Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase

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Abbreviations

AIM2	Absent in melanoma 2
BCR	B-cell receptor
BTK	Bruton tyrosine kinase
cAMP	Cyclic adenosine monophosphate
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase-recruitment domain
CLL	Chronic lymphocytic leukemia
CLRs	C-type lectin receptors
DLBCL	Diffuse large B-cell lymphoma
DMARDs	Disease-modifying antirheumatic drugs
EM	Electron microscopy
FADD	FAS-associated death domain protein
FCAS	Familial cold autoinflammatory syndrome
FDA	Food and Drug Administration
GBP5	Guanylate-binding protein 5
HIN	Haematopoietic interferon-inducible nuclear protein
HMGB1	High mobility group box 1 protein
IAPP	Islet amyloid polypeptide
IFI16	IFNγ-inducible protein 16
IFN	Interferon
IgH	Immunoglobulin heavy
IL-1β	Interleukin-1β
IL-1Ra	IL-1 receptor antagonist
IL-18	Interleukin-18
ITAMs	Immunoreceptor tyrosine-based activation motifs
LeuLeuOMe	L-Leucyl-L-leucine methyl ester
LPS	Lipopolysaccharides
LRRs	Leucine-rich repeats
MAMP	Microbe-associated molecular pattern

MAPK	Mitogen activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MCL	Mantle cell lymphoma
MSU	Monosodium urate
MWS	Muckle-Wells syndrome
Nek7	NIMA-related Kinase-7
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLRs	NOD-like receptors
NLRC4	NLR Family CARD Domain Containing 4
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	Nitric oxide
NOD	Nucleotide-binding-and-oligomerization domain
NOMID	Neonatal onset multisystem inflammatory disorder
NSAIDs	Non-steroidal anti-inflammatory drugs
PH	Pleckstrin homology domain
PIDs	Immunodeficiencies
PIP3	Phosphatidylinositol (3, 4, 5)-trisphosphate
PKR	Double-stranded RNA-dependent protein kinase
PRRs	Pathogen- or pattern-recognition receptors
PYD	Pyrin domain
RLRs	RIG-I-like receptors
ROS	Reactive oxygen species
SYK	Spleen tyrosine kinase
Tc	T cytotoxic
TEC	Transient erythroblastopenia of childhood
Th	T helper
TIR	Toll/IL-1 receptor
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
XID	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

1 Introduction

1.1 The human immune system

The human body regularly encounters and combats a vast array of pathogenic organisms and toxic molecules. The immune system has evolved to protect the host from and also helps the host eliminate these pathogenic microbes, toxic or allergenic substances. Immunity is divided into two parts, innate and the adaptive responses, determined by the speed and specificity of the reaction. Both of these mechanisms possess the ability of self-nonself discrimination to detect pathogens or toxins, and meanwhile to avoid responses that produce excessive damage of self-tissues. Although the innate and adaptive immune responses are fundamentally different in their mechanisms of action, they provide an intact, fully effective immune response based on synergy and interaction between them.

1.1.1 Innate immune response

Innate immune response corresponds to the first line of host defense against any invading microbe (nonself) or agent (damaged self) the immune system perceives as dangerous to the body's cells and tissues. The innate immune system comprises chemic-physical barriers (e.g., epidermal cells, mucous membranes, and pH), immune cells (e.g., macrophages, dendritic cells, and neutrophils) and soluble molecules (e.g., Antimicrobial enzymes, complement system, antimicrobial peptides)[3]. The skin represents a physical barrier preventing pathogens from invading internal tissues. Digestive enzymes destroy microbes that enter the stomach with food. Innate immune cells including macrophages, equipped with molecular detectors such as Toll-like receptors (TLRs), sense and engulf microbes, damaged cells, and other foreign materials in the body.

Innate immunity relies on a defense strategy that involves a set of defined germline encoded receptors referred to as pathogen- or pattern-recognition receptors (PRRs) that recognize microbe-associated molecular patterns (MAMPs) or endogenous danger-associated molecular patterns (DAMPs)[4]. Activation of PRRs triggers intracellular signaling cascades leading to the production of inflammatory cytokines, chemokines, and type 1 interferons, the induction of antimicrobial responses, pyroptosis and the

recruitment of phagocytic cells. These innate responses are responsible for the efficient elimination of pathogens and other molecular threats and instructing the development of an appropriate pathogen-specific adaptive immune response.

Four main classes of pattern recognition receptors are recognized, Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs)[5]. TLRs are membrane-bound glycoproteins. Each TLR is composed of a ligand-binding motif which contains leucine-rich repeats, a transmembrane domain and a cytoplasmic signaling Toll/IL-1 receptor (TIR) homology domain[6]. CLRs comprise a large family of transmembrane receptors characterized by a calcium-dependent carbohydrate-binding domain[7]. Retinoic acid-inducible gene-I-like receptors (RIG-like receptors, RLR) and Nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, NLR) are two major classes of the cytoplasmic pattern recognition receptors. In particular, the cytoplasmic signaling complexes which involve certain NLR family members and the adaptor protein ASC, commonly called inflammasomes, are under intense investigation[8-12]. However, aberrant activation of PRRs has been implicated in various autoimmune and inflammatory conditions including inflammatory bowel diseases[13,14], septic shock, cryopyrin-associated periodic syndromes (CAPS) and type 2 diabetes[15-17].

1.1.2 Adaptive immune response

The adaptive immune system, different from the innate immune system, exhibits high specificity to a particular pathogen. This specificity of adaptive responses relies on the T- and B-lymphocytes characterized by antigen-specific receptors on the surfaces.

The dominant role of the T cells is to identify and destroy infected cells, as well as recognize peptide fragments of antigens presented on MHC molecules on the surface of antigen presenting cells (APC). Despite these common features, T cells perform several, quite different functions in the body. Two major types of effector T cells, $CD8^+$ cytotoxic T cells (Tc) and $CD4^+$ helper T cells (Th), have been identified[18]. The function of Tc is killing the cells that are infected with viruses or other pathogens. Th mediates immune response by directing other cells. B cells produce antibodies which serve to neutralize toxins, prevent organisms adhering to mucosal surfaces, activate complement, opsonize bacteria for phagocytosis, and sensitize tumor and infected cells

for antibody-dependent cytotoxic attack by killer cells. Defination of subgroup of B cells based on the types of antibody they produce, and the types of antigen they respond. Although antibody is the terminally secreted product of activated B cells, early in B-cell development it is a transmembrane protein that locates on the B cell surface, acting as the B-cell receptor (BCR)[3,19].

The recognization and binding of antigen by the BCR triggers two separate functional mechanisms of BCR. One is to turn on a set of signaling pathway associated with B cell activation, survival, development, or proliferation. The other one is to internalize the antigen to lysosomes, degrade them to peptides, and present these peptides to T cells via major histocompatibility complex class II. Thus B cells can initiate immune response as antigen presenting cells via BCR. Dysregulation in BCR signal transduction may result in immune deficiency or B-cell malignancy[20]. The physiology of B cells is intimately connected with the function of their B-cell receptors. In B cells, the balance of initiation, amplitude, and duration of BCR activation can be influenced by the activity of kinases (like BTK, LYN, SYK, PI3K), the expression adaptor molecules (like GAB1, BLNK, GRB2, CARD11), or phosphatases (like SHIP-1, SHP-1 and PTEN)[21,22].

1.2 Inflammasomes are a group of cytosolic protein complexes to mediate critical host immune response

The inflammasomes are a group of multimeric signaling complexes that consist of an inflammasome sensor molecule, the adaptor protein ASC and caspase-1. Inflammasome formation is triggered by a range of pathogenic and physiological stimuli that emerge during infections, tissue damage or metabolic imbalances. Activation and formation of inflammasome causes the cleavage of pro-caspase-1 to active caspase-1, which results in the maturation and secretion of inflammatory cytokines interleukin-1ß (IL-1ß) and interleukin-18 (IL-18). Additionally, inflammasome activation triggers a caspase-1-dependent, highly inflammatory type of programmed cell death, pyroptosis.

1.2.1 Nod-like receptor family

NOD-like receptors are a recently found group member of the PRR superfamily. NLR family members share a tripartite domain organization comprising the following: (1) an amino-terminal domain which could be a pyrin domain, a CARD (caspase activation

and recruitment) domain, a BIR (baculovirus inhibitor of apoptosis protein repeat) domain, or a transactivation domain. These diverse pyrin domains serve a similar function for downstream protein-protein interaction; (2) a centrally located NOD (nucleotide-binding oligomerization) domain which is important for self-oligomerization; (3) and a carboxy-terminal LRR (leucine-rich repeat) domain which is supposed to provide recognition of MAMP and DAMP[23]. To date, 22 NLR family members have been identified in humans. The 22 human NLRs can be subdivided into NLRP or NLRC (Figure 1.1) based on whether the N terminus contains a pyrin or caspase activation and recruitment domain (CARD), respectively. Only about half of the NLRs have been characterized in any detail. Most of the NLRs which have been studied can be divided into 4 different groups based on their signaling function: activators of inflammasome; activators of NF-κB (nuclear factor-κB) and MAPK (mitogen activated protein kinase), inhibitors of inflammatory signaling, and trans-activators of MHC expression[24,25].

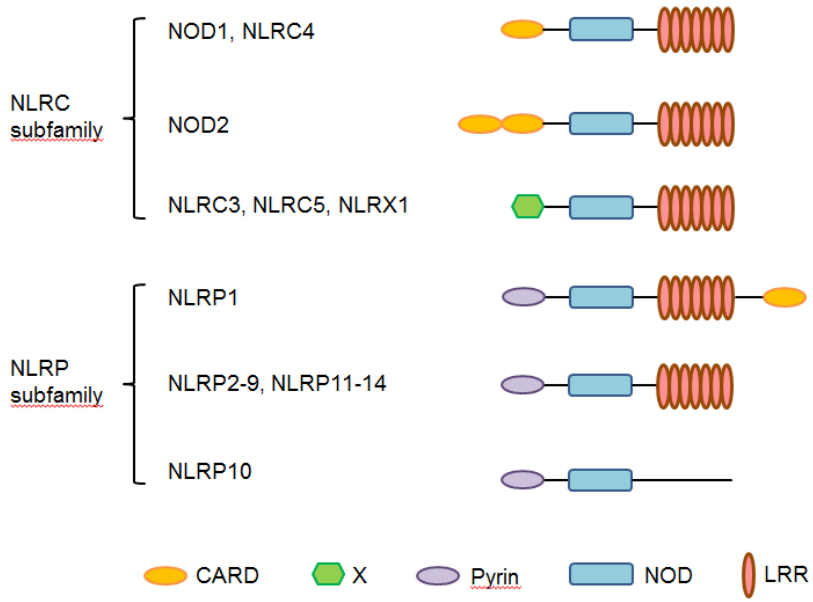


Figure 1.1: Schematic representation of individual NLR domains.

Figure adapted from Claes *et al.*[2]. NLRs, except NLRP10, contain an N-terminal death-fold domain, a central NOD domain and a C-terminal LRR domain. For the N-terminal, the NLRC subfamily contain either a CARD, or a X domain which is uncharacterized, whereas the NLRP subfamily contain a pyrin domain. CARD, caspase activation and recruitment domain; NOD, nucleotide-binding oligomerization domain; LRR, leucine-rich repeat.

1.2.2 Members of Inflammasome-forming proteins

To date, several NOD-like receptors (NLR) are involved in the formation of inflammasomes, and serve as inflammasome sensor molecules in inflammasome signaling pathway. These inflammasome sensor molecules include NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP3, NLRP6, NLRP7, NLRP12 or NLRC4 (NOD-, LRR- and CARD-containing 4). It remains to be seen if other members of the NLR family are capable of forming or regulating inflammasome assembly in response to some unknown stimuli. Two other inflammasomes that contain the absent in melanoma 2 (AIM2) and IFNγ-inducible protein 16 (IFI16) have been described as well. Besides, Retinoic acid-inducible gene I (RIG-I) protein, together with ASC and caspase-1, is also thought to trigger an inflammasome assemble possibly via its CARDs[26,27].

There are other functions been described for these molecules. Indeed, NLRP12 can function as a negative regulator of non-canonical NF-κB signaling, or as a positive regulator of dendritic cell migration. NLRP6 can negatively regulate innate immunity and host defense against bacterial pathogens. RIG-I can function as a PRR that senses viruses, and that triggers an interferon (IFN) response via mitochondrial antiviral signaling protein (MAVS). IFI16 has been suggested to play a role in sensing DNA that transmit a IFN response signal through the protein STING (stimulator of IFN genes; also known as TMEM173)[28,29].

1.2.3 Tertiary structure of NLRP3 inflammasome

Unlike other inflammasomes, NLRP3 inflammasomes are activated by endogenous or exogenous DAMPs and are involved in the process of sterile inflammation[1]. As described above, NLRP3 contains three domains: C-terminal leucine-rich repeats, a central nucleotide domain namely NACHT domain, and an N-terminal effector domain which is a pyrin domain.

NLRP3 inflammasomes are composed of NLRP3, ASC, and caspase-1 which form a ring-like structure. Lu *et al.*[30] (Cell, 2014) addressed assembly mechanisms for ASC-dependent NLRP3 inflammasomes using in vitro reconstitution, electron microscopy (EM) and polymerization assays. NLRP3 oligomerized through its NBD nucleate ASC^{PYD} filaments. The flexibly linked ASC^{CARD} clusters along the ASC^{PYD} filament to

act as the platform for caspase-1^{CARD} filament formation, leading to proximity-induced caspase dimerization and activation. The ternary inflammasome complex showed star-shaped branched filamentous morphology and exhibited unequal stoichiometries among the component proteins (Figure 1.2).

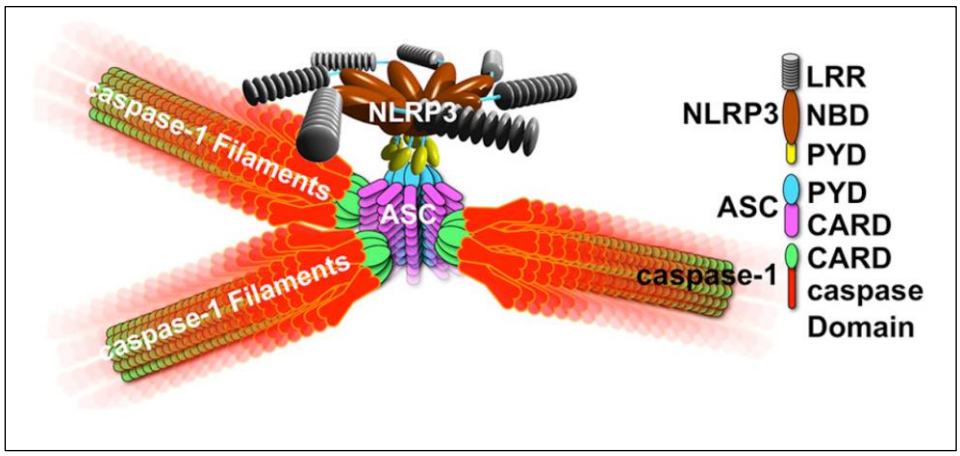


Figure 1.2: Model of NLRP3 inflammasome assembly.

Figure adapted from Lu *et al.*[30]. NLRP3 oligomerizes upon activation to form a platform of PYDs that induces ASC filament assembly through PYD/PYD interactions. Multiple ASC molecules cluster to promote caspase-1 filament formation through CARD/CARD interactions. Proximity induced dimerization of the caspase domain activates the enzyme followed by autocleavage.

1.2.4 Comparison of NLRP3, NLRC4, and AIM2 in structure and signaling pathway

The NLRC4 inflammasome can be triggered by bacterial flagellin and the inner rod protein of the type III secretion systems (T3SS) of diverse bacterial species. NLRC4 can directly activate caspase-1 through their CARD-CARD interaction without recruiting ASC (Figure 1.3). Activation of NLRC4/caspase-1 results in the maturation and secretion of IL-1β and IL-18, the downstream caspase-7 activation and the induction of other cellular events that are poorly understood[27]. The NLRC4 inflammasome recognizes structurally distinct ligands via a group of NLR family members, termed apoptosis inhibitory proteins (NAIPs)[31,32].

AIM2 belongs to the hematopoietic interferon-inducible nuclear protein (HIN) family and consists of an N-terminal pyrin domain to recruit ASC and a DNA-binding HIN domain. AIM2 is an intracellular DNA receptor that recognizes double-stranded DNA through its HIN domain. Interaction of its two domains maintains itself in an autoinhibitory conformation. Recognition of dsDNA by the HIN domain subsequently liberates the Pyrin domain, which recruits ASC to form an inflammasome complex[33].

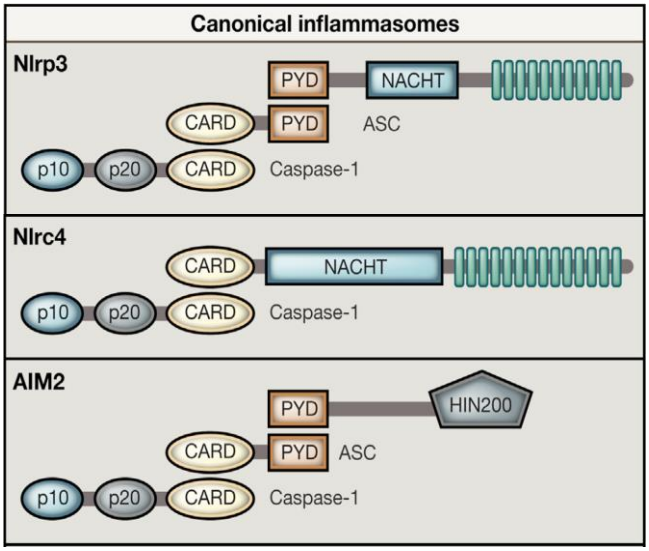


Figure 1.3: Domain architecture of NLRP3, NLRC4 and AIM2 Inflammasome components.

Figure adapted from Lamkanf *et al.*[1]. Schematic description of NLRP3, NLRC4, AIM2 receptors and additional inflammasome components. NLRP3 and NLRC4 have the typical tripartite structure of NLRs, but NLRP3 contains an N-terminal PYD, whereas NLRC4 is characterized by an N-terminal CARD. AIM2 comprises the characteristic N-terminal PYD and a C-terminal HIN domain.

1.3 Mechanism and regulation of NLRP3 inflammasome activation

Among NLR inflammasome complexes, the NLRP3 inflammasome is best characterized. Via caspase-1 self-cleavage, the NLRP3 inflammasome complex triggers processing of immature form pro-IL-1β and pro-IL-18 to their active forms that are secreted proinflammatory cytokines: IL-1ß and IL-18. NLRP3 inflammasome activation initiates as well as pyroptosis, a form of programmed inflammatory cell death, via Gasdermin D. When NLRP3 inflammasome assembles, homotypic interactions of NLRP3 N-terminal PYD domain and ASC PYD occur. Similarly, ASC recruits caspase-1 via the CARD/CARD interaction. However, the mechanisms of NLRP3 inflammasome activation continue to be debated.

1.3.1 Chemical and structural diversity of NLRP3 ligands

A wide range of DAMPs has been currently recognized as NLRP3 agonists that induce NLRP3 inflammasome formation, including endogenous stimuli of cellular and metabolic stress such as amyloid-β, monosodium urate (MSU) crystals, islet amyloid polypeptide (IAPP) which contribute respectively to the pathogenesis of Alzheimer's disease, gout, type 2 diabetes, or mitochondrial dysfunction, exogenous agents such as extracellular ATP, silica, asbestos, or alum and obesity-related factors such as fatty acids, ceramide, hyperglycemia or reactive oxygen species (ROS). Several PAMPs or microbial stimuli can also activate this inflammasome such as pore-forming toxins, staphylococcus aureus, influenza virus or sendai virus. Caspase-1 was initially identified as the protease responsible for the processing and maturation of IL-1β in response to ATP[34,35]. However, more evidence is emerging that most microbial stimuli, such as TLR ligands, can prime the NLRP3 inflammasome through TLR signaling rather than directly activate NLRP3[36].

1.3.2 Basic concepts of NLRP3 inflammasome activation

For NLRP3 inflammasome activation, a two-signal model has been proposed in macrophages as the key mechanism. In this model, microbial (MAMPs) or endogenous (DAMP) molecules provide the first signal (priming) to enhance the NF-κB dependent *NLRP3* and *IL1B* expression; the second signal (activation) normally induced by pore-

forming toxins, extracellular ATP, or particulate matter triggers assembly into the NLRP3 inflammasome complex.

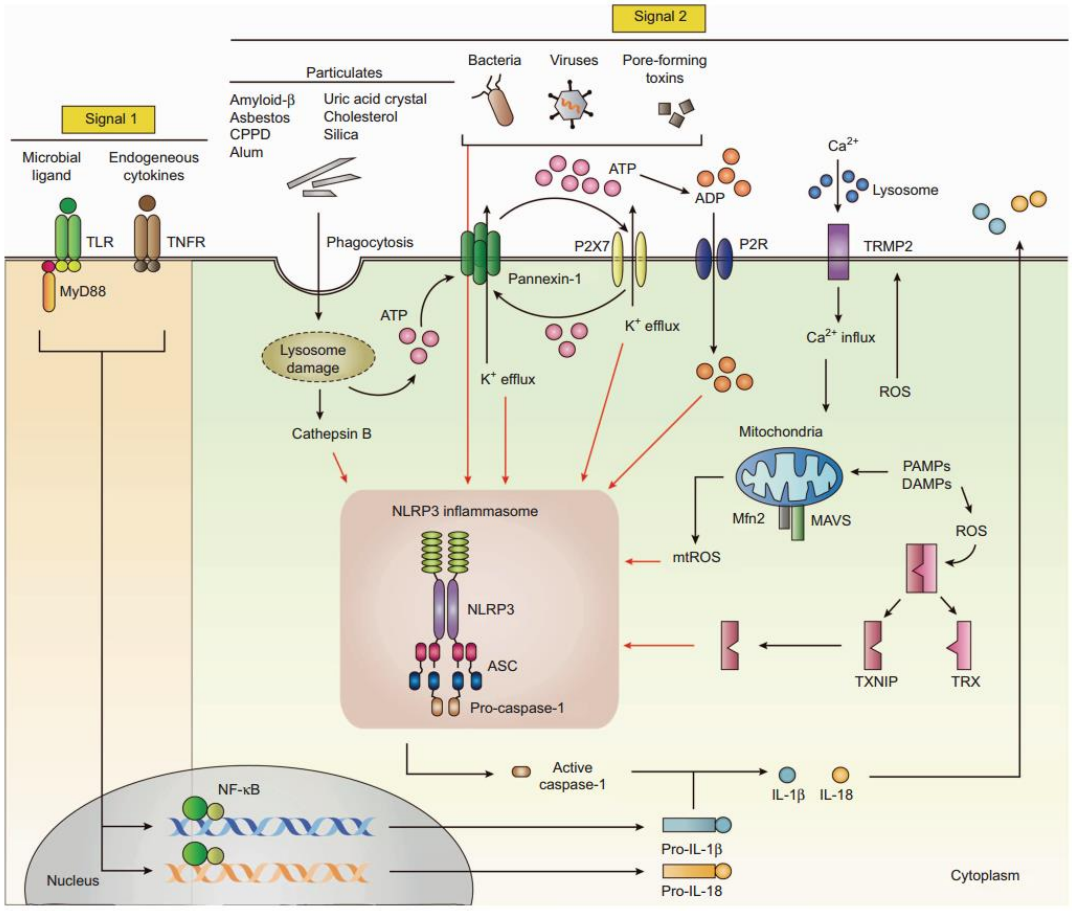


Figure 1.4: Two-signal model for NLRP3 inflammasome activation.

Figure adapted from Jo *et al.*[37]. Both signal 1 and signal 2 are required for NLRP3 inflammasome activation. The effect of Signal 1 (priming, left) is upregulation of NLRP3 and pro-IL-1ß via NF-κB activation. Signal 2 (activation, right) is provided by a great diversity of stimuli, such as particulate matter, ATP, viral RNA, and pore-forming toxins, The effect of signal 2 is to promote the assembly of ASC and pro-caspase-1, leading to NLRP3 inflammasome complex activation. K^+ efflux, induced by most NLRP3 activators, is necessary and sufficient for NLRP3 activation. Ca^{2+} signaling and mitochondrial dysfunction have been implicated to mediate NLRP3 inflammasome activation. Phagocytosis of particulate matter leads to lysosomal rupture and thus activates NLRP3 through K^+ efflux and perhaps the release of cathepsin B. TLR, Toll-like receptor; TNFR, tumor necrosis factor receptor. ADP, adenosine diphosphate; ATP, adenosine triphosphate; K^+, potassium; ASC, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain; CPPD, calcium pyrophosphate dehydrate; DAMPs, damage-associated molecular patterns; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B

cells; P2X7, P2X purinoceptor 7; P2R, purinergic receptor; PAMPs, pathogen-associated molecular patterns; ROS, reactive oxygen species; TLRs, toll-like receptors; TNF-a, tumor necrosis factor-a; TXNIP, thioredoxin (TRX)-interacting protein.

1.3.2.1 Priming: first signal

Activation of the NLRP3 inflammasome in response to only NLRP3 activators in macrophages, such as mouse bone marrow-derived macrophages, is minimal, whereas NLRP3 inflammasome activation can be strongly increases by pretreatment with microbial ligands[36]. Therefore, activation of NLRP3, especially in mouse myeloid cells, requires a "priming" step, also known as the signal 1. The signal 2 in inflammasome activation involves the toll-like receptor (TLR)/nuclear factor (NF)-κB pathway. Unlike ASC and caspase-1, it is believed that resting macrophages constitutively express insufficient NLRP3 to promote inflammasome. Microbial components such as TLR ligands, or endogenous molecules such as tumor necrosis factor enhance expression of NLRP3 (and pro-IL-1ß) through the NF-κB activation. Also FAS-associated death domain protein (FADD) and caspase-8 have been recently shown to induce NLRP3 expression during priming[38]. Thus, priming (signal 1) positively regulates the NLRP3 inflammasome through the NF-κB dependent induction of NLRP3 and pro-IL-1ß expression. In addition to NLRP3 expression, priming has also been proposed to play a role in a posttranscriptional regulatory mechanism controlling the activation of NLRP3: TLR4-MyD88-dependent deubiquitination of NLRP3 by BRCC3. As a critical regulator, BRCC3 does not affect NLRP3 expression levels whereas potentiates NLRP3-specific activation of caspase-1 at short time points[39].

1.3.2.2 Activation: second signal

Signal 2, a specific activating step, induced by various PAMPs and DAMPs, activates the functional NLRP3 inflammasome by initiating assembly of a multi-protein complex consisting of NLRP3, the adaptor protein ASC, and pro-caspase-1. Upon recognition of NLRP3 activators, presumably via the LRR domain of NLRP3, NLRP3 monomers induce oligomerization and interact with the pyrin domain (PYD) domain of ASC through homophilic interactions. The adaptor protein ASC then recruits the cysteine protease pro-caspase-1 via a caspase recruitment domain (CARD). The resulting

autocatalysis and activation of caspase-1 lead to maturation and secretion of pro-inflammatory cytokines IL-1ß and IL-18, as well as pyroptosis via Gasdermin D. N domain of Gasdermin D disrupts cellular functions by forming pores on the membrane. Caspase-1 also promotes the release of High mobility group box 1 protein (HMGB1)[40].

Since the NLRP3 activators are of great chemical and structural diversity, it has been hypothesized that its activators do not directly interact and activate NLRP3; instead, NLRP3 activation is triggered through an intermediate cellular signal elicited by all these stimuli. Several activating molecular mechanisms have been suggested including potassium (K^+) efflux, lysosomal destabilization and rupture in response to particulates, and mitochondrial dysfunction and reactive oxygen species (ROS) generation.

K^+ efflux has been proposed as a common step in the activation of the NLRP3 inflammasome. K^+ efflux is induced in response to most or all NLRP3 stimuli, including nigericin, ATP, and particulate matter, and decreasing cytosolic K^+ concentration is sufficient to activate the NLRP3 inflammasome. The precise mechanism by which low K^+ levels affect NLRP3 activation is not understood. While K^+ efflux in NLRP3 activation is well established, Ca^{2+} mobilization and Ca^{2+}-mediated signaling have also been suggested for NLRP3 activation, but this remains controversial.

A second mechanism proposed to contribute to NLRP3 activation involves lysosomal rupture and leakage of lysosomal contents in the cytosol. Endocytosis of particulate matter leads to lysosomal damage and cathepsin B release into the cytosol, promoting NLRP3 inflammasome activation. In addition to the cytosolic release of cathepsin B, particulate matter also triggers K^+ efflux, which is dependent on phagocytosis and required for NLRP3 activation. Consistent with this concept, treatment of macrophages with the lysosomotropic agent Leu-Leu-O-methyl ester alone induces rapid K^+ efflux which subsequently triggers NLRP3 activation.

The role of mitochondria, mitochondria-associated adaptor MAVS, and mitochondria-derived molecules, including mROS in NLRP3 inflammasome activation, is widely conflicting throughout the literature and remained to be determined[37].

1.3.3 Non-canonical NLRP3 inflammasome pathway

Besides canonical NLRP3 inflammasome activation in murine macrophages, a caspase-11-dependent NLRP3 activation pattern which is called non-canonical inflammasome pathway has been characterized. In this non-canonical NLRP3 inflammasome activation, cytosolic detection of lipopolysaccharides (LPS) induced by most Gram-negative bacteria (i.e., *Citrobacter rodentium, Escherichia coli, and Salmonella typhimurium*) activates caspase-11, which results in NLRP3-dependent maturation and release of IL-1ß/IL-18, whereas NLRP3-independent pyroptosis (Figure 1.5)[41].

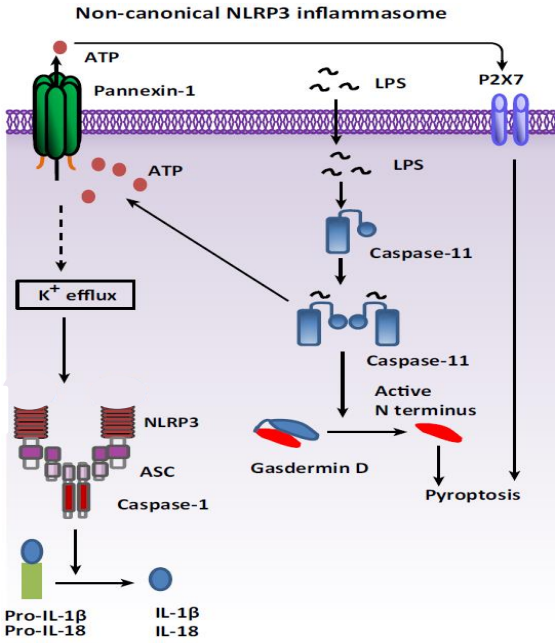


Figure 1.5: Mechanism of TLR4-independent Non-canonical NLRP3 inflammsome activation in murine macrophages.

Figure adapted from He *et al.*[41]. Lipopolysaccharide (LPS) from Gram-negative bacteria is delivered into the cytosol through transfection or infection, rather than recognized by membrane anchored TLR4. Caspase-11 detects the cytosolic LPS. Active caspase-11 promotes the activation of NLRP3-ASC-caspase-1 pathway by inducing the pannexin-1 channel. Caspase-11 activation also triggers pyroptosis through the cleavage of gasdermin D.

1.3.4 Alternative NLRP3 inflammasome pathway

In human but not in murine monocytes, an alternative NLRP3 inflammasome pathway is also activated in response to TLR ligands alone, such as LPS-induced TLR4 response (Figure 1.6). Unlike classical NLRP3 inflammasome signaling, in this pathway, K^+ efflux is not required, which contrasts with NLRP3 activation induced by pore-forming toxins, ATP, or particulate matter. Mechanistically, alternative inflammasome activation proceeds independently of potassium efflux, pyroptosome formation, and pyroptosis, while it employs the molecules RIPK1, FADD, and CASP8 signaling downstream of TLR4-TRIF to activate NLRP3 after LPS treatment[42]. Interestingly, the involvement of this signaling cascade is limited to alternative inflammasome activation and does not extend to classical NLRP3 activation.

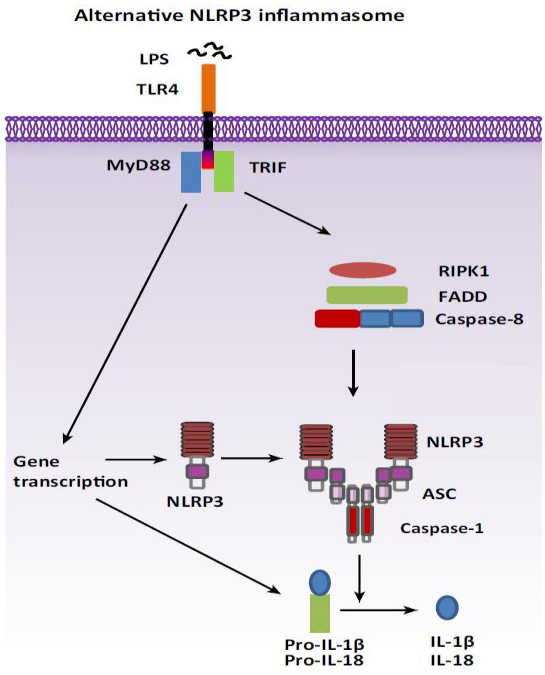


Figure 1.6: Mechanism of TLR4-driven alternative NLRP3 inflammsome activation in human monocytes.

Figure adapted from He *et al.*[41]. The alternative NLRP3 inflammasome pathway is triggered in human monocytes upon LPS challenge on TLR4. TLR4-TRIF-RIPK1-FADD-CASP8 signaling amplifies this pathway activation upstream of NLRP3. This pathway does not engage in any classical inflammasome characteristics, such as K^+ efflux, pyroptosis induction.

1.3.5 Aberrant IL-1ß in disease and therapeutics

By activating lymphocytes, promoting leukocyte transmigration into sites of infection or injury, and generating fever, IL-1ß regulates local and systemic responses to infection, injury and immunological challenge[43]. Furthermore, IL-1ß can induce upregulated expression of the IL-2 receptor on the surface of lymphocytes, promote B-cell proliferation, instruct B cells to enhance antibody production, and increase T-cell survival. However, immune dysregulation of IL-1ß contributes to the pathogenesis prevalent diseases in Western societies such as type 2 diabetes, atherosclerosis, neurodegenerative diseases and cancer. Thus, a fine balance must be maintained between the activation and inhibition of inflammation to allow the immune system to remove any sources of danger without causing harm to the host.

By identifying IL-1β play a key role in the pathogenesis of several autoinflammatory diseases, the important function of NLR molecules in autoinflammatory disorders has recently been partially recognized. The first autoinflammatory disorders found to be directly mediated by dysfunctional inflammasome activation were Cryopyrin-associated periodic syndromes (CAPS)[44-46].

Characterized by constitutive activation of the NLRP3 inflammasome and resultant excessive IL-1β production, Cryopyrin-associated periodic syndromes (CAPS) or cryopyrinopathies refer to at least three phenotypically distinct disorders which are caused by mutations in the *NLRP3* gene. These three CAPS disorders range from the relatively mild familial cold autoinflammatory syndrome (FCAS), to the intermediate Muckle-Wells syndrome (MWS), and to the severe neonatal onset multisystem inflammatory disorder (NOMID). These conditions share overlapping features of inflammation, including fever, rash, conjunctivitis, and arthralgia in spite of clinical heterogeneity,. NACHT domain has been shown to contain approximately 60 MWS disease-associated mutations, suggesting that alteration of the binding properties results in spontaneous, unrestrained assembly of the inflammasome and mature IL-1β production. Indeed, in the basal state, more mature IL-1β is secreted by monocytes from MWS patients[47], and mice harboring mutations of NLRP3 which are equivalent to the human disease present inflammasome hyperactivation leading to deregulated IL-1β production[48].

Since corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying antirheumatic drugs (DMARDs) have not provided clinically significant benefit, therefore treatment of CAPS has been difficult. However, patients with treatment targeting IL-1 have exhibited dramatic improvement. The best known IL-1 inhibitor is anakinra which is a recombinant human IL-1 receptor antagonist (IL-1Ra). Other available inhibitors include canakinumab, a humanized anti-IL-1β monoclonal antibody, and rilonacept, a dimeric fusion protein consisting of extracellular portions of the IL-1 receptor and the Fc region of IgG1[49].

1.3.6 Reported regulators of NLRP3 inflammasome activation

Several regulators including Cyclic adenosine monophosphate (cAMP), double-stranded RNA-dependent protein kinase (PKR), guanylate-binding protein 5 (GBP5), and NIMA-related Kinase-7 (Nek7) have been proposed to affect NLRP3 activation in response to all or select stimuli. However, in some cases, the mechanism and functional study of these regulators are not as well-established, and further studies are required.

According to the universal NLR model, NLRP3 likely exists in an inactive, auto-inhibited conformation, which is maintained by the interaction with the ubiquitin ligase SGT1, the heat shock chaperon HSP90[50], and the deubiquitinating enzyme BRCC3[51]. Therefore, deubiquitination of NLRP3 is essential for its activation. Another mechanism to preserve an inactive conformation or to prevent oligomerization has been proposed to be interacted with cAMP. cAMP binds to NLRP3 directly via its NACHT domain to inhibit inflammasome assembly, and downregulation of cAMP relieves this inhibition[52].

PKR was reported to regulate the activation of all known inflammasomes, including NLRP1, NLRP3, NLRC4, and AIM2. Deletion or inhibition of PKR leads to reduced activation of caspase-1 and maturation of IL-1ß and IL-18 in response to a wide array of stimuli. GBP5 was suggested to promote NLRP3 inflammasome activation in response to non-crystalline stimuli, such as ATP, nigericin, and bacteria but not particulate matter[41].

Nek7 has been shown to be essential for NLRP3 inflammasome activation, but not NLRC4 and AIM2 inflammasomes, induced by NLRP3 stimuli including nigericin, ATP, MSU crystals, and alum. Upon NLRP3 stimulation, Nek7 directly interacts with

NLRP3 through the LRR domain of NLRP3 and the catalytic domain of Nek7. Nek7-mediated NLRP3 activation does not require the kinase activity of Nek7. The NLRP3/NEK7 interaction was shown to be downstream of potassium efflux and promote NLRP3 oligomerization, ASC speck formation, and caspase-1 activation [53].

Recently, Ito *et al.*[54] identified Bruton's tyrosine kinase (BTK) as a critical component of the NLRP3 inflammasome. Inhibition of BTK by pharmacological or genetic means severely impaired activation of the NLRP3 inflammasome. BTK inhibitor suppressed infarct volume growth and neurological damage in a brain ischaemia/reperfusion model.

1.4 Bruton tyrosine kinase

The Bruton's tyrosine kinase was originally identified in 1993 as a non-receptor protein-tyrosine kinase that is defective in an immune deficiency called X-linked agammaglobulinemia (XLA) or Bruton´s agammaglobulinemia. BTK is a 76-kDa polypeptide with 659 amino acid residues. The gene encoding the BTK molecule was isolated in 1993 and was recognized to be located on the X chromosome in the region Xq21.3-22.1. The *BTK* gene is composed of 19 exons and spans over 36 kb. BTK was named independently at the time as B cell progenitor kinase and agammaglobulinemia tyrosine kinase. [55,56].

1.4.1 Architecture of BTK

BTK belongs to the transient erythroblastopenia of childhood (TEC) family of non-receptor protein tyrosine kinases including BTK, TEC, ITK (IL2-inducible T cell kinase), RLK (resting lymphocyte kinase), and BMX (bone marrow tyrosine kinase gene in chromosome X), all of which are highly expressed in hematopoietic cells. BTK contains a short N-terminal pleckstrin homology (PH) domain, a TEC homology (TH) domain, followed by an SH3, SH2, and carboxyterminal protein kinase domain (Figure 1.7), which is an architecture shared with the other members of TEC family of protein kinases[57].

PH domains are found in several intracellular signaling molecules that are shown be able to bind phospholipid head groups and in some cases also mediate protein-protein interaction[58]. In BTK, the PH domain is attracted to membrane-associated PIP3, which is responsible for their membrane-localization. The membrane-localized BTK is active

and associated with transient phosphorylation of two tyrosine residues, Y551 and Y223. Y551 within the kinase domain is trans-phosphorylated by the Src family tyrosine kinases, resulting in the auto-phosphorylation of Y223 within the SH3 domain. In particular, the functional importance of SH3 Y223 auto-phosphorylation remains unclear because phosphorylation of Y223 does not seem to affect BTK activity. The PH domain mutation E41K increases BTK binding to PIP3, independent of PI3K activity, resulting in increased membrane localization and robust transformation potential. E41K-*BTK* expression in the B-cell lineage leads to selective expansion or survival of B-1 B cells, whereby residual B cells are hyper-responsive and are spontaneously driven into germinal-center-independent IgM plasma cell differentiation[59].

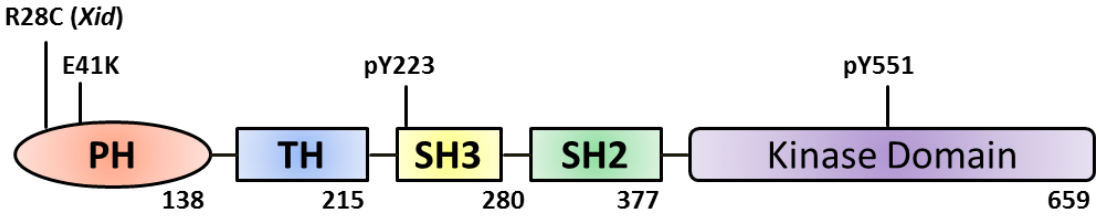


Figure 1.7: The structure of Bruton tyrosine kinase (BTK).

BTK has a PH, TH, SH3, SH2 and kinase domain. The relative size of each domain is indicated by the amino acid residue number given below. The positions of the R28C *Xid* loss-of-function mutation, E41K gain-of-function mutation are indicated, as well as the phosphorylation sites Y223 and Y551.

1.4.2 BTK functions

BTK is expressed in the cells of all hematopoietic lineages, except for T and plasma cells, and plays an essential role in various biologic functions of different cell types.

1.4.2.1 The role of BTK in B cell antigen receptor signaling

BTK is a critical component of BCR signaling. BTK is involved in all aspects of B cell development, including maturation, proliferation, apoptosis, differentiation.

During development, each B cell recombines immunoglobulin variable (V), diversity (D), and junction genes (J) thereby forming a unique sequence that establishes the specific antigen-binding site of the B cell receptor. On B cell membrane, the B cell

receptor complex binds to a disulfide-linked Igα-Igß heterodimer. After antigen is specifically recognized by the receptor, the Src family kinase LYN catalyzes the phosphorylation of pairs of tyrosine residues in Igα-Igß ITAMs (immunoreceptor tyrosine-based activation motifs) thus creating a docking site for the two SH2 domains of SYK (spleen tyrosine kinase). SYK recruits and activates PI3 kinase, which triggers the conversion of membrane-associated PIP2 (phosphatidyl inositol 4, 5 bis-phosphate) to (PIP3phosphatidyl inositol 3,4,5-trisphosphate). SYK and Lyn catalyze the trans-phosphorylation of BTK at Tyr551, which leads to the interaction of BTK and PIP3 through the amino-terminal PH domain. BTK catalyzes the phosphorylation of PLCγ2 at residues Tyr753 and Tyr759 and then proceeds the downstream signaling via the Ras/RAF/MEK/ERK module which promotes proliferation, survival and also NF-κB activation which is essential for B cell maturation and differentiation[60-62] (Figure 1.8).

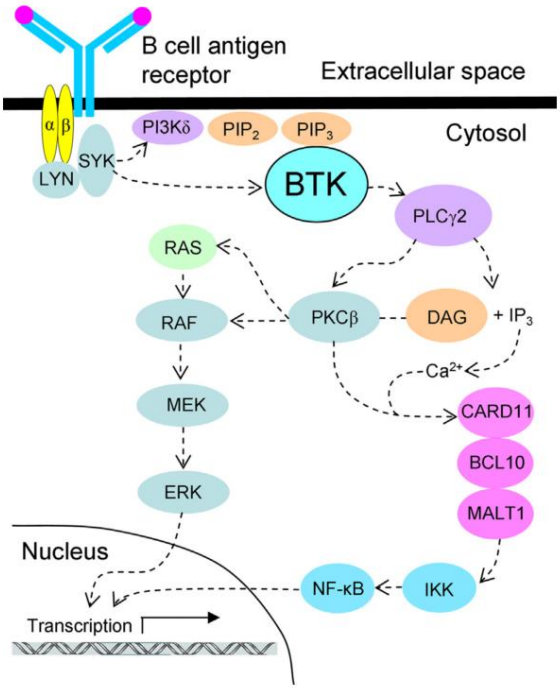


Figure 1.8: Function of Bruton tyrosine kinase in B cell receptor signaling.

Figure adapted from Roskoski Jr.[61]. B cell receptor signaling requires a network of protein kinases and adaptors that mediate antigen stimulation to intracellular responses. BCR activation results in the activation of SYK tyrosine kinase. SYK together with the Src family kinase LYN phosphorylates several downstream signaling molecules including BTK as described above. The dashed arrows indicate that several steps may be involved.

1.4.2.1.1 Disease relevance of BTK: X-linked agammaglobulinemia (XLA)

The importance of the PH domain of BTK is also proved by the R28C missense mutation in the well-characterized mouse model X-linked immunodeficiency (*xid*). B cells with the *xid* mutation lack essential signals for B cell activation and maturation. Therefore, B cells from *xid* mice have abnormal responses to a variety of activation signals, and do not respond to thymus-independent type 2 antigens. Similar to the *xid* mutation, X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by mutations in the *BTK* gene. X-linked agammaglobulinemia (XLA) results in the production of few B cells and severe humoral immunodeficiency because XLA patients are characterized by an almost complete block of B cell development at the pre-B cell stage. The XLA phenotype in humans can be explained by the function of BTK in signaling pathways downstream of the BCR. The pre-BCR is an immature form of the BCR that monitors for functional immunoglobulin heavy (IgH) chain rearrangement by deposition of the IgHμ protein on the cell surface, thereby providing signals for survival, proliferation and cellular differentiation. As previously described, activation of BTK upon pre-BCR or BCR stimulation requires two key steps: firstly membrane association, a process that critically depends on interaction of the PH domain with the products of PI3K, and secondly phosphorylation of the Y551 tyrosine in the kinase domain by tyrosine kinases, such as Lyn, resulting in BTK autophosphorylation at Y223. Loss of BTK function disrupts this pre-BCR checkpoint function; therefore, in patients with XLA, clonal expansion and developmental progression of IgHμ chain-expressing pre-B cells are abrogated.

Patients with XLA are usually markedly susceptible to infections with encapsulated bacteria, particularly *Streptococcus pneumonia* and *Haemophilus influenza type B.* In addition patients with XLA have an elevated risk of infection with *Giardia*, mycoplasma, and enteroviruses. Most infections are in the respiratory and gastrointestinal tract. Current treatment is not curative. Because these patients are unable to mount specific antibody responses, immunoglobulin replacement therapy through both intravenous or subcutaneous infusion, and treatment of infections are available nowadays to help XLA patients survive into adulthood[57,63,64].

1.4.2.1.2 Further disease relevance of BTK: B-cell malignancies

BTK-dependent B-cell antigen receptor signaling is likely to be involved in maintaining the malignant phenotype in B-cell lymphomas and leukemias, including diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, mantle cell lymphoma (MCL), and B-cell chronic lymphocytic leukemia (CLL). Therefore targeting of BCR signaling has become a field of major interest and intense drug development. As a critical effector molecule that governs normal B-cell development, differentiation, and functioning, BTK activity is dysregulated in human B-cell malignancies and exploited by tumor cells for increased proliferative potential, evasion of apoptosis, enhanced survival, and tumor progression[65].

Survival of various B-cell malignancies is also controlled by chemokine-mediated homing and interacting with lymphoid microenvironments. These interactions are also dependent on BTK due to its role in signaling downstream of these malignancy chemokines. The potential for therapeutic BTK-targeting is currently being tested in clinical settings[66].

1.4.2.2 The role of BTK in innate immune response

Although BTK is most intensely studied in the context of BCR signaling and thus adaptive immunity, it is also present in innate immune cells such as macrophages, neutrophils, natural killer cells, mast cells, and platelets. It has been recently indicated that Bruton tyrosine kinase (BTK) plays an essential role in various biologic functions of these different cell types. In particular, BTK was linked to the Toll-like receptor (TLR) pathway.

Macrophages recognize the foreign pathogen via a number of pathogen recognition receptors (PRRs) followed by phagocytosis, which leads to the production of inflammatory cytokines like TNF-α and nitric oxide (NO) release. BTK is reported to be activated upon LPS treatment of macrophages, resulting in increased TNF-α secretion. Xid mice-derived macrophages produce less NO, TNF-α and IL1ß, but secrete higher amounts of IL-12[60,67]. Human neutrophil experiments have provided additional information suggesting a role for BTK in the regulation of innate immune functions. Fiedler *et al.* showed BTK is an important regulator of GM-CSF- and Toll-like

receptor-induced neutrophilic granulocyte maturation and function *in vivo*[68]. A report by Honda *et al.* demonstrated production of ROS was significantly increased when XLA neutrophils were stimulated with TLR ligands, together with a neutrophil chemoattractant (fMLP)[69]. In addition, BTK is indicated to be required for the pro-inflammatory cytokine secretion like IL-12, TNF-α and IL-6 in mast cells[70], as well as TLR3-triggered NK cell activation[71]. It was also suggested that BTK interacts with TLR family members TLRs 4, 6, 8, 9 and that BTK is a member of the multiprotein complex that is recruited to the TLR TIR domain upon LPS stimulation[60]. However, the precise mechanisms of BTK regulating inflammasomes activity remain elusive.

1.4.3 FDA-approved BTK inhibitor ibrutinib and others

Given the importance of B-cell receptor signaling in B cell malignancies and the central role of BTK in this pathway, an attractive strategy is to target inhibition of this kinase. In 1999, the first rationally designed BTK small-molecule inhibitor (LFM-A13) was shown to have anti-leukemic activity in vitro[72], but it is very unselective. More selective human BTK inhibitors were subsequently developed including the irreversible inhibitor ibrutinib (also known as PCI-32765). Ibrutinib is the first-in-class, highly potent small molecule inhibitor that selectively binds to a cysteine residue (Cys-481) and irreversibly inhibits BTK phosphorylation and thus its enzymatic activity. Ibrutinib mono-therapy has recently shown encouraging clinical activity in patients with mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL) and Waldenström macroglobulinemia (WM). As a result, ibrutinib received breakthrough designation and was approved for the treatment of relapsed MCL by the US Food and Drug Administration (FDA) in 2013[73].

Second-generation human BTK inhibitors including ACP-196, ONO/GS-4059, and BGB-3111, who are supposed to have fewer off-target effects and are more selective and potent than BTK, are developed and being evaluated clinically aiming to gain less toxic and more targeted therapy for B cell malignancies. Additionally, a newly discovered, highly specific small-molecule BTK inhibitor, CGI1746, can uniquely stabilize BTK in an inactive conformation suppressing inflammation in rheumatoid arthritis. Additionally, CGI1746 holds promise in multiple therapeutic areas, including

SLE, rheumatoid arthritis, severe asthma and B cell malignancies. Future clinic trials addressing tolerability and efficacy of CGI1746 are of great interest[74,75].

1.5 Aim of this project

Since up to now few proteins that directly regulate NLRP3 inflammasome activation in human primary immune cells have been identified and non of these are currently targetable, promising pharmacological strategies clinically targeting NLRP3 inflammasome-driven inflammatory diseases remain elusive. Therefore the overall aim of this project was to identify novel regulators of the NLRP3 inflammasome in human cells and understand more the regulatory mechanism of NLRP3. A global unbiased SILAC phosphoproteomics screen was therefore employed as a starting point to investigate potential regulators. The analysis of the phospho-screen yielded Bruton's tyrosine kinase (BTK) as a very promising candidate. My thesis focused on BTK's role in the NLRP3 inflammasome in myeloid cells for the following work. The questions which I was aiming to answer were (a) does BTK directly and specifically participate in the NLRP3 inflammasome process, especially whether it binds directly to NLRP3 or ASC; (b) whether interfering BTK, e.g. using BTK inhibitors, would influence NLRP3 inflammasome function, in particular mature IL-1ß release; (c) whether inhibiting BTK could contribute to the treatment of NLRP3 inflammasome-linked inflammatory disease. To address these questions genetic and pharmacological ablation of BTK in cells from human healthy donors or patients with dysfunctional BTK (XLA patients) or hyperactive NLRP3 (CAPS patients) and from mice with BTK deficiency (Xid point mutations or complete gene knockout) were used in assays such as phospho-flow cytometry to detect BTK phosphorylation, co-immunoprecipitations, ASC speck formation assays, pro-caspase-1 and pro-IL-1 cleavage analysis and caspase-1 or IL-1 release by ELISA.

2 Materials and Methods

2.1 Study subjects and sample acquisition

All human subjects provided written informed consent in accordance with the Declaration of Helsinki and the study was approved by the local ethics committees. Buffy coats from healthy donors were provided by the Tübingen University Hospital Transfusion Medicine Department. Male XLA patients with confirmed genetic and clinical BTK deficiency were recruited at the Center for Immunodeficiency at Freiburg University Hospital. Male healthy donors in a similar age range were recruited at the Department of Immunology, Tübingen, and blood taken on the same day as that from patients with XLA. Samples were processed in the same way and measured together. Patients with MWS were recruited at the Pediatrics Department of the University Hospital Tübingen.

2.2 Molecular biology methods

2.2.1 Plasmids and DNA purification

Plasmids transformations were performed with 100 ng of DNA and 20-100 µl of competent bacteria (*Escherichia coli* DH5α). The transformed bacteria were incubated on ice for 30 min followed by a heat-shock treatment in water bath at 42 °C for 45 s. The tubes were placed back on ice for 2 min and 200 µl of pre-warmed S.O.C or NZY^+ medium was added to cultivate the transformed bacteria for 1 hour at 37 °C. 200 µl of the bacterial suspension was plated onto a 10 cm LB agar plate containing the appropriate antibiotic. The plates were incubated at 37 °C overnight. Several colonies were picked up and transferred into 2-4 mL (miniprep) or 100-200 mL LB medium (midiprep) that was supplemented with the appropriate selectable antibiotics. The bacterial culture was incubated at 37 °C for 12-18 hours in a shaking incubator. Then the plasmid DNA was extracted and purified from bacteria pellets according to the manufacture instructions (PureYield™ Plasmid Miniprep or Midiprep System) using a silica membrane column. Additionally, with a unique Endotoxin Removal Wash in this kit, protein, RNA and endotoxin contaminants were removed from purified plasmid DNA.

2.2.2 Enzyme linked immunosorbent assay (ELISA)

IL-1ß, IL-2, TNF-α, IFN-γ, and caspase-1 levels in supernatants were detected using Enzyme-Linked Immunosorbent Assay (ELISA). Human IL-1ß, IL-2, TNF-α, IFN-γ ELISA kits were from BioLegend, human caspase-1 and mouse IL-1ß ELISA kits were from R&D system. 96 well plates were pre-coated with monoclonal capture antibodies overnight at 4°C. According to the protocol which was provided by the company, the plates were blocked with 1× assay buffer for 1 hour. Samples, including a two-fold serial diluted standard, control specimens, and unknowns with/without proper dilution, are pipetted into these wells. Each sample was analyzed in triplicates and incubated at room temperature for 2 hours. After washing, a detection antibody was added to the wells for 1 hour, and this antibody bound to the immobilized protein captured during the first incubation. After removal of excess detection antibody, an HRP conjugate (secondary antibody or streptavidin) was added and bound to the detection antibody. After a third incubation and washing to remove the excess HRP conjugate, a substrate solution was added and was converted by the enzyme to a detectable form (color signal). The concentrations of the protein of interest were measured at the wave length of 450 nm on a standard plate-reader (Fluostar, BMG).

2.2.3 Quantitative real-time PCR

mRNA was isolated using the RNeasy Mini Kit on a Qiacube robot (both from Qiagen) and transcribed to cDNA (High Capacity RNA-to-cDNA Kit from Life Technologies), and the mRNA expression of *IL1B and NLRP3* was quantified in triplicates relative to that of the housekeeper *TBP* (TATA-box binding protein) using TaqMan primers (Life Technologies) on a real-time cycler (Applied Biosystems; 7500 fast).

2.3 Biochemical method

2.3.1 Cell preparation for mass spectrometry analysis

Null THP-1 cells (Invitrogen) were grown in “light” (L-lysine/Lys0, L-arginine/Arg0), “medium-heavy” (D4-L-lysine/Lys4, $^{13}C_6$-L-arginine/Arg6) and “heavy” ($^{13}C_6{}^{15}N_2$-L-lysine/Lys8, $^{13}C_6{}^{15}N_4$-arginine/Arg10) SILAC medium for three passages. Incorporation of the labeled amino acids was in each case confirmed to be over 97%. For the

experiment cells were primed with 300 ng/mL PMA for three hours and left to rest overnight. The next day the cells were detached and either left unstimulated (light) or stimulated with 15 μM Nigericin for 5 minutes (medium) or 10 minutes (heavy). After washing with ice-cold PBS (containing phosphatase and protease inhibitors, Roche), cells pellets were snap-frozen and stored at -80 ℃ prior to analysis.

2.3.2 SDS-PAGE electrophoresis for Pro-IL-1β and caspase-1 cleavage

Equal numbers of cells were primed by PMA (100 ng/mL, Invivogen) overnight or LPS (300 ng/mL, Invivogen) for 3 hours, and then stimulated with indicated stimuli in Opti-MEM (Gibco). Protein in 500 mL supernatants was precipitated by 500 mL methanol (VWR International) and 125 mL chloroform (Sigma). The interface layer containing protein was washed and further purified by centrifuging with 500 mL methanol. Then the protein pellet was resuspended in 1× NuPAGE LDS loading buffer (Invitrogen) supplemented with 1× NuPAGE Sample Reducing Agent (Invitrogen) and denatured for 5 minutes at 95°C. Where applicable, 10 ng recombinant Protein A was added prior to precipitation as a control. The cell fractions were lysed in a RIPA buffer with protease inhibitors (Sigma). 15% and 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels were used for protein from supernatants and whole cell lysates respectively. PageRuler^{TM} Prestained Protein Ladder (Life Technologies) was used as molecular size marker.

The proteins were transferred onto a nitrocellulose membrane (0.45 μm pore size, GE healthcare) by semi-dry blot (Bio-Rad Trans-Blot Turbo^{TM} Transfer System) using the "Standard" protocol for 35-38 min depending on the protein molecular weight. The transfer buffer comprised diluted NuPAGE Transfer Buffer (20 ×) and 10% methanol. For immunoblotting, an automatic process of BlotCycler™ Touch (Precision Biosystems) was used at 4°C overnight with blocking in 5% BSA (Bovine serum albumin) TBST buffer (TBS containing 0.05% Tween 20) for 1.5 hours, primary antibody incubation for 7 hours (anti-IL-1ß antibodies were diluted in 1:500, anti-caspase-1 antibodies were diluted in 1:1000), secondary antibody incubation (anti-rabbit 1:5000, anti-mouse 1:10000 dilution) for 4 hours, and three 3 × 5 min washing steps after each antibody binding. The membranes were incubated in the AceGlow^{TM}

chemiluminescense substrate, and the signal was captured and analyzed by the Peqlab Fusion SL charge-coupled device (CCD) system.

2.3.3 Cell preparation for crosslinking assay for ASC oligomers

2×10^6 null THP-1 or ASC-mCerulean cells (from Veit Hornung) were plated in a 6 well format (Greiner Bio One), primed with 100 ng/mL PMA for overnight or with 300 ng/mL LPS for 2 hours, respectively, then treated with 60 µmol/L ibrutinib for 1 hour and then with 15 µmol/L Nigericin for 1 hour; unless otherwise stated.

2.4 Cell biology assays

2.4.1 Cell line and cultivation

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Life Technologies). THP-1 (DSMZ no.: ACC 16) were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Life Technologies). Null THP-1 cells (stably expressing a non-targeting shRNA) and NLRP3-deficient THP-1 cells (stably expressing an NLRP3-targeting shRNA; both from InvivoGen) were cultured in RPMI-1640 supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Life Technologies), sodium pyruvate (1 mmol/L; from Invitrogen), HEPES buffer (10 mmol/L; from Sigma), Normocin (100 µg/mL; from InvivoGen), and Hygromycin B (100 µg/mL; from Invitrogen). iGluc THP-1 cells were a kind gift of V. Hornung, Institute of Molecular Medicine, Munich, Germany, and cultured in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Life Technologies), and sodium pyruvate (1 mmol/L; from Invitrogen). BTK-shRNA- and mock control-THP-1 cells were a kind gift of R. Morita, Keio University School of Medicine, Tokyo, Japan, and were cultured in RPMI-1640 supplemented with 10% FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Life Technologies), in the presence of 1.5 and 2 mg/mL G418, respectively. ASC-

mCerulean-expressing immortalized macrophages or THP-1 cells were in RPMI-1640 medium supplemented with 10% FCS penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Life Technologies). All cell lines were cultured at 37°C and 5% CO2.

2.4.2 PBMCs isolation and monocyte-derived macrophages differentiation

Peripheral blood mononuclear cells (PBMCs) from healthy donors (or XLA, Muckle-Wells, and ibrutinib treated cancer patients) were isolated from whole blood using Ficoll (Merck Millipore) density gradient purification and washed three times with RPMI to reduce platelet contamination. To generate primary macrophages, PBMCs were isolated from buffy coats by Ficoll and then $CD14^+$ monocytes were isolated and purified from PBMCs using CD14 magnetic beads (MACS Miltenyi Biotec; >90% purity assessed by anti-CD14-PE flow cytometry) for positive selection. The cells were seeded at a concentration of 1×10^6 cells/mL in 96-well tissue culture plates in RPMI-1640 supplemented with 10% FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Life Technologies). Cells were differentiated into macrophages in the presence of 25 ng/mL recombinant human GM-CSF (Peprotech) for 5 days.

PBMCs were seeded in RPMI-1640 (Sigma), 10% FCS (GE Healthcare), 2 mmol/L L-Glutamine and 1% PenStrep (Life Technologies). Cells were then treated with 10 ng/ml LPS for 3 hours, and stimulated with 15 µmol/L Nigericin for 1 hour or instead with 50 ng/ml PMA and 1 µmol/L Ionomycin for 4 hours. PBMC from MWS were seeded at a concentration of 1×10^6 cells/mL in 24-well tissue culture plates. Cells were then treated with 10 ng/mL LPS, 1 mmol/L ATP concomitantly with 60 µmol/L ibrutinib or a DMSO control for 4 hours and supernatants collected for ELISA. The resulting Monocyte-derived macrophages (MoMacs) were primed with 300 ng/ml LPS for 3 hours and pre-treated with ibrutinib at 20 µM or 60 µM for 10 minutes before stimulation with 15 µmol/L Nigericin or the indicated amounts of LukAB or Panton Valentine Leukocidin (PVL) for 1 hour.

2.4.3 Bone marrow derived macrophages generation

Btk KO and wild type littermates (all C57BL/6 background) were used at an age of 8 to 12 weeks. Bone marrow cells were isolated from femurs and tibiae using standard procedures (details available on request) and 3×10^6 cells/mL plated in 10 cm non-tissue culture coated dishes in 10 mL complete RPMI media containing 10% GM-CSF (M1 polarization) or M-CSF (M2 polarization) conditioned medium for 5-7 days. Cells were always counted and re-seeded prior to in vitro assays to ensure equal cell numbers. Ex vivo animal experiments were in accordance with institutional guidelines and German animal protection laws. All mouse colonies were maintained in specific-pathogen free conditions.

2.4.4 Flow cytometry and phospho-flow

For whole blood analysis of healthy donors and XLA patients 200 µL of whole blood from patients and health controls was stained with a mix of antibodies detecting cell surface antigens: anti-CD3-FITC, -CD19-Pacific Blue, -CD14-PE and -CD11b-APC for 30 minutes at room temperature in the dark. Samples were then fixed and permeabilized (Lyse/Fix Buffer, BD) for 20 minutes, washed and resuspended in 200µl of PBS 0.5% BSA for analysis (BD Fortessa). A standardized protocol and identical flow cytometer settings were used for all donors. For phosflow analysis, the indicated primed cells were treated and then fixed (Lyse/Fix Buffer, BD). LIVE⁄DEAD Fixable Aqua was used to stain dead cells (Life Technologies). Cells were permeabilized with 1 mL of cold methanol, Fc-receptors were blocked (Human AB serum) and cells were stained with antibodies against anti-Btk (pY551) PE, anti-Btk (pY223) BV421, and anti-total BTK Alexa Fluor 647 (all from BD). Corresponding isotype controls were from Immunotools.

2.5 Data analysis and statistics

Data were analyzed in GraphPad Prism version 6.0 using 2 tailed Student *t* tests and non-parametric Mann-Whitney-U or Wilcoxon matched-pairs signed rank tests. A *P* value of less than 0.05 was generally considered statistically significant and marked by an asterisk throughout the figure legends, even if considerably lower.

3 Results

Parts of the results are already published in "Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase", Liu et al., J Allergy Clin Immunol. 2017 Feb 16. pii: S0091-6749(17)30232-4. doi: 10.1016/j.jaci.2017.01.017.

3.1 BTK is rapidly phosphorylated upon NLRP3 inflammasome activation

The NLRP3 inflammasomes are cytosolic multi-protein complexes that are formed to mediate host immune responses to microbes and danger signals. Although under intensive investigation NLRP3 inflammasome has been recognized to be important in immunity and pathology of human diseases, the mechanism and regulation of its activation remain elusive. The main aim of this thesis was to identify and characterize novel regulators of the NLRP3 inflammasome complexes that is activating or inhibiting the inflammasome assembly, modulate aberrant inflammasome activity via such regulators, and provide a route to developing therapeutic agents for NLRP3 inflammasome-associated diseases.

3.1.1 BTK is early up-regulated in a global phospho-proteomics screen upon Nigericin

The NLRP3 inflammasome can be activated by diverse stimuli. Among these activators, the most rapid and potent responses of NLRP3 inflammsome are elicited by the microbial potassium ionophore Nigericin and extracellular ATP respectively. Additionally, as mentioned above, activation of NLRP3 needs a first signal, namely the "priming" step. Therefore, the human monocyte cell line THP-1 was differentiated into macrophages (THP-1-Mφs) by 300 ng/mL phorbol 12-myristate 13-acetate (PMA) priming (signal 1) for 3 hours; this treatment induced intracellular pro-IL-1β accumulation without engaging TLR signalling pathways. Next, THP-1-Mφs were treated with Nigericin for 5 or 10 minutes to activate the NLRP3 inflammasome (signal 2), in order to capture early, NLRP3-proximal events. These THP-1 cells were then subjected to an unbiased triple SILAC phospho-proteomics[76]. We compared the phospho-proteome of unstimulated cells vs. cells stimulated for 5 or 10 minutes and

performed cluster analyses of all the candidates from this screen. As shown below, the candidates could be sorted and defined into 4 groups: late phosphorylated, early dephosphorylated, late dephosphorylated and early phosphorylated (Figure 3.1). Intriguingly, a phospho-peptide of Bruton tyrosin kinase (BTK) was found in cluster 4 "early phosphorylated", and this phospho-peptide contained the well-known BTK regulatory site tyrosine 551. Y551 was significantly up-regulated 2.6-fold within 5 min of stimulation (Figure 3.2).

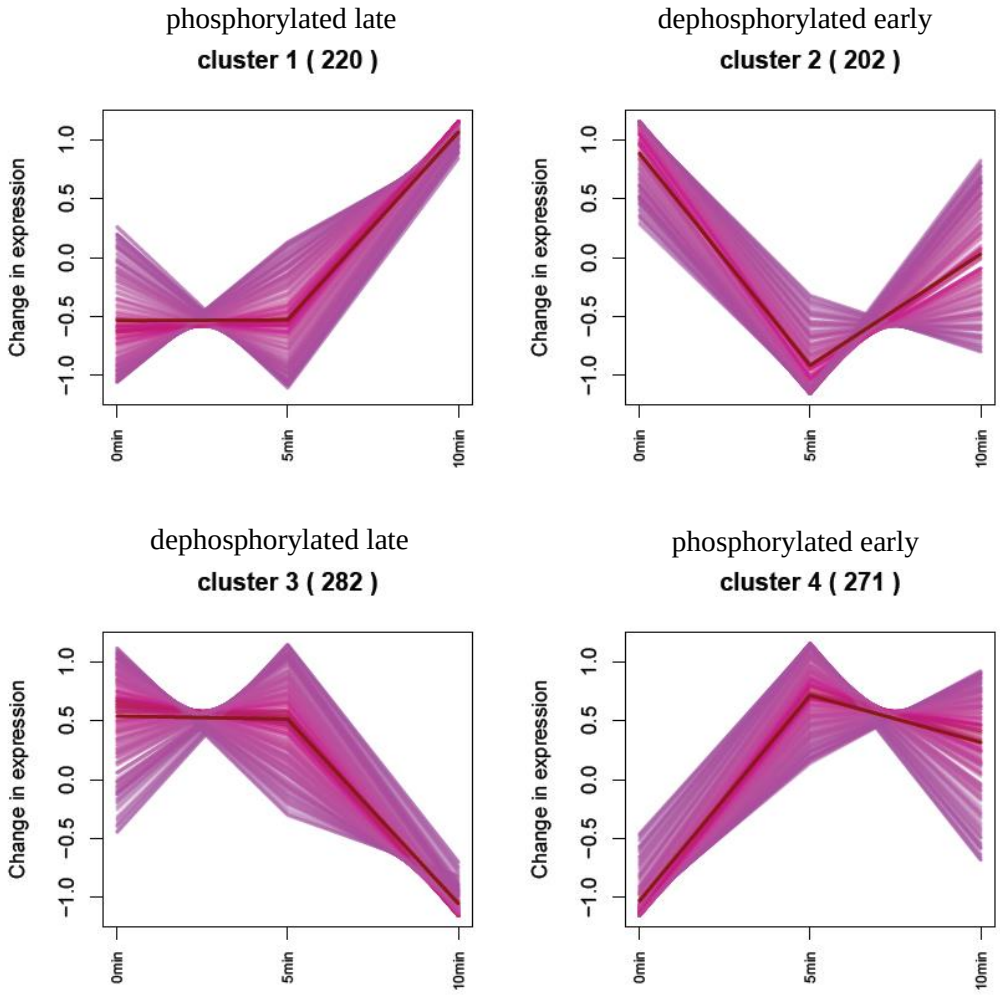


Figure 3.1 Cluster analyses of the candidates from phospho-proteomics screen. Cluster 1: phosphorylated late; cluster 2: dephosphorylated early; cluster 3: dephosphorylated late; cluster 4: phosphorylated early.

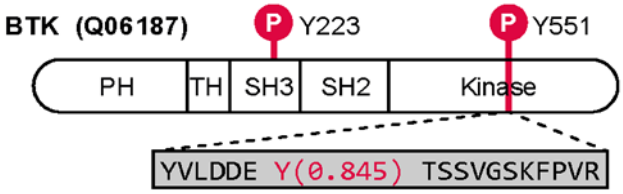


Figure 3.2 Schematic description of BTK domains, phospho-sites (red) and the regulated phospho-peptide (grey) in BTK. (Liu *et al.*[77])

3.1.2 Phosphorylation of BTK in THP-1 and primary macrophages is validated by phospho-flow cytometry analysis

To confirm this phosphorylation effect on BTK by another experimental technic, PMA primed and Nigericin treated THP-1 cells were subjected to phospho-specific flow cytometry (Figure 3.3A). This analysis revealed rapid, but transient, phosphorylation at site Y551 of BTK after Nigericin addition. The Nigericin-induced phosphorylation effects robustly increased at 5 min, and then greatly decreased at 10 min stimulation, with even further reduction at 20 min. These data indicated BTK was rapidly activated by an NLRP3 inflammasome trigger.

Activation of BTK correlates with an increase in the sequential phosphorylation of two regulatory BTK tyrosine residues. Y551 (site 1) within the Src homology type 1 (SH1) domain is transphosphorylated by the Src family tyrosine kinases in B cell receptor signaling pathway. A second major phosphorylated tyrosine residue Y223 (site 2) is located within the BTK SH3 domain. Phosphorylation of Y223 (site 2) occurs by a Btk kinase-dependent mechanism, i.e., autophosphorylation[78]. However, in our data (Figure 3.3A), Y223 showed only subtle phosphorylation. To investigate the role of BTK in a more physiological condition, we generated human monocyte-derived macrophages (MoMacs). Briefly, we isolated $CD14^+$ monocytes from buffy coats using anti-CD14 magnetic beads, differentiate them into macrophages and tested BTK phosphorylation in these cells upon Nigericin stimulation. Importantly, similar results were observed in human primary macrophages (Figure 3.3B).

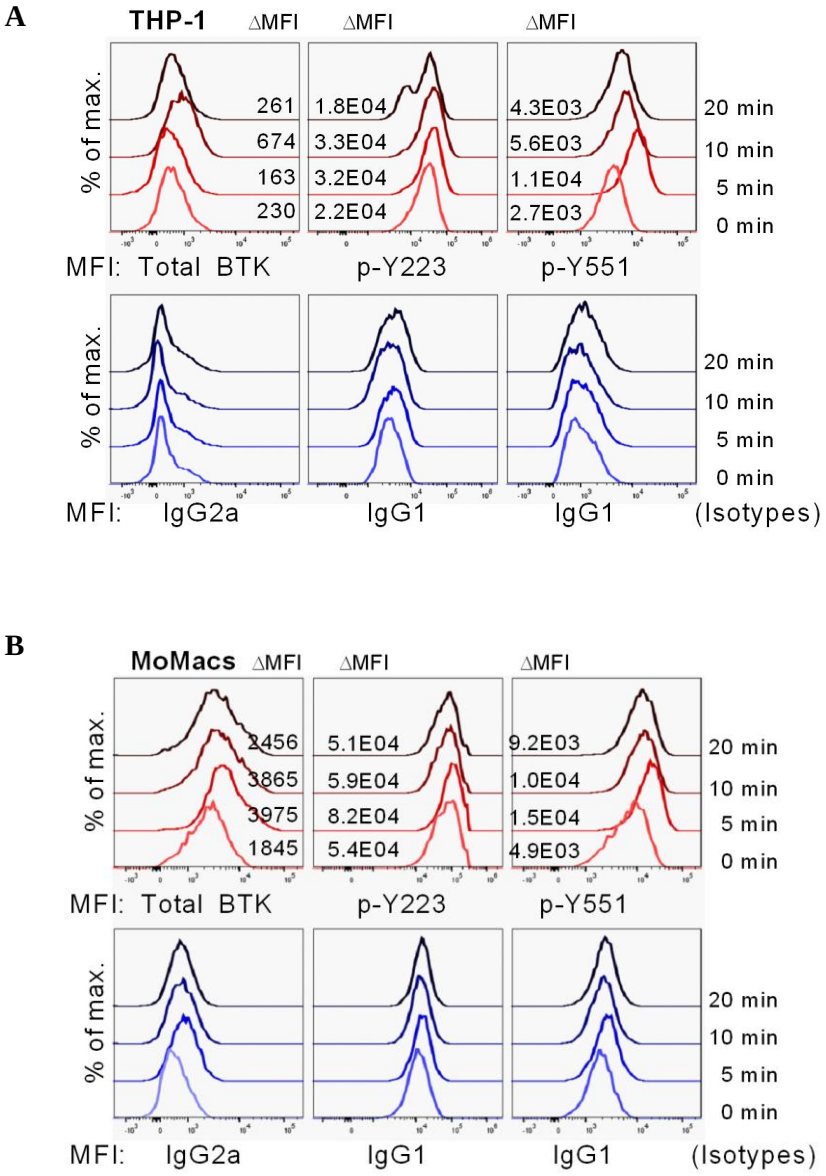


Figure 3.3 Representative phospho-flow plot of phosphorylation responses of BTK Y551 and Y223. PMA-primed THP-1 cells (**A**) or LPS-primed human primary MoMacs (**B**) were stimulated without or with Nigericin for 5, 10, or 20 minutes, stained with indicated phospho-specific antibodies and analyzed by flow cytometry. ΔMFI differences for each antibody-isotype pair are given. Data are representative of three experiments. (Liu *et al.*[77])

3.2 Pharmacological inhibition of BTK attenuates NLRP3 inflammasome activation

3.2.1 Ibrutinib inhibits IL-1β release from THP-1 cells

The NLRP3 inflammasome responds to microbes and danger signals by processing and activating proinflammatory cytokines, including interleukin 1ß (IL-1ß) and IL-18. Therefore we could assess NLRP3 inflammasome activation by measuring IL-1ß release. Our previous data showed that Y551 of BTK is phosphorylated upon NLRP3 inflammasome activation, and it has been demonstrated by Rawlings *et al.*[79] that the transphosphorylation of BTK on Tyr551 in the catalytic domain resultes in a 5-10-fold increase in BTK enzymatic activity. We thus wondered whether kinase activity of BTK has functional significance of inflammasome activation. To address this question, PMA primed THP-1 cells were pre-treated with or without the FDA-approved inhibitor of BTK, ibrutinib (PCI-32765), and then stimulated with Nigericin. DMSO served as control. As we expected, IL-1β secretion was substantially reduced (Figure 3.4A) in ibrutinib treated THP-1 cells.

Since it is reported that BTK binds to several members of the Toll-like receptor (TLR) family (namely, TLRs 4, 6, 8, and 9) and participates in TLR4-mediated NF-κB activation. Inhibition of BTK could decrease NF-κB dependent induction of *NLRP3* and *IL1B* expression during priming of NLRP3 inflammasome[62]. To minimize this potential effect on priming, we incubated THP-1 with ibrutinib for 1 hour - a relatively short time comparing to the 16 hours of PMA priming. Notably, which nicely meet the requirement of our experimental design, pharmacological inhibition of BTK had no regulatory effects on mRNA levels of PMA-primed NLRP3 expression, and IL-1ß mRNA level was even higher with the ibrutinib pre-treatment (Figure 3.4B).

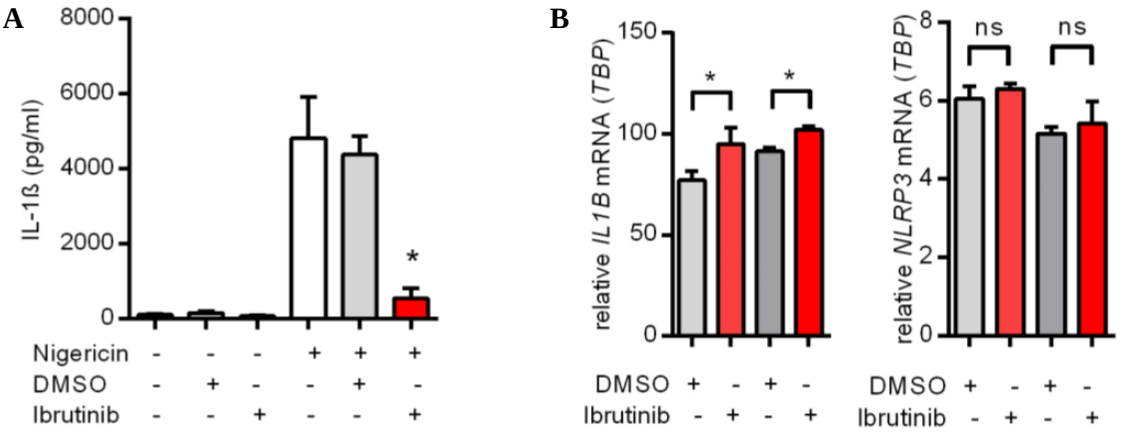


Figure 3.4 (**A**) Enzyme-linked immunosorbent assay (ELISA) of IL-1ß in supernatants from THP-1 cells primed with PMA overnight, pre-incubated with ibrutinib (60 µM) and followed by stimulation with Nigericin for 1 hour. (**B**) qPCR analysis of mRNA levels before Nigericin addition. A is representative of two experiments and B of three. DMSO control is shown in the grey bar. Means + SDs are shown, and Two-sided Student's *t*-test was used. **P*<0.05. *ns*, not significant. (Liu *et al.*[77])

Caspases are a family of intracellular cysteine proteases that cleave target protein substrates only after aspartic acid residues. Caspases carry a variety of cellular functions. Especially, their essential roles have been intensively suggested in programmed cell death, such as apoptosis, necroptosis and pyroptosis[80]. These forms of cell death are important for inflammatory response to protect an organism from pathogenic attack and stress signals. Moreover, several "pro-inflammatory caspases", including the human caspase-1, -4, and -5 and the murine caspase-1, -11 and -12, participate in the processing and secretion of pro-inflammatory molecules[80,81]. Caspase-1 is the first caspase to be identified. Caspase-1 is present as an inactive zymogen in the cytosol of phagocytic cells. The secretion of proinflammatory cytokines IL-1β and IL-18 caused by NLRP3 inflammasome activation is mediated via cleavage and activation of caspase-1. To confirm the inhibitory role of ibrutinib on IL-1ß secretion, we employed the so-called iGluc THP-1 cell[82], which contains a transgene-encoded biosensor reporting the proteolytic activity of caspase-1 in the course of inflammasome activation. This protease reporter is based on the biological activity of a pro-interleukin (IL)-1β-Gaussia luciferase (iGLuc) fusion construct, in which pro-IL-1β-dependent formation of protein aggregates renders GLuc enzyme inactive. Caspase-1 and IL-1 cleavage leads to monomerization of this biosensor protein, resulting in a strong gain in luciferase activity. Thus IL-1ß release can be quantified in the form of a Gaussia luciferase assay. Gaussia

luciferase activity measurement showed that IL-1ß cleavage in iGLuc THP-1 cells was significantly reduced with the treatment of ibrutinib (Figure 3.5).

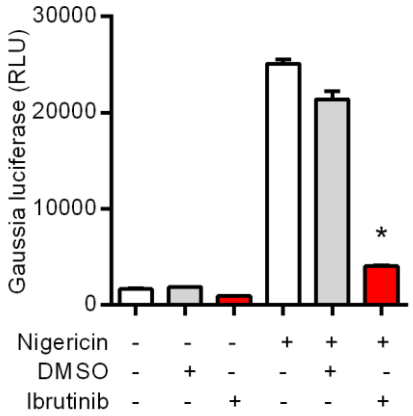


Figure 3.5 Gaussia luciferase assay of IL-1ß from supernatants of PMA differentiated iGLuc THP-1 cells, pre-incubated with ibrutinib (60 µM), and stimulated with Nigericin. Data are representative of three experiments. DMSO control is shown in the grey bar. Means + SDs are shown. Two-sided Student's *t*-test was used. **P*<0.05. (Liu *et al.*[77])

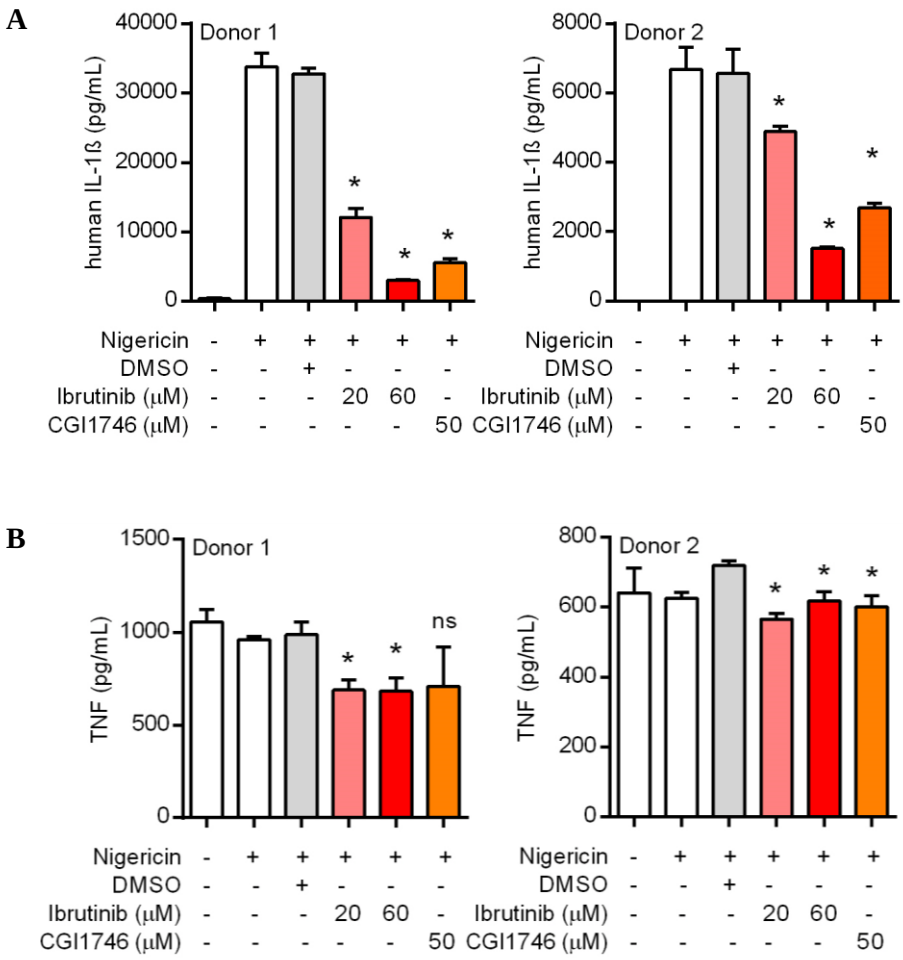
3.2.2 Ibrutinib and CGI inhibit IL-1β release from primary macrophages

The priming step of NLRP3 activation involves the TLR/NF-κB signaling pathway. The activation of TLRs by microbial stimuli, such as LPS which we used to prime macrophages here, leads to production of type I IFNs that are important for antiviral immune responses and secretion of pro-inflammatory cytokines including TNF-α. Thus TNF-α secretion is a critical checkpoint for the priming step of NLRP3. Additionally, we found that pharmacological BTK inhibition strongly decreased IL-1β release in primary MoMacs (Figure 3.6A) whereas the effect on TNF-α release was greatly weaker (Figure 3.6B). Similar results were observed with an additional specific BTK inhibitor, CGI1746 (Figure 3.6A, B).

Then we wondered whether this reduction effect is due to inhibitors-induced cytotoxicity. To address this question here, we employed Cell Counting Kit-8 (CCK8), for quantitation of viable cell number in proliferation and cytotoxicity assays. Importantly, ibrutinib and CGI1746 did not show cytotoxicity at the concentrations used

here as assessed by simultaneous CCK8 viability testing (Figure 3.6C). Therefore, the cytotoxicity effect from these BTK inhibitors can be excluded.

Taken together, given the non- or minor- effects of BTK inhibition on the level of pro-IL-1β or NLRP3 mRNA or secreted TNF-α, these data indicated that the obtained influence related directly to IL-1β maturation of NLRP3 inflammasome activation rather than priming process.



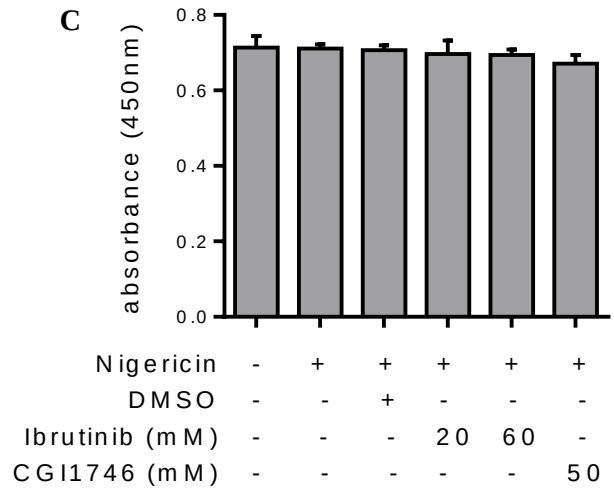


Figure 3.6 ELISA of human IL-1ß (A), TNF-α (B) in supernatants and CCK8 cell viability assay (C) of LPS-primed human primary macrophages that were pretreated with DMSO (mock), ibrutinib (20 µM, 60 µM) or CGI1746 (20 µM) for 10 min and then stimulated with Nigericin for 1 h. In Figure 3.6 (**A**) and (**B**), 2 of 5, and in Figure 3.6 (**C**), 1 of 3 donors is shown. Means +SD are shown and two-sided Student's *t*-tests were used. Comparison to the DMSO control is shown in the grey bar. **P*<0.05. *ns*, not significant. (A) and (B) are from Liu *et al.*[77]

3.2.3 Ibrutinib and CGI inhibit caspase-1 activation and IL-1β secretion from primary macrophages

It is known that upon NLRP3 inflammasome activation, the inactive pro-caspase-1 zymogen is self-activated by proteolytic cleavage into the enzymatically active heterodimer composed of two 10- and 20-kDa subunits. Active caspase-1 is essential for the cleavage of pro-IL-1β and pro-IL-18 into their mature, biologically active forms. Thus, caspase-1 cleavage is a critical step for the NLRP3 inflammasome activation. Therefore, we further investigated the effects of BTK inhibition on inflammasome activity by measuring caspase-1 cleavage and subsequent IL-1 secretion. The proteins in supernatants were precipitated by methanol and chloroform, resolved in NuPAGE loading buffer, and then denatured by boiling. The denatured protein samples from supernatants were loaded on a SDS-PAGE gel and then blotted with anti-caspase-1 (cleaved) and anti-IL-1ß (cleaved) antibodies. Meanwhile, the protein samples from whole cell lysates were blotted with anti-pro-caspase-1, anti-pro-IL-1ß and anti-BTK antibodies. The western blot results showed that both ibrutinib and CGI strongly

reduced caspase-1 cleavage in LPS-primed human primary MoMacs treated with Nigericin. Accordingly, less cleaved IL-1ß was also detected in primary human macrophages pre-treated with ibrutinib and CGI (Figure 3.7).

Collectively, these data indicated that pharmacological inhibition of BTK specifically suppresses NLRP3 inflammasome activation and subsequent IL-1ß secretion.

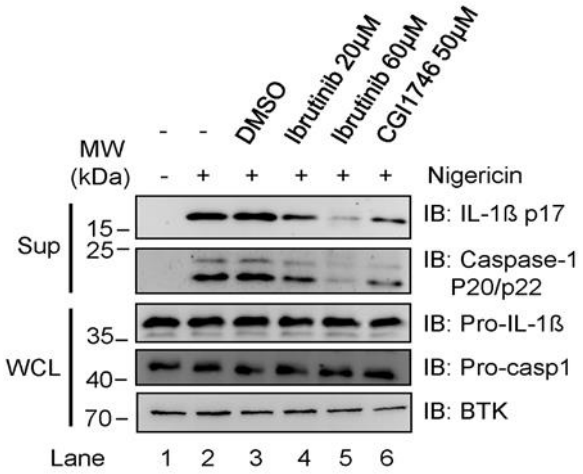


Figure 3.7 Immunoblot analysis for cleaved IL-1ß and caspase-1 in supernatants, pro-IL-1ß, pro-caspase-1, and BTK in whole cell lysates (WCL) from human primary macrophages that were pretreated with DMSO (mock), ibrutinib (20 μM, 60 μM) or CGI1746 (20 μM) for 10 min and then stimulated with Nigericin for 1 h. Data are representative of three experiments. (Liu *et al.*[77])

3.3 Genetic ablation of BTK attenuates NLRP3 inflammasome activation

3.3.1 IL-1β release is reduced in *Btk* knockout bone marrow derived macrophages

As with any pharmacological kinase inhibitor, ibrutinib can produce off-target effects, for example, the possibility of other kinases apart from BTK (LYN, LCK, TEC, etc.) can be affected by ibrutinib[83]. To rule out this off-target effect, we further explored the possibility that knocking out *Btk* would also influence the IL-1ß release from murine bone-marrow derived macrophages (BMDMs). Thus GM-CSF- and M-CSF-differentiated *Btk* KO and WT BMDMs were generated respectively and the effects of BTK deficiency on IL-1ß release upon Nigericin stimulation were detected from those LPS-primed macrophages. Remarkably, IL-1ß release was obviously suppressed in both

GM-CSF- and M-CSF- differentiated *Btk* KO vs. WT BMDMs (Figure 3.8A). Next, we examined the LPS-primed *Il1b* and *Nlrp3* mRNA expression before Nigericin treatment. However, no significant decrease was observed in *Btk* KO macrophages (Figure 3.8B). Additionally, same trend was obtained in xid BTK-deficient BMDMs by my colleague Zsofia Bittner.

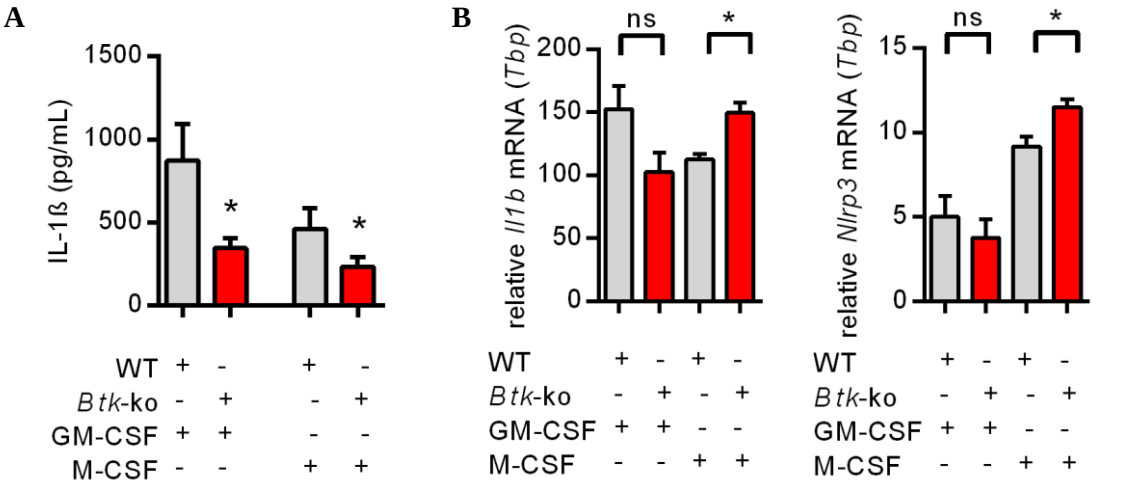


Figure 3.8 (**A**) ELISA of IL-1β in supernatants from equal numbers of GM-CSF or M-CSF-differentiated, LPS-primed and Nigericin treated WT or *Btk* KO BMDMs (mean+SEM). (**B**) RT-qPCR (mean+SEM) prior to Nigericin addition. Pooled data from 3 mice (biological replicates)/group (one out of two identical experiments) are shown. *=p<0.05. Student's *t*-test was used. (Liu *et al.*[77])

3.3.2 IL-1β release is reduced in *BTK* knockdown THP-1 cells

To further investigate the effects of genetic ablation of *BTK* on NLRP3 inflammasome activity in human system, we tested IL-1ß release from THP-1 cells in which BTK was constitutively downregulated by shRNA. Compared to the corresponding mock THP-1 cells, *BTK*-shRNA THP-1 cells showed a reduced IL-1ß secretion in response to NLRP3 inflammasome activating stimuli Nigericin, monosodium urate (MSU) and ATP. Additionally, IL-1ß secretion stimulated by poly(dA:dT) (an AIM2 inflammasome activator) was not influenced by BTK knockdown (Figure 3.9).

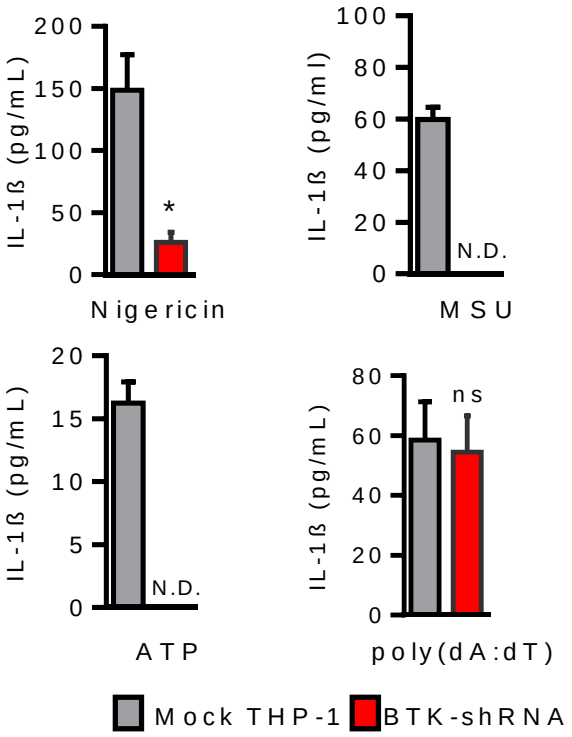


Figure 3.9 ELISA of IL-1β in supernatants from primed THP-1 cells expressing either a non-targeting (Mock) or *BTK*-shRNA. *=p<0.05. Student's *t*-test was used. Data are representative of two experiments. ns, not significant. N.D., not detected. (Liu *et al.*[77])

3.3.3 IL-1β release is down-regulated in XLA PBMCs in response to Nigericin

Previous experimental evidence has proved the effects of genetic BTK ablation on inflammasome activation in the murine system and human cell line, and it would be more interesting to investigate whether these effects occur in human blood samples. As previously reported, X-linked agammaglobulinemia (XLA) results from mutations in the *BTK* gene[64]. Thus we examined PBMCs from XLA patients with genetically and cytometrically confirmed BTK deficiency in comparison with matched healthy donors. AS shown in Figure 3.10, by measuring B cell surface marker CD19, the XLA patients failed to generate mature B cells compare to the healthy donors.

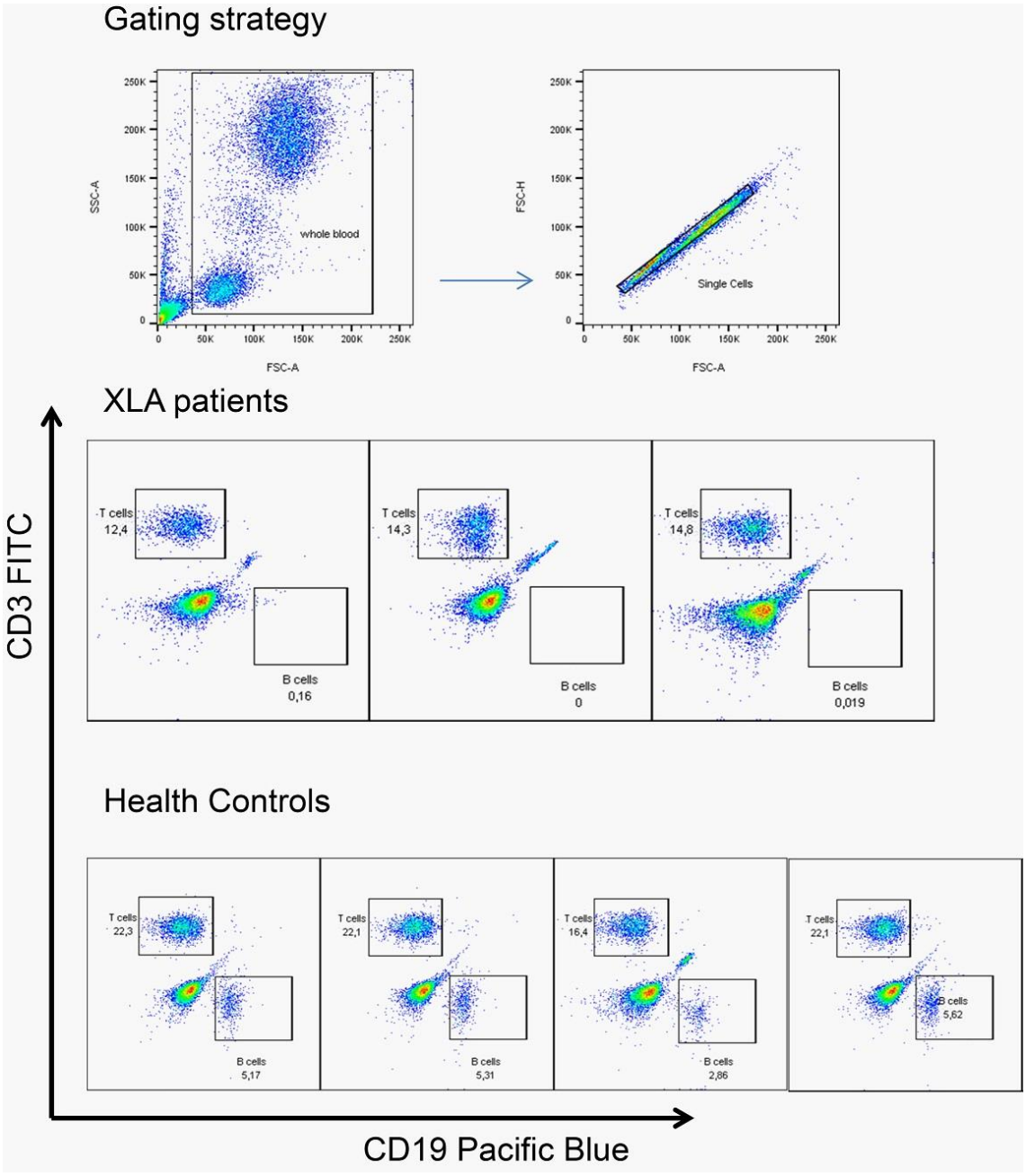


Figure 3.10 Flow cytometry analysis of whole blood from donors with XLA and healthy donors. Before PBMCs purification, whole blood from the 3 donors with XLA and 4 out of 6 healthy donors (shown in Figure 3.11 and 3.12) was stained with anti-CD3-FITC and anti-CD19-Pacific blue monoclonal Abs and analyzed by flow cytometry as indicated. Figure is adapted from Liu *et al.*[77]

To monitor cell viability and cytotoxicity during preparation, PBMCs were treated with PMA plus ionomycin, which triggers various NLRP3-independent cytokines production in T cells. As expected, IL-1ß was poorly produced from this stimulus (Figure 3.11). Since *BTK* is not expressed in T cells[60], PMA plus ionomycin is functionally irrelevant to BTK-dependent effect on NLRP3 inflammasome activity. In addition, no significant difference of IL-2 and IFNγ release from T cells was obtained, thus indicating similar overall cellular viability in PBMC preparations from healthy and XLA donors.

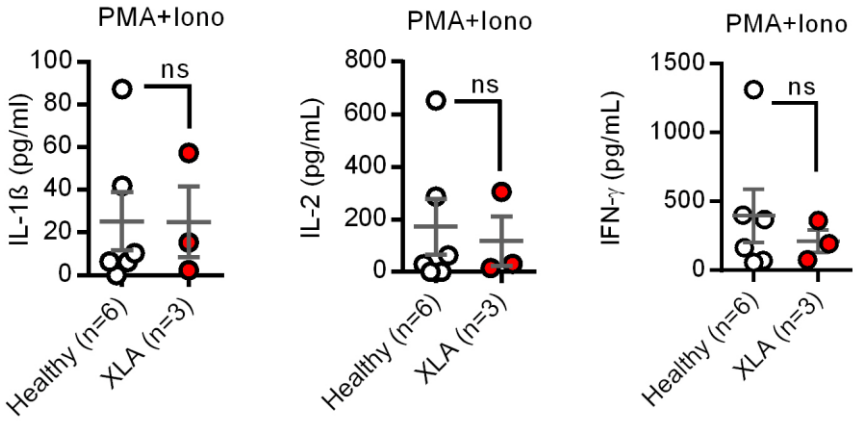


Figure 3.11 ELISA of PMA (50 ng/mL) plus ionomycin (1µmol/L) induced IL-1β, IL-2 and IFNγ release of PBMCs from male XLA patients and age-matched male healthy donors (mean ±SEM of biological replicates, each symbol represents one donor). Pooled data from six vs. three donors (biological replicates; mean+SEM in grey) are shown. *=p<0.05. Mann-Whitney U test was used. ns, not significant. (Liu *et al.*[77])

However, IL-1β release in response to Nigericin was substantially lower from LPS-primed XLA PBMCs compared to those from healthy donors. And BTK deficiency in XLA also decreased Nigericin stimulated IL-1ß secretion in R848-primed PBMCs (Figure 3.12A). Furthermore, when we used ATP as an alternative trigger, although differences in IL-1β levels in response to ATP were not statistically significant due to donor-to-donor variation, a similar trend towards lower IL-1β in XLA PBMCs was clear (Figure 3.12B). However, IL-1ß level from LPS-priming was substantially lower and comparable from XLA PBMCs versus healthy donors.

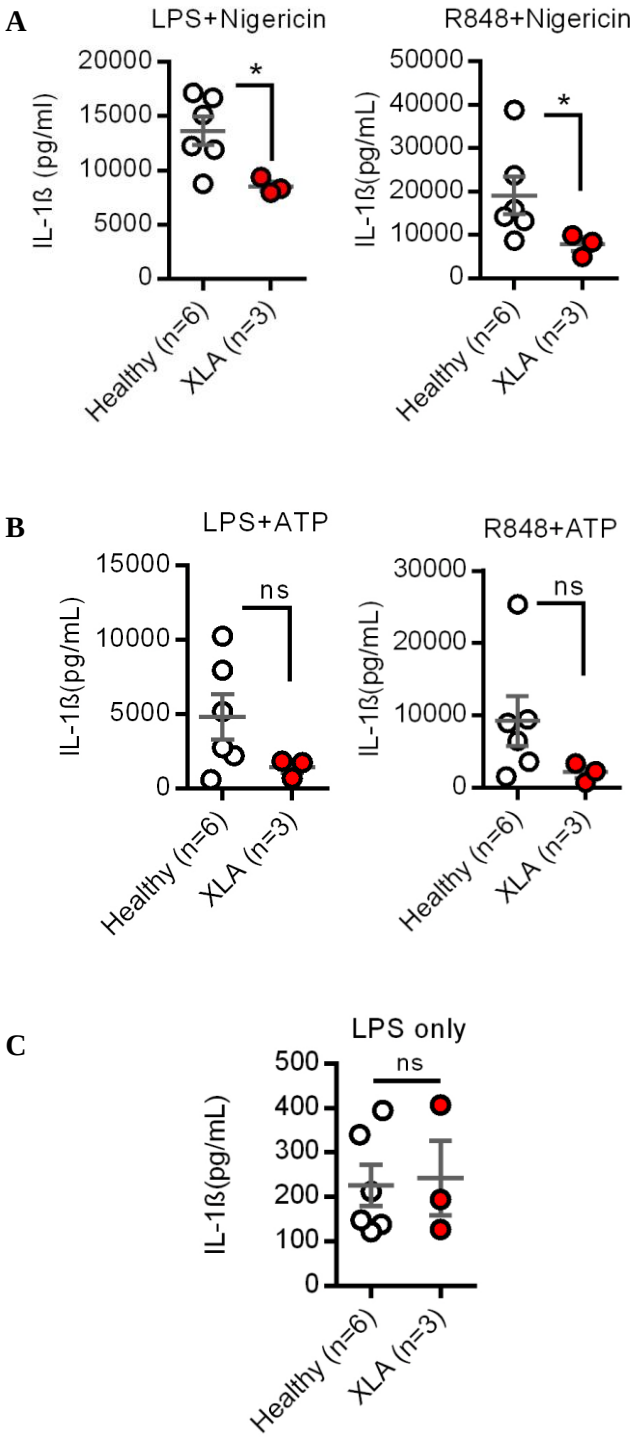


Figure 3.12 (**A, B**) ELISA of IL-1β in supernatants from PBMCs treated with LPS or R848 for 3 hours followed by a second stimuli Nigercin or ATP for 1 hour. (**C**) IL-1β release from only LPS treatment for 3 hours. Pooled data are from 3 male XLA patients and 6 age-matched male healthy donors (mean ±SEM of biological replicates, each symbol represents one donor). *=p<0.05. Mann-Whitney U test was used. ns, not significant. (Liu *et al.*[77])

As expected, western blot analysis showed that secreted mature caspase-1 and IL-1β was also reduced in XLA PBMCs compared to PBMCs from healthy donors (Figure 3.13A), although LPS-primed *IL1B* and *NLRP3* mRNA levels at the time of NLRP3 agonist addition were even higher (Figure 3.13B).

Taken together, these data suggested that genetic disruption of BTK in both mice and humans attenuates NLRP3 inflammasome activation and confirms a role of BTK in the NLRP3 inflammasome.

For whole-cell-lysate immunoblotting, actin, tubulin or GAPDH are normally used as loading control. However, for supernatant-precipitation immunoblotting, there are no other publications on the NLRP3 inflammasome where loading controls for supernatants would have been shown, possibly due to this technical difficulty. Notably, to rule out unequal efficiency of protein precipitation across different supernatants, here we employed recombinant Protein A as a control protein in the supernatant precipitations. Basically, 10 ng recombinant Protein A was added into each supernatant aliquot from the same samples for IL-1 and caspase-1 precipitation. Then these Protein A containing supernatant samples were precipitated and blotted along with endogenously present IL-1 and caspase-1 as control. As shown in Figure 3.13A, precipitation levels were highly comparable. Protein A was also used in Figure 3.20 for the same reason as western blot loading control.

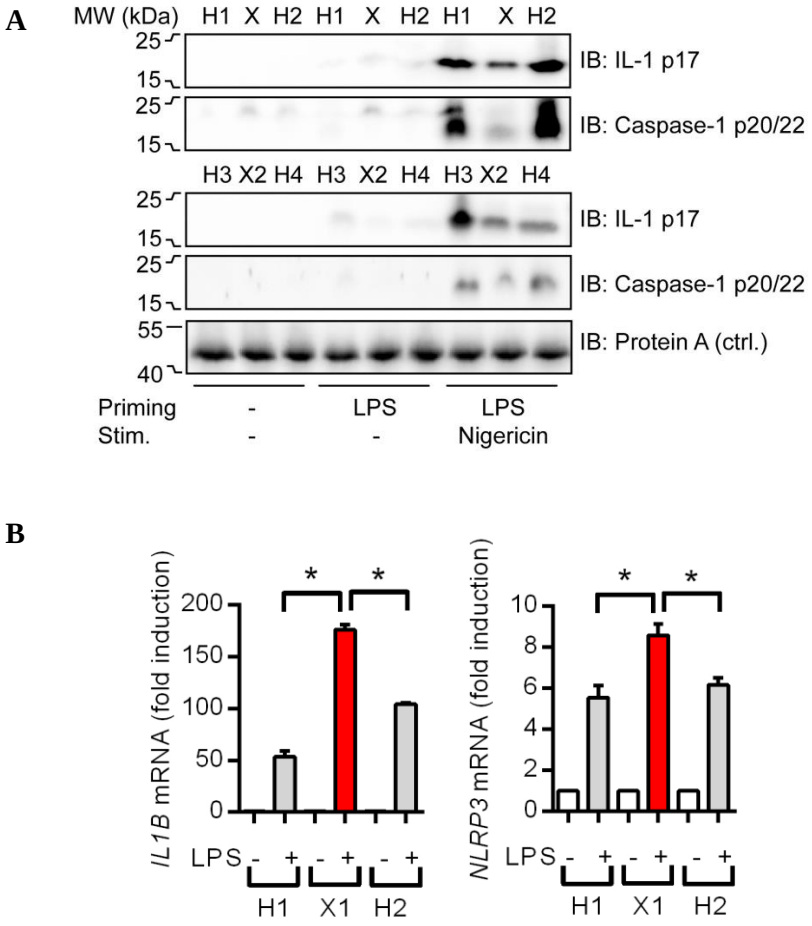


Figure 3.13 Supernatant immunoblot (**A**) or RT-qPCR relative to TBP (mean+SD) (**B**) of the stimulated PBMCs. Two vs. four donors are shown, respectively. Protein A served as blotting control. In (**B**), Student's *t*-test was used. *=p<0.05. (Liu *et al.*[77])

3.4 BTK interacts with ASC and NLRP3 and promotes inflammasome formation

3.4.1 BTK physically interacts with both ASC and NLRP3 in Co-IP

Previous study has demonstrated that BTK plays a critical role in NLRP3 activity and the subsequent caspase-1 cleavage and IL-1ß secretion. We wondered how BTK promotes NLRP3 inflammasome activation. Therefore we sought to determine if these two proteins interact with one another or BTK might also bind to other inflammasome components when co-expressed exogenously in HEK293T cells. Indeed, as we expected, co-immunoprecipitation (CoIP) assay showed that BTK could directly interact with NLRP3 and also ASC (Figure 3.14).

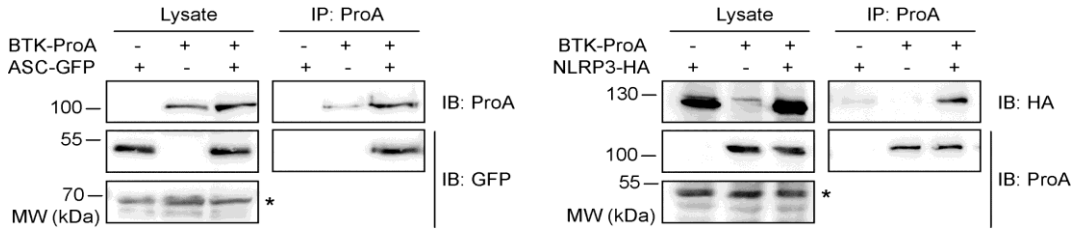


Figure 3.14 Co-immunoprecipitation of HEK293T cells transfected with BTK-Protein A and ASC-GFP or NLRP3-HA. * = non-specific loading control. Data are representative of two identical experiments. (Liu *et al.*[77])

3.4.2 BTK enhances ASC speck formation and ibrutinib reduces speck formation

Caspase-1 activation in response to NLRP3 trigger requires the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain. Additionally, as previously reported, after inflammasome activation, ASC assembles into a large protein complex, which is termed "speck". ASC specks can be observed as they reach a size of around 1 μm and in most cells only one speck forms upon inflammasome activation. Thus ASC speck formation can be used as a visual readout for inflammasome activation[84]. Notably, co-expression of BTK significantly enhanced the over-expression induced speck formation in HEK293T cells transfected with ASC-GFP (Figure 3.15A, quantified in Figure 3.15B).

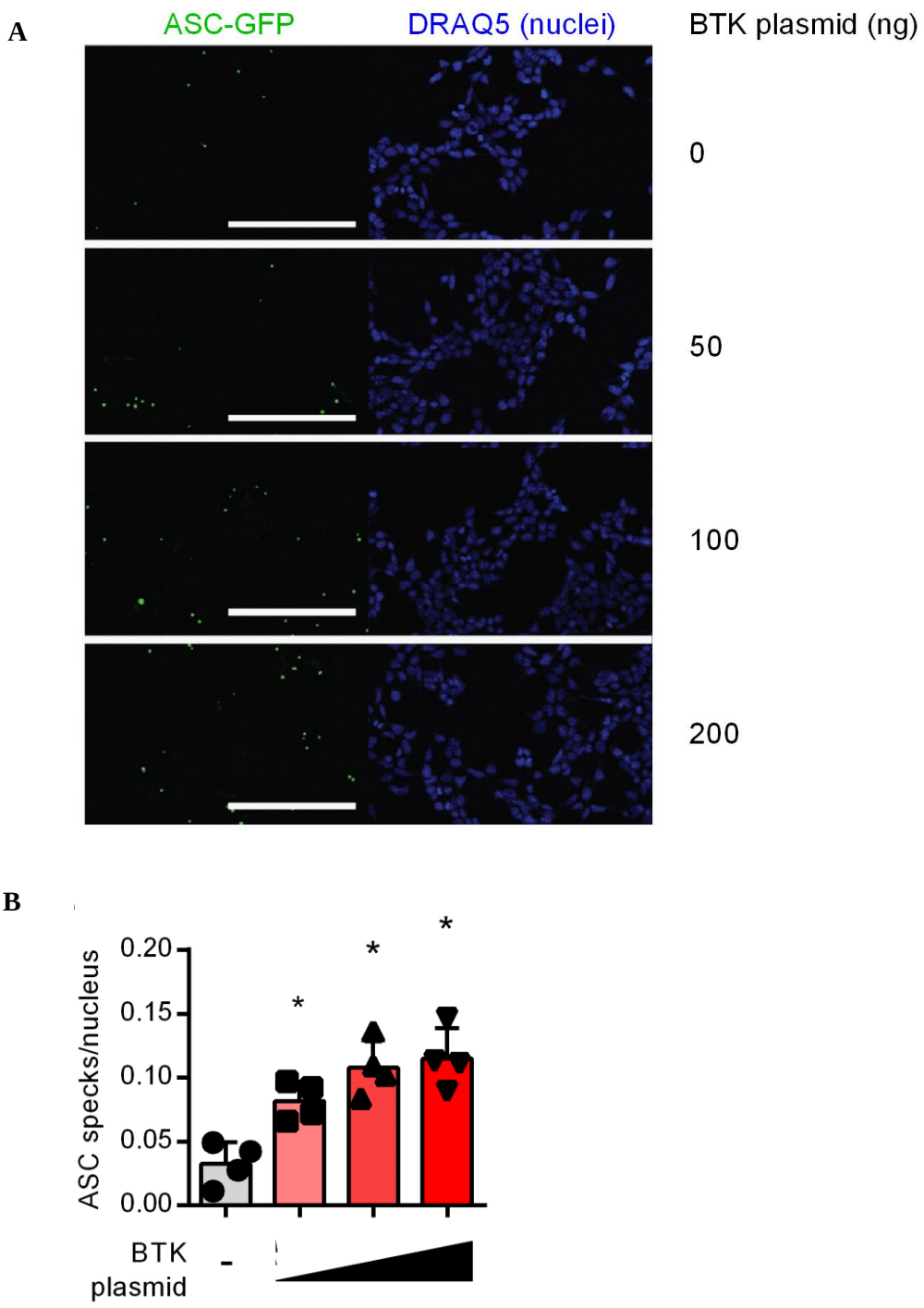
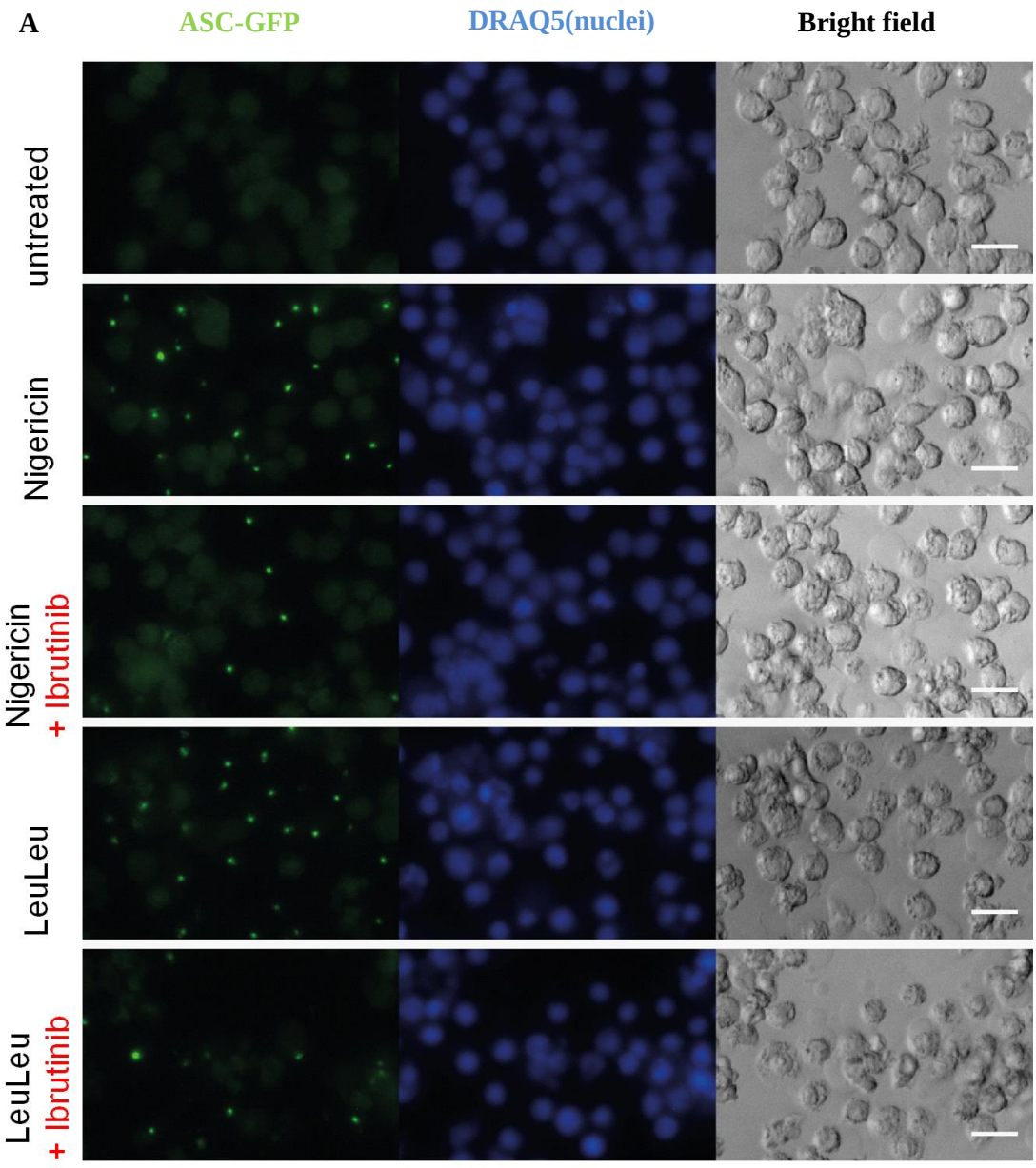


Figure 3.15 (**A**) Confocal microscopy of ASC-GFP specks in fixed and stained transfected HEK293T cells with various dose of BTK plasmids (Scale bar = 200 μm). (**B**) is the quantification of (**A**). In (**B**), Student's *t*-test was used. *=p<0.05. Data are representative of two identical experiments. (Liu *et al.*[77])

Consistent with previous experiments, Fluorescence microscopy images showed that ASC speck formation, the number but not the size of ASC specks, in stably ASC-mCerulean expressing murine macrophages was substantially attenuated by BTK inhibitor pretreatment when these cells were stimulated with Nigericin or another NLRP3 trigger, Leu-Leu-OMe, a lysosomal destabilizing agent (Figure 3.16A, quantified in Figure 3.16B).



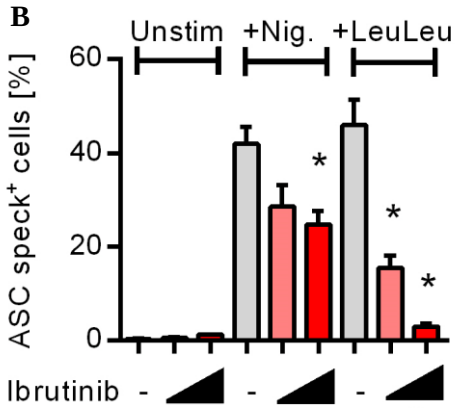


Figure 3.16 (**A**) Representative fluorescence microscopy images of ASC specks with or without pretreatment of ibrutinib from immortalized *Nlrp3* KO macrophages overexpressing NLRP3-FLAG ASC-mCerulean. Scale bar = 20 μm. (**B**) is the quantification of (**A**). In (**B**), Student's *t*-test was used. *=p<0.05. Data are representative of three experiments. (**B**) shows the combined analysis of 3 experiments. (Liu *et al.*[77])

Furthermore, in stably expressing ASC-mCerulean THP-1 cells, Nigericin stimulation enhanced NLRP3-mediated ASC cross-linking/oligomerization, whereas BTK inhibitor suppressed ASC oligomerization (Figure 3.17).

Collectively, these data indicated that BTK directly influenced inflammasome activation at the level of NLRP3 and ASC.

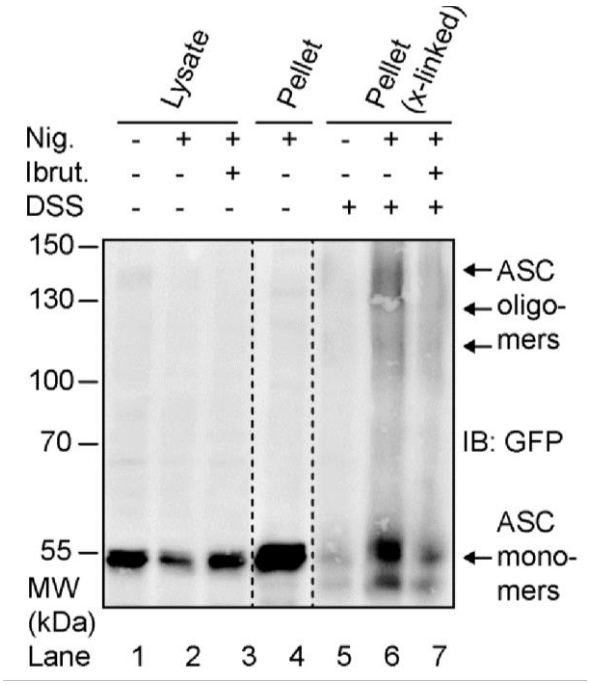


Figure 3.17 DSS cross-linking of ASC from cell lysates of LPS-primed THP-1-ASC-mCerulean cells that were pretreated with ibrutinib for 1 hour and then Nigericin-treated for 1 hour. Data are representative of three experiments. (Liu *et al.*[77])

3.5 BTK inhibition blocks IL-1β release in Muckle-Wells-Syndrome and ibrutinib-treated cancer patients *ex vivo*.

3.5.1 Caspase-1 and IL-1β release from MWS patient PBMCs are reduced by ibrutinib

Our results so far posed the question whether BTK may rather be a plausible point of therapeutic intervention to target the many human inflammasome/IL-1β-related inflammatory processes or disorders. As described before, Muckle-Wells Syndrome is an autoinflammatory disease caused by gain-of-function mutations in the NLRP3 gene and is characterized by excessive and constitutive IL-1β release compared to healthy donors. We next aimed to determine whether this NLRP3-associated inflammation could be ameliorated by BTK inhibition. We triggered IL-1β release by LPS (no second stimulus required due to NLRP3 auto-activation)[85] in PBMCs from four MWS patients with or without ibrutinib treatment. As shown here, LPS-dependent IL-1β (Figure 3.18A) and caspase-1 (Figure 3.18B) release was strongly reduced by BTK inhibitor for all assessed patients, and the level of inhibition corresponded to the dose of ibrutinib

(Figure 3.18C). Thus, pharmacological BTK inhibition blocked excessive IL-1β release that characterizes the autoinflammatory MWS.

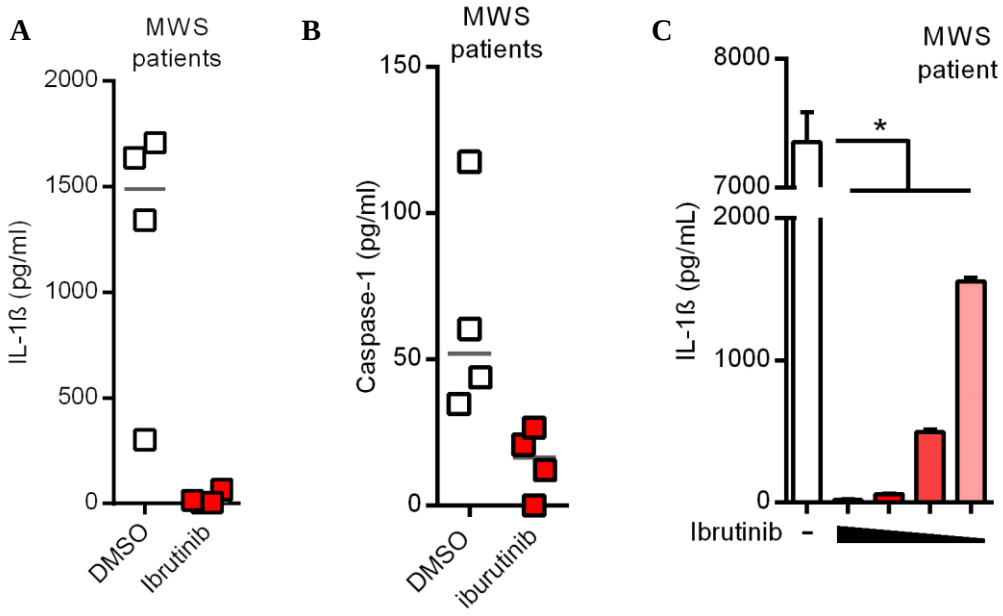


Figure 3.18 (**A**) and (**B**) ELISA of LPS-induced IL-1β or caspase-1 release from LPS-stimulated PBMCs of 4 MWS patient. (**C**) ELISA of IL-1ß secretion with the titration of ibrutinib from 60, to 30, to 15, to 7.5 µM for two MWS patients; one patient is shown (mean +SD). In (**A**) and (**B**), the Wilcoxon matched-pairs signed-rank test was used. In (**B**), Student's *t*-test was used. *=p<0.05. (Liu *et al.*[77])

3.5.2 PBMCs from cancer patients with ibrutinib therapy show impaired IL-1β processing and release *ex vivo*

Ibrutinib (PCI-32765), as a potent covalent inhibitor of BTK, has recently been approved by the Food and Drug Administration (FDA) for the treatment of chronic lymphocytic leukemia and mantle cell lymphoma. NLRP3-mediated caspase-1 and IL-1β release was shown to be suppressed by BTK inhibitors. We also confirmed that BTK knockout mice were functionally deficient in inflammasome activation. To address whether application of ibrutinib in human patients would affect inflammasome activity *in vivo*, we stimulated PBMCs from male cancer patients receiving ibrutinib daily *ex vivo* and compared their ability to process (caspase-1 activity) or release IL-1β in response to NLRP3 triggers Nigericin and/or ATP by ELISA or immunoblot,

respectively. We found that *in vivo* application of ibrutinib specifically correlated with lower IL-1ß release in these patients compared with subjects not receiving this drug administration (Figure 3.19A). In contrast, TNF-α secretion was not influenced by ibrutinib application (Figure 3.19B).

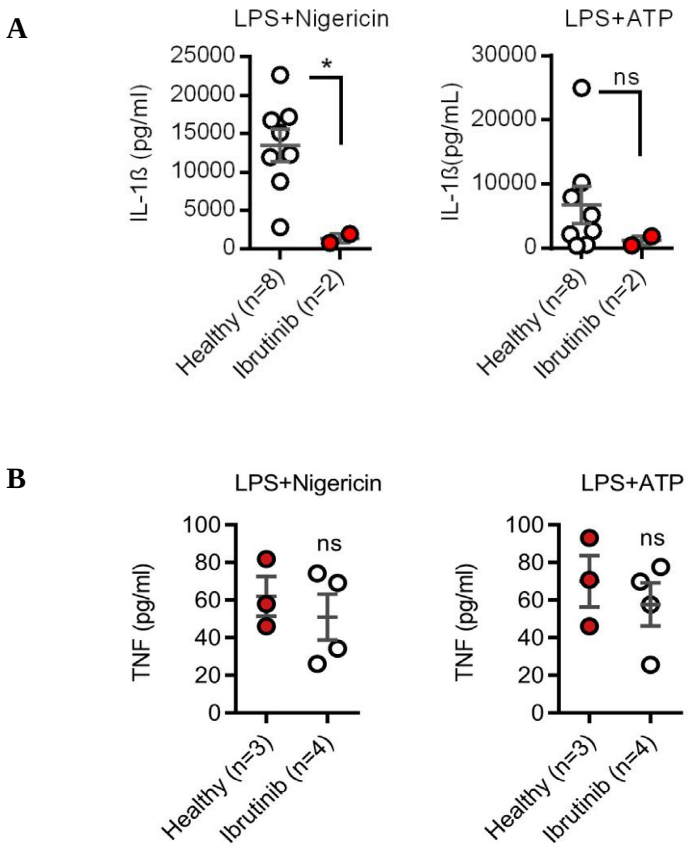


Figure 3.19 ELISA of IL-1ß (**A**) secretion, TNF-α (**B**) release (mean ±SEM of biological replicates, each symbol represents one donor) from PBMCs of male cancer patients daily receiving ibrutinib and male healthy donors. Pooled data from eight vs. two donors are shown in (**A**) and from three vs. four donors in (**B**). In (**A**) and (**B**) a Mann-Whitney U test was used, *=p<0.05. (Liu *et al.*[77])

In addition, we performed protein precipitation from the supernatants and the protein samples were subjected to western blotting detection for cleaved IL-1ß and caspase-1. Same as Figure 3.13A, recombinant Protein A here served as precipitation and loading control. Western blot results showed that *in vivo* application of ibrutinib specifically alleviated caspase-1 activation and IL-1β secretion *ex vivo* in these patients (Figure

3.20). Taken together, our data therefore suggest that BTK inhibitor may reduce the NLRP3 inflammasome activity *in vivo* in patients.

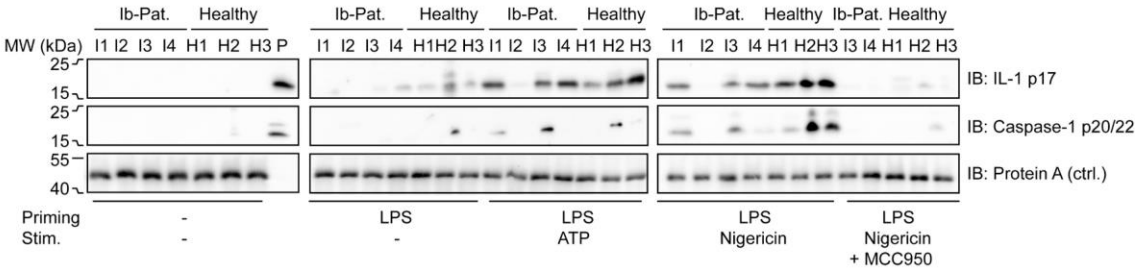


Figure 3.20 Supernatants immunoblot analysis of caspase-1 and IL-1ß cleavage from PBMCs of male cancer patients daily receiving ibrutinib and male healthy donors. Protein A served as blotting control. Pooled data from four vs. three donors are shown. (Liu *et al.*[77])

3.6 BTK is essential for full *S. aureus* toxin-induced inflammasome activity

3.6.1 LukAB and PVL trigger NLRP3-dependent IL-1ß release in a dose-dependent manner

The most common presentation of BTK deficient patients with XLA is increased susceptibility to encapsulated pyogenic bacteria. Skin infections in patients with XLA are mostly caused by *Staphylococcus aureus* and group A streptococci, and they can present as impetigo, cellulitis, abscesses, or furuncles[86]. In addition, it has been reported that the *Staphylococcus aureus* pore-forming toxin Leukocidin A/B (LukAB)[87] and Panton-Valentine leucocidin (PVL)[88] induce inflammatory responses in human phagocytes via the NLRP3 inflammasome. Given that IL-1ß processing and secretion were significantly reduced by BTK deficiency in response to Nigericin in immune cells from XLA patients, we wondered whether BTK plays a role in immune response against LukAB and PVL via NLRP3. To address this question, for a start, we detected NLRP3 activation by measuring IL-1ß release in THP-1 and primary human macrophages upon LukAB and PVL stimulation. NLRP3-deficient and null (control) THP-1 cells were primed with PMA overnight and stimulated with the indicated amounts of LukAB for 60 min. Nigericin here served as positive control. Human

monocytes derived macrophages were primed with 300 ng/mL LPS for 3 hours and then stimulated with the indicated amounts of LukAB, PVL, Nigericin for 60 min. We found that both LukAB and PVL trigger NLRP3-dependent IL-1ß release in a dose dependent manner (Figure 3.21).

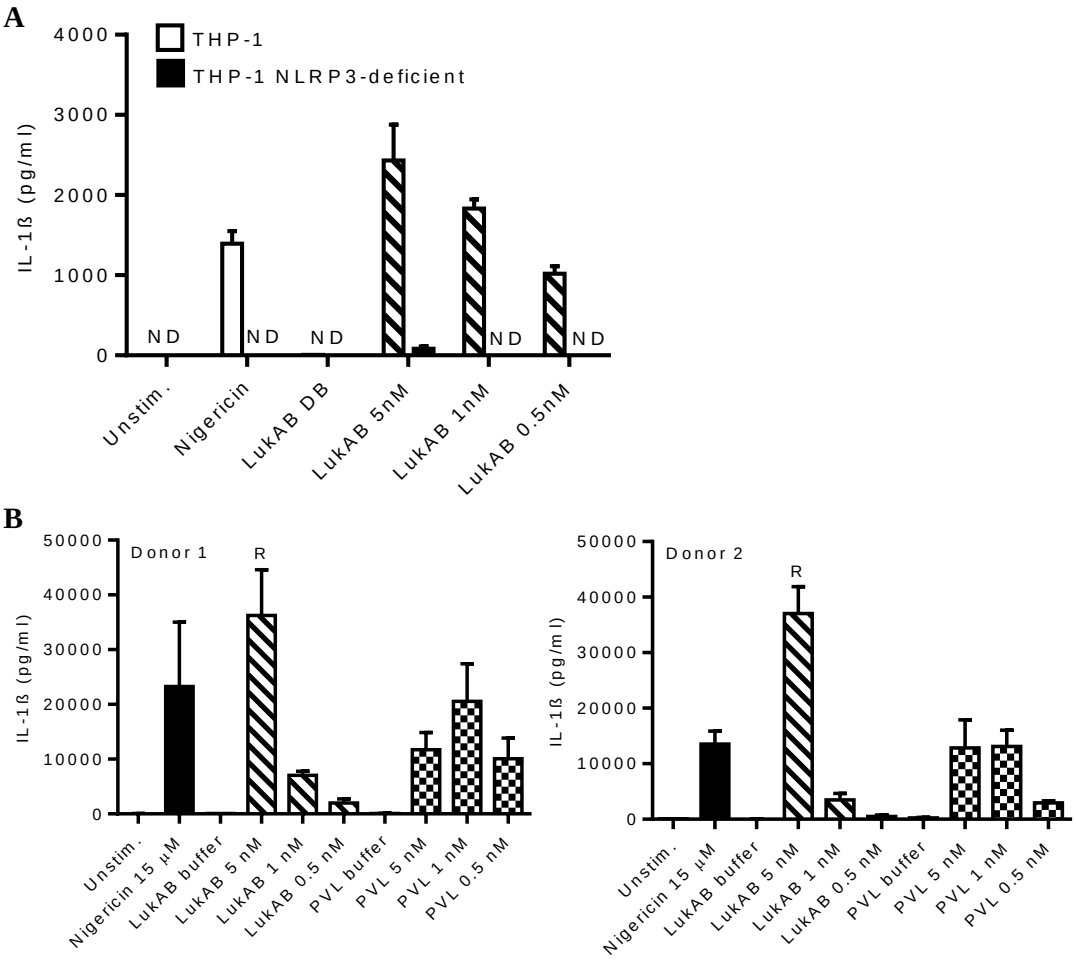


Figure 3.21 ELISA of IL-1β release in supernatants from (A) PMA-differentiated null and NLRP3-deficient THP-1 cells, and (B) LPS-primed human MoMacs from two donors, with indicated stimulation. DB, LukAB dialysis buffer. Means +SD are shown. R, out of range. Data are representative of two experiments. (Liu *et al.*[77])

3.6.2 Ibrutinib treatment and *BTK* knock-down decrease IL-1β release in response to LukAB and PVL

Given that LukAB and PVL activate NLRP3 inflammasome, we went on to investigate the effects of pharmacological inhibition of BTK and genetic *BTK* knock-down on NLRP3 inflammasome activation. Results obtained from PMA-primed THP-1

macrophages that were pretreated with ibrutinib or CGI1746 and then stimulated with LukAB showed that BTK inhibitors strongly reduced IL-1ß release in response to LukAB (Figure 3.22A). Additionally, shRNA-mediated *BTK* knockdown THP-1 cells also showed substantially decreased IL-1ß secretion in response to LukAB compared with mock THP-1 cells harboring a non-targeting shRNA (Figure 3.22B). Furthermore, in response to both LukAB and PVL, IL-1ß levels of the human primary macrophages pretreated with ibrutinib were largely lower than that of DMSO control (Figure 3.22C).

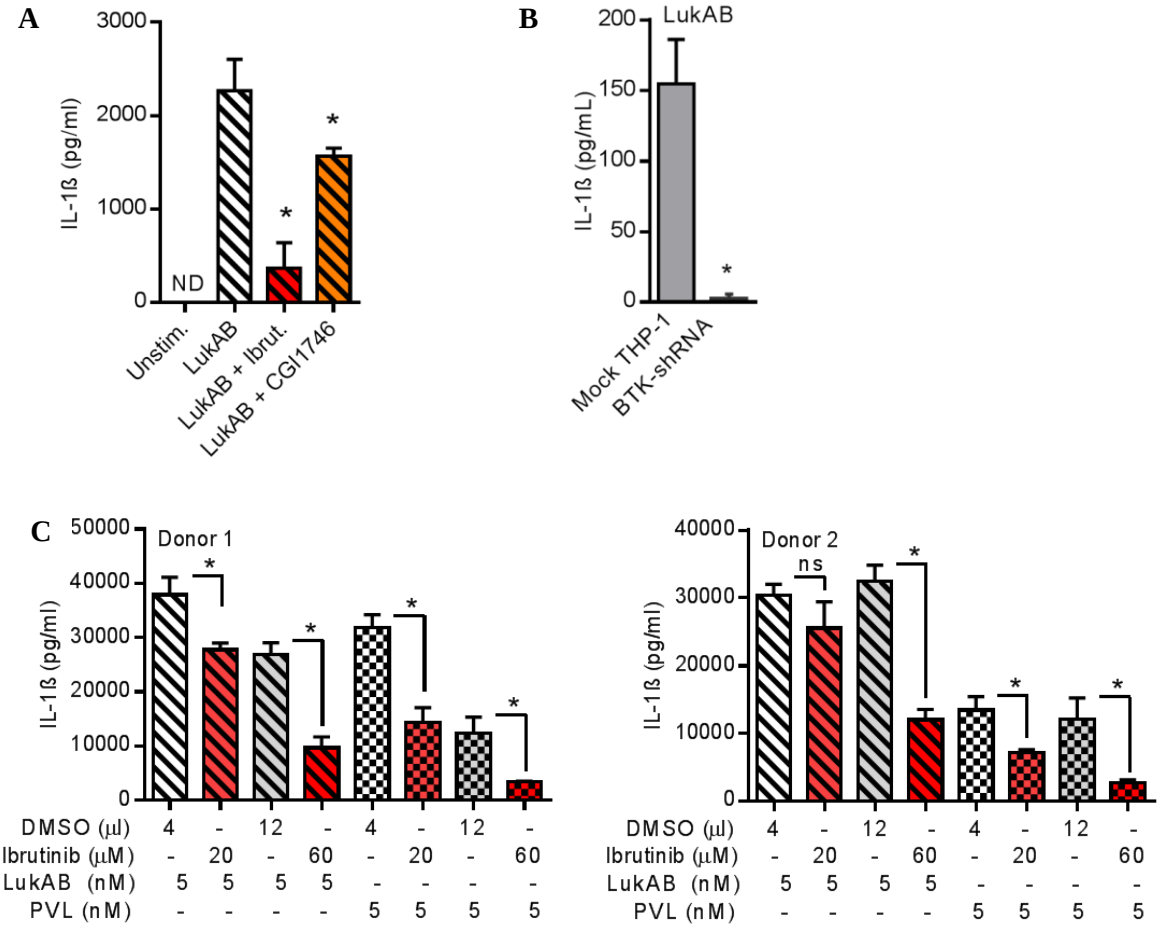


Figure 3.22 ELISA of IL-1β release in supernatants from **(A)** THP-1 cells pretreated with ibrutinib or CGI1746 for 10 min, followed with 5nM LukAB stimulation for 1 h, **(B)** PMA primed BTK-shRNA and mock THP-1 cells stimulated with 5nM LukAB for 1 h, and **(C)** LPS-primed human MoMacs from two donors, pretreated with ibrutinib and then stimulated with 5nM LukAB or PVL for 1 h. Means +SD are shown. Student's *t*-test was used. *=p<0.05. *ND*, not detectable; *ns*, not significant. In **(A)** and **(B)**, Data are representative of two experiments. **(C)**, 2 of 2 identical experiments are shown. (Liu *et al.*[77])

3.6.3 BTK deficiency in XLA PBMCs leads to reduced caspase-1 activity and IL-1β cleavage in response to LukAB

Our next aim was to investigate the how BTK deficiency influence NLRP3 inflammasome activity in response to the *Staphylococcus aureus* toxin LukAB. Thus, LPS primed PBMCs from XLA patients and age-matched healthy donors were stimulated with LukAB. Caspase-1 and IL-1ß cleavage were measured using supernatant immunoblot. Previously, LPS was used for priming PBMCs by activating TLR4. However, *Staphylococcus aureus* is a gram-positive bacterium which does not contain LPS. Instead, *Staphylococcus aureus* activates TLR2[89]. Thus, here the TLR2 agonist Pam3CSK4 was also used for priming. We found that upon the stimulation of LukAB, with either LPS-priming or Pam3CSK4-priming, both caspase-1 cleavage and IL-1ß secretion were highly reduced in PBMCs from XLA patients compared to those from healthy donors (Figure 3.23).

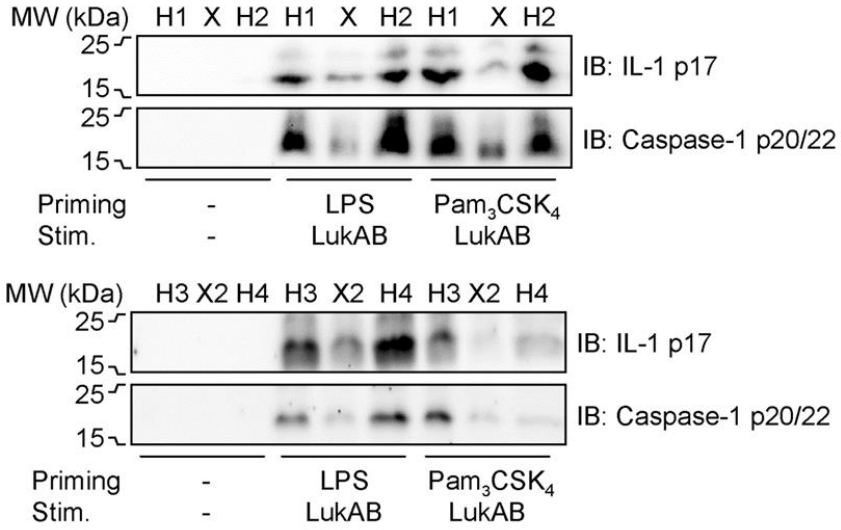


Figure 3.23 Supernatant immunoblot of PBMCs from male patients with XLA and age-matched male healthy donors, primed with LPS (10 ng/mL) or Pam3CSK4 (1µg/mL) for 3 h, and then stimulated with LukAB (5nM) for 1 h. 2 of 2 identical experimentas are shown. X, X2: XLA patient number 1, 2. H1, H2, H3, H4: healthy donor number 1, 2, 3, and 4. (Liu *et al.*[77])

Collectively, both pharmacological inhibition and genetic deficiency of BTK altered NLRP3 inflammasome activation in response to LukAB. These data indicate that BTK might be required for the NLRP3 inflammasome/IL-1ß mediated host immune response.

4. Discussion

The NLRP3 inflammasome is an essential component of innate immunity and contributor to the pathology of several human diseases. However, dysregulated activation of the NLRP3 inflammasome and subsequent IL-1ß secretion has been implicated in the pathogenesis of inherited and acquired inflammatory diseases. BTK is a member of the Tec family of non-receptor TKs that is structurally related to SYK. The well-known role of BTK is its essential involvement in B cell development and function. BTK also appears to be important for myeloid cells[60]. However, the role of BTK in innate immunity is only emerging[60]. This study describes a crucial novel role for BTK in a key process of innate immunity namely NLRP3 inflammasome activation. This is elucidated by providing the experimental evidence that both pharmacological inhibition and genetic ablation of BTK impaired NLRP3 inflammasome activation in primary human macrophages; IL-1ß processing and release were reduced in *Btk* knockout BMDMs from mice and *BTK*- deficient cells from XLA patients; BTK directly bound to NLRP3 and ASC; BTK inhibition strongly reduced caspase-1 and IL-1ß release in PBMCs from MWS patients; decreased IL-1ß processing and release was also observed in PBMCs from patients receiving ibrutinib *in vivo*. These results provide a new understanding of the function of BTK and provide a compelling rationale for targeting BTK to treat inflammatory diseases linked to NLRP3 inflammasome activation.

In this chapter, I will mainly discuss (i) the BTK function at a molecular level, including a comparison with previous findings; the relationship between K^+ efflux and BTK; the potential role of BTK phosphorylation; the kinase activity and scaffolding function of BTK; the effect of SYK on NLRP3 inflammasome activation, (ii) the relationship between BTK and other reported direct NLRP3 regulators, (iii) the role of BTK in cell types other than macrophages, (iv) therapeutic implications.

4.1 Molecular role of BTK

4.1.1 Additional information on BTK and the NLRP3 inflammasome

An independent study published by Ito *et al.*[54] in 2015 showed that BTK is an essential component of the NLRP3 inflammasome and contributes to ischaemic brain injury. Similar to our results, they found that BTK interacted with ASC and NLRP3. Inhibition of BTK by pharmacological or genetic means severely impaired NLRP3 inflammasome activity. Additional to our data, they demonstrated that ibrutinib suppressed infarct volume growth and neurological damage in the ischaemic brain *in vivo*. They also proved that Ca^{2+} was not involved in the BTK-dependent activation of NLRP3 inflammasome. The results were mainly observed in the murine system. Notably, our results expand the above findings by focusing on the human system including using human primary macrophages, blood samples from XLA patients, MWS patients, ibrutinib-treated cancer patients. Moreover, we identify BTK as a critical regulator modulating the NLRP3 inflammasome activation via an unbiased proteomics screen, rather than Ito´s screen of several pharmacological signal inhibitors. Furthermore, we use an additional inhibitor of BTK, namely CGI1746, in human primary macrophages, and also blood samples from XLA, which could be a potential inflammasomopathy. Thus, our study is complementary to Ito *et al.´*s approach despite of limited amount of overlap which should be noted positively regarding scientific reproduction.

4.1.2 Is BTK downstream of K^+ efflux?

Although the role of Ca^{2+} signaling, mitochondrial dysfunction and lysosomal leakage in NLRP3 inflammasome activation remains still unclear, an agreement has been reached that K^+ efflux is a common step in the activation of the NLRP3 inflammasome[41]. Most or all NLRP3 activators, including Nigericin, ATP, and particular matter induce NLRP3 inflammasome assembly through K^+ efflux. Therefore, some obvious questions are: Do the BTK phosphorylation and BTK-NLRP3 interaction require K^+ efflux? Will K^+ efflux affect BTK-associated ASC speck formation? To address these questions, one could check the influence of e.g. BTK-NLRP3 interaction in the presence of a high extracellular concentration of K^{+90}. If the BTK-NLRP3 interaction was abolished, then K^+ efflux would be required for this binding. It is possible that a decrease in

intracellular K^+ causes conformational changes in NLRP3 that allow the binding of BTK to NLRP3. To assess this hypothesis, one approach would be to investigate whether a mutation in NLRP3 (e.g. gain-of-function mutation $NLRP3^{R260W}$)[91] that allows K^+ efflux-independent activation still requires BTK for inflammasome activation.

4.1.3 The role of phosphorylation site Tyr223 (site 2) of BTK

Through K^+ efflux or not, our data revealed that BTK was phosphorylated upon NLRP3 inflammasome activation. However, a critical, but unresolved, feature of BTK activation is the pattern of phosphorylation of individual BTK molecules after NLRP3 stimulation, i.e., are BTK molecules phosphorylated on both regulatory tyrosine residues or only one? Our study presented a rapid and robust phosphorylation of Tyr551 (site 1) in the kinase domain of BTK, but not Tyr223 (site 2) in the SH3 domain. It is possible that Tyr223 auto-phosphorylation is not required for the regulation of inflammasome activation upon NLRP3 triggers in macrophages. Over a long period, it has been assumed that, upon BCR stimulation, phosphorylation of Y551 can lead to autophosphorylation at Y223 and the double sites phosphorylation are critical for BTK activity. However, according to a recent study, in contrast to site 1, site 2 phosphorylation has little discernible influence on BTK catalytic activity[92]. And other study found that Y223 auto-phosphorylation was not required for the regulation of differentiation of pre-B cells or peripheral B cells *in vivo*[93]. It is also possible that Tyr223 phosphorylation occurred later than 10 min Nigerin stimulation, or even earlier during the priming step, and was therefore not detected here. Ito *et al*[54] observed PMA treatment activated BTK by phosphorylating its Tyr223, inducing BTK to interact with both ASC and NLRP3. Therefore, the role of Y223 phosphorylation for BTK function in NLRP3 inflammasome activation in the human system remains to be further investigated. Nevertheless, future studies aiming to identify the phosphorylation pattern of individual BTK molecules in NLRP3 inflammasome signaling will clarify how BTK is activated and how it functions. For example, reconstituted mutants Y223F and Y551F (non-phosphorylatable) can be transfected into THP-1 cells. Then IL-1ß release and ASC speck formation should be assessed in comparison with those from wild type BTK and empty vector transfect THP-1 cells.

4.1.4 Does the kinase activity or scaffolding function of BTK contribute to inflammasome activiy? or both?

Although Tyr223 showed only subtle phosphorylation in response to Nigericin in our results, the observed robust and rapid phosphorylation of Tyr551 encouraged us to make use of the pharmacological BTK inhibitors. It is interesting that inhibition of BTK even works to prevent the inflammasome activation due to the stimulus-independent NLRP3-activating mutation found in MWS patients. If BTK is upstream of NLRP3 and only the kinase activity is required, ibrutinib should not alter the inflammasome activity since MWS patients carry the gain-of-function mutation which leads to NLRP3 auto-activation[45]. This suggests that BTK is not exclusively upstream of NLRP3, and may have a scaffolding role in the NLRP3 inflammasome. That means BTK partially functions as a platform protein or acts as an adapter molecule for the interaction between NLRP3 and ASC, independent of its catalytic activity. Actually, our results have shown the direct interaction of BTK-ASC and BTK-NLRP3. It has been proposed by Ito *et al*[54] that activated BTK physically interacts with ASC probably due to signal 1, then signal 2 induces recruitment of NLRP3 to this BTK-ASC complex, further inducing ASC oligomerization. However, because ibrutinib inhibits BTK phosphorylation and thus its enzymatic activity, it is likely that the kinase activity of BTK is involved in the regulation of NLRP3 inflammasome. Indeed, our results showed ibrutinib suppressed ASC oligomerization. Then its substrate could be NLRP3 or ASC who is a direct interactor of BTK. Further studies could focus on investigating the substrate of BTK in the NLRP3 inflammasome signaling. Whole cell lysates from primed THP-1 stimulated with Nigericin in the presence or absence of BTK inhibitors could be subjected to immunoblot analysis using anti-phospho-tyrosine antibodies. The missing bands in inhibitor-treated lysates could be substrate proteins whose phosphorylation is suppressed by the BTK inhibitor. The protein in these bands could be identified by mass spectrometry. In conclusion, our data suggest that BTK might regulate NLRP3 inflammasome activation by at least two mechanisms: enhancing inflammasome signaling directly by kinase activity and serving as a platform protein to recruit the core components of NLRP3 which allows inflammasome assembly. To test this hypothesis, constructs harboring various types of mutations in BTK, for example, mutation targeting the kinase domain, truncated constructs, and BTK-shRNA could be

expressed in THP-1 or primary macrophages by lentivirus-mediated transduction. NLRP3 inflammasome activation could then be analyzed by measuring IL-1ß processing and release, ASC speck formation, and caspase-1 activity. If the scaffold function of BTK is required, NLRP3 inflammasome activation would be largely impaired in BTK-shRNA cells compared to WT, whereas this reduction-effect would be much less in kinase-inactive cells. Then the PH domain, SH2, or SH3 domain truncated reconstitution can be further assayed to explore which is critical for the scaffolding role.

4.1.5 The role of SYK in inflammasome activation?

Since SYK is an upstream regulator of BTK in B cell receptor signaling, we wondered whether SYK played a role in NLRP3 inflammasome activity. Our preliminary data observed from THP-1 macrophages showed that, without causing cell death, inhibition of SYK, attenuated NLRP3 inflammasome activity monitored by IL-1ß release at the concentration of 5µM and 10µM, suggesting that SYK and BTK exhibit overlapping functions on regulating inflammasome activation (Figure 5.1). In line with our observation, Ito *et al*[54] also showed the SYK inhibitor R406 reduced Alum-induced IL-1ß secretion in murine peritoneal macrophages. It is also shown that SYK kinase signaling couples to the NLRP3 inflammasome for anti-fungal host defense[94]. One explanation is SYK may influence the priming step in terms of pro-IL-1ß accumulation and NLRP3 expression. But this possibility can be excluded by our experimental design since inhibitor treatment was fairly short (1 hour) comparing to the priming period (16 hours), and also can be excluded by Ito´s experimental evidence showing no pro-IL-1ß difference with R406 treatment versus DMSO. Second, based on our results and those previous reports, it is unlikely that SYK directly promotes NLRP3 oligomerization by binding to NLRP3 or phosphorylating ASC. It is more likely that K^+ efflux leads to downstream sequential activation SYK and BTK, and SYK alters inflammasome activity though BTK. Further experiments are necessary to decipher this puzzle. Binding assay for SYK-NLRP3 or SYK-ASC would be an interesting beginning point.

4.2 BTK and other reported regulators

Collectively, several reported NLRP3 regulators including NIMA-related kinase 7 (NEK7), double-stranded RNA-dependent protein kinase (PKR), guanylate-binding protein 5 (GBP5), tripartite motif-containing protein 31 (TRIM31) could positively or negatively regulate NLRP3 inflammasome activation at different levels and via different mechanisms[53,95-99]. Are these regulators better or less well-characterized than BTK? Is any of them involved in the BTK-dependent NLRP3 inflammasome activation?

NEK7 belongs to the NIMA-related kinase family, which regulates the mitotic progression and DNA damage response[100]. The requirement of NEK7 in NLRP3 inflammasome activation induced by NLRP3 stimuli including ATP, Nigericin, MSU crystals, has been shown in three independent studies[53,97,98]. NEK7 is also required for macrophages containing the CAPS-associated NLRP3^{R258W} activating mutation to activate caspase-1. Our results show that BTK interacts with ASC and NLRP3, and Ito´s paper demonstrates that the NLRP3-NACHT and -leucine-rich repeat domains bind to both the BTK-SH2/3 and -TK domains. However, unlike BTK, NEK7 only binds to the leucine-rich repeat domain of NLRP3, through its catalytic domain. The interaction between NEK7 and ASC is not described in these NEK7 papers. Additionally, the kinase activity of NEK7 is not required for the NEK7-mediated NLRP3 activation. Phosphorylation of NEK7 was not observed in these three studies and a scaffolding role of NEK7 which facilitates the interaction of NLRP3 and ASC was hypothesized. Nek9, a known upstream regulator of Nek7, is dispensable for NLRP3 inflammasome activation upon Nigericin stimulation.

Our study suggests that BTK is dispensable for the activation of the NLRC4 and AIM2 inflammasomes. By contrast, PKR regulates the activation of all known inflammasomes, including NLRP1, NLRP3, NLRC4, and AIM2[96]. In response to a wide array of stimuli, genetic deletion or pharmacological inhibition of PKR results in reduced caspase-1 activation and secretion of IL-1β and IL-18. However, another study with macrophages from two different PKR-deficient mice, including even the mutant mice in the former study, could not independently prove the role of PKR in inflammasome activation[101]. Thus, the function of PKR in NLRP3 inflammasome activation needs to be validated by further studies.

Similar to PKR, the role of GBP5 in NLRP3 inflammasome activation remains controversial. With the stimulation of nigericin, ATP, and bacteria, but not particulate matter, GBP5 was reported to enhance NLRP3 inflammasome activation[95]. By contrast, another study observed GBP5-deficiency did not have any effect on canonical and non-canonical inflammasome activation in murine macrophages[102]. It is unclear what accounts for the discrepancies in these studies.

Unlike BTK and the other regulators, TRIM31 limits NLRP3 inflammasome activation, both in resting macrophages and NLRP3 inflammasome-activated macrophages, by promoting proteasomal degradation of NLRP3 as a feedback suppressor of NLRP3 inflammasome[99]. TRIM31 also could be immunoprecipitated with NLRP3, but not with ASC or caspase-1.

Taken together, how do all the reported molecules, especially BTK, NEK7, and TRIM31, regulate NLRP3 inflammasome activation together? Which is the most prominent regulator? Does any interaction or influence among each other? For example, BTK-NLRP3 binding assays can be performed in NEK7 knockdown macrophages to explore whether the interaction between NEK7 and NLRP3 could be disrupted by NEK7 depletion. Presumably, since they all directly bind to NLRP3, the known regulators of NLRP3 could work together to fine tune the activation of inflammasome to maintain immune homeostasis. More importantly, since BTK directly interacts with both NLRP3 and ASC, it is more likely that BTK regulates NLRP3 inflammasome activation by directly promoting NLRP3 oligomerization rather than recruiting other regulators. Additionally, it is important and could be the focus of future work to check the function of BTK in non-canonical (caspase-11-dependent) and alternative (K^+-independent) NLRP3 inflammasome pathways. Interestingly, there is one study providing a novel link between BTK and the regulation of caspase-11 expression[103].

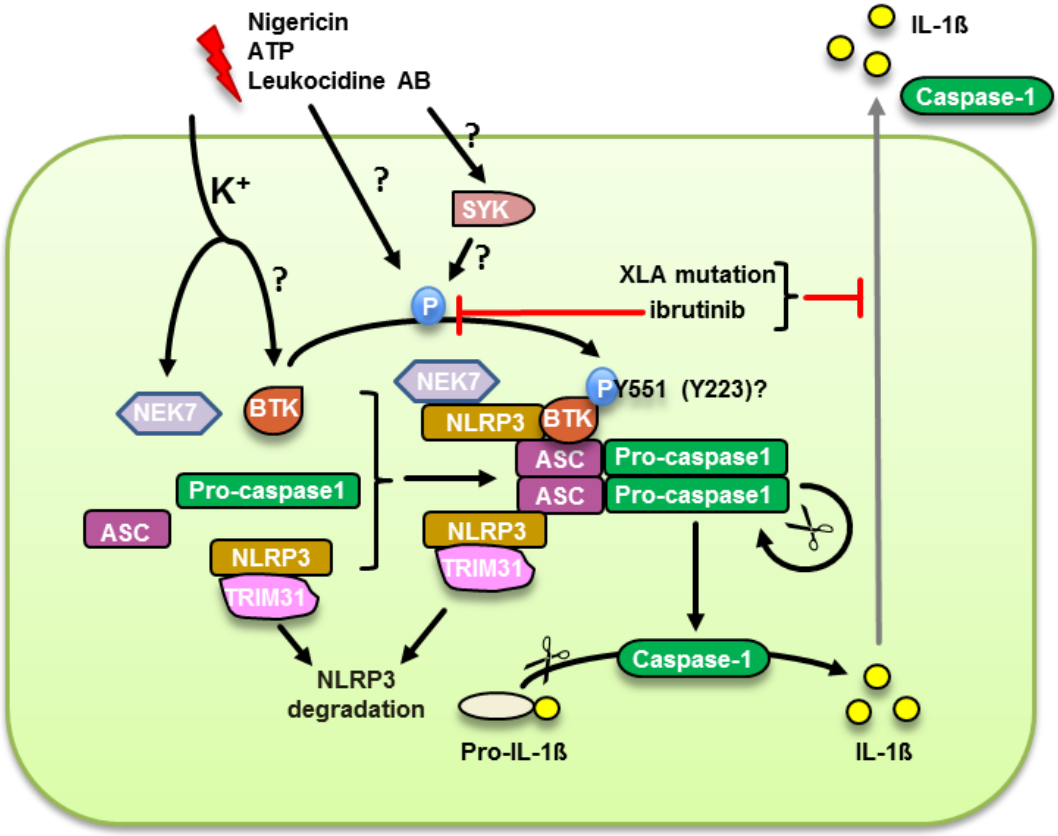


Figure 4.1 Working model for BTK and another two reported regulators NEK7 and TRIM31 regulating NLRP3 inflammasome activation.

Figure adapted from Liu *et al.*[77]. NLRP3, NACHT, LRR and PYD domains-containing protein 3; BTK, Bruton's Tyrosine Kinase; ASC, Apoptosis-associated speck-like protein containing a CARD; XLA, X-linked agammaglobulinemia; TRIM31, tripartite motif-containing protein 31; NEK7, NIMA-related kinase 7.

4.3 The role of BTK in other cell types besides of macrophages

Although it is well known that the NLRP3 inflammasome controls interleukin-1β maturation in innate immune cells, there are very few reports on a direct role for NLRP3 in human adaptive immune cells. As described above, BTK is a critical component for B-lymphocyte development, differentiation, and signaling, and the role of BTK in B cell receptor signaling has been well studied. If NLRP3 was expressed and formed inflammasomes in B cells, would these two structurally and functionally diverse signaling pathways converge at a certain level since they both employ BTK? Will the molecules upstream or downstream of BTK in BCR signaling pathway provide any hint

for studying mechanism of NLRP3? Exploring these questions could help elucidate the mystery the NLRP3 inflammasome pathway further. In a recent study, the expression as well as the function of TLRs on human B cells has been extensively and systematically investigated, showing that TLRs 1, 6, 7, 8, 9, and 10 are expressed on human B cells[104]. Therefore, these data suggest that a priming step of NLRP3 inflammasome could occur via TLRs. Notably, there is a first study that verified the expression of NLRP3 in murine B cells[105]. In this study, NLRP3 expression was inducible in B cells by curdlan stimulation. B cells were directly activated in response to curdlan, and this activation required NLRP3 but not TLR and IL-1 receptor signaling, suggesting a critical role of B cell-intrinsic NLRP3 in Ab responses[105]. Thus, further investigation on the role of BTK in NLRP3 inflammasome pathway in the context of BCR signaling in the human system could be of great interest. Although not tested in this study, IL-1ß release assay from B cells in response to NLRP3 stimuli is necessary.

BTK is not restricted to B cells and macrophages. It is also expressed in platelets neutrophils, dendritic cells (DCs), etc. Platelets are one component of the blood-vascular axis responsible for preventing hemorrhage. Activated platelets initiate hemostatic plug formation and provide scaffolding for coagulation activation. Interestingly, consistent with the BTK function shown in our results, our collaborators revealed that NLRP3 inflammasome activation in platelets promotes platelets activation and aggregation in a BTK-dependent manner[106]. Platelets have assumed a role in the development of focal cerebral ischemia by virtue of their participation in thromboemboli that may initiate stroke symptoms[107]. This indicates that targeting BTK might represent an alternative to antiplatelet therapy for ischemic stroke. NLRP3 inflammasome also exists in neutrophils[108,109], dendritic cells[110,111], and to explore the role of BTK in NLRP3 inflammasome activity and consequent BTK-mediated inflammasome function is necessary to expand our knowledge of BTK in innate immunity.

4.4 Therapeutic implications

Several studies suggest the detrimental consequences of aberrant NLRP3 inflammasome-mediated IL-1ß release is driving inflammatory disorders. Macrophages play a critical role in the initiation, maintenance, and resolution of a variety of human

inflammatory disorders, such as sepsis-related multiple organ dysfunction/multiple organ failure, microbial infection, acute brain/lung/hepatic/renal injuries, neurodegenerative disorders, tumorigenesis, osteoporosis/osteonecrosis, cardiovascular and metabolic diseases, and autoimmune diseases[112,113]. In *in vivo* model of Alzheimer's disease, deposition of amyloid-β peptide driven NLRP3/caspase-1 axis contribute to the pathogenesis of Alzheimer's disease[114]. Similarly, recent studies suggest that MSU-crystal induced inflammatory gout attack is dependent on the proinflammatory cytokine IL-1β[115]. Additionally, monocytes derived from newly identified untreated type 2 diabetic patients display elevated expression of inflammasome components *NLRP3*, along with increased caspase-1 activation[116]. So far, three IL-1-targeted agents have been approved by the US Food and Drug Administration (FDA) for the treatment of CAPS: the IL-1 receptor antagonist anakinra, the soluble decoy receptor rilonacept and the neutralizing monoclonal anti-IL-1β antibody canakinumab[117]. This approach can therefore be effective, and trials targeting a broad spectrum of new indications are underway, including metabolic syndrome, type 1 and type 2 diabetes mellitus, stroke, and myocardial infarction[118]. Our findings that BTK inhibitors reduce NLRP3-dependent IL-1ß release suggest that NLRP3 inflammasome inhibition through BTK represents a new therapeutic intervention for these inflammatory diseases. Moreover, as mentioned previously, since the BTK inhibitor ibrutinib with high safety has been FDA-approved, and other novel BTK inhibitors (LFM-A13, GDC-0834, HM-71224, CGI-560, CGI-1746, ONO-4059, CC-292, and CNX-774) has been in clinical development for the treatment of B-cell malignancies and autoimmune disorders, it would be an attractive, time-and-money saving strategy if these BTK inhibitors could directly contribute to the treatment of NLRP3-driven inflammatory diseases. Besides, with completing this project, another collaboration project contributes to the strength of this clinical application possibility. Murthy *et al*[119] recently proved that platelet activation, aggregation, and thrombus formation could be promoted by BTK-dependent NLRP3 inflammasome activation in platelets, and these effects could be reduced by pharmacological or genetic deletion of BTK, indicating functional relevance between BTK and platelet-related inflammation.

It should be noted that the strategy of targeting IL-1 axis via BTK might present certain advantages over the previously indicated approach. MCC950, a small-molecule

inhibitor of NLRP3, effectively reduces interleukin-1β (IL-1β) production *in vivo* and attenuates the severity of experimental autoimmune encephalomyelitis (EAE)[120]. However, the precise mechanism of action of MCC950 remains unclear. Furthermore, unlike the BTK inhibitor ibrutinib, MCC950 has not been FDA-approved. To invest on evaluating potential clinical application of MCC950 will take considerable time and refill hurdles to overcome. Anakinra, as an IL-1 receptor antagonist, inhibits both IL-1ß and its close homologue IL-1α. Although the physiological role of IL-1α is still not well defined, the IL-1α constitutive production in epidermis has been verified, and the essential role of IL-1α in the maintenance of skin barrier function has been implicated, especially with increasing age[121]. Therefore, application of Anakinra might cause IL-1α-deficiency mediated detrimental consequences in IL-1ß driven diseases. Furthermore, Anakinra cannot ameliorate the HMGB1-driven inflammatory and autoimmune diseases[122]. Since IL-1ß is the terminal product of NLRP3 inflammasome, targeting upstream BTK might be more effective. IL-1β maturation can be mediated by a number of different enzymes, including serine proteases and caspase-8[26], and we have demonstrated that ibrutinib does not block the major antimicrobial inflammasomes NLRC4 and NLRP1. Thus, specific targeting of NLRP3 will not result in the complete blockade of IL-1β *in vivo* during infection. Although the effect of BTK on pyroptosis and additional cytokines activated by the NLRP3 inflammasome, such as IL-18 and HMGB1, must also be evaluated, some of the pathologies of NLRP3-associated disorders may be due to IL-18 and pyroptosis[123], which ibrutinib could block while Anakinra cannot.

Given that BTK is important for antibodies production from B cells, ibrutinib can be applied in a critical period of NLRP3-driven chronic inflammation when ibrutinib could effectively and actively inhibit the pathology, whereas the B cells and antibodies production could be rescued afterward[124]. Further clinical trials to explore detailed application might be necessary to establish the full benefits of ibrutinib as a therapeutic agent in the clinical environment.

4.5 Conclusion

In summary, intensive efforts have been put into the investigation of the mechanism of BTK regulating NLRP3 inflammasome activation. Base on the already obtained results, we could expand our findings further by investigating more of the function of BTK at a molecular level. By comparing with the other identified regulators, BTK might most directly and prominently promote NLRP3 inflammasome activation. As next steps, exploration of the BTK/NLRP3 mechanism in macrophages and other cell types would be of great help to understand the role of BTK in inflammation and thus in the whole immune system. Advantages of the BTK inhibition for therapeutically targeting the inflammasome in prevalent diseases might draw interests of clinicians and may in turn fuel research into some of the open questions prompt by this study.

5 Appendixes

5.1 Supplemental data

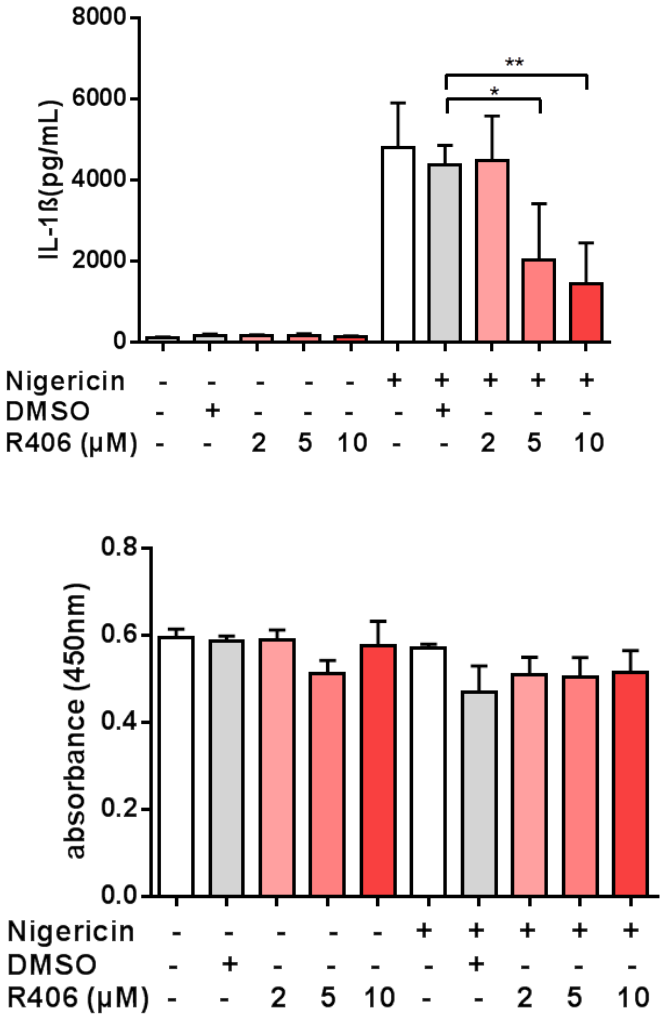


Figure 5.1 (A) ELISA of IL-1ß in supernatants from THP-1 cells primed with PMA overnight, pre-incubated with R406 (2, 5, 10 µM) for 1 hour and followed by stimulation by Nigericin to trigger NLRP3 activation for 1 hour. (B) Absorbance of CCK8 assay to measure cytotoxicity after inhibitors pre-incubation and then Nigericin treatment. Data are representative of two experiments. Means +SD are shown, and Student´s t-tests were used. *P<0.05.

5.2 Recipes of buffers

Table 5.1: Buffer solutions that were used in this study

Buffer name	Recipe
PBS (phosphate buffered saline)	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, add dH_2O to a final volume 1 L, adjust to pH 7.4
TBS (Tris buffered saline)	50 mM Tris HCl, pH 7.4 and 150 mM NaCl
TBS-Tween 0.1% (immunoblot washing buffer)	TBS, 0.1% Tween-20
stop solution	2N H_2SO_4
SDS running buffer	Tris 25 mM, glycine 250 mM, SDS 0.1%
RIPA lysis buffer	50 mM HEPES, 150 mM NaCl, 0.1% NP-40, 20 mM ß-glycerophosphate, 2 mM DTT, adjust to PH 6.9, freshly supplemented with Roche inhibitor tablets Complete Mini Protease Inhibitor

5.3 List of antibodies in immunoblot analysis

Table 5.2: Antibodies used in this study

Antibody	Species	Dilution	Company	Catalog number
Anti-mouse HRP conjugated		1:10000	Promega	W4028
Anti-rabbit HRP conjugated	goat	1:5000	Vector	PI-1000
Anti-IL-1ß (for both pro- and cleaved IL-1ß)	mouse	1:500	R&D	MAB201
Anti-caspase-1 (for both pro- and cleaved caspase-1)	rabbit	1:1000	Cell Signaling Technology	CST3866s
Anti-BTK	mouse	1:1000	BD	611117
Anti-Protein A	rabbit	1:625000	Sigma Aldrich	P3775
Anti-HA	rabbit	1:1000	Cell Signaling Technology	#3724
Anti-GFP	rabbit	1:5000	Sigma	G1544

Abstract

The Nod-like receptor, NLRP3, has been described as a key immune sensor of microbial, environmental and endogenous insults or stress. The multimeric NLRP3 inflammasome recruits the adaptor protein ASC to activate caspase-1, leading to the maturation of the pro-inflammatory cytokines Interleukin (IL)-1β and IL-18. The NLRP3 inflammasome has a fundamental role in host defense against microbial pathogens but its deregulation is also implicated in sterile inflammation and the pathogenesis of several inflammatory diseases including Muckle-Wells Syndrome (MWS) caused by inherited NLRP3 mutations. NLRP3 inflammasome activity is tightly controlled to maintain immune homeostasis and avoid detrimental effects. However, the regulatory mechanism of this complex and medically relevant process are poorly understood. In this study, we identified Bruton's tyrosine kinase (BTK), a non-receptor protein tyrosine kinase of the TEC family of proteins, as a novel regulator of the NLRP3 inflammasome by an unbiased triple SILAC phospho-proteomics. Further results revealed that pharmacological (using the Food and Drug Administration (FDA)-approved inhibitor, ibrutinib) and genetic (in *BTK*-mutated patients and *Btk*-knockout mice) BTK ablation attenuated caspase-1 activation and IL-1β maturation in response to Nigericin and the *Staphylococcus aureus* toxin, LukAB. Additionally, BTK directly interacted with NLRP3 and ASC, and promoted ASC speck formation and caspase-1 cleavage. Consistently, ibrutinib inhibited IL-1β release from immune cells of MWS patients. More importantly, obtained results from immune cells of ibrutinib therapy treated cancer patients provide evidence that *in vivo* application of ibrutinib affects IL-1ß processing and release in response to Nigericin. Thus, our data suggest that BTK is a critical regulator of NLRP3 inflammasome activation and that NLRP3 inflammasome-associated inflammatory diseases could potentially be targeted via BTK.

Zusammenfassung

Der Nod-like Rezeptor NLRP3 ist als ein wichtiger Sensor des Immunsystems für mikrobiellen, umwelt- und endogenen Gefahrensignale sowie zellulären Stress beschrieben worden. Nach Multimerisierung rekrutiert das NLRP3 Inflammasom das Adaptorprotein ASC und Caspase-1, welche nach Aktivierung Prozessierung der pro-inflammatorischen Zytokine IL-1β und IL-18 führt. Das NLRP3 Inflammasom spielt eine bedeutende Rolle in der Wirtsabwehr gegen mikrobielle Pathogene aber auch steriler Entzündung, und seine Fehlregulierung ist in der Pathogenese mehrerer inflammatorischer Krankheiten, einschließlich dem sogenannten Muckle-Wells Syndrom (MWS), welches durch Mutationen in NLRP3 verursacht wird, assoziiert. Daher ist die Aktivität des NLRP3 Inflammasoms strikt reguliert. Jedoch sind die regulatorischen Mechanismen und die medizinisch relevanten Prozesse auf molekularer Ebene kaum verstanden. In dieser Studie haben wir die Bruton`s Tyrosin Kinase (BTK), eine nicht-Rezeptor Tyrosinkinase der TEC Familie, in einem SILAC Phospho-Proteom Ansatz als einen neuen Regulator des NLRP3 Inflammasoms identifizieren können. Weitere Ergebnisse zeigten, dass pharmakologisches (durch Verwendung des FDA-zugelassenen Inhibitors Ibrutinib) und genetisches (in BTK-mutierten Patienten und BTK-Knockout Mäusen) Ausschalten von BTK, Caspase-1 Aktivität und Interleukin (IL)-1β Reife in Antwort auf Nigericin und einem Toxin aus *Staphylococcus aureus*, LukAB, stark herabsetzten. Zudem, interagierte BTK direkt mit NLRP3 und ASC, und förderte die Inflammasom-Komplexbildung und Caspase-1 Spaltung. Zudem blockierte Ibrutinib die Ausschüttung von IL-1β von Immunzellen von MWS Patienten. Immunzellen von Patienten auf Ibrutinib-Therapie zeigten ebenfalls eine auf Nigerizin hin verringerte IL-1β Prozessierung und Ausschüttung. Somit beeinflusst die *in vivo* Anwendung von Ibrutinib die Aktivität des NLRP3 Inflammasoms auch beim Menschen. Unsere Ergebnisse deuten darauf hin, dass BTK ein überaus wichtiger Regulator der Aktivierung des NLRP3 Inflammasoms ist und dass NLRP3 Inflammasom- assoziierte inflammatorische Krankheiten möglicherweise über BTK gezielt therapeutisch behandelt werden könnten.

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Contribution declaration for data presented in the results of this thesis:

Contribution of others:

1. Figure 3.1: the Mass spectrometry and cluster analysis were done by the collaboration group Proteomics & Proteome Center Tuebingen, and the cells were prepared by Tica Pichulik, Ph.D. and me.
2. Figure 3.3 and Figure 3.10: the Flow cytometry were done by Magno Garcia Delmiro.
3. Figure 3.8: the mice were provided by Prof. Dr. Cornelia Brunner in Ulm University.
3. Figure 3.14 and Figure 3.15: experiments were done by Dr. Olaf-Oliver Wolz.
4. Figure 3.16: experiments were done by Andrea Stutz, Ph.D., from our collaboration group of Institute of Innate Immunity, University Hospital Bonn.
5. Figure 3.17: ASC cross-link immunoblots were obtained by Dr. Olaf-Oliver Wolz.
6. Figure 3.21 and Figure 3.22: experiments were done by Tica Pichulik, Ph.D. and me.

Own contribution: Experimental work that is not indicated above, data acquisition, analysis of data and preparation of the figures presented in the results of this thesis.

Publication

The data presented in this thesis (**Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase**) is partially adapted from the published manuscript:

Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase.

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