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Effects of Small Molecules and Cytokine Signalling on NLRP3 Inflammasome Activation

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za
moje roditelje Svetlanu i Vojina
i mog brata Aleksandra

for
my parents Svetlana and Vojin
and my brother Aleksandar

SUMMARY

NLRP3 is a Nod-like receptor sensing a broad variety of intracellular danger- or pathogen-associated molecular patterns, that often act through K^+ efflux or production of reactive oxygen species (ROS). Upon activation, NLRP3 forms an inflammasome complex, consisting of ASC polymer fibres that recruit caspase-1. In this process caspase-1 is autocleaved and leaves the so-called ASC speck. Activated capase-1 is processing on one hand pro-IL-1β to a pro-inflammatory, mature cytokine IL-1β and on the other hand gasdermin D, which builds pores in the cell membrane leading to a special form of lytic cell death called pyroptosis. However, the exact molecular mechanisms of NLRP3 activation remain even after years of research still puzzling.

In this work NLRP3 activation mechanisms were investigated using two different approaches both trying to shed light on NLRP3 upstream signalling. By using a range of small molecules, some of which were published to activate NLRP3 in a K^+ efflux independent manner, we tried to understand the involvement of K^+ efflux and ROS production in NLRP3 engagement. Both imatinib and masitinib, two structurally similar tyrosine-kinase inhibitors (TKIs), triggered robust NLRP3 inflammasome activation in bone-marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) depending on K^+ efflux and ROS production. Interestingly, we found that imatinib and masitinib are leading to another form of unconventional lytic cell death, which was inhibited by the osmoprotectant PEG. This unconventional cell death most likely induces indirectly NLRP3 activation by membrane destabilization and consequently also pyroptosis. This observation is of great clinical interest, as imatinib is commonly used for the treatment of chronic myeloid leukemia.

Furthermore, we focussed on the recently published NLRP3 interaction partner NEK7, which was shown to play a pivotal role in NLRP3 oligomerization and inflammasome activity in BMDMs. Surprisingly, we observed NEK7-independent NLRP3 inflammasome assembly in BMDCs, that was induced in BMDMs by GM-CSF-treatment prior to inflammasome stimulation. While GM-CSF did not prime the cells itself, it led to enhanced NLRP3 expression and increased cytokine secretion without affecting pyroptosis. In conclusion, these findings emphasize the

importance of GM-CSF as a pro-inflammatory mediator and raise awareness to differences in inflammasome signalling in BMDM and BMDC cultures.

The diverse effects of the small molecular TKIs imatinib and masitinib and the cytokine GM-CSF on NLRP3 upstream signalling, once more reveal the complexity of NLRP3 inflammasome activation. While the TKIs trigger NLRP3 inflammasome assembly indirectly by an unconventional cell death, GM-CSF enhances NLRP3 signalling by increasing its transcription.

ZUSAMMENFASSUNG

NLRP3 ist ein NOD-ähnlicher Rezeptor, der eine Vielzahl verschiedenster intrazellulärer Pathogen-assoziierter Moleküle erkennt. Diese haben meist einen starken K^+-Ausstrom und eine erhöhte ROS Produktion zur Folge. Stimuliertes NLRP3 bildet das Inflammasom aus, welches durch lange ASC-Fasern Caspasen in unmittelbare Nähe zueinander bringt und so deren Autoprozessierung und Aktivierung ermöglicht. Aktive Caspase-1 verlässt den sogenannten „ASC speck" und prozessiert einerseits das entzündungsfördernde Zytokin IL-1β und andererseits Gasdermin D, das Zellmembranporen bildet und so Pyroptose, einen lytischen Zelltod, induziert. Trotz langjähriger Forschung an NLRP3 bleibt der genaue Aktivierungsmechanismus jedoch ungeklärt. Um NLRP3 Aktivierungsmechanismen besser verstehen zu können, wurden in dieser Arbeit zwei verschiedene Upstream-Signale untersucht.

Da kürzlich bekannt wurde, dass einige kleine, synthetische Moleküle unabhängig vom K^+-Ausstrom NLRP3 aktivieren können, arbeiteten wir mit zwei strukturell sehr ähnlichen Tyrosin-Kinase-Inhibitoren (TKIs), Imatinib und Masitinib, um die Rolle vom K^+-Ausstrom und ROS Produktion besser verstehen zu können. Wir konnten zeigen, dass beide TKIs eine K^+-Ausstrom und ROS abhängige IL-1β-Sekretion in Makrophagen und dendritischen Zellkulturen induzieren, die NLRP3-Inflammasom abhängig ist. Dabei rufen Imatinib und Masitinib einen unkonventionellen, lytischen Zelltod hervor, der die Zellmembran destabilisiert und so NLRP3 und folglich Pyroptose aktiviert. Unsere Beobachtungen haben große klinische Relevanz, da Imatinib ein gängiges Mittel in der Therapie von chronisch myeloischen Leukämien ist.

Außerdem beschäftigten wir uns mit dem kürzlich publizierten NLRP3-Interaktionspartner NEK7, dessen unabdingbare Rolle für die NLRP3-Oligomerisierung und Inflammasomaktivierung in kultivierten Knochenmarks-Makrophagen bewiesen wurde.

Interessanterweise konnten wir zeigen, dass NEK7 in dendritischen Zellkulturen für die NLRP3 Aktivierung nur eine kleine Rolle spielt. Die NEK7-Unabhängigkeit konnten wir auch in Makrophagen-Zellkulturen durch das kolonie-stimulierende Zytokin GM-CSF bewirken. GM-CSF führt dabei zu einer erhöhten NLRP3

Expression und IL-1β Sekretion, beeinflusst aber nicht die Pyroptose. Unsere Ergebnisse demonstrieren die wichtige entzündungsfördernde Funktion von GM-CSF und lenken gleichzeitig das Augenmerk auf die Unterschiede zwischen Inflammasom-assoziierten Signalwegen in Makrophagen und dendritischen Zellkulturen, die normalerweise wenig Beachtung finden.
Die unterschiedlichen Effekte von den kleinen TKI Molekülen Imatinib und Masitinib und dem GM-CSF Zytokin auf die Upstream-Signalwege von NLRP3, veranschaulichen die Komplexität der NLRP-Aktivierung. Während die TKIs diese indirekt durch einen unkonventionellen Zelltod bewirken, beeinflusst GM-CSF die NLRP3 Transkription und erhöht so die NLRP3-Inflammasome Signale.

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ABBREVIATION

°C	degrees Celsius
AIM2	absent in melanoma 2
APC	antigen-presenting cells
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosintriphosphate
BIR	baculoviral inhibition of apoptosis protein repeat
BMDCs	bone marrow-derived dendritic cells
BMDMs	bone marrow-derived macrophages
CARD	caspase-recruitment domain
CLR	C-type lectin receptors
CpG	cytosine triphosphate deoxynucleotide-guanine triphosphate deoxynucleotide
DAMP	damage-associated molecular pattern
DC	dendritic cell
DFOA	deferoxamine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ds	double-stranded
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein
fmk	fluoromethylketone
Flt3	fms-like tyrosine kinase 3
g	gram

GM-CSF	granulocyte-macrophage colony-stimulating factor
GSDM	gasdermin
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIN	hematopoietic interferon-inducible nuclear antigen
IFN	interferon
IL	interleukin
ILCs	innate lymphoid cells
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factors
L	litre
LDH	lactate dehydrogenase
LGP2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide
LRR	leucine rich repeat
M	molar
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MDA-5	melanoma differentiation-associated gene 5
MDP	muramyl-dipeptide
MHC	mayor histocompatibility complex
min	minutes
MSU	mosodium urate crystals
MyD88	myeloid differentiation factor 88
NEK	NIMA-related kinase
NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nima	never in mitosis gene a
NK	natural killer
NAD+/NADH	nicotinamide adenine dinucleotide
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
PAMP	pattern-associated molecular pattern
PCR	polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
poly(dA:dT)	poly(deoxyadenylic-deoxythymidylic)
PRR	pattern recognition receptor
PYD	pyrin N-terminal homology domain
RBC	red blood cell
RIG-I	retinoic acid-inducible gene I
RIPK	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
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
Syk	spleen tyrosine kinase
TEMED	N,N,N',N'-Tetramethylethylendiamine
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TRIF	TIR-domain-containing adaptor protein inducing IFNβ
U	units
V	volt

INTRODUCTION I

1.1. THE INNATE IMMUNE SYSTEM

Living organisms are exposed to infectious agents and their toxins throughout their lifetime. In this regard, different immune mechanisms evolved to defend the body from those and from the harm they cause. These are divided into the innate immune system and the adaptive immune system. While the adaptive immune system induces immunological memory and relies thereby on very specific antigen-receptors and specialised cells, the innate immune system serves as a rapid answer to a broad range of infection patterns and a first-line of defence.

1.1.1 AN OVERVIEW

The very first innate, protective barrier in humans is the skin and mucosa that prevent the direct invasion of pathogens into the body. If pathogens, however, evade the host's anatomic barrier, different antimicrobial enzymes at those barriers as well as the complement fight the early infection. The complement is a system of a variety of

humoral proteins, many of which are proteases. When complement proteins bind to pathogens, they get activated and process other complement proteins. This complement cascade will lead in the end to the membrane lysis of certain pathogens, an inflammation and the recruitment of innate immune cells.
The recruited cells are phagocytes which engulf and digest the complement labelled pathogens and thus build the next line of defence. Phagocytes cannot only detect complement but can also sense pathogen-associated molecular patterns (PAMPs) with their pattern recognition receptors (PRRs). PRRs are germ-line encoded receptors, which recognize a broad range of danger molecules that can also be of endogenous origin (danger-associated molecular patterns DAMPs). Most activated PRRs induce an intracellular signalling cascade that activates the secretion of pro-inflammatory cytokines and chemokines that again recruit other immune cells, both innate and adaptive, and thus shape the inflammatory response.

1.1.2 Cells of the innate immune system

One of the first cell types recruited to the infection sites are neutrophils. Neutrophils are the most numerous innate immune cells as well as the most abundant subgroup of granulocytes, which belong to the myeloid leukocyte lineage. They circulate in the blood but transmigrate into infected tissue in a process called extravasation (Steven, 1892; Borregaard, 2010). This multi-step process involves adhesive interactions between leukocytes and the endothelium that are induced by cytokines and chemokines secreted by phagocytes at the inflammation site (Ley et al., 2007). Neutrophils recognise complement and PAMPs, so that pathogens can be taken up and attacked by their cytoplasmic granules eventually. These granules contain superoxide or antimicrobial peptides and enzymes (Kolaczkowska & Kubes, 2013). Furthermore, extracellular pathogens can be defeated by neutrophils by degranulation of different antimicrobial granules or by the fatal release of neutrophil extracellular traps (NETs). NETs consist of nuclear chromatin that traps pathogens and enables an easier engulfment of those by other phagocytic immune cells (Brinkmann et al., 2004).

Two more granulocyte cell types exist, which play a role in innate immunity - eosinophils and basophils. Like neutrophils, both cell types have granules that are degranulated upon activation. They can fight bigger pathogens like ticks, parasites or helminths. While the granules of eosinophils are acidophilic and contain peroxidases, ribonucleases, lipases and the toxic major basic protein (Voehringer, 2013), basophil granules are filled with central inflammatory mediators like histamine, serotonin and heparin (Rosenberg et al., 2013). Nevertheless, basophils and eosinophils play an essential role in allergic reaction and asthma, too.

Together with eosinophils and basophils, mast cells are also involved in allergic inflammation. They are best known for their tremendous influence on IgE-mediated allergic responses. Mast cells develop, like granulocytes, from common myeloid progenitors in the bone marrow and migrate in an immature state to peripheral tissue, where they undergo maturation (Kirshenbaum, 2000). They are commonly found in the skin or mucosa. Similar to basophils their granules contain histamine and heparin, allowing them to fight parasitic infections as well as to participate in wound healing (Urb & Sheppard, 2012).

Natural Killer (NK) cells are innate immune cells that derive, unlike the already mentioned cell types, from common lymphoid progenitors (Geiger & Sun, 2016). They have, however, also granules with cytotoxic proteins and additionally the pore-forming protein perforin. When the granules are released, perforin induces pores in the cell membrane of the target cell, so that the cytotoxic proteins can enter and induce apoptosis (Paul & Lal, 2017). What makes NK cells so unique is, however, their ability to detect stressed and virus-infected cells by a missing-self mechanism. Thus, if the activation of inhibitory/self-receptors and activating/stress-receptors is disbalanced, NK cells induce a cytotoxic response in the target cell (Jaeger & Vivier, 2012). As NK cells not only recognize virally infected cells but also tumor cells, they have a huge inhibitory effect on tumor development (Vivier et al., 2012).

Recently, NK cells have been grouped to innate lymphoid cells (ILCs). ILCs belong to the innate immune lineage that derive from lymphoid progenitors but lack B and T cell markers (Mazzurana et al., 2018). ILCs are contributing to the rapid, innate immune response and amplifying it by secreting different cytokines. Lymphoid

tissue-inducer (LTi) cells are regulating lymphoid tissue development before birth and their architecture later on (Mazzurana et al., 2018).

Together, with macrophages and dendritic cells (DCs), two antigen-presenting innate immune cell-types, ILCs are interacting with adaptive immune cells and directing their response.

1.1.3 Macrophages and Dendritic Cells

Macrophages and dendritic cells are phagocytes of the innate immune system that derive from common myeloid progenitors in the bone marrow. They are tissue-resident cells that can be found in almost all tissues where they screen their environment for any pathogen or danger associated molecular patterns (Steven, 1892; Steinman & Cohn, 1973).

A central dogma in macrophage research was that tissue-resident macrophages are highly differentiated cells that lost their ability to proliferate and thus must be renewed by circulating progenitors, which are monocytes (Davies & Taylor, 2015). Monocytes are myeloid cells, that circulate the blood and can migrate to tissue to differentiate into macrophages or dendritic cells (Geissmann et al., 2010). However, it was recently shown that different tissue-resident macrophages develop during embryogenesis and migrate to the peripheral tissue, where they self-renew during adulthood (Gomez Perdiguero et al., 2015; Röszer, 2018).

Nevertheless, a large influx of circulating monocytes into inflamed tissue leads to an increased monocyte differentiation into macrophages or dendritic cells during infection (Geissmann et al., 2010). Upon PAMP or DAMP sensing via their scavenger, pattern recognition or phagocytic receptors, macrophages engulf the pathogens or target cells in a phagosome that eventually fuses with intracellular lysosomes, filled with digestive enzymes and toxic peroxides (Brüne et al., 2013). As a response to their PRR activation they additionally secrete different inflammatory cytokines like TNFα, IL-12, IL-1 or IL-6 to induce further immune responses, both innate as well as adaptive. Furthermore, macrophages possess the ability to interact with adaptive immune cells directly by displaying the digested antigen on their major histocompatibility complex class II (MHCII) receptors, known as antigen

presentation. Apart from their pro-inflammatory role, macrophages can also act in an anti-inflammatory way, thereby secreting IL-10 or TGFβ to induce wound-healing or tissue repair during later inflammation phases. The pro-inflammatory phenotype is defined as the M1 macrophage polarization, while the anti-inflammatory phenotype is referred to as M2 macrophage polarization (Murray et al., 2014; Murray & Wynn, 2011).

Like macrophages also dendritic cells can fight infections by phagocytosis. However, their main role is the antigen-presentation to T cells to initiate adaptive immune responses. Most dendritic cells derive from common myeloid progenitors in the bone marrow, however, some can also develop from the common lymphoid progenitor lineage (Collin & Bigley, 2018). Dendritic cells migrate to tissue and lymphoid organs in an immature state via blood circulation, where they scan their environment for pathogen or danger associated molecules and phagocytose them. When their PRRs get activated, they become mature, upregulate MHCII receptors and co-stimulatory molecules on their surface and migrate to the draining lymph nodes or spleen for antigen-presentation (Solano-Gálvez et al., 2018). Apart from exogenous antigen display on MHCII receptors, dendritic cells can also present these on MHC class I (MHCI) receptors in a process called cross-presentation. Thus, dendritic cells can activate both $CD4^+$ T helper cells as well as cytotoxic $CD8^+$ T cells and are key mediators of the innate and adaptive immune cross-talk and thus important initiators of the adaptive immune response (Gutiérrez-Martínez et al., 2015). This rare and heterogenous cell type can be divided into three major subsets, which are conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs) and monocyte-derived cell populations (moDCs) (Collin & Bigley, 2018). While pDCs secrete high amounts of type I interferons upon stimulation, cDCs respond to PRR activation like M1 macrophages with TNFα, IL-12, IL-1 or IL-6 secretion (Durai & Murphy, 2016). As pDCs secrete type I interferons and express rather low levels of MHCII receptors compared to mature cDCs upon stimulation, they are less suitable for antigen-presentation and were linked to anti-viral immune responses (Collin & Bigley, 2018). Most steady-state DCs derive from DC-precursors, however, during infection, monocytes are recruited to the inflamed tissue, where they may

differentiate, dependent on the environmental signals, also to dendritic cells. However, their exact role and function in the immune response remains unclear.

1.1.4 GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF)

As the isolation of tissue-resident DCs and macrophages is very difficult and results in low numbers of vital cells, they were generated *in vitro* for decades of myeloid cell research. Thereby, granulocyte-macrophage colony-stimulating factor (GM-CSF) is used to treat bone marrow isolated cells in order to differentiate DCs and macrophage colony-stimulating factor (M-CSF) for macrophage development. GM-CSF and M-CSF are cytokines that belong to the colony-stimulating superfamily that are responsible for myelopoiesis of monocytes, macrophages, dendritic cells (Ushach & Zlotnik, 2016). Another canonical member is of high relevance, granulocyte (G)-CSF. *In vivo* M-CSF-deficient mice show the strongest phenotype with a severe lack of osteoclasts and tissue macrophages (Dai et al., 2002; Wiktor-Jedrzejczak et al., 1990), while G-CSF-deficiency causes low numbers of neutrophils (Lieschke et al., 1994).

The importance of GM-CSF under physiological conditions is so far not major, cells lacking GM-CSF only display diminished alveolar macrophage numbers. Especially the small effect of GM-CSF on DC populations in vivo is surprising, as it is *in vitro* crucial for generation of bone marrow derived DCs (BMDCs). In this regard, GM-CSF deficiency in mice does not influence the numbers of lymphoid-tissue-resident cDCs and effects only the numbers of non-lymphoid-tissue cDCs predominantly in lung, dermis and intestine (Vremec et al., 1997; Greter et al., 2012).

In a healthy organism, GM-CSF is virtually absent. These changes, however, in inflammation where GM-CSF is produced and secreted locally as a monomeric glycoprotein. It has been reported that T helper type 17 (Th17) cells respond to IL-23R activation by GM-CSF expression (McGeachy, 2011). As Th17 cells are also involved in autoimmunity, GM-CSF seems to contribute to healthy tissue inflammation. Furthermore, blockage or deficiency of GM-CSF contributes to better multiple sclerosis and arthritis progression in mouse models (Shiomi et al. 2016). The cellular and molecular mechanism of this observation is however poorly understood.

While the M-CSF receptor is a tyrosine kinase receptor, the GM-CSF receptor (GM-CSFR) is a heterodimeric transmembrane protein without intrinsic catalytic activity. It belongs to the family of type I cytokine receptors and shares structural similarity to the IL-3 and IL-5 receptor. Upon ligand binding, GM-CSFR triggers the JAK-STAT pathway for differentiation and inflammation responses and uses PI3K-MAPK signalling for proliferation and cell survival (Becher, Tugues, & Greter, 2016). The receptor itself is mostly expressed on DCs and in a lower percentage on macrophages.

The GM-CSF cell culture was recently under critical observation. It displays a high heterogeneity with cells resembling cDCs and monocyte-derived macrophages, expressing CD11c and MHCII surface molecules (see 1.1.3), but still they differ from their physiological counterparts (Helft et al., 2015). Future research on DCs should be ideally restricted to a standardized, specific subpopulation of the BMDC culture.

1.2. Pattern Recognition receptors (PRRs)

To increase the chances of pathogen-recognition by germ-line encoded receptors in the best possible way, PRRs are very diverse in structure as well as in their location within the innate immune cell. While the membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CTLs) target PAMPs or DAMPs from exogenous sources, RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) bind cytosolic ones. Usually pathogens carry more than just one PAMP, so that more than just one PRR gets activated at the same time. Furthermore, different PRRs can recognize overlapping PAMPs. By this interplay of PRRs the invading pathogens can be attacked through different pathways and the infection can be eliminated more efficiently.

1.2.1 Toll-like receptors (TLRs)

Toll-like receptors (TLR) were the first identified PRRs and are thus the best characterized so far, with 13 different variants described in mice and 11 in humans. All TLRs are type I transmembrane proteins with an amino (N) terminus pointing outwards that contains multiple leucine-rich repeat (LRR) domains. Their carboxy (C) terminus have a Toll-IL-1 receptor (TIR) domain, that interacts with TIR-containing adapters and induces downstream signalling upon activation (M. Yamamoto, Takeda, & Akira, 2004). As some TLRs are localized in the cell membrane while others can be found in membranes of intracellular vesicles, like ER, lysosome or endosome, they react to distinct stimuli and sense a broad range of intra- and extracellular PAMPs from different bacteria, viruses, fungi or parasites.
The cell membrane bound TLRs, TLR1, 2, 4, 5 and 6, are mainly activated by components of the microbial membrane, while the intracellular TLRs 3, 7, 8 or 9 react upon binding of different nucleic acids.
TLR3 recognizes double-stranded DNA, while TLR7 is specialised for single-stranded DNA. This is also true for TLR8, which shares a great homology with TLR7. Both TLR7 and 8 were shown to be also activated by synthetic ligands, like imidazoquinolines. TLR9 is signalling upon receptor-binding of unmethylated 2’-

deoxyribo (cytidine-phosphateguanosine) (CpG) rich DNA sequences, motifs that are commonly found in bacterial genomic DNA (Kawai & Akira, 2010; Kawasaki & Kawai, 2014).

TLR2 can bind together with TLR1 and 6 different lipoproteins of gram negative as well as gram-positive bacteria. As heterodimers these TLRs can also interact with Dectin-1, which is a C-type lectin receptor and will be described in more detail in chapter 1.2.2, to discriminate different lipopeptide PAMPs. TLR5 is activated upon the interaction with bacterial flagellin, which is also recognised by the intracellular TLR5 homologue TLR11 (Kawai & Akira, 2010; Kawasaki & Kawai, 2014).

Lipopolysaccharides (LPS) of gram-negative bacteria are stimulating TLR4, which is the only TLR that is activating two different downstream signalling pathways. Myeloid Differentiation Primary Response 88 (MyD88) and TIR-domain-containing adaptor protein inducing IFNβ (TRIF) are the adapter proteins that are activated by the TIR domain of TLRs upon PAMP association. While MyD88 interacts with all TLRs except TLR3, TRIF acts only downstream of TLR3 and 4 (M. Yamamoto et al., 2004). MyD88 induces nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein (MAP) kinase activation, which will lead to the transcription of pro-inflammatory cytokines like pro-IL-1β, TNFα, and IL-6. The TRIF pathway would also signal via NFκB for pro-inflammatory cytokine secretion. Additionally, it would induce type I interferon upregulation, by activating interferon regulatory factors (IRFs) (Akira & Hoshino, 2003; Hoebe et al., 2003). TLR7 and 9 are exceptions within this system as they are able to induce type I interferon production in a MyD88-dependent manner (Uematsu et al., 2005).

TLR4 is, as mentioned above, acting in a MyD88-dependent and TRIF-dependent manner. Here, MyD88 induces an early-phase activation of NFκB, while TRIF gets only activated after the endocytosis of TLR4, leading to the late-phase activation of NFκB and type I interferon expression.

1.2.2 C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) belong to a superfamily of both membrane and soluble proteins, that is defined by the C-type lectin-like domain (CTLD). Even

though these proteins share the CTLD motif, they are not all Ca^{2+}-dependent (C-type) or carbohydrate binding (lectin) proteins (Zelensky & Gready, 2005). CTLD containing proteins have a wide range of ligands, that are still poorly characterised. However, some CTLD containing proteins serve as PRRs on innate immune cells like neutrophils, dendritic cells or macrophages. These CLRs are known to bind carbohydrates in a Ca^{2+}-dependent way by their carbohydrate recognition domain (CRD) (Geijtenbeek & Gringhuis, 2009). There are two different types of membrane-bound CLRs, distinguished by their N-terminal orientation. While the N terminus of type I CLRs faces outwards of the cell and contains 8-10 CRDs, the N terminus of type II CLRs points into the cytoplasm and the one CRD is located at the extracellular C-terminal site (Figdor, van Kooyk, & Adema, 2002).

A well-studied type I CLR is the mannose receptor (MR). It recognises mannosylated ligands, which can be found on glycans of different pathogens like fungi, bacteria or viruses, but also on host glycoproteins. This is why MRs also contribute to the resolution of inflammations as they play an important role in extracellular clearance during inflammation, when glycoproteins are increased extracellularly (Martinez-Pomares, 2012). Upon receptor activation phagocytosis is initiated, so that the pathogen bound to it can be degraded by lysosomes. As the MRs do not possess any intracellular signalling motif, they need the interact with TLRs for cytokine secretion and signalling (van de Veerdonk et al., 2009).

Type II CLRs share quite similar extracellular C-terminal sites, differ however in their intracellular N-terminal region, that is able to induce cell signalling upon receptor activation. The most prominent type II CLRs are DC-associated C-type lectin 1 (Dectin-1), Dectin-2 and macrophage-inducible C-type lectin (Mincle), each recognizing different carbohydrate patterns of the fungal cell wall. Upon receptor binding, spleen tyrosine kinase (Syk) activation occurs directly over the tyrosine-based activation motif (ITAM) in the case of Dectin-1 signals or indirectly via ITAM-containing adaptors in the case of Dectin-2 and Mincle. Syk signals over the caspase recruitment domain 9 (Card9) containing adapter protein to NFκB to induce pro-inflammatory IL-6 and IL-23 secretion (Dambuza & Brown, 2015; Figdor et al., 2002; Patin, Orr, & Schaible, 2017).

However, some CLRs can modulate or supress the signalling of other PRRs like TLRs. While the DC-specific intracellular adhesion molecule 3 (ICAM3)-grabbing nonintegrin (DC-SIGN) is enhancing NFκB signalling and IL-10 secretion upon TLR activation (den Dunnen, Gringhuis, & Geijtenbeek, 2009), DC-inhibitory receptor (DCIR), myeloid inhibitor C-type lectin receptor (MICL) and blood DC antigen 2 (BDCA-2) have an inhibitory effect on TLR signalling (Redelinghuys & Brown, 2011). DCIR and MICL contain the tyrosine-based inhibitory motif (ITIM) that depletes TLR8 and TLR9 activation and IL-12 secretion upon Lipopolysaccharide (LPS) and zymosan treatment (Meyer-Wentrup et al., 2009). Even though, BDCA-2 has an ITAM, it also inhibits TLR9-dependent type I interferons and TLR-mediated IL-6 and TNFα production in a process that is so far not fully understood (Cao et al., 2007; Dzionek et al., 2001; Röck et al., 2007).

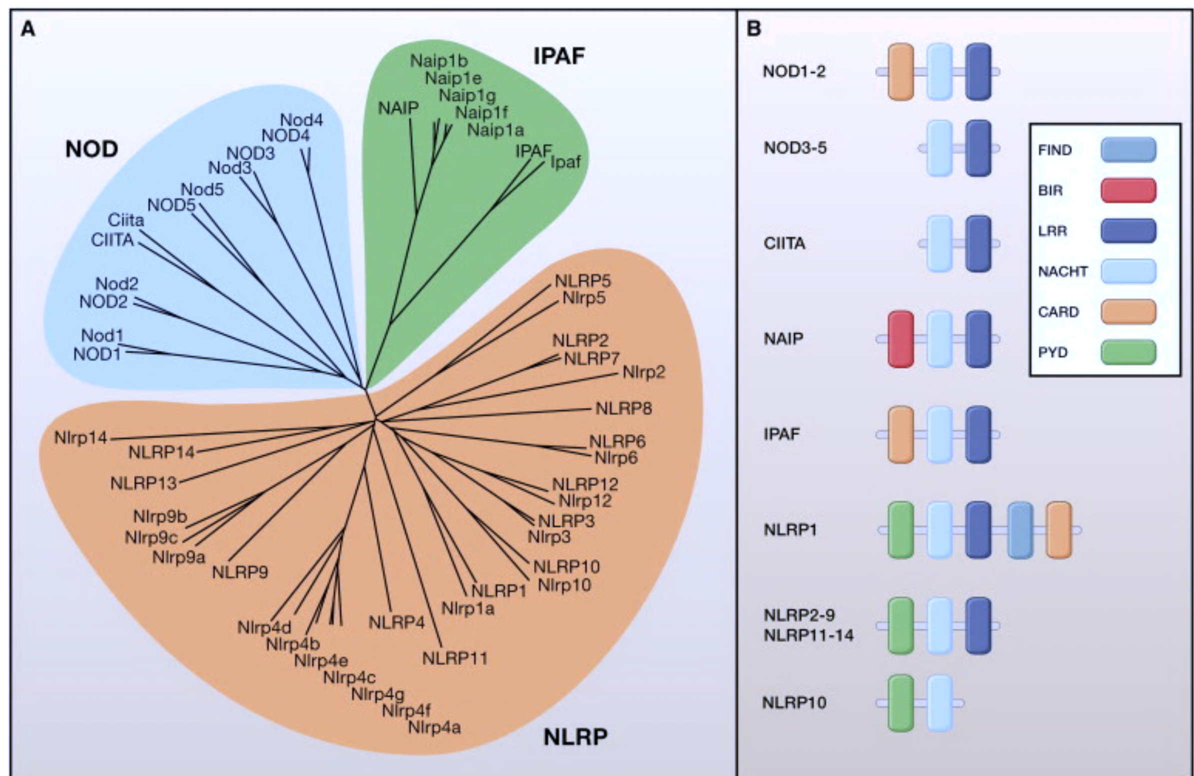
1.2.3 RIG-I-like receptors (RLRs)

In contrast to the described TLRs and CLRs, retinoic acid-inducible gene-I-like receptors (RIG-I-like receptors, short RLRs) represent together with nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, short NLRs) the main two cytosolic PRRs.

The RLR family consists of three different members, which are retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA-5), and laboratory of genetics and physiology 2 (LGP2) (Luo et al., 2011). RIG-I and MDA5 share a similar structure. The DExD/H-box RNA helicase domain and the autoregulatory C-terminal domain (CTD) scan the cytosolic environment for viral double-stranded RNA, while the two N-terminal caspase activation and recruitment domains (CARDs) are responsible for downstream signalling. In an inactive state the CTD and CARD domains interact and unfold upon activation to induce NFκB and IRF phosphorylation and thus type I interferon and pro-inflammatory cytokine expression (Kato et al., 2006).

Apart from the lacking CARDs, LGP2 has a similar structure like RIG-I and MDA5 with an DExD/H-box and CTD. It was believed that LGP2 has only a negative effect on RIG-I and MDA5 signalling (Quicke, Diamond, & Suthar, 2017). Recent studies

showed, however, that low concentrated LGP2 also can lead to an enhanced MDA5 signalling, by increasing the MDA5 affinity to target RNA (Childs, Randall, & Goodbourn, 2013).



Picture 1: Human and Mouse NLR Family Members from (Schroder & Tschopp, 2010a).
A) Phylogenetic Tree of murine (lowercase) and human (uppercase) NACHT domains, showing the distinct subfamilies: NLRP, IPAF, NOD. B) Schematic structures of human NLR, revealing structural similarities within the NLRP subfamily.

1.2.4 NOD-LIKE RECEPTORS (NLRS)

Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) represent a big family of cytosolic PRRs with 22 members in human and 34 in mice. Nevertheless, they share great similarity in all vertebrate phyla and can thus be directly compared between different mammalian organisms.

NLRs can be further divided into 3 different subfamilies based on the phylogenetic variation of their NACHT domain: the NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5,

NOD5/NLRX1, CIITA), the NLRPs (NLRP1-14) and the IPAFs (IPAF/NLRC4 and NAIP) (Schroder & Tschopp, 2010b).

The members of all subfamilies share a central NOD (also referred to as NACHT) and a C-terminal LRR domain, which is only missing in NLRP10. In an ATP dependent manner, the NACHT domain oligomerizes upon NLR activation and is responsible for signal transduction. The LRR domains are believed to be involved in ligand sensing and autoregulation. Differences in the N-terminal region are observed among the NOD and IPAF family members, while the NLRP family shows consistently a C-terminal pyrin domain (PYD) (Schroder & Tschopp, 2010b).

NOD1 and NOD2 have a N-terminal CARD, which interacts with the CARD-containing Receptor-interacting serine/threonine-protein kinase 2 (RIP2) upon activation, which eventually leads to NFκB signalling (Inohara & Nuñez, 2003) . They are activated by different motifs of bacterial cell wall components. In particular, NOD1 recognizes γ-glutamyl diaminopimelic acid (iE-DAP) (Girardin et al., 2003a), found in some gram-negative and some gram-positive bacteria, and NOD2 muramyl dipeptide (MDP) (Girardin et al., 2003b), that is part of the peptidoglycans of most bacteria. They play an important role in the gut, where they keep the bacterial burden and microbiome homeostasis in check (G. Y. Chen & Núñez, 2009). This is why, mutations in these proteins are often associated to Crohn's disease (S. Yamamoto & Ma, 2009).

MHC class II transactivator (CIITA) the only PRR that has regulatory effects on MHCII transcription upon IFNγ exposure (Steimle et al., 1994). It has a N-terminal acidic transactivation domain that is responsible for the binding and thus activation of the transcriptional machinery of MHCII. NLRC5 has a similar structure to CIITA and was recently reported to play an important role in the regulation of the MHCI transcription machinery (Y. Yao et al., 2012). The functions of NLRC5 and NLRX1 are not characterized yet.

NAIPs carry characteristically a N-terminal baculoviral inhibition of apoptosis protein repeat (BIR) domain. NAIP2 and -5 can induce a conformational change in inactivated NLRC4 and thus assist in its activation and oligomerization upon bacterial type 3 secretion system or flagellin sensing in the cytoplasm (Kofoed & Vance, 2011; Y. Zhao et al., 2011). NLRC4 contains a N-terminal CARD, which

interacts with ASC or directly with caspase-1 in a complex called inflammasome to induce pro-inflammatory cytokine secretion. As NAIPs do not have the ability to oligomerize, only one NAIP molecule is found in each NAIP inflammasome complex (Zhang et al., 2015).

Some of the NLRP family members are also activating caspase-1 in an inflammasome-dependent manner, which will be explained in more detail in 1.3. As mentioned above, all NLRPs have an N-terminal PYD. NLRP1 has additionally a domain with function to find (FIIND) and CARD at the C-terminal end. Thus, NLRP1 and NLRC4 are the only CARD-containing NLRs that form an inflammasome and can interact with different caspases at the same time (Schroder & Tschopp, 2010b).

1.3. THE INFLAMMASOMES

Inflammasomes are cytosolic, oligomeric multiprotein complexes that lead on the one hand to interleukin-1β (IL-1β) maturation and on the other hand to pyroptosis, a specific form of cell death, by recruiting and activating caspase-1 (Martinon, Burns, & Tschopp, 2002; Schroder & Tschopp, 2010b). IL-1β is a potent pro-inflammatory cytokine, which plays a crucial role in host defence against infections. It can also, however, cause pathological inflammation if its maturation is deregulated.

1.3.1 CANONICAL INFLAMMASOMES

Various receptors from different families are described as canonical inflammasome forming proteins. These are NLRP1, NLRP3, NLRP6, NLRP12 and NLRC4 of the NLR family, absent in melanoma 2 (AIM2) and interferon inducible protein 16 (IF16) from the PHYIN family and RIG-I of the RLR family.

The PHYIN family consists of PRRs that can sense cytosolic DNA, both from microbial origin as well as from host cancer cells. They contain a N terminal PYD that is responsible for downstream signalling and a C terminal HIN200 that can directly bind DNA molecules. AIM2 recognizes double-stranded DNA from bacteria, viruses and host that is present in the cytoplasm (Bürckstümmer et al., 2009; Rathinam et al., 2010). Upon activation it induces ASC polymerization by pyrin-pyrin interactions. *In vitro*, AIM2 can be stimulated by transfection of poly(dA:dT), a synthetic, double-stranded DNA (Hornung et al., 2009). RIG-I is activated by double-stranded RNA and NLRC4 by bacterial type 3 secretion components or flagellin. They are described in more detail in chapters 1.2.3 and 1.2.4, respectively.

NLRP1 was the first inflammasome-inducing PRR to be described. In human, it has, as described in chapter 1.2.4, besides the PYD, NACHT and LRR domain an additional C terminal FIIND and CARD. There are multiple paralogues of human NLRP1 found in mice, that are activated by an endopeptidase, called anthrax lethal factor of *Bacillus anthracis* and are missing the PYD (Boyden & Dietrich, 2006). Thus, mouse NLRP1 paralogues are interacting with ASC by the C terminal CARD domain upon activation, that is induced by direct cleavage by the lethal factor (Levinsohn et

al., 2012). This cleavage may lead to a conformational change and loss of the autoinhibition that is characteristic for the inactivated state of NLRPs.

NLRP6, 7 and 12 inflammasome formation is not completely understood yet. It is believed that NLRP6 plays an important role in microbiome homeostasis, as its loss results in an altered gut flora (Elinav et al., 2011; Wlodarska et al., 2014). NLRP7 is distinct to human macrophages, where it signals in an inflammasome-dependent manner upon microbial acylated lipopeptide sensing (Khare et al., 2012; Radian et al., 2015). The role of NLRP12 is, however, still undefined.

NLRP3 is probably the most investigated inflammasome-forming NLR and was also of special interest in this thesis, as the exact molecular mechanisms of NLRP3 activation remain undefined. It is activated upon a variety of different PAMPs or DAMPs, solidifying the hypothesis that a central common process must be induced by those that can then be sensed by NLRP3 (Shao et al., 2015). Different possible activation mechanisms will be introduced in depth in chapter 1.4. Downstream signalling of NLRP3 is comparable to the one of the other canonical inflammasome receptors.

Inflammasome signalling needs in either case a first priming signal to pre-activate the cell and happens thus in a 2-step process. Priming can be induced by stimulation of TLRs, IL-1 receptor, TNFα receptor or NODs upon which NFκB engagement causes a transcriptional upregulation of the NLRs themselves and also of cytokine precursors like pro-IL-1β (Hiscott et al., 1993; Bauernfeind et al., 2009). *In vitro*, cells are, for the purpose of NLRP3 inflammasome analysis, usually primed by TLR4 signalling, which is stimulated by extracellular LPS. LPS-priming has also an additional rapid, transcription-independent enhancing effect on NLRP3 activation, that is not fully understood yet. However, an additional PAMP or DAMP motif is crucial for inflammasome formation.

1.3.2 Downstream signalling of canonical inflammasome receptors

Upon PRR oligomerization, ASC is recruited by PYD-PYD or CARD-CARD interactions (Masumoto et al., 1999) in the case of NLRC4 (Li et al., 2018). This induces homotypic ASC oligomerization into one macromolecule, called ASC speck, which is built of long helical ASC filaments (Lu et al., 2014a). Within ASC specks, the

ASC^{CARD} is protruding from the filaments and enables pro-caspase-1 recruitment by CARD-CARD interaction (Dick et al., 2016).

Pro-caspase-1 consists of a CARD and two subunits, the smaller p10 and bigger p20, where the active centre is located (Miller et al., 1993; Walker et al., 1994; Wilson et al., 1994). Due to the close proximity of caspases within the ASC speck, pro-caspase-1 can cleave itself at the linker between CARD and p20 and hence gets fully active. This cleavage leads on the one hand to conformational rearrangement and flexibility and on the other hand to the interruption of the CARD-CARD interaction between ASC and caspase-1. This process induces p20/p10 heterotetramer stabilization of two activated caspases (Thornberry et al., 1992; Cerretti et al., 1994; Yamin, Ayala, & Miller, 1996; Elliott et al., 2009). As caspase-1 is a strongly inflammatory protease, the half-life of the potent p20/p10 heterotetramers is very short, restricting its potent effect (Walsh et al., 2011).

When active caspase-1 leaves the ASC speck, it has two distinct effects for the progression of inflammatory signalling. The first one is the cleavage of the IL-1β precursor pro-IL-1β and the IL-18 precursor pro-IL-18, the second the induction of pyroptosis (Thornberry et al., 1992). IL-18, like IL-1ß, belongs to the IL-1 cytokine superfamily signals to T helper and NK cells. Interestingly, ASC-deficient macrophages show weak IL-1β secretion upon NLRC4 activation, showing that the CARD-CARD interaction can develop between NLRC4 and Caspase-1 (Li et al., 2018). Moreover, it becomes clear that ASC filament formation is enhancing the initial signal for downstream signalling.

Only the mature IL-1β can bind the IL-1β receptor (IL1ßR), this is why it needs to get processed in a tightly regulated manner before secretion. Two Caspase-1 cleavage sites are known in immature pro-IL-1β, one leading to a minor 26kDa product with so far unknown function and the other to the mature 17kDa form (Afonina et al., 2015). It was long debated how IL-1β is secreted, as it happens in an “unconventional” way (Nickel & Rabouille, 2009). Normally, secretion proteins carry two signal peptides: one for the endoplasmatic reticulum (ER) and the other one for the Golgi. However, pro-IL-1β does not have any signal peptides, but is secreted in a regulated pathway (Rubartelli et al., 1990).

Recently, the exact mechanism of caspase-1 dependent pyroptosis was unravelled, which also shed light upon the secretion pathway of IL-1β.

Gasdermin-D (GSDMD) is a protein that consists of a 31kDa N-terminal and a 22kDa C-terminal part, which are kept by conformational folding in an autoinhibited, inactivated state (Sborgi et al., 2016a). After proteolytic cleavage, the N-terminus locates to the inner leaflet of the cell membrane where it charge-dependently interacts with phosphatidylinositol, which is missing in the outer leaflet. The N-terminus of GSDMD integrates into the membrane, where it oligomerizes and forms pores of 10-14nm (Chen et al., 2016; Ding et al., 2016; Liu et al., 2016). These pores lead to cell swelling and lysis, two observations characteristics for pyroptosis. Furthermore, pyroptotic cells show DNA fragmentation, depending on nucleases that differ from those of apoptosis, and condensation of the nucleus (Bergsbaken et al., 2009; Fink & Cookson, 2005; Miao et al., 2011).

It is debated whether IL-1β is secreted passively via these pores or in a rather more active process. Earlier observations showed that glycine, which stabilizes the osmotic gradient, can prevent pyroptotic cell lysis, but not IL-1β secretion (Conos et al., 2017a). This could lead to the assumption that another secretion mechanism must be involved. Interestingly, GSDMD-deficient cells show an intact cell membrane and no pyroptotic cell burst upon inflammasome activation. Thereby, pro-caspase-1 processed, mature IL-1β is accumulating within the cell, strongly pointing towards the passive IL-1β secretion hypothesis (Schneider et al., 2017; Shi et al., 2015). Furthermore, IL-1α, which is activated by calpains and not caspase-1 (Howard et al., 1991), is also secreted in a GSDMD-dependent matter upon caspase-1 activating signalling (Schneider et al., 2017).

1.3.3 THE NON-CANONICAL INFLAMMASOME

Nevertheless, IL-1β secretion and pyroptosis can also be induced in a PRR-independent manner. During non-canonical inflammasome activation, murine caspase-11 and its human orthologues caspase-4 and 5 serve as the PAMP sensor and signal transducer (Hagar et al., 2013; Kayagaki et al., 2011). It was reported that intracellular LPS, an essential component of gram-negative bacterial cell walls,

directly binds to inactive capase-11 . This binding leads to proteolytic autocleavage, probably by conformational change, causing caspase-11 dimerization and activation (Shi et al., 2014).

Active Caspase-11 is, like caspase-1, able to cleave GSDMD and provoke pyroptosis (Kayagaki et al., 2015; Aglietti et al., 2016) but different to capase-1 it cannot process pro-IL-1β. The N-terminal GSDMD subunit is forming pores in the cell membrane in the same way it happens during canonical inflammasome signalling, as described in chapter 1.3.2. This process eventually leads to pyroptosis and osmotic pressure destabilization, inducing water influx and ion fluctuations of potassium and calcium for example. K^+ efflux is seen as a potential key player of NLRP3 activation, as it is often observed prior to NLRP3 inflammasome activation (Shao et al., 2015). With this in mind, the non-canonical NLRP3 inflammasome assembly upon caspase-11-dependent LPS sensing, that can be observed as ASC specks and measured by mature IL-1β secretion (Rühl & Broz, 2015), could be stimulated by the K^+-efflux induced by GSDMD pores. NLRP3 is thus activated by the by-products of pyroptosis, discriminating IL-1 secretion and pyroptosis even further. The mechanisms involved in NLRP3 activation will be described in further detail in chapter 1.4.

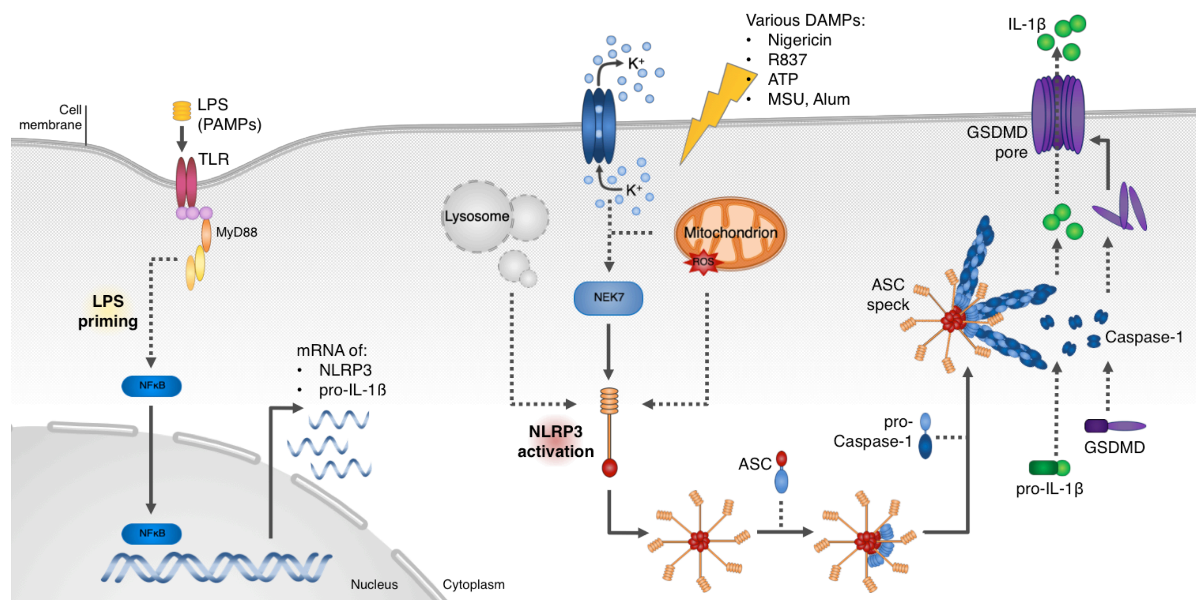
1.4. NLRP3 Activation

While most inflammasomes are stimulated by specific PAMPs, the NLRP3 inflammasome responds to a plethora of different stress signals, including extracellular ATP, potassium ionophores, crystals, insoluble particles, and certain pathogens (Broz & Dixit, 2016). This broad variety of NLRP3 inflammasome activators, raises the question if it is rather sensing a common signal downstream of these PAMPs. Different stress conditions can by observed prior to speck formation. These include K^+ efflux, ROS, and endolysosomal leakage, but the exact mechanistic details and common traits of NLRP3 activation remain however elusive. K^+ efflux was the first NLRP3 upstream signal to be described and is now seen as the most common activation mechanism (Franchi et al., 2014; Muñoz-Planillo et al., 2013a; Rivers-Auty & Brough, 2015). A strong argument is the substantial inhibition of NLRP3 signalling when extracellular KCl is added to the cells before PAMP stimulation (Walev et al., 1995). Moreover, an intracellular drop in K^+ concentration is induced shortly after treatment with common NLRP3 activators like nigericin or extracellular ATP. Nigericin is a pore-forming bacterial toxin, while extracellular ATP leads to opening of the purinergic receptor P2X7 (Perregaux et al., 2001), both allowing K^+ efflux.

As inhibition of ROS produced by NADPH oxidase also impaired IL-1β secretion in THP1 cells (Dasu, Devaraj, & Jialal, 2007), a role in NLRP3 activation was suggested. Furthermore, mitochondrial ROS production by a dysfunctional respiratory chain, induced by complex I or complex III inhibitors, increases inflammasome signalling (Groß et al., 2016a). Specific mitoROS inhibition would indeed negatively affect stretch-induced IL-1β secretion in alveolar macrophages (Wu et al., 2013). The crystal structure gives further evidence that NLRP3 inflammasome formation could be triggered by ROS. A highly redox-state sensitive disulfide bond between PYD and NOD, which is conserved in NLRP3 among six different species (including human and mice), strengthens the hypothesis additionally (Bae & Park, 2011).

Lysosomal leakage occurs when phagocytosed pathogens, crystals or particles manage to disturb phagolysosome integrity and the lysosomal membrane gets permeable. Thereby the most abundant hydrolases, the cathepsins, enter the cell

plasma and induce apoptosis or necrosis, depending on the severity of the leakage (Aits & Jaattela, 2013).



Picture 2: NLRP3 inflammasome formation pathway according to Toldo & Abbate, 2017. Inflammasome formation happens in a two-step process. The first priming signal is dependent on the NFκB pathway, which is activated by DAMP stimulation of TLRs. NfκB activity leads to transcription and translation of inflammasome components, mainly NLRP3 and pro-IL-1β. *In vitro*, LPS is used to trigger TLR4 signalling and prime the cell.
The second signal triggers NLRP3 activation by a not fully understood mechanism. NLRP3 senses a huge variety of PAMPs and DAMPs. Depicted here is the NLRP3 activation by extracellular ATP as an example. Many NLRP3 activators induce K^+ efflux, mitochondrial ROS, lysosomal leakage or a combination of these. Recently NEK7 was found to act downstream of K^+ efflux and upstream of NLRP3, directly binding to it and leading to NLRP3 oligomerization and ASC speck formation. ASC fibres recruit Pro-Caspase-1 is then autoprocessed, leaving the speck in an activated form. Active Caspase-1 is inducing maturation of pro-IL-1β and pro-IL-18. Furthermore caspase-1 cleaves GSDMD, which oligomerizes in the cell membrane and forms pores, leading to pyroptosis and cytokine secretion.

As cathepsin inhibitors, lysosome-destabilizing agents or neutralization of lysosomal acidification were reported to modulate NLRP3 activation, it was postulated that lysosomal leakage could be involved in inflammasome formation (He et al., 2016). However, further studies revealed that caspase-1 activation is observed before lysosomal leakage, indicating independent NLRP3 signalling (Fujisawa et al., 2006;

Heid et al., 2013). Furthermore, it was shown that lysosomal leakage could lead to K^+ efflux or ROS production (Heid et al., 2013; Katsnelson et al., 2015), two mechanisms convincingly reviewed as possible upstream NLRP3 stimulating events.

1.4.1 NEK7: A NEW UPSTREAM REGULATOR OF NLRP3

Three simultaneous reports from different research groups provided strong evidence that NEK7 (never in mitosis A (NIMA)-related kinase 7), a kinase so far only associated with mitotic spindle apparatus formation, plays a crucial role in NLRP3 inflammasome activation.

In a phosphorylation cascade downstream of NEK9, the serine/threonine kinase NEK7 induces together with NEK6 the phosphorylation of Kinesin family member 11 (KIF11), influencing its accumulation at the microtubules and the separation of the centrosomes for mitotic progression (Fry et al., 2012; O'Regan & Fry, 2009; Yissachar et al., 2006). Recently, a role in neuronal dendrite morphogenesis was also attributed to NEK7, whereby it effects the cytoskeletal microtubules in a KIF11-dependent manner as well (Freixo et al., 2018).

The additional role of NEK7 in inflammasome activation was surprising, but emerging evidence of diminished NLRP3 signalling in NEK7-deficient bone marrow derived macrophages (BMDMs) was very convincing. The NEK-7 dependent NLRP3 activation and oligomerization can be observed for both canonical and non-canonical NLRP3 stimuli downstream of K^+ efflux, but not for activators of the other inflammasomes (He et al., 2016). NEK7 binds directly to the LRR domain of NLRP3, thereby releasing its autoinhibition (He et al., 2016; Shi et al., 2016). For this interaction the active site of NEK7 is crucial, but not the kinase activity (Shi et al., 2016). Notably, Caspase-1 processing downstream of the gain-of-function NLRP3 mutation NLRP3-R258W is still NEK7-dependent (He et al., 2016). Nek7-dependency was also observed *in vivo*, as mice lacking NEK7 secreted less IL-1β both after MSU injections as well as in an experimental multiple sclerosis model.

Moreover, mitotic cells are responding less to NLRP3 stimuli and show diminished NLRP3-NEK7 interaction, indicating that NEK7 is strongly contributing to the exclusivity of these two opposing events, pyroptosis and mitosis (Shi et al., 2016).

1.4.2 SMALL MOLECULES: NEW TOOLS TO STUDY NLRP3 ACTIVATION

Previous work of our group showed that small molecules like imidazoquinoline imiquimod or some TKIs can induce robust NLRP3-dependent IL-1β secretion in BMDMs and BMDCs.

Imiquimod, is an imidazoquinoline derivate drug (also known as R837), commonly used as therapeutic medication in condyloma conditions, basal cell carcinoma, and actinic keratosis. It was described that Imiquimod binds and inhibits the non-kinase NQO2 (Bantscheff, Eberhard, et al., 2007). NQO2 is a promiscuous quinone oxidoreductase that catalyses a two-electron detoxification of quinones to form hydroquinones with unclear physiological function (Vella et al., 2005). The quinone oxidoreductases NQO2 and mitochondrial Complex I were shown to be ROS-producing molecular targets of Imiquimod. Imiquimod can bind NQO2 and activate the NLRP3 inflammasome without the need of K^+ efflux, but by an intense production of mitochondrial ROS (Groß et al., 2016a).

NQO2 is also bound by some tyrosine kinase inhibitors (TKIs), of which a few also show NLRP3 inflammasome stimulating effects. It seems however, that NQO2 binding is not mandatory linked to the ability to activate the NLRP3 inflammasome (Groß, PhD Thesis, 2016).

The family of TKIs includes small molecule inhibitors selectively targeting the ATP-binding pocket of proto-oncogenic tyrosine kinases. By blocking constitutive cellular signalling all TKIs have anti-neoplastic activity and promote growth arrest and cell death of cancer cells (Levitzki, 2013). Imatinib was also shown to bind and inhibit NQO2 and induce IL-1β secretion in BMDMs and BMDCs (Winger et al., 2009; Groß, PhD Thesis, 2016; Magnani, PhD Thesis, 2017).

Imatinib, initially developed as a PDGFR kinase blocker, was later found to be a potent inhibitor of the BCR-ABL fusion kinase and KIT (Hantschel et al., 2008). Consequently, it was the first TKI commercially used as a cytostatic drug under the trade name Glivec (Gleevec in US), which is still most successfully used for treatment

of chronic myeloid leukemia (Hunter et al., 2007). Masitinib, is a TKI structurally related to imatinib, inhibiting specifically PDGFR and c-KIT. Masitinib is currently being tested in several clinical trials and has been so far used in treatment of canine mastocytosis (Adenis et al., 2014; Lortholary et al., 2017).

Small molecules are interesting new tools to study NLRP3 inflammasome activation, as emerging evidence shows their ability to modulate its responses. Furthermore, findings observed by this approach could have clinical relevance and lead to new therapies using small molecules for NLRP3 associated inflammatory diseases.

1.5. Aim of this Thesis

Even after years of research, the exact molecular mechanism of NLRP3 activation remains unsolved. With regard to many pathological roles of NLRP3 in diseases like gout, arthritis or autoinflammatory diseases such as cryopyrin-associated period syndrome (CAPS) , it would be of therapeutic interest to answer this question (Mangan et al., 2018).

As small molecules like imiquimod represent a new NLRP3 targeting approach, they are of special interest for a better understanding of upstream signals triggering inflammasome formation. The finding of TKI-induced inflammasome activation introduces novel small molecule activators of NLRP3 to the field of inflammasome research.

To study the molecular details of inflammasome activation by TKIs, we focussed on imatinib and masitinib. This extends the toolbox of clearly defined molecular triggers of the inflammasome and thus facilitates the study of its fine mechanistic aspects. Furthermore, the reported pro-inflammatory site of TKI-treatment is of significant clinical importance and may relate to the immunomodulatory effects elicited by imatinib in patients (Zitvogel et al., 2016).

In a second approach we tried to investigate the recently identified mediator of NLRP3 activation NEK7 in further detail. Hereby we tried to focus on differences between BMDM and BMDC cultures, as NEK7 dependency was only observed in macrophages so far. A better understanding of NEK7 activation may provide a new targeting strategy for treatment of NLRP3-associated diseases.

MATERIALS & METHODS II

2.1. MATERIALS

All salts, reagents and fine chemicals were purchased from either Sigma-Aldrich, Carl Roth, or Enzo. Tyrosine kinase inhibitors were purchased from Selleckchem. R837 was purchased from Invivogen. Tissue culture media, supplements and antibiotics were from Thermo Scientific. GM-CSF and M-CSF were obtained from ImmunoTools. Tissue culture plasticware was from TPP or Sarstedt.

2.1.1 ANTIBODIES

Antibody	Host species	Company
ASC (AL177)	rabbit	Adipogen AG
Caspase-1 p20 (Casper-1)	mouse	Adipogen AG
IL-1β (AF-401)	goat	R&D Systems Inc.
NEK7 (EPR4900)	rabbit	Abcam PLC
NLRP3 (Cryo-2)	mouse	Adipogen AG
Tubulin (B512)	mouse	Sigma-Aldrich Co. LLC

Conjugated Antibody	Reactivity	Company
CD11b PE/Cy7-conjugated (M1/70)	mouse/human	BioLegend Inc.
CD11c PerCP/Cy5.5-conjugated (N418)	mouse	BioLegend Inc.
CD135 APC-conjugated (A2F10)	mouse	BioLegend Inc.
F4/80 FITC-conjugated (BM8)	mouse	Abcam PLC
KI-67 PE conjugated	mouse	BioLegend Inc.
MHCII APC/Cy7-conjugated (M5/114.15.2)	mouse	BioLegend Inc.

2.2. MICE

Mice were housed under SOPF or SPF (specifying pathogen free) facilities at Klinikum rechts der Isar in Munich, Germany or Charles River Laboratories in Calco, Italy in accordance with local and European guidelines.

Used for inflammasome experiments were following mice: Nlrp3$^{-/-}$ (Martinon et al., 2006), ASC$^{-/-}$ (Mariathasan et al., 2004), caspase-1$^{-/-}$ (Kuida et al., 1995); Nek7$^{-/-}$ (Shi et al., 2016), kindly provided by Prof. Bruce Beutler and colleagues; and GSDMD$^{-/-}$ (He et al., 2015), kindly provided by Prof. Petr Broz and colleagues.

Colonies were maintained by inbreeding of heterozygous mice and kept under strict observation for manifestation of disease symptoms. Offspring were genotyped from biopsy material by PCR or Western blot analysis in the case of Nek7$^{-/-}$ breeding.

2.3. CELL LINES

Cell lines were cultured at 37°C with 5% CO_2 in humidified incubators and handled under a HEPA-filter cell culture sterile hood. NIH-3T3 murine embryonic fibroblasts, HeLa human epithelial cells and HEK293T human embryonic kidney cells were cultured in DMEM (1mM pyruvate, 25mM glucose, 4mM glutamine) supplemented with 10% FCS and 100U/mL penicillin and 100mg/mL streptomycin (abbreviated Pen/Strep). Every 2-3 days, the cells were passaged by washing with phosphatebuffered saline (PBS) and treated for 5-10 min at 37°C with Trypsin and 0.05% EDTA solution. Cultivation media were prepared as recommended by the German collection of microorganisms and cell cultures (DSMZ).

2.4. BMDC and BMDM culture

Bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) were isolated from the murine tibia and femur of age and sex matched mice, as published in detail by our group (Schneider et al., 2013). Cells were grown for 6-8 days in humidified incubators at 37°C and 5% CO_2 in the presence of recombinant murine (rm) M-CSF (BMDMs) or GM-CSF (BMDCs). For the NEK7 project, both BMDCs as well as BMDCs were cultured for 6-8 days in RPMI-1640 (11.1mM glucose with GlutaMAX) supplemented with 10% FCS and 100U/mL penicillin and 100mg/mL streptomycin in the presence of M-CSF (BMDMs) or GM-CSF (BMDCs).

2.5. Inflammasome Assay

After 6-8 days of differentiation, cells were harvested using 0.05% EDTA in HBSS buffer. BMDCs and BMDMs were plated in 96-well plates at a density of 0.12-0.2 x 10^6 cells per well in medium containing growth factor. All stimulations were performed in triplicates.

Before inflammasome stimulation, cells were primed with 20ng/mL *E.coli* K12 ultrapure LPS for 3 h. Typical inflammasome activator concentrations and times were as follows: 5mM ATP 180 min, 100µM imiquimod (R837), 5µM nigericin 60 min, 400µg/mL MSU/alum 180 min, 1µg/mL poly(dA:dT) 180 min (transfected with Lipofectamine2000 in OptiMEM, Invitrogen). MSU crystals were prepared as previously described (Martinon et al., 2006). All inflammasome activators were titrated before first usage and the lowest dose and the shortest time required to cause significant IL-1 secretion.

Inhibitors were added after 3 h of priming, and 30 min before stimulation with inflammasome activators. Inhibitor concentrations were as follows, unless indicated otherwise: 200µM ammonium pyrolidinedithiocarbamate (APDC or PDTC), 40µM ebselen, 25mM KCl, 5µM MCC950, 30µM necrostatin-1, 3000g/mol polyethylene glycol (PEG), or 20µM zVAD-fmk. MCC950 was provided by Prof. Matthew A. Cooper (University of Queensland) (Coll et al., 2015a). To minimize off-target effects

of extracellular KCl, it was mixed by careful pipetting immediately after administration. Inhibitors were also titrated, and the lowest effective dose was used. When the GM-CSF effects was of interest, 20ng/mL GM-CSF was added directly to the cell culture medium, either 1 h prior to LPS-priming or the night before the experiment. The procedure was described in further detail by our group (Schneider et al., 2013).

2.6. Biochemical analysis upon inflammasome stimulation

2.6.1 Cytokine Quantification

For cytokine quantification of cell-free supernatants, enzyme-linked immunosorbent assay (ELISA) kits for murine IL-1α, IL-1β, IL-6, IL-10 and TNFα (Ready-Set-Go from eBioscience) were used according to manufacturer's protocol with half the volumes used (50µL/well instead of 100µL/well). The supernatants were diluted 1:3 – 1:5 prior to loading. ELISA data is depicted as mean ± SEM of technical triplicates. Absorbance at 450 nm was measured using a Tecan Sunrise instrument, and concentrations were interpolated from the standard curve using the accompanying Tecan Magellan software. The standard curve was prepared by serial dilution of recombinant cytokine provided in the kit and was added to each plate when the samples were loaded as well.

2.6.2 Immunoblotting

For protein analysis of cell-free supernatant and cell lysates the samples were collected in sodium dodecyl sulfate (SDS)- and dithiothreitol (DTT)-containing sample buffer and the triplicates pooled. Proteins were separated according to Laemli (Laemli, 1970) in a discontinuous SDS polyacrylamide gel (acrylamide content 10% or 12%). The SDS Page was run in BioRad electrophoresis cells with 1× SDS running buffer (15 g/L Tris base, 72 g/L Glycine, 5 g/L SDS) for 1-2 h at 100-120V.

SDS-PAGE separated proteins were transferred to a nitorcellulose membrane (GE Healthcare) using wet blotting (Towbin et al., 1992). The blot was run at 110V per gel for 1.5 h, cooled with ice. The nitrocellulose membrane was then washed in PBS-Tween (PBST) and protein loading visualised by Ponceau S stain. Upon incubation with blocking buffer (PBST with 5% skim milk) for 1 h at room temperature with gentle agitation, a primary antibody (in PBST with 2% skim milk and 0.05% sodium azide) was added overnight at 4°C with gentle agitation (antibodies are listed in 2.1.1). This was followed by at least three washing steps in PBST each lasting 10 min. Afterwards the membrane was incubated with the secondary antibody conjugated with horseradish peroxidase (HRP) (diluted in PBST with 2% skim milk) 1-1.5h at room temperature. After five times washing with PBST for a total time of 1-2 h, the membrane was covered with Enhanced chemiluminescence substrate (Western Lightning Plus from PerkinElmer or ECL Ultra from Lumigen) and chemiluminescence was captured with cooled charge-coupled device (CCD) camera (ChemoCam from Intas). The procedure was described in further detail by our group (Schneider et al., 2013).

2.6.3 Cytotoxcity assay

Lytic cell death was quantified with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Assays were conducted following manufacturer's instructions, with the exception that half the volume was used. Maximum cell death (100%) was determined by lysing example cells with the lysis buffer provided in the kit. Cell-free medium was used to determine the background, which was subtracted from all values before calculating the percentage of cell death.

2.7. Immunofluorescence imaging

Cells of interest were seeded in 8-chamber culture slides for immunofluorescence imaging at a density of 8 x 10^5 cells/chamber. Cells were treated like it is described in 2.5, with an exemption for nigericin of which double the concentration was used (10 µM).

Subsequently, the cells were washed with 1x DPBS, fixed for 10 min in 4% paraformaldehyde, and extracted for 5 min in DPBS supplemented with 0.1% Triton X- 100. Inflammasome components were visualized by antibodies (listed in 2.1.1) diluted 1:200 in blocking buffer (1x DPBS + 5% FBS + 0.1% Triton X-100). After washing three times with 1x DPBS supplemented with 0.1% Triton X-100, the staining was completed by incubation with an secondary antibody conjugated to a fluorescent dye. Finally, slides were mounted in Vectashield containing DAPI (Vector Laboratories). Images were taken with constant laser settings on a Leica SP8 confocal microscope equipped with a 63x/1.40 oil objective. To detect all specks in a image frame, a z-stack of the region of interest was captured. Thus, the depicted images are maximum projections of the respective z-stacks.

2.8. FLOWCYTOMETRIC ANALYSIS

BMDMs and BMDCs were treated and collected as described in 2.5. To determine the total cell number, cells were counted with trypan blue and annotated. A maximum of 2 x 10^6 cells was pipetted to a V-bottom 96-well plate and washed with FACS buffer (1x PBS with 2%FCS). Cells were then treated with conjugated antibodies listed in 2.1.1, which were diluted in FACS buffer following manufacturer's protocol and incubated for 1 h at room temperature and in the dark. Afterwards, cells were washed twice with FACS buffer. Cells were used immediately or resuspended in IC fixation buffer (eBioscience) and subsequently stored for a maximum of 48 hours.

All samples were measured on a FACS Aria II (BD Biosciences) and data analysis was done using the FlowJo (Threestar) software.

Results III

3.1. NLRP3 Inflammasome Activation by the Tyrosine Kinase Inhibitors Imatinib and Masitinib

Tyrosine kinase inhibitors (TKIs) are commonly used in the clinics and have been proven highly successful therapeutics for various neoplastic disorders. While some TKIs have a narrow range of very specific molecular targets, others are capable of inhibiting a set of kinases when applied systemically and thus harbour the potential of complex side effects. Some TKIs were found to bind the flavoprotein and quinone oxidoreductase NQO2 (Bantscheff, Eberhard, et al., 2007), which is a known target of imiquimod (R837), a small molecule acting as a potent inflammasome activator (Groß et al., 2016b).

3.1.1 Imatinib and Masitinib induce IL-1ß secretion in primary murine myeloid cells

To test, whether part of the adverse effects could potentially be attributed to systemic inflammation and inflammasome activation, we treated LPS-primed bone

marrow-derived dendritic cells (BMDCs) with imatinib, a well-established TKI, and masitinib, with a related molecular structure to imatinib currently being tested in clinical trials (Adenis et al., 2014). Both TKIs induced robust IL-1β secretion in BMDCs at concentrations comparable to levels of well-established inflammasome activators like, ATP, nigericin, imiquimod (R837), MSU, alum, and poly(dA:dT) (Figure 1A). We determined an optimal working concentration of both TKIs by titration and observed peak production of IL-1β for a TKI concentration ranging between 5-100 µM (Figure 1A). Interestingly, IL-1β secretion is observed only after a threshold TKI concentration of about 20-30 µM. Indeed, a similar all-or-none response has been observed for other established inflammasome activators. Also, IL-1β secretion levels induced by imatinib and masitinib treatment are largely comparable to those of canonical NLRP3 activators. One hallmark of inflammasome activation is represented by the cleavage and the subsequent secretion of the caspase-1 residual p20 to the extracellular space (Martinon et al., 2002). We detected secretion of cleaved caspase-1 p20 in supernatants of LPS-primed BMDCs treated with either of the two selected TKIs, again to a comparable extend as with established inflammasome activators (Figure 1B). When performing experiments with stimulation kinetics we identified for 60µM TKI concentration an optimal IL-1β secretion after 3 and 4 hours of stimulation (Figure 1C, data from Giovanni Magnani).

Taken together, our data indicate that both imatinib and masitinib can induce prominent IL-1β release after passing a threshold concentration of about 20-30 µM. Also, the secretion of caspase-1 p20 to the supernatant strongly suggests that the measured IL-1β secretion derives from the action of an active inflammasome in the tested BMDCs.

3.1.2 Imatinib and Masitinib activate the NLRP3 Inflammasome

As imatinib and masitinib are inducing significant secretion of IL-1β and caspase-1 cleavage, we investigated what inflammasome sensor protein might be responsible for this response. To this end, we tested LPS-primed BMDCs generated from either wildtype or knockout mice of different inflammasome components. Comparable to

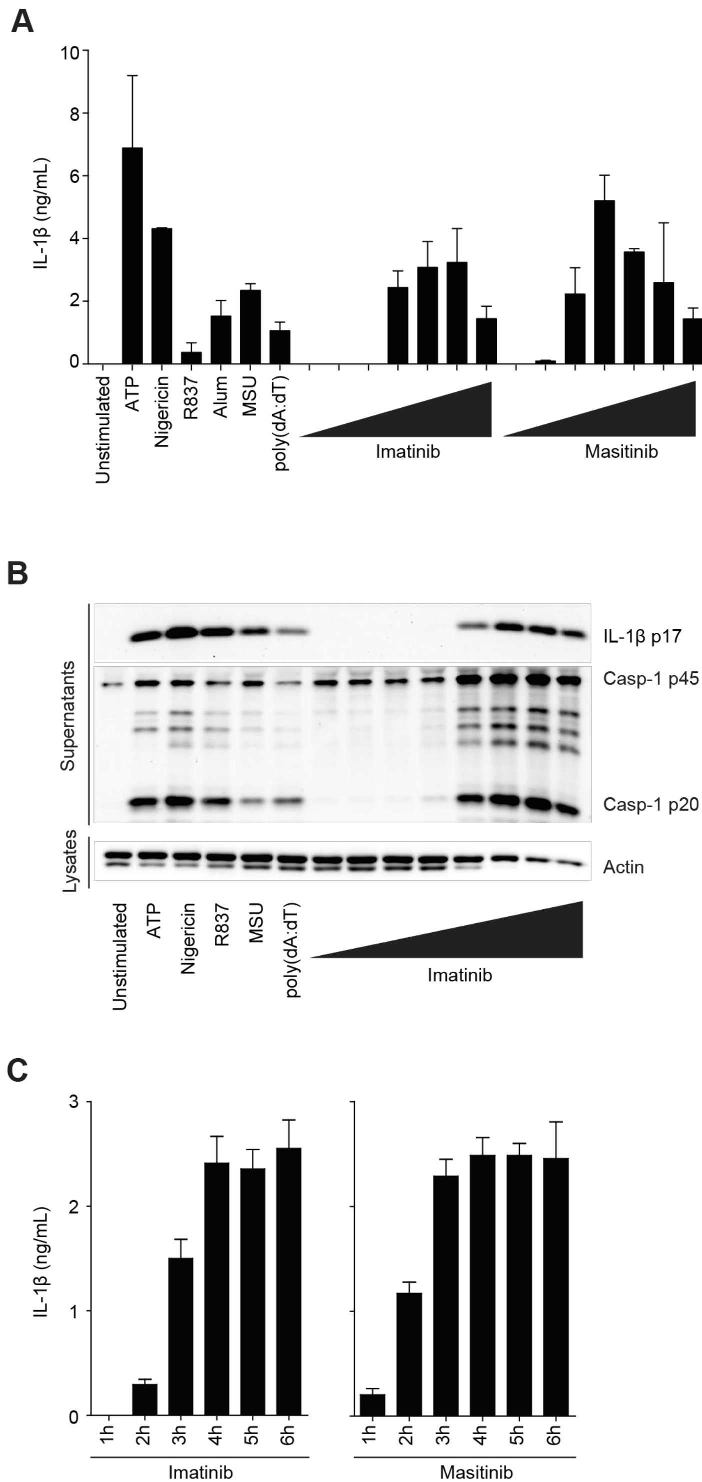


Figure 1: The Tyrosine-kinase inhibitors (TKIs) imatinib and masitinib induce strong caspase-1 cleavage and IL-1β secretion in bone marrow derived dendritic cells (BMDCs).
(A) BMDCs were primed 3 h with 20ng/ml LPS, and then stimulated with imatinib and masitinib (5, 10, 20, 40, 60, 80, 100 µM) for 3h and NLRP3 inflammasome activators, whose concentrations are listed in the experimental procedures. IL-1β was measured from supernatant of LPS-primed cells by ELISA. (B) Immunoblot analysis of lysates and cell-free supernatants from (A). (C) IL-1β secretion time course from LPS-primed BMDCs treated with 60µM imatinib and masitinib. IL-1β was measured from supernatant of LPS-primed cells by ELISA (done by Giovanni Magnani).
Cytokine secretion data is depicted as mean ± SD of technical triplicates. The results from each experiment in this figure are representative of at least three independent experiments.

control inflammasome activators nigericin and R837, IL-1β secretion was strongly reduced in NLRP3 and ASC-deficient cells as compared to wildtype (Figure 2A).
The compound MCC950 is a potent and selective small molecule inhibitor of the NLRP3 inflammasome capable of abolishing both IL-1β and pyroptotic cell death (Coll et al., 2015b). We therefore tested both imatinib and masitinib in combination

with MCC950 pre-treatment and observed a significantly reduced secretion of IL-1β (Figure 2B). The IL-1β secretion induced by the TKIs and the reported NLRP3 activators, nigericin, R837, and monosodium urate crystals (MSU) was inhibited by pre-treatment with MCC950. However, poly(dA:dT), an activator of the AIM2 inflammasome, still elicited IL-1β secretion unaffected by MCC950 (Figure 2B). This observation supports the hypothesis that NLRP3 plays a major role in TKI-induced IL-1β secretion.

Another hallmark of inflammasome activation is the generation of microscopically detectable speck structures of multiprotein aggregates with a size of about 1 µm (Fernandes-Alnemri et al., 2007). To assess speck formation, LPS-primed wildtype BMDCs were either left untreated or were treated with nigericin, imatinib, or masitinib. With exception of LPS only controls, all samples treated with the TKIs or nigericin, exhibited speck formation, visible by ASC-staining and confocal microscopy (Figure 2C). Remarkably, masitinib acted as a very potent inflammasome stimulus, with ASC-specks formed after incubation times even shorter than those of K^+ ionophore nigericin.

Recently, a serine/threonine kinase termed NEK7, known to be taking part in mitotic processes, has been introduced as an upstream interaction partner of NLRP3. Two groups independently showed that NEK7 is critically required for NLRP3 inflammasome activation. On one hand NEK7 transduces the potassium efflux signal upstream of the NLRP3 inflammasome, on the other hand it senses mitochondrial ROS production. Both events trigger NEK7 association with NLRP3 and drive inflammasome assembly, independent of its kinase activity (He et al., 2016; Shi et al., 2016). To investigate if NEK7 also transduces the TKI-driven activation signal to NLRP3, we stimulated $Nek7^{-/-}$ BMDCs with imatinib and masitinib. We observed that inflammasome activation by both TKIs partially depends on NEK7, as IL-1β secretion was diminished in $Nek7^{-/-}$ BMDCs (Figure 2D). However, we observed some residual IL-1β secretion from $Nek7^{-/-}$ BMDCs, when stimulated with imatinib and masitinib in comparison to stimulatory conditions with ATP, MSU and Imiquimod (R837) in which IL-1β was largely absent.

So far, both TKIs demonstrated a NLRP3-dependent mode of action comparable to that of small molecule inhibitor Imiquimod. We therefore tested if imatinib and

masitinib triggered production of reactive oxygen species (ROS) to a similar extend as the imidazoquinoline. Indeed, IL-1β secretion triggered by both compounds was inhibited when cells were pre-incubated with the glutathione peroxidase mimetic ebselen (Figure 2E). These results indicate that both TKIs induce intracellular ROS production that eventually could lead to NLRP3 inflammasome activation and subsequent IL-1β secretion.

K^+ efflux is recognized as one of the major upstream events of NLRP3 activation and was proposed as an indispensable trigger for inflammasome assembly upon stimulation with particulate compounds (Franchi et al., 2007; Muñoz-Planillo et al., 2013). However, the small molecule imiquimod was found to also activate NLRP3 in the absence of K^+ efflux (Groß et al., 2016b). To test whether imatinib and masitinib require K^+ efflux to trigger NLRP3 activation, we prevented K^+ efflux by treating LPS-primed BMDCs with high doses of extracellular KCl prior to stimulation with TKIs or control stimuli. As previously reported, this treatment fully inhibited IL-1β secretion upon stimulation with the bacterial pore-forming toxin and K^+ ionophore nigericin (Pétrilli et al., 2007). Both imatinib and masitinib activated the inflammasome K^+ efflux-dependent, as increased extracellular concentrations of KCl reduce significantly IL-1β secretion (Figure 2E). As observed during time-lapse microscopy, masitinib, showed faster activation kinetics (Figure 1C and 2C) and was therefore less inhibited by KCl and ebselen after long time exposures. This effect points to a positive feedback loop of an axis which integrates events of membrane lysis, K^+ efflux, and eventually inflammasome activation, driven by masitinib and imatinib acting on BMDCs.

Taken together, these data demonstrate that imatinib and masitinib can trigger the NLRP3 inflammasome in a NEK7-dependent manner, and induce intracellular ROS production and K^+ efflux that eventually trigger inflammasome activation in BMDCs.

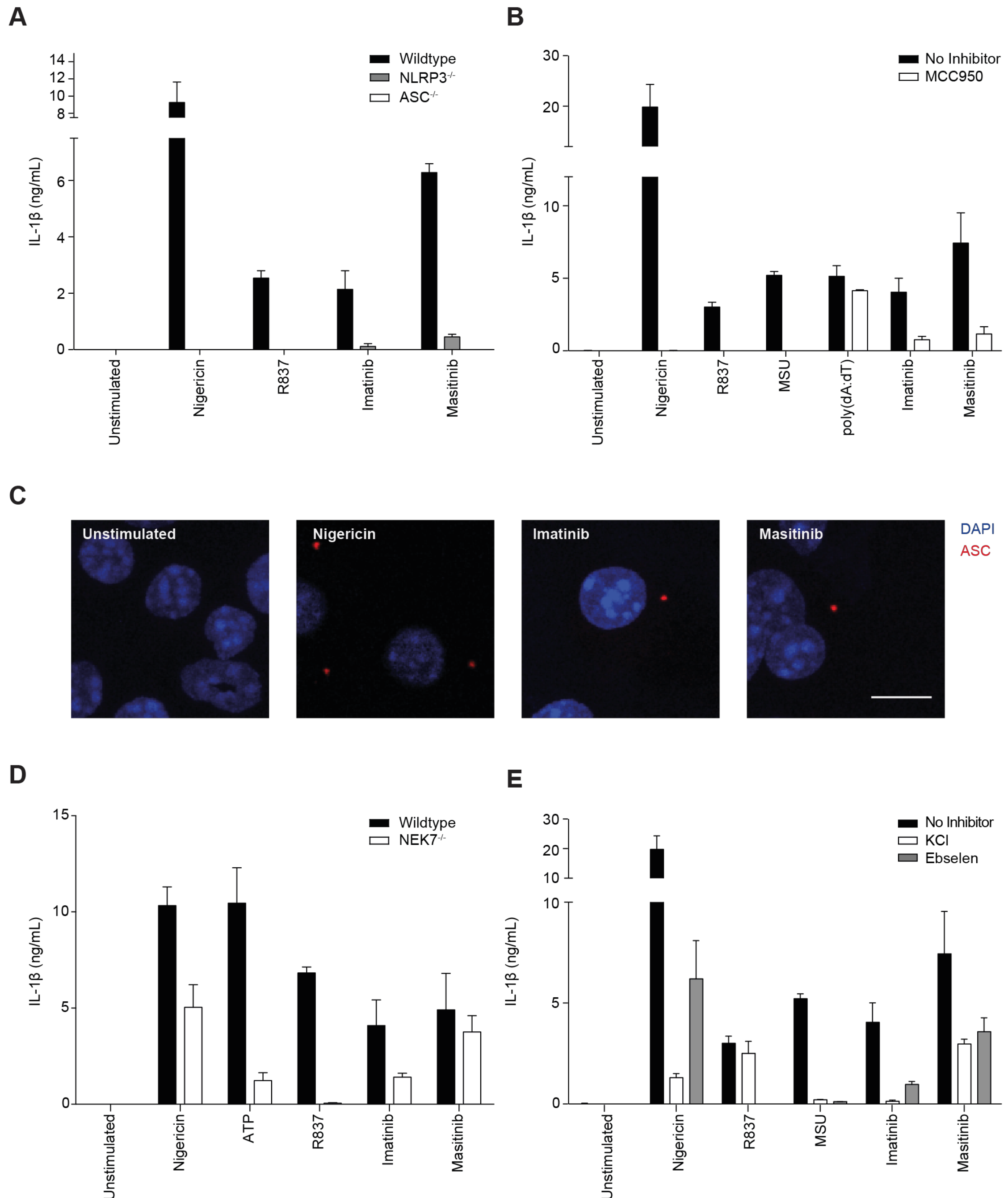


Figure 2: Imatinib and masitinib activate the NLRP3 inflammasome. (A) LPS-primed BMDCs from wildtype, NLRP3- and ASC-deficient mice were treated with 40µM imatinib and 20µM masitinib for 3 h. (B) BMDCs were treated with MCC950 30 min prior stimulation with in methods indicated concentrations of nigericin, imiquimod, MSU, poly(dA:dT), imatinib (60µM) or masitinib (40µM). (C) Immunofluorescence staining of ASC (red) in fixed, LPS-primed BMDCs stimulated with 10µM nigericin, 60µM imatinib or 40µM masitinib. Nuclei were counterstained with DAPI (blue). Scale bar represents 10 µm. (D) LPS-Primed BMDCs from wildtype or NEK7-deficient mice were stimulated with control compounds and with imatinib (40µM) or masitinib (20µM). (E) LPS-primed BMDCs were treated with 25mM KCl or 40µM ebselen for 30 min and subsequently stimulated with control compounds and with imatinib (60µM) or masitinib (40µM). (A-E) IL-1β was quantified from cell-free supernatants. Cytokine secretion data is depicted

Figure 2 continued: as mean ± SD of technical triplicates. The results from each experiment in this figure are representative of at least three independent experiments.

3.1.3 Imatinib and Masitinib do not require inflammasome function to trigger cell death

Gasdermin-D (GSDMD) was recently proposed as the protein responsible for pore formation during pyroptosis upon cleavage by caspase-1 or caspase-11 (Liu et al., 2016a). Additionally, GSDMD regulates the release of IL-1β from living hyperactivated macrophages, independent of its pyroptotic capacity (Evavold et al., 2018). Caspase-11 recognizes intracellular LPS and cleaves GSDMD provoking membrane pores. K^+ ions are surging through the leaky membrane and lead to the activation of NLRP3 in a non-canonical way, by involving K^+ efflux first and inflammasome activation after (Aglietti & Dueber, 2017). We therefore sought to test whether imatinib and masitinib trigger lytic cell death in a caspase-1, caspase-11 and GSDMD-dependent manner. To this end, we stimulated LPS-primed BMDCs from GSDMD-deficient or caspase-1/caspase-11 double knockout mice with TKIs and known NLRP3 inflammasome activators and measured both IL-1β secretion and LDH release, monitoring the percentage of lytic cell death. In line with previous reports, R837, ATP and MSU showed strongly reduced IL-1β secretion and cell death in knockout cells (Figure 3A). In response to imatinib and masitinib, BMDCs from both caspase-1/11 and GSDMD knockout mice displayed a similar LDH release profile, showing prominent residual signal even with the highest TKI concentration tested (Figure 3A). We detected also a high residual LDH signal of Caspase1/11$^{-/-}$ and GSDMD$^{-/-}$ cells treated with the crystal particle MSU, which was not observed before but most likely can be attributed to the membrane compromising nature of the crystalline agent. Nevertheless, it is well established that NLRP3 activation by MSU and other crystal particles is inhibited by the phagocytosis inhibitor cytochalasin D (Hornung et al., 2008). However, when we pre-incubated BMDCs with cytochalasin D and subsequently stimulated with TKIs, we did not observe inhibition of IL-1β secretion or LDH release, contrary to the outcome in MSU-treated cells. This indicates that neither imatinib nor masitinib is activating the

inflammasome in a crystal particle-like manner (Figure 3B). These data also indicate that both TKIs trigger an unconventional form of cell death that does not require caspase-1, caspase-11 and GSDMD cleavage and is thus clearly distinguishable from both canonical as well as non-canonical pyroptosis. IL-1β secretion was likewise strongly reduced from GSDMD$^{-/-}$ cells as well as caspase-1/11-deficient cells upon TKIs treatment. Yet, higher drug concentrations could cause IL-1β secretion even in GSDMD$^{-/-}$ BMDCs (Figure 3A). As those cells still have a functional caspase-1 that cleaves pro-IL-1β, retention of mature IL-1β within the cell was detectable (Figure 3C). Given the fact that we observed a strong residual LDH signal in GSDMD$^{-/-}$ BMDCs treated with TKIs, we assume that the IL-1β secretion derived from pores introduced by another form of TKI-induced lytic cell death independent of pyroptosis. This hypothesis is strongly supported by western blotting for IL-1β and Caspase-1 from the same experiments (Figure 3C). Here we detected cleavage of pro-IL-1β in TKI-treated GSDMD$^{-/-}$ BMDCs. Caspase1/11$^{-/-}$ cells were unable to cleave pro-IL-1β, hence they did not secrete the mature form p20 (Figure 3B). Also, a faint IL-1β p20 signal was still detectable in GSDMD knockouts, matching with the hypothesis outlined above.

IL-1α secretion followed the same trend over the range of TKI concentrations tested as observed for IL-1β, indicating that both cytokines are released due to a similar cellular activation event (Figure 3A). This is in line with previous observations, that IL-1α secretion is a concomitant phenomenon of inflammasome activation even though its pro-form is cleaved by calpains and not by active caspase-1 (Gross et al., 2012; Howard et al., 1991; Schneider et al., 2017). These data show that TKIs are causing a lytic form of cell death which significantly differs from pyroptosis and suggest, that inflammasome activation may be rather a consequence than the primary cause of cell death in TKI-treated BMDCs.

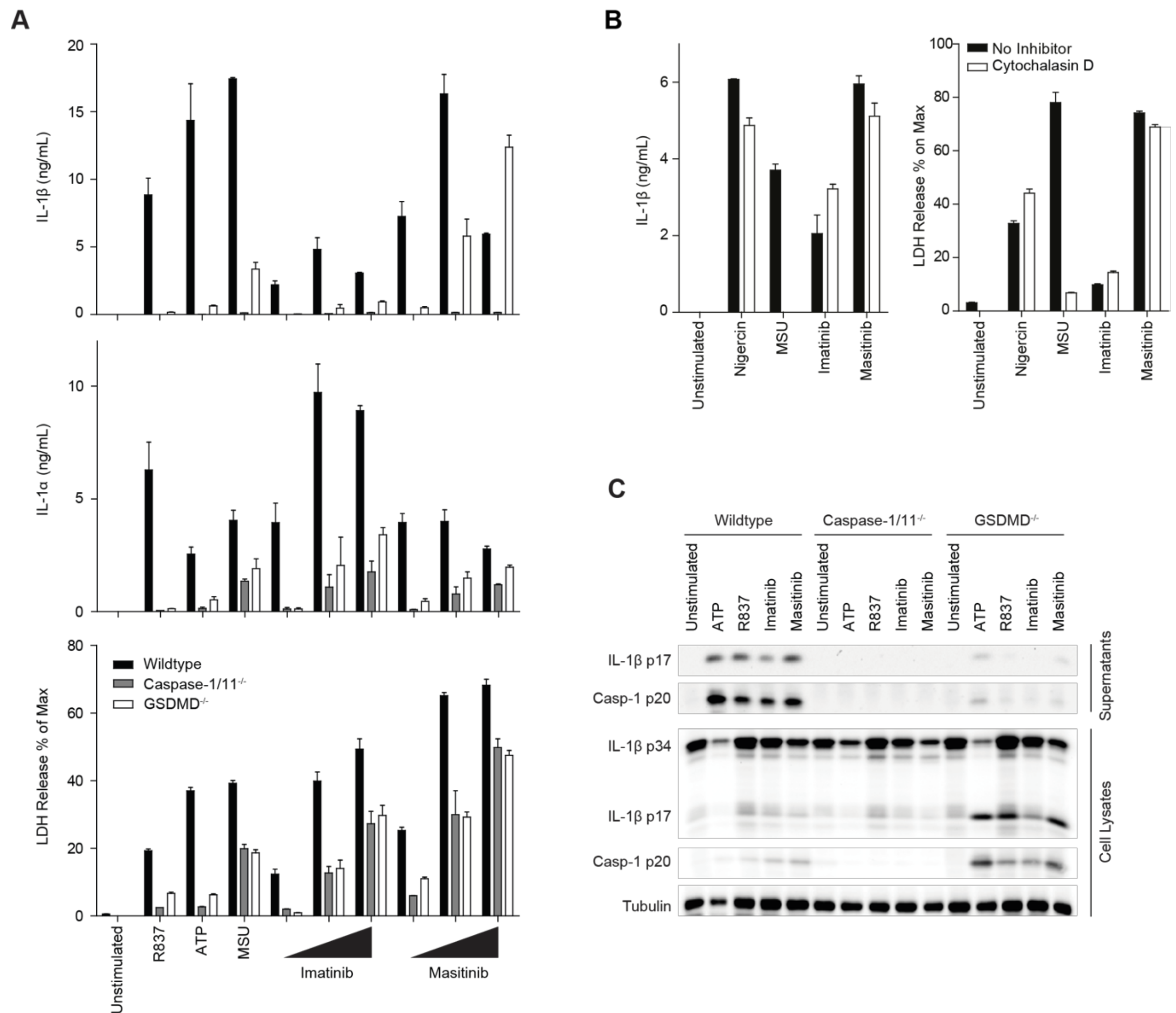


Figure 3: Imatinib and masitinib trigger a lytic from of cell death that is GSDMD and caspase-1 independent. (A) LPS-Primed BMDCs from wildtype, caspase-1/11- or GSDMD-deficient mice were stimulated with control compounds and with imatinib (40, 60, 80 µM) or masitinib (20, 40, 60 µM). IL-1β (top), IL-1α (middle) and lactic dehydrogenase (LDH) (bottom) were quantified from cell-free supernatants. (B) (A) LPS-Primed BMDCs were treated with 3µM cytochalasin D for 30 min and subsequently stimulated with control compounds and with imatinib (60µM) or masitinib (40µM). IL-1β (left) and LDH (right) were quantified from cell-free supernatants. (C) Immunoblot analysis of lysates and cell-free supernatants from (A). Cytokine secretion and LDH release data is depicted as mean ± SD of technical triplicates.

3.1.4 Imatinib and Masitinib induce a zVAD-fmk resistant and unconventional form of lytic cell death

To study the mechanisms behind TKI-induced cell death and inflammasome activation in more detail, we tested the pan-caspase inhibitor zVAD-fmk. The inhibitor was applied prior to inflammasome stimulation and effectively blocked IL-1β secretion induced by all compounds tested (Figure 4A). The LDH release showed that zVAD-fmk could not prevent cell death for neither TKIs nor canonical inflammasome stimuli. This is in line with published data showing that cells which were inhibited with zVAD-fmk are driven toward other forms of programmed cell death thus overcoming the loss of a pyroptotic pathway (Cullen et al., 2015; Gross et al., 2012). Recently, a link between necroptosis and inflammasome activation has been proposed by several groups. It was shown that RIPK3 can activate NLRP3 through caspase-8 catalytic activity in a MLKL-independent manner (Lawlor et al., 2015). Also, MLKL-driven necroptosis leads to K^+ efflux and NLRP3 inflammasome activation (Conos et al., 2017a; Gutierrez et al., 2017). To test an involvement of necroptosome-driven cell death upon TKI-stimulation, we tested BMDCs pre-treated with necrostatin-1, a small molecule inhibitor of RIPK1, commonly used to block necroptosis (Vandenabeele et al., 2013). Necrostatin-1 was unable to block IL-1β release and cell death induced by treatment with imatinib, masitinib or other inflammasome activators (Figure 4B). Moreover, cell death is not affected by the presence of necrostatin-1 as LDH release levels are always comparable to wild-type controls (Figure 4B and C). Together, these observations imply that imatinib and masitinib are not activating NLRP3 through engagement of the necroptosome.

The highly regulated signalling pathway of necroptosis, revolutionized the view on necrosis as an unprogrammed lytic cell death (Berghe et al., 2014). Different other lytic cell death pathways were described, some of which are parthanatos and ferroptosis. Both are highly regulated, but are independent of the activity of caspases and cannot be rescued by pan-caspase inhibitors like zVAD-fmk (Koh et al., 2005; Dixon et al., 2012). Parthanatos is a poly-(ADP-ribose) polymerase-1 (PARP-1) mediated cell death pathway that is activated by DNA damage and leads to an enhanced synthesis of the toxic poly-ADP-ribose (PAR). PAR directly binds the apotosis-inducing factor (AIF), which is then translocated from the mitochondria to

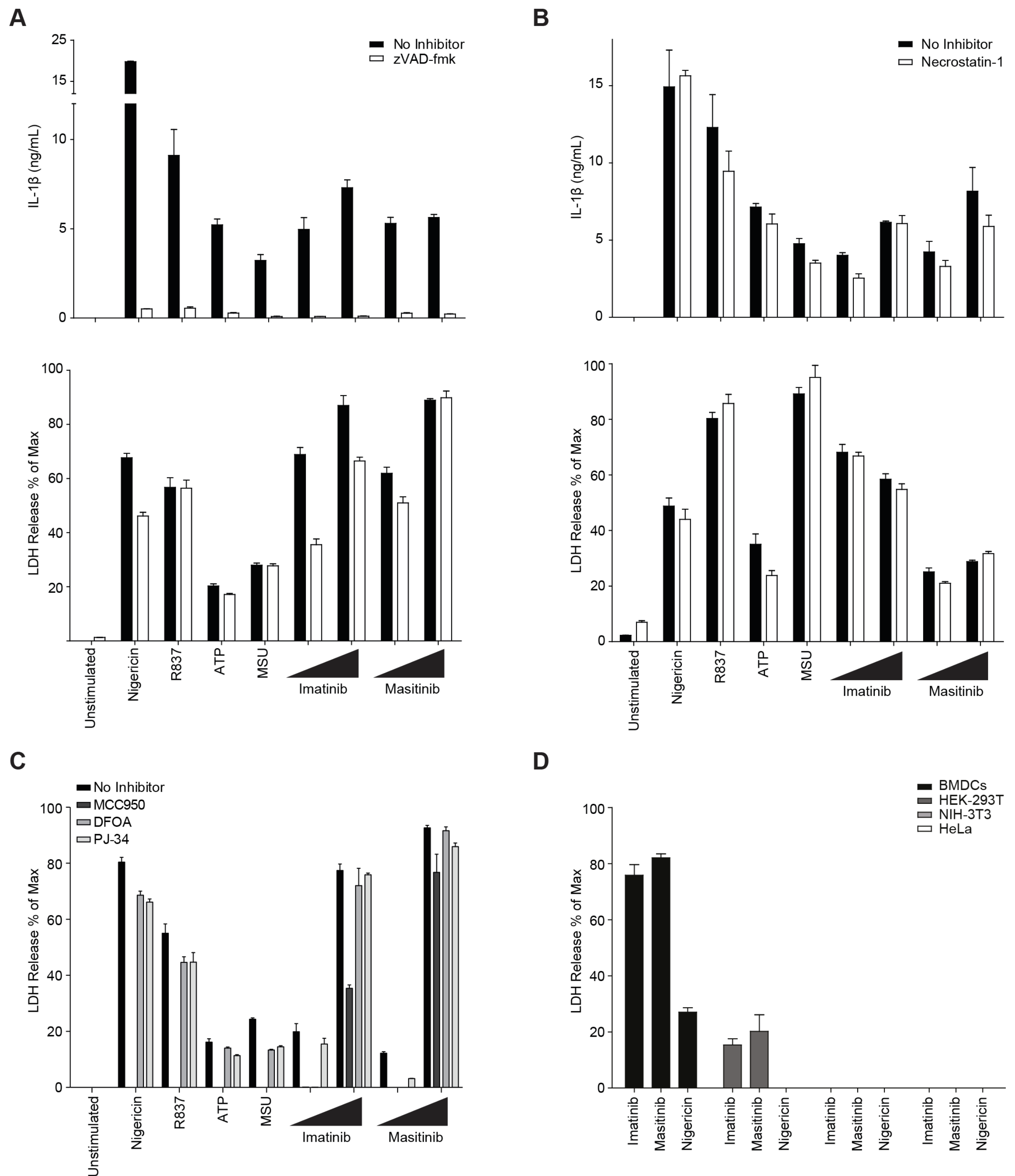


Figure 4: Imatinib and masitinib induce a non-conventional form of programmed cell death that is caspases-independent. (A, B) LPS-Primed BMDCs were treated with (A) zVAD-fmk 20μM or (B) Necrostatin-1 30μM for 30 min and subsequently stimulated with control compounds and with imatinib (40, 60 μM) or masitinib (20, 40 μM). IL-1β (upper) and LDH (lower) were quantified from cell-free supernatants. (C) LPS-Primed BMDCs were treated with MCC950 5μM, DFOA 100μM, PJ-34 5μM for 30 min and subsequently stimulated with control compounds and with imatinib (40, 60 μM) or masitinib (20, 40 μM). LDH was quantified from cell-free supernatants. (D) LDH release of LPS-primed BMDCs, HEK293T, NIH-3T3 and HeLa cells after stimulation with 60μM imatinib, 30μM masitinib or 5μM nigericin. Cytokine secretion and LDH release data is depicted as mean ± SD of technical triplicates. The results from each experiment in this figure are representative of at least three independent experiments.

the nucleus, where it induces chromatin condensation and DNA fragmentation (Zhang et al., 1994). PJ-34 inhibits PARP-1 by direct interaction and has an strongly suppressive effect on parthanatos (Huang et al., 1994). Ferroptosis is, however, induced by the accumulation of lipid peroxides in the cell. These are generated by excessive intracellular iron and a lacking lipid repair mechanism, which is, under physiological conditions, regulated by a cysteine/glutamate antiporter. Deferoxamine (DFOA) acts as an iron chelator and can thus specifically inhibit ferroptosis. (Dixon et al., 2012)

To test, whether these two cell death pathways are activated by imatinib and masitinib when caspases are blocked by zVAD-fmk (Figure 4A), DFOA and PJ-34 were applied prior to inflammasome activation. Even though DFOA was effective in lower concentrations of both TKIs, the effect was completely lost for 20µM higher concentrations (Figure 4C).

To reject the hypothesis that we were observing a general cytotoxicity event, we tested imatinib and masitinib on HEK-293T cells, HeLa cells, and 3T3 fibroblasts, and analysed LDH release. Both TKIs were relatively ineffective in causing cell death in the tested cells lines (Figure 4D). Taken together, our data show that both imatinib and masitinib are causing a form of zVAD-resistant lytic cell death and do neither rely on necroptosome assembly nor on parthanatos or ferroptosis. It is intriguing to speculate that both TKIs tested here instead provoke another unconventional form of cell death, yet to be characterized in full mechanistical details.

3.1.5 High molecular weight PEG rescues Imatinib- and Masitinib-driven Cell Death

When challenging distinct forms of programmed cell death by specific inhibition or by using cells for various knock-out mouse strains, we did not achieve complete reduction in LDH release upon TKI stimulation. Polyethylene glycols (PEGs) were shown to protect cells from death by preserving and maintaining membrane integrity (Dutheil et al., 2009; Malhotra et al., 2011). Moreover, high molecular weight PEG was reported to prevent cell swelling and inflammasome activation in a *S. typhimurium* infection model (Fink et al., 2008; Fink & Cookson, 2006). Thus, we

preincubated LPS-primed BMDCs with PEG 3000 before treatment with either nigericin, ATP, MSU, or the two TKIs imatinib and masitinib. PEG was highly effective in inhibiting IL-1β secretion induced by both TKIs, whereas control activators engaged inflammasome assembly even in the presence of PEG (Figure 5A and B). While the pyroptotic decay induced by nigericin, ATP, and MSU remained unaffected, the PEG polyether compound specifically protected BMDCs from a TKI-induced cell death (Figure 5A). In contrast, PEG showed no effect on TNFα production and secretion by BMDCs upon stimulation (Figure 5B).

To address the question if PEG is inhibiting ion fluctuations triggered by membrane stabilization upon cell death activation, we transfected LPS with Lipofectamine2000. In this way we could test whether NLRP3 can be activated by K^+ efflux in a non-canonical manner in the presence of PEG. We observed unaltered IL-1β secretion upon LPS transfection in the presence of PEG (Figure 5C), indicating an specific effect on TKI triggered cell death independent of ion flux.

Taken together, these data show that the tested TKIs are inducing a cell death pathway which is distinguishable from pyroptosis induced by common NLRP3 stimuli. Also, we observed that PEG protects the cells from TKI-induced inflammasome activation and subsequent cell death. This observation suggests that NLRP3 inflammasome assembly upon TKI-treatment might be a consequence of an unconventional mode of cell death mechanism and a cell type specific mode of action restricted to inflammasome competent cells of the organism.

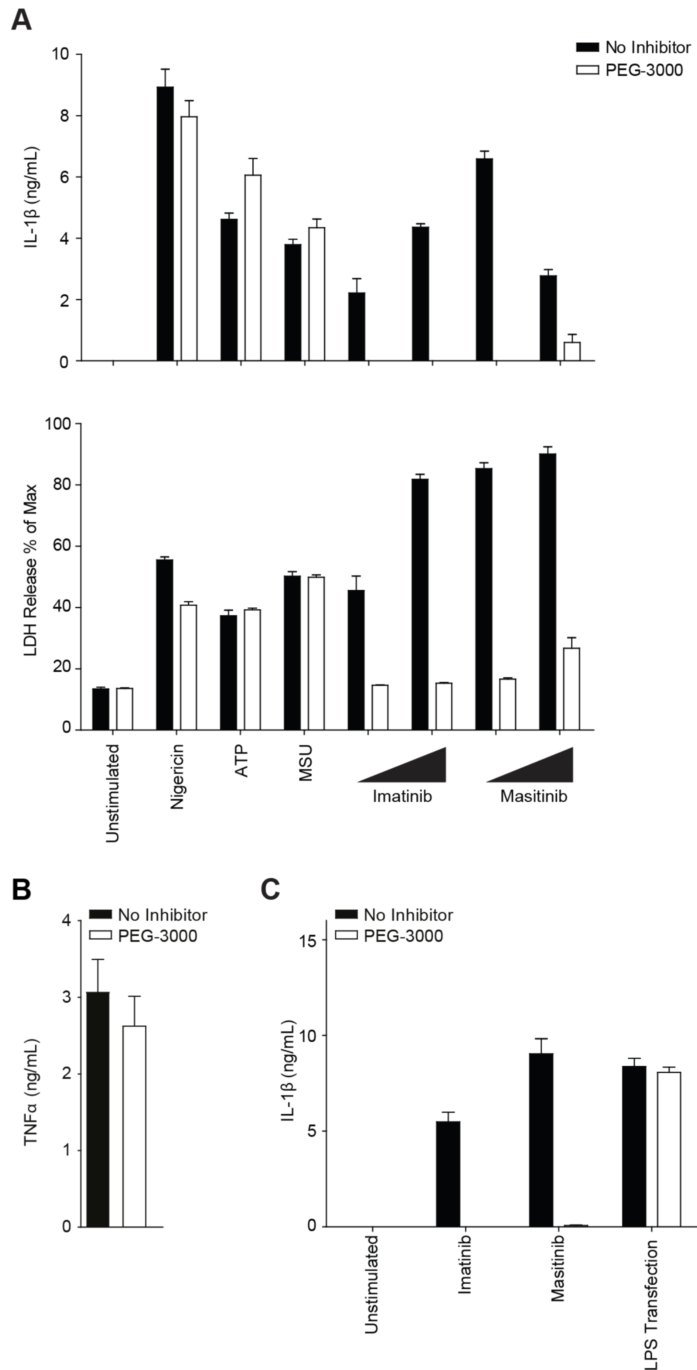


Figure 5: High molecular weight polyethylene glycol (PEG) rescues imatinib and masitinib-induced lytic cell death. (A) LPS-primed BMDCs were treated with 5mM PEG-3000 for 30 min and subsequently stimulated with control compounds and with imatinib (60, 80μM) or masitinib (40, 60μM). IL-1β (upper) and LDH (lower) were quantified from cell-free supernatants. (B) Primed BMDCs were left untreated or treated 5mM PEG-3000. TNF secretion was quantified from cell-free supernatants by ELISA.
(C) LPS-Primed BMDCs were treated with 5mM PEG-3000 for 30 min and subsequently stimulated with imatinib (50μM) or masitinib (30μM) for 3h or were transfected with 2 μg LPS per well on a 96-well plate and stimulated 16h. IL-1β was quantified from cell-free supernatants. Cytokine secretion and LDH release data is depicted as mean ± SD of technical triplicates. The results from each experiment in this figure are representative of at least three independent experiments.

3.2. NEK7 DEPENDENCY IN NLRP3 INFLAMMASOME ACTIVATION

The NIMA-related kinase 7 (NEK7) is a serine-threonine kinase, which was previously associated with the assembly of the spindle apparatus during mitosis. However, recently, an unexpected role of NEK7 in NLRP3 inflammasome formation was reported by three groups independently (He et al., 2016; Schmid-burgk et al., 2016; Shi et al., 2016). NEK7 was shown to sense both K^+ efflux as well as mitochondrial ROS and transmit these signals to NLRP3 directly, by binding to the LRR domain of NLRP3. It is thus the key player upstream of NLRP3 that was searched for in the past decade.

3.2.1 NLRP3 ACTIVATION IS INDEPENDENT OF NEK7 IN BMDCS

To test, whether we could observe NEK7 dependency in our inflammasome assays as well, we treated LPS-primed wildtype, Nek7$^{-/-}$ or Caspase-1$^{-/-}$ BMDMs and BMDCs with nigericin, a standard NLRP3 activator. Prior to nigericin stimulation we added different NLRP3 inflammasome inhibitors or left the cells untreated. As expected, we could only detect IL-1β secretion in wildtype BMDMs, while Nek7$^{-/-}$ and Caspase-1$^{-/-}$ BMDMs did not show any IL-1β secretion (Figure 6A). Surprisingly, we observed prominent IL-1β in the supernatant culture medium of Nek7$^{-/-}$ BMDCs (Figure 6B), while Caspase-1$^{-/-}$ BMDCs did not show any IL-1β secretion. The IL-1β signal in NEK7-deficient BMDCs was in fact lower than the one of wildtype cells, however, still very robust. Like under wildtype conditions, the IL-1β secretion was inhibited by the well-established NLRP3 inflammasome inhibitors, namely KCl, MCC950, PDTC and ebselen (Figure 6B).

When investigating this observation on protein level, we observed very similar behavior in both cell types. Wildtype BMDMs showed cleaved Caspase-1 and mature IL-1β in the supernatant, while Nek7$^{-/-}$ and Caspase-1$^{-/-}$ BMDMs did not show any IL-1β or Caspase-1 processing (Figure 6C). This was clearly different in BMDCs, where Nek7$^{-/-}$ cells showed high levels of cleaved Caspase-1 and mature IL-1β in

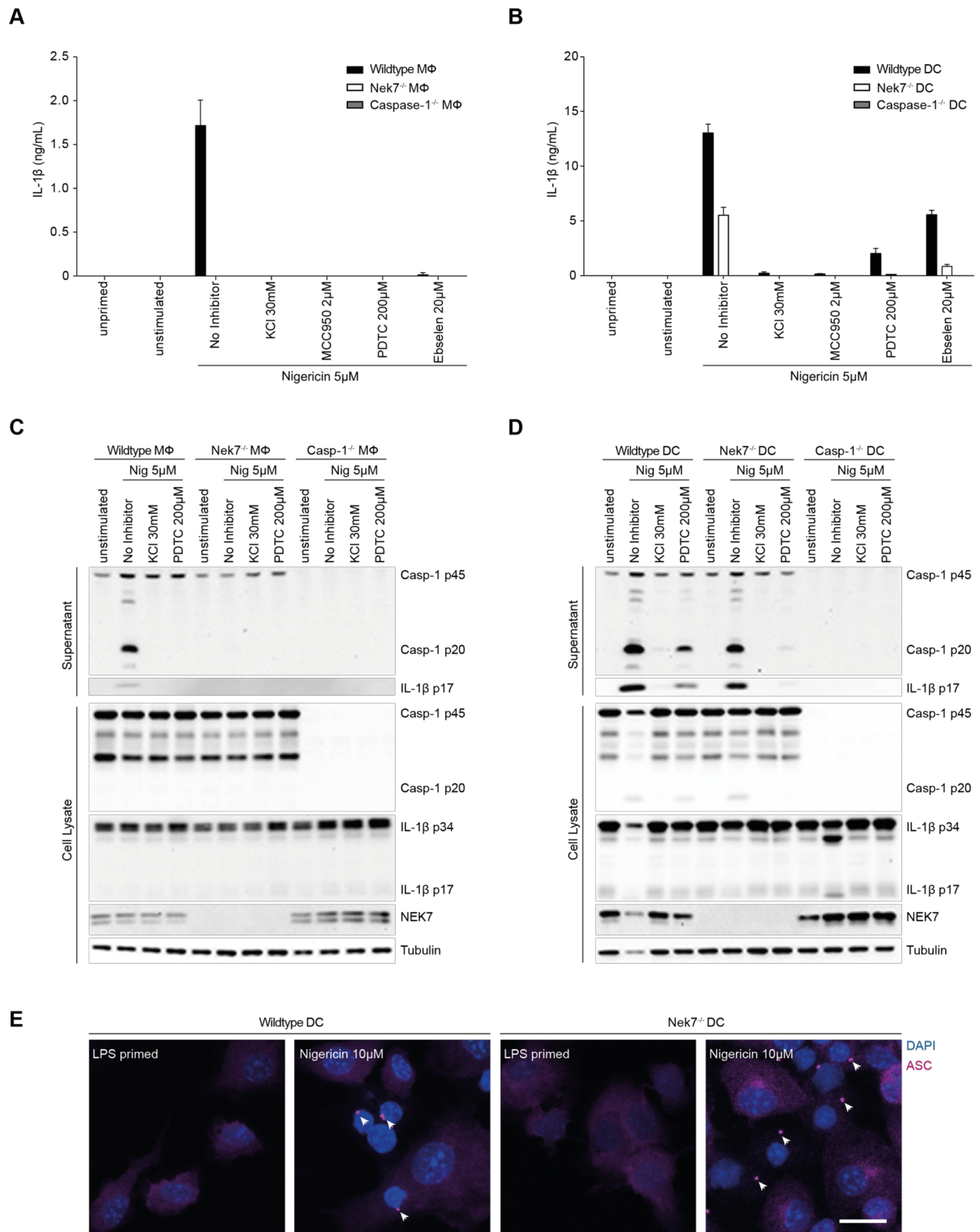


Figure 6: Nek7 independent NLRP3 activation by Nigericin in BMDCs. A, B) Wildtype, NEK7- and Caspase-1-deficient (A) BMDMs or (B) BMDCs were LPS-primed and treated with 30mM KCl, 2µM MCC950, 200µM PDTC or 20µM ebselen for 30 min and subsequently stimulated with nigericin. IL-1β was quantified from cell-free supernatants. Cytokine secretion data is depicted as mean ± SD of technical triplicates. (B) Immunoblot analysis of lysates and cell-free supernatants from (A). (C) Immunoblot analysis of lysates and cell-free supernatants from (D). (E) Immunofluorescence staining of ASC (magenta) in fixed, LPS-primed wildtype or NEK7-deficient BMDCs stimulated with 10µM nigericin. Nuclei were counterstained with DAPI (blue).

Figure 6 continued: Scale bar represents 10 µm. The results from each experiment in this figure are representative of at least three independent experiments

the supernatant which were comparable to results obtained with wildtype BMDCs (Figure 6D).

As NEK7 was reported to be crucial for ASC speck formation in BMDMs (Y. He, Zeng, et al., 2016), we analysed ASC speck formation in LPS-primed BMDCs upon nigericin stimulation. Also here we observed frequent ASC speck formation in NEK7-deficient BMDCs that was not different from those forming in wildtype cells (Figure 6E).

Taken together, our data confirm the reports that NEK7 is playing a crucial role for NLRP3 inflammasome formation in BMDMs. Interestingly, BMDCs show a strong NEK7 independency for NLRP3 activation and ASC speck formation, arising the question what the difference is between these two cell types that normally behave in a very similar way in regard to NLRP3 activation.

3.2.2 GM-CSF LEADS TO A STRONGER RESPONSIVENESS OF BMDMS TO NLRP3 INFLAMMASOME ACTIVATORS

As nigericin is inducing strong IL-1β secretion in NEK7-deficient BMDCs, we investigated if we could observe the same effect after stimulation with different NLRP3 activators or if it is nigericin specific. Thus, we treated LPS-primed wildtype, Nek7$^{-/-}$ and Caspase-1$^{-/-}$ BMDCs with gramicidin, ATP, R837 and the TKIs Imatinib, Masitinib and Pazopanib. Pazopanib is a TKI that is used in the clinic for renal cell carcinomas and soft tissue sarcomas (Sternberg et al., 2010; van der Graaf et al., 2012). Our group showed that Pazopanib, unlike Imatinib and Masitinib, is inducing NLRP3 activation by mitochondrial ROS but not K^+ efflux (Magnani, 2016; unpublished PhD thesis). Consequently, Pazopanib may have close similarity to the mechanism of NLRP3 activation of R837.

Interestingly, also other NLRP3 activators were able to induce robust IL-1β in NEK7-deficient BMDCs (Figure 7A). As expected, Caspase-1$^{-/-}$ BMDCs did not show any IL-1β signal after activation independent of the stimulus used (Figure7A).

To address the differences between BMDM and BMDC cultures, we tested first the different cell culture media first. While BMDMs are grown in Gibco DMEM medium with 40ng/mL of M-CSF, BMDCs are kept in Gibco RPMI media with HEPES, ß-Mercaptoethanol and 20ng/mL GM-CSF. Therefore, we changed the medium of wildtype, Nek7$^{-/-}$ and Caspase-1$^{-/-}$ BMDMs and BMDCS either for overnight incubation or right before LPS-priming and nigericin stimulation with the other cell culture medium respectively (Figure 7B). We observed a strong increase in IL-1β secretion in wildtype BMDMs after medium change, especially when leaving the BMDMs overnight in DC medium. Surprisingly, we could also detect high IL-1β in the supernatant of Nek7-deficient BMDMs. As expected, the control Caspase-1$^{-/-}$ BMDMs were not able to process IL-1β Caspase-1 independent even after medium change (Figure 7B). BMDCs were negatively affected by the MΦ medium, even when the medium was changed right before LPS-priming (Figure 7B).

Even though the groups, observing Nek7 dependency for NLRP3 activation, used DMEM medium with M-CSF as a growth factor, other groups culture BMDMs in RPMI medium with M-CSF (Camell et al., 2017; Zhao et al., 2018; Aranda-Souza et al., 2019).

To test, whether the growth factor GM-CSF or the medium is responsible for the boosting effect, we tested the kinetic effect of GM-CSF on BMDMs grown in RPMI medium (Figure 7C) as well as the responsiveness of BMDMs, cultured in different medium backgrounds (Figure 7D).

First, GM-CSF was either added to the medium of BMDMs already containing M-CSF or the growth factor was changed to GM-CSF for different time periods-overnight, 6h, 3h and 1h before LPS-priming. The IL-1β signal was strongly increased when GM-CSF was added prior NLRP3 activation, independent of the presence of M-CSF in the medium (Figure 7C). An overnight incubation induced the highest IL-1β secretion, while no difference between 6h or 1h before LPS-priming was observed (Figure 7C). For all following experiments only two time points were used, overnight and 1h before priming.

Second, we tested different media conditions, with or without GM-CSF incubation, for their influence on IL-1β secretion. Therefore, BMDM were cultured in 4 different media conditions: DMEM with two different M-CSF concentrations or RPMI with or

without HEPES plus 80ng/mL M-CSF. Surprisingly, we could also detect differences in IL-1β secretion after standard nigericin activation in the four culture medium conditions without GM-CSF (Figure 7D). IL-1β secretion was highest in RPMI medium without HEPES. GM-CSF led to the same enhancing effect in all four culture medium conditions, and was thus acting independent of the medium (Figure 7D).

As a stronger NLRP3 inflammasome responsiveness of BMDMs was observed in RPMI medium and because we wanted to minimize the differences of BMDM and BMDC cultures to be able to isolate the effect of the growth factor itself, we decided to culture the BMDMs in RPMI medium with 80ng/mL M-CSF for the remaining experiments. Under these aligned culture conditions, we tested the effect of GM-CSF on LPS-primed BMDMs treated with different NLRP3 activators or poly(dA:dT), a well-established AIM2 inflammasome activator. An enhancing effect of GM-CSF on IL-1β secretion was even visible when added 1h before priming, it was far stronger when GM-CSF was added to the medium overnight (Figure 7E). Furthermore, the increase in IL-1β secretion induced by GM-CSF was activator independent, and thus also observed for the AIM2 inflammasome (Figure 7E). The effect was missing for BMDCs grown in RPMI medium supplemented with GM-CSF. To exclude that GM-CSF is inducing stronger IL-1β secretion in an unspecific way, we tested the standard NLRP3 inhibitors on nigericin stimulated BMDMs treated with GM-CSF. The strong IL-1β signal was completely diminished in the presence of each inhibitor, which was not the case when IL-1β secretion was induced by the AIM2 inflammasome (Figure 7F).

Finally, these data show that GM-CSF as well as RPMI medium itself induce higher IL-1β secretion by BMDMs, which is diminished by standard NLRP3 inhibitors. Because the response to nigericin is stronger when cells are preactivated with GM-CSF, even right before priming, Nek7 independent IL-1β secretion can be observed in BMDMs. The enhancing effect of GM-CSF is, however, not NLRP3 inflammasome specific, but is also influencing AIM2 inflammasome signalling.

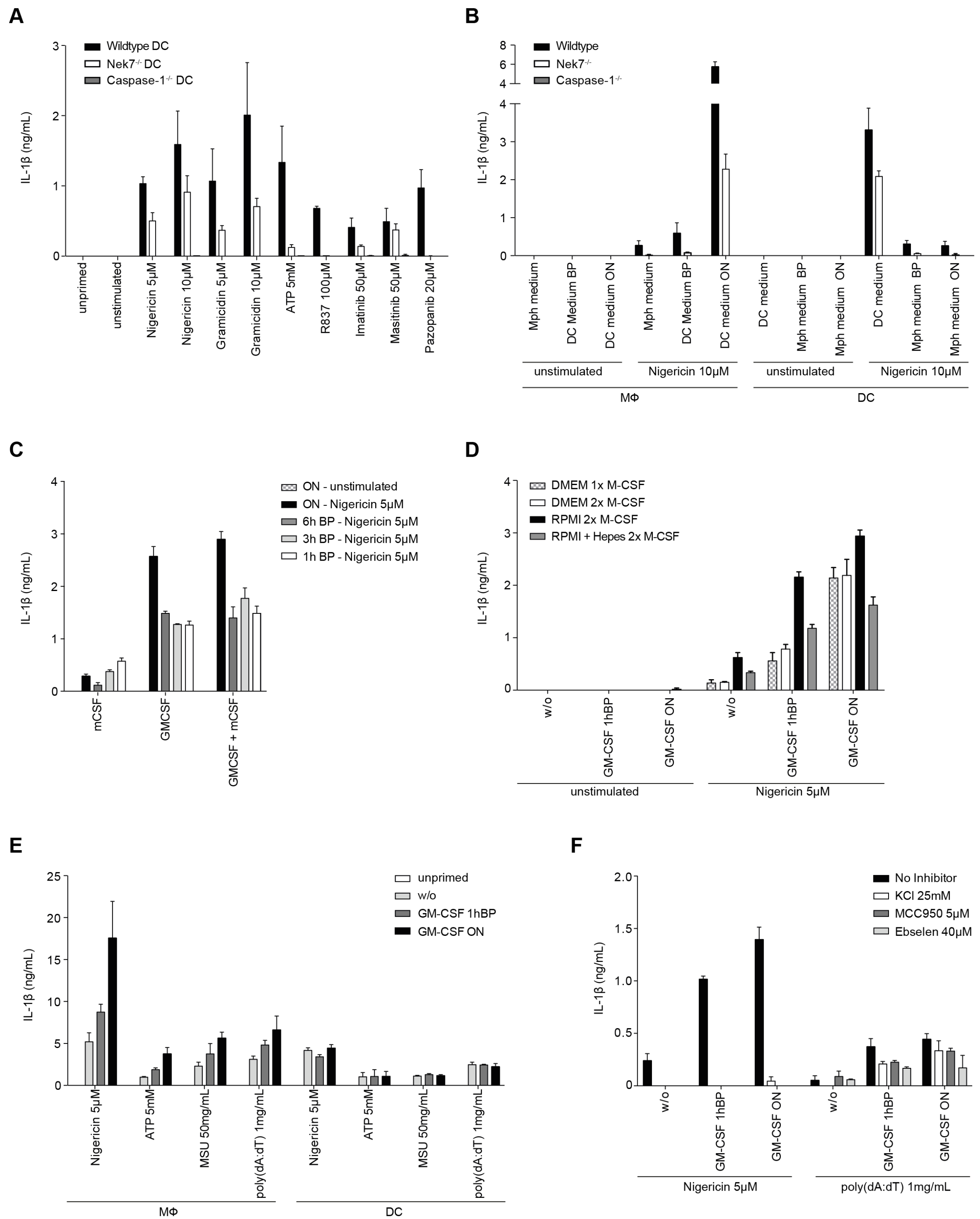


Figure 7: GM-CSF induces a stronger inflammasome response in BMDMs. (A) LPS-primed wildtype, NEK7- or Caspase-1 deficient BMDCs were treated with two different concentrations of nigericin or gramicidin (5μM and 10μM), control compounds in standard concentrations and the TKIs imatinib 50μM, masitinib 50μM or pazopanib 20μM. (B) LPS-primed wildtype, Nek7$^{-/-}$ or Caspase-1$^{-/-}$ BMDMs and BMDCs were stimulated with 10μM nigericin after an medium change either the night before or right before priming. (C) LPS-primed BMDMs were treated with nigericin 5μM after M-CSF, GM-CSF or both were supplemented to the standard Macrophage medium without growth factor (DMEM + 10% FCS +1% P/S). The growth factor

Figure 7 continued: was added overnight, 6 h, 3 h or 1 h before priming. (D) BMDMs were cultured in four different medium conditions, before they were LPS-primed and stimulated with Nigericin 5µM. Either a night before or 1 h before priming GM-CSF was added to the medium. (E) BMDMs or BMDCs were cultured in RPMI + 10% FCS +1% P/S supplemented with either M-CSF or GM-CSF. 1 h before LPS-priming (1h BP) or overnight (ON) additionally GM-CSF was added and the cells were stimulated with nigericin 5µM, ATP 5mM, MSU 50mg/mL or poly(dA:dT) 1mg/mL. (F) LPS-primed BMDMs were treated with KCl 25mM, MCC950 5µM or ebselen 40µM for 30 min and subsequently stimulated with nigericin 5µM or poly(dA:dT) 1mg/mL after GM-CSF was added 1 h before priming or overnight.
IL-1β was quantified from cell-free supernatants. Cytokine secretion data is depicted as mean ± SD of technical triplicates. The results from each experiment in this figure are representative of at least three independent experiments.

3.2.3 GM-CSF induces NEK7 independent NLRP3 activation in BMDMs

To analyse the isolated effect of GM-CSF on NLRP3 activation, we tested if GM-CSF alone is responsible for the NEK7 independency that was induced by the complete medium switch shown in Figure 7B. Furthermore, we were wondering if GM-CSF is acting as a priming signal, inducing a stronger inflammasome response in general. To answer these questions, we treated wildtype, Nek7$^{-/-}$, Nlrp3$^{-/-}$ and Caspase-1$^{-/-}$ BMDMs, which were cultured in RPMI medium supplemented with M-CSF, with GM-CSF instead of LPS or additionally to LPS, before stimulating them with nigericin. Interestingly, we did not observe any IL-1β without LPS-priming after GM-CSF preactivation (Figure 8A). However, Nek7$^{-/-}$ BMDMs showed IL-1β secretion even in the absence of GM-CSF, indicating a role of RPMI as a culture medium. GM-CSF additionally boosted IL-1β secretion in wildtype and Nek7$^{-/-}$ BMDMs, when added prior to LPS-priming and nigericin stimulation, showing clearly a NLRP3 activation pathway that is NEK7 independent (Figure 8A).
The GM-CSF effect was similar in AIM2 inflammasome activated BMDMs. Here, wildtype, Nek7$^{-/-}$, Nlrp3$^{-/-}$ BMDMs showed comparable IL-1β secretion after poly(dA:dT) activation, demonstrating the complete independence of AIM2 activation on NLRP3 and NEK7 (Figure 8B). As a control, caspase-1$^{-/-}$ BMDMs were never capable to secrete mature IL-1β (Figure 8A and B).
With the knowledge that GM-CSF cannot prime BMDMs, but is needed for their strong responsiveness, we were questioning if the presence of GM-CSF at any time

is sufficient to induce stronger IL-1β secretion and if it acts independent of LPS-priming. Therefore, we added GM-CSF to wildtype BMDMs at different time points before or during LPS-priming, but before nigericin stimulation. We observed that GM-CSF has only an enhancing effect on IL-1β secretion when added prior to LPS-priming and stayed ineffective when added after. This was also the case for TNFα secretion of wildtype BMDMs (Figure 8C). However, while TNFα secretion was dependent on the GM-CSF incubation period prior to LPS-priming, IL-1β was secreted in an all- or nothing response to GM-CSF.

To investigate the effect of GM-CSF on the NLRP3 signalling proteins, we analysed unprimed (Figure 8D) and LPS-primed (Figure 8E) BMDMs via western blot. As different NLRP3 splicing variants were found in human tissue[1], we compared also wildtype to Nlrp3$^{-/-}$ BMDMs and BMDCs, to test whether GM-CSF is influencing NLRP3 splicing or if the splicing variants could be cell specific.

We did not find any bigger differences between NLRP3 of BMDMs and BMDCs, (Figure 8D and E). However, we observed alterations in NLRP3 expression upon GM-CSF treatment in unprimed BMDMs (Figure 8D), indicating an enhancing effect of GM-CSF on transcriptional NLRP3 priming. Pro-Caspase-1 cleavage was increased in unprimed BMDMs upon nigericin treatment following GM-CSF administration (Figure 8D). Pro-IL-1β transcription was only induced by LPS-priming, being stronger in BMDMs treated with GM-CSF prior to LPS-priming (Figure 8E).

Taken together, this data demonstrate that GM-CSF is inducing a Nek7 independent IL-1β secretion. We can conclude that GM-CSF is not priming the cell but rather influences transcriptional priming of NLRP3 without effecting pro-IL-1β expression.

[1] See https://www.proteinatlas.org/ENSG00000162711-NLRP3/tissue

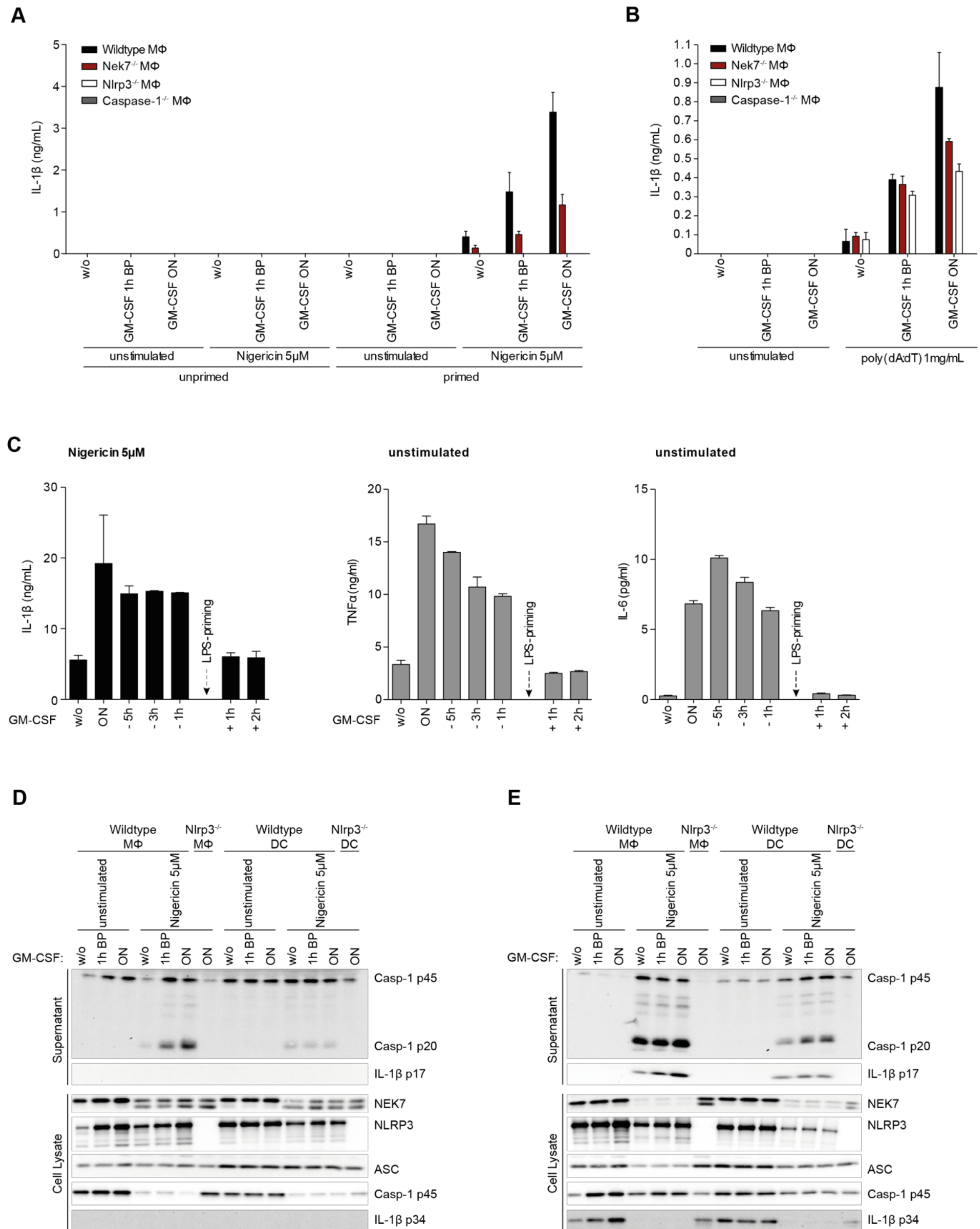


Figure 8: GM-CSF induces a Nek7 independency in BMDMs by licensing pro-Caspase-1. (A, B) Wildtype, NEK7-, NLRP3- or Caspase-1 deficient BMDMs were either left unprimed or LPS-primed after GM-CSF was added to the medium overnight (ON) or 1 h before priming (1h BP). The cells were then either stimulated with (A) nigericin 5μM or (B) poly(dA:dT) 1mg/mL. (C) LPS-primed BMDMs were treated with GM-CSF overnight, 5 h, 3 h, 1 h before priming or 1h and 2h after priming then stimulated with nigericin (left) or left unstimulated (middle and right). IL-1β, TNFα and IL-6 were quantified from cell-free supernatants. Cytokine secretion data is depicted as mean ± SD of technical triplicates. (D, E) Immunoblot analysis of lysates and

Figure 8 continued: cell-free supernatants from unprimed cells (D) and primed cells (E) of (A). The results from each experiment in this figure are representative of at least three independent experiments.

3.2.4 GM-CSF IS NOT EFFECTING THE CELL IDENTITY OF BMDMS

To reject the hypothesis that GM-CSF could induce a cell identity shift, whereby cultured BMDMs would become more DC-like and would therefore show a stronger inflammasome response, we performed FACS analysis of BMDMs treated with GM-CSF. M-CSF cultured BMDMs are known to differentiate in a rather homogenous fashion (Weischenfeldt & Porse, 2008). GM-CSF is, however, known to induce a very heterogeneous cell population, consisting of dendritic cells and macrophages (Helft et al., 2015b). Thus, macrophages can be found to some extend in the adherent fraction of a differentiation culture, while dendritic cells stay loosely adherent.

We analysed different DC and Mph marker of loosely adherent (Figure 9A) and adherent M-CSF cultured BMDMs (Figure 9B) and their changes in $CD11c^{high}MHCII^{high}$ population upon GM-CSF treatment.

Indeed, GM-CSF incubation overnight induced a bigger $CD11c^{high}MHCII^{high}$ population in loosely adherent cohorts of BMDMs (Figure 9A). However, no difference was observed when GM-CSF was added to the BMDMs culture 1h before LPS-priming (Figure 9A and B). CD11b and F4/80, two established macrophage surface marker, were also only downregulated in the loosely adherent $CD11b^{high}MHCII^{high}$ population when GM-CSF was present overnight (Figure 9A). Dendritic surface marker like CD11c or CD135 are unchanged. This was also true for the $CD11c^{high}MHCII^{high}$ population as a control. In adherent $CD11b^{high}MHCII^{high}$ populations, GM-CSF had no effect, even when added overnight (Figure 9B).

Because GM-CSF can by definition induce M1 macrophage polarity (Mantovani et al., 2004; Martinez & Gordon, 2014), we tested in the adherent and loosely adherent BMDM culture besides IL-1β and TNFα, the two M1 macrophage cytokines, also IL-10, which is secreted by M2 macrophages (Verreck et al., 2004). We either activated the NLRP3 inflammasome of loosely adherent or adherent wildtype BMDMs or left them unprimed or primed while supplementing GM-CSF before. Here, we could see, that while TNFα and IL-1β secretion is induced by GM-CSF,

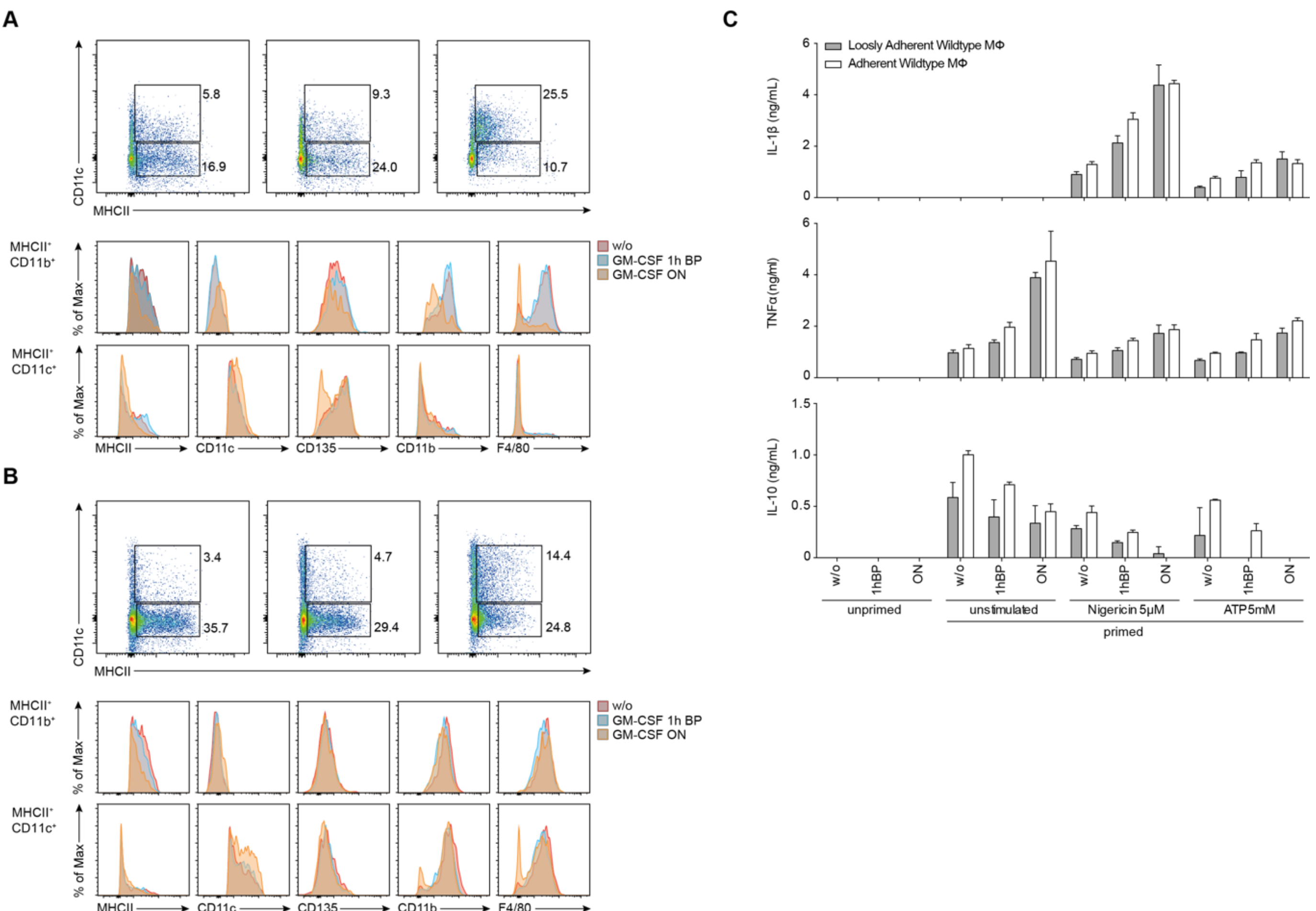


Figure 9: GM-CSF is not effecting BMDM cell identity, but increases M1 cytokines while decreasing IL-10 secretion. (A, B) Population and Surface marker analysis of (A) loosely adherent and (B) adherent BMDM culture cells were stained with several sets of antibodies to detect dendritic cell and macrophage population after GM-CSF was supplemented to the medium overnight (ON) or 1h before LPS-priming (1hBP). (C) LPS-Primed BMDMs were stimulated with nigercin 5µM or ATP 5mM after GM-CSF was added to the medium overnight or 1h before priming. IL-1β, TNFα and IL-10 were quantified from cell-free supernatants. Cytokine secretion data is depicted as mean ± SD of technical triplicates. The results from each experiment in this figure are representative of at least two independent experiments.

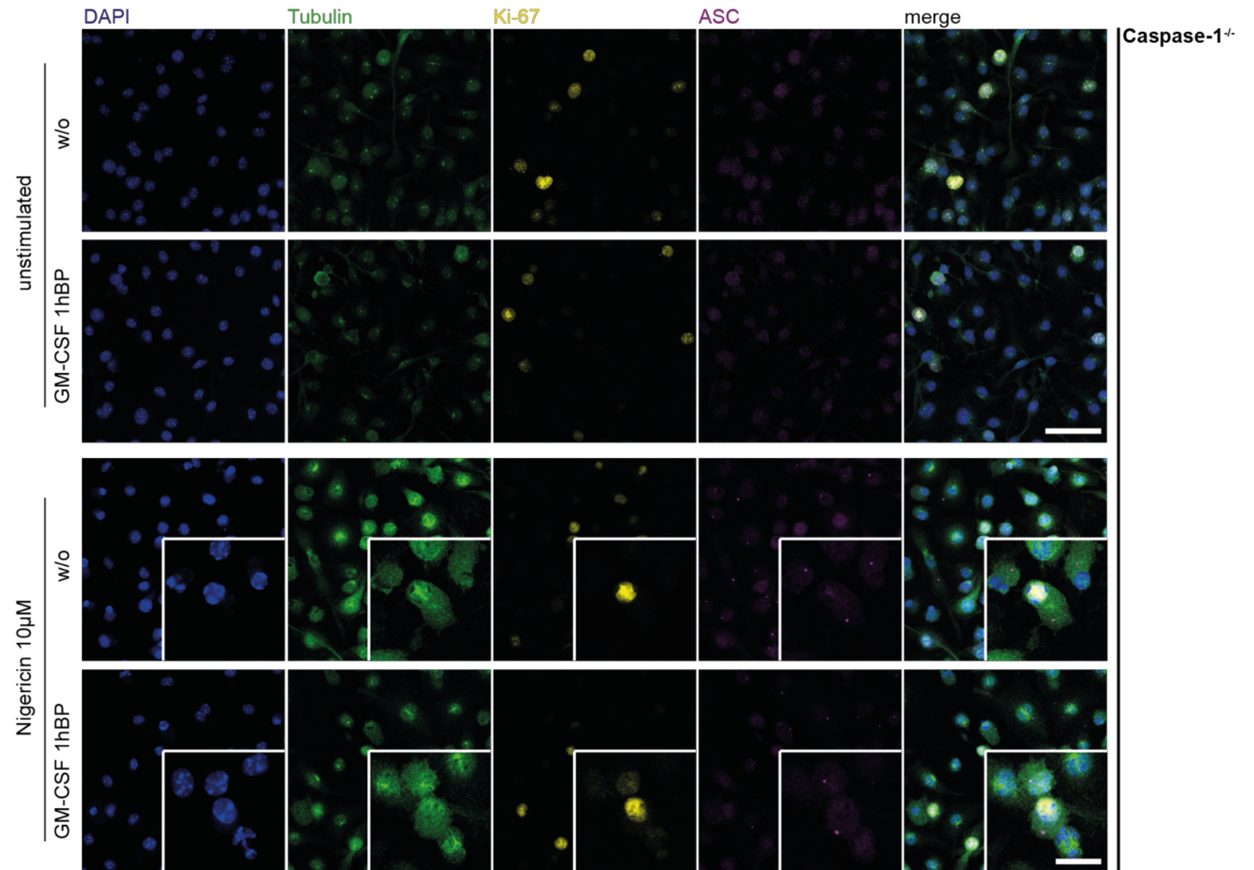
IL-10 is decreasing in the presence of GM-CSF (Figure 9C). Especially so, when the BMDMs were stimulated with inflammasome activators like nigericin or ATP (Figure 9C). Only a minor difference was observed between loosely adherent and adherent BMDMs IL-10 secretion, the later even secreting slightly more IL-10. IL-1β and TNFα secretion was comparable between these two cohorts (Figure 9C).

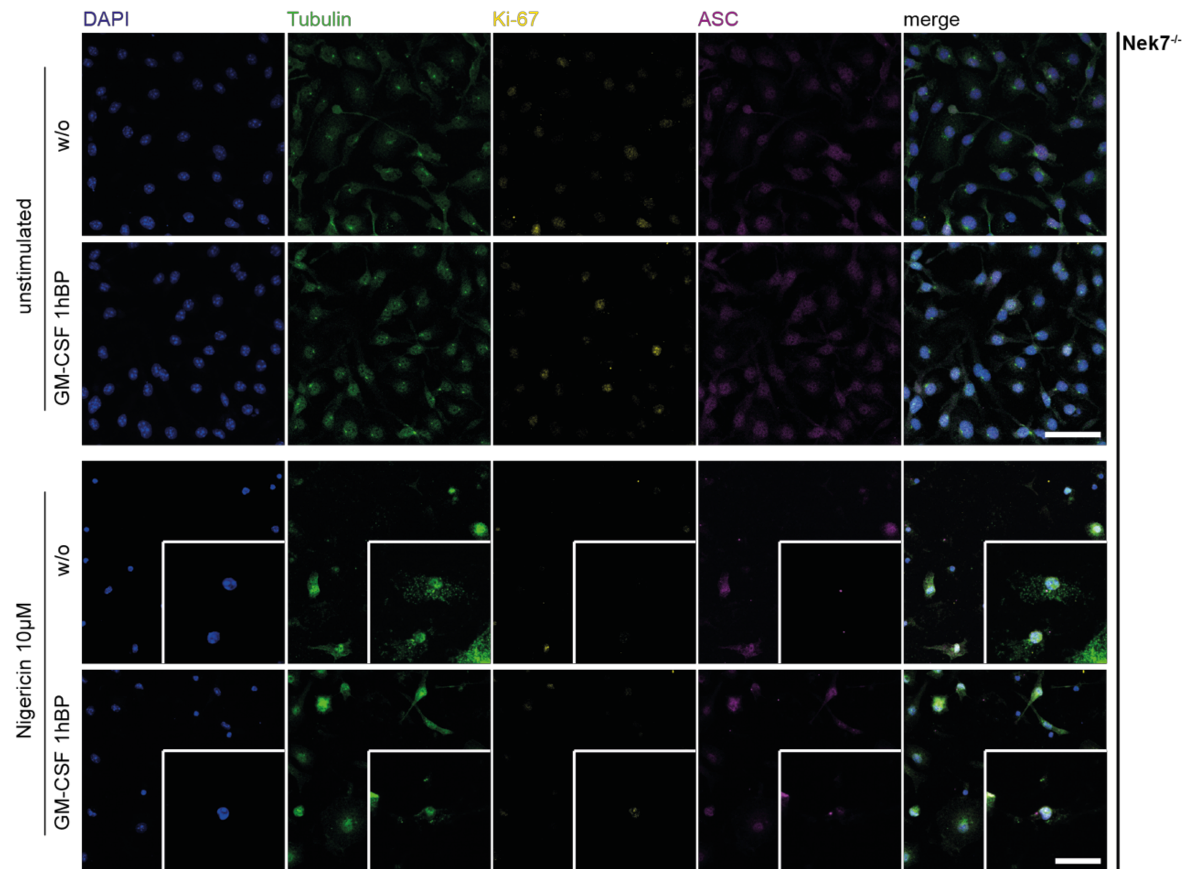
Finally, we can conclude that GM-CSF is not effecting the cell identity when added 1h before LPS-priming neither in loosely adherent nor in adherent BMDMs. After 1h of GM-CSF treatment an enhancement in IL-1β secretion and NLRP3 expression were observed. A small shift towards a DC population was only found in loosely adherent BMDMs and adherent CD11c^{high}MHCII^{high} BMDMs when GM-CSF was added overnight. The adherent CD11b^{high}MHCII^{high} BMDMs were not effected by overnight GM-CSF supplementation. GM-CSF had however a inhibiting effect on IL-10 secretion, which is normally induced in M2 macrophages.

3.2.5 GM-CSF effects IL-1β secretion without enhancing ASC speck frequencies or pyroptosis

As NEK7 is known for its pivotal role in microtubule organization during spindle apparatus formation (Yissachar et al., 2006), it was not surprising to see data showing that NEK7 is involved in the decision making process whether a cell would go into mitosis or pyroptosis (Shi et al., 2016). GM-CSF has also been associated to cell proliferation and survival by PI3K signalling (van de Laar et al., 2012). To see how GM-CSF and especially NEK7-deficiency is influencing cell proliferation and ASC speck formation in BMDMs, we stimulated caspase-1$^{-/-}$ and Nek7$^{-/-}$ LPS-primed cells with nigericin and stained for DAPI, tubulin, ASC and Ki-67, a cellular proliferation marker that marks the chromosome surface during mitosis.

Caspase-1-deficient BMDMs showed, like expected, ASC speck formation upon nigericin treatment (Figure 10). As the cells lack caspase-1, GSDMD is not being processed and pyroptosis is not induced. Ki-67 positive nuclei could be detected in these ASC positive cells (Figure 10), indicating that speck formation and mitosis are not exclusive events within one cell. NE7-deficient BMDMs also developed ASC specks upon nigericin treatment (Figure 10), even though in a much lower frequency.


A
DAPI
Tubulin
Ki-67
ASC
merge
Caspase-1-/-
unstimulated
w/o
GM-CSF 1hBP
Nigericin 10µM
w/o
GM-CSF 1hBP


B
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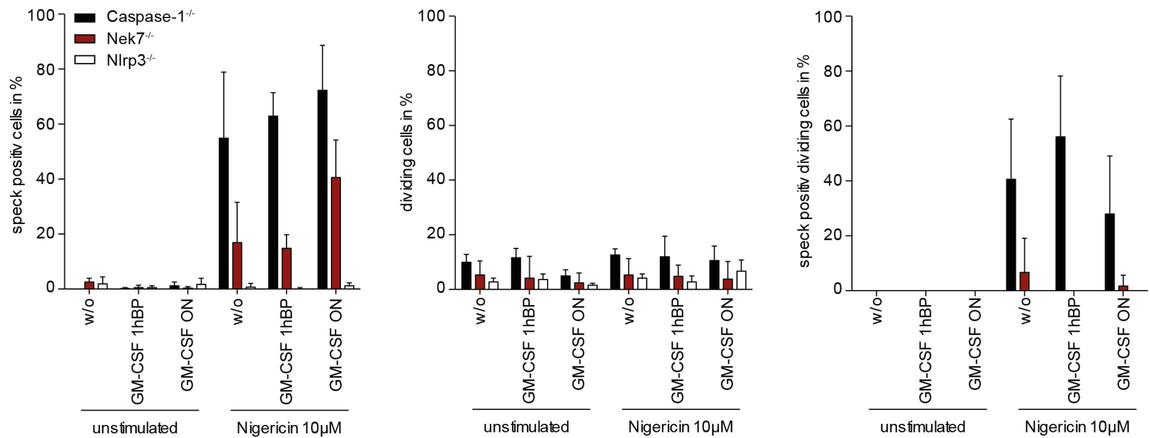
unstimulated
w/o
GM-CSF 1hBP
Nigericin 10µM
w/o
GM-CSF 1hBP

Figure 10: BMDMs form ASC specks independent of GM-CSF. Immunofluorescence staining of ASC (magenta), Tubulin (green) and KI-67 (yellow) in fixed, LPS-primed Caspase-1- or NEK7-deficient BMDMs stimulated with 10µM nigericin. Before LPS-priming cells were treated with GM-CSF for 1h (1hBP) or left untreated. Nuclei were counterstained with DAPI (blue). Scale bar in the bigger picture represents 50µm, while the scale bar in the smaller picture represents 10µm. The pictures are representative of two independent experiments.

Furthermore, Nek7$^{-/-}$ BMDMs displayed the typical pyroptotic phenotype showing a highly condensed nucleus. However, the Ki-67 signal was very weak in Nek7$^{-/-}$ (Figure 10), which may be explained by the reported NEK7 role in mitosis (REF).
GM-CSF seemed to have no big influence on the behaviour of the investigated cells, this is why we evaluated the microscopy pictures by count. Therefore, at least 400 cells were counted. Our assumption proved true, the percentage of speck positive cells did not increase upon GM-CSF-treatment for 1h before priming, and only very weakly when GM-CSF was added overnight (Figure 11A). This provides further evidence of the GM-CSF effect on inflammasome signalling itself and the missing one on cell identity. Furthermore, GM-CSF did not induce increased cell proliferation in this experimental setting (Figure 11A). Interestingly, NEK7-deficient ASC-positive BMDMs showed very rarely a KI-67 signal. This observation should be further investigated.
As we did not see any effect of GM-CSF on ASC speck formation, we analysed LDH release of wildtype, Nek7$^{-/-}$ and Caspase-1$^{-/-}$ BMDMs of the same experiment. It is of note, that nigericin treated wildtype as well as NEK7-deficient BMDMs that were stimulated with nigericin, were not effected by GM-CSF supplementation (Figure 11B). Thus, LDH release did not increase upon GM-CSF treatment 1h before priming, and only very weakly upon overnight administration (Figure 11B). This is in line with the microscopy analysis (Figure 10) and strengthens the hypothesis of specific inflammasome signalling interference by GM-CSF. Moreover, it becomes clear that GM-CSF is not influencing pyroptosis.
Taken together, this findings show that Nek7$^{-/-}$ BMDM have the ability to form ASC specks, even though it was published differently. Moreover, GM-CSF can further increase IL-1β secretion by effecting inflammasome cytokine signalling without

interfering with the pyroptotic part of the signalling cascade. Furthermore, mitosis is not supressing NLRP3 activation.

A



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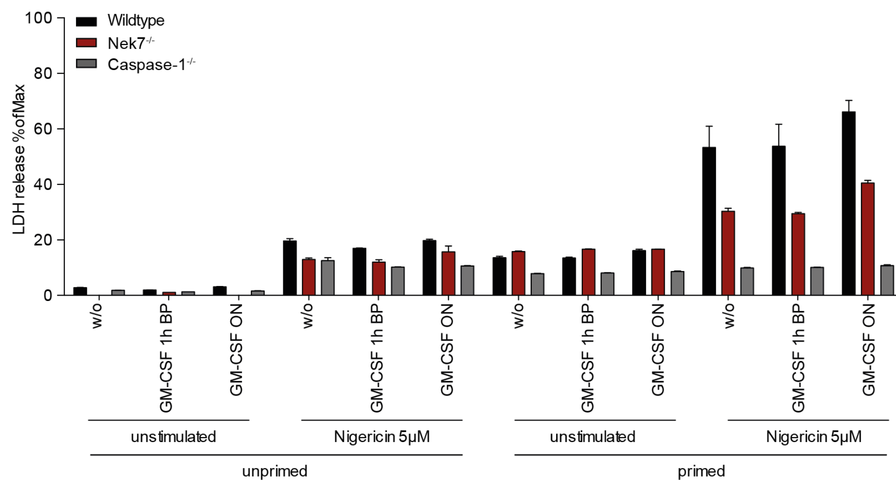

Figure 11: GM-CSF is neither influencing ASC speck frequency, cell proliferetation nor pyroptosis. (A, B) Wildtype, NEK7- or NLRP3-deficient LPS-primed BMDMs were treated with GM-CSF 1h before priming (1h BP) or overnight (ON) and stimulated with nigericin (A) 10µM or (B) 5µM. (A) Count of the immunofluorescence staining from figure 10. At least 300 nuclei in four images were counted. Data is depicted as mean ± SD of technical quadruplets. (B) LDH was quantified from cell-free supernatants. LDH release data is depicted as mean ± SD of technical triplicates. The results from each experiment in this figure are representative of at least three independent experiments.

DISCUSSION IV

4.1. IMATINIB AND MASITINIB TWO NLRP3 ACTIVATING SMALL MOLECULES

Several clinically relevant TKIs are robust and fast acting inflammasome stimuli (Magnani, PhD thesis, 2017). Focusing on the structurally related TKIs imatinib and masitinib, we observed triggering of a non-conventional cell death in myeloid cells that eventually led to the secretion of inflammatory mediators and NLRP3 inflammasome activation. The cytokines released during this process may affect leukemic cell survival and differentiation during TKI-treatment and might contribute to both immunomodulatory effects and adverse reactions.
As both imatinib and masitinib do not cause pyroptosis exclusively, they could be of special interest for analysis of programmed lytic cell deaths and their interplay in research studies.

4.1.1 IMATINIB AND MASITINIB INDUCE IL-1β SECRETION BY NLRP3 ACTIVATION

Imatinib and masitinib induced strong IL-1β secretion and caspase-1 activation in wildtype BMDCs upon incubation times comparable to those of common NLRP3

stimulators (Figure 1). Inflammasome knockout mice, including $Nlrp3^{-/-}$, showed strongly diminished IL-1β secretion (Figure 2). Moreover, the specific NLRP3 inflammasome inhibitor MCC950 (Coll et al., 2015b), whose exact targeting mechanism is still unknown, inhibited IL-1β secretion upon stimulation by both TKIs (Figure 2). This observation suggests that NLRP3 acts as a sensor responsible for inflammasome activation upon imatinib and masitinib treatment. Another hallmark of inflammasome activation is the ASC speck formation, in which ASC polymerizes in a filamentous manner enhancing the surface for downstream signalling by caspase-1 recruitment (Dick et al., 2016; Lu et al., 2014b). ASC specks were as well observed upon TKI treatment, confirming the possibility of conventional activation of NLRP3 (Figure 2).

The small molecule imiquimod is inducing NLRP3 stimulation by mitochondrial ROS following Complex I inhibition and is acting in a K^+ efflux independent manner (Groß et al., 2016a). As imatinib like imiquimod binds the flavoprotein NQO2 (Winger et al., 2009),we tested the NLRP3 upstream signalling upon TKI treatment. NQO2 binding, which we first believed to be a possible ROS sensing NLRP3 activator and thus the missing link between ROS and NLRP3, did not correlate with inflammasome activation (Groß, PhD Thesis, 2016; Magnani, PhD Thesis, 2017). This is supporting previous findings demonstrating that NQO2 is not involved in the NLRP3 activating ROS production (Groß et al., 2016a). The TKI effect on NLRP3 can however be inhibited by the ROS inhibitor ebselen (Figure 2) like it is the case for imiquimod (Groß et al., 2016a), indicating ROS production upon TKI treatment. Besides ROS production, conventional NLRP3 activators cause K^+ efflux and/or lysosomal leakage (Franchi et al., 2007; Muñoz-Planillo et al., 2013b; Rivers-Auty & Brough, 2015). Here we observe that elevated extracellular K^+ levels, which inhibit K^+ efflux and thus NLRP3 activation by many common stimuli like the K^+ ionophore nigericin (Walev et al., 1995), prevented inflammasome activation by imatinib and to a lower extant also by masitinib (Figure 2 and data not shown). This weaker inhibition of masitinib by standard NLRP3 inflammasome inhibitors could be explained by faster activating kinetics of masitinib, which were observed especially during microscopic analysis of ASC specks.

In addition, imatinib and masitinib both depend on NEK7 for full inflammasome activation (Figure2), which is a recently identified direct interaction partner of NLRP3 acting downstream of K^+ efflux (He et al., 2016). As nigericin induced IL-1β secretion was not completely diminished in NEK7-deficient BMDCs, we decided to investigate this phenomenon in further experiments, which are presented in the second part of this thesis.

Taken together, these findings prove that imatinib and masitinib trigger NLRP3 inflammasome activation leading to IL-1β secretion, that is comparable to those induced by common NLRP3 stimulator.

4.1.2 Imatinib and masitinib do not lead to cytokine secretion by pyroptosis

A downstream consequence of inflammasome activation, beside IL-1β processing, is the caspase-1 dependent cleavage of GSDMD. GSDMD forms pyroptotic pores along the cytosolic membrane through which IL-1β and other alarmins exit the cells (X. Chen et al., 2016; Ding et al., 2016; Liu et al., 2016b; Sborgi et al., 2016b). Interestingly, even though TKI-induced cytokine secretion of IL-1β and IL-1α, which is processed by calpains and not caspase-1 (Howard et al., 1991), was as expected dependent on GSDMD, LDH release was not inhibited by GSDMD-deficiency (Figure 3). Therefore, a secondary lytic cell death must be activated in GSDMD knockout BMDCs, that also leads to the secretion of mature cytokines downstream of caspase-1 or calpains which are still active in GSDMD-deficient cells (Figure 3). This could be explained by caspase-1 cleavage of another gasdermin family member or by a non-conventional cytokine secretion that is independent of GSDMD pores. The later phenomenon was described before as a secondary form of pyroptosis which is dependent on apoptotic caspases (especially caspase-8) and differs from apoptosis (Schneider et al., 2017).

Despite several reports showing that imatinib and masitinib can induce a conventional form of apoptosis in different types of cells (Belloc et al., 2007; Fahey et al., 2013; Milovancev et al., 2016), we were unable to confirm these observations in our experimental setting using BMDCs (Magnani, PhD thesis, 2017).

As the tested TKIs induced also strong K^+ efflux but were not entirely dependent on ASC (Magnani, PhD Thesis, 2017), a mechanism resembling the so-called non-

canonical inflammasome (Kayagaki et al., 2011) seemed to be a possible upstream stressor leading to NLRP3 inflammasome formation. Thereby, K^+ efflux following membrane destabilization induced by Caspase-11 cleaved GSDMD, activated NLRP3 (Kayagaki et al., 2015b; Rühl & Broz, 2015). However, when testing this hypothesis, we could not completely inhibit cell death in caspase-1/11 double knock-out cells and assumed that TKIs can induce besides pyroptosis also other forms of lytic cell death (Figure 3).

Surprisingly, LDH levels of Caspase1/11$^{-/-}$ and GSDMD$^{-/-}$ BMDCs treated with the crystal particle MSU were still elevated (Figure 3). MSU is most likely activating cell death by membrane destabilization that is attributed to crystalline agents (Hari et al., 2015).To exclude that TKIs are forming crystals, we treated the cells additionally with cytochalasin D, a phagocytosis inhibitor that is reported to inhibit NLRP3 activation by MSU and other crystal particles (Hornung et al., 2008). Cytochalasin D treatment did not influence the cytokine secretion upon TKI stimulation, while MSU induced IL-1β and cell death were strongly diminished, indicating that phagocytosis of crystalline particles is not the mode of action here (Figure 3). A recent study, showed that redundant cathepsins are inducing a caspase-1 independent cell death upon MSU stimulation of macrophages. This cell death can be inhibited by K777, a broad cathepsin inhibitor (Orlowski et al., 2017). As K777 did not inhibit cell death by imatinib and masitinib (data not shown), we can conclude that both TKIs are not activating NLRP3 in a crystal particle manner. Interesting is, however, that NLRP3 activation by MSU seems to be only induced by secondary effects caused by the cathepsin-dependent cell death (Orlowski et al., 2017).

Taken together, these results indicate that imatinib and masitinib in BMDCs could also engage a form of cell death which is not dependent on the inflammasome itself but can activate it in turn.

4.1.3 Imatinib and masitinib are activating an unconventional cell death

Because we observed a GSDMD-independent lytic cell death, a central question of this work was which cell death mechanism is triggered by TKIs such as imatinib and masitinib. Other forms of programmed cell death require the action of different caspases and may be either immunologically silent like apoptosis or highly

inflammatory like pyroptosis (McIlwain et al., 2013). We therefore tested zVAD-fmk, necrostatin-1 and other cell death inhibitors but were unable to completely abolish cell death and inflammasome activation induced by the TKIs at once (Figure 5 and Magnani, PhD Thesis, 2017).

The pan-caspase inhibitor zVAD-fmk can consequently block many caspase-dependent cell death pathways. We did indeed see a drop in cytokine secretion upon zVAD-fmk treatment, which did not, however, stop the cell from dying in a lytic form (Figure 4). This is in line with published reports showing a shift toward other forms of programmed cell death thus overcoming the loss of a pyroptotic pathway upon zVAD-fmk treatment (Lemaire et al., 1998; Vandenabeele et al., 2006). It also further supports our hypothesis that imatinib and masitinib are inducing another lytic cell death, that is then again leading to NLRP3 activation only as a consequence.

As necroptosis can be activated in a caspase independent way by RIP kinase dependent MLKL processing (Kang et al., 2013; Murphy et al., 2013; Rickard et al., 2014; Sun et al., 2012; J. Zhao et al., 2012), we were wondering if the necroptosome is activated by imatinib and masitinib. The downstream necroptosome signalling recruits MLKL, which is like GSDMD building pores in the cell membrane, leading to membrane destabilization and K^+ efflux. As a frequently observed mechanism upstream of NLRP3, the MLKL pore induced K^+ efflux is effecting an interplay between necroptosis and pyroptosis (Conos et al., 2017b). Nevertheless, the specific inhibition of RIPK1 by necrostatin-1 (Vandenabeele et al., 2013) did not have any effect on neither cytokine secretion nor cell viability, leaving our question unanswered (Figure 4). This is why we also tested inhibitors of other two regulated necrotic cell death pathways: parthanatos and ferroptosis (reviewed in Berghe et al., 2014). PJ-34 inhibits PARP1 directly, which is mediating parthanatos by the accumulation of toxic PAR upon DNA damage (David et al., 2009; Huang et al., 1994; Zhang et al., 1994). Deferoxamine (DFOA) is an ion chelator that is inhibiting ferroptosis stimulating lipid peroxides, which are generated by increased intracellular iron concentrations (Dixon et al., 2012). DFOA had comparable effects to MCC950 treatment when lower imatinib and masitinib concentrations were administered (Figure 4). As ROS is inducing lipid peroxide development (Barrera, 2012), and lipid peroxides are inhibited by DFOA, DFOA could indirectly limit the

effects ROS. The above discussed inhibition of imatinib and partially also masitinib induced IL-1β secretion by ebselen (Figure 2), emphasizing once again the ROS-inducing character of the two tested TKIs. Interestingly, the inhibitory effect of DFOA was completely lost for 20μM higher TKI concentrations (Figure 4), indicating either different cell death cross-talks, an unconventional mode of cell death involvement or simply necrosis. We could however exclude a necrotic cell death induction by both TKIs, as they were ineffective in NIH-3T3, HeLa and showed low lytic cell death rates in HEK-293T cells.

So far, our observation could not pinpoint to one distinct cell death pathway induced upon imatinib and masitinib treatment.

However, indicated by the strong increase in K^+ efflux (Magnani, PhD Thesis, 2017), we concluded that cell death was occurring most likely through membrane destabilization. By using osmoprotective agents we observed that BMDCs can be protected from TKI-induced lytic cell death when pretreated with PEG (Figure 5). Polyether treatment has previously been used to protect cells from *Salmonella spp.*-induced pyroptotic cell death (Pomerantz et al., 2001). Remarkably, cellular responses to classic NLRP3 activators like nigericin and ATP, or the non-canonical inflammasome activator intracellular LPS, were not affected by PEG treatment (Figure 5). However, it still remains unclear what factor is inhibited by PEG that would lead to imatinib and masitinib-induced cell death.

In summary, both imatinib and masitinib seem to trigger a mixed cell death form in BMDCs which depend on substantial cell membrane destabilization. This destabilization is in turn sensed by NLRP3 and eventually triggers inflammasome assembly. From our data, we can conclude that imatinib and masitinib cause neither apoptosis, pyroptosis nor necroptosis alone. However, the tested TKIs might activate a combination of these or a yet unknown specific form of regulated necrosis. At the same time, we can exclude TKI-mediated non-specific membrane damage and non-regulated necrosis since toxicity experiments conducted on other cell lines exhibited no substantial LDH release (Figure 4). Therefore, it appears more likely that those drugs cause a new regulated form of lytic cell death. Our findings are in line with previous reports where imatinib induced a caspase-independent necrotic form of cell death on BCR-ABL-positive CML cells (Deininger et al., 1997). As the

panorama of programmed necrosis is constantly gaining definition and expanding numbers of programmed cell death modalities become defined, further research urges to define the molecular switches responsible for mixed forms of cell death and to identify regulators common to each of these pathways (Berghe et al., 2014).

4.1.4 Are TKIs useful tools for analysis of the fine mechanisms of NLRP3 activation?

Several TKIs were tested to assess their ability to induce inflammasome activation and lytic cell death (Magnani, PhD Thesis, 2017). Depending on their action, TKIs can be potentially used as tools to study the fine mechanistic aspects of inflammasome regulation. There are TKIs which are not lethal to cells, others induce cell death and inflammasome activation independent of NLRP3 (Magnani, PhD Thesis, 2017). The ones of interest for NLRP3 research purposes are however those that activate NLRP3 and cause either exclusively pyroptosis or another form of lytic cell death, like imatinib and masitinib. Specific NLRP3 and pyroptosis activators should be investigated in further detail in upcoming studies.

One of these is pazopanib, a TKI that is used in the clinics for renal cell carcinomas and soft tissue sarcomas. Pazopanib is very specific in its NLRP3 activation, relying also on NEK7 (Figure 7) and GSDMD (data not shown). Interestingly, it is like imiquimod acting downstream of mitochondrial ROS, but its signalling is not influenced by K^+ efflux (data not shown). The exact mechanism of pazopanib induced NLRP3 inflammasome formation is not understood yet and could hint to the missing link between ROS production and NLRP3.

In conclusion, future research will focus on TKIs and their exact mechanisms of NLRP3 activation. Here, it is likely that a combination of the reported on- and off-target effects of a respective TKI can trigger cellular stress responses that eventually lead to NLRP3 activation (Zitvogel et al., 2016). These interactions may represent the cause of several side effects. Therefore, as TKIs are currently used to treat several types of cancers, it will be crucial to understand if the inflammasome activation has any contribution in their mode of action or in the immunomodulatory effects elicited by these drugs.

4.1.5 COULD TKI INDUCED CELL DEATH AND CYTOKINE SECRETION BE OF RELEVANCE FOR MEDICAL TREATMENT OF CML?

Our present work reports how imatinib and masitinib can trigger an unconventional mode of lytic cell death in dendritic cells and activate the NLRP3 inflammasome eventually leading to IL-1β secretion. This is an event which might easily be present *in vivo* as well if we consider the levels of imatinib and other TKIs in patients undergoing this drug therapy. Detectable blood levels of imatinib were reported in both human patients and murine cancer models by several independent works, showing the same concentration ranges and high variability in different blood plasma (Larson et al., 2008; Nassar et al., 2009; Peng et al., 2004; Picard et al., 2007). The peak imatinib concentration in the bloodstream was observed between 1 and 3 hours after administration (Peng et al., 2004). Depending on the given dosage (400 or 600 mg), the peak concentration was ranging between 1-4 µg/ml and 6-12 µg/ml, respectively. Assuming a mean concentration of 2 µg/ml for a 400 mg and 8 µg/ml for a 600 mg dosage, blood levels could range between 4µM and 16µM for imatinib mesylate. These concentrations are slightly below the threshold of inflammasome activation we observed but might lead to inflammasome induction in the course of a longer treatment regimen (Larson et al., 2008). To prove this hypothesis, it would be interesting to measure blood levels of IL-1β directly from patients administered imatinib.

In a recent report, CML patients treated with nilotinib (a second generation BCR-Abl inhibitor) rather than those receiving imatinib indeed displayed detectable IL-1β levels in their bloodstream (Sukegawa et al., 2017). While nilotinib did not elicit significant IL-1β in BMDCs (data not shown), the observed effect could potentially be attributed to a secondary immune response or to the altered behaviour of CML cells during the course of treatment. Further analysis is required to correlate imatinib and IL-1β, as well as other inflammatory cytokine levels and their impact on leukemia development and progression.

Cytokines and particularly IL-1 family cytokines have an extraordinary immunomodulatory potential and can play pivotal roles in cancer progression and therapy (Dinarello, 2010, 2011; Lewis et al., 2006). In our work we showed how

human PBMCs can secrete IL-1β in vitro when stimulated with imatinib and masitinib (Magnani, PhD Thesis, 2017).

There were some reports showing high hematic levels of IL-1β in CML patients, especially those with more advanced cancer stages (Wetzler et al., 1994; Zhang et al., 2014). In a recent study, it was proposed that IL-1 secretion is increased in CML bone marrow and that CML leukemic stem cells (LSCs) have a higher expression of IL-1 receptor (IL-1R1). Blockade of IL-1 signalling could modulate the inflammatory milieu and help during the elimination of CML LSCs (Zhang et al., 2016). Another study strongly supported the hypothesis that IL-1β is crucial in modulating CML progression by showing how imatinib-resistant CML cells secrete high IL-1β levels, thus affecting stromal cell production of CXCL11, which in turn promoted migration of CML cells into the stromal tissues (Lee et al., 2016).

Yet, IL-1β is a crucial regulator of AML progression and development. In a recent study, AML tumor onset was accelerated when leukemic precursor cells lost expression of crucial necroptosis and inflammasome components. Therefore, the lack of RIPK3 and an impaired IL-1β secretion can also foster tumor growth (Höckendorf et al., 2016). There are many publications on the role of IL-1β in hematologic malignancies that were recently reviewed in detail (Arranz, Arriero, & Villatoro, 2017).

In general, leukemic patients display enhanced IL-1β signalling which has very freuquently a negative impact on tumor growth and treatment. Many of the immunomodulatory functions of imatinib may be caused as consequence of the off-targets interaction that were identified in chemical proteomics screenings (Bantscheff et al., 2007; Breitkopf, Oppermann et al., 2010; Rix et al., 2007; Zitvogel et al., 2016). As the role of the inflammasome in immunomodulation is still unclear, further investigation and additional *in vivo* data is required. A future goal will be to test inflammasome knockouts in CML, GIST or other cancers models upon treatment with imatinib and other TKIs, which could further elucidate the role of the inflammasome, lytic cell death and IL-1β in cancer progression and therapy.

4.2. NEK7-INDEPENDENT NLRP3 ACTIVATION

NEK7 is a serine/threonine kinase known for its pivotal role in mitosis as it is involved in the phosphorylation cascade of the Kinesin family member 11 KIF11, regulating spindle apparatus formation (Fry et al., 2012; Haq et al., 2015; O'Regan & Fry, 2009). A role in neuronal dendrite morphogenesis was also attributed to NEK7, whereby it effects the cytoskeletal microtubules in a KIF11-dependent manner as well (Freixo et al., 2018).

Unexpectedly, three independent studies recently showed that NLRP3 formation strongly depends on NEK7 in BMDMs (He et al., 2016; Schmid-Burgk et al., 2016; Shi et al., 2016). Thereby, NEK7 was shown to act downstream of NLRP3 inflammasome triggering K^+ efflux and mitochondrial ROS. It transduces these signals by directly binding to the LRR domain, inducing NLRP3 oligomerization (He et al., 2016; Shi et al., 2016).

Surprisingly, throughout our investigations on TKI-induced NLRP3 activation, we observed IL-1β secretion in NEK-deficient BMDCs stimulated with common canonical inflammasome activators like nigericin and ATP (Figure 2). When we looked into this phenomenon in further detail, we could confirm our first observation, showing NEK7-independent NLRP3 activation exclusively in BMDC cultures. Furthermore, we were able to induce this independency also in BMDMs by GM-CSF treatment shortly before LPS-priming. This raises once more the question what the exact role of GM-CSF is, both in cell culture as well as in its physiological environment. In the context of innate immune signalling *in vitro*, we postulate that GM-CSF is enhancing NLRP3 expression in unprimed macrophages, which leads to the subsequent increase in IL-1β secretion.

4.2.1 NEK7 INDEPENDENCY IN BMDCS

NEK7-deficient BMDMs were shown to be NLRP3 inflammasome incompetent (He et al., 2016; Schmid-Burgk et al., 2016; Shi et al., 2016). In line with these publications, we were able to detect IL-1β secretion and caspase-1 cleavage upon nigericin treatment only in the supernatant of wildtype BMDMs but not in those of

Nek7$^{-/-}$ BMDMs, confirming the NEK7 dependency for NLRP3 inflammasome formation in BMDM cultures.

Astonishingly, BMDCs showed in the same experimental setting, a strong signal for both inflammasome activation parameters. As this finding is contrary to the literature, we analysed this observation in further detail. We found that IL-1β secretion in NEK7-deficient BMDCs has similar features to NLRP3 downstream signalling in wildtype BMDCs (Figure 6). In this regard, we detected lower IL-1β concentration in the supernatant of Nek7$^{-/-}$ BMDCs. IL-1β secretion did follow caspase-1 cleavage, that was strongly diminished by the specific NLRP3 inhibitor MCC950, and was also dependent on common inflammasome upstream signalling mechanisms like K^+ efflux and ROS production (Figure 6B and D). Furthermore, the lack of NEK7 did not influence ASC speck formation in DC culture cells, further supporting the strong evidence that BMDCs do not need NEK7 for NLRP3 oligomerization and signalling. This is not only the case for nigericin induced NLRP3 activation, but also for other common canonical inflammasome activators like gramicidin or ATP (Figure 2D and 7A). It was reported that NEK7 senses K^+ efflux induced by nigericin and transmits the signal to NLRP3 in a so far unknown mechanism (He et al., 2016). Interestingly, the small molecules imiquimod and pazopanib, which are specific NLRP3 and pyroptosis activators, are thereby strictly NEK7 dependent also in BMDCs. Both imiquimod and pazopanib induce K^+ efflux independent NLRP3 activation that relies on ROS production (Groß et al., 2016c and Magnani, PhD Thesis, 2017) and differ in this manner from nigericin or gramicidin, which are K^+ ionophores. Nevertheless, ebselen and PDTC, two ROS inhibitors, diminished the amount of secreted IL-1β upon nigericin treatment in Nek7$^{-/-}$ BMDCs (Figure 6), showing ROS is still sensed in these cells. Respiratory chain inhibitors are strongly debated in their potential to induce NLRP3 activation by mitochondrial ROS development, as some groups observed IL-1β secretion upon complex I inhibition by rotenone treatment and others did not (Groß et al., 2016b; Muñoz-Planillo et al., 2013a; Nakahira et al., 2011). Future experiments with NEK7-deficient BMDCs should include rotenone or piericidin A, another complex I inhibitor. In conclusion, in BMDCs NEK7 is dispensable for NLRP3 inflammasome activation upon treatment with K^+ efflux inducing agents like nigericin or gramicidin.

The difference between BMDMs and BMDCs should, in the framework of NLRP3 inflammasome activation, not only be reviewed in detail but also investigated scientifically. Attention should be payed to variations in upstream NLRP3 activation mechanisms like K^+ efflux or ROS production as well as priming signalling. So far, only superficially, direct comparison was made between these two cell type cultures with differences being only reported for NFκB signalling kinetics upon LPS stimulation in the context of inflammasome activation (He et al., 2013).

As NLRP3 interaction with NEK7 was only investigated in BMDMs and an association with NEK6, a NEK-7 related paralogue, and NEK9, another NEK family member, was excluded within the same (He et al., 2016), it could be of interest to repeat this experiment in a BMDC setting.

Furthermore, with the knowledge of the involvement of NEK7 in microtubule organization and the reported microtubule-dependent NLRP3 activation (Gao et al., 2016; Misawa et al., 2015), the reported missing cytoskeletal role in inflammasome formation (He et al., 2016), should be reevaluated in BMDCs. Especially because it was reported that the microtubule-driven spatial arrangement of mitochondria promotes NLRP3 activation (Misawa et al., 2013). Maybe this observation can explain the NEK7-dependency in BMDCs upon R837 and pazopanib-induced NLRP3 stimulation.

4.2.2 GM-CSF INDUCES NEK7 INDEPENDENCY IN BMDMS

We tried to approach the most obvious difference between BMDCs and BMDMs, which is their culture medium. It was very interesting to see, that the BMDM reactivity to nigericin was highly increased when the medium was exchanged to BMDC culturing conditions one night before the experiment (Figure 7B). Surprisingly, this medium change also induced NEK7-independent IL-1β secretion in BMDMs. We assumed that this observation must be an effect induced by the colony stimulating factor GM-CSF and confirmed this hypothesis in subsequent experiments. Nevertheless, RPMI already led to a favorable culture condition of BMDMs, as bone marrow-derived cells grown in RPMI, supplemented with M-CSF for macrophage differentiation, showed a higher and to some extent also NEK7-independent NLRP3 activation, which could also be observed by ASC speck

formation (Figure 8A, 10B and C). Even though the role of NEK7 in NLRP3 inflammasome formation was in all three cases reported in BMDMs cultured in DMEM conditions (He et al., 2016; Schmid-Burgk et al., 2016; Shi et al., 2016), we decided to minimize the differences in BMDM and BMDC cultures and focus on the effect of GM-CSF alone by using the same culture condition with RPMI as a standard medium background for all bone marrow-derived cells. Moreover, different inflammasome research groups are using RPMI as a standard culturing condition for BMDM cultures and observe normal behaviour (Aranda-Souza et al., 2019b; Camell et al., 2017; Zhao et al., 2018).

Compared to DMEM, RPMI displays highly increased concentrations of PO_4^{2-} (DMEM: 0.91mM, RPMI: 5.63mM, human serum: 0.97-1.45mM, mouse serum: 1.26-1.52mM) and lower Ca^{2+} (DMEM: 1.8mM, RPMI: 0.42mM, human serum: 1.11-1.32mM, mouse serum: 0.97-1.09mM) and Glucose (DMEM: 25mM, RPMI: 11.1mM, human serum: 3.9-7.8mM, mouse serum: 3.4-7mM) (Mckee & Komarova, 2017). These differences could also play a role in cell responsiveness, even though the K^+ concentration is comparable in both media (DMEM and RPMI: 5.4mM, human serum: 3.5-5.6mM, mouse serum: 4.3-5.8mM) (Mckee & Komarova, 2017). Recently, the small adaptations of media conditions to different cell types and their physiological environment is debated and should be kept in mind for future experimental approaches (Mckee & Komarova, 2017; T. Yao & Asayama, 2017).

GM-CSF enhances IL-1ß secretion

The role of GM-CSF in *in vitro* cell differentiation and in physiological immune responses was recently reevaluated as its signalling is only poorly understood and increasing evidence show its importance in chronic inflammatory disease development and cancer therapy (Bhattacharya et al., 2015; Hamilton, 2002). A reason for this lack in characterization could be the missing impact of GM-CSF-deficiency on myeloid development *in vivo*. Meanwhile, the strong immunomodulatory role of GM-CSF is however well accepted. GM-CSF is secreted by IL-23R activated Th17 cells, which are known mediators of autoimmunity and tissue inflammation (Codarri et al., 2011; Korn et al., 2009). Furthermore, the initiation of tissue inflammation by self-reactive Th17 cells is strictly dependent on

their GM-CSF production. GM-CSF secretion can be induced *in vitro* by IL-2 or IL-23 in combination with IL-1 (Hartmann et al., 2014; Noster et al., 2014), which is in turn secreted by myeloid cells upon NLR activation. Interestingly, it has been reported that myeloid cells react to GM-CSF with increased expression of inflammasome components like IL-1β, ASC or NLRP3 (Croxford et al., 2015). Thus, GM-CSF is a key player in the communication between pathogenic T cells and the myeloid compartment, inducing mutual immune responses in both cell types. It remains however unclear which myeloid cell type exactly is responding to GM-CSF. In line with this is our observation of a boosted IL-1β response upon GM-CSF treatment even for as short periods as 1h before LPS-priming and nigericin treatment. The enhancing effect is also measured for other common NLRP3 activators as well as for the AIM2 inflammasome stimulator poly(dA:dT) (Figure 7E). Thus, GM-CSF is not only influencing NLRP3 but also the AIM2 inflammasome. It is doing so in a specific manner, as the IL-1β signal can be inhibited with standard NLRP3 inflammasome inhibitors (Figure 7F).

GM-CSF is enhancing NLRP3 expression but not pro-IL-1ß synthesis

We could show that GM-CSF is no stronger priming agent than LPS, as BMDMs treated with GM-CSF without LPS-priming did not show any IL-1β secretion neither upon NLRP3 activation nor upon AIM2 stimulation (Figure 8A and B). However, GM-CSF is only inducing stronger cytokine secretion before LPS-priming, if added after the boosting effect is lost as in the absence of GM-CSF (Figure 8C). This could mean that LPS gives a predominant signal which determines the amount of IL-1β being secreted through a yet unknown factor. Indeed, a NLRP3 desensitization was reported upon prolonged LPS-priming, tightly controlling inflammasome activity and inappropriately high cytokine secretion (Groslambert & Py, 2018a). Interestingly, while IL-1β was secreted in an all- or nothing response shortly after supplementation, both TNFα and IL-6 secretion was dependent on the GM-CSF incubation period, only being higher when GM-CSF was applied overnight (Figure 8C).

Upon TLR4 activation LPS-priming leads to MyD88 and TRIF recruitment, NFκB signalling and transcriptional upregulation of NLRP3 and pro-IL-1β. It has been shown, that GM-CSF can increase NFκB signalling in this context, thus enhancing the expression of inflammasome components (Furuya et al., 2018; Vento-Tormo et al., 2017). Nevertheless, LPS can also act in a more rapid and transient way and induce non-transcriptional priming of NLRP3 independent of protein synthesis, signalling through MyD88 and IRAK-1 (Lin et al., 2014). As GM-CSF is acting in a rapid manner before LPS-priming, it could interfere with non-transcriptional priming of NLRP3. This hypothesis can be tested by the use of inhibitors of transcriptional protein synthesis, e.g. cyclohexamide, before GM-CSF and LPS treatment. We tried to approach this question by western blot analysis of unprimed and LPS-primed BMDMs treated with GM-CSF prior to inflammasome stimulation. While ASC and pro-IL-1β expression was not induced by GM-CSF, NLRP3 protein concentration was enhanced in GM-CSF-treated, unprimed BMDMs (Figure 8D). Probably this is the reason why nigericin alone - without any additional priming signal - is enough to activate caspase-1 cleavage and secretion (Figure 8D). Pro-IL-1β expression is also increased upon GM-CSF incubation, however only when LPS-priming occurs hereafter (Figure 8E). The first and initiating priming signal is referred to as “licensing” when it induces non-transcriptional modifications of NLRP3 and as “priming” when NLRP3 and pro-IL1ß transcription is activated (Groslambert & Py, 2018b; Juliana et al., 2012; Stutz et al., 2017). Our observations however show that GM-CSF is causing NLRP3 protein synthesis without pro-IL-1β expression. In general, further analysis of TLR4 as well a GM-CSF receptor signalling is needed to investigate the crosstalk between these two pathways.

GM-CSF is not inducing a population identity shift but stronger M1 macrophage polarization

Because GM-CSF is used for cell differentiation *in vitro* and GM-CSF-derived macrophages are known to respond stronger to NLRP3 activation (Shaw et al., 2014), we wanted to exclude a population identity shift in the BMDM culture. Indeed, macrophage surface marker of both adherent and loosely adherent BMDMs were not influenced by GM-CSF when added for 1h (Figure 9A and B). Even the overnight

GM-CSF treatment increased the number of CD11c$^+$ cells only slightly and thus weakly diminished macrophage marker expression in loosely adherent cells (Figure 9A). NLRP3 expression and IL-1β secretion can be detected in supernatants of BMDMs which were supplemented with GM-CSF 1h before LPS-priming, providing strong evidence that GM-CSF is influencing NLRP3 activation itself.
However, macrophage polarization was affected by GM-CSF according to changes in their cytokine secretion profile (Figure 9C). IL-10 is a cytokine secreted by M2 macrophages, which have an anti-inflammatory role, while pro-inflammatory M1 macrophages respond with TNFα, IL-6 and IL-1 cytokines (Arango Duque & Descoteaux, 2014). As IL-10 secretion decreases with GM-CSF treatment and M1 macrophage cytokines increase, we can conclude that GM-CSF is inducing a rather pro-inflammatory state (Figure 9C). This is in line with published data, showing that GM-CSF is inducing a M1-like transcriptome in cultured BMDM (Lacey et al., 2012). IL-4 is having an opposite effect, leading to M2 macrophage polarization and an anti-inflammatory phenotype (Arango Duque & Descoteaux, 2014). Interestingly, IL-4 was shown to inhibit non-transcriptional NLRP3 licensing, which is postulated to induce microtubule-associated trafficking of NLRP3 to stressed mitochondria and then ROS sensing (Hwang et al., 2015). Keeping in mind, that NEK7 has an important role in microtubule formation, analysis of the cytoskeleton upon GM-CSF could be of interest for a better understanding of NLRP3 activation processes. In first microscopy experiments, we did not detect any correlation between ASC specks and microtubule changes upon GM-CSF treatment (Figure 10). This is in line with our previous study, where we were unable to detect colocalization of NLRP3 with mitochondria upon R837 treatment (Groß et al., 2016b)
In summary, we can conclude that GM-CSF is influencing the pro-inflammatory state of the cell. Consequently, it has also a boosting effect on NLRP3 signalling. It is noteworthy that GM-CSF is not influencing pyroptosis, not even in NEK7-deficient BMDMs (Figure 11B), showing once more that cytokine maturation and pyroptosis are two distinct events only linked by the activity of caspase-1. Consequently, caspase-1 activity is likely to be unaffected by GM-CSF. As the speck morphology and number were also not dependent on GM-CSF (Figure 10 and 11A), the

enhanced cytokine secretion may be result of the GM-CSF-induced NLRP3 expression. The exact mechanism still needs to be investigated.

4.3. Conclusion

NLRP3 is an intracellular PRR that reacts to a huge variety of PAMPs and DAMPs. What the common NLRP3 triggering mechanism is, remains unknown and the complexity of its activation is tremendous. NEK7, a kinase known for years for its pivotal role in cell proliferation, is suddenly and convincingly reported to be a crucial activator of NLRP3. By direct binding to the NLR itself, NEK7 triggers NLRP3 oligomerization and inflammasome formation. This interaction is not only of scientific interest as described in this thesis, but it also has clinical relevance with first reports demonstrating a clinical significance of the NEK7-NLRP3 inflammasome signalling in patients with lupus erythematosus (Ma et al., 2018).

Inducing small molecules to the field of inflammasome research, offers us the opportunity to analyse and shape NLRP3 upstream signalling in further detail. TKIs are small molecules that induce robust NLRP3 inflammasome-dependent cytokine secretion. As they are commonly used for therapy of haematological malignancies, research on pro-inflammatory immune modulation by TKIs could reveal whether this effect is beneficial or disadvantageous for cancer treatment.

Furthermore, it is clear that inflammasome activation could also differ between myeloid cells like macrophages and dendritic cells, introducing another variable in NLRP3 signalling. It would be interesting to investigate this variance in further detail, especially in BMDM and BMDC cultures. Here, GM-CSF cytokine signalling could influence culture BMDMs in a physiological manner.

The diverse effects of the small molecular TKIs imatinib and masitinib and the cytokine GM-CSF on NLRP3 upstream signalling, once more reveal the complexity of NLRP3 inflammasome activation.

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References

Adenis, A., Blay, J.-Y., Bui-Nguyen, B., Bouché, O., Bertucci, F., Isambert, N., … Le Cesne, A. (2014). Masitinib in advanced gastrointestinal stromal tumor (GIST) after failure of imatinib: a randomized controlled open-label trial. *Annals of Oncology : Official Journal of the European Society for Medical Oncology*, *25*(9), 1762–9. http://doi.org/10.1093/annonc/mdu237

Afonina, I. S., Mü, C., Martin, S. J., & Beyaert, R. (2015). Review Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. *Immunity*, *42*, 991–1004. http://doi.org/10.1016/j.immuni.2015.06.003

Aglietti, R. A., & Dueber, E. C. (2017). Recent Insights into the Molecular Mechanisms Underlying Pyroptosis and Gasdermin Family Functions. *Trends in Immunology*, *38*(4), 261–271. http://doi.org/10.1016/J.IT.2017.01.003

Aglietti, R. A., Estevez, A., Gupta, A., Ramirez, M. G., Liu, P. S., Kayagaki, N., … Dueber, E. C. (2016). GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. *Proceedings of the National Academy of Sciences*, 201607769. http://doi.org/10.1073/PNAS.1607769113

Aits, S., & Jaattela, M. (2013). Lysosomal cell death at a glance. *Journal of Cell Science*, *126*(9), 1905–1912. http://doi.org/10.1242/jcs.091181

Akira, S., & Hoshino, K. (2003). *Myeloid Differentiation Factor 88-Dependent and-Independent Pathways in Toll-Like Receptor Signaling*. *The Journal of Infectious Diseases* (Vol. 187). Retrieved from https://academic.oup.com/jid/article-abstract/187/Supplement_2/S356/814390

Aranda-Souza, M. Â., Lorena, V. M. B. de, Correia, M. T. dos S., Pereira-Neves, A., & Figueiredo, R. C. B. Q. de. (2019a). A C-type lectin from Bothrops leucurus snake venom forms amyloid-like aggregates in RPMI medium and are efficiently phagocytosed by peritoneal macrophages. *Toxicon*, *157*, 93–100. http://doi.org/10.1016/J.TOXICON.2018.11.309

Aranda-Souza, M. Â., Lorena, V. M. B. de, Correia, M. T. dos S., Pereira-Neves, A., & Figueiredo, R. C. B. Q. de. (2019b). A C-type lectin from Bothrops leucurus snake venom forms amyloid-like aggregates in RPMI medium and are efficiently phagocytosed by peritoneal macrophages. *Toxicon*, *157*, 93–100. http://doi.org/10.1016/J.TOXICON.2018.11.309

Arango Duque, G., & Descoteaux, A. (2014). Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in Immunology*, *5*, 491. http://doi.org/10.3389/fimmu.2014.00491

Arranz, L., Arriero, M. del M., & Villatoro, A. (2017). Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications. *Blood Reviews*, *31*(5), 306–317. http://doi.org/10.1016/J.BLRE.2017.05.001

Bae, J. Y., & Park, H. H. (2011). Crystal structure of NALP3 protein pyrin domain (PYD) and its implications in inflammasome assembly. *The Journal of Biological Chemistry*, *286*(45), 39528–36. http://doi.org/10.1074/jbc.M111.278812

Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hobson, S., … Drewes, G. (2007). Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nature Biotechnology*, *25*(9), 1035–1044. http://doi.org/10.1038/nbt1328

Bantscheff, M., Schirle, M., Sweetman, G., Rick, J., & Kuster, B. (2007). Quantitative mass spectrometry in proteomics: a critical review. *Analytical and Bioanalytical Chemistry*, *389*(4), 1017–1031. http://doi.org/10.1007/s00216-007-1486-6

Barrera, G. (2012). Oxidative Stress and Lipid Peroxidation Products in Cancer Progression and Therapy. *ISRN Oncology*, *2012*, 1–21. http://doi.org/10.5402/2012/137289

Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., … Latz, E. (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *Journal of Immunology (Baltimore, Md. : 1950)*, *183*(2), 787–91. http://doi.org/10.4049/jimmunol.0901363

Becher, B., Tugues, S., & Greter, M. (2016). GM-CSF: From Growth Factor to Central Mediator of Tissue Inflammation. *Immunity*, *45*(5), 963–973. http://doi.org/10.1016/j.immuni.2016.10.026

Belloc, F., Moreau-Gaudry, F., Uhalde, M., Cazalis, L., Jeanneteau, M., Lacombe, F., … Mahon, F.-X. (2007). Imatinib and nilotinib induce apoptosis of chronic myeloid leukemia cells through a Bim-dependant pathway modulated by cytokines. *Cancer Biology & Therapy*, *6*(6), 912–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17538248

Berghe, T. Vanden, Linkermann, A., Jouan-Lanhouet, S., Walczak, H., & Vandenabeele, P. (2014). Regulated necrosis: The expanding network of non-apoptotic cell death pathways. *Nature Reviews Molecular Cell Biology*, *15*(2), 135–147. http://doi.org/10.1038/nrm3737

Bergsbaken, T., Fink, S. L., & Cookson, B. T. (2009). Pyroptosis: host cell death and inflammation. *Nature Reviews. Microbiology*, *7*(2), 99–109. http://doi.org/10.1038/nrmicro2070

Bhattacharya, P., Budnick, I., Singh, M., Thiruppathi, M., Alharshawi, K., Elshabrawy, H., … Prabhakar, B. S. (2015). Dual Role of GM-CSF as a Pro-Inflammatory and a Regulatory Cytokine: Implications for Immune Therapy. *Journal of Interferon & Cytokine Research : The Official Journal of the International Society for Interferon and Cytokine Research*, *35*(8), 585–99. http://doi.org/10.1089/jir.2014.0149

Borregaard, N. (2010). Neutrophils, from Marrow to Microbes. *Immunity*, *33*(5), 657–670. http://doi.org/10.1016/j.immuni.2010.11.011

Boyden, E. D., & Dietrich, W. F. (2006). Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nature Genetics*, *38*(2), 240–244. http://doi.org/10.1038/ng1724

Breitkopf, S. B., Oppermann, F. S., Kéri, G., Grammel, M., & Daub, H. (2010). Proteomics Analysis of Cellular Imatinib Targets and their Candidate Downstream Effectors. *Journal of Proteome Research*, *9*(11), 6033–6043. http://doi.org/10.1021/pr1008527

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., … Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science (New York, N.Y.)*, *303*(5663), 1532–5. http://doi.org/10.1126/science.1092385

Broz, P., & Dixit, V. M. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. *Nature Reviews Immunology*, *16*(7), 407–420. http://doi.org/10.1038/nri.2016.58

Brüne, B., Dehne, N., Grossmann, N., Jung, M., Namgaladze, D., Schmid, T., … Weigert, A. (2013). Redox control of inflammation in macrophages. *Antioxidants & Redox Signaling*, *19*(6), 595–637. http://doi.org/10.1089/ars.2012.4785

Bürckstümmer, T., Baumann, C., Blüml, S., Dixit, E., Dürnberger, G., Jahn, H., … Superti-Furga, G. (2009). An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome A R t i C L e S. http://doi.org/10.1038/ni.1702

Camell, C. D., Sander, J., Spadaro, O., Lee, A., Nguyen, K. Y., Wing, A., … Dixit, V. D. (2017). Inflammasome-driven catecholamine catabolism in macrophages blunts lipolysis during ageing. *Nature*, *550*(7674), 119–123. http://doi.org/10.1038/nature24022

Cao, W., Zhang, L., Rosen, D. B., Bover, L., Watanabe, G., Bao, M., … Liu, Y.-J. (2007). BDCA2/Fc epsilon RI gamma complex signals through a novel BCR-like pathway in human plasmacytoid dendritic cells. *PLoS Biology*, *5*(10), e248. http://doi.org/10.1371/journal.pbio.0050248

Cerretti, D. P., Hollingsworth, L. T., Kozlosky, C. J., Valentine, M. B., Shapiro, D. N., Morris, S. W., & Nelson, N. (1994). Molecular characterization of the gene for human interleukin-1 beta converting enzyme (IL1BC). *Genomics*, *20*(3), 468–73. http://doi.org/10.1006/geno.1994.1202

Chen, G. Y., & Núñez, G. (2009). Gut Immunity: A NOD to the Commensals. *Current Biology*, *19*(4), R171–R174. http://doi.org/10.1016/J.CUB.2008.12.027

Chen, X., He, W.-T., Hu, L., Li, J., Fang, Y., Wang, X., … Han, J. (2016). Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. *Cell Research*, *26*(9), 1007–20. http://doi.org/10.1038/cr.2016.100

Childs, K. S., Randall, R. E., & Goodbourn, S. (2013). LGP2 plays a critical role in sensitizing mda-5 to activation by double-stranded RNA. *PloS One*, *8*(5), e64202. http://doi.org/10.1371/journal.pone.0064202

Codarri, L., Gyülvészi, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., … Becher, B. (2011). RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nature Immunology*, *12*(6), 560–567. http://doi.org/10.1038/ni.2027

Coll, R. C., Robertson, A. A. B., Chae, J. J., Higgins, S. C., Muñoz-Planillo, R., Inserra, M. C., … O'Neill, L. A. J. (2015a). A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nature Medicine*, *21*(3), 248–55. http://doi.org/10.1038/nm.3806

Coll, R. C., Robertson, A. A. B., Chae, J. J., Higgins, S. C., Muñoz-Planillo, R., Inserra, M. C., … O'Neill, L. A. J. (2015b). A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nature Medicine*, *21*(3), 248–55. http://doi.org/10.1038/nm.3806

Collin, M., & Bigley, V. (2018). Human dendritic cell subsets: an update. *Immunology*, *154*(1), 3–20. http://doi.org/10.1111/imm.12888

Conos, S. A., Chen, K. W., De Nardo, D., Hara, H., Whitehead, L., Núñez, G., … Vince, J. E. (2017a). Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(6), E961–E969. http://doi.org/10.1073/pnas.1613305114

Conos, S. A., Chen, K. W., De Nardo, D., Hara, H., Whitehead, L., Núñez, G., … Vince, J. E. (2017b). Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(6), E961–E969. http://doi.org/10.1073/pnas.1613305114

Croxford, A. L., Lanzinger, M., Hartmann, F. J., Schreiner, B., Mair, F., Pelczar, P., … Becher, B. (2015). The Cytokine GM-CSF Drives the Inflammatory Signature of CCR2+ Monocytes and Licenses Autoimmunity. *Immunity*, *43*(3), 502–14. http://doi.org/10.1016/j.immuni.2015.08.010

Cullen, S. P., Kearney, C. J., Clancy, D. M., & Martin, S. J. (2015). Diverse Activators of the NLRP3 Inflammasome Promote IL-1β Secretion by Triggering Necrosis. *Cell Reports*, *11*(10), 1535–1548. http://doi.org/10.1016/J.CELREP.2015.05.003

Dai, X.-M., Ryan, G. R., Hapel, A. J., Dominguez, M. G., Russell, R. G., Kapp, S., … Stanley, E. R. (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood*, *99*(1), 111–20. http://doi.org/10.1182/BLOOD.V99.1.111

Dambuza, I. M., & Brown, G. D. (2015). C-type lectins in immunity: recent developments. *Current Opinion in Immunology*, *32*, 21–27. http://doi.org/10.1016/J.COI.2014.12.002

Dasu, M. R., Devaraj, S., & Jialal, I. (2007). High glucose induces IL-1beta expression in human monocytes: mechanistic insights. *American Journal of Physiology. Endocrinology and Metabolism*, *293*(1), E337-46. http://doi.org/10.1152/ajpendo.00718.2006

David, K. K., Andrabi, S. A., Dawson, T. M., & Dawson, V. L. (2009). Parthanatos, a messenger of death. *Frontiers in Bioscience (Landmark Edition)*, *14*, 1116–28. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19273119

Davies, L. C., & Taylor, P. R. (2015). Tissue-resident macrophages: then and now. *Immunology*, *144*(4), 541–8. http://doi.org/10.1111/imm.12451

Deininger, M. W., Goldman, J. M., Lydon, N., Melo, J. V, Toyokuni, S., Ueno, M., … Nakahata, T. (1997). The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL-positive cells. *Blood*, *90*(9), 3691–8. http://doi.org/10.1182/blood-2003-05-1605

den Dunnen, J., Gringhuis, S. I., & Geijtenbeek, T. B. H. (2009). Innate signaling by the C-type lectin DC-SIGN dictates immune responses. *Cancer Immunology, Immunotherapy*, *58*(7), 1149–1157. http://doi.org/10.1007/s00262-008-0615-1

Dick, M. S., Sborgi, L., Rühl, S., Hiller, S., & Broz, P. (2016). ASC filament formation serves as a signal amplification mechanism for inflammasomes. *Nature Communications*, *7*(1), 11929. http://doi.org/10.1038/ncomms11929

Dinarello, C. A. (2010). Why not treat human cancer with interleukin-1 blockade? *Cancer Metastasis Reviews*, *29*(2), 317–29. http://doi.org/10.1007/s10555-010-9229-0

Dinarello, C. A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood*, *117*(14), 3720–32. http://doi.org/10.1182/blood-2010-07-273417

Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., … Shao, F. (2016). Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature*, *535*(7610), 111–116. http://doi.org/10.1038/nature18590

Dixon, S. J., Lemberg, K. M., Lamprecht, M. R., Skouta, R., Zaitsev, E. M., Gleason, C. E., … Stockwell, B. R. (2012). Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*, *149*(5), 1060–72. http://doi.org/10.1016/j.cell.2012.03.042

Durai, V., & Murphy, K. M. (2016). Functions of Murine Dendritic Cells. *Immunity*, *45*(4), 719–736. http://doi.org/10.1016/j.immuni.2016.10.010

Dutheil, D., Underhaug Gjerde, A., Petit-Paris, I., Mauco, G., & Holmsen, H. (2009). Polyethylene glycols interact with membrane glycerophospholipids: is this part of their mechanism for hypothermic graft protection? *Journal of Chemical Biology*, *2*(1), 39–49. http://doi.org/10.1007/s12154-009-0014-x

Dzionek, A., Sohma, Y., Nagafune, J., Cella, M., Colonna, M., Facchetti, F., … Schmitz, J. (2001). BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *The Journal of Experimental Medicine*, *194*(12), 1823–34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11748283

Elinav, E., Strowig, T., Kau, A. L., Henao-Mejia, J., Thaiss, C. A., Booth, C. J., … Flavell, R. A. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*, *145*(5), 745–57. http://doi.org/10.1016/j.cell.2011.04.022

Elliott, J. M., Rouge, L., Wiesmann, C., & Scheer, J. M. (2009). Crystal Structure of Procaspase-1 Zymogen Domain Reveals Insight into Inflammatory Caspase Autoactivation. *Journal of Biological Chemistry*, *284*(10), 6546–6553. http://doi.org/10.1074/jbc.M806121200

Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., & Kagan, J. C. (2018). The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. *Immunity*, *48*(1), 35–44.e6. http://doi.org/10.1016/J.IMMUNI.2017.11.013

Fahey, C. E., Milner, R. J., Kow, K., Bacon, N. J., & Salute, M. E. (2013). Apoptotic effects of the tyrosine kinase inhibitor, masitinib mesylate, on canine osteosarcoma cells. *Anti-Cancer Drugs*, *24*(5), 519–526. http://doi.org/10.1097/CAD.0b013e32836002ba

Fernandes-Alnemri, T., Wu, J., Yu, J.-W., Datta, P., Miller, B., Jankowski, W., … Alnemri, E. S. (2007). The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death and Differentiation*, *14*(9), 1590–604. http://doi.org/10.1038/sj.cdd.4402194

Figdor, C. G., van Kooyk, Y., & Adema, G. J. (2002). C-type lectin receptors on dendritic cells and langerhans cells. *Nature Reviews Immunology*, *2*(2), 77–84. http://doi.org/10.1038/nri723

Fink, S. L., Bergsbaken, T., & Cookson, B. T. (2008). Anthrax lethal toxin and Salmonella elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(11), 4312–7. http://doi.org/10.1073/pnas.0707370105

Fink, S. L., & Cookson, B. T. (2005). Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and Immunity*, *73*(4), 1907–16. http://doi.org/10.1128/IAI.73.4.1907-1916.2005

Fink, S. L., & Cookson, B. T. (2006). Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cellular Microbiology*, *8*(11), 1812–25. http://doi.org/10.1111/j.1462-5822.2006.00751.x

Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., Ozkurede, U., Kim, Y.-G., Chakrabarti, A., … Núñez, G. (2014). Cytosolic Double-Stranded RNA Activates the NLRP3 Inflammasome via MAVS-Induced Membrane Permeabilization and K^+ Efflux. *The Journal of Immunology*, *193*(8), 4214–4222. http://doi.org/10.4049/jimmunol.1400582

Franchi, L., Kanneganti, T.-D., Dubyak, G. R., & Núñez, G. (2007). Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *The Journal of Biological Chemistry*, *282*(26), 18810–8. http://doi.org/10.1074/jbc.M610762200

Freixo, F., Martinez Delgado, P., Manso, Y., Sánchez-Huertas, C., Lacasa, C., Soriano, E., … Lüders, J. (2018). NEK7 regulates dendrite morphogenesis in neurons via Eg5-dependent microtubule stabilization. *Nature Communications*, *9*(1), 2330. http://doi.org/10.1038/s41467-018-04706-7

Fry, A. M., O'Regan, L., Sabir, S. R., Bayliss, R., Goh, B., Kim, A. C., & Quarmby, L. M. (2012). Cell cycle regulation by the NEK family of protein kinases. *Journal of Cell Science*, *125*(Pt 19), 4423–33. http://doi.org/10.1242/jcs.111195

Fujisawa, A., Kambe, N., Saito, M., Nishikomori, R., Tanizaki, H., Kanazawa, N., … Miyachi, Y. (2006). Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. *Blood*, *109*(7), 2903–11. http://doi.org/10.1182/blood-2006-07-033597

Furuya, M. Y., Asano, T., Sumichika, Y., Sato, S., Kobayashi, H., Watanabe, H., … Migita, K. (2018). Tofacitinib inhibits granulocyte–macrophage colony-stimulating factor-induced NLRP3 inflammasome activation in human neutrophils. *Arthritis Research & Therapy*, *20*(1), 196. http://doi.org/10.1186/s13075-018-1685-x

Gao, W., Yang, J., Liu, W., Wang, Y., & Shao, F. (2016). Site-specific phosphorylation and microtubule dynamics control Pyrin inflammasome activation. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(33), E4857-66. http://doi.org/10.1073/pnas.1601700113

Geiger, T. L., & Sun, J. C. (2016). Development and maturation of natural killer cells. *Current Opinion in Immunology*, *39*, 82–9. http://doi.org/10.1016/j.coi.2016.01.007

Geijtenbeek, T. B. H., & Gringhuis, S. I. (2009). Signalling through C-type lectin receptors: shaping immune responses. *Nature Reviews Immunology*, *9*(7), 465–479. http://doi.org/10.1038/nri2569

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science (New York, N.Y.)*, *327*(5966), 656–61. http://doi.org/10.1126/science.1178331

Girardin, S. E., Boneca, I. G., Carneiro, L. A. M., Antignac, A., Jéhanno, M., Viala, J., … Philpott, D. J. (2003). Nod1 Detects a Unique Muropeptide from Gram-Negative Bacterial Peptidoglycan. *Science*, *300*(5625), 1584–1587. http://doi.org/10.1126/science.1084677

Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., … Sansonetti, P. J. (2003). Nod2 Is a General Sensor of Peptidoglycan through Muramyl Dipeptide (MDP) Detection. *Journal of Biological Chemistry*, *278*(11), 8869–8872. http://doi.org/10.1074/jbc.C200651200

Gomez Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., … Rodewald, H.-R. (2015). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid

progenitors. *Nature*, *518*(7540), 547–51. http://doi.org/10.1038/nature13989

Greter, M., Helft, J., Chow, A., Hashimoto, D., Mortha, A., Agudo-Cantero, J., … Merad, M. (2012). GM-CSF Controls Nonlymphoid Tissue Dendritic Cell Homeostasis but Is Dispensable for the Differentiation of Inflammatory Dendritic Cells. *Immunity*, *36*(6), 1031–1046. http://doi.org/10.1016/j.immuni.2012.03.027

Groslambert, M., & Py, B. F. (2018a). Spotlight on the NLRP3 inflammasome pathway. *Journal of Inflammation Research*, *11*, 359–374. http://doi.org/10.2147/JIR.S141220

Groslambert, M., & Py, B. F. (2018b). Spotlight on the NLRP3 inflammasome pathway. *Journal of Inflammation Research*, *11*, 359–374. http://doi.org/10.2147/JIR.S141220

Groß, C. J., Mishra, R., Schneider, K. S., Médard, G., Wettmarshausen, J., Dittlein, D. C., … Groß, O. (2016a). K+ Efflux-Independent NLRP3 Inflammasome Activation by Small Molecules Targeting Mitochondria. *Immunity*, *45*(4), 761–773. http://doi.org/10.1016/J.IMMUNI.2016.08.010

Groß, C. J., Mishra, R., Schneider, K. S., Médard, G., Wettmarshausen, J., Dittlein, D. C., … Groß, O. (2016b). K+ Efflux-Independent NLRP3 Inflammasome Activation by Small Molecules Targeting Mitochondria. *Immunity*, *45*(4), 761–773. http://doi.org/10.1016/j.immuni.2016.08.010

Groß, C. J., Mishra, R., Schneider, K. S., Médard, G., Wettmarshausen, J., Dittlein, D. C., … Groß, O. (2016c). K + Efflux-Independent NLRP3 Inflammasome Activation by Small Molecules Targeting Mitochondria. *Immunity*, *45*(4), 761–773. http://doi.org/10.1016/j.immuni.2016.08.010

Gross, O., Yazdi, A. S., Thomas, C. J., Masin, M., Heinz, L. X., Guarda, G., … Tschopp, J. (2012). Inflammasome activators induce interleukin-1α secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity*, *36*(3), 388–400. http://doi.org/10.1016/j.immuni.2012.01.018

Gutiérrez-Martínez, E., Planès, R., Anselmi, G., Reynolds, M., Menezes, S., Adiko, A. C., … Guermonprez, P. (2015). Cross-Presentation of Cell-Associated Antigens by MHC Class I in Dendritic Cell Subsets. *Frontiers in Immunology*, *6*, 363. http://doi.org/10.3389/fimmu.2015.00363

Gutierrez, K. D., Davis, M. A., Daniels, B. P., Olsen, T. M., Ralli-Jain, P., Tait, S. W. G., … Oberst, A. (2017). MLKL Activation Triggers NLRP3-Mediated Processing and Release of IL-1β Independently of Gasdermin-D. *Journal of Immunology (Baltimore, Md.: 1950)*, *198*(5), 2156–2164. http://doi.org/10.4049/jimmunol.1601757

Hagar, J. A., Powell, D. A., Aachoui, Y., Ernst, R. K., & Miao, E. A. (2013). Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science (New York, N.Y.)*, *341*(6151), 1250–3. http://doi.org/10.1126/science.1240988

Hamilton, J. A. (2002). GM-CSF in inflammation and autoimmunity. *Trends in Immunology*, *23*(8), 403–8. http://doi.org/10.1016/S1471-4906(02)02260-3

Hantschel, O., Rix, U., & Superti-Furga, G. (2008). Target spectrum of the BCR-ABL inhibitors imatinib, nilotinib and dasatinib. *Leukemia & Lymphoma*, *49*(4), 615–619. http://doi.org/10.1080/10428190801896103

Haq, T., Richards, M. W., Burgess, S. G., Gallego, P., Yeoh, S., O'Regan, L., … Bayliss, R. (2015). Mechanistic basis of Nek7 activation through Nek9 binding and induced dimerization. *Nature Communications*, *6*(1), 8771. http://doi.org/10.1038/ncomms9771

Hari, A., Zhang, Y., Tu, Z., Detampel, P., Stenner, M., Ganguly, A., & Shi, Y. (2015). Activation of NLRP3 inflammasome by crystalline structures via cell surface contact. *Scientific Reports*, *4*(1), 7281. http://doi.org/10.1038/srep07281

Hartmann, F. J., Khademi, M., Aram, J., Ammann, S., Kockum, I., Constantinescu, C., … Becher, B. (2014). Multiple sclerosis-associated IL2RA polymorphism controls GM-CSF production in human TH cells. *Nature Communications*, *5*(1), 5056. http://doi.org/10.1038/ncomms6056

He, W., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., … Han, J. (2015). Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. *Cell Research*, *25*(12), 1285–1298. http://doi.org/10.1038/cr.2015.139

He, Y., Franchi, L., & Núñez, G. (2013). TLR agonists stimulate Nlrp3-dependent IL-1β production independently of the purinergic P2X7 receptor in dendritic cells and in vivo. *Journal of Immunology (Baltimore, Md. : 1950)*, *190*(1), 334–9. http://doi.org/10.4049/jimmunol.1202737

He, Y., Hara, H., & Núñez, G. (2016). Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends in Biochemical Sciences*, *41*(12), 1012–1021. http://doi.org/10.1016/j.tibs.2016.09.002

He, Y., Zeng, M. Y., Yang, D., Motro, B., & Núñez, G. (2016). Nek7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature*, *530*(7590), 354. http://doi.org/10.1038/NATURE16959

Heid, M. E., Keyel, P. A., Kamga, C., Shiva, S., Watkins, S. C., & Salter, R. D. (2013). Mitochondrial Reactive Oxygen Species Induces NLRP3-Dependent Lysosomal Damage and Inflammasome Activation. *The Journal of Immunology*, *191*(10), 5230–5238. http://doi.org/10.4049/jimmunol.1301490

Helft, J., Böttcher, J., Chakravarty, P., Zelenay, S., Huotari, J., Schraml, B. U., … Reis e Sousa, C. (2015a). GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c+MHCII+ Macrophages and Dendritic Cells. *Immunity*, *42*(6), 1197–1211. http://doi.org/10.1016/J.IMMUNI.2015.05.018

Helft, J., Böttcher, J., Chakravarty, P., Zelenay, S., Huotari, J., Schraml, B. U., … Reis e Sousa, C. (2015b). GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of

CD11c+MHCII+ Macrophages and Dendritic Cells. *Immunity*, *42*(6), 1197–1211. http://doi.org/10.1016/J.IMMUNI.2015.05.018

Hiscott, J., Marois, J., Garoufalis, J., D'Addario, M., Roulston, A., Kwan, I., … Bensi, G. (1993). Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Molecular and Cellular Biology*, *13*(10), 6231–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8413223

Höckendorf, U., Yabal, M., Herold, T., Munkhbaatar, E., Rott, S., Jilg, S., … Jost, P. J. (2016). RIPK3 Restricts Myeloid Leukemogenesis by Promoting Cell Death and Differentiation of Leukemia Initiating Cells. *Cancer Cell*, *30*(1), 75–91. http://doi.org/10.1016/j.ccell.2016.06.002

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., … Beutler, B. (2003). *Identification of Lps2 as a key transducer of MyD88-independent TIR signalling*. Retrieved from www.nature.com/nature

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D. R., … Fitzgerald, K. A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature*, *458*(7237), 514–518. http://doi.org/10.1038/nature07725

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., … Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature Immunology*, *9*(8), 847–56. http://doi.org/10.1038/ni.1631

Howard, A. D., Kostura, M. J., Thornberry, N., Ding, G. J., Limjuco, G., Weidner, J., … Mumford, R. A. (1991). IL-1-converting enzyme requires aspartic acid residues for processing of the IL-1 beta precursor at two distinct sites and does not cleave 31-kDa IL-1 alpha. *Journal of Immunology (Baltimore, Md. : 1950)*, *147*(9), 2964–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1919001

Huang, S.-H., Xiong, M., Chen, X.-P., Xiao, Z.-Y., Zhao, Y.-F., & Huang, Z.-Y. (1994). PJ34, an inhibitor of PARP-1, suppresses cell growth and enhances the suppressive effects of cisplatin in liver cancer cells. *Oncology Reports*, *20*(3), 567–572. http://doi.org/10.3892/or_00000043

Hunter, T. (2007). Treatment for chronic myelogenous leukemia: the long road to imatinib. *The Journal of Clinical Investigation*, *117*(8), 2036–43. http://doi.org/10.1172/JCI31691

Hwang, I., Yang, J., Hong, S., Ju Lee, E., Lee, S.-H., Fernandes-Alnemri, T., … Yu, J.-W. (2015). Non-transcriptional regulation of NLRP3 inflammasome signaling by IL-4. *Immunology and Cell Biology*, *93*(6), 591–9. http://doi.org/10.1038/icb.2014.125

Inohara, N., & Nuñez, G. (2003). NODs: intracellular proteins involved in inflammation and apoptosis. *Nature Reviews Immunology*, *3*(5), 371–382. http://doi.org/10.1038/nri1086

Jaeger, B. N., & Vivier, E. (2012). Natural killer cell tolerance: control by self or self-control? *Cold Spring Harbor Perspectives in Biology*, *4*(3). http://doi.org/10.1101/cshperspect.a007229

Juliana, C., Fernandes-Alnemri, T., Kang, S., Farias, A., Qin, F., & Alnemri, E. S. (2012). Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *The Journal of Biological Chemistry*, *287*(43), 36617–22. http://doi.org/10.1074/jbc.M112.407130

Kang, T.-B., Yang, S.-H., Toth, B., Kovalenko, A., & Wallach, D. (2013). Caspase-8 Blocks Kinase RIPK3-Mediated Activation of the NLRP3 Inflammasome. *Immunity*, *38*(1), 27–40. http://doi.org/10.1016/j.immuni.2012.09.015

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., … Akira, S. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. http://doi.org/10.1038/nature04734

Katsnelson, M. A., Rucker, L. G., Russo, H. M., & Dubyak, G. R. (2015). K+ efflux agonists induce NLRP3 inflammasome activation independently of Ca2+ signaling. *Journal of Immunology (Baltimore, Md. : 1950)*, *194*(8), 3937–52. http://doi.org/10.4049/jimmunol.1402658

Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology*, *11*(5), 373–384. http://doi.org/10.1038/ni.1863

Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. *Frontiers in Immunology*, *5*, 461. http://doi.org/10.3389/fimmu.2014.00461

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., … Dixit, V. M. (2015a). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*, *526*(7575), 666–671. http://doi.org/10.1038/nature15541

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., … Dixit, V. M. (2015b). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*, *526*(7575), 666–671. http://doi.org/10.1038/nature15541

Kayagaki, N., Warming, S., Lamkanfi, M., Walle, L. Vande, Louie, S., Dong, J., … Dixit, V. M. (2011). Non-canonical inflammasome activation targets caspase-11. *Nature*, *479*(7371), 117–121. http://doi.org/10.1038/nature10558

Khare, S., Dorfleutner, A., Bryan, N. B., Yun, C., Radian, A. D., de Almeida, L., … Stehlik, C. (2012). An NLRP7-Containing Inflammasome Mediates Recognition of Microbial Lipopeptides in Human Macrophages. *Immunity*, *36*(3), 464–476. http://doi.org/10.1016/J.IMMUNI.2012.02.001

Kirshenbaum, A. (2000). REGULATION OF MAST CELL NUMBER AND FUNCTION. *Hematology/Oncology Clinics of North America*, *14*(3), 497–516. http://doi.org/10.1016/S0889-8588(05)70293-8

Kofoed, E. M., & Vance, R. E. (2011). Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature*, *477*(7366), 592–5. http://doi.org/10.1038/nature10394

Koh, D. W., Dawson, T. M., & Dawson, V. L. (2005). Mediation of cell death by poly(ADP-ribose) polymerase-1. *Pharmacological Research*, *52*(1), 5–14. http://doi.org/10.1016/J.PHRS.2005.02.011

Kolaczkowska, E., & Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, *13*(3), 159–175. http://doi.org/10.1038/nri3399

Korn, T., Bettelli, E., Oukka, M., & Kuchroo, V. K. (2009). IL-17 and Th17 Cells. *Annual Review of Immunology*, *27*(1), 485–517. http://doi.org/10.1146/annurev.immunol.021908.132710

Kuida, K., Lippke, J., Ku, G., Harding, M., Livingston, D., Su, M., & Flavell, R. (1995). Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science*, *267*(5206). Retrieved from http://science.sciencemag.org/content/267/5206/2000.long

Lacey, D. C., Achuthan, A., Fleetwood, A. J., Dinh, H., Roiniotis, J., Scholz, G. M., … Hamilton, J. A. (2012). Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models. *Journal of Immunology (Baltimore, Md. : 1950)*, *188*(11), 5752–65. http://doi.org/10.4049/jimmunol.1103426

Laemli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, *227*(5259), 680–685. http://doi.org/10.1038/227680a0

Larson, R. A., Druker, B. J., Guilhot, F., O’Brien, S. G., Riviere, G. J., Krahnke, T., … IRIS (International Randomized Interferon vs STI571) Study Group. (2008). Imatinib pharmacokinetics and its correlation with response and safety in chronic-phase chronic myeloid leukemia: a subanalysis of the IRIS study. *Blood*, *111*(8), 4022–8. http://doi.org/10.1182/blood-2007-10-116475

Lawlor, K. E., Khan, N., Mildenhall, A., Gerlic, M., Croker, B. A., D’Cruz, A. A., … Vince, J. E. (2015). RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nature Communications*, *6*, 6282. http://doi.org/10.1038/ncomms7282

Lee, C.-R., Kang, J.-A., Kim, H.-E., Choi, Y., Yang, T., & Park, S.-G. (2016). Secretion of IL-1β from imatinib-resistant chronic myeloid leukemia cells contributes to *BCR - ABL* mutation-independent imatinib resistance. *FEBS Letters*, *590*(3), 358–368. http://doi.org/10.1002/1873-3468.12057

Lemaire, C., Andréau, K., Souvannavong, V., & Adam, A. (1998). Inhibition of caspase activity induces a switch from apoptosis to necrosis. *FEBS Letters*, *425*(2), 266–270. http://doi.org/10.1016/S0014-5793(98)00252-X

Levinsohn, J. L., Newman, Z. L., Hellmich, K. A., Fattah, R., Getz, M. A., Liu, S., … Moayeri, M. (2012). Anthrax Lethal Factor Cleavage of Nlrp1 Is Required for Activation of the Inflammasome. *PLoS Pathogens*, *8*(3), e1002638. http://doi.org/10.1371/journal.ppat.1002638

Levitzki, A. (2013). Tyrosine Kinase Inhibitors: Views of Selectivity, Sensitivity, and Clinical Performance. *Annual Review of Pharmacology and Toxicology*, *53*(1), 161–185. http://doi.org/10.1146/annurev-pharmtox-011112-140341

Lewis, A. M., Varghese, S., Xu, H., & Alexander, H. R. (2006). Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *Journal of Translational Medicine*, *4*, 48. http://doi.org/10.1186/1479-5876-4-48

Ley, K., Laudanna, C., Cybulsky, M. I., & Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews Immunology*, *7*(9), 678–689. http://doi.org/10.1038/nri2156

Li, Y., Fu, T.-M., Lu, A., Witt, K., Ruan, J., Shen, C., & Wu, H. (2018). Cryo-EM structures of ASC and NLRC4 CARD filaments reveal a unified mechanism of nucleation and activation of caspase-1. *Proceedings of the National Academy of Sciences*, *115*(43), 10845–10852. http://doi.org/10.1073/pnas.1810524115

Lieschke, G., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., … Dunn, A. (1994). Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood*, *84*(6).

Lin, K.-M., Hu, W., Troutman, T. D., Jennings, M., Brewer, T., Li, X., … Pasare, C. (2014). IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. *Proceedings of the National Academy of Sciences*, *111*(2), 775–780. http://doi.org/10.1073/pnas.1320294111

Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., & Lieberman, J. (2016a). Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature*, *535*(7610), 153–8. http://doi.org/10.1038/nature18629

Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., & Lieberman, J. (2016b). Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature*, *535*(7610), 153–158. http://doi.org/10.1038/nature18629

Lortholary, O., Chandesris, M. O., Bulai Livideanu, C., Paul, C., Guillet, G., Jassem, E., … Hermine, O. (2017). Masitinib for treatment of severely symptomatic indolent systemic mastocytosis: a randomised, placebo-controlled, phase 3 study. *Lancet (London, England)*, *389*(10069), 612–620. http://doi.org/10.1016/S0140-6736(16)31403-9

Lu, A., Magupalli, V. G., Ruan, J., Yin, Q., Atianand, M. K., Vos, M. R., … Egelman, E. H. (2014a). Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. *Cell*, *156*(6), 1193–206. http://doi.org/10.1016/j.cell.2014.02.008

Lu, A., Magupalli, V. G., Ruan, J., Yin, Q., Atianand, M. K., Vos, M. R., … Egelman, E. H. (2014b). Unified Polymerization Mechanism for the Assembly of ASC-Dependent Inflammasomes. *Cell*, *156*(6), 1193–1206. http://doi.org/10.1016/J.CELL.2014.02.008

Luo, D., Ding, S. C., Vela, A., Kohlway, A., Lindenbach, B. D., & Pyle, A. M. (2011). Structural insights into RNA recognition by RIG-I. *Cell*, *147*(2), 409–22. http://doi.org/10.1016/j.cell.2011.09.023

Ma, Z.-Z., Sun, H.-S., Lv, J.-C., Guo, L., & Yang, Q.-R. (2018). Expression and clinical significance of the NEK7-NLRP3 inflammasome signaling pathway in patients with systemic lupus erythematosus. *Journal of Inflammation (London, England)*, *15*, 16. http://doi.org/10.1186/s12950-018-0192-9

Malhotra, R., Valuckaite, V., Staron, M. L., Theccanat, T., D'Souza, K. M., Alverdy, J. C., & Akhter, S. A. (2011). High-molecular-weight polyethylene glycol protects cardiac myocytes from hypoxia- and reoxygenation-induced cell death and preserves ventricular function. *American Journal of Physiology. Heart and Circulatory Physiology*, *300*(5), H1733-42. http://doi.org/10.1152/ajpheart.01054.2010

Mangan, M. S. J., Olhava, E. J., Roush, W. R., Seidel, H. M., Glick, G. D., & Latz, E. (2018). Targeting the NLRP3 inflammasome in inflammatory diseases. *Nature Reviews Drug Discovery*, *17*(8), 588–606. http://doi.org/10.1038/nrd.2018.97

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology*, *25*(12), 677–686. http://doi.org/10.1016/J.IT.2004.09.015

Margolin, N., Raybuck, S. A., Wilson, K. P., Chen, W., Fox, T., Gu, Y., & Livingston, D. J. (1997). Substrate and inhibitor specificity of interleukin-1 beta-converting enzyme and related caspases. *The Journal of Biological Chemistry*, *272*(11), 7223–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9054418

Mariathasan, S., Newton, K., Monack, D. M., Vucic, D., French, D. M., Lee, W. P., … Dixit, V. M. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*, *430*(6996), 213–218. http://doi.org/10.1038/nature02664

Martinez-Pomares, L. (2012). The mannose receptor. *Journal of Leukocyte Biology*, *92*(6), 1177–1186. http://doi.org/10.1189/jlb.0512231

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime Reports*, *6*, 13. http://doi.org/10.12703/P6-13

Martinon, F., Burns, K., & Tschopp, J. (2002). The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-β. *Molecular Cell*, *10*(2), 417–426. http://doi.org/10.1016/S1097-2765(02)00599-3

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., & Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*, *440*(7081), 237–241. http://doi.org/10.1038/nature04516

Masumoto, J., Taniguchi, S., Ayukawa, K., Sarvotham, H., Kishino, T., Niikawa, N., … Sagara, J. (1999). ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *The Journal of Biological Chemistry*, *274*(48), 33835–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10567338

Mazzurana, L., Rao, A., Van Acker, A., & Mjösberg, J. (2018). The roles for innate lymphoid cells in the human immune system. *Seminars in Immunopathology*, *40*(4), 407–419. http://doi.org/10.1007/s00281-018-0688-7

McGeachy, M. J. (2011). GM-CSF: the secret weapon in the TH17 arsenal. *Nature Immunology*, *12*(6), 521–522. http://doi.org/10.1038/ni.2044

McIlwain, D. R., Berger, T., & Mak, T. W. (2013). Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology*, *5*(4), a008656. http://doi.org/10.1101/cshperspect.a008656

Mckee, T. J., & Komarova, S. V. (2017). Is it time to reinvent basic cell culture medium? http://doi.org/10.1152/ajpcell.00336.2016

Meyer-Wentrup, F., Cambi, A., Joosten, B., Looman, M. W., de Vries, I. J. M., Figdor, C. G., & Adema, G. J. (2009). DCIR is endocytosed into human dendritic cells and inhibits TLR8-mediated cytokine production. *Journal of Leukocyte Biology*, *85*(3), 518–525. http://doi.org/10.1189/jlb.0608352

Miao, E. A., Rajan, J. V, & Aderem, A. (2011). Caspase-1-induced pyroptotic cell death. *Immunological Reviews*, *243*(1), 206–14. http://doi.org/10.1111/j.1600-065X.2011.01044.x

Miller, D. K., Ayala, J. M., Egger, L. A., Raju, S. M., Yamin, T. T., Ding, G. J., … Rolando, A. M. (1993). Purification and characterization of active human interleukin-1 beta-converting enzyme from THP.1 monocytic cells. *The Journal of Biological Chemistry*, *268*(24), 18062–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8349684

Milovancev, M., Helfand, S. C., Marley, K., Goodall, C. P., Löhr, C. V, & Bracha, S. (2016). Antiproliferative effects of masitinib and imatinib against canine oral fibrosarcoma in vitro. *BMC Veterinary Research*, *12*, 85. http://doi.org/10.1186/s12917-016-0712-x

Misawa, T., Saitoh, T., Kozaki, T., Park, S., Takahama, M., & Akira, S. (2015). Resveratrol inhibits the acetylated α-tubulin-mediated assembly of the NLRP3-inflammasome. *International Immunology*, *27*(9), 425–434. http://doi.org/10.1093/intimm/dxv018

Misawa, T., Takahama, M., Kozaki, T., Lee, H., Zou, J., Saitoh, T., & Akira, S. (2013). Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. *Nature Immunology*, *14*(5), 454–460. http://doi.org/10.1038/ni.2550

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., & Núñez, G. (2013a). K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity*, *38*(6), 1142–53. http://doi.org/10.1016/j.immuni.2013.05.016

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., & Núñez, G. (2013b). K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity*, *38*(6), 1142–53. http://doi.org/10.1016/j.immuni.2013.05.016

Murphy, J. M., Czabotar, P. E., Hildebrand, J. M., Lucet, I. S., Zhang, J.-G., Alvarez-Diaz, S., … Alexander, W. S. (2013). The Pseudokinase MLKL Mediates Necroptosis via a Molecular Switch Mechanism. *Immunity*, *39*(3), 443–453. http://doi.org/10.1016/j.immuni.2013.06.018

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., … Wynn, T. A. (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, *41*(1), 14–20. http://doi.org/10.1016/j.immuni.2014.06.008

Murray, P. J., & Wynn, T. A. (2011). Obstacles and opportunities for understanding macrophage polarization. *Journal of Leukocyte Biology*, *89*(4), 557–63. http://doi.org/10.1189/jlb.0710409

Nakahira, K., Haspel, J. A., Rathinam, V. A. K., Lee, S.-J., Dolinay, T., Lam, H. C., … Choi, A. M. K. (2011). Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nature Immunology*, *12*(3), 222–230. http://doi.org/10.1038/ni.1980

Nassar, I., Pasupati, T., Judson, J. P., & Segarra, I. (2009). Reduced exposure of imatinib after coadministration with acetaminophen in mice. *Indian Journal of Pharmacology*, *41*(4), 167–72. http://doi.org/10.4103/0253-7613.56071

Nickel, W., & Rabouille, C. (2009). Mechanisms of regulated unconventional protein secretion. *Nature Reviews Molecular Cell Biology*, *10*(2), 148–155. http://doi.org/10.1038/nrm2617

Noster, R., Riedel, R., Mashreghi, M.-F., Radbruch, H., Harms, L., Haftmann, C., … Zielinski, C. E. (2014). IL-17 and GM-CSF Expression Are Antagonistically Regulated by Human T Helper Cells. *Science Translational Medicine*, *6*(241), 241ra80-241ra80. http://doi.org/10.1126/scitranslmed.3008706

O'Regan, L., & Fry, A. M. (2009). The Nek6 and Nek7 Protein Kinases Are Required for Robust Mitotic Spindle Formation and Cytokinesis. *Molecular and Cellular Biology*, *29*(14), 3975–3990. http://doi.org/10.1128/MCB.01867-08

Orlowski, G. M., Sharma, S., Colbert, J. D., Bogyo, M., Robertson, S. A., Kataoka, H., … Rock, K. L. (2017). Frontline Science: Multiple cathepsins promote inflammasome-independent, particle-induced cell death during NLRP3-dependent IL-1b activation. *J. Leukoc. Biol*, *101*, 0-000. http://doi.org/10.1189/jlb.3HI0316-152R

Patin, E. C., Orr, S. J., & Schaible, U. E. (2017). Macrophage Inducible C-Type Lectin As a Multifunctional Player in Immunity. *Frontiers in Immunology*, *8*, 861. http://doi.org/10.3389/fimmu.2017.00861

Paul, S., & Lal, G. (2017). The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Frontiers in Immunology*, *8*, 1124. http://doi.org/10.3389/fimmu.2017.01124

Peng, B., Hayes, M., Resta, D., Racine-Poon, A., Druker, B. J., Talpaz, M., … Capdeville, R. (2004). Pharmacokinetics and pharmacodynamics of imatinib in a phase I trial with chronic myeloid leukemia patients. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *22*(5), 935–42. http://doi.org/10.1200/JCO.2004.03.050

Perregaux, D. G., Labasi, J., Laliberte, R., Stam, E., Solle, M., Koller, B., … Gabel, C. A. (2001). Interleukin-1? posttranslational processing?exploration of P2X7 receptor involvement. *Drug Development Research*, *53*(2–3), 83–90. http://doi.org/10.1002/ddr.1175

Pétrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., & Tschopp, J. (2007). Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death & Differentiation*, *14*(9), 1583–1589. http://doi.org/10.1038/sj.cdd.4402195

Picard, S., Titier, K., Etienne, G., Teilhet, E., Ducint, D., Bernard, M.-A., … Mahon, F.-X. (2007). Trough imatinib plasma levels are associated with both cytogenetic and molecular responses to standard-dose imatinib in chronic myeloid leukemia. *Blood*, *109*(8), 3496–3499. http://doi.org/10.1182/BLOOD-2006-07-036012

Pomerantz, B. J., Reznikov, L. L., Harken, A. H., & Dinarello, C. A. (2001). Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1. *Proceedings of the National Academy of Sciences*, *98*(5), 2871–2876. http://doi.org/10.1073/pnas.041611398

Quicke, K. M., Diamond, M. S., & Suthar, M. S. (2017). Negative regulators of the RIG-I-like receptor signaling pathway. *European Journal of Immunology*, *47*(4), 615–628. http://doi.org/10.1002/eji.201646484

Radian, A. D., Khare, S., Chu, L. H., Dorfleutner, A., & Stehlik, C. (2015). ATP binding by NLRP7 is required for inflammasome activation in response to bacterial lipopeptides. *Molecular Immunology*, *67*(2), 294–302. http://doi.org/10.1016/j.molimm.2015.06.013

Rathinam, V. A. K., Jiang, Z., Waggoner, S. N., Sharma, S., Cole, L. E., Waggoner, L., … Fitzgerald, K. A. (2010). The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nature Immunology*, *11*(5), 395–402. http://doi.org/10.1038/ni.1864

Redelinghuys, P., & Brown, G. D. (2011). Inhibitory C-type lectin receptors in myeloid cells. *Immunology Letters*, *136*(1), 1–12. http://doi.org/10.1016/j.imlet.2010.10.005

Rickard, J. A., O'Donnell, J. A., Evans, J. M., Lalaoui, N., Poh, A. R., Rogers, T., … Silke, J. (2014). RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell*, *157*(5), 1175–88. http://doi.org/10.1016/j.cell.2014.04.019

Rivers-Auty, J., & Brough, D. (2015). Potassium efflux fires the canon: Potassium efflux as a common trigger for canonical and noncanonical NLRP3 pathways. *European Journal of Immunology*, *45*(10), 2758–2761. http://doi.org/10.1002/eji.201545958

Rix, U., Hantschel, O., Durnberger, G., Remsing Rix, L. L., Planyavsky, M., Fernbach, N. V., … Superti-Furga, G. (2007). Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. *Blood*, *110*(12), 4055–4063. http://doi.org/10.1182/blood-2007-07-102061

Röck, J., Schneider, E., Grün, J. R., Grützkau, A., Küppers, R., Schmitz, J., & Winkels, G. (2007). CD303 (BDCA-2) signals in plasmacytoid dendritic cellsvia a BCR-like signalosome involving Syk, Slp65 and PLCγ2. *European Journal of Immunology*, *37*(12), 3564–3575. http://doi.org/10.1002/eji.200737711

Rosenberg, H. F., Dyer, K. D., & Foster, P. S. (2013). Eosinophils: changing perspectives in health and disease. *Nature Reviews. Immunology*, *13*(1), 9–22. http://doi.org/10.1038/nri3341

Röszer, T. (2018). Understanding the Biology of Self-Renewing Macrophages. *Cells*, *7*(8). http://doi.org/10.3390/cells7080103

Rubartelli, A., Cozzolino, F., Talio, M., & Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *The EMBO Journal*, *9*(5), 1503. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC551842/

Rühl, S., & Broz, P. (2015). Caspase-11 activates a canonical NLRP3 inflammasome by promoting K^+ efflux. *European Journal of Immunology*, *45*(10), 2927–2936. http://doi.org/10.1002/eji.201545772

Sborgi, L., Rühl, S., Mulvihill, E., Pipercevic, J., Heilig, R., Stahlberg, H., … Hiller, S. (2016a). GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death. *The EMBO Journal*, *35*(16), 1766–1778. http://doi.org/10.15252/embj.201694696

Sborgi, L., Rühl, S., Mulvihill, E., Pipercevic, J., Heilig, R., Stahlberg, H., … Hiller, S. (2016b). GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death. *The EMBO Journal*, *35*(16), 1766–1778. http://doi.org/10.15252/embj.201694696

Schmid-burgk, J. L., Chauhan, D., Schmidt, T., Ebert, T. S., Reinhardt, J., Endl, E., & Hornung, V. (2016). A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component of NLRP3 Inflammasome, *291*(1), 103–109. http://doi.org/10.1074/jbc.C115.700492

Schneider, K. S., Groß, C. J., Dreier, R. F., Kuster, B., Broz, P., & Correspondence, O. G. (2017). The Inflammasome Drives GSDMD-Independent Secondary Pyroptosis and IL-1 Release in the Absence of Caspase-1 Protease Activity. *CellReports*, *21*, 3846–3859. http://doi.org/10.1016/j.celrep.2017.12.018

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 (pp. 117–135). http://doi.org/10.1007/978-1-62703-523-1_10

Schroder, K., & Tschopp, J. (2010a). The inflammasomes. *Cell*, *140*(6), 821–32. http://doi.org/10.1016/j.cell.2010.01.040

Schroder, K., & Tschopp, J. (2010b). The Inflammasomes. *Cell*, *140*(6), 821–832. http://doi.org/10.1016/J.CELL.2010.01.040

Shao, B.-Z., Xu, Z.-Q., Han, B.-Z., Su, D.-F., & Liu, C. (2015). NLRP3 inflammasome and its inhibitors: a review. *Frontiers in Pharmacology*, *6*, 262. http://doi.org/10.3389/fphar.2015.00262

Shaw, O. M., Steiger, S., Liu, X., Hamilton, J. A., & Harper, J. L. (2014). Brief Report: Granulocyte-Macrophage Colony-Stimulating Factor Drives Monosodium Urate Monohydrate Crystal-Induced Inflammatory Macrophage Differentiation and NLRP3 Inflammasome Up-Regulation in an In Vivo Mouse Model. *Arthritis & Rheumatology*, *66*(9), 2423–2428. http://doi.org/10.1002/art.38730

Shi, H., Wang, Y., Li, X., Zhan, X., Tan, M., Fina, M., … Beutler, B. (2016). NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nature Immunology*, *17*(3), 250. http://doi.org/10.1038/NI.3333

Shi, H., Wang, Y., Li, X., Zhan, X., Tang, M., Fina, M., … Beutler, B. (2016). NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nature Immunology*, *17*(3), 250–8. http://doi.org/10.1038/ni.3333

Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., … Shao, F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature*, *526*(7575), 660–665. http://doi.org/10.1038/nature15514

Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., … Shao, F. (2014). Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature*, *514*(7521), 187–192. http://doi.org/10.1038/nature13683

Shiomi, A., Usui, T., & Mimori, T. (2016). GM-CSF as a therapeutic target in autoimmune diseases. *Inflammation and Regeneration*, *36*, 8. http://doi.org/10.1186/s41232-016-0014-5

Solano-Gálvez, S. G., Tovar-Torres, S. M., Tron-Gómez, M. S., Weiser-Smeke, A. E., Álvarez-Hernández, D. A., Franyuti-Kelly, G. A., … Vázquez-López, R. (2018). Human Dendritic Cells: Ontogeny and Their Subsets in Health and Disease. *Medical Sciences (Basel, Switzerland)*, *6*(4). http://doi.org/10.3390/medsci6040088

Steimle, V., Siegrist, C. A., Mottet, A., Lisowska-Grospierre, B., & Mach, B. (1994). Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science (New York, N.Y.)*, *265*(5168), 106–9. http://doi.org/10.1126/SCIENCE.8016643

Steinman, R. M., & Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *The Journal of Experimental Medicine*, *137*(5), 1142–62. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4573839

Sternberg, C. N., Davis, I. D., Mardiak, J., Szczylik, C., Lee, E., Wagstaff, J., … Hawkins, R. E. (2010). Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *28*(6), 1061–8. http://doi.org/10.1200/JCO.2009.23.9764

Steven, J. L. (1892). Metchnikoff on the Comparative Pathology of Inflammation. *Glasgow Medical Journal*, *38*(3), 195–205. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/30435737

Stutz, A., Kolbe, C.-C., Stahl, R., Horvath, G. L., Franklin, B. S., Ray, O. van, … Latz, E. (2017). NLRP3 inflammasome assembly is regulated by phosphorylation of the pyrin domain. *Journal of Experimental Medicine*, *214*(6), 1725–1736. http://doi.org/10.1084/JEM.20160933

Sukegawa, M., Wang, X., Nishioka, C., Pan, B., Xu, K., Ohkawara, H., … Ikezoe, T. (2017). The BCR/ABL tyrosine kinase inhibitor, nilotinib, stimulates expression of IL-1β in vascular endothelium in association with downregulation of miR-3p. *Leukemia Research*, *58*, 83–90. http://doi.org/10.1016/j.leukres.2017.05.005

Sun, L., Wang, H., Wang, Z., He, S., Chen, S., Liao, D., … Wang, X. (2012). Mixed Lineage Kinase Domain-like Protein Mediates Necrosis Signaling Downstream of RIP3 Kinase. *Cell*, *148*(1–2), 213–227. http://doi.org/10.1016/j.cell.2011.11.031

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., … Tocci, M. J. (1992). A novel heterodimeric cysteine protease is required for interleukin-1βprocessing in monocytes. *Nature*, *356*(6372), 768–774. http://doi.org/10.1038/356768a0

Toldo, S., & Abbate, A. (2017). The NLRP3 inflammasome in acute myocardial infarction. *Nature Reviews Cardiology*, *15*(4), 203–214. http://doi.org/10.1038/nrcardio.2017.161

Towbin, H., Staehelin, T., & Gordon, J. (1992). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. *Biotechnology (Reading, Mass.)*, *24*(9), 145–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1422008

Uematsu, S., Sato, S., Yamamoto, M., Hirotani, T., Kato, H., Takeshita, F., … Akira, S. (2005). Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. *The Journal of Experimental Medicine*, *201*(6), 915–23. http://doi.org/10.1084/jem.20042372

Urb, M., & Sheppard, D. C. (2012). The role of mast cells in the defence against pathogens. *PLoS Pathogens*, *8*(4), e1002619. http://doi.org/10.1371/journal.ppat.1002619

Ushach, I., & Zlotnik, A. (2016). Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage. *Journal of Leukocyte Biology*, *100*(3), 481–9. http://doi.org/10.1189/jlb.3RU0316-144R

van de Laar, L., Coffer, P. J., & Woltman, A. M. (2012). Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. *Blood*, *119*(15), 3383–3393. http://doi.org/10.1182/blood-2011-11-370130

van de Veerdonk, F. L., Marijnissen, R. J., Kullberg, B. J., Koenen, H. J. P. M., Cheng, S.-C., Joosten, I., … Netea, M. G. (2009). The Macrophage Mannose Receptor Induces IL-17 in Response to Candida albicans. *Cell Host & Microbe*, *5*(4), 329–340. http://doi.org/10.1016/J.CHOM.2009.02.006

van der Graaf, W. T., Blay, J.-Y., Chawla, S. P., Kim, D.-W., Bui-Nguyen, B., Casali, P. G., … Hohenberger, P. (2012). Pazopanib for metastatic soft-tissue sarcoma (PALETTE): a randomised, double-blind, placebo-controlled phase 3 trial. *The Lancet*, *379*(9829), 1879–1886. http://doi.org/10.1016/S0140-6736(12)60651-5

Vandenabeele, P., Grootjans, S., Callewaert, N., & Takahashi, N. (2013). Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. *Cell Death and Differentiation*, *20*(2), 185–7. http://doi.org/10.1038/cdd.2012.151

Vandenabeele, P., Vanden Berghe, T., & Festjens, N. (2006). Caspase inhibitors promote alternative cell death pathways. *Science's STKE : Signal Transduction Knowledge Environment*, *2006*(358), pe44. http://doi.org/10.1126/stke.3582006pe44

Vella, F., Ferry, G., Delagrange, P., & Boutin, J. A. (2005). NRH:quinone reductase 2: an enzyme of surprises and mysteries. *Biochemical Pharmacology*, *71*(1–2), 1–12. http://doi.org/10.1016/j.bcp.2005.09.019

Vento-Tormo, R., Alvarez-Errico, D., Garcia-Gomez, A., Hern andez-Rodr ıguez, J., Buj an, S., Basaga, M., … Ballestar, E. (2017). Immune deficiencies, infection, and systemic immune disorders DNA demethylation of inflammasome-associated genes is enhanced in patients with cryopyrin-associated periodic syndromes. *Journal of Allergy and Clinical Immunology*, *139*, 202–211.e6. http://doi.org/10.1016/j.jaci.2016.05.016

Verreck, F. A. W., de Boer, T., Langenberg, D. M. L., Hoeve, M. A., Kramer, M., Vaisberg, E., … Ottenhoff, T. H. M. (2004). Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(13), 4560–5. http://doi.org/10.1073/pnas.0400983101

Vivier, E., Ugolini, S., Blaise, D., Chabannon, C., & Brossay, L. (2012). Targeting natural killer cells and natural killer T cells in cancer. *Nature Reviews. Immunology*, *12*(4), 239–52. http://doi.org/10.1038/nri3174

Voehringer, D. (2013). Protective and pathological roles of mast cells and basophils. *Nature Reviews Immunology*, *13*(5), 362–375. http://doi.org/10.1038/nri3427

Vremec, D., Lieschke, G. J., Dunn, A. R., Robb, L., Metcalf, D., & Shortman, K. (1997). The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. *European Journal of Immunology*, *27*(1), 40–44. http://doi.org/10.1002/eji.1830270107

Walev, I., Reske, K., Palmer, M., Valeva, A., & Bhakdi, S. (1995). Potassium-inhibited processing of IL-1 beta in human monocytes. *The EMBO Journal*, *14*(8), 1607–14. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7737113

Walker, N. P., Talanian, R. V, Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., … Hammill, L. D. (1994). Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. *Cell*, *78*(2), 343–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8044845

Walsh, J. G., Logue, S. E., Lüthi, A. U., & Martin, S. J. (2011). Caspase-1 promiscuity is counterbalanced by rapid inactivation of processed enzyme. *The Journal of Biological Chemistry*, *286*(37), 32513–24. http://doi.org/10.1074/jbc.M111.225862

Weischenfeldt, J., & Porse, B. (2008). Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. *CSH Protocols*, *2008*(12), pdb.prot5080. http://doi.org/10.1101/pdb.prot5080

Wetzler, M., Kurzrock, R., Estrov, Z., Kantarjian, H., Gisslinger, H., Underbrink, M., & Talpaz, M. (1994). Altered levels of interleukin-1 beta and interleukin-1 receptor antagonist in chronic myelogenous leukemia: clinical and prognostic correlates. *Blood*, *84*(9). Retrieved from http://www.bloodjournal.org/content/84/9/3142.long?sso-checked=true

Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A. W., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W., … Stanley, E. R. (1990). Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(12), 4828–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2191302

Wilson, K. P., Black, J.-A. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., … Livingston, D. J. (1994). Structure and mechanism of interleukin-Iβ converting enzyme. *Nature*, *370*(6487), 270–275. http://doi.org/10.1038/370270a0

Winger, J. A., Hantschel, O., Superti-Furga, G., & Kuriyan, J. (2009). The structure of the leukemia drug imatinib bound to human quinone reductase 2 (NQO2). *BMC Structural Biology*, *9*(1), 7. http://doi.org/10.1186/1472-6807-9-7

Wlodarska, M., Thaiss, C. A., Nowarski, R., Henao-Mejia, J., Zhang, J.-P., Brown, E. M., … Flavell, R. A. (2014). NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell*, *156*(5), 1045–59. http://doi.org/10.1016/j.cell.2014.01.026

Wu, J., Yan, Z., Schwartz, D. E., Yu, J., Malik, A. B., & Hu, G. (2013). Activation of NLRP3 inflammasome in alveolar macrophages contributes to mechanical stretch-induced lung inflammation and injury. *Journal of Immunology (Baltimore, Md. : 1950)*, *190*(7), 3590–9. http://doi.org/10.4049/jimmunol.1200860

Yamamoto, M., Takeda, K., & Akira, S. (2004). TIR domain-containing adaptors define the specificity of TLR signaling. *Molecular Immunology*, *40*(12), 861–868. http://doi.org/10.1016/J.MOLIMM.2003.10.006

Yamamoto, S., & Ma, X. (2009). Role of Nod2 in the development of Crohn's disease. *Microbes and Infection*, *11*(12), 912–8. http://doi.org/10.1016/j.micinf.2009.06.005

Yamin, T. T., Ayala, J. M., & Miller, D. K. (1996). Activation of the native 45-kDa precursor form of interleukin-1-converting enzyme. *The Journal of Biological Chemistry*, *271*(22), 13273–82. http://doi.org/10.1074/JBC.271.22.13273

Yao, T., & Asayama, Y. (2017). Animal-cell culture media: History, characteristics, and current issues. *Reproductive Medicine and Biology*, *16*(2), 99–117. http://doi.org/10.1002/rmb2.12024

Yao, Y., Wang, Y., Chen, F., Huang, Y., Zhu, S., Leng, Q., … Qian, Y. (2012). NLRC5 regulates MHC class I antigen presentation in host defense against intracellular pathogens. *Cell Research*, *22*(5), 836–847. http://doi.org/10.1038/cr.2012.56

Yissachar, N., Salem, H., Tennenbaum, T., & Motro, B. (2006). Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. *FEBS Letters*, *580*(27), 6489–6495. http://doi.org/10.1016/J.FEBSLET.2006.10.069

Zelensky, A. N., & Gready, J. E. (2005). The C-type lectin-like domain superfamily. *FEBS Journal*, *272*(24), 6179–6217. http://doi.org/10.1111/j.1742-4658.2005.05031.x

Zhang, B., Chu, S., Agarwal, P., Campbell, V. L., Hopcroft, L., Jørgensen, H. G., … Bhatia, R. (2016). Inhibition of interleukin-1 signaling enhances elimination of tyrosine kinase inhibitor-treated CML stem cells. *Blood*, *128*(23), 2671–2682. http://doi.org/10.1182/blood-2015-11-679928

Zhang, B., Ho, Y., McDonald, T., Lin, A., Snyder, D. S., Ngo, V. N., … Bhatia, R. (2014). Role of Enhanced Microenvironmental Interleukin-1 (IL-1) Expression and Increased IL-1 Responsiveness in Persistence of Leukemia Stem Cells in TKI Treated CML Patients. *Blood*, *124*(21). Retrieved from http://www.bloodjournal.org/content/124/21/4357?sso-checked=true

Zhang, J., Dawson, V. L., Dawson, T. M., Snyder, S. H., Bowers, W. J., Federoff, H. J., … Dawson, V. L. (1994). Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science (New York, N.Y.)*, *263*(5147), 687–9. http://doi.org/10.1126/science.8080500

Zhang, L., Chen, S., Ruan, J., Wu, J., Tong, A. B., Yin, Q., … Wu, H. (2015). Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization. *Science (New York, N.Y.)*, *350*(6259), 404–9. http://doi.org/10.1126/science.aac5789

Zhao, J., Jitkaew, S., Cai, Z., Choksi, S., Li, Q., Luo, J., & Liu, Z.-G. (2012). Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proceedings of the National Academy of Sciences*, *109*(14), 5322–5327. http://doi.org/10.1073/pnas.1200012109

Zhao, X., Huang, X.-H., Dong, X.-H., Wang, Y.-H., Yang, H.-X., Wang, Y., … Jiang, X.-X. (2018). Deubiquitinase Mysm1 regulates macrophage survival and polarization. *Molecular Biology Reports*, *45*(6), 2393–2401. http://doi.org/10.1007/s11033-018-4405-3

Zhao, Y., Yang, J., Shi, J., Gong, Y.-N., Lu, Q., Xu, H., … Shao, F. (2011). The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature*, *477*(7366), 596–600. http://doi.org/10.1038/nature10510

Zitvogel, L., Rusakiewicz, S., Routy, B., Ayyoub, M., & Kroemer, G. (2016). Immunological off-target effects of imatinib. *Nature Reviews Clinical Oncology*, *13*(7), 431–446. http://doi.org/10.1038/nrclinonc.2016.41

Other sources

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PhD Thesis:

Giovanni Magnani:
Roles of the NLRP3 Inflammasome in the development and therapy of hematologic malignancies, accepted 22.02.2017 by TUM Medical Faculty

Christina Groß:
Characterization of TLR7-independent effects of Imidazoquinolines, accepted 01.02.2016 by the TUM Medical Faculty