(B, C) Relative mass spectrometry signal for specific (*i.e.* competed by the indicated concentrations of imiquimod) binding of NQO2 (B) or non-specific binding of Sfxn3 (C) to GM019.

(D, E) Surface representation of the active site of NQO2 in complex with imiquimod (D) or CL097 (E). Both compounds (in green) can form an aromatic stack with the isoalloxazine rings of the FAD cofactor (in orange). Imiquimod has two possible binding modes (D). The two subunits of the NQO2 homodimer are highlighted in blue and red (E).

Appendix 3 – *Curriculum vitae*

CHRISTINA J. GROß

Date of Birth: 27.05.1986
Place of Birth: Victoria, BC, Canada

HIGH SCHOOL EDUCATION

2002-2004 Mount Douglas Senior Secondary School, Victoria, BC, Canada

UNIVERSITY EDUCATION

2004-2010	*University of Victoria*	***BSc Microbiology (Honours)***
	Cumulative Grade	8.49 / 9.00
	Honours Thesis:	The role of integrin-associated cortical signalling complexes and focal adhesion kinase in sea urchin embryogenesis Supervisor: Prof. Robert D. Burke
2010-2011	*Université de Lausanne*	***MSc Medical Biology***
	Cumulative Grade:	5.97 / 6.00
	Master's Thesis:	Expression profiling of murine NLRs identifies NALP12 and a novel regulator of neutrophil function. Supervisors: Prof. Jürg Tschopp, Dr. Kate Schroder
2011-2012	*Université de Lausanne*	***PhD Program in Immunology and Cancer*** Supervisor: Prof. Jürg Tschopp †
2012-2016	*Technische Universität München*	***PhD Program Medical Life Science and Technology*** Supervisor: Prof. Jürgen Ruland

AWARDS AND SCHOLARSHIPS *(selection)*

2012 TUM Graduate School Stipend (declined)
2011 NSERC Canada Graduate Scholarship (PhD)
2010 Roche Continents participant, Salzburg
2009 NSERC Canada Graduate Scholarship (MSc)
2008 Keystone Symposia Travel Scholarship
2008 Co-operative Education Student of the Year, University of Victoria
2007 British Columbia Cancer Studentship, BC Cancer Agency
2007 JM Warren Award, BC Cancer Agency
2007 Terry Fox Scholarship, University of Victoria
2007 Howard Petch Scholarship for Highest Academic Standing, University of Victoria
2006 Science Undergraduate Research Award, University of Victoria
2004-6 The President's Scholarship, University of Victoria

ADDITIONAL RESEARCH EXPERIENCE

Department of Developmental Immunology, Max-Planck-Institut für Immunbiologie
May 2008 - August 2008

Position: International Summer Intern. *Supervisor:* Prof. Thomas Boehm. *Project:* Studies of thymus development in a murine model and evolution of genetic networks underlying thymus development in lower vertebrates.

Terry Fox Laboratory, British Columbia Cancer Research Centre
May 2007 - April 2008

Position: Research Student. *Funding:* BC Cancer Studentship and JM Warren Award. *Supervisor:* Prof. Gerald Krystal/Dr. Laura Sly. *Project:* The role of SHIP and the PI3K pathway in the alternative activation of macrophages.

Department of Biochemistry and Microbiology, University of Victoria
May 2006 - April 2007

Position: Research Student. *Funding:* Science Undergraduate Research Award (Faculty of Science, University of Victoria). *Supervisor:* Prof. Claire Cupples. *Project:* Protein interactions in *E. coli* DNA repair pathways.

CONFERENCE PRESENTATIONS

Thomas CJ, Mishra R, Dittlein DC, Traidl-Hoffman C, Ruland J, Perocchi F, Groß O. *Poster Presentation* – A mitochondrial target of imiquimod is involved in Nlrp3 inflammasome activation and cancer cell growth arrest. *Keystone Symposium on Immunometabolism and Tumor Metabolism.* Banff, Canada. Feb 2016

Thomas CJ, Schroder K, and Tschopp J. *Oral Presentation* - NLR Expression and Inflammasome Activation in Neutrophils. *36th FEBS Congress*. Torino, Italy. June 2011

Thomas CJ, Schroder K, and Tschopp J. *Poster Presentation* - NLR Expression and Inflammasome Activation in Neutrophils. *TOLL 2011*. Riva del Garda, Italy. May 2011

Thomas CJ, Ho VW, Feldman ME, Williams O, Shokat KM, Krystal G, and Sly LM. *Poster Presentation* – PI3K is required for alternative activation of macrophages. *Keystone Symposium on Inflammation, Microenvironment and Cancer*. Snowbird, Utah. Mar 2008.

Thomas CJ, Ho VW, Kuroda E, Krystal G, and Sly LM. *Poster Presentation* – SHIP inhibits the alternative activation of macrophages. *BC Cancer Agency Annual Cancer Conference*. Vancouver, Canada. Nov 2007.

Appendix 4 - *Publication List*

Submitted

Groß C.J., Mishra R., Dittlein D.C., Gorka O., Schneider K.S., Wettmarshausen J., Robertson A.A.B, Cooper M.A., Schroder K., Ruland J., Traidl-Hoffmann C., Perocchi F., Groß O. (2015) Imiquimod Inhibits Mitochondrial Complex I Activity and Induces K^+ efflux-independent Activation of the NLRP3 Inflammasome. Immunity, *manuscript number D-15-00890.*

Published

Chen K.W., Bezbradica, J.S., **Groß C.J**., Wall A.A., Sweet M.J., Stow J.L., Schroder K. (2016) The neutrophil NLRP3 inflammasome is activated by soluble but not particulate or crystalline agonists. Eur J Immunol, *Published online ahead of print.*

Zamoshnikova, A., **Groß, C.J.,** Schuster, S., Chen, K.W., Wilson, A., Tacchini-Cottier, F., and Schroder, K. (2015). NLRP12 is a neutrophil-specific, negative regulator of in vitro cell migration but does not modulate LPS- or infection-induced NF-κB or ERK signalling. Immunobiology *221,* 341-346*.*

Groß, C.J., and Groß, O. (2015). The NLRP3 inflammasome admits defeat. Trends Immunol *36*, 323–324.

Semper, R.P., Mejías-Luque, R., **Groß, C.**, Anderl, F., Müller, A., Vieth, M., Busch, D.H., Prazeres da Costa, C., Ruland, J., Gross, O., et al. (2014). Helicobacter pylori-induced IL-1β secretion in innate immune cells is regulated by the NLRP3 inflammasome and requires the cag pathogenicity island. The Journal of Immunology *193*, 3566–3576.

Chen, K.W., **Groß, C.J.**, Sotomayor, F.V., Stacey, K.J., Tschopp, J., Sweet, M.J., and Schroder, K. (2014). The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute Salmonella challenge. Cell Reports *8*, 570–582.

Yabal, M., Müller, N., Adler, H., Knies, N., **Groß, C.J.**, Damgaard, R.B., Kanegane, H., Ringelhan, M., Kaufmann, T., Heikenwälder, M., et al. (2014). XIAP restricts TNF- and RIP3-dependent cell death and inflammasome activation. Cell Reports *7*, 1796–1808.

Chan, D., **Thomas, C.J.**, Taylor, V.J., and Burke, R.D. (2013). Integrins on eggs: focal adhesion kinase is activated at fertilization, forms a complex with integrins, and is necessary for cortex formation and cell cycle initiation. Mol. Biol. Cell *24*, 3472–3481.

Thomas, C.J., and Schroder, K. (2013). Pattern recognition receptor function in neutrophils. Trends Immunol *34*, 317–328.

Roth, S., **Thomas, C.J.**, & Ruland, J. (2013). Immunobiology of C-Type Lectin Receptors. In G. Hartmann & H. Wagner (Eds.), Innate immunity: resistance and disease-promoting principles (Vol. 4, pp. 11–14).

Schneider, K. S., **Thomas, C.J.**, & Gross, O. (2013). Inflammasome activation and inhibition in primary murine bone marrow-derived cells, and assays for IL-1α, IL-1β, and caspase-1. Methods in Molecular Biology *1040*, 117–135.

Gross, O., Yazdi, A.S., **Thomas, C.J.**, Masin, M., Heinz, L.X., Guarda, G., Quadroni, M., Drexler, S.K., and Tschopp, J. (2012). Inflammasome activators induce interleukin-1α secretion via distinct pathways with differential requirement for the protease function of caspase-1. Immunity *36*, 388–400.

Gross, O., **Thomas, C.J.**, Guarda, G., and Tschopp, J. (2011). The inflammasome: an integrated view. Immunol Rev *243*, 136–151.

Weisser, S.B., McLarren, K.W., Voglmaier, N., **van Netten-Thomas, C.J.**, Antov, A., Flavell, R.A., and Sly, L.M. (2011). Alternative activation of macrophages by IL-4 requires SHIP degradation. Eur J Immunol *41*, 1742–1753.

Gross, O., **Thomas, C.J.**, and Layland, L.E. (2011). Inflammasome Activation in Response to Eukaryotic Pathogens. In I. Couillin, V. Petrilli, and F. Martinon (Eds.), Progress in Inflammation Research: The Inflammasomes, 65-83.

Sly, L.M., Hamilton, M.J., Kuroda, E., Ho, V.W., Antignano, F.L., Omeis, S.L., **van Netten-Thomas, C.J.**, Wong, D., Brugger, H.K., Williams, O., et al. (2009). SHIP prevents lipopolysaccharide from triggering an antiviral response in mice. Blood *113*, 2945–2954.

van Netten, J.P., **van Netten-Thomas, C.J.**, and Fletcher, C.L. (2007). Mammographic breast density, Comment on: Mammographic density and the risk and detection of breast cancer. N Engl J Med *356*, 1885–1887.