



**Fakultät für Medizin**

**Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar**

# **Roles of the NLRP3 Inflammasome in the development and therapy of hematologic malignancies**

**Giovanni Magnani**

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

**Doctor of Philosophy (Ph.D.)**

genehmigten Dissertation.

**Vorsitzender:** Prof. Dr. Roland M. Schmid

**Betreuer:** Priv.-Doz. Dr. Olaf Groß

**Prüfer der Dissertation:**

1. Priv.-Doz. Dr. Philipp Jost

2. apl. Prof. Dr. Florian Bassermann

Die Dissertation wurde am 13.12.2016 bei der Fakultät für Medizin der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 22.02.2017 angenommen.

This work is dedicated to my family

*Accidere ex una scintilla incendia passim*

Titus Lucretius Carus - De rerum natura (circa 55 BC)

# TABLE OF CONTENTS

ABSTRACT	7
ZUSAMMENFASSUNG	8
RIASSUNTO	9
LIST OF ABBREVIATIONS	10
INTRODUCTION	11
The Inflammasomes in the context of Pattern Recognition Receptors	11
The different types of Inflammasomes	17
The NLRP1 Inflammasome	18
The NLRP3 Inflammasome	19
The NLRC4 Inflammasome	21
The AIM2 inflammasome	23
The emerging inflammasomes	23
The inflammasome independent cleavage of interleukin-1	25
A mechanistic overview of NLRP3 inflammasome assembly	27
Canonical NLRP3 activation	27
Inflammasome signal propagation	30
NLRP3 inflammasome regulation mechanisms	32
Non-canonical NLRP3 activation	34
Small molecules to study the NLRP3 inflammasome, the unexpected new tools: tyrosine kinase inhibitors (TKIs)	37
Modulation of NLRP3 inflammasome: small molecules at the forefront	38
Tyrosine Kinase Inhibitors	40
The role of the inflammasome in cancer	43
Inflammation driving cancer development	43
ASC as a tumor suppressor?	45
AIM OF THE PROJECT	47
Project one: imatinib and masitinib activate the NLRP3 inflammasome	47
Project two: The role of ASC in hematologic malignancies	49
MATERIAL AND METHODS	51
Materials	51
Mice	51
Cell lines	52
Methods	53
Isolation of murine BMDMs and BMDCs	53
Inflammasome assays with BMDM and BMDCs	53
Measurement of secreted cytokines by ELISA	54
Measurement of cell death	54
Western blot for inflammasome experiments	55

ASC speck visualization assays	56
Residual potassium assays	56
Flow cytometric measurement of ROS and lysosomal leakage	56
Isolation of human PBMCs and inflammasome stimulation	57
Isolation of murine intestinal organoids	58
Mouse model analysis	58
Flow cytometric assays of sick and control mouse organs	59
Isolation of murine B and T cells	59
Survival experiments with B and T cells	60
CFSE experiments with B and T cells	60
Isolation of murine keratinocytes	60
Apoptosis experiments with murine keratinocytes	61
Bone marrow transplantation to induce oncogenesis	61
<b>RESULTS</b>	<b>63</b>
Project one results	63
Imatinib induces IL-1 $\beta$ secretion in primary murine myeloid cells	63
Imatinib activates the NLRP3 inflammasome	66
Imatinib activates NLRP3 through a K <sup>+</sup> dependent mechanism	69
Imatinib triggers mild ROS production	73
Caspase protease activity-independent cell death induced imatinib	76
Imatinib does not induce RIPK3/MLKL-dependent necroptosis	78
Osmoprotectant high molecular weight PEG protects from imatinib induced cell death	80
Imatinib activates the NLRP3 though an unconventional mechanism	82
Evaluation of the therapeutic relevance for imatinib-induced inflammasome activation	87
Other TKIs can activate the inflammasome with different mechanisms	91
Pazopanib induces inflammasome without causing K <sup>+</sup> efflux	93
Project two results	95
ASC is dispensable for Burkitt's lymphoma progression	95
A flow cytometric analysis of the lymphoma model	97
Lymphoma B cells spread and colonize all the lymphoid organs	99
ASC knockout B cells survive and proliferate normally	101
ASC is dispensable in a peripheral T cell lymphoma model	103
A preliminary test to analyze the role of ASC in myeloid leukemia	106
<b>DISCUSSION</b>	<b>108</b>
Discussion for project one	108
The ability of TKIs to activate the inflammasome does not correlate to their binding of NQO2	108
Four different effects of TKIs on myeloid cells	109
Imatinib activates the NLRP3 inflammasome	109
Upstream requirements for Imatinib-induced NLRP3 inflammasome activation	110
Is there a role for caspase-8 in TKI induced inflammasome activation?	112

The mode of IL-1 $\beta$ release induced by Imatinib	113
Type of Cell death induced by Imatinib	114
Masitinib is a stronger stimulus than imatinib	117
Pazopanib activates NLRP3 without causing K <sup>+</sup> efflux	117
Inflammasome-driven immunomodulatory effects induced by imatinib	118
Discussion for project two	123
ASC does not alter the tested hematologic malignancies	123
Inflammasome, pyroptosis and the role of ASC in cancer	124
ACKNOWLEDGEMENTS	126
CURRICULUM VITAE WITH PUBLICATION LIST	127
REFERENCES	129

## ABSTRACT

The NLRP3 inflammasome is an oligomeric protein complex that is mainly formed within myeloid cells in response to many danger signals. The mechanisms of activation consist of two distinct events: a first priming step activates NF $\kappa$ B which induces the transcription of the inactive pro-forms of IL-1 $\beta$  and IL-18. Experimentally, the first stimulus is frequently provided by the TLR4 ligand LPS. The second, independent signal drives the nucleation of NLRP3 and is provided by either pathogens or sterile stresses like crystalline particles. Upon activation, NLRP3 binds and activates the adaptor protein ASC, leading to the formation of the characteristic ASC “speck” structure. Finally, caspase-1 binds to the speck and becomes enzymatically active by autocatalytic cleavage. Active caspase-1 cleaves the pro-forms of IL-1 $\beta$  and IL-18 which are secreted from the cell through an unconventional mechanism that is still not fully understood. The inflammasome also activates a special form of cells death called “pyroptosis”, that has mixed characteristics of both apoptosis and necroptosis, and is mainly driven by cleavage of gasdermin-D by caspase-1. Therefore, the strong physiological activities of IL-1 family cytokines both on the local and systemic level, is tightly regulated by the two-step process of inflammasome activation. Consequently, patients with activating mutations in NLRP3 frequently suffer from autoinflammatory diseases. The inflammasome is activated by a multitude of structurally different exogenous or endogenous signals that are not likely to bind NLRP3 directly. The exact mechanism how NLRP3 is activated is unknown. However, there are three major upstream events that can drive the assembly: potassium efflux, (mitochondrial) ROS production and lysosomal leakage. This thesis work is divided in two parts, in the first one, we found that certain tyrosine kinase inhibitors can activate NLRP3 through a non-conventional mechanism that involves potassium efflux and membrane instability without causing massive ROS production. The second part is aimed to investigate the role of the central inflammasome adaptor protein ASC as an oncosuppressor in hematologic malignancies, suggested by its epigenetic silencing in several types of tumors. To test a role of ASC in leukemia, we crossed different oncogene mouse models with ASC-deficient animals. We did not observe differences in cancer progression or entity, indicating that a lack of ASC does not further promote cancer development for the tested cancers.

## ZUSAMMENFASSUNG

Das NLRP3-Inflammasom ist ein oligomärer Proteinkomplex, der sich, als Antwort auf verschiedenste Gefahrensignale, in myeloiden Zellen hauptsächlich ausbildet. Der Mechanismus, der dieser Aktivierung zugrunde liegt, ist durch zwei unabhängige Vorgänge bestimmt: im ersten Schritt, dem sogenannten „Priming“, wird durch den NF $\kappa$ B-Signalweg die Transkription der inaktiven Proformen von IL-1 $\beta$  und IL-18 induziert. Im zweiten Schritt leitet ein weiteres Signal die Polymerisation von NLRP3-Proteinen ein. Dieser Stimulus kann sowohl von Pathogenen als auch sterilen Substanzen, wie Kristallpartikeln, stammen. Die Aktivierung von NLRP3 bewirkt die Interaktion zwischen diesem und dem Adapterprotein ASC. Dabei entsteht die für das Inflammasom bezeichnende Struktur des „ASC-specks“. Schließlich bindet Caspase-1 an ASC und wird dann durch autoproteolytischen Verdau enzymatisch aktiv. Aktive Caspase-1 spaltet IL-1 $\beta$  und IL-18, die durch einen bisher nicht vollständig aufgeklärten Mechanismus sezerniert werden. So ist die starke, physiologische Rolle von Zytokinen der IL-1-Familie lokal und systemisch durch diese zwei Vorgänge der Inflammasom-Aktivierung stark reguliert. Das Inflammasom verursacht auch Pyroptose, eine bestimmte Art von programmiertem Zelltod, der sowohl Eigenschaften von Apoptose als auch Nekroptose aufweist und hauptsächlich durch die Caspase-1 induzierte Prozessierung von Gasdermin-D hervorgerufen wird. Folglich leiden Patienten mit aktivierenden Mutationen von NLRP3 häufig an Autoimmunerkrankungen. Das Inflammasom ist durch eine Vielzahl von strukturell unterschiedlichen exo- und endogenen Stimuli aktivierbar. Der genaue Mechanismus der NLRP3 Aktivierung ist weiterhin unklar. Jedoch sind drei Vorgänge, die die Inflammasom-Assemblierung einleiten können, bekannt: Kaliumausstrom, (mitochondriale) ROS-Produktion und lysosomale Perforation. Diese Arbeit behandelt zwei unterschiedliche Fragestellungen. Einerseits konnten wir zeigen, dass bestimmte Tyrosinkinase-Inhibitoren NLRP3 durch einen unkonventionellen Mechanismus aktivieren können, der Kaliumausstrom und Membranstabilität, jedoch kaum ROS-Produktion aufweist. Andererseits untersuchten wir die Rolle von ASC als Tumorsuppressor in Leukämie, das ASC in einigen Tumoren dem Gen-Silencing unterliegt. Diese Arbeit lässt darauf schließen, dass Fehlen von ASC in den ausprobierten Leukämiemodellen die Tumorentwicklung nicht weiter fördert.



## RIASSUNTO

L'inflammasoma NLRP3 è un complesso proteico oligomero che si forma principalmente nelle cellule mieloidi in risposta a diversi stimoli. Il meccanismo di attivazione consiste in due eventi distinti: il primo è detto "priming", e serve ad attivare NFκB che regola la trascrizione delle forme inattive di IL-1β e IL-18. Sperimentalmente, il primo stimolo è fornito da LPS come ligando di TLR4. Il secondo stimolo indipendente, permette la nucleazione di NLRP3 e può essere dato sia da patogeni che da stress sterili come particelle cristalline. Successivamente, l'inflammasoma si assembla grazie alla proteina adattatore ASC nella caratteristica forma detta "speck". Infine, la caspasi-1 si lega ad ASC e diventa cataliticamente attiva attraverso auto-proteolisi. La caspasi-1 attivata taglia le forme immature di IL-1β e IL-18 che vengono poi secrete dalla cellula mediante un meccanismo non convenzionale e non completamente compreso. L'inflammasoma attiva anche uno speciale tipo di morte cellulare detta "piroptosi", che ha caratteristiche miste tra apoptosi e necrosi ed è principalmente indotta dalla gasdermina-D, tagliata anch'essa da caspasi-1. Le citochine secrete hanno importanti effetti fisiologici sia a livello locale che a livello sistemico ed è per questo che sono strettamente regolate dal meccanismo a due passaggi di NLRP3. Di fatto, pazienti con mutazioni che attivano sregolatamente NLRP3, soffrono frequentemente di malattie autoinfiammatorie. L'esatto meccanismo di attivazione di NLRP3 è ancora dibattuto, però sono noti tre diversi eventi che portano al suo assemblaggio: l'efflusso di potassio, la produzione di ROS (mitocondriali) e la rottura dei lisosomi. Questo lavoro di tesi è suddiviso in due parti, nella prima abbiamo osservato come alcuni inibitori di tirosina chinasi possono attivare NLRP3 attraverso un meccanismo non convenzionale, che coinvolge l'efflusso di potassio e la destabilizzazione della membrana senza causare massiccia produzione di ROS. La seconda parte mira ad investigare il ruolo di ASC come oncosoppressore in tumori ematologici, funzione suggerita dal fatto che il gene è frequentemente silenziato epigeneticamente in diversi tipi di cancro. Per testare il ruolo di ASC nella leucemia, abbiamo incrociato diversi modelli oncogeni con ASC knockout ma non abbiamo individuato differenze nella progressione o nell'entità dei tumori. Questo indica che la mancanza di ASC non accelera la progressione tumorale perlomeno nelle leucemie che abbiamo sperimentato.

## LIST OF ABBREVIATIONS

Absent in melanoma 2 (AIM2)	Mitochondrial antiviral-signaling protein (MAVS)
Acute lymphoblastic leukemia (ALL)	mixed lineage kinase domain like (MLKL)
Acute myeloid leukemia (AML)	NACHT, LRR and PYD domains-containing protein 3 (NLRP3)
AIM2-like receptor (ALR)	NAD(P)H dehydrogenase quinone 2 (NQO2)
Apoptosis-associated speck-like protein containing a CARD (ASC)	Nod-like receptor (NLR)
Breakpoint cluster region-Abelson fusion kinase (BCR-Abl)	Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)
Caspase recruitment domains (CARD)	Nucleotide-binding oligomerization domain-containing protein (NOD)
Chronic lymphoid leukemia (CLL)	Pathogen-associated molecular patterns (PAMPs)
Chronic myeloid leukemia (CML)	Pattern recognition receptors (PRRs)
Cryopyrin-associated periodic syndrome (CAPS)	Peptidoglycan (PGN)
C-type lectin (CLR)	Platelet-derived growth factor receptors (PDGFR)
Cytokine Release Inhibitory Drug (CRID3)	Protein tyrosine kinase (PTK)
Damage-associated molecular patterns (DAMPs)	Pyrin domain (PYD)
Enzyme-linked immunosorbent assay (ELISA)	Reactive oxygen species (ROS)
Epidermal growth factor receptor (EGFR)	Receptor-interacting serine/threonine-protein kinase 1 (RIPK1)
Fluorescence-activated cell sorting (FACS)	Receptor-interacting serine/threonine-protein kinase 3 (RIPK3)
Gasdermin-D (GSDMD)	Retinoic acid-inducible gene I (RIG-I)
Gastrointestinal stromal tumor (GIST)	RIG-I-like receptor (RLR)
Green fluorescent protein (GFP)	Toll-like Receptor (TLR)
Interleukin 18 (IL-18)	Tyrosine kinase inhibitor (TKIs)
Interleukin-1β (IL-1β)	Tyrosine-protein kinase Kit (KIT)
Leucine-rich repeat (LRR)	
Lipopolysaccharide (LPS)	

# INTRODUCTION

## The Inflammasomes in the context of Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are primarily expressed by cells of the innate immune system, (including granulocytes, macrophages, and dendritic cells) but also by several types of non-immune cells like epithelial or mesothelial cells like those of the peritoneum. The function of these proteins is to recognize so-called pathogens-associated molecular patterns (PAMPs) derived from microbes, or damage-associated molecular patterns (DAMPs), derived mainly by cell components released during lytic cell death, for example upon trauma (Brubaker, Bonham, Zanoni, & Kagan, 2015). The existence of such pattern-recognition proteins was first predicted in 1989 by Charles A. Janeway, a pioneer in the field of immunology, when he suggested the principles of innate control on adaptive immunity in two postulates: 1) the cells with innate function can recognize microbes as non-self and discriminate them from self-antigens, 2) the cells of innate immunity can lead to the activation of cell with adaptive function (Janeway, 1989).

After more than 20 years of research on this field, numerous distinct families of PRRs have been identified and their number is still growing. An important challenge for the development of future therapeutics is to more precisely understand the functions and the regulation mechanisms that control PRRs signaling and generate an immune response. In general, PRR can be either located on the cell membrane and in the intracellular space but there are also PRRs that are secreted by the immune cells. PRRs located on myeloid innate immune cells can be classified in two main categories on the basis of their cellular localization: membrane bound (either cytoplasmic or in endosomal membranes) or cytosolic receptors (Brubaker et al., 2015).

Two major families of membrane-bound PRRs are the Toll-like receptors (TLRs) and the C-type lectin like receptors (CLRs). The TLRs are the most studied and well understood class of PRRs. Members of the TLR family are found associated to the plasma membrane and/or to endosomal membranes. The name goes back to Toll, a gene and protein first identified in the late 90s in the model organism *Drosophila melanogaster* by the group of Christiane Nüsslein-Volhardt. Some years later, the

group of Jules Hoffmann discovered that Toll has an immune function in the adult fly in addition to its function in development. Several scientists including Bruce Beutler, Shizuo Akira, and Ruslan Medzhitov (the latter together with Charles Janeway himself) discovered structurally similar receptors in mammals that became known as Toll-like receptors. This work, had a fundamental impact on the field of immunology and therefore, Hoffmann and Beutler were awarded with the Nobel prize in physiology and medicine in 2011 (O'Neill, Golenbock, & Bowie, 2013).

So far, 10 functional TLRs were found in humans and 12 in mice but the endeavor to characterize their functions is still ongoing. Since TLRs can recognize a plethora of different pathogen-derived and damage signals, mutations in their genes or of proteins involved in their signal transduction pathways are associated with either severe immunodeficiency or autoimmune disorders (Maglione, Simchoni, & Cunningham-Rundles, 2015). For inflammasome research, TLR4 is the most prominent of these receptors since it is responsible of sensing extracellular lipopolysaccharide (LPS). LPS stimulation represents the first of the two activations steps required for NLRP3 inflammasome assembly. After sensing LPS, TLR4 engages intracellular downstream signaling adaptors including MyD88 and TRIF to transduce the signal, leading to the activation of the transcription factors including NF $\kappa$ B and Jun/FOS respectively. In particular, these transcription factors control numerous pro-inflammatory cytokine as well as several of the proteins that are components of the different types of inflammasomes (Takeuchi & Akira, 2010).

The CLRs are a large and structurally diverse protein family having up to 17 different phylogenic groups. Multiple CLRs contribute to the recognition of eukaryotic pathogens including fungi and helminths. Like Dectin-1, they can recognize exogenous carbohydrates in a calcium dependent or independent manner. Dectin-1 (encoded by the CLEC7A gene), plays a fundamental role in the recognition of the opportunistic fungal pathogens of the genera *Aspergillus*, *Candida*, *Pneumocystis*, *Coccidioides*, and *Penicillium* (Hoving, Wilson, & Brown, 2014; Huysamen & Brown, 2009).. Other CLRs recognize endogenous danger signals, for example released during cell death. They can engage both activating and regulating signaling pathways, thereby helping to fine-tune immune responses.

Three major classes cytosolic PRRs are the RIG-I-like receptors (RLRs), the NOD-like receptors (NLRs) and the AIM2-like receptors (ALRs). The RLRs are a small category

of PRRs comprising only three proteins in total: RIG-I, MDA5, and LGP2, which all share a similar structure and the ability to bind viral RNAs. Through the activation of NF $\kappa$ B and other transcription factors, RLR ligand binding leads the production of potent anti-viral cytokines, especially of type I interferons (type-I IFNs). The role of these receptors has been highlighted in the recognition of several different viral pathogens and especially of influenza orthomyxoviridae (Gack, 2014).

The NOD-like receptors are particularly important in the context of this thesis work, as they include the sensor proteins that take part in the formation of inflammasome complexes. The NLRs are a big family of PRRs and contain more than 20 members for humans and even more in mice. Although they share a similar structure, they can be classified in 4 different subfamilies: NLRA (member: CIITA), NLRB (member: NAIP), NLRC (members: NOD1, NOD2, NLRC3, NLRC4, NLRC5, NLRX1) and finally the NLRP (members: NLRP1 – 14). The NLRs are listed and depicted with their domain combinations in Table 1 (Motta, Soares, Sun, & Philpott, 2015; Schroder & Tschopp, 2010).

Seven different types of domains can be found in NLRs, but only 2 of them are present on all the receptors. One is the leucine-rich-repeat (LRR) domain, usually located at the C-terminus and, in analogy to TLRs, thought to be responsible for the recognition of danger signals (as an exception, it is absent only from NLRP10). The other is the so-called NACHT domain whose name refers to some of the proteins that contain it, namely they are NAIP, CIITA, HET-E, and TEP1 (NACHT, also sometimes referred to as nucleotide binding domain, NBD). It is usually found at the center of the structure, and has ATP/GTPase activity and contains a Mg<sup>2+</sup> ion binding pocket. The N-terminal domains can vary in their combination and are therefore used for categorizing the NLRs: caspase recruitment domain (CARD), baculovirus IAP repeat (BIR), pyrin domain (PYD), and acidic transactivation domain (TA). Currently, only NLRs bearing a CARD or PYD domain have been observed to form inflammasomes. Both the PYD and CARD domains can engage in homotypic interactions that are necessary for binding to the same type of the domain in either the adaptor protein ASC or in caspase-1 to complete inflammasome formation (G. Chen, Shaw, Kim, & Nunez, 2009). NLRs can perform many different roles in immunity and can be divided in four functional categories: signal transduction, autophagy, transcriptional activation, and inflammasome assembly (Motta et al., 2015).

- 1) NOD1 and NOD2 were the first two NLRs to be discovered. They have a crucial role in the recognition of intracellular peptidoglycan moieties (PGN). NOD1 binds meso-diaminopimelic acid (meso-DAP) mostly found on cell walls of Gram-negative bacteria. NOD2 binds muramyl dipeptide (MDP) and therefore, it was shown to protect the host against *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* bacteria (Girardin et al., 2003; Inohara et al., 2003). Consequently, both these NLRs were found pivotal in maintaining intestinal homeostasis and protecting the gastrointestinal tract from infections. In fact, mutations or polymorphisms affecting NOD-encoding genes are correlated with an altered immune response toward commensal microbiota and to Crohn's disease, as well as other gut related disturbs (Kobayashi et al., 2005).
- 2) Autophagy is a normal cellular function. It works through the formation of the so-called autophagosome, which helps to clean up superfluous or damaged cell components to maintain homeostasis. For its discovery, Yoshinori Ohsumi was awarded with the 2016 Nobel prize in physiology or medicine. Several classes of autophagy have been described: autophagy of mitochondria (known as mitophagy), of the endoplasmic reticulum, of peroxisomes, and even of the proteasome (Deretic, 2012). In addition, autophagy can contribute to immune defenses by elimination of invading pathogens. NOD1 and NOD2 were showed to induce the formation of an autophagosome during intracellular bacterial infections. They can recruit autophagy-related protein 16-1 (ATG16L1) to the plasma membrane, at the site of bacterial entrance, to initiate autophagy (Travassos et al., 2010).
- 3) NLRs can also drive expression of major histocompatibility complex (MHC) class I and II, surface protein that are essential receptors needed to elicit the adaptive immune response by presenting antigen peptides to cells of adaptive immunity. CIITA is a well-known and indispensable component of the MHC-II transcription machinery (Scholl, Mahanta, & Strominger, 1997). In addition, NLRC5 was recognized more recently as an important member of the MHC-I transcription machinery (Meissner, Li, & Kobayashi, 2012).
- 4) The fourth functional category of NLRs is represented by the inflammasomes, and they will be reviewed in the next section.

The last PRRs category to be mentioned here is constituted by the AIM2-like receptors (ALRs). Humans have four members: AIM2, IFI16, PYHIN1, and MDA5, instead

murine ALRs are much higher in number encoding for up to 13 different members. Particularly, the AIM2 and the IFI16 receptors of both human and mice can drive the assembly of functional inflammasomes in response to double strand DNA with concomitant IL-1 $\beta$  maturation and secretion (Gray et al., 2016).

SUBFAMILY	PROTEIN STRUCTURE	GENE	FUNCTION	ACTIVATOR	HUMAN DISEASE
NLRA	N-term C-term 	CIITA	MHC-II expression	IFN- $\gamma$	Bare lymphocyte syndrome
NLRB		NAIP	Control of <i>Legionella pneumophila</i> infection	Flagellin	Spinal muscular atrophy
NLRC		NOD1	NF $\kappa$ B	DAP	Asthma, IBD (IBS)
		NOD2	NF $\kappa$ B, autophagy, Type I-IFN production	MDP	Crohn's disease, Blau syndrome
		NLRC3	Negative regulator of T cells in response to LPS	unknown	
		NLRC4	Caspase-1 activation, cell death, phagosome maturation	Rod proteins, flagellin	
		NLRC5	Inflammatory response and MHC-I upregulation	IFN- $\gamma$ , IFN- $\beta$	
		NLRX1	ROS production and antiviral response	Unknown	
NLRP		NLRP1	Response to anthrax bacteria	Lethal toxin, MDP	Vitiligo, Type I diabetes
		NLRP2	Embryonic development, Caspase1 activation		
		NLRP3	Caspase1 activation	Many PAMPs and DAMPs	CAPS
		NLRP4	Embryogenesis, Type I-IFN production, autophagy	Unknown	
		NLRP5	Embryogenesis	Unknown	
		NLRP6	NF $\kappa$ B inhibition, caspase1 activation	Unknown	
		NLRP7	Embryogenesis, caspase1 activation	Lipopeptides	Hydatidiform mole
		NLRP10	Dendritic cell migration	Unknown	
		NLRP12	Inhibition of non-canonical NF $\kappa$ B, ERK and AKT pathway, caspase1 activation	Yersinia	Hereditary periodic fever
		NLRP14	Spermatogenesis		Spermatogenesis failure
Domains:  NACHT  LRR  TA  CARD  BIR  PYD  Undefined					

**Table 1: Classification, protein structure, and function of the human NOD-like receptor family.**

Members of the NLR family are subdivided into four subfamilies based on the nature of the NH<sub>2</sub>-terminal domain: caspase recruitment and activation domain (CARD), baculovirus IAP repeat (BIR), pyrin domain (PYD), and acidic transactivation domain (TA). The C-terminal is most frequently occupied by a leucine-rich repeat domain. Other abbreviations: lipopolysaccharide (LPS), muramyl dipeptide (MDP), diaminopimelic acid (DAP), inflammatory bowel disease (IBD) and cryopyrin-associated periodic syndromes (CAPS). Inspired and adapted from Motta et al. (2015), *Physiological reviews*, 95(1).



## The different types of Inflammasomes

Inflammasomes are oligomeric protein complexes that are assembled upon stimulation by a multitude of DAMPs and PAMPs, primarily in different types of myeloid innate immune cells, such as monocytes, macrophages, dendritic cells and neutrophils. All the different types of inflammasomes share the same mechanism of assembly that requires three distinct events. The first event is the activation of the NLR and its oligomerization. Next, the adaptor protein ASC is recruited to the NLR to create a supramolecular structure with rounded shape called “speck” which is easily detectable via immunofluorescent microscopy. Finally, caspase-1 binds to the ASC speck, inducing proximity-induced autocatalytic cleavage. Caspase-1 is responsible for two distinct effector functions representing the hallmarks of inflammasome activation (Broz & Dixit, 2016).

The first function of caspase-1 is to catalyze the proteolysis of interleukin 1 beta (IL-1 $\beta$ ) and interleukin 18 (IL-18), generating their mature and biologically active forms (Lamkanfi & Dixit, 2014). The cleaved cytokines are secreted from the cells in an unconventional way, the exact mechanism of which is still under debate. The mature form of IL-1 $\beta$  is effective on the local level as it activates the survival and effector functions of myeloid and lymphoid cells, especially of T<sub>H</sub>17 lymphocytes. On the systemic level, IL-1 $\beta$  provokes fever and the secretion of a variety of pro-inflammatory effectors, like the acute phase proteins released by the liver (Garlanda, Dinarello, & Mantovani, 2013). Caspase-1 protease activity also controls this unconventional release of IL-1 $\beta$ , but the connection is as unclear as the release mechanisms itself. The second main function of caspase-1 is to cleave a protein called gasdermin-D (GSDMD) which causes a specific form of cell death called pyroptosis that, in contrast with apoptosis, causes membrane instability and is pro-inflammatory due to release of many alarmins (X. Liu et al., 2016).

Understanding the mechanisms of inflammasome biology has become an important field of research due to the remarkable inflammatory potential of IL-1 $\beta$  and its striking effects on the body physiology. Indeed, the interest in therapeutic approaches to target this complex is continuously increasing with the number of connections established between inflammasome activity and pathology. These complexes were already correlated and mechanistically connected to several pathologic conditions including

infections, metabolic dysfunctions, neurodegenerative diseases, cardiovascular diseases, intestinal and other forms of cancer (Guo, Callaway, & Ting, 2015; Terlizzi, Casolaro, Pinto, & Sorrentino, 2014). Several modalities of treatment neutralizing IL-1 are currently available that are based on biologicals, but the possibility to modulate inflammasome activity with small molecule compounds is at the center of discussion to develop new drugs. To support this process, researchers need to expand the knowledge on the activator and regulatory mechanisms at the basis of this pathway, that are still largely unknown and require more comprehensive elucidation.

There are different types of inflammasomes that are defined by the sensor protein that initiates its formation upon stimulation. Each sensor can recognize specific pathogen- or danger-related signal. The first inflammasome discovered was NLRP1 (Martinon, Burns, & Tschopp, 2002).

### The NLRP1 Inflammasome

Humans carry only a single copy for NLRP1, whereas mice have three different variants, NLRP1a, NLRP1b, and NLRP1c. However, so far, only the NLRP1a and NLRP1b orthologues have been shown to support inflammasome formation (Sastalla et al., 2013).

The structure of NLRP1 is unique as compared to other inflammasome-forming sensors, and there is a substantial difference between the human and the murine receptors. The human version is organized starting from the N-terminus with the sequence PYD domain, NACHT, FIIND, LRR, and CARD at the C-terminus (Table1). The murine versions (both NLRP1a and NLRP1b), have a domain sequence that starts at the N-terminus with a CARD, a FIIND, a LRR, and a NACHT domain at the C-terminus (Lamkanfi & Dixit, 2014). The function of the FIIND domain is to date unknown but a Q593P point mutation in this domain activates the receptor constitutively and causes excessive pyroptosis in hemopoietic progenitors, resulting in a severe neutrophilia (Masters et al., 2012). Given the fact that both the human and the murine variant bear a CARD domain, they can contact caspase-1 directly without the need for the ASC adaptor (Faustin et al., 2007; Guey, Bodnar, Manie, Tardivel, & Petrilli, 2014). However, another report shows that ASC is indispensable to reach normal levels of IL-1 $\beta$  maturation (Van Opdenbosch et al., 2014).

NLRP1 is involved in the inflammatory response against *Bacillus anthracis* spores as NLRP1 deficient mice are not fully protected against the pathogen (Terra et al., 2010). Later, researchers found that this inflammasome senses the lethal toxin (LeTx) secreted by *B. anthracis* than the bacterium itself. Specifically, researchers found that LeTx has a metalloprotease subunit that cleaves NLRP1 close to its N-terminus. The cleavage, allows a conformational change that switches NLRP1 into the active state (Chavarria-Smith & Vance, 2013). Another pathogen sensed by NLRP1 is *Toxoplasma gondii*, although it does so without requiring the cleavage at the N-term to be activated (Ewald, Chavarria-Smith, & Boothroyd, 2014).

NLRP1 is also involved within other pathogenic conditions, as researchers found mutations that were correlated with different inflammatory diseases in humans. For instance, they reported vitiligo (Y. Jin et al., 2007), Addison's disease (Zurawek et al., 2010), rheumatoid arthritis, systemic sclerosis, and Crohn's disease (Sharma & Kanneganti, 2016). The exact mechanisms of activity and the whole spectrum of functions of NLRP1 are clearly not yet understood. It is therefore required to improve the knowledge of this inflammasome, since it is involved in the defense against some of the deadliest pathogens (Ewald et al., 2014).

### The NLRP3 Inflammasome

The NLRP3 inflammasome is to date the most well-studied of all. Its discovery was prompted by the observation that mutations in its encoding-gene (CIAS1) correlate with a group of rare, auto-inflammatory syndromes, as outlined below in detail (Hoffman, Mueller, Broide, Wanderer, & Kolodner, 2001). Soon, scientists realized that this NLR can assemble an inflammasome upon stimulation with a multitude of chemically and physically very different agents (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon, Petrilli, Mayor, Tardivel, & Tschopp, 2006). NLRP3 is therefore the inflammasome with the broadest activation spectrum, as it responds to a plethora of PAMPs and DAMPs (Vanaja, Rathinam, & Fitzgerald, 2015).

NLRP3 has a simple structure as compared to NLRP1. It has a LRR at the C-terminus, a central NACHT domain that facilitates oligomerization and a PYD at the N-terminus that forms homotypic binding with the PYD domain of the adaptor protein ASC upon activation. As outlined above, ASC then forms large specks and binds caspase-1 through homotypic binding between the CARD domains of both proteins. Caspase-1

gets active by auto-cleavage and then proteolyzes the IL-1 $\beta$  and IL-18 pro-forms to their active, secreted forms (Lechtenberg, Mace, & Riedl, 2014).

A wide variety of pathogens and their PAMPs can be sensed by NLRP3. These include bacteria (*Staphylococcus aureus*, group B *Streptococcus* and *Listeria monocytogenes*), viruses (through recognition of viral RNA) fungi (together with CTLs recognition), bacterial pore-forming toxins (like nigericin) and protozoan proteins (plasmodial haemozoin) (Guo et al., 2015). In addition to PAMPs, NLRP3 is also able to sense many DAMPs, either sterile endogenous stresses like extracellular ATP, monosodium urate crystals (MSU, found in patients suffering gout disease), or environmental particles like alum, asbestos, silica, or nanoparticles (Broz & Dixit, 2016). The way NLRP3 can be activated by so many stimuli is still a matter of debate, but researchers tend to agree that it can sense general, cellular danger signals rather than being able to bind to each of these molecules (Fitzgerald, 2010).

There are several accepted mechanisms through which NLRP3 can be activated, but different stimuli are not inducing all of them at the same time or to the same degree. To date, no master regulator was found, but more than one activation mechanisms was discovered: 1) potassium (K<sup>+</sup>) efflux (Petrilli et al., 2007), 2) loss of mitochondrial stability with ROS production (Misawa et al., 2013; Shimada et al., 2012; Zhou, Yazdi, Menu, & Tschopp, 2011) and 3) lysosomal destabilization, potentially via the release of cathepsins (Hornung et al., 2008). In addition to these canonical regulators, there is also a non-canonical way to activate NLRP3 which goes through caspase-11. This caspase is a homologue of caspase-1 that, instead of being activated through ASC, can sense intracellular LPS and is required to activate NLRP3 during intracellular infections by Gram-negative bacteria. Caspase-11 induces NLRP3 by cleaving GSDMD, causing potassium efflux through the N-terminus of GSDMD that integrates into the cytoplasmic membrane, forming pores (Kayagaki et al., 2015). These mechanisms will be described in the next chapter with more detail.

Dysregulated NLRP3 activity is at the basis of many pathologic conditions. Dominant somatic mutations in NLRP3 were found to cause different autoinflammatory diseases named cryopyrin-associated periodic syndromes (CAPS), cryopyrin being an alias for the NLRP3 gene. These include chronic infantile neurologic cutaneous and articular syndrome (CINCA), Muckle-Wells syndrome (MWS) and familial cold auto-inflammatory syndrome (FCAS) (C. A. Yang & Chiang, 2015). In total, circa 90 different

point mutations within the NLRP3 encoding gene were correlated with autoinflammatory diseases (Masters, Simon, Aksentijevich, & Kastner, 2009). Moreover, the dysregulated activation of NLRP3 caused by genetic alterations is also implicated with inflammatory bowel diseases (IBDs) (Gagliani, Palm, de Zoete, & Flavell, 2014). A correlation was even found with neurodegenerative diseases. Researchers showed that in a murine model of experimental autoimmune encephalomyelitis (EAE), those animals lacking the NLRP3 had a milder phenotype (Jha et al., 2010). NLRP3 was linked to Alzheimer's disease when researchers discovered it can be activated by the presence of  $\beta$ -amyloid plaques (Halle et al., 2008). Furthermore, the  $\alpha$ -synuclein ( $\alpha$ Syn) plaques found in the brain of Parkinson's disease patients were shown to induce the activation of NLRP3 (Yan et al., 2015). The NLRP3 inflammasome was also connected to several metabolic disorders, like gout, where MSU is the activator (Liu-Bryan, 2010), atherosclerosis where the stimulus is provided by oxidized low-density lipoprotein (LDL) (Düweil et al., 2010) and type 2 diabetes, where it responds to fatty acids like palmitate or ceramide (present at the onset of type-2 diabetes) and islet amyloid polypeptide (IAPP), a protein secreted by  $\beta$ -cells that can form prion-like structures (Masters et al., 2010).

In sum, NLRP3 forms an inflammasome with a complicated mechanism of activation that can trigger a strong response upon many different stimuli. However, the actual mechanism behind NLRP3 assembly is not yet understood, and the number of activators is continuously increasing, testifying that this complex may indeed be a universal but indirect danger sensor. More work is still required to better understand the complexity of NLRP3 molecular biology and the impact it has on different diseases. I believe the following questions will be crucial to answer: is there a unified mechanism behind NLRP3 assembly? Can this mechanism be selectively inhibited? Are NLRP3 inflammasome inhibitors promising drugs that can reach the clinic?

### The NLRC4 Inflammasome

The case of NLRP3 is unusual, as it can sense many stimuli. The NLRC4 inflammasome, as in the case of NLRP1, can sense only a limited number of molecular patterns. However, unlike NLRP1, for this receptor there is no major difference between the human and mouse version. NLRC4 has a LRR (the biggest in size among the NLRs having 12 repeats) at its C-terminus, followed by a NACHT domain, and

finally a CARD domain at the N-terminus that, as in the case of NLRP1, can contact caspase-1 directly, without the need of ASC (Hu et al., 2013). However, researcher showed how ASC enhances the NLRC4 inflammasome to secrete more IL-1 $\beta$  since ASC is involved in autocatalytic processing of caspase-1 and efficient maturation of IL-1 $\beta$  (Broz, von Moltke, Jones, Vance, & Monack, 2010; O. Gross et al., 2012).

NLRC4 can respond to a good variety of pathogens including *Salmonella Typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Burkholderia thailandensis* and *Legionella pneumophila* (Lamkanfi & Dixit, 2011). Molecularly, NLRC4 can sense bacterial flagellin in general as well as type 3 secretion systems (T3SS) which are used by Gram-negative bacteria to inject virulence factors into the host cell (J. Yang, Zhao, Shi, & Shao, 2013). However, NLRC4 cannot sense either the flagellin or the T3SS directly but first needs other NLRs to recognize the activators. Specifically, NAIP1 binds to the T3SS needle protein, NAIP2 binds to the T3SS rod protein and NAIP5/NAIP6 bind bacterial flagellin. Once this binding takes place, NLRC4 oligomerizes with the respective NAIP protein, driving ASC speck formation (Vance, 2015). Humans have only one NAIP protein, which seem to be able to bind the needle protein of T3SS only, this appears to reduce the activation repertoire of this inflammasome in human cells (J. Yang et al., 2013).

NLRC4 activating mutations were found in patients who developed a recurrent macrophage activation syndrome (Canna et al., 2014), and others were presenting a syndrome of enterocolitis and autoinflammation (Romberg et al., 2014). There are still many obscure points in the regulation mechanism of NLRC4. Nevertheless, NLRC4 and NLRP3 were found in one inflammasome, indicating that they can participate in the formation of the same macromolecular complex (Man et al., 2014). This is a first hint of cross-talk between different inflammasomes and it will be interesting to see if other types and inflammasome sensors can also cooperate and if this reduces the activation threshold of the individual receptors or has other biological effects.

Notably, other groups showed that in a model of intraperitoneal flagellin injection, which should kill the mouse within half of an hour, NLRC4 was indispensable to observe the phenotype. Together with it, the authors reported a rapid eicosanoid storm. Eicosanoid secretion is a phenomenon that was not yet put in relation with inflammasome activity (von Moltke et al., 2012). Understanding the fine mechanism of

NLR4 assembly and how this and potentially other inflammasomes provoke eicosanoid production, will be important points to address in future research.

### The AIM2 inflammasome

Absent in melanoma 2 (AIM2), despite not being a NLR, can assemble a fully functional inflammasome in response to double stranded DNA (Hornung et al., 2009). AIM2 has a structure distinct from NLRs, lacking the typical C-terminal LRR, instead bearing a HIN-200 domain that is responsible for dsDNA recognition. The N-terminus has a PYD domain that can bind the ASC adaptor and then contact caspase-1 through its CARD domain (T. Jin et al., 2012).

AIM2 can bind to the DNA of several pathogens to induce inflammasome assembly, making it a crucial component of the immune response against *Listeria monocytogenes*, *Francisella tularensis*, cytomegalovirus, and vaccinia viruses (Rathinam et al., 2010). However, AIM2 can in principle bind any double stranded DNA, including artificial poly(dA:dT) DNA. Binding of AIM2 to transfected vector DNA and subsequent activation of pyroptosis appears to contribute to the difficulties transfecting myeloid cells for transient protein expression (Fernandes-Alnemri, Yu, Datta, Wu, & Alnemri, 2009). There are also pathological implications for AIM2, as it was found involved in development of systemic lupus erythematosus (Panchanathan et al., 2011) and psoriasis (Dombrowski et al., 2011).

There is another ALR, named interferon- $\gamma$ -inducible protein 16 (IFI16), that was recently shown to form a functional inflammasome and secrete IL-1 $\beta$ . This inflammasome is induced upon IFN- $\gamma$  signaling and assembled in response to several types of viral infections like Epstein-Barr viruses (EBV), Kaposi sarcoma herpesviruses (KSHV) and even HIV-1 (Dell'Oste et al., 2015). One research group has recently found that IFI16 acts as a host DNA sensor required for CD4 T cell death upon abortive HIV infection. IFI16 senses the HIV-1 viral DNA, drives inflammasome assembly and provokes pyroptosis (Monroe et al., 2014).

### The emerging inflammasomes

The members of the NLRs family share structural similarities, making many of them candidates for inflammasome activation and other proinflammatory signaling

pathways. Several of them belong to the subfamilies of NLRs activating NF $\kappa$ B or autophagy, making it somewhat unlikely that they would also form an inflammasome, although there is some evidence that this could in principle be possible. However, the specific ability to form an inflammasome was reported for several other NLRs, although the mode of activation remains unclear.

The NLRP6 receptor is only expressed in the gastrointestinal tract and can form an inflammasome in intestinal epithelial cells. It was put in relation with inflammatory pathways affecting intestinal homeostasis. For instance, the loss of NLRP6 results in an altered microbial flora of the gut, making mice more susceptible to spontaneous intestinal hyperplasia and dextran sulfate sodium (DSS) induced colitis (Elinav et al., 2011; Nowarski et al., 2015). In contrast, another study indicated NLRP6 as an inhibitor of the clearance mechanisms against *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* (Anand et al., 2012).

The NLRP7 inflammasome was characterized in human macrophages and found involved in response to microbial acylated lipopeptides (Khare et al., 2012).

NLRP12 is present in innate immune cells and seems particularly expressed in neutrophils (Ulland et al., 2016). NLRP12 role is still under debate. Initially was found induced in a mouse model of *Yersinia pestis* infection. Then, they found NLRP12 colocalized with NLRP3 inflammasome complexes in monocytes of patients suffering from malaria infections (Ataide et al., 2014; Vladimer et al., 2012). Contrastingly, in a model of DSS induced colitis, mice were found more susceptible to the treatment due to an impaired NF $\kappa$ B signaling (Allen et al., 2012).

In addition to NLRs, there are also other two emerging inflammasomes assembled by non-NLRs sensor proteins, those are pyrin and RIG-I. Pyrin is a protein expressed predominantly in neutrophils, monocytes, and eosinophils. It has a N-terminal PYD domain, two central B-box zinc-finger domains that can bind DNA and a C-terminal B30.2-SPRY domain, whose ligand is not known (Yu et al., 2006). Pyrin was first discovered as the protein mutated in familial Mediterranean fever (FMF) and similar autoinflammatory diseases. Soon after, researchers understood that pyrin can assemble a functional inflammasome, as those patients could be healed with anti-IL-1 $\beta$  therapy (Chae et al., 2011). The pyrin inflammasome can detect modifications of Rho GTPase signaling. So far, the following bacteria were shown to cause Rho GTPases modifications and consequent pyrin inflammasome assembly: *Clostridium*



*difficile*, *Vibrio parahemolyticus*, *Histophilus somni*, *Clostridium botulinum*, and *Burkholderia cenocepacia* (Xu et al., 2014). These pathogens secrete Rho-modifying toxins that can cause glycosylation, adenylation, and ADP ribosylation to many different Rho-GTPases family members (Xu et al., 2014). Nonetheless, a group worked with pyrin knockout animals in an animal model of EAE fostered with pertussis toxin (PTX). Researchers observed that pyrin assembles an inflammasome in response to PTX and leads to IL-1 $\beta$  secretion, accelerating the disease onset (Dumas et al., 2014).

Retinoic acid-inducible gene I protein (RIG-I, encoded by DDX58) is an RNA helicase ubiquitously expressed. RIG-I binds 5'-triphosphate uncapped RNA from many different viruses and is the most representative protein of its PRR family. The primary signaling pathway of RIG-I passes through mitochondrial antiviral-signaling protein (MAVS) and triggers type-I IFN expression. RIG-I can also contact BCL10 and CARD9 to activate NF $\kappa$ B (I. Y. Chen & Ichinohe, 2015). However, in myeloid cells, RIG-I can also assemble a functional inflammasome through its CARD domain which interacts with ASC to trigger caspase-1 activity and IL-1 $\beta$  maturation and secretion.

### The inflammasome independent cleavage of interleukin-1

The discovery of the inflammasome has been ground-breaking and initiated a real revolution over the last 10 years of research in the field of innate immunity and inflammation. However, interleukin-1 was already found in the mid-80s as the cytokine that induces fever and related physiological effects. Its cloning and initial characterization studies in murine models and human patients followed quickly (Dinarello, 1994). Since then, many other studies on IL-1 were conducted and the family was enlarged to a total of 11 different cytokine members (Garlanda et al., 2013). Despite the biggest attention aims on the mechanisms that drive inflammasomes assembly, studies on IL-1 itself are still moving the interest of the scientific community especially for the applications in translational medicine. In fact, there are already several treatments for autoinflammatory diseases that target inflammasome-dependent cytokines. Moreover, there is evidence showing how IL-1 $\beta$  and IL-18 can also be cleaved by inflammasome-independent mechanisms (Netea, van de Veerdonk, van der Meer, Dinarello, & Joosten, 2015). For instance, neutrophils and macrophages can cleave IL-1 $\beta$  and IL-18 with serine proteases like proteinase 3

(PR3), elastase, cathepsins, chymase, and chymotrypsin (Netea et al., 2015). Also, metalloproteinases meprin  $\alpha$  or meprin  $\beta$  were found to cleave those cytokines (Herzog et al., 2009). Two of the most significant *in vivo* studies that confirmed the role of those proteases, were carried out in different pathogenic conditions like intestinal inflammation (Banerjee & Bond, 2008) or *C. albicans* infections (Kullberg, van 't Wout, & van Furth, 1990)

Some *in vitro* studies showed that peripheral blood mononucleated cells (PBMCs) have a constitutively active caspase-1, meaning that the release of IL-1 $\beta$  is different between primary human monocytes and macrophages or dendritic cells (DCs). Indeed, macrophages and DCs require the classic two-hit inflammasome activation to trigger IL-1 $\beta$  secretion. Monocytes instead, can process IL-1 $\beta$  already upon TLR stimulation. This diversity may be because monocytes are floating in the sterile environment of peripheral blood and macrophages and DCs reside in a tissue environment. The two-step activation of macrophages and DCs was selected to avoid dysregulated inflammasome activation (Gaidt et al., 2016; Netea et al., 2009). As a counterproof for this finding, the same group reported, in another work, that inhibiting both caspase-1 and PR3 had the best therapeutic potential in a murine model of inflammatory arthritis (Joosten et al., 2009).

Many studies were conducted through animal models and patient data have demonstrated that IL-1 induced pathologies are largely inflammasome-dependent. Therefore, it is currently unclear if inhibiting alternative mechanisms of IL-1 $\beta$  maturation and release can be fruitful for the development of therapeutic strategies. Nonetheless, more effort is still required to comprehend these mechanisms and their eventual cross-talk with the inflammasomes.

## **A mechanistic overview of NLRP3 inflammasome assembly**

The NLRP3 inflammasome can respond to a multitude of physically and chemically diverse PAMPs and DAMPs and the mechanisms behind its activation are correspondingly complex. In macrophages and DCs, which are most commonly used to study inflammasome biology *in vitro*, the full activation is achieved by a two-step process. The first step is priming, where an initial stimulus activates the transcription and translation of inflammatory cytokines, one of which is the pro-form of IL-1 $\beta$ . The second step, the actual inflammasome activation, is triggered by a stimulus that induces NLRP3 to initiate the assembly of the inflammasome through the adaptor protein ASC, leading to the activation of the effector caspase-1. To control its activity, the NLRP3 inflammasome has regulation checkpoints at both the transcriptional and post-translational level. These complex mechanisms tightly control the secretion of IL-1 $\beta$  and thereby, its strong physiologic effects that include fever and tissue destruction. The effects of dysregulated NLRP3 activation are highlighted by gain-of-function mutations affecting patients with CAPS and metabolic diseases including gout, atherosclerosis or type 2 diabetes (Guo et al., 2015). In this chapter, I review the current knowledge of the molecular mechanisms of inflammasome activation and its regulation. Of note, the scientific community has not agreed or found yet a unique and common trigger event that can indisputably explain how NLRP3 is assembled that would be shared by all known NLRP3 activators (Rathinam & Fitzgerald, 2016).

### **Canonical NLRP3 activation**

Priming, also known as signal one, is a very general stimulus and any ligand that can trigger activation of NF $\kappa$ B is in theory suitable for inflammasome priming. Ligands for TLRs, NLRs (NOD1 and NOD2), the IL-1 receptor itself (as a paracrine inflammasome signal) as well as TNF- $\alpha$  receptors TNFR1 and TNFR2 (Franchi, Eigenbrod, & Nunez, 2009) can provide signal one. NF $\kappa$ B is a transcription factor that directs the expression of pro-IL-1 $\beta$  together as well as other pro-inflammatory cytokines. Moreover, even though NLRP3 is already present at basal level in the cytoplasm, NF $\kappa$ B can boost its expression levels in different cell types, apparently increasing the susceptibility of the cell for inflammasome activation. The remaining components of the inflammasome

ASC, caspase-1 are already present in the cytoplasm and are largely unaffected by priming in terms of their expression level (Sutterwala, Haasken, & Cassel, 2014).

Studies conducted with LPS as priming agent show that inflammasome activation is abrogated when cells are missing the MyD88 adaptor protein because of suppression of NF $\kappa$ B activation (Embry, Franchi, Nunez, & Mitchell, 2011). However, it seems that TRIF and IRAK are also involved in a transcription-independent priming mechanism which is still poorly understood and involves deubiquitination of NLRP3 (Lin et al., 2014). It is also very recently becoming appreciated that phosphorylation and dephosphorylation events at distinct sites are an effect of priming bringing NLRP3 into a state where it remains inactive but is ready for activation by signal two. Despite not being the actual inflammasome formation event, the priming is a key event and it is still unclear how many and what pathways may be involved in it. Other pathways were implied already before, like ERK signaling (activated in parallel with NF $\kappa$ B), autophagy, and even tubulin/actin rearrangements (Jo, Kim, Shin, & Sasakawa, 2016). As discussed before, it is important to mention that PBMCs can bypass the two-step process and activate the inflammasome directly by detecting LPS, suggesting that in these cells, caspase-1 has a different regulation mechanism as compared to macrophages and dendritic cells (Gaidt et al., 2016; Netea et al., 2009).

The second signal is provided by a PAMP or DAMP and triggers inflammasome assembly. NLRP3 can sense a plethora of pathogens (including viruses, bacteria, fungi or protozoans), and danger signals (e.g. ATP, monosodium urate crystals (MSU), silica, asbestos, or alum) (Man & Kanneganti, 2015). NLRP3 recognize such a heterogeneous spectrum of stimuli but it is extremely unlikely that it does it by direct contact with them. Researchers rather believe that the different activators cause similar cellular stress conditions and an imbalance in homeostasis that is then sensed by NLRP3 by unknown mechanisms (Vanaja et al., 2015). The evidence for this is that inhibition or prevention of these conditions can dampen NLRP3 activation in response to most if not all known NLRP3 activators. Based on a multitude of inhibitor studies, there are now three categories of upstream event: 1) potassium efflux, 2) deregulated mitochondrial activity, and 3) lysosomal instability.

- 1) Decrease in intracellular potassium ( $K^+$ ) is recognized as the most common activation mechanism as inhibition of  $K^+$  efflux by increasing the extracellular concentration of KCl results in substantial inflammasome dampening. There are

studies suggesting it may be the unique or primary trigger for all NLRP3 stimuli (Munoz-Planillo et al., 2013). Some of the most commonly used NLRP3 activators are directly causing K<sup>+</sup> efflux. Pore-forming toxins like nigericin or tetanolysin-O (TLO) are good potassium ionophores causing efflux and consequent inflammasome activation. Also, ATP induces NLRP3 activation by binding to the purinergic receptor P2X7, thus provoking its opening, which causes K<sup>+</sup> efflux. As a consequence, intracellular potassium levels drop and rapid inflammasome formation takes place (Pelegrin & Surprenant, 2006). Together with K<sup>+</sup> efflux, a role for Ca<sup>2+</sup> influx has been reported to be involved in NLRP3 activation. Extracellular calcium is sensed by G protein calcium sensing receptors (GPCR6A or CSRA) that in turn activate phospholipase C (PLC) that then triggers release of calcium from the endoplasmic reticulum, which was suggested to contribute to the activation of NLRP3 (G. S. Lee et al., 2012). However, the role of calcium in inflammasome activation is still controversial as other groups reported that NLRP3 does not require Ca<sup>2+</sup> signaling for its assembly (Katsnelson, Rucker, Russo, & Dubyak, 2015).

- 2) Mitochondrial dysfunction, accompanied, for example, with reduction of the normal negative mitochondrial inner membrane potential ( $\Delta\Psi_m$ ) and with ROS production is a well-established upstream event of inflammasome activation (Heid et al., 2013). Moreover, ROS production seems to contribute to optimal LPS priming (Ives et al., 2015). How NLRP3 senses elevated ROS levels and if it does so directly or indirectly is unclear. However, one group showed that mitochondrial dysfunction leads to accumulate oxidized mitochondrial DNA in the cytosol which was suggested to activate NLRP3 (Shimada et al., 2012). Another study reported that the dispersion of cardiolipin during  $\Delta\Psi_m$  may be the direct cause of inflammasome activation (Gonzalvez et al., 2008). More recently, a group showed how the enzyme hexokinase, normally involved in glycolysis, can act bind to Gram-positive bacteria. Hexokinase is then released from the mitochondrial surface and drives assembly of NLRP3 (Wolf et al., 2016).

Another aspect is the spatial rearrangement of mitochondria. It was proposed that NLRP3 is associated with the membrane of the endoplasmic reticulum during resting state, and that ASC is instead associated with the mitochondrial membrane. Mitochondrial damage diminishes the concentration of oxidized NAD<sup>+</sup>, thus

inactivating sirtuin-2 (SIRT2), which leads to the accumulation of acetylated  $\alpha$ -tubulin. This event increases microtubules motility to create a contact between the endoplasmic reticulum and mitochondria (Misawa et al., 2013).

- 3) Lysosomal rupture can activate NLRP3. In this context, it is caused either by phagocytosed pathogens able to escape the endo-lysosome or because of particles or crystals causing endo-lysosomal damage upon phagocytosis (Hornung et al., 2008). Proton pump inhibitors (to neutralize lysosome acidic pH) and blockade of cathepsins could remarkably prevent NLRP3 inflammasome activation (Hornung et al., 2008; Qi et al., 2016). In line with this, the lysosome-destabilizing agents Leu-Leu-OMe (LLO) is a potent, NLRP3-dependent inflammasome inducer, although at concentrations far higher than what is required for lysosomal rupture (Lima et al., 2013). The role of lysosomal rupture is still controversial, as it seems that this event may be a trigger for either ion flux or mitochondrial ROS production, thereby potentially acting through the other mechanisms mentioned (Katsnelson, Lozada-Soto, Russo, Miller, & Dubyak, 2016).

In the most recent literature, the kinase NEK7 was proposed as “new” regulator involved in NLRP3 activation. NEK7 is a serine/threonine kinase that plays an essential role at the onset of mitosis, where it regulates formation of the mitotic spindle and cytokinesis (O'Regan & Fry, 2009). NEK7 was recently found also to bind the LRR domain of NLRP3 independently of its kinase activity. This work showed that mitosis and NLRP3 are two mutually exclusive events that determine the fate of the cell (H. Shi et al., 2016). Further work is needed to understand the significance of this interplay between inflammasome activation and the cell cycle. The activation mechanisms of NLRP3 are complicated and elusive, some were identified but there are still many points to be clarified.

### Inflammasome signal propagation

The inflammasome is an oligomeric supramolecular complex and its complete formation requires the assembly of many monomers of its components (NLRP3, ASC and caspase-1). I will here describe the events that happen after both the stimuli necessary for NLRP3 activation reached the cells. Of note, the current knowledge was achieved by studying all the known inflammasome complexes and therefore what follows applies to all conventional inflammasomes of them.

The first event in inflammasome assembly is the nucleation of the receptor. Structural insights into this process were gained using NLRC4 and AIM2, where crystal structure data could be obtained. In one study, the authors observed that binding of the NAIP2 adaptor drives the assembly of NLRC4 oligomers that nucleate themselves in a disc-like shape (L. Zhang et al., 2015). In two separate studies, researchers observed how AIM2 tends to form filaments that bind to dsDNA directly (T. Jin et al., 2012; Morrone et al., 2015).

The second event that takes place is the assembly of the ASC speck. Normally, the cells with an active inflammasome display one speck only with a diameter of circa 0,8 to 2  $\mu\text{M}$ . Speck formation is a hallmark of inflammasome activation (Fernandes-Alnemri et al., 2007). A recent study reported formation of the ASC speck in detail. They found a two-step assemblage process where both PYD and CARD contribute to the formation of filaments that are organized by the PYD and that expand to form a speck with a rod-like shape. Binding to the ASC CARD by the CARD of caspase-1 leads to proximity-induced activation of the protease by autocatalytic cleavage. These events were explored for both NLRP3 and AIM2 complexes and, in conclusion, the authors suggest that this mechanism can be shared among all the inflammasomes (A. Lu et al., 2014). Finally, another report shows how ASC specks are released into the extracellular space where they can be phagocytosed by neighboring cells, thus driving once again ASC nucleation and expansion to induce inflammasome activation and pyroptosis in the next cell. These results indicate that ASC can act like a prion protein, although it is unclear if this signal propagation is occurring *in vivo*. (Baroja-Mazo et al., 2014).

Returning to the ASC speck, once it is formed and caspase-1 joins the structure, it is still a matter of debate what other proteins can join the structure. However, some reports indicate that caspase-8 may play a role. Caspase-8 is primarily known for its role in apoptotic pathways, although recently, it was demonstrated to be involved in priming and activation of the canonical NLRP3 inflammasome (Gurung et al., 2014). Some other reports put caspase-8 in relation to NLRP3 assembly, recently reviewed by Kanneganti and collaborators (Sharma & Kanneganti, 2016). One paper suggested a mechanism by which the protease localizes around the ASC speck (Sagulenko et al., 2013). Recently, another group found that caspase-8 can also associate with ASC PYD domain through its tandem death effector domain (tDED). These works constitute

milestones, showing how caspase-8 has an interplay between apoptosis and pyroptosis.

Inflammasome activation is accompanied by pyroptosis, a unique form of cell death that shares aspects of both apoptosis and necroptosis. Nuclear condensation and DNA fragmentation are apoptotic hallmarks. Cytoplasmic swelling and plasma membrane rupture with alarmin release are features of pyroptosis shared with necroptosis (Fink & Cookson, 2006). Another protein named gasdermin-D (GSDMD) was recently found fundamental for cell death driven by the inflammasome. It was reported as a substrate of caspase-1 and caspase-11, inducing pyroptosis in both canonical and non-canonical inflammasomes. Both caspases cleave GSDMD, releasing its N-terminal fragment which is responsible for inducing pyroptotic cell death by forming pores in the cytoplasmic membrane (Aglietti et al., 2016; Kayagaki et al., 2015). Until the discovery of GSDMD, the link between caspase-1 and pyroptosis was missing.

### NLRP3 inflammasome regulation mechanisms

There are many reports exploring the mechanisms by which cells can dampen inflammasome assembly. These are non-overlapping results and sometimes come to different conclusions. It is therefore difficult to gain a comprehensive overview but I will focus here on those mechanisms that were most reproducible and observed by several groups independently. A first regulation step takes place at the translational level, where micro-RNA miR-223 binds to a conserved site in the 3' UTR of the NLRP3 transcript and suppresses protein expression, resulting in impaired inflammasome activation. Other micro-RNAs were found to modulate NLRP3 and IL-1 $\beta$  transcription, but these still require more in-depth characterization (Pedraza-Alva, Perez-Martinez, Valdez-Hernandez, Meza-Sosa, & Ando-Kuri, 2015). Most mechanisms reported on inflammasome regulation act at the post-transcriptional level, and can be divided into two categories:

- 1) Regulation by phosphorylation: as we saw, the first step needed to assemble the inflammasome, is NLRP3 oligomerization. NLRP3 was found phosphorylated by protein kinase R (PKR) but two different groups drew different conclusions on this result. In one study PKR was required for inflammasome activation (B. Lu et al., 2012). However, another work reports opposite results (Hett et al., 2013).



Phosphorylation also regulates ASC speck formation. ASC phosphorylation is required for speck assembly. The kinases Syk and Jnk can phosphorylate ASC on specific tyrosine residues (Hara et al., 2013). However, there is also a report showing a fully functional inflammasome even in Syk knockout cells (J. Yang, Liu, & Xiao, 2016). Bruton's tyrosine kinase (BTK), well known for B-cell development, was recently shown to phosphorylate ASC and NLRP3 to allow inflammasome assembly. The tyrosine kinase inhibitor ibrutinib could successfully inhibit IL-1 $\beta$  secretion (Ito et al., 2015). Controversially, the I $\kappa$ B kinase  $\alpha$  (IKK $\alpha$ ) was reported as a negative regulator of the inflammasome because it phosphorylates ASC and prevents its association with NLRP3 (Martin et al., 2014).

- 2) Regulation by ubiquitination: BRCC3, and its human counterpart BRCC36, were identified as NLRP3 deubiquitinating enzymes, which are essential for inflammasome assembly. BRCC3 is a metalloproteinase that cleaves Lys-63 linked polyubiquitin on inactive NLRP3, unleashing the protein to assume the right tridimensional conformation needed for the nucleation step. Deubiquitination occurs upon TLR stimulation and requires production of mitochondrial ROS (Py, Kim, Vakifahmetoglu-Norberg, & Yuan, 2013). Conversely, ASC needs to be ubiquitinated by the linear ubiquitin assembly complex (LUBAC) for its function (Rodgers et al., 2014).

Together with transcriptional and post-translational modifications, there are two other pathways that were related to inflammasome dampening: interferons and autophagy. Type I IFNs (especially IFN $\alpha$  and IFN $\beta$ ) can reduce the expression of IL-1 $\beta$  and of IL-18 leading to an impaired inflammasome response (Guarda et al., 2011). The way how IFNs represses NLRP3 activity is not completely understood but, there are many studies that found that IFNs can improve several diseases driven by NLRP3 mutations. Furthermore, in the case of multiple sclerosis, IFN- $\beta$  therapy is a standard treatment. The administration of IFN- $\beta$  to EAE mice could also prevent NLRP3 activation and ameliorates the animals phenotype (Shao, Xu, Han, Su, & Liu, 2015). However, the role of Type-I IFNs is still controversial as there were other reports showing IFNs are positively influencing the NLRP3 inflammasome. These results were already reviewed elsewhere (Malireddi & Kanneganti, 2013).

Autophagy is a normal cellular process used to degrade superfluous or dysfunctional cellular components. Autophagy can also inhibit the inflammasome by different

mechanisms, mainly through clearance of damaged mitochondria (Zhou et al., 2011), degradation of ASC specks and IL-1 $\beta$  sequestration (Jabir et al., 2015). One group showed that small molecules activating autophagy can also inhibit the inflammasome. Vice versa, autophagy inhibitors promote inflammasome activation by accumulation of ROS-producing-mitochondria (Shao et al., 2015).

Finally, there is another class of negative regulators, these are the CARD-only proteins (COP) and the pyrin-only proteins (POP). These proteins are expressed in humans and primates but are absent in rodents. Therefore *in vivo* studies are conducted by administration of recombinant proteins or with genetic knock-in lines (Pedraza-Alva et al., 2015). There are three COPs in total: CARD16 (or COP or PSEUDO-ICE), CARD17 (or INCA) and CARD18 (or ICEBERG), they show high similarity with the CARD domains of caspase-1 and they can inhibit the inflammasome by interfering with the normal ASC - caspase-1 joining (Pedraza-Alva et al., 2015). The POPs are also three in total: POP1, POP2, and POP3. They interfere with PYD-PYD interactions and were shown to reduce IL-1 $\beta$  secretion in both the canonical and the non-canonical inflammasome. Notably, POP-1 could ameliorate LPS-induced peritonitis and the phenotype of a CAPS transgenic mouse model (de Almeida et al., 2015). The ways inflammasome can be regulated are many and can act at several levels both upstream and downstream of its assembly. One of the most important challenges of inflammasome research will be to clarify those results that are still controversial and to find missing parts of regulatory mechanisms.

### Non-canonical NLRP3 activation

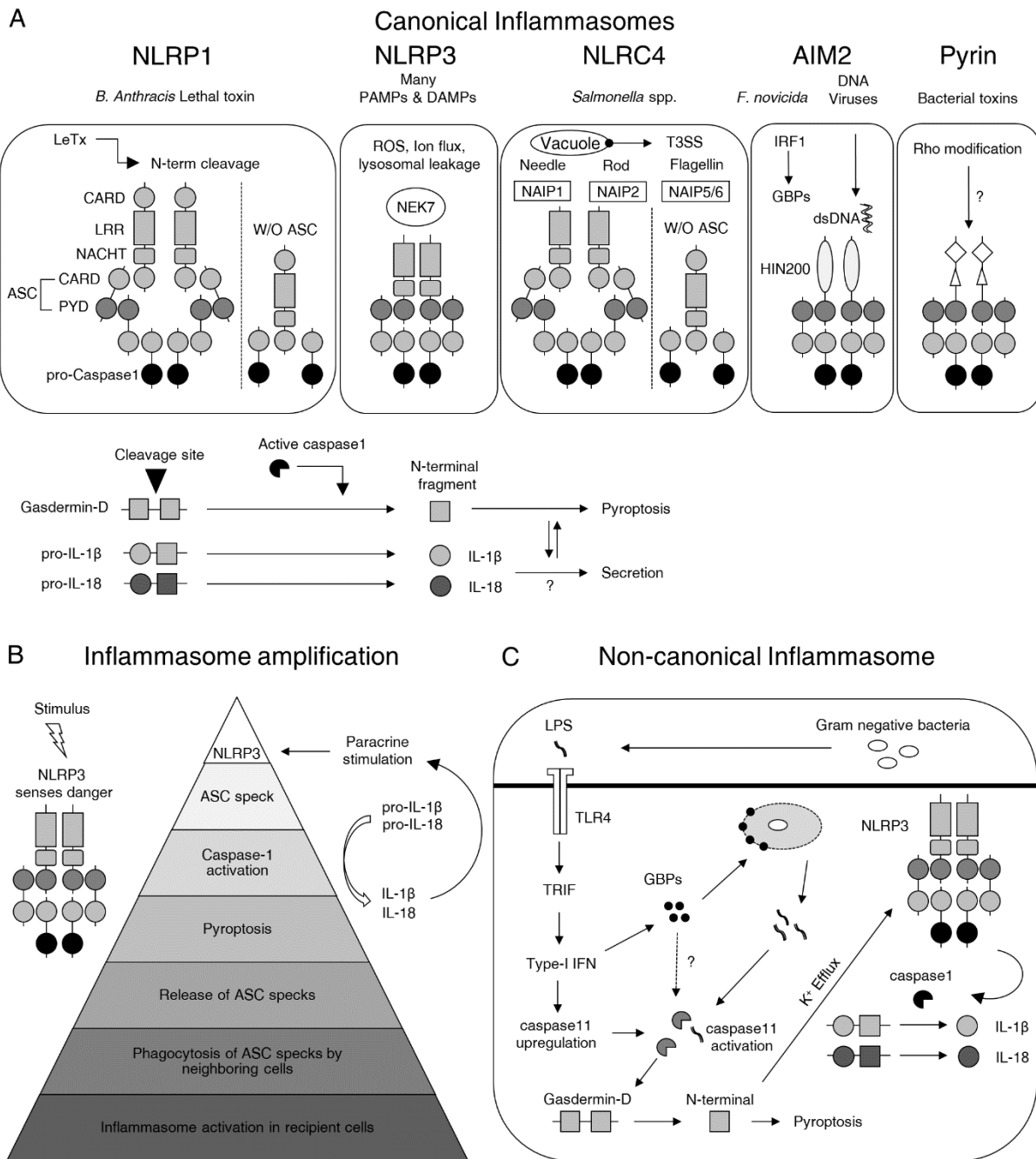
The NLRP3 inflammasome can also be activated by an alternative, non-canonical pathway which relies on the ability of caspase-11 (human orthologues are caspases 4 and 5) to sense intracellular LPS. The pathway is activated in response to Gram-negative bacteria as caspase-11 can sense LPS, inducing its switch to the active state. The observation that prompted the description of this pathway, was made by researchers who observed how naturally occurring caspase-11-deficient mouse inbred strains are protected against LPS-mediated cell death in a model of induced endotoxemia. This work demonstrated that TLR4 is not the only PRR responsible for LPS-induced cell death (Kayagaki et al., 2011). Later, the same group showed that, upon priming with poly(I:C), which is a TLR3 agonist inducing expression of

inflammatory proteins included pro-caspase-11, mice of both wildtype and TLR4 knockout genotype were susceptible to *E. coli* induced endotoxemia. This showed that TLR4 is only responsible for priming and implicated caspase-11 for cell death and inflammasome activation in response to LPS (Kayagaki et al., 2013).

As already discussed, the assembly of the canonical inflammasome requires two independent hits. The first is the priming, it is most of the times achieved experimentally through TLR4 stimulation with LPS. The signal two is provided by the actual inflammasome stimulus and drives nucleation of NLRP3, followed by ASC speck formation and finally by the binding and autocatalytic cleavage of caspase-1. Once it is cleaved, caspase-1 is released from the upstream complex and cleaves pro-IL-1 $\beta$  to its actively secreted form. However, caspase-11 (and its human counterparts) can bind intracellular LPS directly, which is sufficient for its oligomerization without an upstream interaction partner facilitating induced proximity (J. Shi et al., 2014).

Active caspase-11 drives two concatenated events. The first one is induction of pyroptosis by cleavage of GSDMD, which creates pores in the cell membrane. The second is a quick drop of intracellular potassium leading to the activation of the NLRP3 inflammasome (Broz & Dixit, 2016). More recently, a group proposed the mechanosensitive ATP release channel pannexin1 as a key regulator of K<sup>+</sup> efflux required for NLRP3 activation during non-canonical inflammasome response. When caspase-11 is cleaved by intracellular LPS, it also drives the cleavage of pannexin1. The channel opens thus releasing ATP that binds on P2X7 purinergic receptors. The P2X7 channel opens and causes K<sup>+</sup> efflux and consequent NLRP3 activation. (D. Yang, He, Munoz-Planillo, Liu, & Nunez, 2015)

Type-I IFN were discussed previously in the context of canonical NLRP3 activity. However, Type-I IFN signaling also constitutes an important regulatory pathway for the non-canonical inflammasome. Gram-negative bacteria and LPS which bind to TLR4 activate MyD88 signaling for NF $\kappa$ B activation. Moreover, TLR4 has another adaptor named TRIF that supports type-I IFNs response. The type-I IFN response induces the expression of several inflammatory proteins, included the guanylate-binding proteins (GBPs). GBP2 is required for caspase-11 activation as it destabilizes the pathogen-containing vacuoles thereby provoking release of the bacteria (or LPS) in the cytosol. Caspase-11 can sense the pathogen and triggers non-canonical NLRP3 activation and pyroptosis (Meunier et al., 2014).



**Figure 1: An overview of the inflammasomes:** (A) the canonical inflammasomes and their stimuli. The activations mechanisms vary among the different complexes. (B) ASC mediates the amplification of inflammasome signaling. The first stage involves the nucleation and expansion of the ASC speck inside the cells were NLRP3 is stimulated. Next, IL-1 $\beta$  can expand the signaling in a paracrine fashion to adjacent cells. Finally, ASC specks are released and engulfed by neighbor cells thus driving a new inflammasome activation cycle. (C) The mechanism of non-canonical inflammasome goes through type-I IFN signaling and caspase-11 activation by LPS. Abbreviations: LeTx, *B. anthracis* lethal toxin; ROS, reactive oxygen species; PAMPs/DAMPs, pathogen/damage associated molecular patterns; IRF1, interferon regulatory factor 1; GBPs, guanylate binding proteins; T3SS, type-III secretion system; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4. The picture is inspired by Sharma & Kanneganti (2016), *The Journal of Cell Biology*, 213 (6).

## **Small molecules to study the NLRP3 inflammasome, the unexpected new tools: tyrosine kinase inhibitors (TKIs)**

Researchers have been trying for years to find new molecules that could inhibit the inflammasome. On one hand, this serves as a tool to study the heterogeneity of mechanisms that activate and regulate NLRP3. On the other hand, this can lead to the discovery of new drugs that could enter the clinics.

The project I have been following for my thesis focuses on the activity that small molecule inhibitors have on the inflammasome. Particularly, I studied the effects of tyrosine kinase inhibitors (TKIs) on NLRP3 activation. TKIs are a class of drugs that selectively inhibit tyrosine kinases. Protein tyrosine kinase (PTKs) signaling is strongly related to cell survival and division. Therefore, genetic alterations of these kinases are associated with cancer development (Levitzki, 2013).

Most TKIs were designed to bind selectively the ATP binding pocket of protooncogenic PTKs and block their signaling, which results in growth arrest or death of cancer cells (Levitzki, 2013). As discussed in the previous section, phosphorylation is important for NLRP3 activation, and it seems more likely to have an activating rather than an inhibitory effect (Pedraza-Alva et al., 2015). However, as in the case of IKK $\alpha$ , blocking phosphorylation may be an event that positively regulates inflammasome assembly (Martin et al., 2014).

In the results section, I will describe the steps toward the full characterization of the inflammasome-activating potential of two drugs: imatinib and masitinib. Imatinib was the first TKI to be introduced into the market under the name Glivec in the late 90s and is produced by Novartis or other generic pharmaceutical companies. Imatinib is used to treat chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). Masitinib is produced by AB Science and is currently tested in clinical trials for several human cancers. It is already approved for veterinary medicine under the trade name of Masivet to treat mast cell tumors. Before introducing TKIs with special focus on imatinib and masitinib in more detail, I will first describe some small molecules of other substance classes that modulate inflammasome activity.

## Modulation of NLRP3 inflammasome: small molecules at the forefront

As outlined above, there are many endogenous mechanisms that regulate inflammasome activity and can act at both the transcriptional and post-transcriptional level. Many inflammatory diseases derive from genetic alterations or dysregulated activity of NLRP3 (and other inflammasomes) leading to increased IL-1 secretion. The finding that many inflammatory diseases are characterized by altered IL-1 secretion predates the discovery of the inflammasome. Therefore, the first drugs that were developed to cure autoinflammatory diseases are IL-1 interceptors.

The FDA (food and drug administration, US) and EMA (European medicine authority, EU) approved Riloncept, Canakinumab, and Anakinra as human drugs. They can inhibit the action of secreted IL-1. Anakinra (trade name Kineret) is a recombinant IL-1 receptor antagonist (IL-1RA) used to treat rheumatoid arthritis (Fleischmann, 2006). Canakinumab (trade name Ilaris) is a human monoclonal antibody specific for IL-1 $\beta$  that is used to treat several forms of CAPS (Dhimolea, 2010). Riloncept (trade name Arcalyst), is a IL-1 decoy receptor generated by fusing the extracellular part of IL-1R1 to the accessory protein IL-1RAcP and to the F<sub>c</sub> portion of a human IgG. It can neutralize IL-1 and is currently approved for CAPS treatment (Dubois, Rissmann, & Cohen, 2011).

These drugs introduced a significant change for the treatment of IL-1 driven diseases. However, they have many adverse effects and drug agencies raised serious safety concerns (Kotz, 2012). Particularly, Anakinra pharmacokinetic is poor and therefore a patient need a high dosage per day (100 mg). Also, these drugs have high molecular weights and cannot pass the blood brain barrier easily, reducing their therapeutic applications (Lopalco et al., 2016). It also truth that the cost of biological treatments are more expensive than those based on small molecules (Imai & Takaoka, 2006). For these reasons, there is an urgent need for a new class of inhibitors that can block the inflammasome itself, preventing IL-1 from even being released as well as blocking pyroptosis. Small molecules have several advantages as compared to therapy based on biologicals, including that they are often orally available and can pass the cell membrane as well as potentially the blood brain barrier.

Small molecules can block the assembly of the complex and prevent the secretion of IL-1 $\beta$ , IL-18, and all the other alarmins released during pyroptosis. I will herein describe three class of compounds that are most abundant in the literature. For a detailed

overview of all the small molecules that can inhibit the inflammasome there is a recent specialized review (Baldwin, Brough, & Freeman, 2016). The MCC-950 molecule belongs to a class of small molecules called cytokine release inhibitor drugs (CRID), which comprises CRID1, CRID2 and CRID3. CRID3 is also known as MCC-950. CRID3 was shown to be efficient in inhibiting the inflammasome in murine bone marrow derived macrophages/dendritic cells (BMDMs, BMDCs) and human peripheral blood mononucleated cells (PBMCs). CRID3 effectively improved the phenotype of mice bearing a NLRP3 mutation that generates Muckle-Wells syndrome. It also reduced the severity of a EAE multiple sclerosis model and demonstrates a good overall pharmacokinetic profile (Coll et al., 2015).

The ketone metabolite  $\beta$ -hydroxybutyrate (BHB) reduced secretion of IL-1 $\beta$  and IL-18 from NLRP3 inflammasome activation in human monocytes (Youm et al., 2015). Of note, BHB inhibits NLRP3 selectively by interfering with potassium efflux and ASC speck formation, it has no action on mitochondrial ROS, autophagy or lysosomal stability. The compound was used to successfully treat models of MWS and FCAS. Interestingly, ketogenic diet attenuates caspase-1 activation and IL-1 $\beta$  secretion in these model mice to the same degree as BHB administration. The production of ketone bodies normally occurs during starvation or physical exercise and reduces inflammation potency. The authors suggest that anti-inflammatory effects of fasting or ketogenic diets are connected to the inhibitory effect of BHB on the NLRP3 inflammasome (Youm et al., 2015).

Another class of inflammasome inhibitors are those that block ROS production. The ROS scavenger compounds N-acetylcysteine and ammonium pyrrolidine dithiocarbamate (APDC) are frequently found in the literature as potent inflammasome inhibitors (Wen et al., 2011). Another drug called ebselen, a glutathione peroxidase mimic and a broad-spectrum ROS scavenger, is regularly used as an effective inflammasome inhibitor (O. Gross et al., 2012; Jabaut, Ather, Taracanova, Poynter, & Ckless, 2013).

Despite many small molecules that can inhibit inflammasome activation, the literature shows some examples of compounds that can instead trigger activation. Indeed, our group has been working on imiquimod (R837), a compound which is used to treat genital warts, superficial basal cell carcinoma, and actinic keratosis that can also activate the inflammasome. R837 activates the inflammasome without causing

potassium efflux but instead by driving the massive production of mitochondrial ROS (C. J. Gross et al., 2016). Another surprising compound was found recently, it is GB111-NH<sub>2</sub>. This compound is normally a cathepsins inhibitor, but it can also interfere with glycolysis in macrophages thus driving NLRP3 assembly. The authors found that glycolysis blockade impairs NADH production, resulting in the accumulation of mitochondrial ROS that were essential for NLRP3 activation (Sanman et al., 2016). This study corroborates the number of evidences that explain how the inflammasome activation and glycolysis disruption may have a tightly regulated connection (Wolf et al., 2016).

### Tyrosine Kinase Inhibitors

Phosphorylation is the most common post-translational modification. The human genome has 518 kinases and 156 phosphatases. The protein kinases are involved in the regulation of pathways that, if deregulated, are some of the most common factors in cancer development: cell proliferation, survival, motility, metabolism, angiogenesis, and evasion of antitumor responses (S. Gross, Rahal, Stransky, Lengauer, & Hoefflich, 2015). There are three main categories of mutations that can affect kinases to become potentially oncogenic: 1) point mutations that switch the catalytic activity to constitutive, 2) gene amplification and consequent kinase overexpression and 3) gene fusions, mainly driven by chromosomal rearrangements, that fuse kinases within other genome regions and leads to overexpression or the generation of a fusion protein with increased kinase activity. Nonetheless, there are other less frequent mechanisms of kinase mutation that were reviewed recently (S. Gross et al., 2015).

Protein kinase are ATP consuming enzymes with a highly-conserved ATP-binding pocket. The binding to ATP is relatively weak, which, together with the high cytoplasmic concentration of ATP, ensures high rates of enzymatic activity. The weak binding allows for the development of small molecules that bind to the pocket much stronger than ATP, inhibiting activity. Pharmacologists and private companies therefore put their efforts into rational drug design to target selectively block those binding pockets (Levitzki, 2013).

In the late 80s, researcher found the first small molecules that could selectively inhibit epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Kovalenko et al., 1994; Yaish, Gazit, Gilon, & Levitzki, 1988). Despite these



early findings, a new compound named imatinib, initially developed as a PDGFR kinase blocker, was later found as a potent inhibitor of the BCR-Abl fusion kinase. This was the very first TKI put in commerce under the trade name Glivec and is still used to treat chronic myeloid leukemia (Hunter, 2007). At present, there are more than 28 clinically approved TKIs. They can target more than one PTK at the time, increasing their efficacy and therapeutic potential. The currently approved TKIs can either bind directly to the ATP pocket or to an allosteric site to change the enzyme conformation. They have different inhibition modalities but most of them fall in the category of reversible inhibitors, while there are only few approved irreversible inhibitors (Wu, Nielsen, & Clausen, 2015, 2016).

I here review the basics about imatinib and masitinib, another similar TKI which is still under clinical investigation. Imatinib is a selective inhibitor of the fusion kinase BCR-Abl, of PDGFR and of mast/stem cell growth factor receptor (SCFR, KIT, CD117). The fusion kinase BCR-Abl is derived from the fusion of the long arms of chromosomes 9 and 22 creating the so-called Philadelphia chromosome (Ph<sup>+</sup>). The Philadelphia chromosome is found in nearly 100% of patients having CML and of circa 30% patients having acute lymphoblastic leukemia (ALL). This fusion gene generates a constitutively active kinase of circa 210kDa, which transforms cells as a single hit. The cells become completely dependent on BCR-Abl for their survival, indeed, cells with Philadelphia chromosome are referred as oncogene-addicted (Salesse & Verfaillie, 2002). Imatinib binds to the ATP binding pocket of BCR-Abl and blocks the kinase activity of protein phosphorylation. As a consequence, cancer cells undergo apoptotic cell death (Goldman & Melo, 2003). Imatinib can also bind selectively to the ATP-binding pocket of KIT and PDGFR. Therefore, it is also used to treat gastrointestinal stromal tumors (GIST). Gastrointestinal stromal tumors originate from interstitial Cajal's cells as a result of activating KIT mutations which are detected in circa 85% of patients and of PDGFR mutation which are found in another 10% (Miettinen & Lasota, 2006).

The drug was first marketed in 2001 and it is still used and considered the first line treatment for the listed diseases. It was a breakthrough in the history of pharmacology as in the "pre-imatinib era", patients with CML could only get hydroxyurea and interferon treatments that could just slow down the disease but did not block its progression. Currently, the 8-years survival rate is around 85% (Comert, Baran, &

Saydam, 2013). The same is true for GISTs, as before imatinib, the median survival was around 1 year and now it is over 5 years (De Giorgi & Verweij, 2005).

However, despite the high specificity of imatinib, many resistance mutations were found in BCR-Abl and KIT that impair the treatment efficacy. Researchers reported point mutations within the ATP-binding pocket that still allow for enzymatic activity but strongly reduce the affinity for imatinib (Antonescu et al., 2005). As a remedy, pharmaceutical companies developed new TKIs to treat CML and GIST that target the same oncogenes but are effective even when imatinib fails. For example, dasatinib (trade name Sprycel), nilotinib (Tasigna), bosutinib (Bosulif), and ponatinib (Iclusig) are approved to treat CML. Sunitinib (Sutent) is approved for GIST and renal cell carcinoma (RCC) treatment.

Another drug we sought to include in this study is masitinib, developed by AB Pharmaceuticals. It is currently under clinical investigation for GIST, other types of cancer, and neurodegenerative diseases. Masitinib is a selective ligand of KIT and PDGFR but not of BCR-Abl (Dubreuil et al., 2009). The chemical structure differs minimally from that of imatinib, nevertheless the two drugs display a different inflammasome activation profile as outlined in the results section.

Another important aspect of imatinib (and other TKIs) is the (side-)effects it has on other cells of the immune system and on immune responses. Continuing research on Imatinib since its introduction to the market has yielded numerous reports showing that the immune system contributes both to amplify drug efficacy and to reduce the emergence of escape mutations. The group of Guido Kroemer introduced the concept of “immunogenic cell death” and investigated the immunomodulatory effects of imatinib for years. They propose, as a future perspective, that TKIs will be used together with cancer immunotherapy to combine their curative effects. The immunomodulatory effects of imatinib were listed and reviewed recently (Zitvogel, Rusakiewicz, Routy, Ayyoub, & Kroemer, 2016). To our knowledge nobody reported the effects of imatinib on the inflammasome and this is the aim of the thesis work here described.

## The role of the inflammasome in cancer

### Inflammation driving cancer development

There is a strong connection between cancer and inflammation. Cancer occurs due to accumulation of genetic mutations and it is fostered by chronic inflammation that can drive proliferation and promote mutations, for example, through radical production. Furthermore, an inflammatory microenvironment helps malignant cells to escape the immune surveillance mechanisms (Hanahan & Weinberg, 2011). Although acute inflammation helps modulating immune system against tumor cells, chronic inflammation supports tumor progression, growth, metastasization and immune evasion (Shalapour & Karin, 2015). Many inflammatory pathways including the inflammasome were associated with cancer development and progression. However, the role of the inflammasome is still controversial as there are many publications showing contrasting findings (Terlizzi et al., 2014).

Some gastrointestinal tumors are associated with chronic inflammation. A dysregulated inflammasome activity, accompanied by high IL-1 $\beta$  secretion, was reported in a model of colorectal cancer (Kolb, Liu, Janowski, Sutterwala, & Zhang, 2014). However, IL-18 was found to be rather protective by inducing repair of colonic tissue after injury (Dupaul-Chicoine et al., 2015). In another work about inducible colorectal cancer, the authors show that NLRP3, ASC, and caspase-1 knockout mice had a more severe phenotype because of recurrent colitis and associated tumorigenesis (Allen et al., 2010). Controversially, in another study NLRP3 and caspase-1 knockouts had instead decreased DSS-induced inflammation and colitis (Bauer et al., 2010). These opposite results were rather confusing and did not explain the actual role of the inflammasome in gastrointestinal cancers. However, one study convincingly explained, by using a DSS induced colitis models, that the composition of the gut microbiota determines the disease severity and the relative contribution of the inflammasome to inflammation and carcinogenesis. The authors report that ASC knockout females can transmit an enhanced colitis phenotype to wildtype pups through the transmission of a colitogenic microflora (Elinav et al., 2011). The differences in the gut microbiome could be the underlying effect that determined the discordant findings obtained for these models.

The inflammasome was also associated with development and progression of skin cancer. In one study, the authors found that human melanoma cells secrete IL-1 $\beta$  because of a constitutively assembled NLRP3 inflammasome (Okamoto et al., 2010). In a second study, the role of ASC was central in melanoma progression. When ASC is expressed in non-metastatic melanoma cells, it reduces expression of NF $\kappa$ B and reduces tumorigenesis. In metastatic cells, ASC increases NF $\kappa$ B signaling and increases pro-IL-1 $\beta$  transcription which is cleaved and secreted by the inflammasome thus enhancing tumor progression (W. Liu et al., 2013). A third study of a murine model of induced skin-carcinogenesis, showed a differential role for ASC depending on the cell type. By using conditional ASC knockout mice, the authors found that ASC has a tumor-promoting role in myeloid cells by taking part to the inflammasome with consequent IL-1 $\beta$  secretion, driving inflammation. In keratinocytes, it is a suppressor gene, as cancer cell progression is significantly delayed when ASC is knocked out in these cells. The authors finally indicate a physical interaction between ASC and p53 as the basis for the cancer cell-intrinsic phenotype (Drexler et al., 2012).

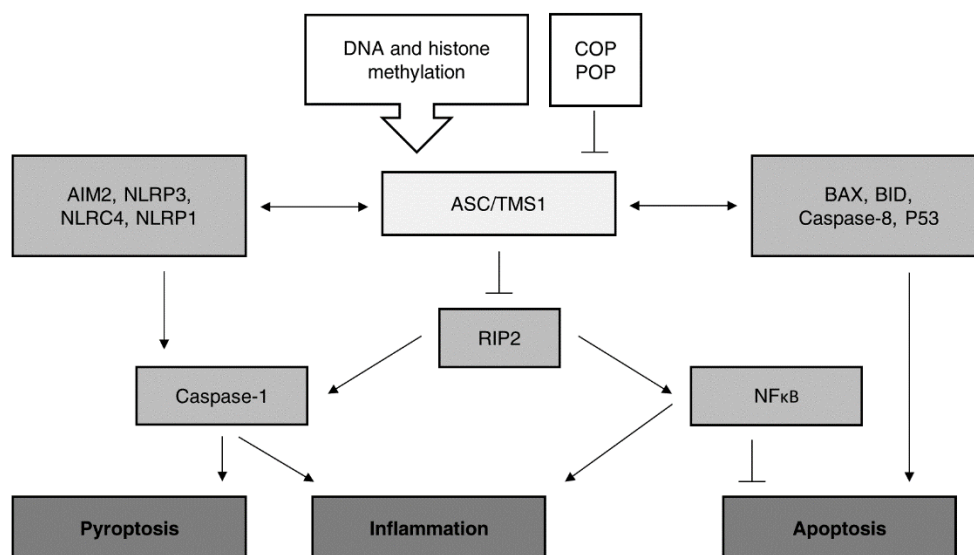
As our research group works prevalently with immune cells we became interested in exploring the role of inflammasome in hematologic malignancies. There are few papers so far that explored in this direction. However, it is a matter of fact that patients suffering from several types of cancers, leukemia included, have high peripheral blood levels of inflammatory cytokines, two of which are IL-1 $\beta$  and IL-18. It was therefore proposed to test IL-1 $\beta$  blocking agents as an anticancer treatment (Dinarello, 2011). In one study, the authors suggest that NLRP3 helps ALL cells to escape glucocorticoid receptor blockade (Paugh et al., 2015). In another one, the oncosuppressor protein PML was proposed to interact physically with NLRP3 to license full inflammasome activation. The authors proposed PML as a new target for inflammasome related diseases (Lo et al., 2013). A recent work from our collaborators, is the first to put the inflammasome in close relation to the progression of acute myeloid leukemia (AML). The necroptosis-related kinase RIPK3 restricts malignant proliferation by activating the inflammasome, which promotes differentiation and cell death through IL-1 $\beta$  in a mouse model of AML (Höckendorf et al., 2016). The link between inflammasome and cancer is still under debate and more studies are required to clarify it.

## ASC as a tumor suppressor?

Although the role of the inflammasome in cancer needs further investigation, there is also another research line that, even before the discovery of the inflammasome, investigated the role of ASC as oncosuppressor gene. The ASC protein was discovered independently by two research groups in the year 2000. The group of Junji Sagar at the Shinshu University of Nagano, coined the name “ASC” which stands for: apoptosis-associated speck-like protein containing a CARD (Masumoto et al., 1999). This work also opened the research line that led the discovery of the inflammasome. In parallel, the group of Paula Vertino at Emory University, identified and named it “target of methylation-induced silencing 1” (TMS1) as the gene’s promoter was found hypermethylated in primary human breast cancer cells (Conway et al., 2000). After this publication, other work showed that ASC/TMS1 has a CpG island in proximity of its promoter that is frequently hypermethylated in primary cells and cell lines of different kind of tumors including brain (Grau et al., 2010; Martinez, Schackert, & Esteller, 2007; Stone et al., 2004), lung (Virmani et al., 2003), liver (C. Zhang et al., 2007), gastrointestinal tract (Ohtsuka et al., 2006), prostate (Collard, Harya, Monzon, Maier, & O’Keefe, 2006; Das et al., 2006), and skin (Guan et al., 2003). Despite these many *in vitro* reports, only two studies reported *in vivo* results so far, and were conducted by using ASC deficient mice. One study identified ASC as pro-oncogenic in a murine model of medulloblastoma (Knight et al., 2014). A second work, already mentioned in the previous paragraph, suggests that ASC has an oncosuppressive role in keratinocytes and a pro-oncogenic one in myeloid cells in model of induced skin-carcinogenesis (Drexler et al., 2012).

These results led the scientific community to think that ASC may have a dual function, one potentially independent from the other. In the first one, it serves as the inflammasome adaptor to trigger IL-1 $\beta$  secretion and promote pyroptotic cell death. In the other function, it might contribute to apoptosis by interacting with other pro-apoptotic proteins, like BAX, BID, caspase-8 and p53. This hypothesis has been recently described in a review article that capitulates the story of ASC/TMS-1 in both pyroptosis and apoptosis (Salminen, Kauppinen, Hiltunen, & Kaarniranta, 2014). The first research branch of ASC, brought important findings like the inflammasome and pyroptosis. The second branch, mainly focusing on apoptosis, is still under question and has not generated breakthrough findings so far.

There are therefore three different aspects to consider. First, ASC might contribute in controlling cell death pathways other than pyroptosis, and this can be apoptosis. Second, a major factor in cancer development is the suppression of cell death pathways which leads to tumor expansion (Hanahan & Weinberg, 2011). Third, ASC was found blocked in its expression in several types of cancers cells. For these reasons, it appears possible that cancer cells may require to block ASC as either an inflammasome or an apoptotic adaptor. Parallely, ASC could have a role in promoting cancer as those inflammasome-competent cells nearby the tumoral area may boost neoplastic growth by undergoing pyroptosis and secreting IL-1 $\beta$  while fighting the malignant cells. In this thesis work, we tried to contribute to this research issue and investigated the role of ASC in hematologic malignancies.



**Figure 2: A diagram of the physical interactions of ASC/TMS1 in cell death pathways.** Inspired and adapted from Salminen et al. (2014), Cellular and Molecular Life Sciences, 71 (10).

## AIM OF THE PROJECT

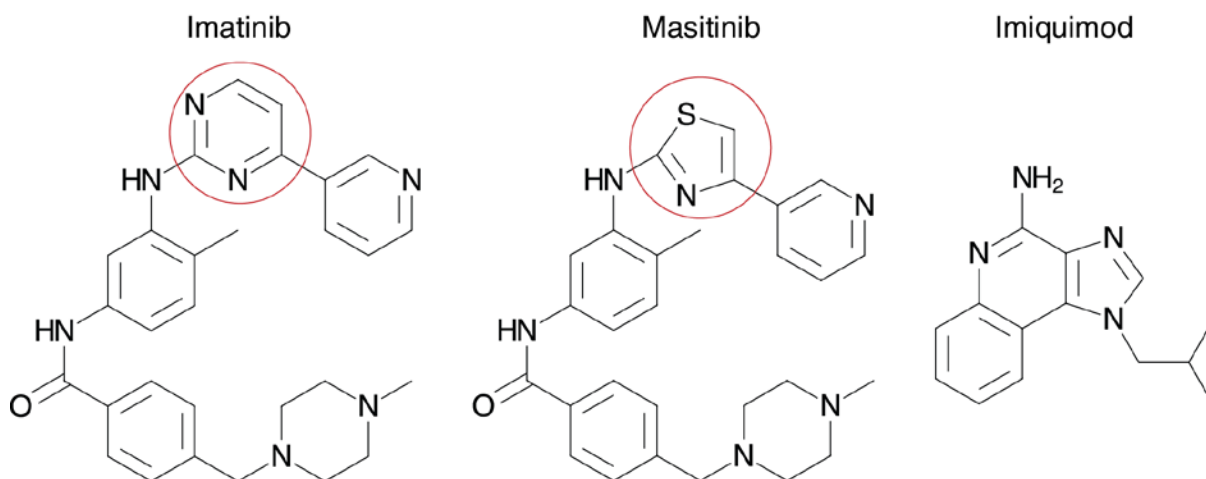
### **Project one: imatinib and masitinib activate the NLRP3 inflammasome**

Our lab focuses on NLRP3 activation mechanisms as well as the mechanisms of downstream signaling through caspase-1. In our recently-published work, we observed that the imidazoquinoline derivate drug R837 (imiquimod) can activate NLRP3 without the need of potassium efflux. Further analysis showed that this compound activates the inflammasome through an intense production of mitochondrial ROS (C. J. Gross et al., 2016). To find cellular targets of R837, eventually involved in NLRP3 activation, we formed a collaboration with the group of Prof. Kuster in Weihenstephan and together, performed a chemical proteomics assay with a synthetic R837 analogue coupled to a bead matrix. The cell extracts of LPS-primed murine bone marrow derived dendritic cells (BMDCs) were either untreated or treated with increasing concentrations of free R837 to compete with the bead-coupled drug. The proteins fished by the beads were isolated and identified by mass spectrometry. One of the best hits was NQO2, meaning it is a target of R837. To prove this results, R837 was co-crystallized with NQO2 and it could be found linked to its active site. Consequently, R837 inhibits the enzymatic activity of NQO2 in a dose-dependent manner (C. J. Gross et al., 2016).

Some years before, our collaborators invented the so-called “kinobead” approach, which is related to the method just mentioned to find R837 as a NQO2 ligand. Here, beads are synthetically coupled with a wide variety of small molecules able to bind virtually the whole kinome. The beads are incubated with a mixture of cell extract of several cancer cell lines, either alone or with a free kinase inhibitor of choice at increasing concentrations, to compete with the bead-coupled compounds. The proteins bound to the beads are identified via mass spectrometry. Those proteins that bind specifically the query (free drug) will lose signal respectively of the increasing dosage (Medard et al., 2015). By competing with increasing concentrations of free drug, the specific affinity of each identified binding partner can be identified. In this way, many known TKIs were tested and in addition to their specific targets, other kinase targets were identified. For imatinib ABL1, KIT and PDGFR were the best kinase hits, which is not surprising as the drug was rationally designed to bind them.

Other kinases that were also found to be inhibited by imatinib through this approach were DDR1 and SYK. Remarkably, also some non-kinase hits were found, the one with the lowest  $K_D$  being NQO2 (Bantscheff et al., 2007). NQO2 is a promiscuous quinone oxidoreductase that catalyzes a two-electron detoxification of quinones to form hydroquinones with unclear physiological function (Vella, Ferry, Delagrangue, & Boutin, 2005). As R837 can bind NQO2 and can activate the inflammasome, we asked ourselves whether imatinib, which is also able to bind NQO2, can trigger NLRP3 assembly as well.

We used imatinib for all the described experiments together with masitinib, a drug with a very similar structure having a thiazole ring in place of the diazine ring (Figure 3). It is currently not approved for treatment in humans but is currently tested in several clinical trials. Remarkably, despite the minimal difference in their structure, imatinib can bind to ABL1, the kinase part of the fusion protein BCR-Abl, but masitinib does not, which allows to easily gain insight as to whether an effect of imatinib could be related to its effect on BCR-Abl. My study of imatinib and masitinib revealed that they can drive inflammasome assembly and induce a mixed form of cell death.



**Figure 3 Structures of imatinib, masitinib and R837:** Structural differences between the TKIs are circled in red. Despite the great difference between the structures of TKIs and R837, they can all activate the NLRP3 inflammasome.



## **Project two: The role of ASC in hematologic malignancies**

In principle ASC, could have two cancer cell intrinsic functions as a tumor suppressor, one in the context of the inflammasome and one independent of it. Inflammasome activation could prevent cancer development through pyroptosis. Inflammasome independent, ASC could play a role through other cells death pathways including apoptosis. Here, I sought to determine whether ASC has any relevant role in controlling cell death pathways and eventually affect cancer progression in B cells, T cells and myeloid cells. The online databases Immunological genome project (IGP) and BioGPS shows that all these cell types express substantial amounts of ASC. Also, they all express detectable levels of caspase-1. Myeloid cells express all the main inflammasome-forming NLRs including NLRP3, NLRC4, NLRP1 and AIM2. However, B and T cells do not express detectable levels of NLRP3, but they still seem to have both NLRC4 and AIM2 and they are potentially inflammasome-competent. Of note, there are already some reports showing that T cells can activate an a functional inflammasome and trigger pyroptosis in response to viral DNA (Dell'Oste et al., 2015). We therefore wanted to understand, in any of these cell type, whether ASC has a function in suppressing cancer progression and if this is related to the pyroptotic or to the apoptotic pathway.

To study the function of ASC in B cells, we crossed ASC knockout mice with a line of Burkitt's lymphoma. These mice bear a human mutated cMyc oncogene under to control of the Ig- $\lambda$  promoter (Kovalchuk et al., 2000). To study the function of ASC in T cells, we crossed ASC conditional knockouts (ALPS) with CD4 Cre mice to delete the gene in T cells only. Mice with ASC-deleted T cell were crossed with another line developing a peripheral T-cell lymphoma (PTCL) disease. The mice bear a human oncogenic fusion kinase originated from a translocation event between chromosome 5 and 9, a recurrent event in PTCL diagnosed patients, the fusion unites interleukin-2-inducible T cell kinase (ITK) with spleen tyrosine kinase (SYK) leading to the fusion protein ITK-SYK. This oncogene leads to a permanent activation of downstream pathways that promote activation and survival of T-cells, thereby inducing lymphoma (Pechloff et al., 2010). Finally, we included a third model of chronic myeloid leukemia (CML) that would represent the cancer progression in fully inflammasome-competent cells. This mouse model requires a bone marrow transduction and transplantation protocol. The construct used bears a p210 version of the BCR-Abl oncogene which

generates a CML disease (Miething et al., 2006). This is a standard model for CML and is frequently found in the literature as a good *in vivo* model. To our knowledge, we are the first who tried to cross ASC deficient lines with these cancer models. The data provide a survival analysis and a comprehensive disease characterization, all accompanied by *in vitro* tests on primary murine lymphocytes.

# MATERIAL AND METHODS

## Materials

All the salts, reagents and fine chemicals were purchased from either Sigma-Aldrich, Carl Roth, or Enzo. Tyrosine kinase inhibitors were purchased from Selleckchem or kindly provided by Dr. Philipp Jost. R837 was purchased from Invivogen. Tissue culture mediums, supplements and antibiotics were from Thermo Scientific. Lab consumables were from TPP or Sarstedt. Other reagents not listed are specified afterwards.

## Mice

Mice were housed in SPF (specifying pathogen free) facilities at Klinikum rechts der Isar with water and food ad libitum. Mice used for inflammasome experiments are NLRP3<sup>-/-</sup> (Martinon et al., 2006), ASC<sup>-/-</sup> (Mariathasan et al., 2004), caspase-1<sup>-/-</sup> (Kuida et al., 1995), P2X7<sup>-/-</sup> (Solle et al., 2001); RIPK3<sup>-/-</sup> (Newton, Sun, & Dixit, 2004) and MLKL<sup>-/-</sup> (Murphy et al., 2013), kindly provided by Dr. Philipp Jost and colleagues; CAR1 transgenic mice (Heger et al., 2015), kindly provided by Prof. Marc Schmidt-Supprian and colleagues; NEK7<sup>-/-</sup> (H. Shi et al., 2016), kindly provided by Prof. Bruce Beutler and colleagues; and GSDMD<sup>-/-</sup> (W. T. He et al., 2015), kindly provided by Prof. Petr Broz and colleagues. SV129 mice naturally lacking caspase-11 activity (Kenneth et al., 2012) were used for non-canonical inflammasome experiments. Mice used for cancers studies are ASC<sup>-/-</sup>, ALPS<sup>fl/fl</sup> (Drexler et al., 2012); λ-Myc (Kovalchuk et al., 2000), kindly provided by Dr. Philipp Jost and colleagues; CD4 Cre (P. P. Lee et al., 2001) and ITK-SYK (Pechloff et al., 2010) were kindly provided by Prof. Jürgen Ruland and colleagues. For the bone marrow transplantation, C57BL/6 recipient mice were purchased from Janvier Labs. All mouse lines were maintained by inbreeding and kept under strict observation for manifestation of disease symptoms. Genotypes were confirmed by PCR analysis of biopsy material. Animals were euthanized ad occurrence in compliance with German and European regulations.

## Cell lines

Cell lines were cultivated at 37°C with 5% CO<sub>2</sub> in humidified incubators and handled under a sterile hood with HEPA-filter. Murine 3T3 embryonic fibroblasts were cultured in DMEM supplemented with pyruvate 1 mM, glucose 25 mM, glutamine 4 mM, FCS 10%, 100 U/mL penicillin and 100 mg/mL strep (abbreviated Pen/Strep). Human HEK293T human embryonic kidney were cultured in same medium as 3T3 cells. All cultures were passaged with the recommended frequency by trypsinization (Trypsin/EDTA 0.05% solution) and counted with hemocytometer and trypan blue solution ad occurrence. Human cancer cell lines were THP-1 (AML M5), CML-1 (CML at blast crisis), EM2 (CML at blast crisis), HL-60 (AML M3), JEKO-1 (B-NHL mantle cell lymphoma), JURKAT (T-ALL), JURL-MK-1 (CML at blast crisis), Kasumi-1 (AML M2), KCL-22 (CML at blast crisis), KG-1 (erythroleukemia), KYO-1 (CML at blast crisis), L428 (Hodgkin lymphoma), MEG-01 (CML at megakaryocytic blast crisis), MHH-preB (B-NHL), MN-60 (B-ALL), MOLM-14 (AML M5), MV4-11 (AML M5), NALM-6 (non-T non-B ALL), OCY-LY3 (B-NHL), PBL-985 (AML M3), ROS-50 (B-ALL), RCH-ACV (B-ALL), RS4;11 (ALL L2), SEM (B-ALL), TOLEDO (B-ALL) and U937 (diffuse histiocytic lymphoma). Cultivation media were prepared as recommended by the German collection of microorganisms and cell cultures (DSMZ). Cells were passaged with the recommended frequency and counted with hemocytometer and trypan blue solution ad occurrence.

## Methods

### Isolation of murine BMDMs and BMDCs

Murine BMDMs and BMDCs were isolated as described in detail (Schneider, Thomas, & Gross, 2013). Briefly, femora and tibiae were collected from mice of the different genotypes with the same age and sex. Bone marrow was flushed with plain RPMI 1640 medium plus Pen/Strep under a laminar flow hood by using sterile forceps, scissors, fine-gauge needles, syringes and 100  $\mu$ m cell strainers. Red blood cell lysis was done with G-DEX II RBC lysis buffer (Intron Biotechnology). Next, cells were either put directly in culture or gradually frozen at  $-80^{\circ}$  in FCS plus 10% DMSO in an isopropanol isolated freezing container (Mr. Frosty, Nalgene). Frozen bone marrow was transferred to liquid nitrogen after overnight freezing for long-term storage. Bone marrow cells were cultivated in two different ways to prepare either BMDMs or BMDCs. To prepare BMDMs, circa  $10 \times 10^6$  cells were seeded on a 10 cm non-tissue culture treated Petri dish in 10 mL of DMEM added with 10% FCS, Pen/Strep, and 20 ng/mL recombinant murine macrophage colony stimulating factor (M-CSF) (Immunotools). To prepare BMDCs, circa  $5 \times 10^6$  cells were seeded on a non-tissue culture treated 10 cm Petri dish in 10 mL of RPMI 1640 supplemented with 10% FCS, Pen/Strep, HEPES 25 mM, and 20 ng/mL recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF) (Immunotools). Additional 10 mL of full medium with cytokines were added after 2 days and a half or full medium change was done every 2 days depending on cell growth and density. Cells reach maturity of differentiation after 6-7 days of cultivation. Cell identity as macrophages or dendritic cells was occasionally confirmed by means of flow cytometry with antibodies against CD11b, CD11c, MHC-II, Gr-1, all antibodies were purchased from eBiosciences. The flow cytometer was a FACS Canto-II (BD Biosciences).

### Inflammasome assays with BMDM and BMDCs

Mature BMDMs or BMDCs were lifted with Hank's balanced salt solution (HBBS) containing 0.05% EDTA and with the help of a cell scraper tool. Cells were counted with trypan blue and plated at a density of 0.12 to 0.15  $\times 10^6$  cells/well in 96 well flat bottom plates in 200  $\mu$ l medium and incubated overnight. Priming was done by stimulating cells with a final concentration of 20 to 50 ng/mL *E. coli* K12 ultrapure LPS

(Invivogen) for 3 hours. Inflammasome stimulation was performed with imatinib and masitinib (Selleckchem) mostly in a range between 20 and 80  $\mu\text{M}$ . Together with TKIs, well-known NLRP3 activators were used as control stimuli. These were 5 mM ATP, 5  $\mu\text{M}$  Nigericin, 100  $\mu\text{M}$  R837, 400  $\mu\text{g/mL}$  MSU/Alum and 1  $\mu\text{g/mL}$  poly(dA:dT) transfected with Lipofectamine 2000 (Invitrogen). Commonly, inhibitors were added circa 30 minutes before inflammasome stimulation. The inhibitors tested were: 5  $\mu\text{M}$  CRID3 kindly provided by Prof. Matthew A. Cooper (Coll et al., 2015), 50 mM KCl, 100  $\mu\text{M}$  2-aminoethoxydiphenyl borate (2-APB), 5 mM glycine, 40  $\mu\text{M}$  ebselen, 40  $\mu\text{M}$  idebenone, 200  $\mu\text{M}$  ammonium pyrrolidinedithiocarbamate (APDC or PDTC), 20  $\mu\text{M}$  zVAD-fmk, 20  $\mu\text{M}$  Ac-YVAD-cmk, 30  $\mu\text{M}$  Necrostatin1 (provided by Dr. Philipp Jost) and polyethylene glycol (PEG) either at 800, 3000 or 9000 g/mol. Classic stimuli and all the inhibitors, were titrated before when first using them and the lowest effective dosage was used to minimize side effects. All inflammasome experiments were conducted in technical replicates, beginning with a triplet of cells for each condition. Cytokine measurements and LDH assay results are calculated as mean  $\pm$  standard deviation of triplicates.

### Measurement of secreted cytokines by ELISA

Cell-free supernatants from stimulated BMDMs and BMDCs were collected and analyzed for IL-1 $\beta$ , IL-1 $\alpha$ , and TNF $\alpha$  secretion with enzyme-linked immunosorbent assay (ELISA) kits (ready-set-go, eBiosciences). BMDC supernatants were diluted 1:3 to avoid that values exceed the upper detection limit, BMDM samples were not diluted. Assays were conducted following manufacturer protocol, with the exception that volumes used were half as indicated (50  $\mu\text{l}$  instead of 100  $\mu\text{l}$  per well) throughout the experiment. Absorbance was measured at 450 nm with a Tecan sunrise benchtop spectrophotometer. Data were analyzed with Tecan Magellan software and concentrations were extrapolated from a standard curve added to each measured plate and prepared with serial dilution of recombinant cytokine provided in the kit.

### Measurement of cell death

Cell death was measured with a CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay (Promega). Assays were conducted following manufacturer protocol, with the exception that volumes were used half as indicated. Maximum cell death (100%) was determined by lysing an unstimulated cell triplet with the lysis buffer provided with the

kit. Medium without cells was used to determine that background value that was subtracted from all values before calculating the percentage of cell death.

### Western blot for inflammasome experiments

Cell-free supernatants and cell lysates from untreated and stimulated BMDMs and BMDCs were prepared in sample buffer containing sodium dodecyl sulfate (SDS) and dithiothreitol (DTT), triplicates used for ELISA measurement were pooled to one sample for western blotting. Samples were loaded on a SDS-PAGE gels with polyacrylamide concentrations comprised between 8 and 15% (gels prepared by Susanne Weiß in the group, using Bio-Rad equipment). Gels were run at circa 100V until the front was just about to leave the gel and blot transfer was done at 100V for 2 hours on nitrocellulose membranes (GE healthcare). Membranes were then incubated in Ponceau S stain to confirm protein transfer. Ponceau stained membranes were scanned to record blot quality. Stained membranes were washed with PBST to wash off the Ponceau stain and then blocked with PBST plus 5% skim milk powder for at least 30 minutes at room temperature with gentle agitation. Blots were thoroughly washed with PBST and incubated with primary antibody prepared following manufacturer instructions, either in PBST plus skim milk or in BSA. To all primary antibodies, 0.05% sodium azide was added to preserve solutions from bacteria contamination. Antibodies solutions were stored in 50 mL falcons at -20°C when not in use. Membranes were incubated with primary antibody either overnight at 4°C or for at least 2 hours at room temperature on a shaker. After incubation, membranes were washed several times for a total of 30 minutes and then incubated with secondary antibodies conjugated with a horseradish peroxidase (HRP) for 2 hours. Secondary antibodies were diluted in PBST plus 2% skim milk. Finally, membranes were washed several times for at least 2 hours. Development was conducted by exposing membranes to chemiluminescence substrate (Perkin Elmer or Lumigen, with different intensities). Emitted luminescence was recorded with a cooled charge-coupled device (Intas, ChemoCam), exposure was in the range of 5 to 20 minutes and regulated depending on signal intensity (16 bit, 64K grayscale). These primary antibodies used were: NLRP3 (cryo-2, Adipogen), ASC (AL177, Adipogen), caspase-1 p20 (casper-1, Adipogen), IL-1 $\beta$  (AF-401, R&D systems), actin (A2066, Sigma-Aldrich), and caspase-8 (1C12, Cell signaling, kindly provided by Dr. Philipp Jost and colleagues).

### ASC speck visualization assays

Mature BMDCs were seeded at  $8 \times 10^5$  cell/mL in an 8-chamber culture slides (Ibidi). Cells were primed with LPS and then subjected to inflammasome stimulation with 5  $\mu$ M nigericin or 50  $\mu$ M imatinib or masitinib for 90 minutes. After stimulation, cells were fixed with IC fixation buffer (eBiosciences) for 30 minutes. Cells were stained with anti-ASC antibody (AL177, Adipogen) diluted in blocking buffer made of PBST and FCS 5%. Following the primary antibody, cells were incubated with an anti-rabbit secondary antibody conjugated with a PE dye and then mounted in Vectashield mounting medium containing DAPI (Vector Laboratories). Confocal microscopy was used to detect ASC specks with a Leica SP8 confocal microscope equipped with a 63 $\times$ /1.40 oil objective (Leica Microsystems). Experiments were conducted in collaboration with Tamara Cíková from our lab.

### Residual potassium assays

Measurement of cellular potassium was conducted with total reflection x-ray fluorescence analysis (TXRF). After BMDCs were stimulated for inflammasome activation, supernatants were used for ELISA cytokine analysis and residual medium was carefully removed from the cells. The cells were then carefully washed with isotonic NaCl solution, and after complete removal, cell on the original plate were stored at -20°C until the next step. Cells were lysed in 25  $\mu$ L of analytical grade HNO<sub>3</sub> 3% solution containing 5  $\mu$ g/mL vanadium as internal standard. From every well, 5  $\mu$ L of lysates were spotted on a silicon wafer and dried by heating. Samples were measured with an Atomika TXRF 8010 device and analyzed using the software Spectra Picofox (Bruker). This experiments were done in collaboration with Stephan Fromm, AG Schuster, Faculty of Chemistry, TUM.

### Flow cytometric measurement of ROS and lysosomal leakage

BMDMs were generated from Vav-Cre - CAR1 transgenic mice and transduced 24 hours with Ad5 adenoviruses (MOI 50) bearing an expression construct for the redox sensing roGFP2 probe that localizes in the cytosol (ViraQuest Inc.). The assay was performed using a FACS Aria II instrument (BD Biosciences) by time-lapse acquisition at 37°C. The first acquisition lasted 3 minutes and recorded the baseline. Next, acquisition was paused, stimuli were added to the cells, mixed well and reloaded on the device and acquisition was continued for another 20 minutes, allowing to detect



ROS generation in real time. Finally, cells were treated with H<sub>2</sub>O<sub>2</sub> to induce maximum ROS formation and acquire the 100% reference signal. Changes in the roGFP2 redox state is detected from a change in emission intensity at different excitation wavelengths. The fluorescent signal has two excitation maxima, at 405 nm for the oxidized form and 488 nm for the reduced form. An increase in the 405/488 nm emission ratio indicates roGFP2 oxidation and indicates a shift in the redox potential due to cytosolic ROS production. To detect lysosomal leakage, BMDMs from ASC knockout mice were incubated for 30 minutes with acridine orange 1 µg/mL. Cells were washed 3 times with phenol red-free HBSS supplemented with 5 mM EDTA and 3% FCS. The analysis was performed with the same procedure described above with the exception that LLOMe 1 µM was used to induce maximal lysosomal disruption. Acridine orange emits a red fluorescent signal (650 nm) when in acidic environment such as the lysosome and green (525 nm) signal when bound to nucleic acids in the cytoplasm or nucleus or other cellular compartments. Any loss of red fluorescence in favor of the green one indicates lysosomal leakage. All samples were measured on a FACS Aria II (BD Biosciences) and data analysis was done using FlowJo (Threestar) software. These experiments were done in collaboration with Dr. Ritu Mishra from our lab.

### Isolation of human PBMCs and inflammasome stimulation

Human PBMCs were isolated from freshly collected venous blood of a healthy volunteer (biosafety level 1) in EDTA tubes. Blood was kept at RT to avoid clogging until extraction of PBMCs. Blood was placed in 50 mL falcons and under layered with Ficoll ( $\rho=1.077$  g/mL, Biochrom AG) at separated interphases. Cells were centrifugated at 400 xg for 30 minutes at 20°C without brakes. After spin, the white buffy coat was collected and cell suspension subjected to RBC lysis. Cells were resuspended in plane RPMI 1640 (without FCS and Pen/Strep) and counted with trypan blue. Cells were seeded at  $4 \times 10^6$  per well on a 96-wells flat bottom tissue-culture treated plate. The plates were incubated for at least 2 hour to allow mononuclear cells to stick to the plate's bottom (other cells will not adhere). Medium was aspirated and wells were washed once with PBS and cultured in 150 µL of RPMI 1640 added with FCS 10%, Pen/Strep, Hepes 25 mM, glutamine 4 mM overnight. PBMCs were primed with 100 ng/mL *E. coli* K12 ultrapure LPS (Invivogen) for 3 hours.

Inflammasome stimulation was carried with nigericin 5  $\mu\text{M}$ , imatinib 50  $\mu\text{M}$  and masitinib 50  $\mu\text{M}$  for 6 hours of incubation.

### Isolation of murine intestinal organoids

Murine intestinal organoids were isolated and cultivated by Dr. A. Pastula from AG Quante, Klinikum rechts der Isar, TUM. The isolation technique was described before by Dr. Pastula (Pastula et al., 2016). Briefly, the small intestines (SI) of wildtype mice were collected and cut in pieces on ice cold PBS plus 10% FCS. Intestinal villi were scraped off from the intestinal biopsies with the help of a microscope glass slide. The remaining tissue was digested in PBS with EDTA 2 mM for 15 minutes at 4°C on a shaker. After digestion, the tissue was washed several times with PBS plus 10% FCS and filtered through a 70  $\mu\text{m}$  cell strainer. The flow through was collected and used to isolate the crypts. The digested biopsies that did not pass the strainer were repeated subjected to the same procedure to extract more material. The crypts were pelleted by centrifugation at 800 xg, 4°C for 8 minutes. The supernatant was removed completely and the pelleted crypts were resuspended in 200  $\mu\text{L}$  of Matrigel (BD Biosciences). Drops of 20  $\mu\text{L}$  crypt-containing Matrigel per well were plated in a pre-warmed 96-wells plate. Culture medium was added on top of every Matrigel drop to support crypts budding and growth. The crypt culture medium is composed of advanced DMEM/F12 (Life Technologies), supplemented with serum free B27 (1:50, Life Technologies), N2 (1:100, Life Technologies), N-acetylcysteine (50 mM, Sigma), recombinant murine epithelial growth factor (EGF 50 ng/mL, Peprotech), Noggin (100 ng/mL, Peprotech), R-Spondin (1  $\mu\text{g}/\text{mL}$ , Peprotech), Glutamax-I supplement (1:100, Life Technologies), Pen/Strep (500  $\mu\text{g}/\text{mL}$ , Life Technologies), and Hepes (10  $\mu\text{M}$ , Life Technologies). Organoids were cultivated for approximately 14 days until medium was changed completely and added with propidium iodide (PI) to a final concentration of 1  $\mu\text{g}/\text{mL}$ . Organoids were stimulated with TKIs at several concentrations ranging from 1  $\mu\text{M}$  to 30  $\mu\text{M}$  for 24 hours. Cell death was analyzed by PI staining with a confocal microscope (Leica).

### Mouse model analysis

Experimental mice developing cancer were left in cages with water and food ab libitum in SPF facilities. Mice were controlled at regular intervals for manifestation of disease symptoms. Mice with Burkitt's lymphoma had evident symptoms of splenomegaly, loss

of body weight and fatigue. Mice with T cell lymphoma were progressively losing control of their rear legs and showed delayed response to gentle teasing. BCR-Abl transplanted mice showed a progressive loss of the walking ability and unresponsiveness. Mice were euthanized when they reached the terminal stage in compliance with the specific score sheets and with European and German rules on experimental animals.

### Flow cytometric assays of sick and control mouse organs

Lymphoid organs (bone marrow, spleen, thymus and lymph nodes) and, ad occurrence, other organs (liver especially) were collected from euthanized mice with cancer and from healthy controls. Lymphoid organs were mashed on a cell strainer to make a single cell suspension. Red blood cell lysis was done with G-DEX II RBC lysis buffer (Intron Biotechnology). Total cell number was counted with trypan blue and annotated. A maximum of  $2 \times 10^6$  cells from each organ were pipetted in wells of a V-bottom 96-wells plate. Cells were washed with FACS buffer (PBS 1x plus FCS 2%) and incubated with 100  $\mu$ L of antibodies stain for 1 hour at 4°C in dark. Cells were washed twice with FACS buffer and optionally fixed, permeabilized, and incubated with intracellular/intranuclear antibodies following manufacturer instructions. Cells were either used immediately or resuspended in IC fixation buffer (eBiosciences) and subsequently stored for a maximum of 48 hours before measurement. Stained cells were collected with a FACS Canto II instrument (BD Biosciences). All antibodies were purchased from eBiosciences and were used following manufacturer recommendations. Antibody mixtures for surface antigens were made in FACS buffer, in permeabilization buffer for intracellular antigens and in FOXP3 buffer for intranuclear antigens.

### Isolation of murine B and T cells

Isolation of B and T lymphocytes was performed with a magnetic separation kit from Miltenyi Biotech. Healthy sex and age matched wildtype and ASC knockout mice were euthanized and spleens and lymphnodes were extracted. After red blood cell lysis, single cell suspensions were counted with trypan blue and used for magnetic separation following kit instructions. Cells were resuspended in MACS buffer and incubated with biotin-conjugated antibodies targeting either CD19 or CD4/CD8. Anti-biotin microbeads were used to bind the antibodies for selective magnetic purification.

Purified cells were resuspended in RPMI 1640 with FCS 10%, Pen/Strep, Hepes 25 mM and glutamine 4 mM, recounted and used for survival experiments. Those cells used for CFSE experiments were stained right after this step.

### Survival experiments with B and T cells

First step is to coat those wells where T cells will be stimulated with anti-CD3 antibody. Plates were incubated overnight at 4°C with 200 µL of 5 µg/mL anti-mouse CD3 antibody dissolved in PBS (BD Biosciences). Coated plates were washed 2 times with PBS before cells were plated. Purified lymphocytes were plated on 96-wells dishes at  $3 \times 10^4$  cells per well in 200 µL of medium. Another 100 µL of medium with or without the selected stimuli were subsequently added. B cells stimuli were anti-mouse CD40 5 µg/mL (Jackson Laboratories), IgM 1 or 10 µg/mL (Jackson Laboratories) and LPS 10 µg/mL (Sigma-Aldrich). Stimuli for T cells were anti-mouse CD3 (BD Biosciences) and CD28 2 µg/mL (BD Biosciences). Cells were harvested after 24, 48 and 72 hours and stained with a AnnexinV/7AAD kit (BD Biosciences). Measurements were acquired with a FACS Canto II instrument (BD Biosciences). Data in the plots derive from the 100% double negative (AnnexinV/7AAD double negative) stained population.

### CFSE experiments with B and T cells

Cells were extracted as described and stained with carboxyfluorescein succinimidyl ester (CFSE) 5 µM in medium for 10 minutes. Cells were washed twice and incubated on 96-wells plates with the same procedure described for survival experiments. CFSE labelled cells were acquired with a FACS Canto II instrument (BD Biosciences) after 48 and 72 hours.

### Isolation of murine keratinocytes

Murine keratinocytes were prepared starting from neonate mice of either wildtype or ASC<sup>-/-</sup> genotype. Skin was peeled off and digested overnight in 10 mL of dispase-HBBS solution (CellnTech). Epidermis was separated from the dermis with forceps and the epidermis was delicately washed in PBS several times to remove completely the remaining dermis, which has a pasty consistence. Before the next step, epidermis sheets were quickly rinsed in ethanol to remove any bacterial contamination and washed in sterile PBS to remove alcohol. Epidermis sheets were digested in accutase (Sigma-Aldrich) for 30 min at room temperature on a Petri dish. Then, sheets were

gently rubbed with forceps on the dish bottom to extract single cells out of the layer. Extracted cells were collected and washed with PBS several times and counted with trypan blue. Cells were seeded on tissue culture-treated 6-wells plates at  $4 \times 10^4$  cells/cm<sup>2</sup> in CnT-07 medium (CellnTech) and kept in culture following the recommendations of the provider (CellnTech).

### Apoptosis experiments with murine keratinocytes

Mature keratinocytes were harvested at confluency and re-plated on separated 6 cm dishes 3 hours before exposition to stimuli. Once cells became adherent, they were dried and quickly stimulated with 200 mJ/cm<sup>2</sup> using a UV Stratalinker (Stratagene). Cells were resuspended in medium and put back in incubator. Dishes of the two genotypes were harvested at 30 minutes, 1, 2 and 4 hours with relative controls. Cells were collected and lysed in Laemmli buffer added with dithiothreitol (DTT). Samples were loaded on a 15% polyacrylamide SDS-PAGE gels and then transferred to nitrocellulose membranes as described above. Blots were developed to detect the phosphorylated H2A.x (2577, Cell Signaling), phosphorylated p53 (P53-CM5P-L, Novocastra), ASC (AL177, Adipogen), and tubulin (T5168, Sigma-Aldrich).

### Bone marrow transplantation to induce oncogenesis

The bone marrow transplantation to induce oncogenesis with a p210 BCR-Abl construct was described in detail before (Miething et al., 2006). Briefly, the pMIG-BCR-Abl p210 construct bearing an EGFP reporter was used to transfect HEK293T cells together with other plasmids encoding a murine ecotropic packaging system (GAG-POL and ENV). Viral supernatants were harvested after 48 hours and filtered with a 0.45 µm filter to remove any residual cell. Virus titer was calculated by transduction of 3T3 murine fibroblasts, analyzed by FACS, as percentage of GFP-positive cells. Bone marrow of C57BL/6 (Janvier) from either wildtype or ASC knockout mice were harvested 4 days after injection with 150 mg/kg 5-fluorouracil (5FU). Stem cells were cultivated and stimulated for 24 prior transplantation in stem cell medium, consisting of IMDM, 20% FCS, Pen/Strep, murine IL-3 10 ng/ml, IL-6 10 ng/ml, stem cell factor (SCF) 50 ng/ml (R&D Systems). Cells were harvested and transduced with spin infection every 12 hours for a total of 4 times with viral supernatants (MOI 5). The medium was replaced after every spin. Prior to transplantation, transduction efficiency was determined by EGFP signal, at least  $2 \times 10^5$  green cells were mixed with  $5 \times 10^6$

healthy wildtype bone marrow per recipient animal. Recipient mice were subjected to 11 Gy total body irradiation and transplanted via tail vein injection. Transplanted mice were put back in cages with oatmeal and antibiotics-enriched water (sulfamethoxazole/trimethoprim 95 mg/kg/24h, and tetracycline 50-60 mg in liter drinking water) *ab libitum* for 1 week, then substituted with normal food and water. Mice were monitored regularly for weight loss/gain and appearance of disease symptoms. Peripheral blood was collected after 10 days and analyzed with flow cytometry (BD Biosciences) to detect EGFP signal in transplanted mice.

# RESULTS

## Project one results

### Imatinib induces IL-1 $\beta$ secretion in primary murine myeloid cells

To address the question whether imatinib is an inflammasome activator or not, we started by testing the drug on primary innate immune cells. Our lab has a long expertise with *in vitro* inflammasome tests. We usually perform experiments using either bone marrow derived dendritic cells (BMDCs) or bone marrow derived macrophages (BMDMs). In general, dendritic cells are preferred to macrophage as they secrete more IL-1 $\beta$  per cell (O. Gross, 2012). I started by testing the drugs on LPS-primed BMDCs in a wide range of concentrations, from 1  $\mu$ M to 100  $\mu$ M, that is the range in which most of the inflammasome stimuli are effective (Schneider et al., 2013).

As expected, upon LPS-priming and treatment with the solvent DMSO alone, BMDCs did not release any detectable IL-1 $\beta$ . However, upon stimulation for 3h, the cells exhibit a prominent IL-1 $\beta$  secretion in response to both TKIs like that induced by established inflammasome stimuli and by R837 (Figure 4A). IL-1 $\alpha$  secretion follows the same trend over the range of concentrations as observed for IL-1 $\beta$ , indicating that both cytokines are released due to of the same cellular activation event. This is in line with previous results from our group, where we showed that IL-1 $\alpha$  secretion is a normal consequence of inflammasome activation even though it is cleaved by calpains and not by caspase-1 (O. Gross et al., 2012) (Figure 4B). By analyzing the same samples by western blot, we can detect the mature form of caspase-1 (p20) in the supernatant of imatinib-treated cells, which is one of the main experimental hallmarks of inflammasome activation (Figure 4C) (Martinon et al., 2002). All these data indicate that these TKIs activate the inflammasome in dendritic cells.

Lactic dehydrogenase (LDH) release is the main marker to quantify lytic cell death. Our samples show a prominent release of LDH, indicating that cells are undergoing lytic cell death to a similar or even higher extent as compared to established inflammasome activators (Figure 4D). Furthermore, we observe a release of  $\beta$ -actin from the cells to the supernatant both with imatinib as well as with established inflammasome activators, but not in untreated control cells (Figure 4C). This shift is

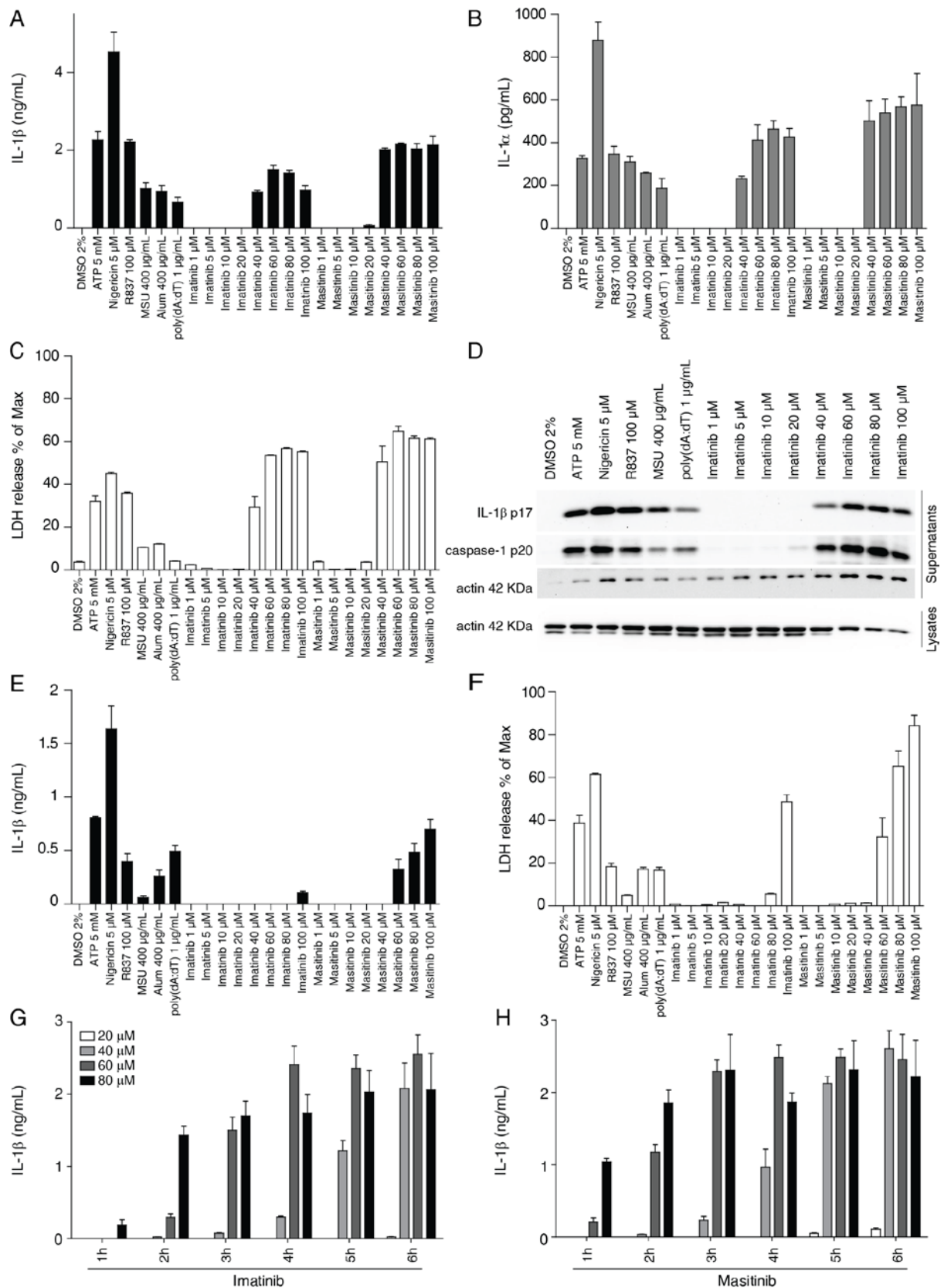
the more evidenced at the higher the TKI concentration tested. This suggests a TKI-induced mechanism of cell death that involves cell membrane destabilization (Figure 4D).

Mouse macrophages are also responding to imatinib and masitinib with the same trend as seen in dendritic cells, although somewhat higher TKI concentrations were required. As expected, macrophages in general secreted less IL-1 $\beta$  than BMDCs despite a similar degree of cell death, indicating that the difference between the two cell types resided in differences in the amounts of pro-IL-1 produced upon priming (Figure 4E, F).

We also performed a kinetic test to identify the best concentrations and incubation times for both drugs. After several repetitions, we decided to continue our tests with an incubation time between 3 and 4 hours. Also, we usually used four different concentrations: 20, 40, 60, and 80  $\mu$ M to see how cells react at higher drug dosage. We started from 20  $\mu$ M because it is usually the lowest drug amount that can generate a detectable IL-1 $\beta$  signal (Figure 4G, H). One remarkable aspect is that IL-1 $\beta$  secretion is triggered with a very tight concentration threshold. Indeed, at 20  $\mu$ M both TKIs make cells secrete little to no IL-1 $\beta$  but already at 40  $\mu$ M they secrete the maximum amount (Figure 4A). This could indicate that TKIs need a trigger event to activate the inflammasome that may be happening when concentrations reach the threshold of circa 40  $\mu$ M.

In general, as observed in repeated experiments, masitinib always leads to a little higher IL-1 $\beta$  secretion at the same compound concentration and is active at a somewhat lower concentration as compared to imatinib. Taken together these data show that imatinib and masitinib are two potent inflammasome activators that need to pass a threshold concentration to induce prominent IL-1 $\beta$  release and lytic cell death.





**Figure 4: imatinib and masitinib stimulate dendritic cells and macrophages to secrete IL-1 $\beta$ .** (A, B) BMDCs from wildtype mice were primed for 3h with 20 ng/ml LPS and subsequently stimulated with typical NLRP3 inflammasome activators, imatinib and masitinib for 3h as indicated. (A) IL-1 $\beta$  and (B) IL-1 $\alpha$  secretion was quantified from cell-free supernatants by ELISA. (D) The same supernatants as in (A), (B) were used to quantify cell death by LDH release, depicted as percentage of complete cell lysis

(100% death = cells lysed in 0.5% Triton X-100). (C) Immunoblot from cell culture supernatants and cell lysates from imatinib-treated BMDCs. Blots were developed to detect the cleaved form of IL-1 $\beta$ , caspase-1 and actin as internal standard. (E, F) LPS-primed BMDMs from wildtype mice were stimulated for 3h with control inflammasome activators, imatinib, and masitinib as indicated. ELISA (E) and LDH release analysis (F) were used to quantify IL-1 $\beta$  secretion and cells death, respectively. (G, H) LPS-primed BMDCs from wildtype mice were stimulated with imatinib (G) and masitinib (H) as indicated. ELISA was used to quantify IL-1 $\beta$  secretion. All data are depicted as mean  $\pm$  SD of technical triplicates.

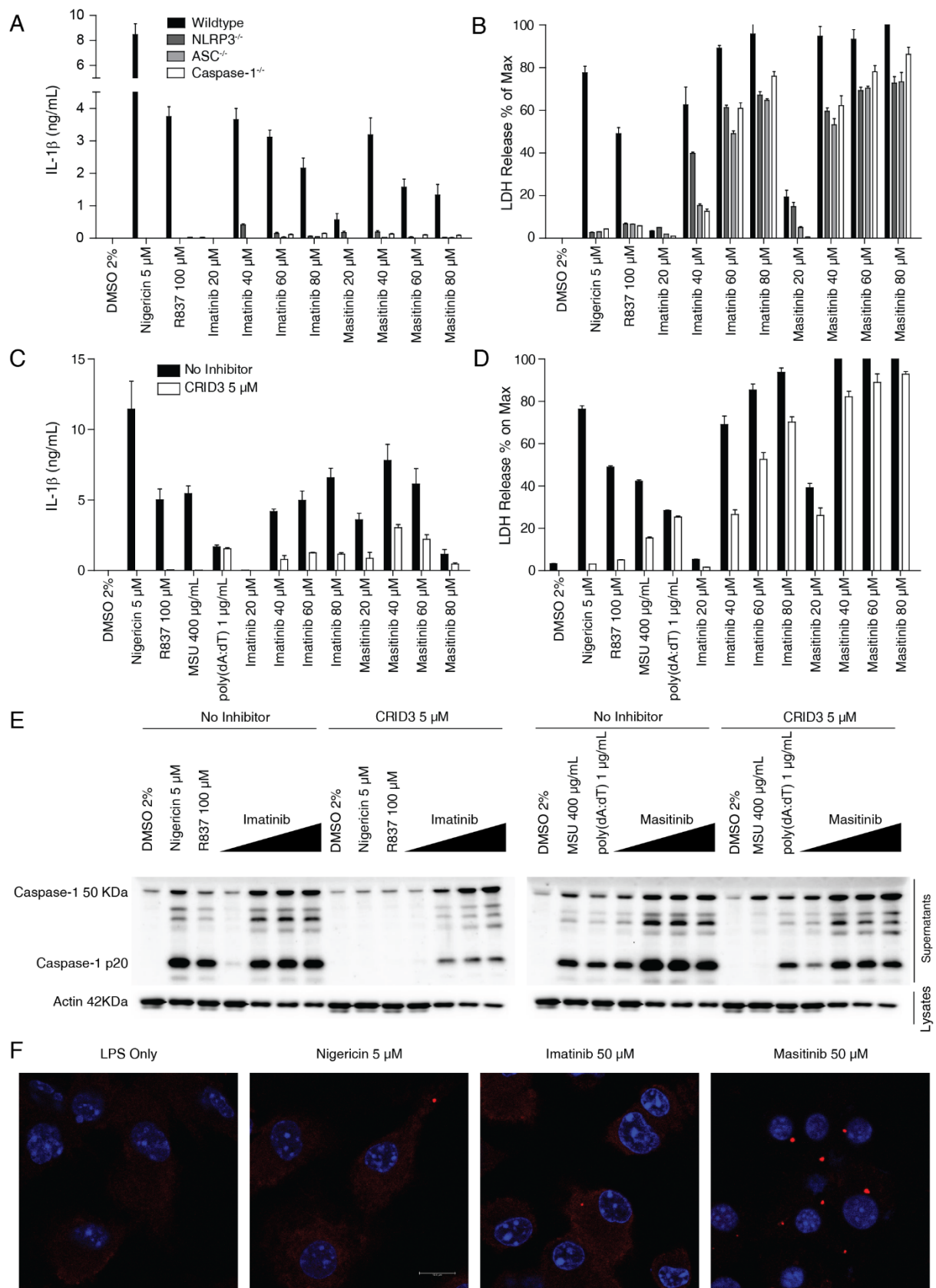
### Imatinib activates the NLRP3 inflammasome

As TKIs are inducing significant secretion of IL-1 $\beta$ , we had next to clarify if this is due to inflammasome activation and what inflammasome sensor might be involved. Since R837 as a small molecule activates NLRP3, we speculated that this sensor is involved. BMDCs generated from either wildtype or different inflammasome knockout mice were tested. The secretion of IL-1 $\beta$  was strongly reduced in NLRP3, ASC, and caspase-1 deficient cells as compared to wildtype even at the highest TKI concentrations (Figure 5A). However, NLRP3 consistently showed a residual signal, suggesting that there might be a second inflammasome sensor involved. Furthermore, there is also a residual signal for caspase-1 knockout cells, that might reflect the involvement of other caspases cleavage, potentially caspase-8 (Figure 5A). The two control compounds Nigericin and R837 show a profile in line with previous results (C. J. Gross et al., 2016). The LDH release assays confirmed that imatinib causes a lytic form of cell death, getting closer to maximum signal as the drug concentration increases (Figure 5B). For most NLRP3 activators, lytic cell death by pyroptosis is inflammasome-dependent. However, remarkably, although LDH release is significantly reduced in inflammasome knockouts upon TKI-treatment, there is a clear residual signal that reaches almost the values of wildtype cells at higher TKI concentrations. This indicates that imatinib and masitinib trigger inflammasome-independent mechanism to induce lytic cell death although IL-1 $\beta$  maturation is clearly inflammasome-dependent (Figure 5A, B). This is emphasized by the observation that, despite both TKIs induce the release of comparable IL-1 $\beta$  levels as the standard activators like nigericin or R837, they consistently induce more cell death (Figure 5A, B, C, D).

The small molecule CRID3 (also known as MCC950) is a potent and selective small molecule inhibitor of the NLRP3 inflammasome (Coll et al., 2015). We tested the TKIs in combination with CRID3 pretreatment and saw a significantly reduced secretion of IL-1 $\beta$  for both TKIs (Figure 5C). However, for both drugs there is a detectable residual

signal as observed in NLRP3 knockout cells. Of note, the control stimuli that activate NLRP3 (nigericin, R837, and MSU) are completely inhibited but poly(dA:dT), an activator of the AIM2 inflammasome, retains complete IL-1 $\beta$  secretion even in the presence of CRID3 (Figure 5C). Cell death with CRID3 pretreatment is only partially reduced for both compounds. In the case of imatinib there is some decrease in LDH release observed at lower concentrations, comparable to the effect CRID3 has on cell death induced by MSU crystals (Figure 5D). Cell death upon masitinib treatment is minimally reduced by CRID3 for each concentration tested. As expected, cell death by classic NLRP3 stimuli is strongly inhibited by CRID3 but those treated with poly(dA:dT) die as much as negative controls (Figure 5D). These data further support the possibility that cell death is mainly driven by an inflammasome-independent mechanism in response to the TKIs (Figure 5B, D). In line with inflammasome inhibition, the western blot analysis of CRID3 pretreated cells reveals an impaired caspase-1 cleavage and secretion (Figure 5E). Caspase-1 cleavage is significantly diminished in the cell lysates, indicating that CRID3 inhibits the inflammasome before caspase-1 associates to it, as reported previously (Perregaux et al., 2001).

A hallmark of inflammasome activation is the generation of detectable speck structures with a size of circa 1  $\mu$ M (Fernandes-Alnemri et al., 2007). Wildtype BMDCs were primed with LPS and then either left untreated or treated with nigericin, imatinib, or masitinib. The speck structures are labeled with anti-ASC antibodies coupled with a fluorescent dye. ASC specks appear as small intense dots detectable by means of fluorescence microscopy. With exception for the LPS control, all the samples where the inflammasome was stimulated exhibited speck formation (Figure 5F). Masitinib is confirmed as a very strong activator as it could generate a higher frequency of specks than any other compound tested in this experiment. Taken together, these data demonstrate that TKIs can trigger the NLRP3 inflammasome, however, a residual signal in NLRP3 but not ASC knockout cells is still detectable. This may come either from another inflammasome, (as ASC knockouts lack any IL-1 $\beta$  secretion) or from a non-inflammasome IL-1 $\beta$  cleavage. Furthermore, cells die because of a strong lytic form of death that seems to be largely independent of the inflammasome. This indicates that inflammasome-dependent pyroptosis is only partially involved in the death mechanism engaged by TKIs.



**Figure 5: Imatinib and Masitinib are NLRP3 inflammasome activators.** (A, B) LPS-primed BMDCs from wildtype, NLRP3, ASC, or caspase-1 knockout mice were stimulated with control activators plus imatinib and masitinib for 3h as indicated. (A) ELISA was used to evaluate IL-1 $\beta$  secretion and (B) LDH

release was used to evaluate cell death. (C, D) LPS-primed BMDCs from wildtype mice were treated with either no inhibitor or CRID3 5  $\mu$ M 30 min prior stimulation. Stimulation was carried for 3h with control activators plus imatinib and masitinib as indicated. Data are depicted as mean  $\pm$  SD of technical triplicates. (E) Immunoblot of supernatants and cell lysates from (C) and (D). Blots were developed to detect the cleaved form of caspase-1 and actin as internal standard. (F) LPS-primed BMDCs from wildtype mice were stimulated with nigericin, imatinib and masitinib as indicated for 90 minutes. Microscopy pictures were taken on fixed BMDCs. Red dye stains for ASC, blue dye is DAPI and stains nuclei. Specks are visible as small intense red dots. The speck experiment was done in collaboration with Tamara Cíková, AG Groß.

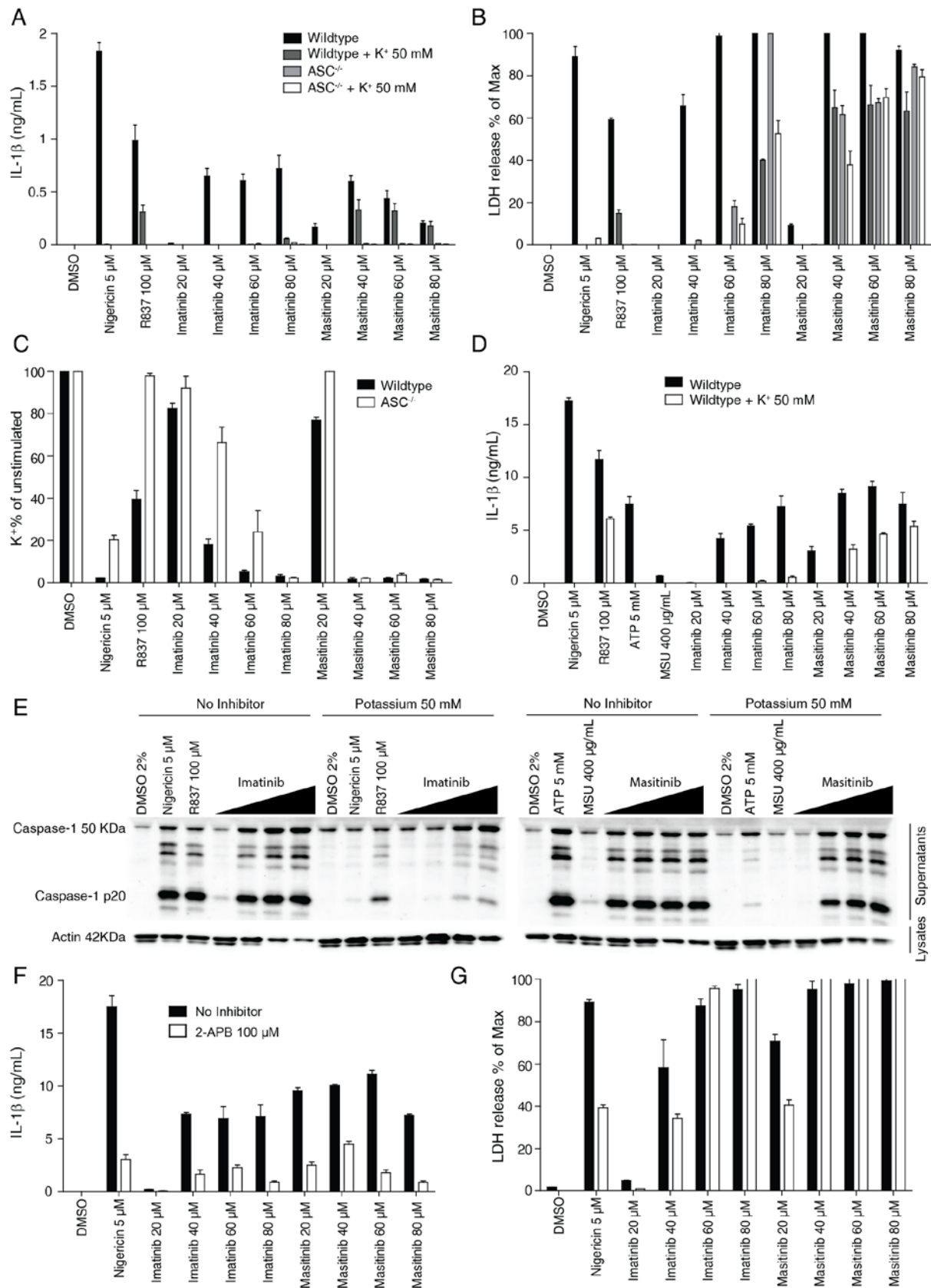
### Imatinib activates NLRP3 through a K<sup>+</sup> dependent mechanism

Potassium (K<sup>+</sup>) efflux was identified early on as a major upstream event and was recently proposed as an indispensable factor in NLRP3 inflammasome assembly (Franchi, Kanneganti, Dubyak, & Nunez, 2007; Munoz-Planillo et al., 2013; Petrilli et al., 2007). However, our group showed that R837 can activate NLRP3 in the absence of K<sup>+</sup> efflux (C. J. Gross et al., 2016). We wanted to see whether imatinib and masitinib require K<sup>+</sup> efflux. To test this, we inhibited K<sup>+</sup> efflux by treating LPS-primed BMDCs with 50 mM KCl for 30 minutes prior to stimulation with TKIs or control stimuli. As previously reported, this treatment fully inhibited IL-1 $\beta$  secretion and LDH release upon stimulation with the bacterial pore-forming toxin and K<sup>+</sup> ionophore nigericin, while inflammasome activation by R837 was still possible. Imatinib was found to be K<sup>+</sup> efflux-dependent, as increased extracellular concentrations of KCl can block IL-1 $\beta$  secretion comparably to ASC deficiency (Figure 6A, B). Surprisingly, masitinib, at most doses tested, displays substantial IL-1 $\beta$  secretion even in presence of high KCl dosage, comparable to R837, while only at the lowest dose that induces little IL-1 $\beta$  secretion the signal appears to be K<sup>+</sup> efflux-dependent (Figure 6A).

To elucidate the connection of K<sup>+</sup> efflux and TKI-induced inflammasome activation in more detail, we directly measured the cellular elemental potassium levels from the same experiments in wildtype and ASC knockout BMDCs stimulated with TKIs or control stimuli. As expected, nigericin induced K<sup>+</sup> efflux was inflammasome independent while R837 did not induce any K<sup>+</sup> efflux in inflammasome-deficient cells, demonstrating that the inflammasome activation observed cannot be a result of K<sup>+</sup> efflux. However, there is a comparatively small loss of K<sup>+</sup> in R837-treated wildtype cells that must be a consequence of pyroptosis and GSDMD pore formation as was reported for the alternative, Caspase-11 mediated NLRP3 activation pathway that involves GSDMD-dependent potassium efflux upstream of NLRP3 activation (cite

papers from Broz, Hornung). Indeed, higher exposure times used in these experiments correspond to a more pronounced feedback loop of pyroptosis, GSDMD, pore formation and potassium efflux that reduces the potassium levels of wildtypes cells. This GSDMD - potassium feedback loop is also the most likely explanation of the somewhat reduced levels of IL-1 $\beta$  upon R837 stimulation in the presence of KCl as compared to our previous study where shorter incubation times were used, which would reduce the influence of potassium-dependent feedback loops (C. J. Gross et al., 2016). Also in line with K<sup>+</sup> efflux-dependent IL-1 $\beta$  secretion, imatinib shows a gradual loss of potassium along with increasing drug concentrations that is somewhat delayed in ASC-deficient cells. This indicates that at lower concentrations, the inflammasome contributes to K<sup>+</sup> efflux through pyroptosis while at higher concentrations, lytic cell death prevails and ion efflux become fully inflammasome independent (Figure 6C). In contrast, although masitinib-induced IL-1 $\beta$  secretion is not K<sup>+</sup> efflux-dependent, it provokes a remarkable potassium loss in most of the conditions tested (Figure 6C). This is in line with masitinib's ability to induces a largely inflammasome-independent cell death that would lead to ion efflux. However, at the minimal concentration of masitinib required (20  $\mu$ M) the pattern of IL-1 $\beta$  secretion and potassium efflux correlates with that of imatinib in that IL-1 $\beta$  secretion is K<sup>+</sup> efflux-dependent and that K<sup>+</sup> efflux is partially inflammasome-dependent (Figure 6A, C, D). The fact that, in contrast to R837, there is substantial potassium efflux independent of the inflammasome at imatinib concentrations of 40 and 60  $\mu$ M together with the fact that inhibition of potassium efflux block inflammasome activation indicates that (like caspase-11 activation) imatinib induced a form of lytic cell death leaning to potassium efflux and subsequent inflammasome activation. Higher concentrations of masitinib overcome the potassium requirement, indicating that this TKI has a second mode of action that does not involve potassium efflux, although lytic cell death for masitinib is largely inflammasome independent. The experiment depicted in 6D was performed to have a new set of cell lysates for western blots as those of figure 6A, B and C were used to measure elemental potassium levels. In this experiment the independency of R837 and masitinib from potassium efflux to trigger IL-1 $\beta$  secretion is confirmed once again (Figure 6D). The blots show clearly the need for potassium efflux by imatinib to activate the inflammasome and that masitinib 20  $\mu$ M has a pattern comparable to that of imatinib as stated previously (Figure 6E).

I next tested if  $\text{Ca}^{2+}$  influx-induced signaling contributes, using 2-aminoethoxydiphenyl borate (2-APB), a compound that inhibits inositol phosphate-3 (IP3) receptors and transient receptor potential channels (TRP). 2-APB was already used to inhibit NLRP3 inflammasome in other studies (G. S. Lee et al., 2012). 2-APB is good in blocking  $\text{IL-1}\beta$  secretion for both TKIs, suggesting TKIs may also trigger calcium signaling to activate NLRP3 (Figure 6F). However, this inhibitor could reduce LDH release only for control stimuli and not for TKIs, indicating that the inflammasome-independent cell death pathway engaged does not require the activation of calcium-dependent protease (Figure 6G). Taken together, these experiments demonstrated that imatinib requires potassium efflux to trigger inflammasome activation but not cell death. Masitinib can activate the inflammasome independently of potassium, however, it does provoke a large efflux of it rather depending on the mechanism of cell death.



**Figure 6: Imatinib activates NLRP3 through a potassium-dependent mechanism.** (A, B) LPS-primed BMDCs from wildtype and ASC knockout mice were either left untreated or treated with KCl 50 mM 30 min prior stimulation. Cells were then stimulated with control activators, imatinib, and masitinib for 3h. The IL-1 $\beta$  secretion (A) was measured by ELISA and cell death (B) was measured by LDH



release. (C) Intracellular potassium concentrations were measured from cells from (A, B). Data are represented as percentages of DMSO control. (D) LPS-primed BMDCs from wildtype were treated with no inhibitor or with potassium. Cells were stimulated as indicated for 3h. ELISA was used to evaluate IL-1 $\beta$  secretion. (E) Immunoblot of supernatants and cell lysates from (D). Blots were developed to detect the cleaved form of caspase-1 as well as b-actin as internal standard. (F, G) LPS-primed wildtype BMDCs were treated with no inhibitor or with potassium 50 mM, 2-APB 100  $\mu$ M or Glycine 5 mM 30 min prior to stimulation as indicated. Cell free supernatants were used to measure (F) IL-1 $\beta$  secretion by ELISA and (G) cell death by LDH release. Data are depicted as mean  $\pm$  SD of technical triplicates. The experiment depicted in (C) was conducted with the collaboration of Stephan Fromm, AG Schuster (TUM).

### Imatinib triggers mild ROS production

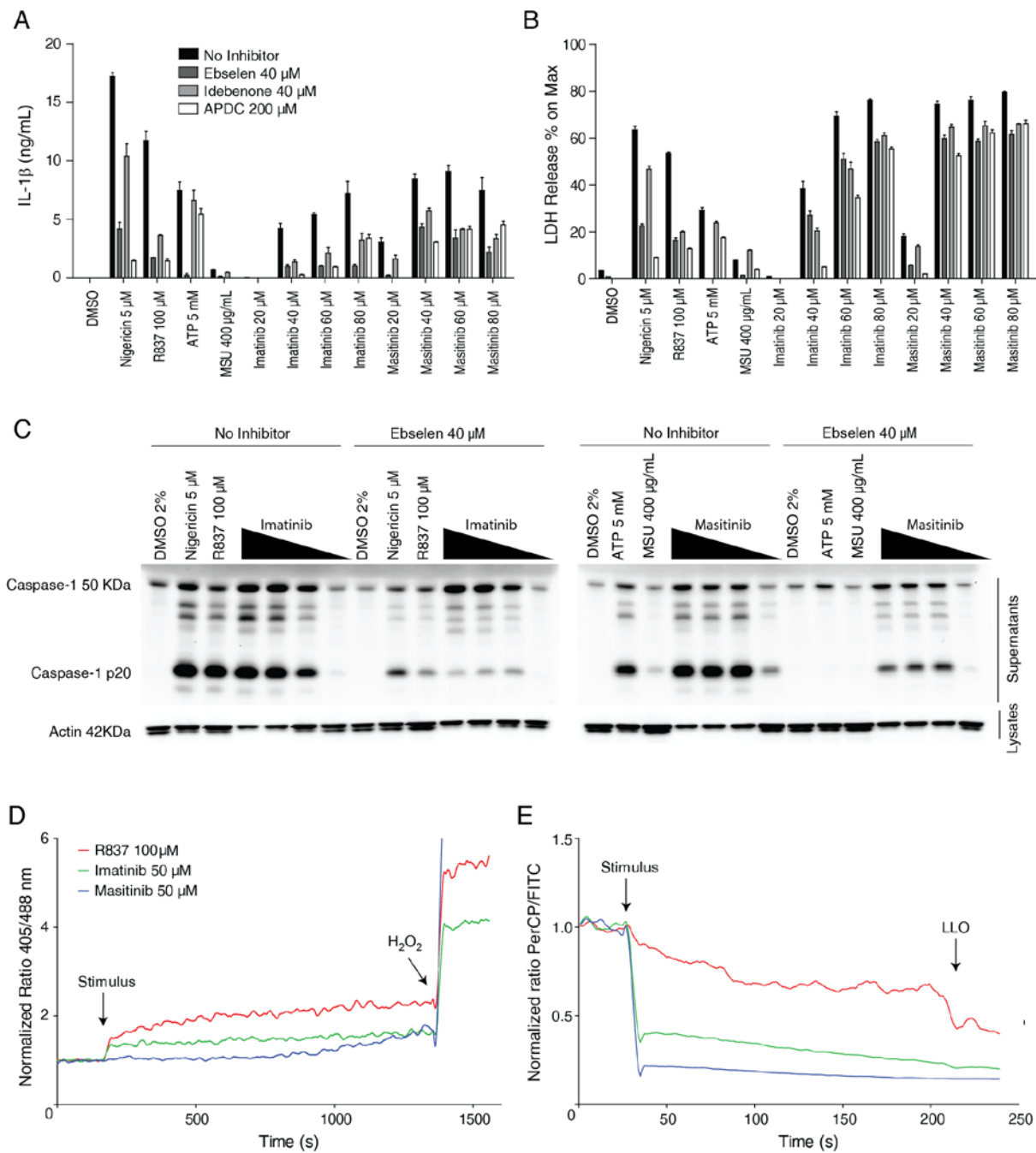
We next sought to determine whether ROS production is involved in inflammasome activation by TKIs. We tested BMDCs stimulated with imatinib or masitinib in the absence or presence of ROS inhibitors, added 30 minutes' prior stimulation (Figure 7A, B). As ROS inhibitors, we had the glutathione peroxidase mimetic ebselen and the ROS scavenger (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC), whose efficacy to inhibit NLRP3 was observed before (O. Gross et al., 2012). Our group found that R837 is a complex-I inhibitor (C. J. Gross et al., 2016), and we asked ourselves if the TKIs may share the same ability. To do so, we used a third compound named idebenone, a synthetic analog of coenzyme Q10 used to treat a rare form of mitochondrial inherited disease named Leber's hereditary optic neuropathy (LHON). These patients have a point mutations in their mitochondrial DNA that causes complex-I dysfunction. Idebenone is a synthetic quinone that can circumvent defects of complex I by delivering electrons to complex III, restoring the full functionality of ATP-synthase (Erb et al., 2012). Cells stimulated with imatinib released less IL-1 $\beta$  in the presence of all the three inhibitors tested (Figure 7A). Masitinib is also inhibited, but to a minor extent at most concentrations. However, as observed before, at 20  $\mu$ M concentration, the pattern of masitinib follows that of imatinib at higher concentrations. These data indicate that ROS production plays a, albeit minor, role in TKIs induced inflammasome activation that is more apparent at lower compound doses and might involve ROS production from mitochondria. Instead, LDH release was not much affected by any of the three inhibitors except maybe at the lowest doses, telling us that ROS production does not play a major role in the cell death mechanisms triggered by the TKIs. We confirmed that R837 induces mainly mitochondrial ROS since bypassing complex-I as well as general ROS inhibition is most effective in blocking both IL-1 $\beta$  secretion and cell death for this activator (Figure 7A, B). Moreover, western blot

analysis reveals that ebselen suppresses the release of cleaved caspase-1 into the supernatants, indicating that the compound can interfere with inflammasome assembly (Figure 7C).

To directly evaluate the amount of ROS production, we transduced CAR1-positive BMDMs with an adenoviral construct bearing a variant of GFP, named roGFP2, that can sense intracellular redox potential (C. J. Gross et al., 2016; Waypa et al., 2010). The roGFP2 bears two extra cysteines in its  $\beta$ -barrel structure that can form a disulfide bond. ROS oxidizes the cysteines and they form a disulfide bond. The ratio between 405/488 nm fluorescence emission changes in function of the redox status of the compartment (Waypa et al., 2010). As expected, R837 induced massive ROS production. Contrastingly, imatinib generates only little cytoplasmic ROS and masitinib even less or only at later time points (Figure 7D). This is in line with results for IL-1 $\beta$ , as they were partially reduced for imatinib and just a little for masitinib when cells were treated with ROS inhibitors (Figure 7A).

As a final test, we used ASC deficient BMDM labeled with acridine orange. This dye accumulates in the acidic compartments of the lysosomes, where it emits a red fluorescent signal (650 nm). The dye that remains in the cytosol or access the nucleus fluoresces green (525 nm). Any loss of red fluorescence indicates lysosomal leakage. In line with published results, R837 showed a good lysosomal leakage (C. J. Gross et al., 2016) but the TKIs showed an immediate loss of signal (Figure 7E). It is difficult to interpret this result but, on a first sight, one may say these compounds provoke a very quick and complete lysosomal rupture. However, such a steep drop in the fluorescence intensity may also mean that TKIs provoke a quick and critical membrane disaggregation and therefore we are observing a side effect. Further experiments are required to clarify this point.

So far, all the data indicate that imatinib activates the NLRP3 inflammasome through a potent potassium (and eventually other ions) efflux with a contribution ROS production. However, for the TKI cell death is largely unaffected by ROS inhibition since we see normal LDH release. Importantly, even though cell death is inflammasome independent at higher TKI concentrations and inhibitors of upstream events of NLRP3 activation are more effective at lower doses, TKIs-induced IL-1 $\beta$  production remains fully inflammasome dependent and largely NLRP3-dependent at any dose and time-point tested (Figure 5-7).



**Figure 7: imatinib and masitinib do not activate massive ROS production.** (A, B) LPS-primed BMDCs from wildtype and ASC knockout mice were treated with no inhibitor or Ebselen 40  $\mu$ M, Idebenone 40  $\mu$ M or APDC 200  $\mu$ M, 30 min prior stimulation. Cells were then stimulated for 3h as indicated. (A) IL-1 $\beta$  secretion was evaluated with ELISA. (B) LDH release was used to quantify cell death. Data are depicted as mean  $\pm$  SD of technical triplicates. (C) Immunoblot of supernatants and cell lysates from (A) and (B). Blots were developed to detect the cleaved form of caspase-1 and actin as internal standard. (D) BMDM from CAR1 mice were transduced with an adenoviral vector encoding roGFP2 and were monitored by flow cytometry for a time-lapse ratiometric measurement upon stimulation with 100  $\mu$ M R837 or imatinib or masitinib 50  $\mu$ M. H<sub>2</sub>O<sub>2</sub> served as a control compound, inducing high ROS production. (E) ASC-deficient BMDMs labeled with acridine orange were analyzed by flow cytometry for a time-lapse ratiometric measurement to evaluate lysosomal leakage. LLO is a control compound that triggers maximal lysosomal rupture. The experiments depicted in (D) and (E) were conducted in collaboration with Dr. Ritu Mishra, AG Groß.

## Caspase protease activity-independent cell death induced imatinib

To study the mechanism behind TKI-induced cell death and inflammasome activation in more detail, we tested the pan-caspase inhibitor zVAD-fmk and the more specific caspase-1 inhibitor YVAD-cmk. The inhibitors were applied 30 min prior inflammasome stimulation. Both inhibitors can block effectively IL-1 $\beta$  secretion in a way comparable to caspase-1 deficient cells, as it was reported previously (O. Gross et al., 2012) (Figure 8A). However, both zVAD and YVAD treated cells retain a IL-1 $\beta$  residual signal like we saw in this experiment and before in the case of caspase-1 deficient cells (Figure 5A).

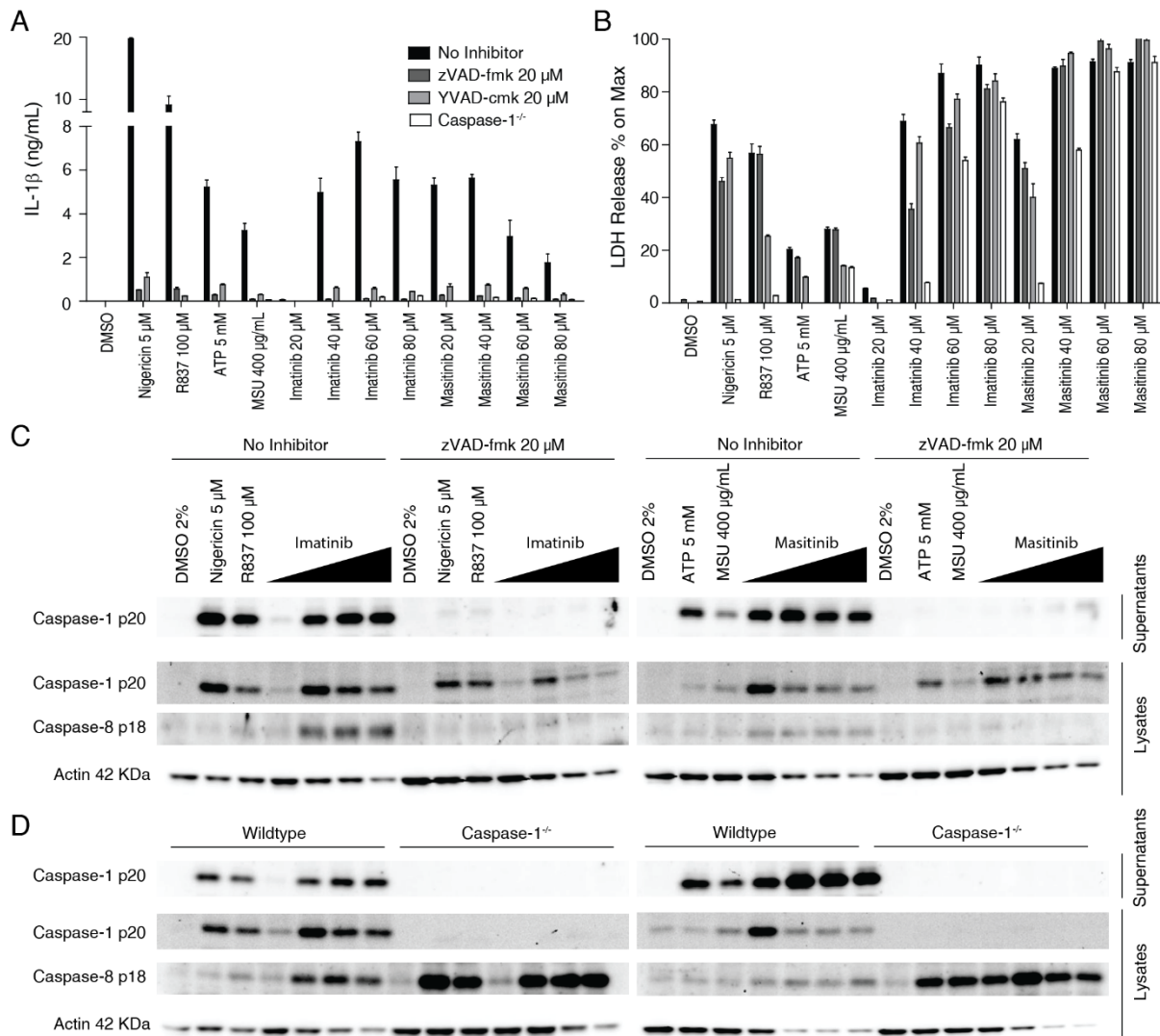
We next analyzed the same samples by western blot to detect caspase-1 cleavage (Figure 8C, D). As expected zVAD potently inhibited the release of the mature form of caspase-1 induced by imatinib and masitinib. I also analyzed caspase-8 cleavage because I thought its activity could be the cause of that IL-1 $\beta$  residual signal found caspase-1 deficient cells. Caspase-8 was initially implicated in FAS-driven extrinsic cell death. However, numerous reports demonstrated that caspase-8 plays a fundamental role in necroptosis and other forms of programmed cell death. More recently, caspase-8 was also proposed as a modulator of several pro-inflammatory processes including NF $\kappa$ B and inflammasome activation (Vince & Silke, 2016).

Imatinib can also trigger caspase-8 cleavage, as detectable in the cell lysates. When cells are pretreated with zVAD, caspase-8 is not cleaved anymore and caspase-1 is less but still cleaved in the cells, and is not released to the supernatant. This may indicate that the inhibitor cannot completely shut down the maturation of caspase-1, but the residual enzyme is not enough to cause complete cell lysis thus preventing its release. However, as some caspase-1 is still cleaved, this may explain why there is little IL-1 $\beta$  signal detected by ELISA (Figure 8C, A). The same is true for masitinib with the exception that caspase-8 cleavage is barely detectable and may not play a determinant role in this death mechanism.

In caspase-1 deficient cells, there is a stronger signal observed for cleaved caspase-8 upon stimulation by both TKIs as well as control inflammasome activators (Figure 8D). The LDH release shows that zVAD and YVAD could not prevent cell death neither for TKIs nor for control stimuli. This is in line with published data, as it was already shown that when the inflammasome is activated, both zVAD and YVAD can divert cells

toward a programmed necrotic cell death to compensate loss of pyroptotic pathway (Cullen, Kearney, Clancy, & Martin, 2015).

These data indicate two things. First, caspase-8 is cleaved upon imatinib stimulation together with inflammasome activation, probably as it joins speck formation as was recently proposed (Fu et al., 2016). Also, I speculate that when caspase-8 joins the speck it contributes, although in minor role, to the maturation of IL-1 $\beta$  because we have a residual signal in caspase-1 knockout cells. Second, caspase-1-deficiency (Figure 5) and caspase-8 blockade does not rescue cell death as BMDCs released high levels of LDH even under these conditions. Ineffectiveness of zVAD and strong, early LDH release support the notion that the cells death pathway induced by the TKIs is not apoptosis. This could mean that the two drugs can trigger several death pathways at once and lead to lytic cell death, even if pyroptosis is blocked. Cumulatively, these results show that caspase-8 may be involved in the inflammasome complex with different modalities as it was already proposed by several research groups or that the TKIs, in contrast to classic NLRP3 activators, activate caspase-8 in an inflammasome-independent manner (Vince & Silke, 2016).



**Figure 8: Imatinib and masitinib do not trigger apoptosis nor another form of cell death based only on caspases.** (A, B) LPS-primed BMDCs from wildtype were treated with no inhibitor or ZVAD-fmk 20  $\mu$ M or YVAD-cmk 20  $\mu$ M 30 min prior stimulation. LPS-primed BMDCs from caspase-1 knockout mice were also included as controls. Cells were then stimulated with control activators plus imatinib and masitinib as indicated for 3h. (A) IL-1 $\beta$  secretion was evaluated by ELISA. (B) An enzymatic assay for LDH activity was used to quantify its release from the cells and thereby, to quantify cell death. LDH release is depicted as percentage of complete cell death. Data are depicted as mean  $\pm$  SD of technical triplicates. (C, D) Immunoblot of supernatants and cell lysates from (A) and (B). Blots were developed to detect the cleaved form of caspase-1, caspase-8, and actin as internal standard.

### Imatinib does not induce RIPK3/MLKL-dependent necroptosis

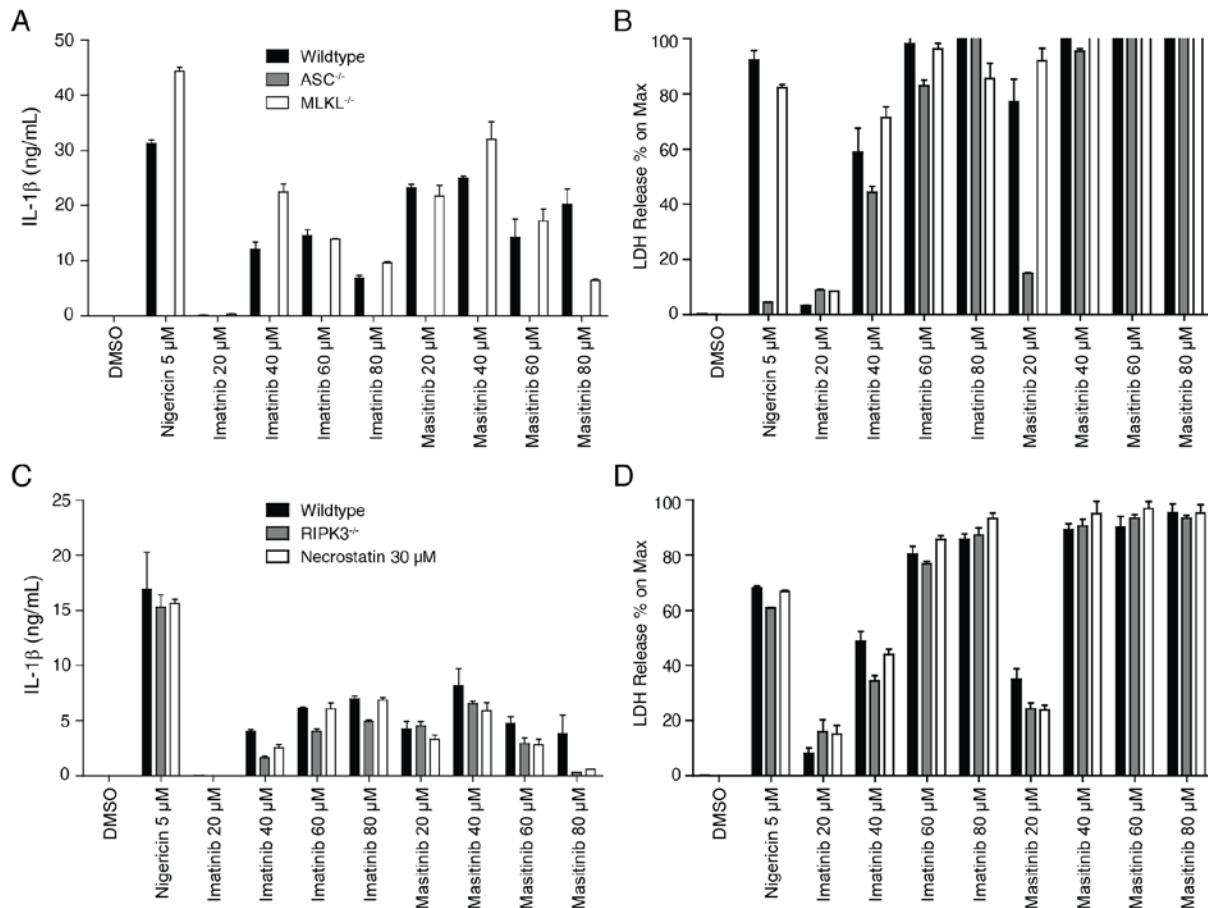
Inflammasome activation, cell death, and secretion of IL-1 family cytokines are intertwined events. Caspase-8 has a pivotal role, being a key adaptor for extrinsic apoptosis and for the programmed necrosis cell death called “necroptosis” (Creagh, 2014). Necroptosis is a lytic form of regulated cell death that requires formation of the so called necroptosome. This complex is activated through TNF-receptor family

members through RIPK1 signaling. However, it is still poorly understood how RIPK1 can modulate the fate decision toward either apoptosis or necrosis. Nevertheless, necroptosis leads to rapid cell rupture like in the case of several other regulated necrotic pathways (Pasparakis & Vandenabeele, 2015). Necroptosis was also related to NLRP3 activation several times, although the exact intersection between these two pathways is still a matter of debate (Vince & Silke, 2016).

We therefore tested BMDCs knockouts for the main component of the necroptosome. This is called mixed lineage kinase domain-like protein (MLKL). MLKL knockout cells do not show any reduction in IL-1 $\beta$  secretion as compared to the wildtype BMDCs. ASC deficient cells proved once again, this an indispensable component for TKIs-induced inflammasome (Figure 9A). Despite of any reduction in inflammasome activation, all the tested knockouts could not protect BMDCs from lytic cell death, since, upon stimulation with both TKIs, the levels of LDH release are comparable with those of the wildtype cells (Figure 9B).

To further analyze the pathway, we tested necrostatin-1, a small molecule inhibitor of RIPK1, frequently used to block necroptosis (Vandenabeele, Grootjans, Callewaert, & Takahashi, 2013) and knockout BMDCs for another fundamental component of the necroptosome named receptor-interacting serine/threonine-protein kinase 3 (RIPK3). As shown in Figure 9C, necrostatin-1 is not able to block inflammasome activation, a result which is in line with previous findings (Cullen et al., 2015). Also, RIPK3 knockouts cells can still activate the inflammasome in the same measure of wildtype controls, implying that TKIs are not activating NLRP3 through the necroptosome. Moreover, cell death is not affected by the presence of necrostatin-1 as LDH release levels together with the wildtype and the RIPK3 knockouts (Figure 9D).

However, with the highest concentration of masitinib, there is some reduction in IL-1 $\beta$  secretion for both MLKL and RIPK3 deficient BMDCs observed (Figure 9A, C). This may mean that, at higher masitinib concentrations, the inflammasome needs the necroptosome to license full activation. This could be because the cells lose membrane integrity and are killed much quicker. Irrespective of the exact underlying mechanisms, these data confirm that TKIs do not activate the necroptosome in parallel or upstream of NLRP3. Moreover, necroptosome-deficiency does not affect cell death induced by imatinib and masitinib.



**Figure 9: Imatinib and masitinib do not induce the necroptosis pathway.** (A, B) LPS-primed BMDCs from wildtype, ASC, and MLKL knockout mice were stimulated as indicated for 3h. (A) IL-1 $\beta$  secretion was evaluated by ELISA (B) and LDH release was evaluated to quantify cell death. (C, D) LPS-primed BMDCs from wildtype and RIPK3 knockout mice were treated with either no inhibitor or necrostatin-1 30  $\mu$ M 30 min prior stimulation. Stimulation was carried out as indicated for 3h. (C) IL-1 $\beta$  secretion was evaluated with ELISA (D) LDH release was measured to quantify cell death. Data are depicted as mean  $\pm$  SD of technical triplicates.

### Osmoprotectant high molecular weight PEG protects from imatinib induced cell death

By testing knockouts cells and inhibitors of several pathways, we could not observe a complete reduction in lytic cell death induced by the TKIs so far. We considered to test osmoprotectants as they can help maintain membrane integrity and might be able to reduce the impact of TKIs on cell lysis. By comparing different polyethylene glycols (PEGs), we observed that molecular weight of 3000 g/mol is the best one to inhibit both TKIs (data not shown).

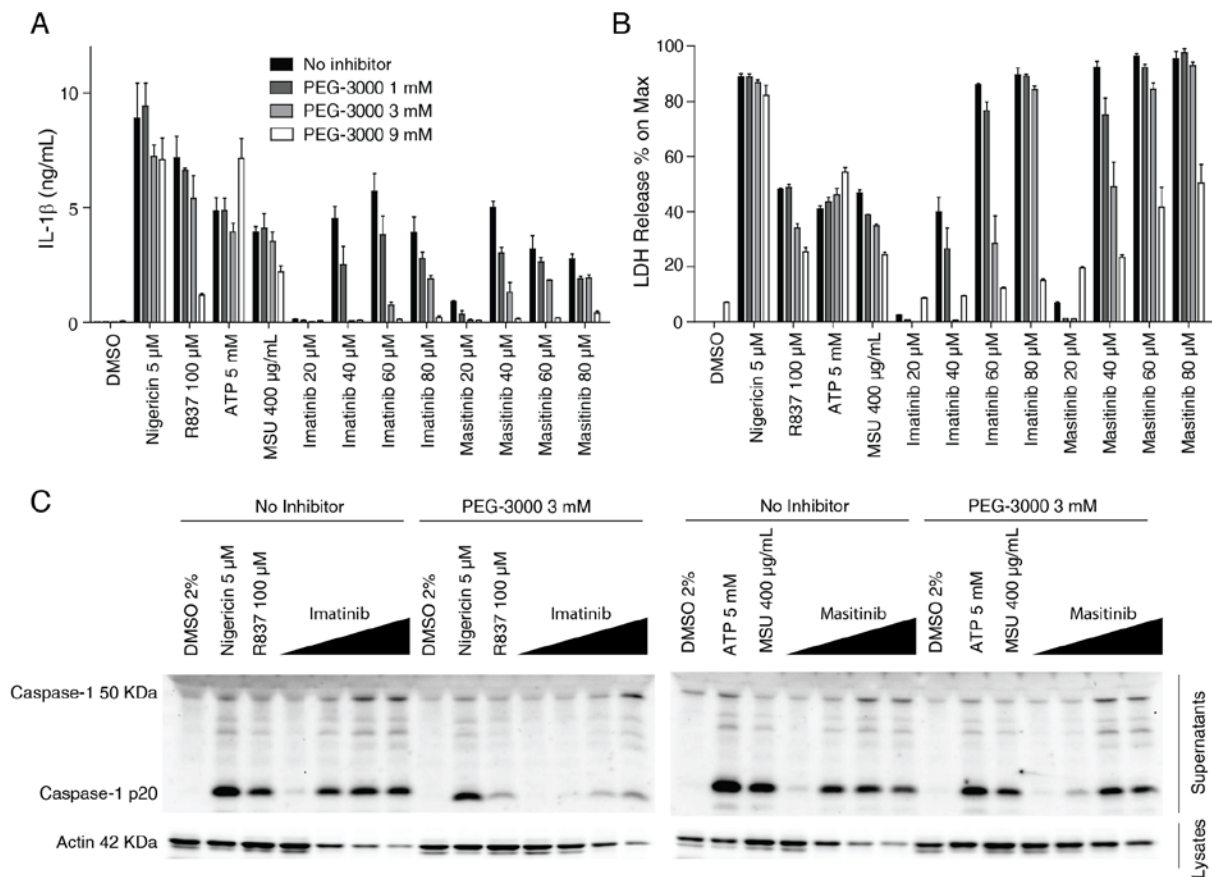
In additional experiments, PEG 3000 was tested at three different concentrations on BMDCs upon TKI stimulation. When PEG 3000 is 3 or 9 mM it is very effective in



inhibiting IL-1 $\beta$  secretion induced by imatinib and, to a lesser extent, also that induced by masitinib. Control activators can still induce inflammasome even in presence of high osmoprotectant concentrations. The only exception is that R837 is significantly inhibited at 9 mM PEG (Figure 10A).

TKI treated cells are protected from death following a similar trend to that of IL-1 $\beta$  secretion dampening (Figure 10B). Death induced by imatinib is well blocked by PEG pretreatment, although cells are only partially protected with masitinib. None of the control stimuli was affected in its cell death profile. A minor exception is visible for R837, in line with what observed for IL-1 $\beta$  secretion. However, PEG seems to have a toxic effect of when at higher concentrations with exposure times like the one used in these experiments (Figure 10B). Indeed, 9 mM PEG 3000 can partially cause cell death by itself as LDH release graphs show a positive signal for DMSO control (Figure 10B). The immunoblots from the same experiments show that caspase-1 release in the supernatant is well reduced for imatinib and, to a lesser extent for masitinib too. In line with IL-1 $\beta$  secretion, caspase-1 release is not affected for the tested control stimuli with exception of R837 (Figure 10C).

Taken together, these data show that osmoprotectants are not effective in defending the cells against nigericin and ATP induced cell death. PEG 3000 can effectively protect BMDCs from TKIs-induced cell death indicating the K<sup>+</sup> efflux they provoke is not through a channel or ionophore like nigericin and ATP, but the result of cell lysis which in turn activates NLRP3. Importantly, IL-1 $\beta$  secretion by R837 is reduced at high PEG concentrations (9 mM). This datum supports the idea that R837 may induce NLRP3 activation and pyroptosis through a positive feedback loop that goes, in succession, via inflammasome activation without K<sup>+</sup> efflux, GSDMD cleavage, cell death, and consequent minor K<sup>+</sup> efflux. I introduced this concept before, after observing wildtype cells stimulated with R837 for longer incubation time, exhibit some loss of intracellular potassium level (Figure 6). This feedback loop would be like that observed during non-canonical inflammasome formation to trigger NLRP3 activation (Kayagaki et al., 2015). To test this hypothesis, we would need to use osmoprotectants in an *in vitro* test of non-canonical inflammasome activation.



**Figure 10: High molecular PEG can partially protect cells from TKIs induced cell death.** (A, B) LPS-primed BMDCs from wildtype mice were treated with either no inhibitor or PEG-3000 at a final concentration of 1, 3, or 9 mM 30 min prior stimulation. Cells were then stimulated for 3h as indicated. (A) IL-1 $\beta$  secretion was quantified by ELISA (B) and LDH release was used to quantify cell death. Data are depicted as mean  $\pm$  SD of technical triplicates. (C) Immunoblot of supernatants and cell lysates from the same experiment. Blots were developed to detect the cleaved form of caspase-1, and  $\beta$ -actin as internal standard.

### Imatinib activates the NLRP3 through an unconventional mechanism

So far, we observed how the TKIs can trigger a lytic cell death event with concomitant inflammasome activation. Many lytic cell death pathways cause release of alarmins, one of which is ATP (Venereau, Ceriotti, & Bianchi, 2015). We therefore speculated that imatinib might engage NLRP3 by causing autocrine or paracrine ATP release from dying cells (Gombault, Baron, & Couillin, 2012). ATP binds to the P2X7 receptor, causing channels opening and potassium efflux, thereby activating the NLRP3 inflammasome (Jo et al., 2016). Furthermore, TKIs are designed as competitive inhibitors of ATP binding sites of oncogenic kinases and might in principle bind ATP binding sites on non-kinases like P2X7 in a non-specific manner. Therefore, we first wanted to address the question whether P2X7 is involved in the NLRP3 activation

mechanism of the TKIs by testing them on P2X7-deficient cells. As expected, P2X7 knockouts could not activate the inflammasome in presence of ATP but could respond normally to all the other control stimuli (Figure 11A). Both TKIs could activate the inflammasome at full potency even in P2X7 knockout cells, except at a lower imatinib concentration (Figure 11A). I may speculate that, at lower concentrations, imatinib can trigger the inflammasome through an autocrine mechanism involving initially unspecific binding of imatinib to P2X7 and then by K<sup>+</sup> efflux with NLRP3 activation and more ATP release. However, at higher imatinib doses, the activation of cell death with membrane destabilization and potassium efflux prevails over any contribution of P2X7. This finding needs confirmation, but it copies the hypothesis that imatinib triggers more than one cell death pathway in parallel of activating NLRP3. In fact, LDH release displays a pattern that follows the same of IL-1 $\beta$  release. The imatinib-induced LDH release signal is strongly reduced at 40  $\mu$ M but not at higher concentrations as it was seen for IL-1 $\beta$ . This also follows the idea that P2X7 could be one of the targets of imatinib that drives NLRP3 assembly, but certainly not the only one. The control stimuli show the predicted LDH release profiles in relation to all tested knockouts (Figure 11B). Masitinib instead, results independent of P2X7 to trigger both inflammasome activation and cell death (Figure 11 A, B).

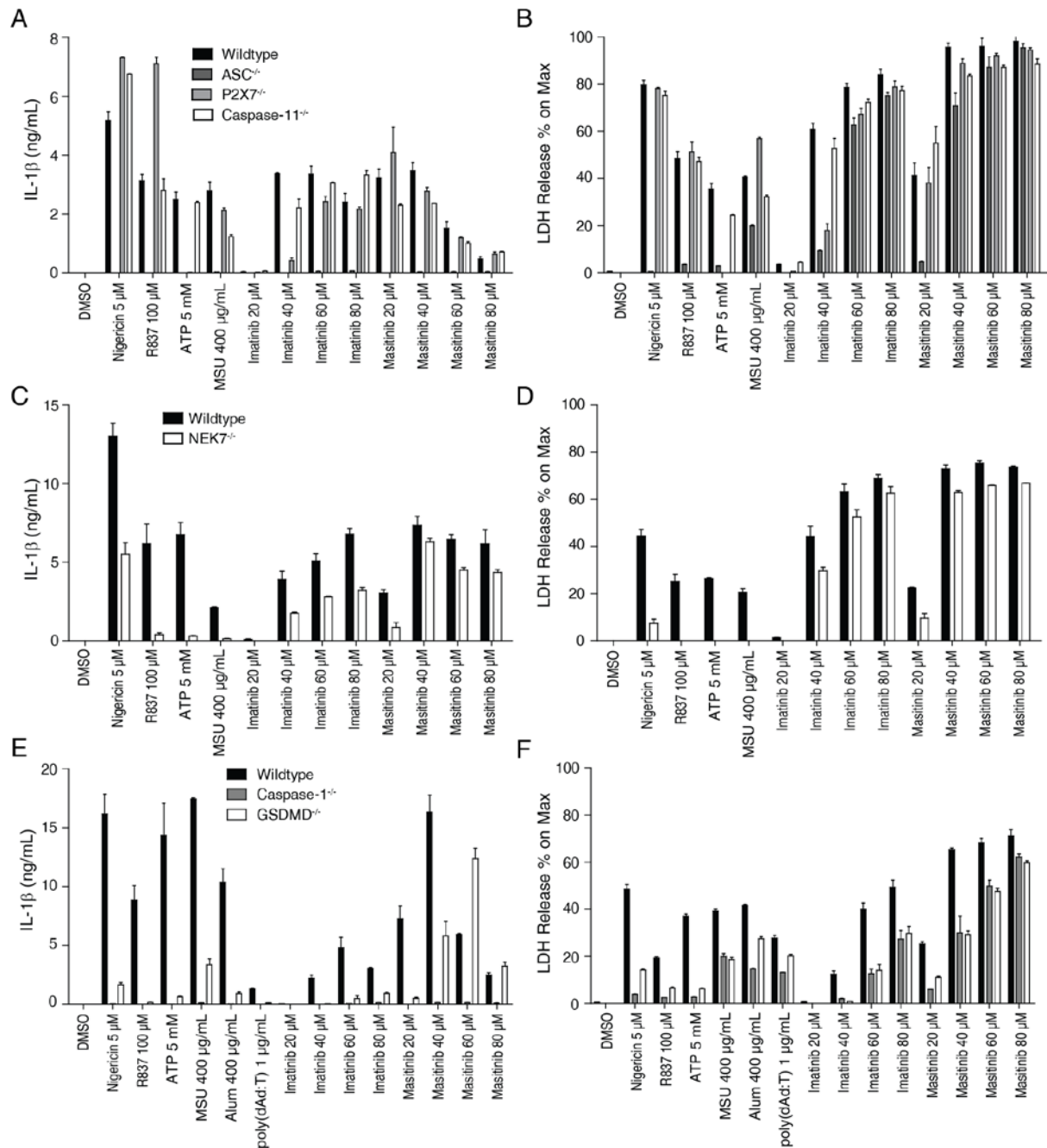
Caspase-11 is responsible for a non-canonical pathway of NLRP3 activation that, like what we see for imatinib, causes cell death that leads to potassium release and causes potassium-dependent NLRP3 activation. Moreover, as outlined in the previous paragraph, we also wanted to test the possibility that TKIs could generate a feedback loop involving in order caspase-11, K<sup>+</sup> efflux, NLRP3, and GSDMD to expand inflammasome activation. For this reason, I tested the TKIs on SV129 primary BMDCs, which are naturally occurring a point mutation that deactivates caspase-11 (Kayagaki et al., 2011). Caspase-11-deficient cells were normally activated by the control stimuli and the TKIs without any loss of signal for both IL-1 $\beta$  secretion and cell death (Figure 11A). This means that TKIs are not using the non-canonical pathway to trigger assembly of NLRP3 inflammasome.

Recently, a new protein named NEK7 has been introduced as an upstream interaction partner of NLRP3. NEK7 is a serine/threonine kinase that takes part in mitosis. Three group independently showed that NEK7 required for NLRP3 activation and one suggested that it can transduce the potassium efflux signal while another suggested

that is involved in sensing ROS production, consequently binding to NLRP3 to drive inflammasome assembly independent of its kinase activity (Y. He, Zeng, Yang, Motro, & Nunez, 2016; H. Shi et al., 2016). We were wondering if the unusual and unclear mode of NLRP3 activation by TKIs does also engage NEK7. Surprisingly, we found that TKIs clearly act NEK7 independent, as they can trigger substantial IL-1 $\beta$  secretion and LDH release even at low concentrations in those knockout cells (Figure 11C, D). Although a slight reduction of inflammasome activity is detectable rather for imatinib than for masitinib, the residual signal is sufficiently high for both drugs as compared to the signal observed in NLRP3-deficient cells (Figure 5) to confirm that they activate NLRP3 independent of NEK7 (Figure 11C). In contrast, R837 which is also a NLRP3 activating small molecule is completely NEK7 dependent as our group recently showed (C. J. Gross et al., 2016). Consistent with my other results, TKIs induced cell death is unaffected by the lack of NEK7, while it depends on it for the control compounds (Figure 11D).

Gasdermin-D was recently proposed as the protein responsible for pore formation during pyroptosis through cleavage by either, caspase-1 or caspase-11 (X. Liu et al., 2016). We tested GSDMD deficient BMDCs since our data indicates that the TKIs induce NLRP3 inflammasome activation through an unconventional form of cell death. Control compounds induce strongly reduced cell death and IL-1 $\beta$  secretion in GSDMD-deficient cells (Figure 11E, F) in line with previous reports (X. Liu et al., 2016). In response to the TKIs, GSDMD knockouts displayed a LDH release profile like that of caspase-1 knockouts, again supporting the notion that new, many programmed death pathways contribute to TKIs-induced cell death (Figure 11F). IL-1 $\beta$  secretion was likewise strongly reduced in GSDMD knockout as much as with the caspase-1 deficient cells upon imatinib treatment. Instead, higher masitinib concentrations can cause IL-1 $\beta$  secretion even in GSDMD knockouts (Figure 11E). A possible explanation is that masitinib is a stronger or faster-acting stimulus than imatinib and the two compounds may have different mode of action. However, imatinib-induced IL-1 $\beta$  secretion appears to be GSDMD-dependent, which is also the case for lower concentrations of masitinib. There are two possible explanations: 1) the role of GSDMD is the same as for conventional NLRP3 activators in that GSDMD controls IL-1 $\beta$  release downstream of inflammasome activation. 2) The TKIs might activate GSDMD (or maybe another gasdermin) in a caspase-1/11 independent fashion,

inducing inflammasome-independent cell death, potassium efflux, and thereby NLRP3 activation. However, since both TKIs do not need GSDMD (or caspase-1) to trigger substantial cell membrane instability and death, it appears more likely that the former is the case and GSDMD plays the same role for the TKIs as for conventional NLRP3 activators. This also suggest once again that TKIs are causing an unconventional form of programmed cell death. To our knowledge there are no similar findings in the literature making this the first ever observation of its kind (NEK7 data comprised). Future research should address what pathways are involved in this form of cell death and what signal transduction features, apart from its reliance on membrane instability, characterize it.



**Figure 11: Evaluating other pathways for imatinib and masitinib induced inflammasome activation.** (A, B) LPS-primed BMDCs from wildtype, ASC, P2X7, or SV129 mice (naturally caspase-11-deficient) were stimulated for 3h as indicated. (A) IL-1 $\beta$  secretion was evaluated with ELISA, (B) LDH release was used to quantify cell death. (C, D) LPS-primed BMDCs from wildtype and NEK7 knockout mice were stimulated as indicated for 3h. (C) ELISA evaluation of IL-1 $\beta$  secretion and (D) LDH release evaluation of cell death. (E, F) LPS-primed BMDCs from wildtype, caspase-1, and gasdermin D knockout mice were stimulated as indicated for 3h. (E) ELISA evaluation of IL-1 $\beta$  secretion and (F) LDH release evaluation of cell death. Data are depicted as mean  $\pm$  SD of technical triplicates.

## Evaluation of the therapeutic relevance for imatinib-induced inflammasome activation

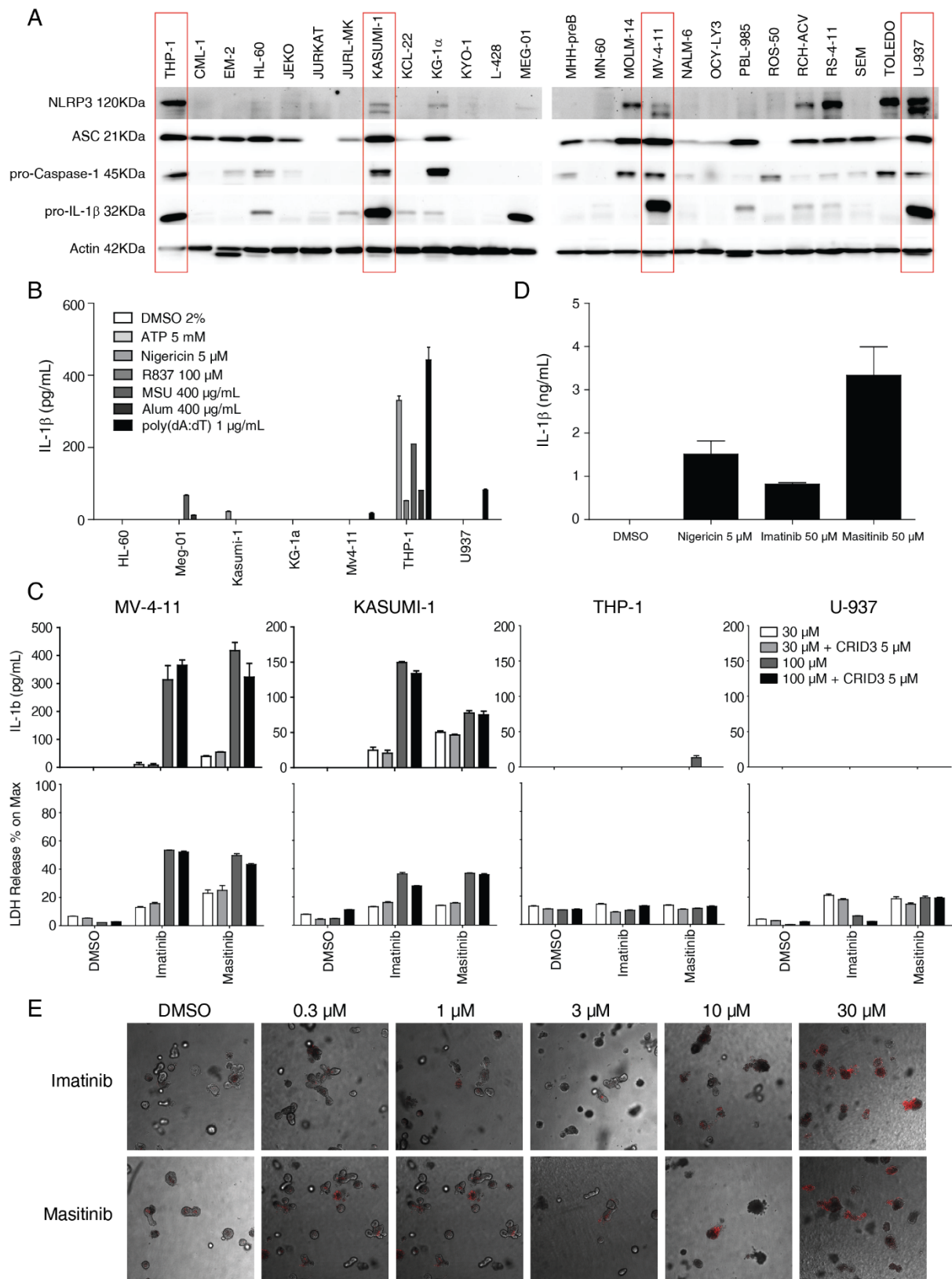
Imatinib is already used and masitinib is under clinical trial to treat several types of cancers (Dubreuil et al., 2009; Zitvogel et al., 2016). To investigate whether the inflammasome could have a concomitant effect in the therapeutic mechanism of these drugs, we tested this hypothesis on leukemic cells directly. A total of 26 different human leukemia cell lines, generated from patients having either acute myeloid leukemia (AML), chronic myeloid leukemia (CML) or lymphoid leukemia, were put into culture in parallel (Figure 12 A). The cells were cultured until they reached a stable growth rate and density. Cells were harvested and differentiated for differentiation with phorbol 12-myristate 13-acetate (PMA), which is a frequently used activator of lymphoid cells and the common standard differentiation stimulus for myeloid cell lines (Carta et al., 2011; Passmore, Lukey, & Ress, 2001). Once cells were primed, they were harvested, lysed, and tested by immunoblot to identify components of the NLRP3 inflammasome. This was done initially to understand which of the cell lines are inflammasome-competent. Of the 26 cell lines, only 4 were clearly positive for NLRP3, ASC, pro-caspase-1, and pro-IL-1 $\beta$  at the same time (Figure 12A red squared). Of note, all the positive lines were generated from patients suffering from AML, among them the standard cell line for inflammasome research, THP-1. AML cells normally acquire cancerogenic mutations at earlier developmental stages respect to those developing CML (Apperley, 2015; Dohner, Weisdorf, & Bloomfield, 2015). AML is characterized by a block in differentiation that we can overcome in cell culture by PMA treatment but that is difficult to achieve in a patient. Surprisingly, none of the CML cells tested carried all the inflammasome components at once. They were consistently negative for at least one, usually more inflammasome components. We speculate this is because CML cells are rather more advanced in differentiation than AML and they could have already accumulated mutations or epigenetic changes blocking the inflammasome and hence pyroptosis, as a survival mechanism. Indeed, one of the hallmarks of cancer is the suppression of cell death pathways (Hanahan & Weinberg, 2011). Inflammasome-dependent pyroptosis is one of several cell death mechanisms that may be selected for inactivating mutations during CML onset. Nevertheless, the lymphoid cell lines were not inflammasome competent, based on the absence of its components (Figure 12A). Although database results show that both B and T cells are

missing NLRP3 expression, they clearly show expression of ASC and caspase-1, albeit weaker than myeloid cells. However, at least for T cells, different groups have identified an inflammasome and pyroptosis pathway engaged by the IFI16 receptor (Dell'Oste et al., 2015). More recently, another group showed that T cells may be able to assemble a NLRP3 inflammasome in response to intracellular effects of complement (Arbore et al., 2016). These findings await confirmations but, in general, they could mean that similar pathways of inflammasome suppression in cancer development are also imaginable in T cells.

Next, we took the four identified cell lines plus two others as negatives controls, to test inflammasome activation by using several classic NLRP3 activators and poly(dA:dT) as an AIM2 stimulus (Figure 12B). Only THP-1 cells seem to respond well to the stimuli. The fact that THP-1 cells activate NLRP3 is in line with the literature, as these cells were used to first discover the inflammasome (Martinon et al., 2002). Furthermore, TKIs were tested several times at different concentrations in the presence or without CRID3 inhibition. As shown in Figure 12C, cells are not sensitive to TKIs because they secrete only minimal IL-1 $\beta$ . THP-1 show a very weak response to masitinib close to the detection limit of the assays, and none for imatinib. Yet, in line with the observation that these are regular inflammasome competent cells, CRID3 could suppress IL-1 $\beta$  production in response to masitinib. However, both TKIs induced IL-1 $\beta$  release in some but not all the other tested AML cell lines, but CRID3 does not reduce this IL-1 $\beta$  secretion, suggesting that this IL-1 $\beta$  release may be resulting from non-inflammasome dependent mechanisms. Also, LDH release does not indicate that cell lines are particularly sensitive to TKIs-induced cell death (Figure 12C). Despite of this, adherent human peripheral blood mononuclear cells (PBMCs) respond well to both TKIs with IL-1 $\beta$  secretion at comparable levels to that of nigericin (Figure 12D). Taken together, our results show that, although primary human immune cells respond to TKIs with IL-1 $\beta$  production it is hard to find a cancer cell line from the hematopoietic compartment that responds to inflammasome activators. Even cell lines that express all three components of the NLRP3 inflammasome were found to be unresponsive, suggesting that other mechanism might be in place to suppress inflammasome activation. This implies that the inflammasome is regularly suppressed during cancer development of normally inflammasome-competent cells and that the activity of TKIs on the inflammasome is not very likely to be involved in their efficacy.



Finally, we wanted to test if this activity is involved in the side effects caused by these drugs. Many TKIs, including imatinib, are administered as pills with different dosage depending on the treatment regimen. Therefore, the drug will reach its highest concentration, albeit temporarily, in the intestine. Intestinal inflammation and diarrhea are a frequent side-effect of TKI treatment. Intestinal epithelial cells express several different types of inflammasomes. For these reasons, we wanted to assess whether TKIs can induce cell death in intestinal epithelial cells. We sought to use intestinal epithelial organoids in collaboration with AG Quante as a model to test the drugs. Intestinal organoids are a tridimensional long-term primary culture with crypts and villus-like epithelial domains representing the complex cell-variety of intestinal epithelium (Mahe et al., 2013). An organoid culture was established and TKIs were tested on the cells for 24 hours of incubation at different concentrations. Propidium iodide was used to track cell death as described before (Grabinger et al., 2014). By judging the PI intensity and the organoid shape by microscopy, we could determine that both the TKIs become toxic on epithelial cells above a concentration of 10  $\mu$ M (Figure 12E). Further tests will be required to test if cell death can be blocked by CRID3 or by using cells from inflammasome-deficient mice. Taken together, these data suggest that inflammasome induction by TKIs may not have a significant role in killing the leukemic cells. However, they may rather be involved in immunomodulatory effects by stimulating the healthy monocytes as well as in the side effect of intestinal inflammation by an effect on the intestinal epithelium, potentially either through inflammasome activation and subsequent cytokine production or through pyroptotic damage of the intestinal barrier.



**Figure 12: Evaluating inflammasome activation by imatinib and masitinib on human cells.** (A) Immunoblots from human leukemia cell lines. Cells were activated with PMA 500  $\mu$ M for 3h and incubated overnight after a full medium change. Blots were developed to detect the full forms of NLRP3, ASC, pro-Caspase-1 and pro-IL-1 $\beta$ . (B) PMA-primed leukemia cell lines selected from (A) were stimulated with NLRP3 and AIM2 activators for 6h. IL-1 $\beta$  secretion was evaluated by ELISA. (C) PMA-primed leukemia cell lines selected from (B) were treated with either no inhibitor or CRID3 5  $\mu$ M 30 min

prior stimulation. Cells were stimulated for 6h with imatinib and masitinib as indicated. IL-1 $\beta$  secretion was evaluated by ELISA, and cell death by LDH release. (D) Human PBMCs were primed with LPS and stimulated with the indicated activators for 24h. IL-1 $\beta$  secretion was evaluated with ELISA. Data are depicted as mean  $\pm$  SD of technical triplicates. (E) Murine organoids were stimulated with imatinib and masitinib as indicated for 24h. Cell death was evaluated with propidium iodide at the confocal microscope. The organoids experiment was conducted in collaboration with Dr. Agnieszka Pastula, AG Quante (TUM).

### Other TKIs can activate the inflammasome with different mechanisms

Imatinib is the first TKI that reached the market and is still used as first line treatment for diseases like CML and GIST (Iqbal & Iqbal, 2014). However, many other TKIs were developed over the years and some already reached the market. For instance Nilotinib, Dasatinib and Bosutinib are used as second line treatments after insurgence of BCR-Abl mutations (Wu et al., 2016).

A set of 13 TKIs were tested several times at different concentrations to select the minimal dose where they generate a robust inflammasome response. We could identify a total of seven compounds inducing a strong IL-1 $\beta$  and concomitant IL-1 $\alpha$  secretion, the secretion of pro-IL-1 $\beta$  was also included in the ELISA assay (Figure 13 A). The 7 drugs that yielded robust and reproducible IL-1 $\beta$  signals with concomitant IL-1 $\alpha$  are classified and described in Table 2.

COMPOUND	TARGET	USED FOR	CLINICAL STAGE
Bosutinib	BCR-ABL, SRC Kinases	CML	Market
Crenolanib	FLT3, PDGFR, Kit	AML, GIST, Glioma, NSCLC, Mastocytosis	I, II, III, many cancer types
Crizotinib	ALK, HGFR, ROS1, RON	NSCLC	Market
Dasatinib	BCR-ABL, SRC Kinases, Kit, EPHA2, PDGFR	CML, Ph+ B-ALL	Market
Imatinib	BCR-ABL, PDGFR, Kit	CML, Ph+ B-ALL, GIST	Market
Masitinib	Kit, PDGFR, FGFR	Mast Cells Tumor (Veterinary Medicine)	Vet: market. Hum: II, III, many diseases
Pazopanib	Kit, PDGFR, VEGFR	RCC, STS	Market

**Table 2 List of inflammasome inducing tyrosine kinase inhibitors.** Abbreviations: CML chronic myeloid leukemia, AML acute myeloid leukemia, GIST gastrointestinal stromal tumor, NSCLC non-small cells lung cancer, Ph+ B-ALL Philadelphia positive B acute lymphoblastic leukemia, RCC renal cell carcinoma, STS soft tissue sarcoma.

3 drugs caused neither IL-1 $\beta$  or concomitant IL-1 $\alpha$  secretion, meaning they don't activate the inflammasome nor induce cell death. These are axitinib, erlotinib and

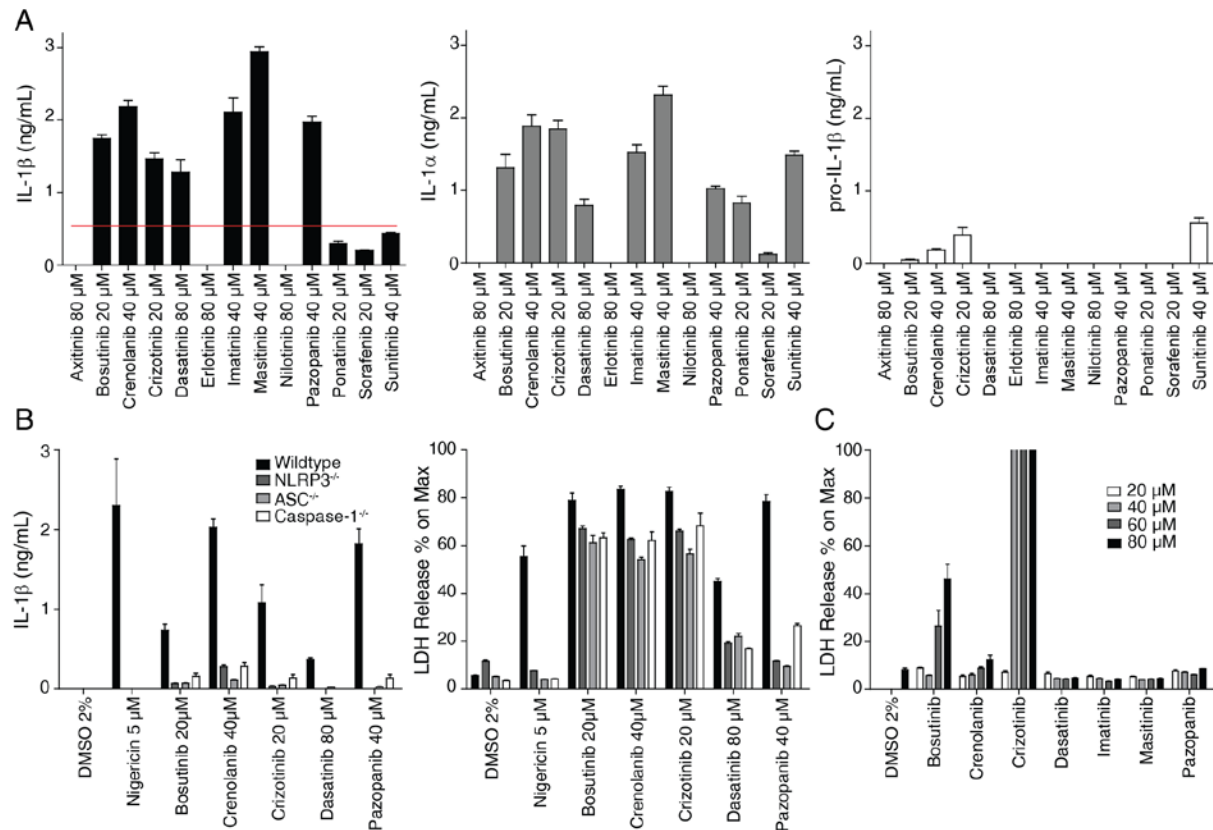
nilotinib. Instead, ponatinib, sorafenib, and sunitinib caused minimal IL-1 $\beta$  secretion, but mostly cell death. This is indicated by the prominent secretion of IL-1 $\alpha$  and, in the case of sunitinib, that the IL-1 $\beta$  signal detected is due to an unspecific release of pro-IL-1 $\beta$  (Figure 13A).

Those 5 drugs that could activate the inflammasome other than imatinib and masitinib were further tested on several knockout-derived cells to determine if the source of IL-1 $\beta$  was from the inflammasome as well as if they were inducing pyroptosis. BMDCs either from wildtype, NLRP3, ASC or caspase-1 knockout mice were tested to this extent. All the drugs showed a IL-1 $\beta$  secretion profile which is dependent on the NLRP3 inflammasome. Remarkably, crenolanib shows a residual signal for NLRP3-deficient cells like in the case of imatinib and masitinib (Figure 13B and 5A). Some TKIs also have a residual signal for caspase-1 knockout cells like it was for imatinib and masitinib, that I guess could come from the activity of the ASC speck recalling another caspase that cleaves IL-1 $\beta$  and that could be caspase-8 as hypothesized before. Cell death is largely inflammasome independent for bosutinib, crenolanib and crizotinib like imatinib and masitinib. Instead, dasatinib and especially pazopanib seem more likely to induce pyroptosis given their dependency on NLRP3 to trigger cell death (Figure 13B).

Another experiment was done by using 3T3 mouse fibroblast treated for 6 hours with the 7 elected inflammasome-inducing compounds to test their cytotoxicity. In general, all the TKIs do not kill the cells with the only exception of crizotinib that results a strong LDH release inducer (Figure 13C). These results imply that TKIs, apart from crizotinib, are causing a lytic but programmed form of cell death that would exclude a general mechanism of non-specific necrosis. Future research will aim to determine what kind of regulated cell death each of these drugs can induce.

Taken together these experiments showed that other TKIs can trigger a strong inflammasome response and we can segregate them in four different categories based on their cytokine secretion and LDH release profiles. First, we have those TKIs that provoke inflammasome-dependent IL-1 $\beta$  secretion and independent cell death that comprises bosutinib, crenolanib, crizotinib, imatinib and masitinib. Second, inflammasome-dependent IL-1 $\beta$  secretion and dependent cell death comprising dasatinib and pazopanib. Third, inflammasome-independent IL-1 $\beta$  secretion and independent cell death comprising ponatinib, sorafenib and sunitinib. Fourth and last

category, non inflammasome and neither cell death inducers comprising axitinib, erlotinib and nilotinib. With this distinction, these drugs represent categories of compounds that can be used to study the fine mechanisms of inflammasome activation and other forms of regulated cell death.

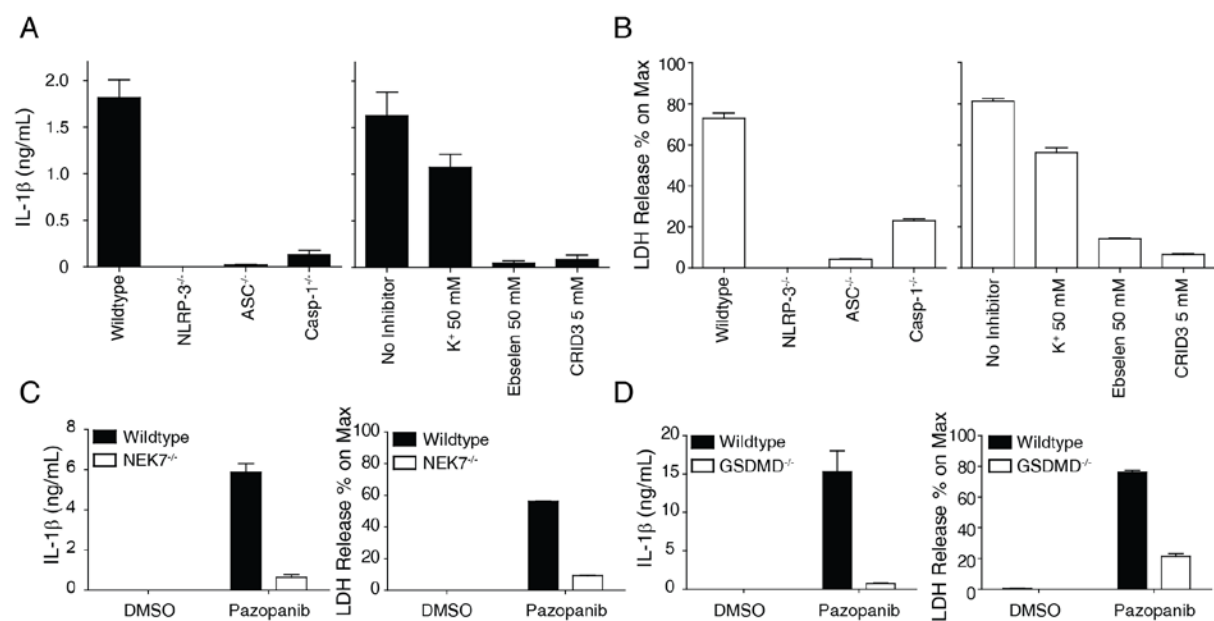


**Figure 13: Other TKIs can strongly induce inflammasome activation.** (A, B) LPS-primed BMDCs from wildtype mice were stimulated with several TKIs as indicated for 3h. (A) IL-1 $\beta$ , IL-1 $\alpha$ , and pro-IL-1 $\beta$  secretion were evaluated with ELISA. The red line traces the threshold for those 7 drugs that induce a remarkable IL-1 $\beta$  secretion. (B) LPS-primed BMDCs from wildtype, NLRP3, ASC and caspase-1 knockout mice were stimulated with several TKIs as indicated for 3h. IL-1 $\beta$  was evaluated with ELISA and cell death with LDH release assay. (C) 3T3 mouse embryonic fibroblasts were treated with several TKIs as indicated for 6h. Cell death was evaluated with LDH release assay. Data are depicted as mean  $\pm$  SD of technical triplicates.

### Pazopanib induces inflammasome without causing K<sup>+</sup> efflux

Pazopanib was sticking out from the field of tested TKIs since it induced robust NLRP3-dependent IL-1 $\beta$  production, but in contrast to imatinib and the other tested TKIs, also displayed inflammasome dependent cell death. This implicated that it has a mode of action distinct from imatinib. We therefore tested the upstream requirements for pazopanib-induced inflammasome activation. The tests conducted on BMDCs revealed that this drug acts like a classic inflammasome activator as both IL-1 $\beta$  and

LDH release are dependent on NLRP3 (Figure 14A, B). The minor residual signal observed in caspase-1 knockout cells is also observed with other, classic inflammasome activators and is most likely related to the activation of caspase-8 at the ASC speck (Sagulenko et al., 2013). Astonishingly, in extensive inhibitor studies, I found that pazopanib is potassium efflux independent but ROS dependent, like what our group recently published for R837 (C. J. Gross et al., 2016). The small molecule inhibitor CRID3 can also block IL-1 $\beta$  secretion and LDH release, differently than what we saw with imatinib and masitinib (Figure 14A, B). Moreover, pazopanib-induced IL-1 $\beta$  secretion and cell death is fully NEK7 and GSDMD dependent (Figure 14C, D). This means that the death mechanism provoked by pazopanib relies exclusively on the inflammasome and triggers pyroptosis only. These results indicate that, although we identified multiple TKIs as potent activators of the inflammasome, they appear to engage distinct upstream mechanisms to cause this effect, and pazopanib appears to be the one with the highest specificity toward activation of NLRP3 and pyroptotic pathway.



**Figure 14: Pazopanib induces pyroptosis and has a potassium efflux independent NLRP3 activation mechanism like R837.** (A, B) LPS-primed BMDCs from wildtype NLRP3, ASC, and caspase-1 knockout mice were treated with either no inhibitor or CRID3 5  $\mu$ M, K<sup>+</sup> 50 mM, or ebselen 40  $\mu$ M 30 min prior to stimulation. Cells were stimulated with pazopanib 40  $\mu$ M for 3h. (A) ELISA was used for evaluation of IL-1 $\beta$  secretion and (B) LDH release for evaluation of cell death. (C, D) IL-1 $\beta$  ELISAs and LDH release assays from wildtype and either NEK7 or GSDMD knockout cells stimulated with pazopanib 40  $\mu$ M for 3h. Data are depicted as mean  $\pm$  SD of technical triplicates.

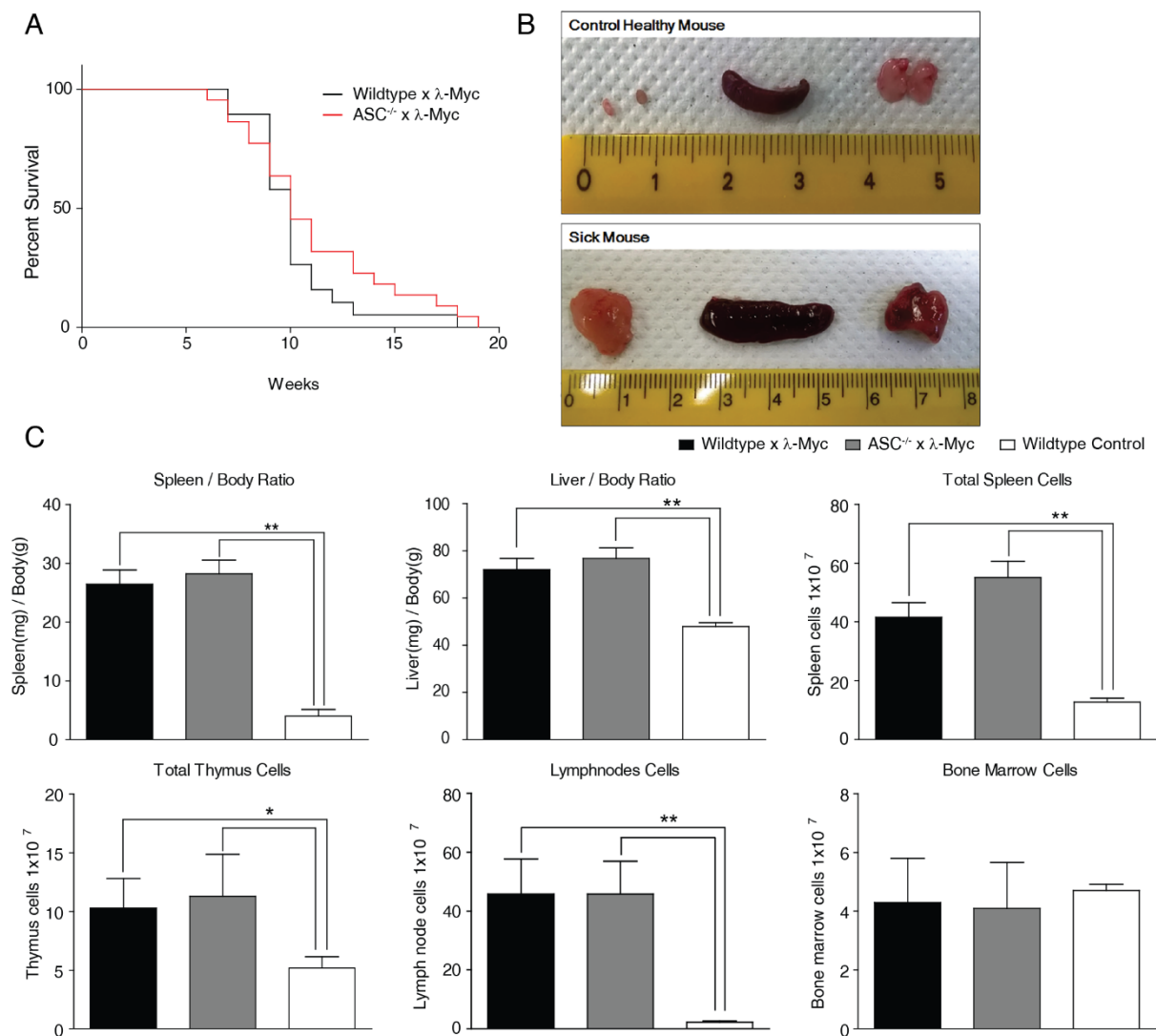
## Project two results

Our observation that hematopoietic malignancies frequently suppress the inflammasome (Figure 12) further encouraged us to directly test a role for the central inflammasome adapter protein ASC in cancer development and progression, either through a role in the context of the inflammasome (through IL-1 production or pyroptosis) or through an inflammasome-independent function in other pathways of cell death and survival. To that end, three different mouse models of cancer in different hematopoietic lineages were used.

### ASC is dispensable for Burkitt's lymphoma progression

Mice deficient for the ASC-encoding gene *Pycard* were crossed with a knock-in line carrying a  $\lambda$ Myc transgene, generating a Burkitt's lymphoma in these mice. Wildtype mice were also crossed with the same line as a control for generating a survival curve. The statistical analysis shows no difference in survival. The log-rank reports a p-value of 0.2271 and the Gehan-Breslow-Wilcoxon test reports a p-value of 0.4662. The median survival rate is of circa 80 days for both the ASC deficient mice and the wildtype controls. The mice analyzed were more than 20 per group (Figure 15A). The lymph nodes, spleens, and thymi of healthy control and sick mice were continuously collected and analyzed. The differences between healthy and sick lymph nodes are easily recognizable as those of healthy mice have a 2-3 mm diameter and the ones from sick animals have over 1 cm in diameter, a condition referred as lymphadenopathy (Figure 15B). The spleen size is also increased. Healthy mice bear spleens that normally reach the length of 1 cm, while those from sick ones can even reach 4 cm, this condition is named splenomegaly. The thymi do not differ in size but many of the analyzed organs showed a red tone rather than the normal pinkish color. This means that there has been a substantial infiltration of cells from the peripheral blood. The peripheral blood carries many lymphoma cells, indicating this tissue could also be infiltrated by them (Figure 15B). Together with the survival rate and the macroscopic evaluation, many other parameters were collected from the animals. The spleen to body weight ratio confirms the splenomegaly. The livers of sick mice were also bigger in size respect to healthy mice. However, for all the observed parameters there is no substantial difference between ASC<sup>-/-</sup> and wildtype mice. (Figure 15C).

The lymphoid organs were collected and meshed for flow cytometric analysis and the total number of cells was also recorded and included as a physiological parameter. Spleens and lymph nodes had a much higher cell count respect of the healthy organs. Again, no differences were evident between the two genotypes. Thymi cell counts confirmed the infiltration hypothesized from the organ inspections. The bone marrow of sick animals did not present an increased number of cells. (Figure 15C). Taken together, this results show that the lymphoma cells, that are originated in the bone marrow, leave it to spread over the blood stream, the lymphoid, and the other organs. These findings are in line with previous reports on this mouse model (Kovalchuk et al., 2000). The mice present the most common symptoms associated with Burkitt's lymphoma that are frequently found in human patients (Molyneux et al., 2012).



**Figure 15: ASC does not protect mice from Burkitt's lymphoma progression.** (A) wildtype and ASC-deficient mice were crossed with the  $\lambda$ -Myc mouse line which generates Burkitt's lymphoma. Mice were kept in IVC cages in a SPF facility with food and water ab libitum. Terminal stage mice were

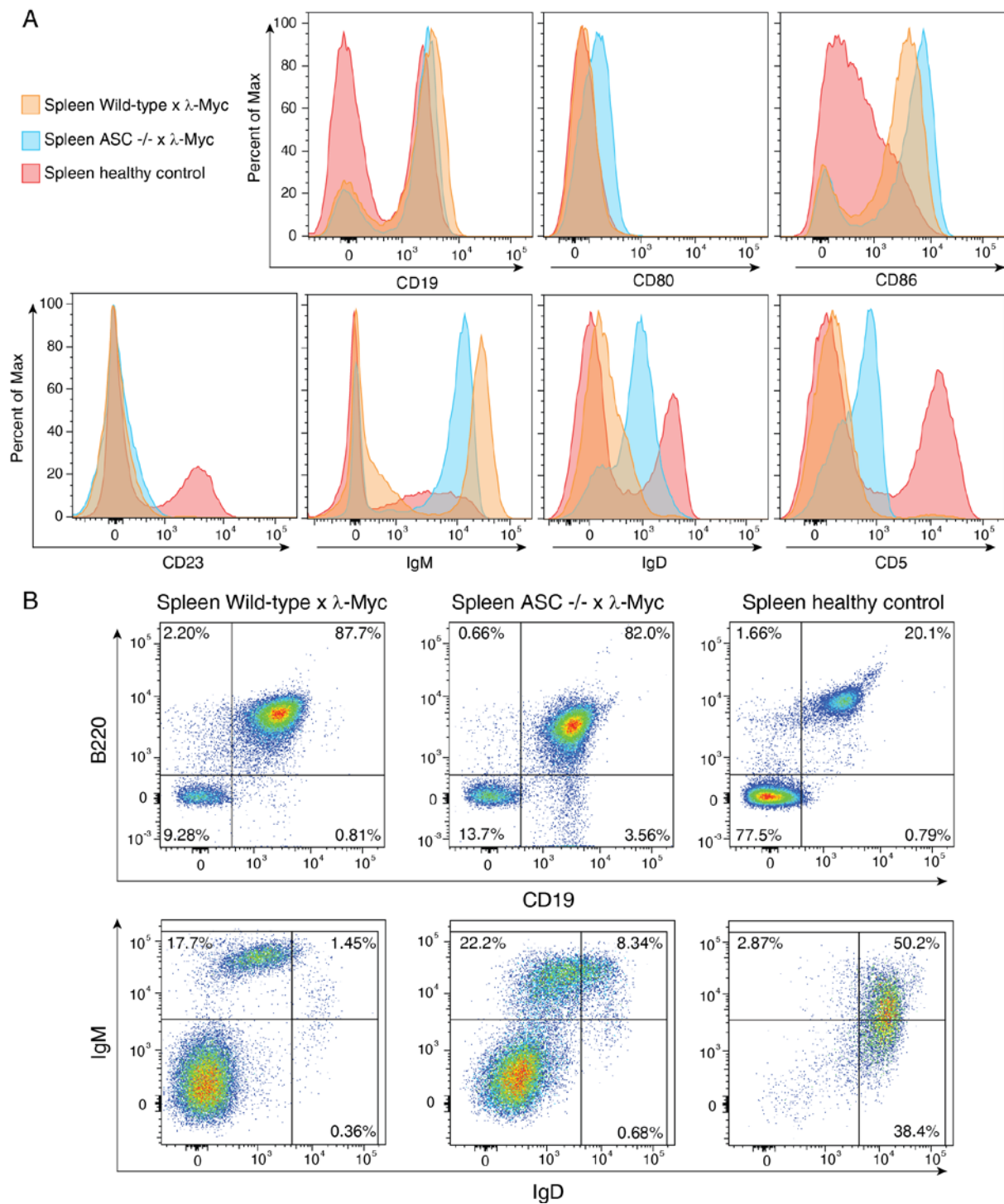


euthanized and a macroscopic evaluation was performed. ASC deficient mice (N =21) were compared in survival with wildtype mice (N=20). (B) Pictures from two wildtype control mice, either sick or healthy, showing lymph nodes, spleens and thymi and compared in size with a ruler. (C) Multiple parameters were collected from euthanized mice as described. Data are representative of all the mice analyzed (N  $\geq$  20 per group)

### A flow cytometric analysis of the lymphoma model

The organs collected from euthanized mice were used to generate a comprehensive flow cytometric analysis to detect eventual differences in disease entity between the ASC and wildtype animals. First, we analyzed the spleen with a set of markers that could confirm the previously reported lineage of lymphoma cells in this model (Kovalchuk et al., 2000). In the original paper the lineage was defined as CD19<sup>+</sup>, IgM<sup>+</sup>, CD23<sup>-</sup> and CD5<sup>-</sup>. The lineage was confirmed as cells from sick mice express CD19 and IgM but lack CD5 and CD3 signal (Figure 16A). Moreover, CD86 was also found highly expressed on B-cells, meaning they have switched to an active state. Moreover, CD80 is not expressed. This confirms previous findings where CD86 was found as a proliferation marker for lymphoma, while these cells were reported to be CD80 negative (Suvas, Singh, Sahdev, Vohra, & Agrewala, 2002) (Figure 16A).

Further analysis showed that there is a clear increase in B220<sup>+</sup> CD19<sup>+</sup> cells in the spleens of sick mice of circa 60%. The normal B cells are all expressing high levels of IgD and low levels of IgM. Lymphoma cells show an opposite antigen composition having expressed high IgM and almost no IgD (Figure 16A, B). This means that the lymphoma was originated from cells that gained a mutation after the pre-B stage and block in differentiation between the immature and the transitional stage according to Hardy's classification of B lymphocytes (Allman & Pillai, 2008). There are two main outcomes of this analysis. First, we verified that this mouse model is characterized by the generation of lineage-defined, immature B cells that spread and colonize lymphoid and other organs. Indeed, B-cell count is increased of 60% respect to the healthy counterparts. Second, there is no difference in lymphoma entity between the two genotypes. These results demonstrate that ASC does not protect from or changes the type of disease in this mouse model. The flow cytometric analysis was expanded to further analyze B cells, T cells, and myeloid cells and a selection of the parameters measured is presented in the following paragraph.



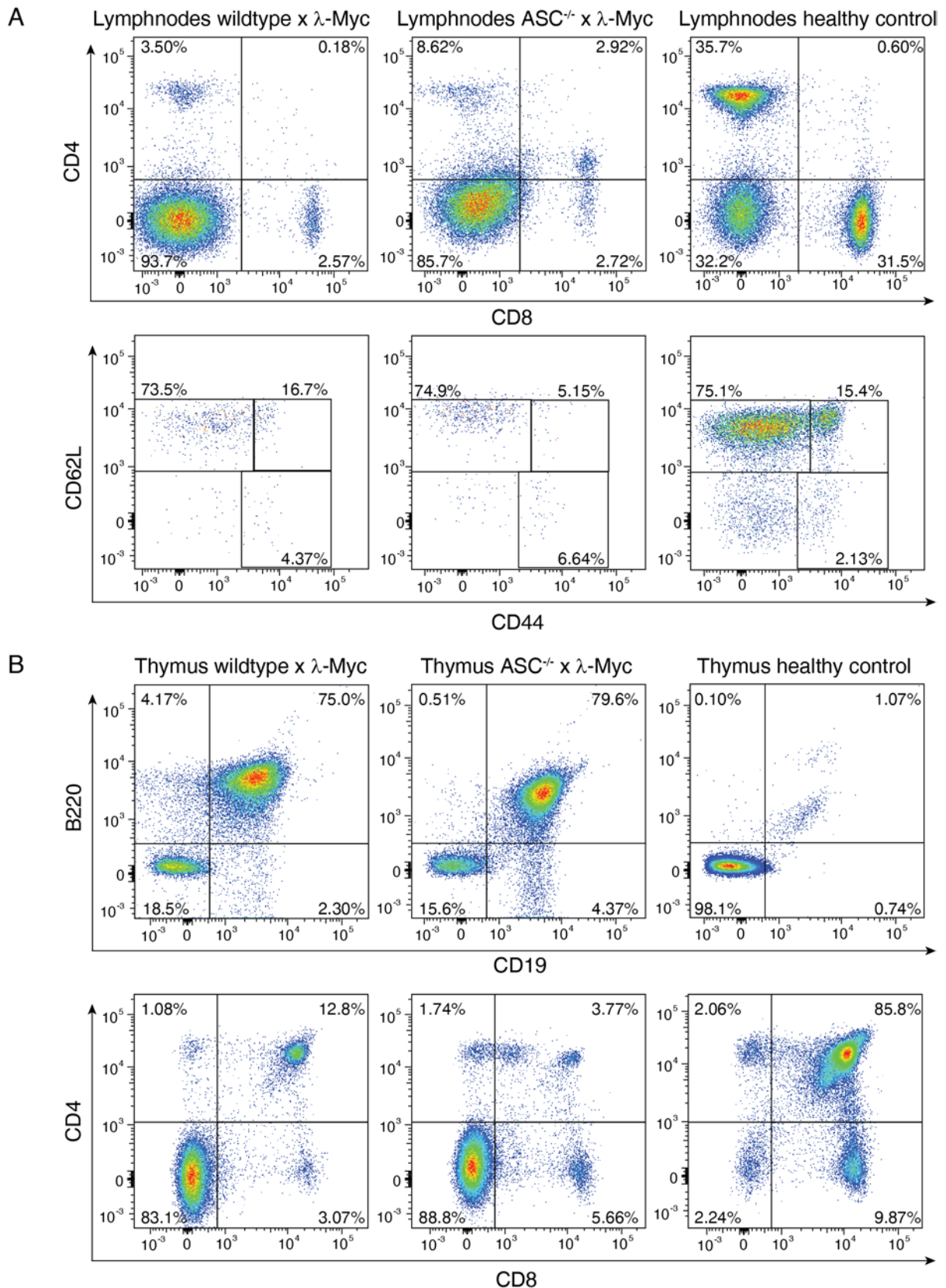
**Figure 16: Flow cytometry analysis reveals no difference between ASC and wildtype mice.** (A) spleens from sick mice were collected, meshed and stained with several sets of antibodies to detect B cells and other immune cells as indicated. Histogram analysis reveals a defined lineage of lymphoma cells. (B) Splenocytes from sick wildtype, ASC $^{-/-}$  and healthy wildtype control mice were collected, stained with antibodies detecting several myeloid and lymphoid markers and finally analyzed by means flow cytometry. Results are displayed as dot blots and compare common B cell maturation markers. B220 and CD19 are expressed almost at every developmental stage of B cells. IgM is expressed by immature B cells, IgD is expressed by mature ones.

## Lymphoma B cells spread and colonize all the lymphoid organs

The analysis of this mouse model was further extended here, to a point beyond what was reported in the original publication or, as to my knowledge, any other study on this mouse model. We analyzed several lymphocytic compartments like lymph nodes, spleen and bone marrow using the most common markers to trace identity and developmental stage of both myeloid and lymphoid cells. In summary, all these measurements confirmed that ASC-deficient cells don't display any differences as compared to wildtype. Exemplary, Figure 17 depicts the analysis of the T cell compartment in lymph nodes and thymi. The lymph nodes of sick mice show a remarkable reduction in the frequency of T cells. Only very low percentages of CD8 and CD4 positive cells are detectable in these organs. In depth analysis of CD62L and CD44 markers, which can detect memory T lymphocytes (Dillon et al., 2004), shows that lymph nodes of sick mice are missing this subset of cells. This indicates that the reduction in T cells frequency does not go at the expense of a specific cell subset (Figure 17A).

Analysis of the thymi reveals that the organs are almost completely infiltrated by lymphoma cells (Figure 17B). The thymus is deputed to the maturation of T cells and normally contains immature lymphocytes positive for the markers CD4 and CD8. Depending on their developmental stage, immature T cells in the thymus could be either double negative, double positive, or single positive. This is appreciable in the control samples. Instead, sick mice have only few T cells remaining (circa 12%). Control thymi do not show presence of B cell, as expected, whereas sick organs display percentages higher than 70% (Figure 17B).

Taken together these data show how the lymphoma cells can colonize other lymphoid tissues, disturbing the normal cellular composition of these organs. This panel, together with the rest of the FACS characterization, is a unique and detailed analysis contributing to a better characterization of this mouse model, and to my knowledge there are no similar dataset in the literature for this mouse model. Finally, no difference between the wildtype and the ASC deficient cells is discernible, an observation that is consistent with the survival curves. In summary, all the data collected shows no significant differences in cell markers between wildtype and ASC-deficient mice.

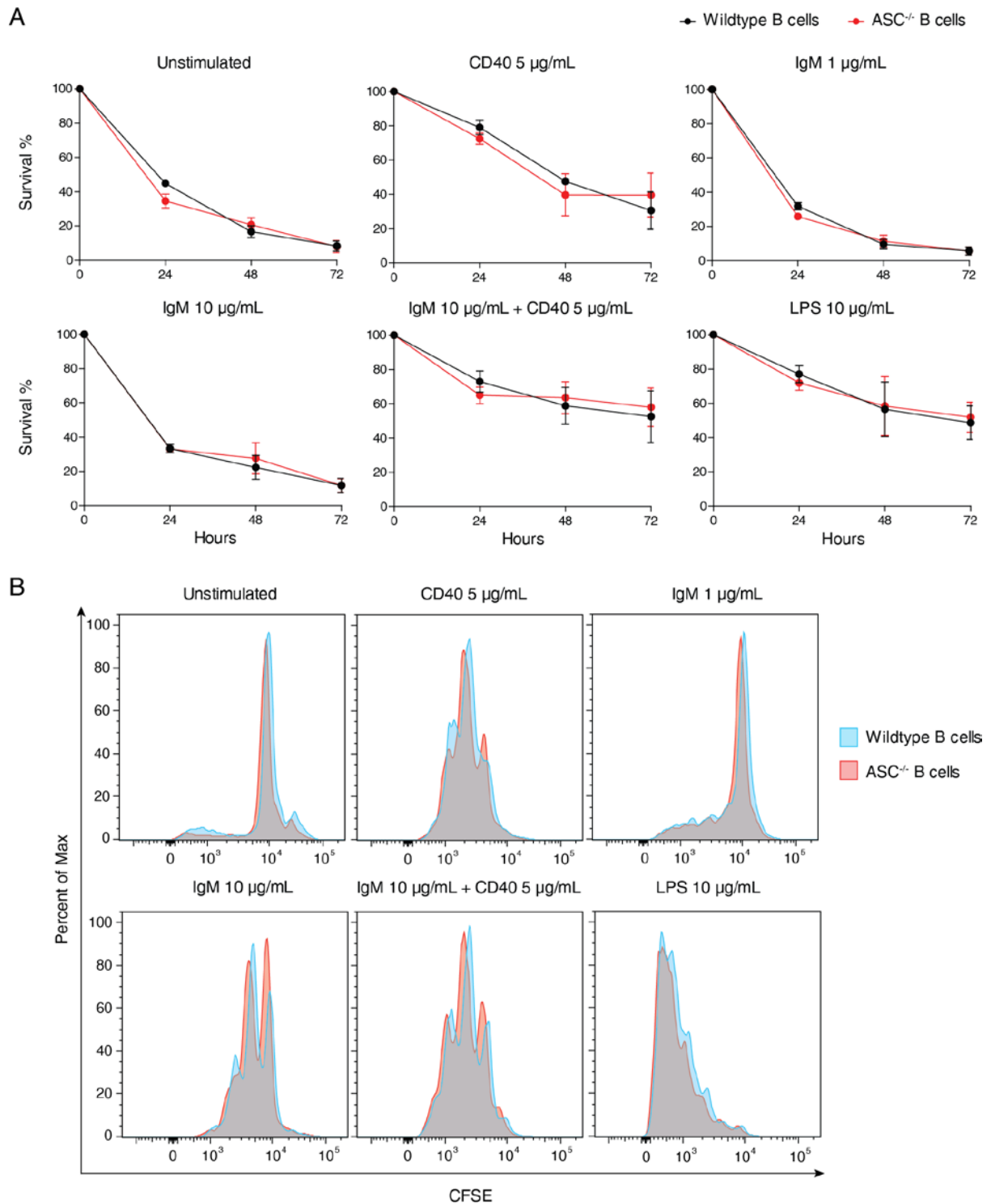


gating is done on CD4<sup>+</sup> CD3<sup>+</sup> cells (B) FACS analysis of the thymus was carried with CD4, CD8 T cell markers and B220, CD19 typical B cell markers.

### ASC knockout B cells survive and proliferate normally

To test whether there are any differences between the ASC knockouts and the wildtype B cells, we performed survival experiments with purified primary cells extracted from spleen and lymph nodes of healthy animals. Cells were cultivated and stimulated with either a soluble IgM F(ab')<sub>2</sub> or an anti-CD40 monoclonal antibody, both well-known activators of B-cell proliferation that can be used separately or in combination (Wortis, Teutsch, Higer, Zheng, & Parker, 1995). LPS was included as a third activator, as it is a potent B cell proliferation stimulus (Donahue & Fruman, 2003). The cells were exposed to the stimuli and survival percentage was evaluated with a combination stain of 7AAD (stains DNA) and AnnexinV (stains for exposed phosphatidylserine upon apoptotic cell death) to exclude dead or dying cells, respectively. The total cell number of double negative 7AAD/AnnexinV expressed as percentage compared to day one, hence only live cells, were used to plot survival curves. The B cells stimulated by LPS showed a good overall survival rate. In contrast, anti-IgM and anti-CD40 induced a minor survival signal. However, when CD40 and IgM are combined, they become very effective in stimulating B cells (Figure 18A). In any case, there is no detectable difference in survival between wildtype and ASC<sup>-/-</sup> B cells as the two cultures show no significant difference for survival under any condition (Figure 18A).

In parallel, some of the B cells were stained with CFSE and subjected to the same stimuli. CFSE analysis allows to detect, by means of FACS, the number of cell division at established timepoints. CFSE experiments are used to have a graphical impact overview of the proliferation rate (Parish, 1999). The CFSE experiment reflects the same pattern of the survival experiment. LPS is the strongest activator and costimulation of IgM and CD40 is more powerful than when the two are used singularly (Figure 18B). These results show that wildtype and ASC deficient B cells do not differ in their ability of activation, survival and proliferation. This results add to the evidences showing there is no difference in cell growth rate between the two genotypes.



**Figure 18: B cells from ASC deficient mice display no difference in activation, survival, and proliferation from wildtype counterparts:** (A) primary B cells were magnet purified from spleen, lymphnodes and peripheral blood. Cells were seeded, stimulated with the described stimuli, and incubated for either 24, 48, or 72 hours. Cells were then harvested, stained with 7AAD and AnnexinV, and analyzed by FACS. Plotted data representative of the 7AAD/AnnexinV double negative total cell numbers expressed as percentage compared to day one. Data are depicted as mean  $\pm$  SD of technical triplicates. The values from the two slopes were all compared by T test to find any significant difference. (B) The same procedure of stimulation was adopted to track proliferation of the lymphocytes. B cells were stained with CFSE and then analyzed by FACS. Data are plotted as percentage of maximal intensity.

### ASC is dispensable in a peripheral T cell lymphoma model

Expression data shows that primary T cells express substantial amounts of ASC, while the Jurkat T cell cancer cell line show no expression of ASC (Figure 12A), indicating that ASC might have an oncosuppressive function in these lymphocytes. We used a model of peripheral T cell lymphoma (PTCL) developed by researchers in our institute to investigate this. The mice bear a human oncogenic fusion kinase generated from a translocation between chromosomes 5 and 9, found in circa 20% of PTCL diagnosed patients. The fusion generates an oncogene named ITK-SYK, which triggers constitutive signaling for T cell activation and survival pathways (Pechloff et al., 2010). The mice bearing ITK-SYK were crossed with a conditional ASC knockout line named ALPS and a CD4-Cre line. The cancer model develops spontaneously in T cells. Therefore, with this breeding scheme we could selectively delete ASC in this lymphocytes subset. This was done to study the role of ASC only in those cells that will generate the lymphoma while excluding a role for ASC in other lineages including myeloid cells, where it might modulate cancer development. Mice were left in cage with water and food at will until they reached the terminal stage. They were then euthanized and a macroscopic evaluation was conducted. The survival curve shows no difference between the ASC-deficient mice and the wildtype counterparts (Figure 19A). The log-rank p-value is 0.8278 and the Gehan-Breslow-Wilcoxon test reports a p-value of 0.7439. The median survival rate is circa 25 weeks for both the ASC deficient mice and the wildtype controls. Circa 30 mice per group were analyzed.

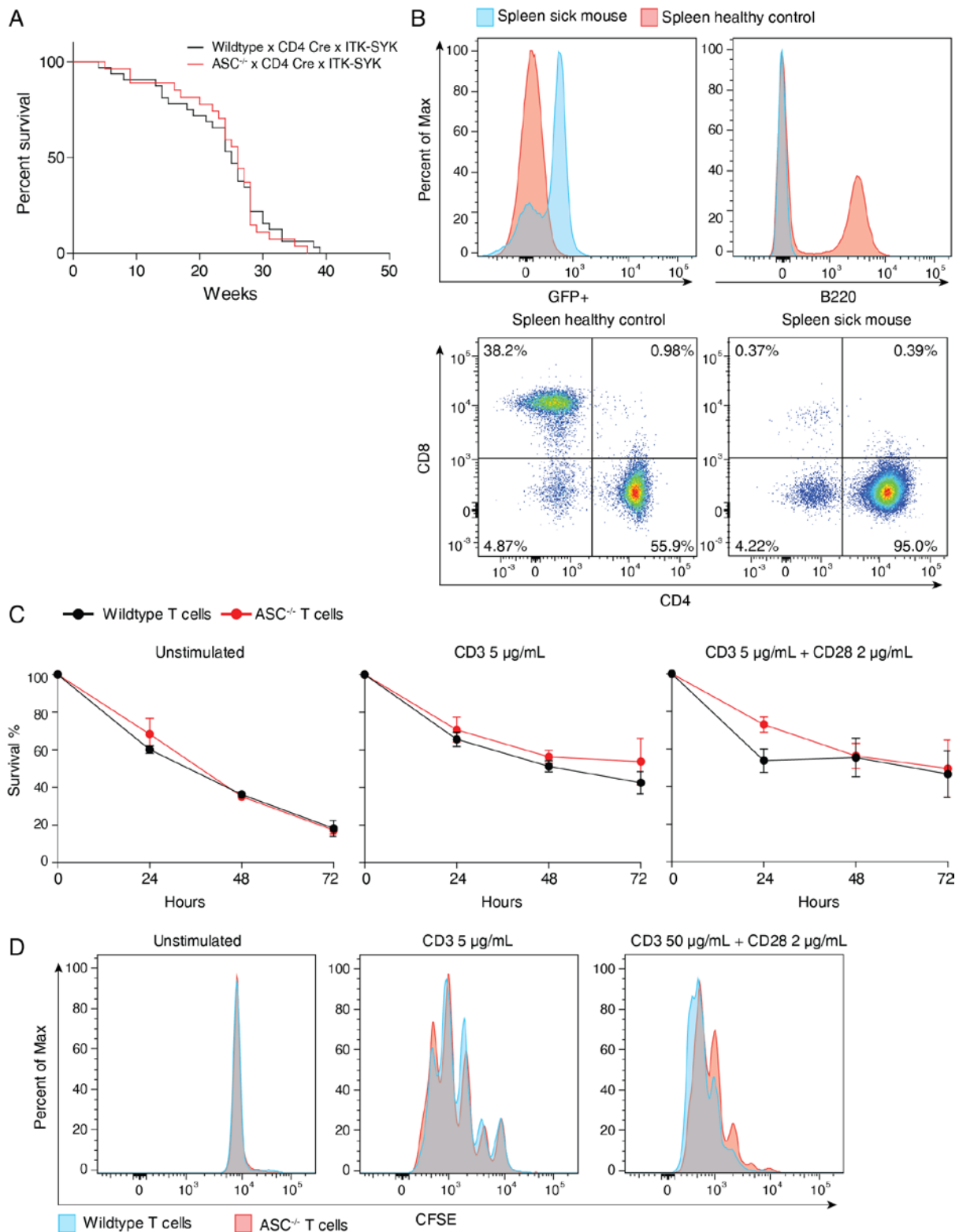
Together with the survival analysis, flow cytometry of lymphoid organs was conducted at time of death to confirm the disease entity and detect any difference between the two genotypes. Of note, the construct bearing ITK-SYK oncogene has a GFP reporter gene as well. Mice who inherited the oncogene were easily detected by the presence of green cells in their lymphoid organs (Figure 19B). Also, the lymphoma entity was confirmed by the analysis of T cells. In fact, sick mice display a drastic reduction in B cell percentage (Figure 19B up-right panel) and an increased expression of CD4 T cell markers with strong reduction of the CD8 (Figure 19B lower panel). More flow cytometric data were collected and they confirm no difference in the kind of lymphoma between the two genotypes. A thorough FACS characterization of the disease was already published by the research group that generated the mouse model and our

data could confirm these previous results (not shown) (Bach et al., 2014; Pechloff et al., 2010).

To further expand the analysis, we did survival experiments comparable to those shown before with B cells. T cells were magnetic bead-purified and seeded. Cells were either left untreated or stimulated with anti-CD3 and anti-CD28 antibodies as described before (Y. Li & Kurlander, 2010). Cells were harvested after 24, 48 and 72 hours and stained with a mix of 7AAD and AnnexinV and analyzed via flow cytometry. The survival profile reveals that T cells get slightly more activated by a mix of CD3 and CD28 rather than CD3 alone as it was with IgM and CD40 for B cells. However, no difference is appreciable between ASC knockout and wildtype cells (Figure 19C).

The same treatment was repeated with CFSE stained T cells to evaluate cell division events. This graphs confirm that CD3 plus CD28 treatment can induce a slightly higher response in T cells but no difference is detectable between the tested genotypes (Figure 19D). In conclusion, ASC does not seem to play a role in T cells for the tested conditions. Other tests are required if we want to clarify what is the function of ASC in T lymphocytes.





**Figure 19: ASC does not protect mice from peripheral T cell lymphoma progression.** (A) survival test of a PTCL malignancy. ASC deficient animals (N =28) do not differ in survival from wildtype mice (N=34). (B) Flow cytometry comparing a wildtype healthy control and a wildtype sick mouse. Sick mice show GFP signal as the reporter gene is on the same oncogenic construct (on the left). A B220 analysis reveals loss of B cells (on the right). CD4 vs CD8 comparison of a control and a sick mouse. (C) Primary T cells were magnetic bead-purified from spleen and lymphnodes. Cells were seeded, stimulated with the described stimuli and incubated for either 24, 48, or 72 hours. Cells were harvested, stained with 7AAD and AnnexinV, and analyzed by FACS. Plotted data representative of the 7AAD/AnnexinV total number of live cells expressed in percentage as compared to day one. Data are depicted as mean  $\pm$

SD of technical triplicates. (D) The same procedure of stimulation was adopted to track proliferation of the lymphocytes. B cells were stained with CFSE and then analyzed by FACS. Data are plotted as percentage of maximal intensity.

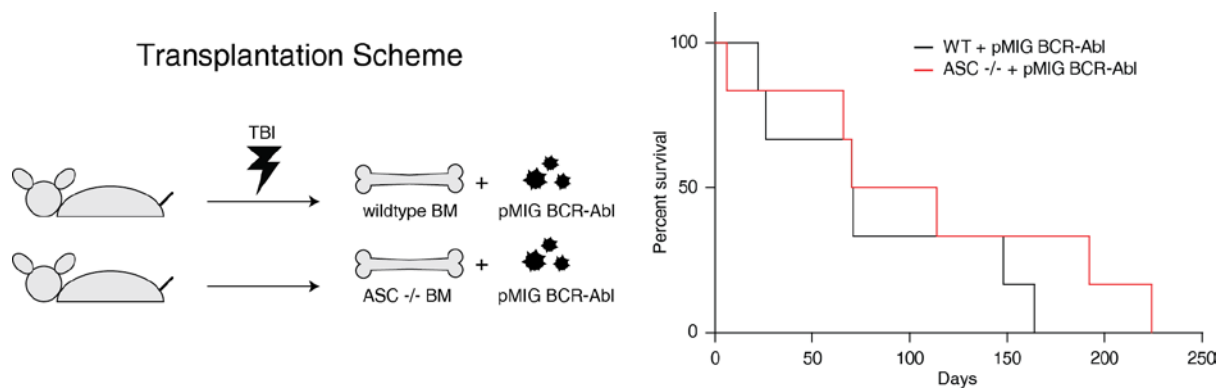
### A preliminary test to analyze the role of ASC in myeloid leukemia

So far, we tested lymphoid cells as the one where we could isolate a putative non-inflammasome and pro-apoptotic function of ASC, without getting positive results. We next switched to a myeloid leukemia model. In myeloid cells, ASC is a component of the inflammasome and deletion of this gene would strongly influence pyroptotic cell death.

We used a transplantation model provoking chronic myeloid leukemia, developed by our collaborators (Miething et al., 2006). The model uses a retroviral murine stem cell virus (MSCV), bearing the p210 form of the BCR-Abl oncogene. BCR-Abl is a strong oncogene that can originate CML malignancies as a single hit, without need of additional mutations (Dingli, Traulsen, Lenaerts, & Pacheco, 2010). The construct is first co-transfected in HEK 293T cells together with an ecotropic packaging vector system to produce retrovirus. The retrovirus is used to transduce cultivated murine bone marrow stem cells. The construct bears an EGFP reporter that allows to quantify efficiency of transduction as percentage of GFP-positive cells in the FITC channel by means of FACS.

Wildtype recipient mice were subjected to total body irradiation. They were then transplanted with either wildtype or ASC knockout bone marrow transduced with the BCR-Abl oncogene (Figure 20A). In this way, the newly reconstituted hemopoietic system will also generate a CML cancer. After mice were transplanted and followed up for complete recovery from the irradiation, they were kept under strict surveillance until they reached the terminal disease stage. The survival curve shows no significant difference between the ASC knockouts and the wildtype transplanted animals (Figure 20B). The log-rank p-value is 0.4048 and the Gehan-Breslow-Wilcoxon test reports a p-value of 0.6921. The median survival rate is circa 80 days for both the genotypes. The transplanted mice were 6 per group. These results indicate another type of blood cancer where ASC does not play a significant role in its progression. Further tests are required to answer the question whether ASC plays a role in CML. Of note, our collaborator of the Jost Group at Klinikum rechts der Isar have used a similar model where the oncogene is FLT3-ITD instead of BCR-Abl. In this model, they identified a

role for ASC in cancer development from the myeloid lineage that is in context to its function within the inflammasome (Höckendorf et al., 2016). These findings underline that ASC can, in addition to skin cancer and other malignancies, indeed also play a role in cancers of the immune system and that other model systems are worth testing to expand this line of research.



**Figure 19: ASC does not protect mice from CML.** (A) transplantation scheme used to generate chimeric mice that will develop CML through transduction with the BCR-Abl oncogene. (B) Transplanted mice (6 animals per group) were left in IVC cages in a SPF facility with food and water ab libitum. Terminal stage mice were euthanized and a macroscopic evaluation was performed.

## DISCUSSION

### Discussion for project one

#### The ability of TKIs to activate the inflammasome does not correlate to their binding of NQO2

NQO2 can bind multiple drugs and xenobiotics, indicating the promiscuity of the enzyme. The binding site is large enough to hold many chemical moieties and the oxidoreductase function may be involved in general xenobiotic detoxification. This activity is implied both in radical detoxification as well as in ROS production through redox cycling. Since ROS production is one of the activation mechanisms of NLRP3, this could be the connection. However, the recently-published data from our group indicates that R837 can bind other flavoproteins in addition to NQO2 and shows mitochondria are a major source of ROS. This indicates that NQO2 is not alone responsible for inflammasome activation (C. J. Gross et al., 2016).

In Figure 13, I show that several other TKIs are inducing the NLRP3 inflammasome. Analysis of kinobeads datasets provided by our colleagues from the Chair of Proteomics and Bioanalytics (Medard et al., 2015), indicate that other TKIs can bind NQO2. The ability of these TKIs to bind NQO2 does not correlate with their ability of inducing the inflammasome. For example, nilotinib and axitinib can bind NQO2 with low  $K_D$  (circa 200 nM for the first and 8  $\mu$ M for the second) but do not activate the inflammasome. Vice versa, crizotinib can induce strong NLRP3 activation but shows no interaction with NQO2 (Table 3). In line with our published data, this supports the hypothesis that NQO2 does not have a primary role as mediator of NLRP3 inflammasome (C. J. Gross et al., 2016).

Drug/Targets	Erlotinib	Axitinib	Sorafenib	Ponatinib	<u>Dasatinib</u>	Sunitinib
$K_D$ for NQO2 (nM)	NA	8612.735	NA	NA	NA	1812.416
Drug/Targets	<u>Crizotinib</u>	Nilotinib	<u>Pazopanib</u>	<u>Crenolanib</u>	<u>Imatinib</u>	<u>Masitinib</u>
$K_D$ for NQO2 (nM)	NA	208.3261	12115.58	22.12974	3.486196	337.7417

**Table 3: the ability of TKIs to bind NQO2 does not correlate with their ability of inducing the inflammasome.** Data were extrapolated from kinobeads assay done by Dr. Guillaume Médard, Chair of Biochemistry (TUM). Bosutinib is missing as it was not included in the assay. Those TKIs that are underlined can also activate the inflammasome.

### Four different effects of TKIs on myeloid cells

Several TKIs were tested together to assess their ability to induce inflammasome activation and cell death (Figure 13). From the obtained results, we can divide the TKIs in four different categories based on inflammasome activation and if cell death is also dependent on pyroptosis or not. The first category comprises those TKIs that could induce the NLRP3 inflammasome but provoked cell death mostly independent of it. Namely these compounds are bosutinib, crenolanib, crizotinib, imatinib, and masitinib. The second category is represented by those TKIs that induce NLRP3 inflammasome and pyroptotic cell death only, these are dasatinib and pazopanib. The third category comprises the TKIs that are inducing cell death and IL-1 $\beta$  secretion independently of the inflammasome. These provoke more secretion of IL-1 $\alpha$  and pro-IL-1 $\beta$  than the mature form of IL-1 $\beta$ , indicating that the ELISA assay for IL-1 $\beta$  may have detected the pro form non-specifically. These drugs are ponatinib, sorafenib, and sunitinib. The fourth category comprises axitinib, erlotinib and nilotinib and these TKIs are neither inducing the inflammasome nor cell death.

Depending on their category, these drugs can be used as “tools” to study cell death and inflammasome activation. Specifically, TKIs that fall into the second category, like pazopanib, can be used to elucidate the fine mechanisms of NLRP3 regulation as they induce cell death completely dependent on it. Future research will be also aimed to expand the study to other TKI compounds to see in which of the four categories they might belong to. Also, 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.

### Imatinib activates the NLRP3 inflammasome

The project derived from results obtained in our lab while working on small molecules that can activate the inflammasome. Since both R837 and imatinib bind NQO2, and R837 is a small-molecule activator of NLRP3, we tested imatinib on BMDCs and found that this drug can induce the inflammasome. This was a new observation and therefore, we became interested to understand the mechanism behind this inflammasome activation.

After testing imatinib on inflammasome-competent cells, we could immediately and astonishingly observe that they are both fast-acting and strong inducers of IL-1 $\beta$  secretion, implicating inflammasome activation. After several repeated experiments, we observed that TKIs have a sort of threshold concentration around 40-50  $\mu$ M, at which they go from no or a weak response to inducing the secretion of maximal amounts of IL-1 $\beta$  (Figure 4). Above the threshold, there is still IL-1 $\beta$  secretion but it reduces gradually as the drug increases in concentration. Indeed, tests conducted over concentrations of 100  $\mu$ M, showed a gradual reduction until loss of signal for IL-1 $\beta$  secretion (not shown). The interpretation I give is that after a certain amount of drug, death occurs so quickly that cells cannot assemble a full inflammasome complex and they die before can secrete IL-1 $\beta$ .

Next, we tested the TKIs on BMDCs deficient for different inflammasome components. TKIs induce strong IL-1 $\beta$  in wildtype cells but not in those derived from inflammasome knockout mice. We can therefore say that NLRP3 is the sensor responsible for inflammasome activation upon imatinib treatment. However, a residual signal for IL-1 $\beta$  secretion was detectable for NLRP3 knockout, whether ASC-deficient cells had no leftover signal (Figure 5). The easiest explanation is that another inflammasome participates in the TKI-induced response. Basing on IGP database expression profiles, NLRP1, NLRC4, and AIM2 are well expressed in both dendritic cells and macrophages. In fact, a group already found NLRC4 associated with NLRP3 upon *S. typhimurium* infection adding to the same macromolecular complex (Man et al., 2014). Further tests on other inflammasome knockout cells will be aimed to clarify this point. Nevertheless, NLRP3 remains the major contributor to the IL-1 $\beta$  secretion induced by TKIs. The next objective was to explore the inflammasome activation events upstream of NLRP3 that are triggered by our compounds.

### Upstream requirements for Imatinib-induced NLRP3 inflammasome activation

Conventional NLRP3 activators cause potassium efflux, ROS production, lysosomal leakage, or a combination thereof. To understand which one of those might be used by imatinib, we had to test the effect of inhibition of all these pathways. Imatinib provokes robust, largely inflammasome-independent potassium efflux and high extracellular concentrations of KCl can effectively block IL-1 $\beta$  secretion even at higher

drug concentrations (Figure 6). Measurements of residual elemental potassium could confirm this result. These findings strongly indicate that potassium efflux is one of the mechanisms engaged by imatinib.

ROS production also appears to be involved in imatinib-induced inflammasome activation, as ROS scavengers and idebenone could reduce IL-1 $\beta$  secretion. This is also evidenced by roGFP2 experiments, where imatinib induces a mild, but still detectable ROS signal as compared to that of R837 (Figure 7). Given these results and compared with other inflammasome literature, it appears there is an important interplay between mitochondrial instability and potassium efflux at the basis of inflammasome activation, in general and upon TKIs stimulation. For instance, there were articles where ebselen was shown to act on voltage-gated K<sup>+</sup> channels (Bubolz, Wu, Larsen, Gutterman, & Liu, 2007; H. Li, Gutterman, Rusch, Bubolz, & Liu, 2004). Moreover, in a recent publication, the authors observed the reduction of IL-1 $\beta$  secretion by ebselen was not totally correlated with loss of ROS production but rather with an overall change of mitochondrial function (Jabaut et al., 2013). Idebenone was also recently used to inhibit the inflammasome and the authors report a mechanism that interferes with potassium efflux as well (Newman et al., 2011). APDC is a good ROS inhibitor but is also a potent and selective agonist of group II metabotropic glutamate receptors (mGluR-2). These receptors regulate opening of potassium channels to trigger synaptic signaling. APDC was shown to interfere with this activity (Hull, Chu, Thanawala, & Regehr, 2013). For these reasons, I believe the cross-talk among the events is fundamental for every activator of the NLRP3 inflammasome. Imatinib and the other TKIs, are the right compounds that can be used to dissect the fine regulatory mechanisms of both potassium efflux and ROS production at the basis of inflammasome activation.

Lysosomal rupture with consequent cathepsins release is another event involved in inflammasome activation (Hornung et al., 2008). We have only one preliminary test available so far and it indicates that imatinib causes a quick and strong lysosomal rupture. However, this test has some intrinsic limitations and future experiments will be conducted with microscopy or with cathepsins inhibitors on BMDCs or BMDMs.

P2X7 is a purinergic receptor and a ligand-gated channel that opens upon binding of ATP and allows potassium efflux for NLRP3 activation (Dubyak, 2012). In P2X7 knockout cells, the IL-1 $\beta$  secretion was significantly reduced upon stimulation by lower

concentrations of imatinib. This could mean that imatinib, when at lower concentrations, shares the same activation mechanism with ATP and binds to P2X7 receptors non-specifically to trigger potassium efflux (Figure 11). However, with increasing imatinib concentrations, IL-1 $\beta$  is secreted normally, as much as from the wildtype cells. This may mean that at higher concentrations, the lytic cell death and subsequent potassium release is enough to overcome any effect on potassium efflux determined by P2X7 and induces NLRP3 without further specificity.

NEK7 is another upstream regulator of NLRP3 and imatinib can activate the inflammasome largely independent of it, which, to our knowledge, is the first time this phenomenon is observed. The control stimuli are mostly NEK7 dependent and this confirms the fact that TKIs activate NLRP3 through a non-conventional method. Curiously, nigericin retains circa half of the IL-1 $\beta$  secretion in NEK7 knockouts. This was unexpected and we are currently investigating this phenomenon.

As introduced in the result section, I developed the hypothesis of a sort of feedback loop involved in NLRP3 activation by TKIs. This would require that imatinib induces first cell lytic cell death, potassium efflux, NLRP3 activation, and GSDMD cleavage that will further destabilize the membrane and foster the circuit. This is the case for non-canonical, caspase-11-mediated inflammasome activation (Sborgi et al., 2016) (Kayagaki et al., 2015). Indeed, all the evidence collected for imatinib points to a similar mechanism of NLRP3-independent lytic cell death leading to potassium efflux and thereby NLRP3 activation. Therefore, I speculated that caspase-11 may play a role in TKIs-induced inflammasome activation and to this extent we tested SV129 cells, naturally having a non-functional caspase-11. In these experiments, we did not observe any reduction of IL-1 $\beta$  secretion, therefore, the non-canonical inflammasome has no role in TKI-induced IL-1 $\beta$  production (Figure 11). This points to a different form of lytic cell death induced by imatinib that couples to NLRP3 activation primarily through potassium efflux which can also boost itself by generating a feedback loop.

### Is there a role for caspase-8 in TKI induced inflammasome activation?

The TKIs were tested in the presence of the pan-caspase inhibitor zVAD and the caspase-1 selective inhibitor YVAD. Both compounds effectively blocked IL-1 $\beta$  secretion in a way comparable to that of caspase-1 knockout cells (Figure 8). By analyzing uninhibited wildtype samples by Western blot, we could detect caspase-8



cleavage in imatinib-treated cells and, very faintly, in those of masitinib. This signal was strongly increased in caspase-1-deficient cells. This can be explained by the fact that caspase-8 associates to the ASC speck and contributes, in minor role, to IL-1 $\beta$  cleavage as it was already shown before (Monie & Bryant, 2015). Another explanation can be that caspase-8 can get activated upstream and independently of inflammasome formation because of imatinib-induced cell death. Indeed, caspase-8 has not only been implicated as caspase acting downstream of ASC, potentially in parallel or instead of caspase-1, but was also reported to contribute to inflammasome activation upstream of NLRP3. To verify whether caspase-8 acts upstream or downstream of ASC, we need to analyze caspase-8 cleavage in ASC deficient cells stimulated with imatinib. With this experiment, we will see if caspase-8 cleavage happens because it associates to ASC specks or independently of it.

### The mode of IL-1 $\beta$ release induced by Imatinib

The way IL-1 $\beta$  is secreted from the cell upon imatinib stimulation can possibly come by two events that do not exclude each other. Either, the active caspase-1 cuts GSDMD, thus provoking IL-1 $\beta$  release through pores or, imatinib-induced cell death causes enough membrane instability to allow passive IL-1 $\beta$  release. The first hypothesis is also supported by recent literature that suggests that IL-1 $\beta$  can exit the cell through pores formed by GSDMD. However, it is still not clear if IL-1 $\beta$  gets out through such pores or if it gets out by the lysis that happens after pore formation (W. T. He et al., 2015; X. Liu et al., 2016).

Imatinib is largely dependent on GSDMD to trigger IL-1 $\beta$  secretion, however, the ELISA experiments show a residual IL-1 $\beta$  signal even in GSDMD knockout cells, very strong in case of masitinib (Figure 11). In caspase-1 knockouts, IL-1 $\beta$  cleavage does not happen (except possibly by some minor contribution from caspase-8 as just discussed) and GSDMD-dependent release is not possible as well. If the cell then dies, it might still be possible the release of pro-IL-1 $\beta$  that the ELISA does not detect. This explains why there is no residual signal for IL-1 $\beta$  secretion in caspase-1 knockouts. If we compare the signal from caspase-1 with that of GSDMD knockout cells, I can say that in GSDMD knockouts, since caspase-1 is still present, IL-1 $\beta$  will still get cleaved in the cells and might then be released by other mechanisms. This

release happens even in absence of GSDMD pores and the easiest explanation for this phenomenon is that imatinib (and even more, masitinib), while provoking lytic cell death and can thereby induce passive release of cleaved IL-1 $\beta$  into the supernatant, thus bypassing the need for GSDMD-dependent release. Another hypothesis is that maybe caspase-1, which is still present and active in GSDMD knockouts, can cleave another gasdermin family protein that is also able to form pores on the cell membrane and allow release of IL-1 $\beta$  but that this process is much slower than the GSDMD-dependent route. Yet another hypothesis would be that IL-1 $\beta$  can use another form of unconventional secretion route independent of lytic pore formation. Further tests will be conducted to explain this phenomenon.

### Type of Cell death induced by Imatinib

A central aspect is the question what is the mechanism by which imatinib triggers cell death. Concerning this, we observed significant differences between imatinib and the control stimuli. The first thing to mention is that, given the concentration-dependence, cell death follows the trend of IL-1 $\beta$  secretion and reaches almost the maximum intensity already after the threshold concentration (discussed before) needed to trigger the inflammasome (Figure 4), which is not unusual. However, inflammasome knockout BMDCs cannot secrete IL-1 $\beta$  but die practically as much as their wildtype counterparts (Figure 5). This is also evidenced by the experiments conducted using the NLRP3 inhibitor CRID3, where BMDCs dye as much as the control untreated cells. These are first observations pointing towards a death mechanism other than pyroptosis that does not depend on the inflammasome and that its activation is most likely a consequence of the imatinib-induced cell death. Also, from the western blots, it is possible to observe a transition of actin from the cell lysates to the supernatant that means cells are undergoing lysis because of an induced membrane instability, which is nonetheless a feature shared among many different programmed cell death pathways (Vanden Berghe, Linkermann, Jouan-Lanhouet, Walczak, & Vandenabeele, 2014). Taken together, these experiments exclude that pyroptosis is the only cell death pathway induced by imatinib.

Experiments conducted with the pan-caspase inhibitor zVAD showed that LDH release can occur even under this condition and that it reaches the level of non-inhibited control samples. This results clearly exclude the classic features of apoptosis and

since imatinib could induce cell death even upon zVAD pretreatment, it activates a form of cell death that does not strictly require the action of caspases. Therefore, other proteases may play a role in imatinib-induced cell death, like for instance serine proteases or calpains (Vandenabeele, Orrenius, & Zhivotovsky, 2005). Another form of programmed cell death that does not strictly require the action of caspases is necroptosis (Moriwaki, Bertin, Gough, Orlowski, & Chan, 2015). Therefore, considering the results just discussed with zVAD, we tested if imatinib induces necroptosis. The results show that the necroptosome is not involved in this cell death events since both RIPK3 and MLKL knockouts as well as necrostatin1 pretreated BMDCs showed the same LDH release profile of controls (Figure 9).

So far, the results could not point us toward the right cell death pathway induced upon imatinib treatment. However, we know that death was occurring through a strong membrane destabilization and one way that could interfere with it might be by protecting the cell membranes with osmoprotective agents. To this extent we tested wildtype cells pretreated with polyethylene glycol, as it was already used successfully to protect cells from lytic cell death (Fink & Cookson, 2006). Our results show that BMDCs were significantly protected from lytic cell death with PEG 3000 3 mM at low and medium TKI concentrations. Remarkably, the classic NLRP3 activators like nigericin and ATP were not affected in their action as LDH release profiles show no reduction in LDH release. These data demonstrate that imatinib induces cell death through a potent cell membrane destabilization that does not require the action of a specific channel like in the case of ATP.

To sum up all the results obtained, we can say that imatinib rather activates a form of mixed cell death that provokes a substantial cell membrane destabilization which is the trigger event for inflammasome assembly. From our data, we could exclude that imatinib causes either apoptosis, pyroptosis, or necroptosis alone but it may either activate a mix of all or it does trigger another specific form of regulated necrosis which is not known. We can also exclude that imatinib damages the membrane non-specifically and provokes non-regulated necrosis since toxicity experiments conducted on 3T3 cells show that imatinib (and the other TKIs), even after 6 hours of exposure, does not induce substantial LDH release (Figure 13C). Therefore, it appears more likely that imatinib causes a new regulated form of lytic cell death (Table 4). There are several other forms of regulated necrosis that were described in the last 10 years by research groups that investigated on programmed cell death, like for instance

ferroptosis, parthanatos or mitochondrial permeability transition (MPT) necrosis (Galluzzi, Lopez-Soto, Kumar, & Kroemer, 2016). Future research will be aimed to understand if imatinib activates one of these pathways specifically, or if it induces a yet undiscovered mechanism of cell death. Importantly, this would be a cell-type specific form of cell death since for example fibroblast are not capable of it.

Apoptosis		Regulated necrosis					
<b>Morphology</b>							
Cytoplasmic shrinkage, chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), blebbing of the plasma membrane, and shedding of apoptotic bodies		Increasingly translucent cytoplasm, swelling of organelles, lysosomal membrane permeabilization, increased cell volume (oncosis), permeabilization of the plasma membrane, mild chromatin condensation, and nuclei remain intact					Loss of nuclear Integrity, massive chromatin decondensation
<b>Death modality</b>							
Intrinsic apoptosis	Extrinsic apoptosis	Necroptosis	Ferroptosis	MPT-mediated regulated necrosis	Parthanatos	Pyroptosis	NETosis/ETosis
<b>Death regulatory factors</b>							
BID, BAX/BAK, Cytochrome c, APAF1, CASP9	RIPK1*, RIPK3†, FADD, CASP8	RIPK1†, RIPK1*, RIPK3*	GPX4	CYPD	PARP1	Inflammasome	NOX
<b>Death execution factors</b>							
CASP3, CASP7		MLKL ion channels	GSH decrease Fe2+	Ca2+ increase	NAD+ increase ATP increase	ROS increase	
		Lipid peroxidation, energetic catastrophe, and lysosomal and plasma-membrane permeabilization					
<b>Synthetic inhibitor (factor they inhibit)</b>							
zVAD-fmk (CASP) q-VD-Oph (CASP)		NEC1/s, Cpd27 (RIPK1), GSK843 (RIPK3), GSK872 (RIPK3), NSA, GW906742X (MLKL)	Fer-1, DFO	SfA (CYPD), CsA (CYPD)	3-AB (PARP1), PJ-34 (PARP1)	CRID3 (?), VX-740 (CASP1), VX-765 (CASP1)	DPI (NOX), GKT137831 (NOX1 and NOX4)
<b>Physiology</b>							
Controlling cell numbers during embryogenesis and homeostasis Immune regulation Pathogen defense		Embryogenesis? Homeostasis? Inflammation IR-injury Thrombosis Neurodegeneration	Glu toxicity IR-injury Neurodegeneration Transplantation	IR-injury Thrombosis Transplantation	DNA damage Neurodegeneration	Inflammation Pathogen defense	Inflammation Extracellular trap formation Pathogen defense

**Table 4: Regulated necrosis pathways.** In this table are listed the most representative pathways of regulated necrotic cell death and their features. Abbreviations: 3-aminobenzamide (3-AB), apoptotic protease-activating factor 1 (APAF1), BH3-interacting domain death agonist (BID), compound 27 or fluro[2,3-d]pyrimidine 27 (Cpd27), cyclosporine A (CsA), diphenylene iodonium (DPI), Fas-associated death domain (FADD), ferrostatin-1 (Fer-1), reduced glutathione (GSH) glutathione peroxidase 4 (GPX4), necrosulfonamide (NSA) necrostatin-1/s (NEC1/s), NADPH oxidase (NOX), quinoly-Val-Asp-Oph (qVD-Oph), sangliferin a (SfA), transient receptor potential cation channel subfamily M member 7 (TRPM7), carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketon (zVAD-fmk). \*Kinase active form, †kinase inactive form. Inspired and adapted from Pasparakis & Vandenabeele (2015), Nature, 517(7534).

### Masitinib is a stronger stimulus than imatinib

In all the experiments performed, masitinib has demonstrated to be a stronger stimulus than imatinib. The primary and surprising difference was that masitinib retains a significant IL-1 $\beta$  residual signal under potassium efflux-inhibition when used at higher concentrations. The easiest explanation for this phenomenon is that masitinib acts somewhat faster than imatinib and therefore provokes cell lysis, hence potassium efflux, in quicker terms despite the presence of high extracellular KCl thereby inducing the NLRP3 inflammasome. However, it might also be that masitinib triggers NLRP3 activation through a mechanism that does not require K<sup>+</sup> efflux like in the case of R837 (Figure 6). By analyzing all the experiments, in general, I believe that masitinib 20  $\mu$ M shows IL-1 $\beta$  secretion and LDH release profiles that are more like imatinib at 40 or 60  $\mu$ M. The difference could be either from a kinetic point of view, meaning that masitinib acts quicker than imatinib, or from a different mode of action.

Masitinib was used as a comparison because it has a very similar structure to that of imatinib but there is a substantial difference between the binding partners of the two compounds (Figure 3). Imatinib was designed to bind selectively BCR-Abl but it can also interact with transmembrane tyrosine kinase KIT and platelet derived growth factor receptor (PDGFR). However, masitinib can bind KIT and PDGFR but not BCR-Abl meaning they have a different binding specificity. However, in the literature there is no suggestion of an involvement of BCR-Abl in NLRP3 activation. As imatinib can bind other proteins (not only kinases) in addition to his specifically designed targets, I can hypothesize that also masitinib has other interactors. Those targeted proteins may differ from those of imatinib and probably cause a stronger impact on cell death and inflammasome activation. To further explain this theme, we would need to determine the differences in interactions of imatinib and masitinib.

### Pazopanib activates NLRP3 without causing K<sup>+</sup> efflux

A major outcome of this project is the finding that TKIs can induce NLRP3 inflammasome in an unconventional way that can be used to elucidate the molecular mechanisms of inflammasome activation. Pazopanib, unlike imatinib and masitinib, shows an inflammasome activation profile and LDH release comparable to that of NLRP3 control stimuli. However, pazopanib is potassium efflux-independent but is well inhibited by ebselen (ROS scavenger) and therefore acts in a way like that of R837

among the NLRP3 inflammasome stimuli. Pazopanib is therefore another good candidate to study the fine mechanisms behind NLRP3 activation. It is also NEK7 and GSDMD dependent and triggers cell death in an inflammasome-dependent manner no matter how high the concentration used (Figure 14 and data not shown). For these reasons, this compound is also a particularly suitable to study the regulation of pyroptosis. In general, all the tested TKIs show some differences in their NLRP3 activation mechanism and they constitute a variegated category of small molecules that can be used to dissect the unknown regulatory aspects of inflammasome and pyroptosis.

### Inflammasome-driven immunomodulatory effects induced by imatinib

As TKIs are used to treat blood cancers, we wanted to understand if those drugs may trigger inflammasome activation and pyroptosis on malignant cells. We tested many different human cell lines representing the main types of myeloid leukemia. Namely, acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and some lines of B or T cell cancers. Basing on database expression profiles of primary, untransformed cells of these lineages, we expected to find inflammasome competent cells in myeloid rather than lymphoid lines. We observed that none of the lymphoid lines were inflammasome-competent and, surprisingly, only a small number of myeloid cell lines had all the components. Curiously, only some of the tested AML lines had full functional NLRP3 inflammasome while none of the CML lines were inflammasome-competent (Figure 12). This initially was unexpected because we thought both CML and AML could be inflammasome-competent as they both belong to the myeloid lineage. Since imatinib is used to cure CML diseases, we wanted to investigate the inflammasome on this specific type of cancer but after these results we moved to study AML cells instead. However, this difference could be due because CML is a slow growing form of leukemia where cells may have the tendency to accumulate genetic or epigenetic alterations that block cell death pathways (as this is one of the cancer survival mechanisms), and one of them could be the inflammasome. Instead, AML is an acute form of leukemia that can develop quite rapidly and cells may retain a transcription profile closer to that of stem cells from where they are generated meaning that they do not express genes and proteins involved in pattern recognition that can induce growth arrest as well as factors for regulated death pathways but are more

committed to self-renewal. In line with this, our collaborators recently showed in a mouse model of AML, that tumor onset is accelerated when leukemic precursor cells lose expression of necroptosis and inflammasome components. The lack of RIPK3 and an impaired IL-1 $\beta$  secretion can foster tumor growth (Höckendorf et al., 2016). To clarify if CML cancers have in general a block in inflammasome activation, we would need to access and analyze patient's expression profiles for the different components of the inflammasome.

Furthermore, by testing TKIs on the inflammasome-competent AML cell lines (based on expression), we saw no strong IL-1 $\beta$  secretion. In addition, the inhibitor CRID3 was not effective in dampening the IL-1 $\beta$  signal (Figure 12). This could be explained either by the fact that some non-inflammasome mechanisms of IL-1 $\beta$  processing are involved or simply that the TKIs, while inducing cell lysis, also provoke release of the pro IL-1 $\beta$  form which is detected non-specifically by the ELISA assay. To verify this hypothesis, we would need to analyze the IL-1 $\beta$  signal on a western blot. Taken together, these results can be summarized in two points: first, most of the cell lines are not inflammasome-competent, and second, TKIs are not good inflammasome stimuli for the tested cell lines. It might be however, that the cell lines tested may have acquired a genetic or epigenetic alteration in other genes than those encoding for inflammasome components which can prevent its activation.

Also, AML cell lines usually require a maturation step to become mature monocytes or macrophages and this is usually done *in vitro* by treating them with PMA. Nonetheless, it is impossible to administrate PMA to a patient and, to my knowledge, nobody ever established a clinical trial for this. Thus, it would be difficult, if not impossible, to activate the inflammasome *in vivo* in leukemic cells within a patient's bloodstream. A subtype of AML, acute promyelocytic leukemia (APL), is characterized by a chromosomal translocation that fuses the retinoic acid receptor-alpha (RARA) with the oncosuppressor transcription factor promyelocytic leukemia protein (PML). The fusion protein binds with enhanced affinity to sites on the cell's DNA, blocking transcription and differentiation of granulocytes (De Braekeleer, Douet-Guilbert, & De Braekeleer, 2014). This disease can be cured by administration of all-trans retinoic acid (ATRA), the acidic form of vitamin A. ATRA induces the release of the fusion oncogene from DNA thereby allowing terminal differentiation of the leukemic promyelocytes. Once promyelocytes have matured into granulocytes, those cells undergo spontaneous

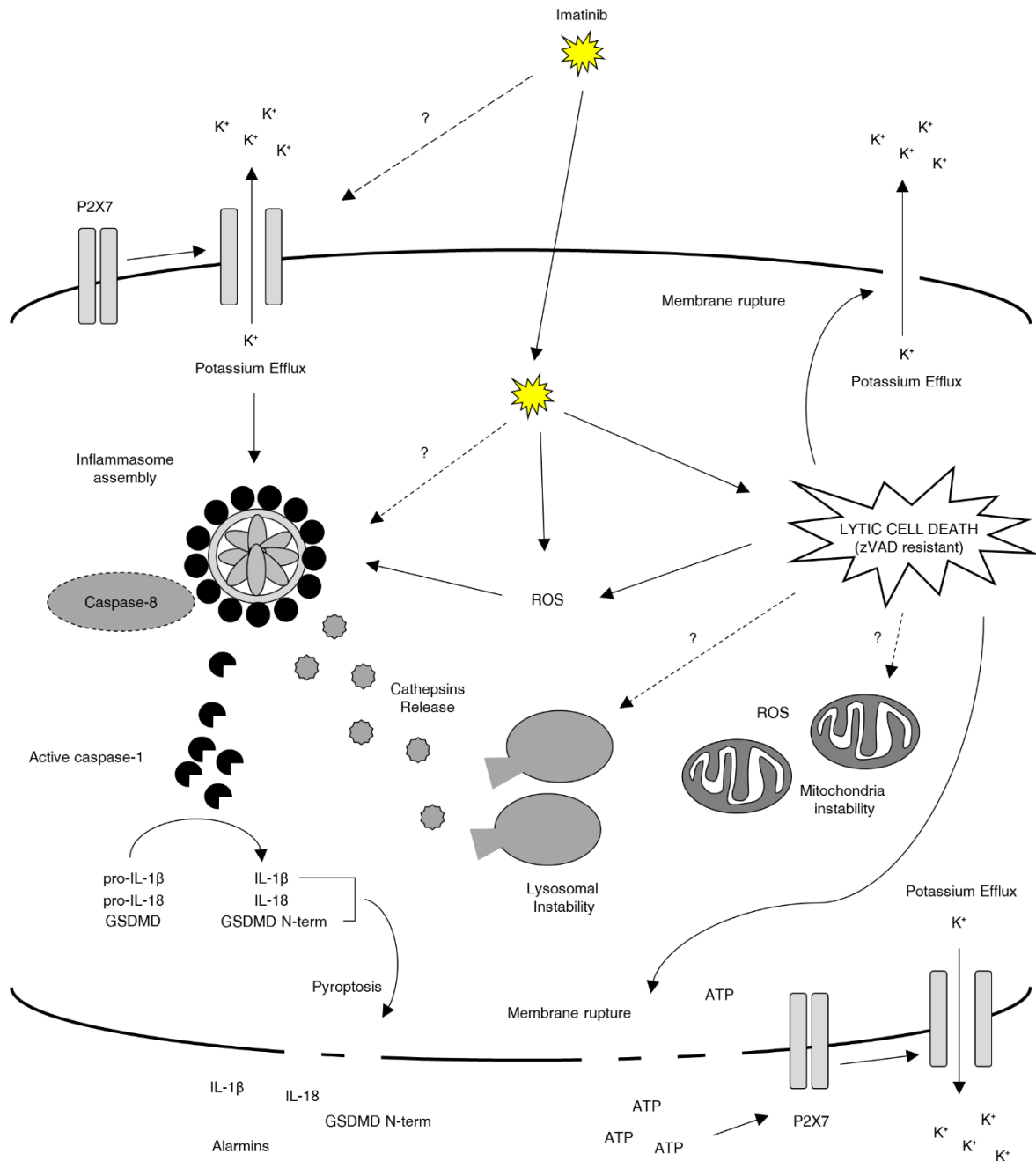
apoptosis. More recently, an arsenic trioxide ( $\text{As}_2\text{O}_3$ ) solution (administered intravenously) was introduced as co-treatment with ATRA to reduce refractory incidences (Lo-Coco et al., 2013). Initially, we thought to examine inflammasome activity in those cells that undergo this ATRA plus  $\text{As}_2\text{O}_3$  treatment. However, after a bibliography research we found that arsenic compounds are potent inflammasome inhibitors (Maier, Crown, Liu, Leppla, & Moayeri, 2014). For these reasons, I may speculate that the inflammasome is, in general, not involved in the therapeutic action of imatinib on cancer cells.

In contrast, PBMCs are responsive to TKIs (Figure 12) and this means that the inflammasome may instead have a role in TKI-driven immunomodulatory effects. So far, many of them were already described for imatinib (Zitvogel et al., 2016). Additionally, a recent work assessed serum levels of IL-1 $\beta$  in patients having CML and being cured with imatinib. The final outcome is that individuals with better prognosis have higher quantities of IL-1 $\beta$  in their bloodstream (Matti, Saleem, & Sabir, 2014). Yet, another study claims that a higher secretion of IL-1 $\beta$  correlates with insurgence of BCR-Abl mutations that can prevent binding of imatinib to its ATP pocket (C. R. Lee et al., 2016). The role of the inflammasome in immunomodulation is therefore still unclear and needs further investigation and *in vivo* data.

Finally, we considered another aspect regarding side effects of imatinib. Those are frequently localized in the gastrointestinal tract and cause nausea, stomach pain, vomiting, and diarrhea. These effects were thoroughly described before for patients having either CML or GIST (Peng, Lloyd, & Schran, 2005). Most of the literature on imatinib side effects is based on blood levels and overall bioavailability measured at long intervals after drug administration. There are no deeper insights that explored the impact of imatinib and of the inflammasome on the intestinal epithelium. Epithelial cells are inflammasome-competent and TKI-induced inflammasome activation might therefore be involved in the side effects. For this reason, we tested imatinib on murine intestinal organoids to see if and at what concentration the drug becomes toxic to the cells. We observed a high toxicity over 10  $\mu\text{M}$  for both TKIs, measured by PI intensity and visual evaluation of their morphology (Figure 12). Based on bioavailability data (Novartis), the peak concentration registered for a 400-mg standard pill is  $2.6 \pm 0.8$   $\mu\text{g}/\text{mL}$  blood which roughly correspond to 5  $\mu\text{M}$  and is about the half of the quantity that induces toxicity in our tests. However, the effective concentration imatinib can



reach in the gut may be higher than this as the pills dissolve (Peng et al., 2005). In line with this results, another group reported that GIST derived human organoids are killed by imatinib over 10  $\mu\text{M}$ , but they do not investigate further on inflammasome (E. C. Chen et al., 2015). Further analysis is required to elucidate this aspect. A possible *in vitro* test to evaluate imatinib toxicity, could be to try wildtype versus inflammasome knockout organoids either healthy or derived from GIST tissue. Also, *in vivo* studies could reveal if the inflammasome plays a role *in vivo* upon imatinib administration and with or without GIST.



**Figure 20: Imatinib causes a mixed form of cell death that triggers inflammasome activation.** a final overview that resumes the death mechanisms triggered by imatinib. The most relevant event is the destabilization of the cytosolic membrane, provoking  $K^+$  efflux and consequent NLRP3 inflammasome activation. Dashed lines represent hypothetical mechanisms.

## Discussion for project two

### ASC does not alter the tested hematologic malignancies

Basing on previous reports, the ASC-encoding gene *Pycard* was found hypermethylated at the promoter level in several types of cancer. Hypermethylation blocks the expression of the targeted gene and it is a very common mechanism adopted by cancer cells to escape regulated cell death (Vogelstein et al., 2013). It was then hypothesized that ASC may play an inflammasome-independent function that would take place in the apoptotic pathways, hence, ASC was also designed as an oncosuppressor protein (Salminen et al., 2014). In addition, ASC controls inflammasome-dependent cell death (pyroptosis) that could likewise suppress cancer development in inflammasome-competent cell types. Inflammasome activation and IL-1 $\beta$  secretion can promote cancer development by driving inflammation but can potentially also have an impact on anti-cancer immune responses (Garlanda et al., 2013). For these reasons, we were interested in studying a cell-intrinsic function of ASC that would contribute to cell death and reduction of cancer growth. We controlled on several databases the expression of inflammasome NLRs, ASC, and caspase-1 in all the main types of immune cells. Basing on databases results, B and T cells do not express NLRP3 but still have high levels of other inflammasome sensors (NLRP1, NLCRC4, or AIM2), of ASC, and of caspase-1. There were also recent reports where the presence of functional inflammasomes in both T and B cells was shown (Arbore et al., 2016; Dell'Oste et al., 2015; Stoecklein et al., 2015). For this reasons, we thought that lymphocytes could be the ideal cells to test an intrinsic function of ASC in regulating tumoral growth, whether it could come from the inflammasome or from another apoptosis-related function of ASC. To this extent, we started by crossing ASC-deficient mice with lines of either B or T cell lymphoma.

The first model used was a mouse line developing a Burkitt's lymphoma. It generates an aggressive B cell malignancy that translates in a short survival interval after birth (circa 80 days). From our results, we could not observe any difference in the survival curves between the ASC knockouts and the wildtype mice (Figure 15). Still we thought it opportune to analyze the cell composition of the cancer in the two genotypes to determine if there was any difference in their tumoral entity. The data collected provide a detailed flow cytometric analysis that defines the cell composition of lymphoid organs

for this cancer model. However, also here we could not see any difference in disease entity between the tested genotypes (Figures 16 and 17). We next sought to test a model of T cell lymphoma to see if possibly T cells could be the right ones to find a more aggressive phenotype in those mice missing ASC expression. This was not the case as both ASC knockouts and relative controls have the same survival curve (Figure 19). Also, the type of cancer was the same for both ASC knockouts and control animals as confirmed by flow cytometry data (Figure 19). The analysis was further implemented with several tests conducted on wildtype and ASC-deficient primary B or T cells. All the experiments revealed a comparable activation and survival rate between wildtype and ASC knockouts cells as appreciable from both 7AAD/AnnexinV and CFSE datasets (Figures 18, and 19).

A third *in vivo* test was conducted with a BCR-Abl-induced CML. This was done to enlarge the chance to find a significant phenotype as myeloid cells are well established inflammasome-competent cells. Here, we used a transplantation model that introduces oncogene-expressing hemopoietic stem cells into irradiated recipient mice (Figure 20). The experiment was done with 6 wildtypes versus 6 ASC-deficient recipients and did not give any variation in survival between the genotypes. Further repetitions would be required to consolidate the phenotype. In conclusion, the data collected show no difference in cancer growth between wildtype and ASC-knockout mice for all the three tested cancer models.

### Inflammasome, pyroptosis and the role of ASC in cancer

The data obtained from project two indicate no difference between controls and ASC knockouts mice and can be interpreted in two distinct ways. As a first interpretation, I can affirm that in those tested models, ASC has no relevant role maybe because the oncogenes are too strong and can bypass any other death pathway. As a second way of interpretation, the inflammasome and any other apoptosis-related function of ASC potentially involved in cancer development in these lineages are not relevant in determining cancer growth and survival in the specific models tested. However, it does not mean that the same is true for all cancers. We could also expand the *in vivo* experiments to other models of solid or blood cancers to increase the possibility of finding a phenotype. Indeed, the first publications where ASC was described as

potential oncogene, were conducted on breast cancer derived cell lines (Conway et al., 2000).

So far, there are only a few papers evidencing a differential phenotype for ASC in cancer disease models and one of them was published by our collaborators (already mentioned before). The authors found that loss of RIPK3 and of the inflammasome accelerates cancer onset in a model of AML driven by FLT3-ITD oncogene (Höckendorf et al., 2016). Further investigation aimed to expand the knowledge about the role of ASC in other cancer models is required and it will also be providential to publish those findings where no phenotype was found.

Another relevant aspect is to understand what is the actual role of ASC in B and T cells from a mechanistic point of view. Does ASC participate in inflammasome only or has it also a role in other cell death pathways? By checking the IGP database, we could see that, B and T cells have high expression levels of NLRP1 and of AIM2. As mentioned above, there are already some works showing that B and T cells could have a functional inflammasome. More interestingly, B cells have high levels of NLRC4. This means that B cells could hypothetically assemble a NLRC4 inflammasome in response to *Salmonella spp* and, by inspecting the literature, there are is evidence in this direction. One of the future goals of our lab will be to expand the experiments to test activation of inflammasomes on B or T lymphocytes and to investigate the possibility of ASC contributing to other cell death pathways.

## ACKNOWLEDGEMENTS

The first acknowledgment goes to my supervisor, Dr. Olaf Groß, as he gave me the opportunity to join his group and offered his supervision during the years of this doctoral work. Together, I thank all my lab colleagues for their collaboration and companionship that contributed to my scientific formation.

Thanks to my thesis committee mentors Dr. Philipp Jost and Prof. Florian Bassermann who gave me important advices and believed in my work and my capabilities. I thank Dr. Katrin Offe and Miss Desislava Zlatanova for their precious support and the discussions we held together both as a student, that helped me to keep track of my PhD, as well as PhD student's representative, which led to the publication of the first student's handbook for the MLST PhD program.

Thanks to Giulia and my family who gave me their understanding, visited me many times, called and encouraged me, so that I could feel the warmth of home even from distance. A special thank goes to all my Munich's friends with whom I had wonderful times outside work and shared some of the best experiences of my life. I will not forget you and visit when I can.

I thank Dr. Marco Conci for his assistance during these years, he helped me to find the right self-confidence in those periods I experienced difficulties. Thanks to Prof. Alessio Peracchi, my first mentor at the University of Parma, he showed me the way to become a scientist and gave me the motivation to pursue this career.

For those friends who studied with me back in Parma, already 5 years ago, and completed their PhDs in different places around the world, I wish we can rejoin together in the future, with our improved knowledge and experiences, to pursue the scientific career we wished for and collaborate once again.

Munich, the 15<sup>th</sup> of November 2016

A handwritten signature in black ink that reads "Giovanni Maguani". The signature is written in a cursive style and is centered below the date.

# CURRICULUM VITAE WITH PUBLICATION LIST

GIOVANNI MAGNANI, PhD

Born 25/09/1987 in Parma (IT)  
Address: Veldener Straße 32, 80687 Munich (DE)  
Mobile: (+49) 17697510984  
Email: magnani.giovanni@outlook.com



---

## Work experience

2013 – Present | Klinikum rechts der Isar (TU-Munich) | Research Fellow

- Research conducted on the NLRP3 inflammasome complex in the context of hematologic malignancies. Several techniques *in vivo* and *in vitro* were used to finalize the project.
- Techniques: applied bioinformatics, mouse breeding/living animal surgeries/necropsy, flow cytometry, cell death assays, inflammasome assays, western blots, ELISA, retrovirus transfection/transduction, bone marrow transplantation.

2011 – 2012 | University of Parma | Master Degree Internship

- Expression and characterization of a *Plasmodium falciparum* parasite B6-dependent enzyme.
- Techniques: applied bioinformatics, gene sub-cloning, recombinant production via *E. coli*, enzymology and biochemical essays, ESI-MS, MALDI TOF-TOF, functional genomics of B6-dependent enzymes.

2011 | University of California, San Francisco | Summer Research Internship

- Research conducted to elucidate the function of *Plasmodium falciparum*'s apicoplast organelle.
- Techniques: cultivation of erythrocytes infected by several strains of *Plasmodium falciparum*, extraction of genomic DNA\RNA, qPCR/RT-qPCR, Southern Blot, bright field/phase contrast/fluorescent microscopy.

2009 | University of Parma | Bachelor Degree Internship

- Expression and characterization of the enzymatic activity of a human B6-dependent enzyme.
- Techniques: PCR, mutagenic PCR, gene cloning and sub-cloning, recombinant production via *E. coli*, functional genomics of PLP-dependent enzymes.

## Education and training

- 2017 **Doctor of Philosophy (PhD)**, Technical University of Munich (DE).  
 2012 **Master Degree (MSc) in Industrial Biotechnology (LM-8)**, University of Parma (IT).  
 2009 **Bachelor Degree (BSc) in Biotechnology (L-2)**, University of Parma (IT).

## Languages

Italian: mother tongue.

English: full oral and written proficiency. TOEFL certificate achieved in 2011.

German: autonomous, basic proficiency, B1 level.

## Publications

- 1) Groß, C. J., Mishra, R., Schneider, K. S., Médard, G., Wettmarshausen, J., Dittlein, D. C., Shi, H., Gorka, O., Koenig, P. A., Fromm, S., **Magnani, G.**, Cikovic, T., Hartjes, L., Smollich, J., Robertson, A. A., Cooper, M. A., Schmidt-Supprian, M., Schuster, M., Schroder, K., Broz, P., Traidl-Hoffmann, C., Beutler, B., Kuster, B., Ruland, J., Schneider, S., Perocchi, F., Groß, O. (2016). K<sup>+</sup> efflux-independent NLRP3 inflammasome activation by small molecules targeting mitochondria. *Immunity*, 45(4), 761-773.
- 2) Höckendorf, U., Yabal, M., Herold, T., Munkhbaatar, E., Rott, S., Jilg, S., Kauschinger, J., **Magnani, G.**, Reisinger, F., Heuser, M., Kreipe, H., Sotlar, K., Engleitner, T., Rad, R., Weichert, W., Peschel, C., Ruland, J., Heikenwalder, M., Spiekermann, K., Slotta-Huspenina, J., Groß, O., Jost, P. J. (2016). RIPK3 Restricts Myeloid Leukemogenesis by Promoting Cell Death and Differentiation of Leukemia Initiating Cells. *Cancer cell*, 30(1), 75-91.
- 3) **Magnani, G.**, Lomazzi, M., & Peracchi, A. (2013). Completing the folate biosynthesis pathway in *Plasmodium falciparum*: p-aminobenzoate is produced by a highly divergent promiscuous aminodeoxychorismate lyase. *Biochemical Journal*, 455(2), 149-155.

## Academic dissertations

- 1) **Magnani, G.**, (2017). Doctor of philosophy thesis: "Roles of the NLRP3 inflammasome in the development and therapy of hematologic malignancies". Technical University of Munich (DE).
- 2) **Magnani, G.**, (2011). Master degree thesis: "Identification and characterization of the 4-amino-4-deoxychorismate lyase from *Plasmodium falciparum*". University of Parma (IT).
- 3) **Magnani, G.**, (2009). Bachelor degree final dissertation title: "Recombinant production of the human multifunctional aminotransferase CCBL1". University of Parma (IT).



## REFERENCES

- 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. *Proc Natl Acad Sci U S A*, *113*(28), 7858-7863. doi:10.1073/pnas.1607769113
- Allen, I. C., TeKippe, E. M., Woodford, R. M., Uronis, J. M., Holl, E. K., Rogers, A. B., . . . Ting, J. P. (2010). The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med*, *207*(5), 1045-1056. doi:10.1084/jem.20100050
- Allen, I. C., Wilson, J. E., Schneider, M., Lich, J. D., Roberts, R. A., Arthur, J. C., . . . Ting, J. P. (2012). NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity*, *36*(5), 742-754. doi:10.1016/j.immuni.2012.03.012
- Allman, D., & Pillai, S. (2008). Peripheral B cell subsets. *Curr Opin Immunol*, *20*(2), 149-157. doi:10.1016/j.coi.2008.03.014
- Anand, P. K., Malireddi, R. K., Lukens, J. R., Vogel, P., Bertin, J., Lamkanfi, M., & Kanneganti, T. D. (2012). NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. *Nature*, *488*(7411), 389-393. doi:10.1038/nature11250
- Antonescu, C. R., Besmer, P., Guo, T., Arkun, K., Hom, G., Koryotowski, B., . . . DeMatteo, R. P. (2005). Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. *Clin Cancer Res*, *11*(11), 4182-4190. doi:10.1158/1078-0432.CCR-04-2245
- Apperley, J. F. (2015). Chronic myeloid leukaemia. *Lancet*, *385*(9976), 1447-1459. doi:10.1016/S0140-6736(13)62120-0
- Arbore, G., West, E. E., Spolski, R., Robertson, A. A., Klos, A., Rheinheimer, C., . . . Kemper, C. (2016). T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4(+) T cells. *Science*, *352*(6292), aad1210. doi:10.1126/science.aad1210
- Ataide, M. A., Andrade, W. A., Zamboni, D. S., Wang, D., Souza Mdo, C., Franklin, B. S., . . . Gazzinelli, R. T. (2014). Malaria-induced NLRP12/NLRP3-dependent caspase-1 activation mediates inflammation and hypersensitivity to bacterial superinfection. *PLoS Pathog*, *10*(1), e1003885. doi:10.1371/journal.ppat.1003885
- Bach, M. P., Hug, E., Werner, M., Holch, J., Sprissler, C., Pechloff, K., . . . Jumaa, H. (2014). Premature terminal differentiation protects from deregulated lymphocyte activation by ITK-Syk. *J Immunol*, *192*(3), 1024-1033. doi:10.4049/jimmunol.1300420
- Baldwin, A. G., Brough, D., & Freeman, S. (2016). Inhibiting the Inflammasome: A Chemical Perspective. *Journal of Medicinal Chemistry*, *59*(5), 1691-1710. doi:10.1021/acs.jmedchem.5b01091
- Banerjee, S., & Bond, J. S. (2008). Prointerleukin-18 is activated by meprin beta in vitro and in vivo in intestinal inflammation. *J Biol Chem*, *283*(46), 31371-31377. doi:10.1074/jbc.M802814200
- 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. *Nat Biotechnol*, *25*(9), 1035-1044. doi:10.1038/nbt1328
- Baroja-Mazo, A., Martin-Sanchez, F., Gomez, A. I., Martinez, C. M., Amores-Iñiesta, J., Compan, V., . . . Pelegrin, P. (2014). The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat Immunol*, *15*(8), 738-748. doi:10.1038/ni.2919
- Bauer, C., Duewell, P., Mayer, C., Lehr, H. A., Fitzgerald, K. A., Dauer, M., . . . Schnurr, M. (2010). Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut*, *59*(9), 1192-1199. doi:10.1136/gut.2009.197822
- Broz, P., & Dixit, V. M. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol*, *16*(7), 407-420. doi:10.1038/nri.2016.58
- Broz, P., von Moltke, J., Jones, J. W., Vance, R. E., & Monack, D. M. (2010). Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe*, *8*(6), 471-483. doi:10.1016/j.chom.2010.11.007
- Brubaker, S. W., Bonham, K. S., Zanoni, I., & Kagan, J. C. (2015). Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol*, *33*, 257-290. doi:10.1146/annurev-immunol-032414-112240
- Bubolz, A. H., Wu, Q., Larsen, B. T., Gutterman, D. D., & Liu, Y. (2007). Ebselen reduces nitration and restores voltage-gated potassium channel function in small coronary arteries of diabetic rats. *Am J Physiol Heart Circ Physiol*, *293*(4), H2231-2237. doi:10.1152/ajpheart.00717.2007

- Canna, S. W., de Jesus, A. A., Gouni, S., Brooks, S. R., Marrero, B., Liu, Y., . . . Goldbach-Mansky, R. (2014). An activating NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome. *Nat Genet*, *46*(10), 1140-1146. doi:10.1038/ng.3089
- Carta, S., Tassi, S., Pettinati, I., Delfino, L., Dinarello, C. A., & Rubartelli, A. (2011). The rate of interleukin-1beta secretion in different myeloid cells varies with the extent of redox response to Toll-like receptor triggering. *J Biol Chem*, *286*(31), 27069-27080. doi:10.1074/jbc.M110.203398
- Chae, J. J., Cho, Y. H., Lee, G. S., Cheng, J., Liu, P. P., Feigenbaum, L., . . . Kastner, D. L. (2011). Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1beta activation and severe autoinflammation in mice. *Immunity*, *34*(5), 755-768. doi:10.1016/j.immuni.2011.02.020
- Chavarría-Smith, J., & Vance, R. E. (2013). Direct proteolytic cleavage of NLRP1B is necessary and sufficient for inflammasome activation by anthrax lethal factor. *PLoS Pathog*, *9*(6), e1003452. doi:10.1371/journal.ppat.1003452
- Chen, E. C., Karl, T. A., Kalisky, T., Gupta, S. K., O'Brien, C. A., Longacre, T. A., . . . Rothenberg, M. E. (2015). KIT Signaling Promotes Growth of Colon Xenograft Tumors in Mice and Is Up-Regulated in a Subset of Human Colon Cancers. *Gastroenterology*, *149*(3), 705-717 e702. doi:10.1053/j.gastro.2015.05.042
- Chen, G., Shaw, M. H., Kim, Y. G., & Nunez, G. (2009). NOD-like receptors: role in innate immunity and inflammatory disease. *Annu Rev Pathol*, *4*, 365-398. doi:10.1146/annurev.pathol.4.110807.092239
- Chen, I. Y., & Ichinohe, T. (2015). Response of host inflammasomes to viral infection. *Trends Microbiol*, *23*(1), 55-63. doi:10.1016/j.tim.2014.09.007
- Coll, R. C., Robertson, A. A., Chae, J. J., Higgins, S. C., Munoz-Planillo, R., Inserra, M. C., . . . O'Neill, L. A. (2015). A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med*, *21*(3), 248-255. doi:10.1038/nm.3806
- Collard, R. L., Harya, N. S., Monzon, F. A., Maier, C. E., & O'Keefe, D. S. (2006). Methylation of the ASC gene promoter is associated with aggressive prostate cancer. *Prostate*, *66*(7), 687-695. doi:10.1002/pros.20371
- Comert, M., Baran, Y., & Saydam, G. (2013). Changes in molecular biology of chronic myeloid leukemia in tyrosine kinase inhibitor era. *Am J Blood Res*, *3*(3), 191-200.
- Conway, K. E., McConnell, B. B., Bowering, C. E., Donald, C. D., Warren, S. T., & Vertino, P. M. (2000). TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. *Cancer Res*, *60*(22), 6236-6242.
- Creagh, E. M. (2014). Caspase crosstalk: integration of apoptotic and innate immune signalling pathways. *Trends Immunol*, *35*(12), 631-640. doi:10.1016/j.it.2014.10.004
- Cullen, S. P., Kearney, C. J., Clancy, D. M., & Martin, S. J. (2015). Diverse Activators of the NLRP3 Inflammasome Promote IL-1beta Secretion by Triggering Necrosis. *Cell Rep*, *11*(10), 1535-1548. doi:10.1016/j.celrep.2015.05.003
- Das, P. M., Ramachandran, K., Vanwert, J., Ferdinand, L., Gopisetty, G., Reis, I. M., & Singal, R. (2006). Methylation mediated silencing of TMS1/ASC gene in prostate cancer. *Mol Cancer*, *5*, 28. doi:10.1186/1476-4598-5-28
- de Almeida, L., Khare, S., Misharin, A. V., Patel, R., Ratsimandresy, R. A., Wallin, M. C., . . . Stehlik, C. (2015). The PYRIN Domain-only Protein POP1 Inhibits Inflammasome Assembly and Ameliorates Inflammatory Disease. *Immunity*, *43*(2), 264-276. doi:10.1016/j.immuni.2015.07.018
- De Braekeleer, E., Douet-Guilbert, N., & De Braekeleer, M. (2014). RARA fusion genes in acute promyelocytic leukemia: a review. *Expert Rev Hematol*, *7*(3), 347-357. doi:10.1586/17474086.2014.903794
- De Giorgi, U., & Verweij, J. (2005). Imatinib and gastrointestinal stromal tumors: Where do we go from here? *Mol Cancer Ther*, *4*(3), 495-501. doi:10.1158/1535-7163.MCT-04-0302
- Dell'Oste, V., Gatti, D., Giorgio, A. G., Gariglio, M., Landolfo, S., & De Andrea, M. (2015). The interferon-inducible DNA-sensor protein IFI16: a key player in the antiviral response. *New Microbiol*, *38*(1), 5-20.
- Deretic, V. (2012). Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. *Curr Opin Immunol*, *24*(1), 21-31. doi:10.1016/j.coi.2011.10.006
- Dhimolea, E. (2010). Canakinumab. *MAbs*, *2*(1), 3-13.
- Dillon, S. R., Sprecher, C., Hammond, A., Bilsborough, J., Rosenfeld-Franklin, M., Presnell, S. R., . . . Gross, J. A. (2004). Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol*, *5*(7), 752-760. doi:10.1038/ni1084
- Dinarello, C. A. (1994). The interleukin-1 family: 10 years of discovery. *FASEB J*, *8*(15), 1314-1325.

- Dinareello, C. A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood*, 117(14), 3720-3732. doi:10.1182/blood-2010-07-273417
- Dingli, D., Traulsen, A., Lenaerts, T., & Pacheco, J. M. (2010). Evolutionary dynamics of chronic myeloid leukemia. *Genes Cancer*, 1(4), 309-315. doi:10.1177/1947601910371122
- Dohner, H., Weisdorf, D. J., & Bloomfield, C. D. (2015). Acute Myeloid Leukemia. *N Engl J Med*, 373(12), 1136-1152. doi:10.1056/NEJMra1406184
- Dombrowski, Y., Peric, M., Koglin, S., Kammerbauer, C., Goss, C., Anz, D., . . . Schaubert, J. (2011). Cytosolic DNA triggers inflammasome activation in keratinocytes in psoriatic lesions. *Sci Transl Med*, 3(82), 82ra38. doi:10.1126/scitranslmed.3002001
- Donahue, A. C., & Fruman, D. A. (2003). Proliferation and survival of activated B cells requires sustained antigen receptor engagement and phosphoinositide 3-kinase activation. *J Immunol*, 170(12), 5851-5860.
- Drexler, S. K., Bonsignore, L., Masin, M., Tardivel, A., Jackstadt, R., Hermeking, H., . . . Yazdi, A. S. (2012). Tissue-specific opposing functions of the inflammasome adaptor ASC in the regulation of epithelial skin carcinogenesis. *Proc Natl Acad Sci U S A*, 109(45), 18384-18389. doi:10.1073/pnas.1209171109
- Dubois, E. A., Rissmann, R., & Cohen, A. F. (2011). Rilonecept and canakinumab. *Br J Clin Pharmacol*, 71(5), 639-641. doi:10.1111/j.1365-2125.2011.03958.x
- Dubreuil, P., Letard, S., Ciufolini, M., Gros, L., Humbert, M., Casteran, N., . . . Hermine, O. (2009). Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. *PLoS One*, 4(9), e7258. doi:10.1371/journal.pone.0007258
- Dubyak, G. R. (2012). P2X7 receptor regulation of non-classical secretion from immune effector cells. *Cell Microbiol*, 14(11), 1697-1706. doi:10.1111/cmi.12001
- Duewell, P., Kono, H., Rayner, K. J., Sirois, C. M., Vladimer, G., Bauernfeind, F. G., . . . Latz, E. (2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*, 464(7293), 1357-1361. doi:10.1038/nature08938
- Dumas, A., Amiable, N., de Rivero Vaccari, J. P., Chae, J. J., Keane, R. W., Lacroix, S., & Vallieres, L. (2014). The inflammasome pyrin contributes to pertussis toxin-induced IL-1 $\beta$  synthesis, neutrophil intravascular crawling and autoimmune encephalomyelitis. *PLoS Pathog*, 10(5), e1004150. doi:10.1371/journal.ppat.1004150
- Dupaul-Chicoine, J., Arabzadeh, A., Dagenais, M., Douglas, T., Champagne, C., Morizot, A., . . . Saleh, M. (2015). The Nlrp3 Inflammasome Suppresses Colorectal Cancer Metastatic Growth in the Liver by Promoting Natural Killer Cell Tumoricidal Activity. *Immunity*, 43(4), 751-763. doi:10.1016/j.immuni.2015.08.013
- 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), 745-757. doi:10.1016/j.cell.2011.04.022
- Embry, C. A., Franchi, L., Nunez, G., & Mitchell, T. C. (2011). Mechanism of impaired NLRP3 inflammasome priming by monophosphoryl lipid A. *Sci Signal*, 4(171), ra28. doi:10.1126/scisignal.2001486
- Erb, M., Hoffmann-Enger, B., Deppe, H., Soeberdt, M., Haefeli, R. H., Rummey, C., . . . Gueven, N. (2012). Features of idebenone and related short-chain quinones that rescue ATP levels under conditions of impaired mitochondrial complex I. *PLoS One*, 7(4), e36153. doi:10.1371/journal.pone.0036153
- Ewald, S. E., Chavarria-Smith, J., & Boothroyd, J. C. (2014). NLRP1 is an inflammasome sensor for *Toxoplasma gondii*. *Infect Immun*, 82(1), 460-468. doi:10.1128/IAI.01170-13
- Faustin, B., Lartigue, L., Bruey, J. M., Luciano, F., Sergienko, E., Bailly-Maitre, B., . . . Reed, J. C. (2007). Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell*, 25(5), 713-724. doi:10.1016/j.molcel.2007.01.032
- 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 Differ*, 14(9), 1590-1604. doi:10.1038/sj.cdd.4402194
- Fernandes-Alnemri, T., Yu, J. W., Datta, P., Wu, J., & Alnemri, E. S. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*, 458(7237), 509-513. doi:10.1038/nature07710
- Fink, S. L., & Cookson, B. T. (2006). Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell Microbiol*, 8(11), 1812-1825. doi:10.1111/j.1462-5822.2006.00751.x

- Fitzgerald, K. A. (2010). NLR-containing inflammasomes: central mediators of host defense and inflammation. *Eur J Immunol*, *40*(3), 595-598. doi:10.1002/eji.201040331
- Fleischmann, R. (2006). Anakinra in the treatment of rheumatic disease. *Expert Rev Clin Immunol*, *2*(3), 331-340. doi:10.1586/1744666X.2.3.331
- Franchi, L., Eigenbrod, T., & Nunez, G. (2009). Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol*, *183*(2), 792-796. doi:10.4049/jimmunol.0900173
- Franchi, L., Kanneganti, T. D., Dubyak, G. R., & Nunez, G. (2007). Differential requirement of P2X7 receptor and intracellular K<sup>+</sup> for caspase-1 activation induced by intracellular and extracellular bacteria. *J Biol Chem*, *282*(26), 18810-18818. doi:10.1074/jbc.M610762200
- Fu, T. M., Li, Y., Lu, A., Li, Z., Vajjhala, P. R., Cruz, A. C., . . . Wu, H. (2016). Cryo-EM Structure of Caspase-8 Tandem DED Filament Reveals Assembly and Regulation Mechanisms of the Death-Inducing Signaling Complex. *Mol Cell*, *64*(2), 236-250. doi:10.1016/j.molcel.2016.09.009
- Gack, M. U. (2014). Mechanisms of RIG-I-like receptor activation and manipulation by viral pathogens. *Journal of Virology*, *88*(10), 5213-5216. doi:10.1128/JVI.03370-13
- Gagliani, N., Palm, N. W., de Zoete, M. R., & Flavell, R. A. (2014). Inflammasomes and intestinal homeostasis: regulating and connecting infection, inflammation and the microbiota. *Int Immunol*, *26*(9), 495-499. doi:10.1093/intimm/dxu066
- Gaidt, M. M., Ebert, T. S., Chauhan, D., Schmidt, T., Schmid-Burgk, J. L., Rapino, F., . . . Hornung, V. (2016). Human Monocytes Engage an Alternative Inflammasome Pathway. *Immunity*, *44*(4), 833-846. doi:10.1016/j.immuni.2016.01.012
- Galluzzi, L., Lopez-Soto, A., Kumar, S., & Kroemer, G. (2016). Caspases Connect Cell-Death Signaling to Organismal Homeostasis. *Immunity*, *44*(2), 221-231. doi:10.1016/j.immuni.2016.01.020
- Garlanda, C., Dinarello, C. A., & Mantovani, A. (2013). The interleukin-1 family: back to the future. *Immunity*, *39*(6), 1003-1018. doi:10.1016/j.immuni.2013.11.010
- Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jehanno, M., Viala, J., . . . Philpott, D. J. (2003). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science*, *300*(5625), 1584-1587. doi:10.1126/science.1084677
- Goldman, J. M., & Melo, J. V. (2003). Chronic myeloid leukemia--advances in biology and new approaches to treatment. *N Engl J Med*, *349*(15), 1451-1464. doi:10.1056/NEJMra020777
- Gombault, A., Baron, L., & Couillin, I. (2012). ATP release and purinergic signaling in NLRP3 inflammasome activation. *Front Immunol*, *3*, 414. doi:10.3389/fimmu.2012.00414
- Gonzalvez, F., Schug, Z. T., Houtkooper, R. H., MacKenzie, E. D., Brooks, D. G., Wanders, R. J., . . . Gottlieb, E. (2008). Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. *J Cell Biol*, *183*(4), 681-696. doi:10.1083/jcb.200803129
- Grabinger, T., Luks, L., Kostadinova, F., Zimberlin, C., Medema, J. P., Leist, M., & Brunner, T. (2014). Ex vivo culture of intestinal crypt organoids as a model system for assessing cell death induction in intestinal epithelial cells and enteropathy. *Cell Death Dis*, *5*, e1228. doi:10.1038/cddis.2014.183
- Grau, E., Martinez, F., Orellana, C., Canete, A., Yanez, Y., Oltra, S., . . . Castel, V. (2010). Epigenetic alterations in disseminated neuroblastoma tumour cells: influence of TMS1 gene hypermethylation in relapse risk in NB patients. *J Cancer Res Clin Oncol*, *136*(9), 1415-1421. doi:10.1007/s00432-010-0796-9
- Gray, E. E., Winship, D., Snyder, J. M., Child, S. J., Geballe, A. P., & Stetson, D. B. (2016). The AIM2-like Receptors Are Dispensable for the Interferon Response to Intracellular DNA. *Immunity*, *45*(2), 255-266. doi:10.1016/j.immuni.2016.06.015
- Gross, C. J., Mishra, R., Schneider, K. S., Medard, G., Wettmarshausen, J., Dittlein, D. C., . . . Gross, O. (2016). K<sup>+</sup> Efflux-Independent NLRP3 Inflammasome Activation by Small Molecules Targeting Mitochondria. *Immunity*. doi:10.1016/j.immuni.2016.08.010
- Gross, O. (2012). Measuring the inflammasome. *Methods Mol Biol*, *844*, 199-222. doi:10.1007/978-1-61779-527-5\_15
- Gross, O., Yazdi, A. S., Thomas, C. J., Masin, M., Heinz, L. X., Guarda, G., . . . Tschopp, J. (2012). Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity*, *36*(3), 388-400. doi:10.1016/j.immuni.2012.01.018
- Gross, S., Rahal, R., Stransky, N., Lengauer, C., & Hoeflich, K. P. (2015). Targeting cancer with kinase inhibitors. *J Clin Invest*, *125*(5), 1780-1789. doi:10.1172/JCI76094
- Guan, X., Sagara, J., Yokoyama, T., Koganehira, Y., Oguchi, M., Saida, T., & Taniguchi, S. (2003). ASC/TMS1, a caspase-1 activating adaptor, is downregulated by aberrant methylation in human melanoma. *Int J Cancer*, *107*(2), 202-208. doi:10.1002/ijc.11376

- Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Forster, I., . . . Tschopp, J. (2011). Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity*, *34*(2), 213-223. doi:10.1016/j.immuni.2011.02.006
- Guey, B., Bodnar, M., Manie, S. N., Tardivel, A., & Petrilli, V. (2014). Caspase-1 autoproteolysis is differentially required for NLRP1b and NLRP3 inflammasome function. *Proc Natl Acad Sci U S A*, *111*(48), 17254-17259. doi:10.1073/pnas.1415756111
- Guo, H., Callaway, J. B., & Ting, J. P. (2015). Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med*, *21*(7), 677-687. doi:10.1038/nm.3893
- Gurung, P., Anand, P. K., Malireddi, R. K., Vande Walle, L., Van Opdenbosch, N., Dillon, C. P., . . . Kanneganti, T. D. (2014). FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. *J Immunol*, *192*(4), 1835-1846. doi:10.4049/jimmunol.1302839
- Halle, A., Hornung, V., Petzold, G. C., Stewart, C. R., Monks, B. G., Reinheckel, T., . . . Golenbock, D. T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol*, *9*(8), 857-865. doi:10.1038/ni.1636
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674. doi:10.1016/j.cell.2011.02.013
- Hara, H., Tsuchiya, K., Kawamura, I., Fang, R., Hernandez-Cuellar, E., Shen, Y., . . . Mitsuyama, M. (2013). Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat Immunol*, *14*(12), 1247-1255. doi:10.1038/ni.2749
- He, W. T., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., . . . Han, J. (2015). Gasdermin D is an executor of pyroptosis and required for interleukin-1beta secretion. *Cell Res*, *25*(12), 1285-1298. doi:10.1038/cr.2015.139
- He, Y., Zeng, M. Y., Yang, D., Motro, B., & Nunez, G. (2016). NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature*, *530*(7590), 354-357. doi:10.1038/nature16959
- Heger, K., Kober, M., Riess, D., Drees, C., de Vries, I., Bertossi, A., . . . Schmidt-Suppryan, M. (2015). A novel Cre recombinase reporter mouse strain facilitates selective and efficient infection of primary immune cells with adenoviral vectors. *Eur J Immunol*, *45*(6), 1614-1620. doi:10.1002/eji.201545457
- 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. *J Immunol*, *191*(10), 5230-5238. doi:10.4049/jimmunol.1301490
- Herzog, C., Haun, R. S., Kaushal, V., Mayeux, P. R., Shah, S. V., & Kaushal, G. P. (2009). Mepripin A and mepripin alpha generate biologically functional IL-1beta from pro-IL-1beta. *Biochem Biophys Res Commun*, *379*(4), 904-908. doi:10.1016/j.bbrc.2008.12.161
- Hett, E. C., Slater, L. H., Mark, K. G., Kawate, T., Monks, B. G., Stutz, A., . . . Hung, D. T. (2013). Chemical genetics reveals a kinase-independent role for protein kinase R in pyroptosis. *Nat Chem Biol*, *9*(6), 398-405. doi:10.1038/nchembio.1236
- Höckendorf, U., Yabal, M., Herold, T., Munkhbaatar, E., Rott, S., Jilg, S., . . . Jost, Philipp J. (2016). RIPK3 Restricts Myeloid Leukemogenesis by Promoting Cell Death and Differentiation of Leukemia Initiating Cells. *Cancer Cell*, *30*(1), 75-91. doi:10.1016/j.ccell.2016.06.002
- Hoffman, H. M., Mueller, J. L., Broide, D. H., Wanderer, A. A., & Kolodner, R. D. (2001). Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet*, *29*(3), 301-305. doi:10.1038/ng756
- 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. doi: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. *Nat Immunol*, *9*(8), 847-856. doi:10.1038/ni.1631
- Hoving, J. C., Wilson, G. J., & Brown, G. D. (2014). Signalling C-type lectin receptors, microbial recognition and immunity. *Cell Microbiol*, *16*(2), 185-194. doi:10.1111/cmi.12249
- Hu, Z., Yan, C., Liu, P., Huang, Z., Ma, R., Zhang, C., . . . Chai, J. (2013). Crystal structure of NLRC4 reveals its autoinhibition mechanism. *Science*, *341*(6142), 172-175. doi:10.1126/science.1236381
- Hull, C. A., Chu, Y., Thanawala, M., & Regehr, W. G. (2013). Hyperpolarization induces a long-term increase in the spontaneous firing rate of cerebellar Golgi cells. *J Neurosci*, *33*(14), 5895-5902. doi:10.1523/JNEUROSCI.4052-12.2013

- Hunter, T. (2007). Treatment for chronic myelogenous leukemia: the long road to imatinib. *J Clin Invest*, 117(8), 2036-2043. doi:10.1172/JCI31691
- Huysamen, C., & Brown, G. D. (2009). The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors. *FEMS Microbiol Lett*, 290(2), 121-128. doi:10.1111/j.1574-6968.2008.01418.x
- Imai, K., & Takaoka, A. (2006). Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer*, 6(9), 714-727. doi:10.1038/nrc1913
- Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., . . . Nunez, G. (2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem*, 278(8), 5509-5512. doi:10.1074/jbc.C200673200
- Iqbal, N., & Iqbal, N. (2014). Imatinib: a breakthrough of targeted therapy in cancer. *Chemother Res Pract*, 2014, 357027. doi:10.1155/2014/357027
- Ito, M., Shichita, T., Okada, M., Komine, R., Noguchi, Y., Yoshimura, A., & Morita, R. (2015). Bruton's tyrosine kinase is essential for NLRP3 inflammasome activation and contributes to ischaemic brain injury. *Nat Commun*, 6, 7360. doi:10.1038/ncomms8360
- Ives, A., Nomura, J., Martinon, F., Roger, T., LeRoy, D., Miner, J. N., . . . So, A. (2015). Xanthine oxidoreductase regulates macrophage IL1beta secretion upon NLRP3 inflammasome activation. *Nat Commun*, 6, 6555. doi:10.1038/ncomms7555
- Jabaut, J., Ather, J. L., Taracanova, A., Poynter, M. E., & Ckless, K. (2013). Mitochondria-targeted drugs enhance Nlrp3 inflammasome-dependent IL-1beta secretion in association with alterations in cellular redox and energy status. *Free Radic Biol Med*, 60, 233-245. doi:10.1016/j.freeradbiomed.2013.01.025
- Jabir, M. S., Hopkins, L., Ritchie, N. D., Ullah, I., Bayes, H. K., Li, D., . . . Evans, T. J. (2015). Mitochondrial damage contributes to *Pseudomonas aeruginosa* activation of the inflammasome and is downregulated by autophagy. *Autophagy*, 11(1), 166-182. doi:10.4161/15548627.2014.981915
- Janeway, C. A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol*, 54 Pt 1, 1-13.
- Jha, S., Srivastava, S. Y., Brickey, W. J., Iocca, H., Toews, A., Morrison, J. P., . . . Ting, J. P. (2010). The inflammasome sensor, NLRP3, regulates CNS inflammation and demyelination via caspase-1 and interleukin-18. *J Neurosci*, 30(47), 15811-15820. doi:10.1523/JNEUROSCI.4088-10.2010
- Jin, T., Perry, A., Jiang, J., Smith, P., Curry, J. A., Unterholzner, L., . . . Xiao, T. S. (2012). Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. *Immunity*, 36(4), 561-571. doi:10.1016/j.immuni.2012.02.014
- Jin, Y., Mailloux, C. M., Gowan, K., Riccardi, S. L., LaBerge, G., Bennett, D. C., . . . Spritz, R. A. (2007). NALP1 in vitiligo-associated multiple autoimmune disease. *N Engl J Med*, 356(12), 1216-1225. doi:10.1056/NEJMoa061592
- Jo, E. K., Kim, J. K., Shin, D. M., & Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol*, 13(2), 148-159. doi:10.1038/cmi.2015.95
- Joosten, L. A., Netea, M. G., Fantuzzi, G., Koenders, M. I., Helsen, M. M., Sparrer, H., . . . van den Berg, W. B. (2009). Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. *Arthritis Rheum*, 60(12), 3651-3662. doi:10.1002/art.25006
- Kanneganti, T. D., Ozoren, N., Body-Malapel, M., Amer, A., Park, J. H., Franchi, L., . . . Nunez, G. (2006). Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature*, 440(7081), 233-236. doi:10.1038/nature04517
- Katsnelson, M. A., Lozada-Soto, K. M., Russo, H. M., Miller, B. A., & Dubyak, G. R. (2016). NLRP3 inflammasome signaling is activated by low-level lysosome disruption but inhibited by extensive lysosome disruption: roles for K<sup>+</sup> efflux and Ca<sup>2+</sup> influx. *Am J Physiol Cell Physiol*, 311(1), C83-C100. doi:10.1152/ajpcell.00298.2015
- Katsnelson, M. A., Rucker, L. G., Russo, H. M., & Dubyak, G. R. (2015). K<sup>+</sup> efflux agonists induce NLRP3 inflammasome activation independently of Ca<sup>2+</sup> signaling. *J Immunol*, 194(8), 3937-3952. doi:10.4049/jimmunol.1402658
- Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., . . . Dixit, V. M. (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signaling. *Nature*. doi:10.1038/nature15541

- Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., . . . Dixit, V. M. (2011). Non-canonical inflammasome activation targets caspase-11. *Nature*, *479*(7371), 117-121. doi:10.1038/nature10558
- Kayagaki, N., Wong, M. T., Stowe, I. B., Ramani, S. R., Gonzalez, L. C., Akashi-Takamura, S., . . . Dixit, V. M. (2013). Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science*, *341*(6151), 1246-1249. doi:10.1126/science.1240248
- Kenneth, N. S., Younger, J. M., Hughes, E. D., Marcotte, D., Barker, P. A., Saunders, T. L., & Duckett, C. S. (2012). An inactivating caspase 11 passenger mutation originating from the 129 murine strain in mice targeted for c-IAP1. *Biochem J*, *443*(2), 355-359. doi:10.1042/BJ20120249
- 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. doi:10.1016/j.immuni.2012.02.001
- Knight, E. R., Patel, E. Y., Flowers, C. A., Crowther, A. J., Ting, J. P., Miller, C. R., . . . Deshmukh, M. (2014). ASC deficiency suppresses proliferation and prevents medulloblastoma incidence. *Oncogene*, *34*(3), 394-402. doi:10.1038/onc.2013.577
- Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., & Flavell, R. A. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*, *307*(5710), 731-734. doi:10.1126/science.1104911
- Kolb, R., Liu, G. H., Janowski, A. M., Sutterwala, F. S., & Zhang, W. (2014). Inflammasomes in cancer: a double-edged sword. *Protein Cell*, *5*(1), 12-20. doi:10.1007/s13238-013-0001-4
- Kotz, J. (2012). The gout pipeline crystallizes. *Nat Rev Drug Discov*, *11*(6), 425-426. doi:10.1038/nrd3748
- Kovalchuk, A. L., Qi, C. F., Torrey, T. A., Taddesse-Heath, L., Feigenbaum, L., Park, S. S., . . . Morse, H. C., 3rd. (2000). Burkitt lymphoma in the mouse. *J Exp Med*, *192*(8), 1183-1190.
- Kovalenko, M., Gazit, A., Bohmer, A., Rorsman, C., Ronnstrand, L., Heldin, C. H., . . . Levitzki, A. (1994). Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. *Cancer Res*, *54*(23), 6106-6114.
- Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. S., & Flavell, R. A. (1995). Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science*, *267*(5206), 2000-2003.
- Kullberg, B. J., van 't Wout, J. W., & van Furth, R. (1990). Role of granulocytes in increased host resistance to *Candida albicans* induced by recombinant interleukin-1. *Infect Immun*, *58*(10), 3319-3324.
- Lamkanfi, M., & Dixit, V. M. (2011). Modulation of inflammasome pathways by bacterial and viral pathogens. *J Immunol*, *187*(2), 597-602. doi:10.4049/jimmunol.1100229
- Lamkanfi, M., & Dixit, V. M. (2014). Mechanisms and functions of inflammasomes. *Cell*, *157*(5), 1013-1022. doi:10.1016/j.cell.2014.04.007
- Lechtenberg, B. C., Mace, P. D., & Riedl, S. J. (2014). Structural mechanisms in NLR inflammasome signaling. *Curr Opin Struct Biol*, *29*, 17-25. doi:10.1016/j.sbi.2014.08.011
- Lee, C. R., Kang, J. A., Kim, H. E., Choi, Y., Yang, T., & Park, S. G. (2016). Secretion of IL-1beta from imatinib-resistant chronic myeloid leukemia cells contributes to BCR-ABL mutation-independent imatinib resistance. *FEBS Lett*, *590*(3), 358-368. doi:10.1002/1873-3468.12057
- Lee, G. S., Subramanian, N., Kim, A. I., Aksentijevich, I., Goldbach-Mansky, R., Sacks, D. B., . . . Chae, J. J. (2012). The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca<sup>2+</sup> and cAMP. *Nature*, *492*(7427), 123-127. doi:10.1038/nature11588
- Lee, P. P., Fitzpatrick, D. R., Beard, C., Jessup, H. K., Lehar, S., Makar, K. W., . . . Wilson, C. B. (2001). A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity*, *15*(5), 763-774.
- Levitzki, A. (2013). Tyrosine kinase inhibitors: views of selectivity, sensitivity, and clinical performance. *Annu Rev Pharmacol Toxicol*, *53*, 161-185. doi:10.1146/annurev-pharmtox-011112-140341
- Li, H., Gutterman, D. D., Rusch, N. J., Bubolz, A., & Liu, Y. (2004). Nitration and functional loss of voltage-gated K<sup>+</sup> channels in rat coronary microvessels exposed to high glucose. *Diabetes*, *53*(9), 2436-2442.
- Li, Y., & Kurlander, R. J. (2010). Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation. *J Transl Med*, *8*, 104. doi:10.1186/1479-5876-8-104
- Lima, H., Jr., Jacobson, L. S., Goldberg, M. F., Chandran, K., Diaz-Griffero, F., Lisanti, M. P., & Brojatsch, J. (2013). Role of lysosome rupture in controlling Nlrp3 signaling and necrotic cell death. *Cell Cycle*, *12*(12), 1868-1878. doi:10.4161/cc.24903

- 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. *Proc Natl Acad Sci U S A*, *111*(2), 775-780. doi:10.1073/pnas.1320294111
- Liu-Bryan, R. (2010). Intracellular innate immunity in gouty arthritis: role of NALP3 inflammasome. *Immunology and Cell Biology*, *88*(1), 20-23. doi:10.1038/icb.2009.93
- Liu, W., Luo, Y., Dunn, J. H., Norris, D. A., Dinarello, C. A., & Fujita, M. (2013). Dual role of apoptosis-associated speck-like protein containing a CARD (ASC) in tumorigenesis of human melanoma. *J Invest Dermatol*, *133*(2), 518-527. doi:10.1038/jid.2012.317
- Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., & Lieberman, J. (2016). Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature*, *535*(7610), 153-158. doi:10.1038/nature18629
- Lo-Coco, F., Avvisati, G., Vignetti, M., Thiede, C., Orlando, S. M., Iacobelli, S., . . . Study Alliance, L. (2013). Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med*, *369*(2), 111-121. doi:10.1056/NEJMoa1300874
- Lo, Y. H., Huang, Y. W., Wu, Y. H., Tsai, C. S., Lin, Y. C., Mo, S. T., . . . Lai, M. Z. (2013). Selective inhibition of the NLRP3 inflammasome by targeting to promyelocytic leukemia protein in mouse and human. *Blood*, *121*(16), 3185-3194. doi:10.1182/blood-2012-05-432104
- Lopalco, G., Rigante, D., Giannini, M., Galeazzi, M., Lapadula, G., Iannone, F., & Cantarini, L. (2016). Safety profile of anakinra in the management of rheumatologic, metabolic and autoinflammatory disorders. *Clin Exp Rheumatol*, *34*(3), 531-538.
- Lu, A., Magupalli, V. G., Ruan, J., Yin, Q., Atianand, M. K., Vos, M. R., . . . Egelman, E. H. (2014). Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. *Cell*, *156*(6), 1193-1206. doi:10.1016/j.cell.2014.02.008
- Lu, B., Nakamura, T., Inouye, K., Li, J., Tang, Y., Lundback, P., . . . Tracey, K. J. (2012). Novel role of PKR in inflammasome activation and HMGB1 release. *Nature*, *488*(7413), 670-674. doi:10.1038/nature11290
- Maglione, P. J., Simchoni, N., & Cunningham-Rundles, C. (2015). Toll-like receptor signaling in primary immune deficiencies. *Ann N Y Acad Sci*, *1356*, 1-21. doi:10.1111/nyas.12763
- Mahe, M. M., Aihara, E., Schumacher, M. A., Zavros, Y., Montrose, M. H., Helmuth, M. A., . . . Shroyer, N. F. (2013). Establishment of Gastrointestinal Epithelial Organoids. *Curr Protoc Mouse Biol*, *3*(4), 217-240. doi:10.1002/9780470942390.mo130179
- Maier, N. K., Crown, D., Liu, J., Leppla, S. H., & Moayeri, M. (2014). Arsenic trioxide and other arsenical compounds inhibit the NLRP1, NLRP3, and NAIP5/NLRC4 inflammasomes. *J Immunol*, *192*(2), 763-770. doi:10.4049/jimmunol.1301434
- Malireddi, R. K., & Kanneganti, T. D. (2013). Role of type I interferons in inflammasome activation, cell death, and disease during microbial infection. *Front Cell Infect Microbiol*, *3*, 77. doi:10.3389/fcimb.2013.00077
- Man, S. M., Hopkins, L. J., Nugent, E., Cox, S., Gluck, I. M., Tourlomousis, P., . . . Bryant, C. E. (2014). Inflammasome activation causes dual recruitment of NLRC4 and NLRP3 to the same macromolecular complex. *Proc Natl Acad Sci U S A*, *111*(20), 7403-7408. doi:10.1073/pnas.1402911111
- Man, S. M., & Kanneganti, T. D. (2015). Regulation of inflammasome activation. *Immunol Rev*, *265*(1), 6-21. doi:10.1111/imr.12296
- 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. doi:10.1038/nature02664
- Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., . . . Dixit, V. M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*, *440*(7081), 228-232. doi:10.1038/nature04515
- Martin, B. N., Wang, C., Willette-Brown, J., Herjan, T., Gulen, M. F., Zhou, H., . . . Li, X. (2014). IKKalpha negatively regulates ASC-dependent inflammasome activation. *Nat Commun*, *5*, 4977. doi:10.1038/ncomms5977
- Martinez, R., Schackert, G., & Esteller, M. (2007). Hypermethylation of the proapoptotic gene TMS1/ASC: prognostic importance in glioblastoma multiforme. *J Neurooncol*, *82*(2), 133-139. doi:10.1007/s11060-006-9264-4
- Martinon, F., Burns, K., & Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*, *10*(2), 417-426.
- Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., & Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*, *440*(7081), 237-241. doi:10.1038/nature04516



- Masters, S. L., Dunne, A., Subramanian, S. L., Hull, R. L., Tannahill, G. M., Sharp, F. A., . . . O'Neill, L. A. (2010). Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1 $\beta$  in type 2 diabetes. *Nat Immunol*, *11*(10), 897-904. doi:10.1038/ni.1935
- Masters, S. L., Gerlic, M., Metcalf, D., Preston, S., Pellegrini, M., O'Donnell, J. A., . . . Croker, B. A. (2012). NLRP1 inflammasome activation induces pyroptosis of hematopoietic progenitor cells. *Immunity*, *37*(6), 1009-1023. doi:10.1016/j.immuni.2012.08.027
- Masters, S. L., Simon, A., Aksentijevich, I., & Kastner, D. L. (2009). Horror aut inflammaticus: the molecular pathophysiology of autoinflammatory disease (\*). *Annu Rev Immunol*, *27*, 621-668. doi:10.1146/annurev.immunol.25.022106.141627
- 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. *J Biol Chem*, *274*(48), 33835-33838.
- Matti, B. F., Saleem, M. A., & Sabir, S. F. (2014). Assessment of interleukin 1 $\beta$  serum level in different responder groups and stages of chronic myeloid leukemia patients on imatinib mesylate therapy. *Indian J Hematol Blood Transfus*, *30*(4), 247-252. doi:10.1007/s12288-014-0339-7
- Medard, G., Pachi, F., Ruprecht, B., Klaeger, S., Heinzlmeir, S., Helm, D., . . . Kuster, B. (2015). Optimized chemical proteomics assay for kinase inhibitor profiling. *J Proteome Res*, *14*(3), 1574-1586. doi:10.1021/pr5012608
- Meissner, T. B., Li, A., & Kobayashi, K. S. (2012). NLRC5: a newly discovered MHC class I transactivator (CITA). *Microbes Infect*, *14*(6), 477-484. doi:10.1016/j.micinf.2011.12.007
- Meunier, E., Dick, M. S., Dreier, R. F., Schurmann, N., Kenzelmann Broz, D., Warming, S., . . . Broz, P. (2014). Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature*, *509*(7500), 366-370. doi:10.1038/nature13157
- Miething, C., Feihl, S., Mugler, C., Grundler, R., von Bubnoff, N., Lordick, F., . . . Duyster, J. (2006). The Bcr-Abl mutations T315I and Y253H do not confer a growth advantage in the absence of imatinib. *Leukemia*, *20*(4), 650-657. doi:10.1038/sj.leu.2404151
- Miettinen, M., & Lasota, J. (2006). Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. *Arch Pathol Lab Med*, *130*(10), 1466-1478. doi:10.1043/1543-2165(2006)130[1466:GSTROM]2.0.CO;2
- 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. *Nat Immunol*, *14*(5), 454-460. doi:10.1038/ni.2550
- Molyneux, E. M., Rochford, R., Griffin, B., Newton, R., Jackson, G., Menon, G., . . . Bailey, S. (2012). Burkitt's lymphoma. *The Lancet*, *379*(9822), 1234-1244. doi:10.1016/s0140-6736(11)61177-x
- Monie, T. P., & Bryant, C. E. (2015). Caspase-8 functions as a key mediator of inflammation and pro-IL-1 $\beta$  processing via both canonical and non-canonical pathways. *Immunol Rev*, *265*(1), 181-193. doi:10.1111/imr.12284
- Monroe, K. M., Yang, Z., Johnson, J. R., Geng, X., Doitsh, G., Krogan, N. J., & Greene, W. C. (2014). IFI16 DNA sensor is required for death of lymphoid CD4 T cells abortively infected with HIV. *Science*, *343*(6169), 428-432. doi:10.1126/science.1243640
- Moriwaki, K., Bertin, J., Gough, P. J., Orłowski, G. M., & Chan, F. K. (2015). Differential roles of RIPK1 and RIPK3 in TNF-induced necroptosis and chemotherapeutic agent-induced cell death. *Cell Death Dis*, *6*, e1636. doi:10.1038/cddis.2015.16
- Morrone, S. R., Matyszewski, M., Yu, X., Delannoy, M., Egelman, E. H., & Sohn, J. (2015). Assembly-driven activation of the AIM2 foreign-dsDNA sensor provides a polymerization template for downstream ASC. *Nat Commun*, *6*, 7827. doi:10.1038/ncomms8827
- Motta, V., Soares, F., Sun, T., & Philpott, D. J. (2015). NOD-like receptors: versatile cytosolic sentinels. *Physiol Rev*, *95*(1), 149-178. doi:10.1152/physrev.00009.2014
- Munoz-Planillo, R., Kuffa, P., Martinez-Colon, G., Smith, B. L., Rajendiran, T. M., & Nunez, G. (2013). K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity*, *38*(6), 1142-1153. doi: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. doi:10.1016/j.immuni.2013.06.018
- Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A., Opitz, B., van der Meer, J. H., . . . Dinarello, C. A. (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1 $\beta$  in monocytes and macrophages. *Blood*, *113*(10), 2324-2335. doi:10.1182/blood-2008-03-146720

- Netea, M. G., van de Veerdonk, F. L., van der Meer, J. W., Dinarello, C. A., & Joosten, L. A. (2015). Inflammasome-independent regulation of IL-1-family cytokines. *Annu Rev Immunol*, 33, 49-77. doi:10.1146/annurev-immunol-032414-112306
- Newman, Z. L., Sirianni, N., Mawhinney, C., Lee, M. S., Leppla, S. H., Moayeri, M., & Johansen, L. M. (2011). Auranofin protects against anthrax lethal toxin-induced activation of the Nlrp1b inflammasome. *Antimicrob Agents Chemother*, 55(3), 1028-1035. doi:10.1128/AAC.00772-10
- Newton, K., Sun, X., & Dixit, V. M. (2004). Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol*, 24(4), 1464-1469.
- Nowarski, R., Jackson, R., Gagliani, N., de Zoete, M. R., Palm, N. W., Bailis, W., . . . Flavell, R. A. (2015). Epithelial IL-18 Equilibrium Controls Barrier Function in Colitis. *Cell*, 163(6), 1444-1456. doi:10.1016/j.cell.2015.10.072
- O'Neill, L. A., Golenbock, D., & Bowie, A. G. (2013). The history of Toll-like receptors - redefining innate immunity. *Nat Rev Immunol*, 13(6), 453-460. doi:10.1038/nri3446
- O'Regan, L., & Fry, A. M. (2009). The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. *Mol Cell Biol*, 29(14), 3975-3990. doi:10.1128/MCB.01867-08
- Ohtsuka, T., Liu, X. F., Koga, Y., Kitajima, Y., Nakafusa, Y., Ha, C. W., . . . Miyazaki, K. (2006). Methylation-induced silencing of ASC and the effect of expressed ASC on p53-mediated chemosensitivity in colorectal cancer. *Oncogene*, 25(12), 1807-1811. doi:10.1038/sj.onc.1209204
- Okamoto, M., Liu, W., Luo, Y., Tanaka, A., Cai, X., Norris, D. A., . . . Fujita, M. (2010). Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. *J Biol Chem*, 285(9), 6477-6488. doi:10.1074/jbc.M109.064907
- Panchanathan, R., Duan, X., Arumugam, M., Shen, H., Liu, H., & Choubey, D. (2011). Cell type and gender-dependent differential regulation of the p202 and Aim2 proteins: implications for the regulation of innate immune responses in SLE. *Mol Immunol*, 49(1-2), 273-280. doi:10.1016/j.molimm.2011.08.022
- Parish, C. R. (1999). Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunology and Cell Biology*, 77(6), 499-508. doi:10.1046/j.1440-1711.1999.00877.x
- Pasparakis, M., & Vandenabeele, P. (2015). Necroptosis and its role in inflammation. *Nature*, 517(7534), 311-320. doi:10.1038/nature14191
- Passmore, J. S., Lukey, P. T., & Ress, S. R. (2001). The human macrophage cell line U937 as an in vitro model for selective evaluation of mycobacterial antigen-specific cytotoxic T-cell function. *Immunology*, 102(2), 146-156.
- Pastula, A., Middelhoff, M., Brandtner, A., Tobiasch, M., Hohl, B., Nuber, A. H., . . . Quante, M. (2016). Three-Dimensional Gastrointestinal Organoid Culture in Combination with Nerves or Fibroblasts: A Method to Characterize the Gastrointestinal Stem Cell Niche. *Stem Cells Int*, 2016, 3710836. doi:10.1155/2016/3710836
- Paugh, S. W., Bonten, E. J., Savic, D., Ramsey, L. B., Thierfelder, W. E., Gurung, P., . . . Evans, W. E. (2015). NALP3 inflammasome upregulation and CASP1 cleavage of the glucocorticoid receptor cause glucocorticoid resistance in leukemia cells. *Nat Genet*, 47(6), 607-614. doi:10.1038/ng.3283
- Pechloff, K., Holch, J., Ferch, U., Schweneker, M., Brunner, K., Kremer, M., . . . Ruland, J. (2010). The fusion kinase ITK-SYK mimics a T cell receptor signal and drives oncogenesis in conditional mouse models of peripheral T cell lymphoma. *J Exp Med*, 207(5), 1031-1044. doi:10.1084/jem.20092042
- Pedraza-Alva, G., Perez-Martinez, L., Valdez-Hernandez, L., Meza-Sosa, K. F., & Ando-Kuri, M. (2015). Negative regulation of the inflammasome: keeping inflammation under control. *Immunol Rev*, 265(1), 231-257. doi:10.1111/imr.12294
- Pelegri, P., & Surprenant, A. (2006). Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J*, 25(21), 5071-5082. doi:10.1038/sj.emboj.7601378
- Peng, B., Lloyd, P., & Schran, H. (2005). Clinical pharmacokinetics of imatinib. *Clin Pharmacokinet*, 44(9), 879-894. doi:10.2165/00003088-200544090-00001
- Perregaux, D. G., McNiff, P., Laliberte, R., Hawryluk, N., Peurano, H., Stam, E., . . . Gabel, C. A. (2001). Identification and characterization of a novel class of interleukin-1 post-translational processing inhibitors. *J Pharmacol Exp Ther*, 299(1), 187-197.

- Petrilli, 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 Differ*, *14*(9), 1583-1589. doi:10.1038/sj.cdd.4402195
- Py, B. F., Kim, M. S., Vakifahmetoglu-Norberg, H., & Yuan, J. (2013). Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Mol Cell*, *49*(2), 331-338. doi:10.1016/j.molcel.2012.11.009
- Qi, X., Man, S. M., Malireddi, R. K., Karki, R., Lupfer, C., Gurung, P., . . . Kanneganti, T. D. (2016). Cathepsin B modulates lysosomal biogenesis and host defense against *Francisella novicida* infection. *J Exp Med*, *213*(10), 2081-2097. doi:10.1084/jem.20151938
- Rathinam, V. A., & Fitzgerald, K. A. (2016). Inflammasome Complexes: Emerging Mechanisms and Effector Functions. *Cell*, *165*(4), 792-800. doi:10.1016/j.cell.2016.03.046
- Rathinam, V. A., 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. *Nat Immunol*, *11*(5), 395-402. doi:10.1038/ni.1864
- Rodgers, M. A., Bowman, J. W., Fujita, H., Orazio, N., Shi, M., Liang, Q., . . . Jung, J. U. (2014). The linear ubiquitin assembly complex (LUBAC) is essential for NLRP3 inflammasome activation. *J Exp Med*, *211*(7), 1333-1347. doi:10.1084/jem.20132486
- Romberg, N., Al Moussawi, K., Nelson-Williams, C., Stiegler, A. L., Loring, E., Choi, M., . . . Lifton, R. P. (2014). Mutation of NLRC4 causes a syndrome of enterocolitis and autoinflammation. *Nat Genet*, *46*(10), 1135-1139. doi:10.1038/ng.3066
- Sagulenko, V., Thygesen, S. J., Sester, D. P., Idris, A., Cridland, J. A., Vajjhala, P. R., . . . Stacey, K. J. (2013). AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ*, *20*(9), 1149-1160. doi:10.1038/cdd.2013.37
- Salesse, S., & Verfaillie, C. M. (2002). BCR/ABL: from molecular mechanisms of leukemia induction to treatment of chronic myelogenous leukemia. *Oncogene*, *21*(56), 8547-8559. doi:10.1038/sj.onc.1206082
- Salminen, A., Kauppinen, A., Hiltunen, M., & Kaarniranta, K. (2014). Epigenetic regulation of ASC/TMS1 expression: potential role in apoptosis and inflammasome function. *Cell Mol Life Sci*, *71*(10), 1855-1864. doi:10.1007/s00018-013-1524-9
- Sanman, L. E., Qian, Y., Eisele, N. A., Ng, T. M., van der Linden, W. A., Monack, D. M., . . . Bogoyo, M. (2016). Disruption of glycolytic flux is a signal for inflammasome signaling and pyroptotic cell death. *Elife*, *5*, e13663. doi:10.7554/eLife.13663
- Sastalla, I., Crown, D., Masters, S. L., McKenzie, A., Leppla, S. H., & Moayeri, M. (2013). Transcriptional analysis of the three Nlrp1 paralogs in mice. *BMC Genomics*, *14*, 188. doi:10.1186/1471-2164-14-188
- Sborgi, L., Ruhl, S., Mulvihill, E., Pipercevic, J., Heilig, R., Stahlberg, H., . . . Hiller, S. (2016). GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death. *EMBO J*, *35*(16), 1766-1778. doi:10.15252/embj.201694696
- Schneider, K. S., Thomas, C. J., & Gross, O. (2013). Inflammasome activation and inhibition in primary murine bone marrow-derived cells, and assays for IL-1alpha, IL-1beta, and caspase-1. *Methods Mol Biol*, *1040*, 117-135. doi:10.1007/978-1-62703-523-1\_10
- Scholl, T., Mahanta, S. K., & Strominger, J. L. (1997). Specific complex formation between the type II bare lymphocyte syndrome-associated transactivators CIITA and RFX5. *Proc Natl Acad Sci U S A*, *94*(12), 6330-6334.
- Schroder, K., & Tschopp, J. (2010). The inflammasomes. *Cell*, *140*(6), 821-832. doi:10.1016/j.cell.2010.01.040
- Shalapour, S., & Karin, M. (2015). Immunity, inflammation, and cancer: an eternal fight between good and evil. *J Clin Invest*, *125*(9), 3347-3355. doi:10.1172/JCI80007
- Shao, B. Z., Xu, Z. Q., Han, B. Z., Su, D. F., & Liu, C. (2015). NLRP3 inflammasome and its inhibitors: a review. *Front Pharmacol*, *6*, 262. doi:10.3389/fphar.2015.00262
- Sharma, D., & Kanneganti, T. D. (2016). The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *J Cell Biol*, *213*(6), 617-629. doi:10.1083/jcb.201602089
- 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. *Nat Immunol*, *17*(3), 250-258. doi:10.1038/ni.3333
- 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. doi:10.1038/nature13683

- Shimada, K., Crother, T. R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., . . . Arditi, M. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity*, *36*(3), 401-414. doi:10.1016/j.immuni.2012.01.009
- Solle, M., Labasi, J., Perregaux, D. G., Stam, E., Petrushova, N., Koller, B. H., . . . Gabel, C. A. (2001). Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem*, *276*(1), 125-132. doi:10.1074/jbc.M006781200
- Stoecklein, V. M., Osuka, A., Ishikawa, S., Lederer, M. R., Wanke-Jellinek, L., & Lederer, J. A. (2015). Radiation exposure induces inflammasome pathway activation in immune cells. *J Immunol*, *194*(3), 1178-1189. doi:10.4049/jimmunol.1303051
- Stone, A. R., Bobo, W., Brat, D. J., Devi, N. S., Van Meir, E. G., & Vertino, P. M. (2004). Aberrant methylation and down-regulation of TMS1/ASC in human glioblastoma. *American Journal of Pathology*, *165*(4), 1151-1161. doi:10.1016/S0002-9440(10)63376-7
- Sutterwala, F. S., Haasken, S., & Cassel, S. L. (2014). Mechanism of NLRP3 inflammasome activation. *Ann N Y Acad Sci*, *1319*, 82-95. doi:10.1111/nyas.12458
- Suvas, S., Singh, V., Sahdev, S., Vohra, H., & Agrewala, J. N. (2002). Distinct role of CD80 and CD86 in the regulation of the activation of B cell and B cell lymphoma. *J Biol Chem*, *277*(10), 7766-7775. doi:10.1074/jbc.M105902200
- Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, *140*(6), 805-820. doi:10.1016/j.cell.2010.01.022
- Terlizzi, M., Casolaro, V., Pinto, A., & Sorrentino, R. (2014). Inflammasome: cancer's friend or foe? *Pharmacol Ther*, *143*(1), 24-33. doi:10.1016/j.pharmthera.2014.02.002
- Terra, J. K., Cote, C. K., France, B., Jenkins, A. L., Bozue, J. A., Welkos, S. L., . . . Bradley, K. A. (2010). Cutting edge: resistance to Bacillus anthracis infection mediated by a lethal toxin sensitive allele of Nalp1b/Nlrp1b. *J Immunol*, *184*(1), 17-20. doi:10.4049/jimmunol.0903114
- Travassos, L. H., Carneiro, L. A., Ramjeet, M., Hussey, S., Kim, Y. G., Magalhaes, J. G., . . . Philpott, D. J. (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol*, *11*(1), 55-62. doi:10.1038/ni.1823
- Ulland, T. K., Jain, N., Hornick, E. E., Elliott, E. I., Clay, G. M., Sadler, J. J., . . . Sutterwala, F. S. (2016). Nlrp12 mutation causes C57BL/6J strain-specific defect in neutrophil recruitment. *Nat Commun*, *7*, 13180. doi:10.1038/ncomms13180
- Van Opdenbosch, N., Gurung, P., Vande Walle, L., Fossoul, A., Kanneganti, T. D., & Lamkanfi, M. (2014). Activation of the NLRP1b inflammasome independently of ASC-mediated caspase-1 autoproteolysis and speck formation. *Nat Commun*, *5*, 3209. doi:10.1038/ncomms4209
- Vanaja, S. K., Rathinam, V. A., & Fitzgerald, K. A. (2015). Mechanisms of inflammasome activation: recent advances and novel insights. *Trends Cell Biol*, *25*(5), 308-315. doi:10.1016/j.tcb.2014.12.009
- Vance, R. E. (2015). The NAIP/NLRC4 inflammasomes. *Curr Opin Immunol*, *32*, 84-89. doi:10.1016/j.coi.2015.01.010
- Vanden Berghe, T., Linkermann, A., Joann-Lanhouet, S., Walczak, H., & Vandenabeele, P. (2014). Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol*, *15*(2), 135-147. doi:10.1038/nrm3737
- 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 Differ*, *20*(2), 185-187. doi:10.1038/cdd.2012.151
- Vandenabeele, P., Orrenius, S., & Zhivotovsky, B. (2005). Serine proteases and calpains fulfill important supporting roles in the apoptotic tragedy of the cellular opera. *Cell Death Differ*, *12*(9), 1219-1224. doi:10.1038/sj.cdd.4401719
- Vella, F., Ferry, G., Delagrèze, P., & Boutin, J. A. (2005). NRH:quinone reductase 2: an enzyme of surprises and mysteries. *Biochemical Pharmacology*, *71*(1-2), 1-12. doi:10.1016/j.bcp.2005.09.019
- Venereau, E., Ceriotti, C., & Bianchi, M. E. (2015). DAMPs from Cell Death to New Life. *Front Immunol*, *6*, 422. doi:10.3389/fimmu.2015.00422
- Vince, J. E., & Silke, J. (2016). The intersection of cell death and inflammasome activation. *Cell Mol Life Sci*. doi:10.1007/s00018-016-2205-2
- Virmani, A., Rathi, A., Sugio, K., Sathyanarayana, U. G., Toyooka, S., Kischel, F. C., . . . Gazdar, A. F. (2003). Aberrant methylation of TMS1 in small cell, non small cell lung cancer and breast cancer. *Int J Cancer*, *106*(2), 198-204. doi:10.1002/ijc.11206
- Vladimer, G. I., Weng, D., Paquette, S. W., Vanaja, S. K., Rathinam, V. A., Aune, M. H., . . . Lien, E. (2012). The NLRP12 inflammasome recognizes Yersinia pestis. *Immunity*, *37*(1), 96-107. doi:10.1016/j.immuni.2012.07.006

- Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A., Jr., & Kinzler, K. W. (2013). Cancer genome landscapes. *Science*, 339(6127), 1546-1558. doi:10.1126/science.1235122
- von Moltke, J., Trinidad, N. J., Moayeri, M., Kintzer, A. F., Wang, S. B., van Rooijen, N., . . . Vance, R. E. (2012). Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. *Nature*, 490(7418), 107-111. doi:10.1038/nature11351
- Waypa, G. B., Marks, J. D., Guzy, R., Mungai, P. T., Schriever, J., Dokic, D., & Schumacker, P. T. (2010). Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circ Res*, 106(3), 526-535. doi:10.1161/CIRCRESAHA.109.206334
- Wen, H., Gris, D., Lei, Y., Jha, S., Zhang, L., Huang, M. T., . . . Ting, J. P. (2011). Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat Immunol*, 12(5), 408-415. doi:10.1038/ni.2022
- Wolf, A. J., Reyes, C. N., Liang, W., Becker, C., Shimada, K., Wheeler, M. L., . . . Underhill, D. M. (2016). Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan. *Cell*, 166(3), 624-636. doi:10.1016/j.cell.2016.05.076
- Wortis, H. H., Teutsch, M., Higer, M., Zheng, J., & Parker, D. C. (1995). B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. *Proc Natl Acad Sci U S A*, 92(8), 3348-3352.
- Wu, P., Nielsen, T. E., & Clausen, M. H. (2015). FDA-approved small-molecule kinase inhibitors. *Trends Pharmacol Sci*, 36(7), 422-439. doi:10.1016/j.tips.2015.04.005
- Wu, P., Nielsen, T. E., & Clausen, M. H. (2016). Small-molecule kinase inhibitors: an analysis of FDA-approved drugs. *Drug Discov Today*, 21(1), 5-10. doi:10.1016/j.drudis.2015.07.008
- Xu, H., Yang, J., Gao, W., Li, L., Li, P., Zhang, L., . . . Shao, F. (2014). Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. *Nature*, 513(7517), 237-241. doi:10.1038/nature13449
- Yaish, P., Gazit, A., Gilon, C., & Levitzki, A. (1988). Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. *Science*, 242(4880), 933-935.
- Yan, Y. Q., Jiang, W., Liu, L., Wang, X. Q., Ding, C., Tian, Z. G., & Zhou, R. B. (2015). Dopamine Controls Systemic Inflammation through Inhibition of NLRP3 Inflammasome. *Cell*, 160(1-2), 62-73. doi:10.1016/j.cell.2014.11.047
- Yang, C. A., & Chiang, B. L. (2015). Inflammasomes and human autoimmunity: A comprehensive review. *J Autoimmun*, 61, 1-8. doi:10.1016/j.jaut.2015.05.001
- Yang, D., He, Y., Munoz-Planillo, R., Liu, Q., & Nunez, G. (2015). Caspase-11 Requires the Pannexin-1 Channel and the Purinergic P2X7 Pore to Mediate Pyroptosis and Endotoxic Shock. *Immunity*, 43(5), 923-932. doi:10.1016/j.immuni.2015.10.009
- Yang, J., Liu, Z., & Xiao, T. S. (2016). Post-translational regulation of inflammasomes. *Cell Mol Immunol*. doi:10.1038/cmi.2016.29
- Yang, J., Zhao, Y., Shi, J., & Shao, F. (2013). Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proc Natl Acad Sci U S A*, 110(35), 14408-14413. doi:10.1073/pnas.1306376110
- Youm, Y. H., Nguyen, K. Y., Grant, R. W., Goldberg, E. L., Bodogai, M., Kim, D., . . . Dixit, V. D. (2015). The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med*, 21(3), 263-269. doi:10.1038/nm.3804
- Yu, J. W., Wu, J., Zhang, Z., Datta, P., Ibrahimi, I., Taniguchi, S., . . . Alnemri, E. S. (2006). Cryopyrin and pyrin activate caspase-1, but not NF-kappaB, via ASC oligomerization. *Cell Death Differ*, 13(2), 236-249. doi:10.1038/sj.cdd.4401734
- Zhang, C., Li, H., Zhou, G., Zhang, Q., Zhang, T., Li, J., . . . Yin, D. (2007). Transcriptional silencing of the TMS1/ASC tumour suppressor gene by an epigenetic mechanism in hepatocellular carcinoma cells. *J Pathol*, 212(2), 134-142. doi:10.1002/path.2173
- 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*, 350(6259), 404-409. doi:10.1126/science.aac5789
- Zhou, R., Yazdi, A. S., Menu, P., & Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature*, 469(7329), 221-225. doi:10.1038/nature09663
- Zitvogel, L., Rusakiewicz, S., Routy, B., Ayyoub, M., & Kroemer, G. (2016). Immunological off-target effects of imatinib. *Nat Rev Clin Oncol*. doi:10.1038/nrclinonc.2016.41
- Zurawek, M., Fichna, M., Januszkiewicz-Lewandowska, D., Gryczynska, M., Fichna, P., & Nowak, J. (2010). A coding variant in NLRP1 is associated with autoimmune Addison's disease. *Hum Immunol*, 71(5), 530-534. doi:10.1016/j.humimm.2010.02.004