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Search for new regulators of MyD88-dependent signaling

Hledání nových regulátorů signalizace proteinu MyD88

Diploma thesis

Supervisor: Mgr. Peter Dráber, Ph.D.

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Abstract

MyD88 plays a crucial role in connecting the signaling pathways of Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs). Its importance lies in its essential function in coordinating host defense against invading pathogens and responding to tissue damage. MyD88-mediated signaling initiates a cascade of events leading to the activation of pro-inflammatory genes and the production of cytokines necessary for an effective immune response. Infections or injuries are often considered as triggers of autoimmune disorders. However, the regulators of MyD88 signaling in disease remain elusive. Here we identified the kinase TBK1 and associated adaptors TANK and AZI2 as new components of the IL-1R-SC. Mechanistically, TANK and AZI2 recruit TBK1 to the signaling complex to inhibit MyD88-dependent signaling and subsequent production of pro-inflammatory cytokines. Moreover, we showed that TBK1-mediated inhibition of MyD88-dependent production of TNF is essential for reducing the severity of TNF-mediated inflammation *in vivo*. Lastly, we propose that MyD88 or its downstream kinase IRAK4 can serve as potential targets in the treatment of inflammatory diseases.

Keywords

Toll-like receptors, IL-1, TNF, MyD88, IRAK4, TBK1, autoimmunity, inflammation, dermatitis

Abstrakt

MyD88 je důležitý adaptorový protein, který propojuje signální dráhy Toll-like receptorů (TLR) a receptorů pro interleukin 1 (IL-1R). Jeho význam tkví v jeho funkci v obraně těla vůči patogenům a v odpovědi na tkáňové poškození. MyD88-mediovaná signalizace spouští kaskádu, která vede k transkripci prozánětlivých genů a produkci cytokinů nutných pro efektivní imunitní odpověď. Infekce a poškození tkání v důsledku úrazů jsou často považovány za spouštěče autoimunitních onemocnění. V této práci jsme identifikovali kinázu TBK1 a s ní asociované adaptory TANK a AZI2 jakožto nové komponenty IL-1R-SC. TANK a AZI2 rekrutují kinázu TBK1 do signálního komplexu za účelem inhibice signalizace a následné produkce prozánětlivých cytokinů. Zároveň jsme ukázali, že TBK1-mediovaná inhibice MyD88-závislé produkce TNF je zásadní pro zmírnění zánětlivé reakce závislé na TNF *in vivo*. Navrhujeme, že MyD88 nebo s MyD88 asociovaná kináza IRAK4 můžou být dobrým terapeutickým cílem v léčbě některých zánětlivých onemocnění mediovaných nadprodukcí TNF.

Klíčová slova

Toll-like receptory, IL-1, TNF, MyD88, IRAK4, TBK1, autoimunita, zánět, dermatitida

Contents

List of abbreviations

AS	ankylosing spondylitis
AZI2	5-azacytidine-induced protein 2
DD	death domain
DIRA	deficiency of IL-1 receptor antagonist
DLBCL	diffuse large B cell lymphoma
DUB	deubiquitinase
EAE	experimental encephalomyelitis
FADD	Fas-associated death domain protein
GPP	general pustular psoriasis
HOIL-1	heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-interacting protein
IBD	inflammatory bowel disease
IKK	inhibitor of nuclear factor kappa-B kinase
IL	interleukin
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
iNOS	inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
ISG	interferon stimulated genes
IκBα	NF-kappa-B inhibitor alpha
JNK	c-Jun N-terminal kinase
KO	knockout
LPS	lipopolysaccharide
LUBAC	linear ubiquitin chain assembly complex
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblasts
MLKL	mixed lineage kinase domain-like
MS	multiple sclerosis
mTOR	mechanistic target of rapamycin

MyD88	myeloid differentiation primary response protein 88
NEMO	NF-κB essential modulator
NET	neutrophil extracellular trap
NF-κB	nuclear factor NF-kappa-B
NLS	nuclear localization signal
OTU	ovarian tumor
OTULIN	OTU domain-containing DUB
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RIPK	receptor-interacting protein kinase
SAPK	stress-activated protein kinase
SF	strep flag tag
SHARPIN	SHANK-associated RH domain-interacting protein
sJIA	systemic-onset juvenile idiopathic arthritis
SLE	systemic lupus erythematosus
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	TANK-binding kinase
TGFβ	transforming growth factor beta
TIR	Toll/IL-1R homology domain
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR1	TNF receptor type 1
TRAF	TNF receptor-associated factor
Ub	ubiquitin

1. Introduction

The immune system is at the forefront of almost all biological processes in our bodies. It plays a crucial role in the elimination of invading pathogens, wound healing, and tissue homeostasis, as well as regulating the sleep-wake cycle, digestion, or hormone production. It is to be expected that when this system is disrupted, we face dire consequences in the form of chronic illnesses, autoimmune disorders, or the development of cancer.

The recognition of pathogens is primarily a responsibility of the innate immune system. In the event of infection, cells that constitutively surveil their surroundings, such as dendritic cells or macrophages, are alerted of foreign invaders via pattern recognition receptors (PRRs), such as Toll-like receptors, NOD-like receptors, or RIG-I-like receptors. After the sensing of microbe-derived molecules, they get rapidly activated and start producing cytokines and chemokines. These prompt the activation of antimicrobial and inflammatory responses to limit the spread of infection, and migration of immune cells to the breached tissue site. In the site of infection, the immune cells ensure effective pathogen clearance. This, however, can lead not only to the destruction of the pathogen, but also of the surrounding tissue (Paludan et al., 2021)*.

The capacity of the immune system to induce cell death is a useful but potentially dangerous feature. On the one hand, it helps keep tissue homeostasis by removing damaged cells, enables the eradication of cells infected with an intracellular pathogen, and can prevent malignant transformation. On the other hand, aberrant death induction can cause extensive tissue damage and loss of its function which can potentially cause life-threatening organ failure. Cell death can be initiated from the inside, when the mechanisms critical for cell survival get disrupted, such as DNA damage, loss of plasma membrane integrity, or oxidative stress. Alternatively, cell death can be initiated from the outside when external stimuli, typically ligands of death domain receptors, trigger cell death pathways in the cytoplasm of the target cell. The main cell death pathways are apoptosis, necrosis, and pyroptosis. Apoptosis is dependent on the action of caspases and preserves the integrity of the plasma membrane, therefore usually not triggering an immune response. Another type of cell death that depends on the action of caspases is pyroptosis. Caspase-mediated cleavage of perforins leads to membrane perforation. The contents of the dying cell are released into its surroundings and alert the immune system. Necrosis (or necroptosis) is independent of caspases and relies on RIPK3 and MLKL. Activation of MLKL by RIPK3 results in the formation of MLKL

oligomers, which get inserted into the plasmatic membrane leading to cell rupture. This type of programmed cell death is also highly proinflammatory (Yuan & Ofengeim, 2024)*.

A tight balance must be kept to protect the body against excessive tissue damage, while also providing sufficient protection from infections. Therefore, the signaling pathways initiating the immune response must be strictly regulated.

1.1. MyD88 at the center of innate immunity

Myeloid differentiation primary response protein 88 (MyD88) is an adaptor protein downstream of a majority of Toll-like receptors (TLRs) and IL-1 receptor family (IL-1Rs) (Wesche et al., 1997). TLRs function as PRRs that detect various of pathogen-derived molecules, for example bacterial lipopolysaccharide, zymosan, or nucleic acids. Furthermore, they recognize host molecules produced and/or released during cellular stress, such as heat-shock proteins or HMGB1 (Yu et al., 2010)*. TLRs are expressed on cells of hematopoietic origin (e.g., neutrophils, dendritic cells, monocytes) and on non-hematopoietic cells (e.g., fibroblasts, epithelial cells) in various combinations and quantities. When an agonistic ligand binds to the receptor, a signaling cascade is initiated resulting in the production of inflammatory cytokines and activation of immune responses (Duan et al., 2022)*. IL-1Rs bind IL-1 family cytokines which generally promote inflammation. IL-1 family cytokines are produced as a precursor and require enzymatic cleavage aided by caspase-1 or other proteases activated in the inflammatory response to yield an active form, with the exception of IL-1α and IL-33 which are biologically active even in their unprocessed form (Inna et al., 2015)*. As such, IL-1Rs can sense damage caused to tissues, recognize ongoing inflammation and amplify it. The expression of IL-1Rs is typically ubiquitous with varying increases for particular receptors in certain cell types (usually immune cell subtypes and epithelial cells) (Boraschi & Tagliabue, 2013)*.

MyD88 is indispensable for TLR and IL-1R signaling. Therefore, it plays a crucial role in the protection against pathogens and the detection of cell damage. Indeed, MyD88 deficiency in humans leads to an increased susceptibility to opportunistic infections with pyogenic bacteria, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa*, and can be lethal if not treated immediately (Picard et al., 2010). On the other hand, several mutations in MyD88 have been linked to the development of cancer. Most notably, a mutation of leucine 265 to proline (L265P) has been found in up to 29 % of cases of diffuse large B cell lymphoma (DLBCL)

(Ngo et al., 2011). This mutation causes the hyperactivation of MyD88 signaling and promotes cancer survival (Yu et al., 2021).

TLRs and IL-1Rs have a similar architecture, with an extracellular domain that binds various ligands, a transmembrane domain, and an intracellular domain with a shared Toll/IL-1 receptor homology domain (TIR) that enables downstream signaling (Akira et al., 2001). While signaling outcomes may vary depending on the cellular context and other stimuli, both TLR and IL-1R signaling generally follow a similar pattern. The receptors exist on the plasma membrane as monomers, however, upon ligand binding they form homo- or heterodimers. The receptor dimerization brings its intracellular TIR domains to close proximity and initiates signaling complex assembly. Aggregated TIR domains interact with the C-terminal TIR domain of MyD88. Meanwhile, the N-terminus of MyD88 contains a death domain (DD), and associates with the DD of interleukin-1 receptor-associated kinases (IRAKs) through homotypic interactions. MyD88 and IRAKs form a closed helical complex termed the myddosome through hierarchal DD clustering (Motshwene et al., 2009). Starting with six molecules of MyD88 at the receptor, followed by recruitment of four IRAK4 molecules, auto- and transphosphorylation of IRAK4 and subsequent recruitment of four molecules of IRAK1/2 (Lin et al., 2010). IRAKs further interact with a diverse array of adaptor proteins and kinases to achieve functional signaling.

Phosphorylated IRAK1 engages E3 ubiquitin ligases Pellino1/2 and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) to generate non-degradative K63-linked ubiquitin chains (K63-Ub) which bind another ubiquitin ligase, linear ubiquitin chain assembly complex (LUBAC) (Emmerich et al., 2013; Goh et al., 2012; Strickson et al., 2017). LUBAC is the only E3 ubiquitin ligase capable of making linear (M1) ubiquitin linkages (Kirisako et al., 2006). The combined action of the E3 ligases creates a robust network of K63-, M1-, and hybrid ubiquitin chains. The ubiquitin linkages serve as a binding platform for other components of the signaling complex. Among the most important are the transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) and inhibitor of κB kinase (IKK) complexes.

TAK1 is a master kinase that activates two major inflammatory signaling pathways – the mitogen activated protein kinase (MAPK) and nuclear factor κB (NF-κB). TAK1-binding protein 1 and 2 (TAB1/2) bind to K63-Ub and anchor TAK1, which then becomes phosphorylated and activates the c-Jun N-terminal kinase (JNK) and stress-activated protein kinase (SAPK) p38

(Bhattacharyya et al., 2010; McDermott & O'Neill, 2002). The lack of ubiquitin-binding motif on TAB2 was reported to severely impair TAK1-mediated MAPK activation (Kanayama et al., 2004). However, another study suggests that TAK1 can function independently of TAB1/2, possibly through direct interaction with TRAF6 (Shim et al., 2005).

The IKK complex consists of enzymatically active IKKα and IKKβ, and a regulatory subunit NF-κB essential modulator (NEMO, or IKKγ). NEMO recognizes K63-Ub and M1-Ub, with a higher affinity for the latter (Rahighi et al., 2009). In the TAK1-dependent NF-κB activation pathway, TAK1 interacts with the IKK complex by phosphorylating IKKβ, which causes rapid phosphorylation of the inhibitor of κBα (IκBα) and its targeting for proteasomal degradation by the addition of K48-Ub (Zhang et al., 2014). Free NF-κB precursor p105 can then be processed into mature NF-κB subunit p50 and translocate into the nucleus (Christian et al., 2016)*. Alternatively, IKKα gets activated through MEKK3 phosphorylation of NEMO. This triggers the release of NF-κB without IκBα degradation (Kim et al., 2007). This process, however, has only been described in the context of MyD88 signaling and shouldn't be confused with the activation of alternative NF-κB pathway which is independent of NEMO and leads to the activation of NF-κB p100 precursor of the NF-κB subunit p52 (Senftleben et al., 2001).

The formation of K63 and M1 ubiquitin chains is counteracted by deubiquitinases CYLD, A20, and recently described OTUD4 and OTUD5. CYLD and A20 both limit MyD88 signaling by cleaving K63-Ub. In addition, CYLD is capable of cleaving M1 ubiquitin, while A20 can bind M1-Ub to protect it from degradation (Tokunaga et al., 2012). Although their functions overlap, CYLD and A20 cannot compensate for each other, as mice deficient in either develop spontaneous MyD88-driven inflammation (Lee et al., 2016; Turer et al., 2008). OTUD4 was formerly only known to cleave K48-Ub (targeting for proteasomal degradation) but it was newly found that after phosphorylation, it becomes capable of switching to K63-Ub substrate (Y. Zhao et al., 2018). OTUD5 has been identified to cleave K11-Ub. However, the knowledge of the importance of K11 ubiquitination in MyD88 signaling remains limited (Liu et al., 2024). Additionally, an *in vitro* study of the myddosome assembly revealed that individual myddosomes cluster together to generate a more potent signal. This potentially creates a rate-limiting step or a threshold for activation. In accord, myddosomes, which were prohibited from associating together proved to be less effective at TRAF6 and LUBAC engagement (Cao et al., 2023).

In summary, the activation of MAPKs and NF-κB by the TAK1 and IKK complexes initiates the expression of pro-inflammatory genes and influences the response to TLR/IL-1R stimuli (Fig.1).

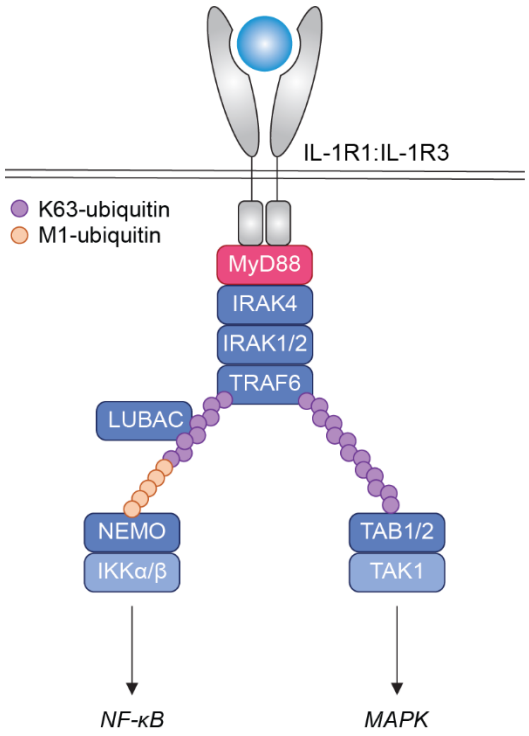


Fig. 1. IL-1 signaling complex

IL-1 receptor signaling is a prototype for the signaling by MyD88-employing receptors. Upon ligand binding, two receptor subunits dimerize and initiate myddosome assembly through sequential binding of MyD88, IRAK4, IRAK1/2. IRAKs then recruit TRAF6 which starts decorating the complex with K63-linked ubiquitin, followed by LUBAC-catalyzed addition of M1-linked ubiquitin. The ubiquitin network facilitates binding of the IKK and TAK1 complexes to trigger NF-κB and MAPK activation, respectively.

1.2. IL-1 in health and disease

The IL-1 family represents a diverse group of cytokines that consists of seven pro-inflammatory cytokines: IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ, and four anti-inflammatory cytokines: IL-1Ra, IL-36Ra, IL-37, IL-38 (Dinarello et al., 2010). Traditionally, they are classified as a part of innate immunity. However, due to their ability to also influence T and B cells directly, they pose more as a bridge between the innate and adaptive immune system (Boraschi, 2022)*.

IL-1α and IL-1β were first noticed for their ability to induce fever and activate lymphocytes (hence the original names endogenous pyrogen or lymphocyte activating factor) and later gave rise to a whole family of IL-1 cytokines. Despite sharing only about 27% sequence homology, both IL-1α and IL-1β are recognized by the same receptor and their signaling outcomes are indistinguishable (Eislmayr et al., 2022)*. The primary distinctions between the two lie in their source and processing. IL-1α is constitutively expressed across mesenchymal and epithelial cell types and is active even in its precursor form. It exists in a membrane-bound form and in a free form. Unprocessed IL-1α contains a nuclear localization signal (NLS), allowing it to translocate to the nucleus, bind chromatin, and positively influence the transcription of IL-6 or IL-8 (Werman et al., 2004; Wessendorf et al., 1993). Upon cellular stress, IL-1α NLS is rapidly acetylated, released from cells, and functions as an alarmin (Cohen et al., 2015). IL-1α seems to be the key initiator of sterile inflammation (Cohen et al., 2010). In comparison, IL-1β is only induced upon inflammatory stimuli and resides in the cytoplasm until it is further processed by caspase 1 in the NLRP3 inflammasome (Martinon et al., 2002; Thornberry et al., 1992). IL-1β is transcribed by various cell types but the main source are activated immune cells, typically monocytes and neutrophils (Boraschi, 2022; Cho et al., 2012).

Due to their capability to trigger and perpetuate inflammation, IL-1 family cytokines are associated with the development or progression of many autoimmune diseases. The most common are described in the following text with the focus on IL-1α/β, since their involvement in autoimmunity is currently the best understood.

Psoriasis is a skin condition characterized by erythematous plaques of thickened skin usually with flaking at the edges, usually with periods of flare-ups and remissions. It affects between 2-3 % of people worldwide, with the highest prevalence in Western Europe, North America, and

Australia. In addition, around 23 % of psoriatic patients also show signs of arthritis (Alinaghi et al., 2019; Damiani et al., 2021). The dominant driver of psoriasis is generally recognized to be the IL-23/Th17 pathway. The aberrant IL-17 production leads to the hyperproliferation of keratinocytes and the attraction of neutrophils to the skin. Neutrophils degranulate, secrete more IL-17, and release neutrophil extracellular trap (NET) *(Lin et al., 2011; Mosca et al., 2021). Furthermore, IL-17 simulates the production of IL-1β, IL-36, and TNF, which perpetuate the pathological activation of Th17. This is in accordance with reported increased levels of these cytokines in lesional skin (Cai et al., 2019; Carrier et al., 2011). Additionally, there is increasing evidence for a strong connection of IL-1β and IL-36 to the pathogenesis of general pustular psoriasis (GPP). This is a rare but potentially life-threatening condition with high fevers and neutrophilia. The disease pathology seems to rely on IL-36-mediated neutrophil activation (Johnston et al., 2017). In support of this, several mutations in the gene coding IL-36 receptor antagonist were observed in patients with GPP, and the administration of antibodies targeting the IL-36 receptor showed disease improvement (Bachelez et al., 2019; Marrakchi et al., 2011; Mateeva et al., 2024). Besides the standard pharmacological interventions, treatments of autoimmune disorders with complex etiologies often utilize so-called biologics, complex molecules derived from cells or living organisms. These are typically recombinant proteins and neutralizing antibodies but also include gene therapy (Rosman et al., 2013)*. Currently, the most utilized biologics for the treatment of psoriasis are neutralizing antibodies against TNF (e.g., etanercept), IL-12/23 (e.g., ustekinumab), and IL-17 (e.g., secukinumab) (Strohl, 2024).

Rheumatoid arthritis (RA) is an autoimmune disorder typically appearing between the ages of 25 and 45, characterized by joint swelling, cartilage degradation, and bone erosion. Both IL-1α and IL-1β have been found in higher amounts in the synovium and circulation of RA patients. IL-1α/β has long been known to contribute to the pathology by stimulating synovial cells to produce proteases and prostaglandins, leading to the infiltration of leukocytes into the joints and resulting in progressive tissue destruction (Krane et al., 1985). RA is often accompanied by comorbidities such as the development of type 2 diabetes and atherosclerosis. Both diseases are accelerated by IL-1 family cytokines, especially IL-18 and IL-33 (Ahmed et al., 2016; England et al., 2018). A recent study investigated the role of IL-38 in RA. Even though it is designated to have anti-inflammatory properties, in RA it can function to both promote inflammation or inhibit it depending

on the presence or lack of IL-1β, respectively (Ding et al., 2024). In addition, IL-1 cytokines are implicated in other arthritic conditions. For example, IL-1β production and release in gout patients exacerbate the condition. This is caused by the activation of the NLRP3 by monosodium urate crystals that have accumulated in the joints of the patients (Joosten et al., 2010). Furthermore, symptoms of systemic sclerosis are alleviated by the neutralization of IL-1R3, a coreceptor utilized by IL-1, IL-33, and IL-36 (Grönberg et al., 2024). Among the approved biologics for RA treatment include blockers of TNF (e.g., certolizumab), IL-6 (e.g., tocilizumab, and IL-1α/β (e.g., canakinumab) (Köhler et al., 2019).

Deficiency of IL-1 receptor antagonist (DIRA) is an inflammatory disease with its roots in biallelic mutations in the gene coding IL-1 receptor antagonist (IL-1Ra). IL-1Ra binds to the IL-1R1 (receptor for IL-1α and IL-1β) and prohibits its association with IL-1R3 and signal transduction (Fields et al., 2019)*. Its deficiency, therefore, leads to unrestrained IL-1 signaling. The disease manifests from birth or within the first few days after birth. Affected children suffer from pustular rash, joint swelling, and often have hepatosplenomegaly. The condition is rare and localized, suggesting its origin from small, isolated populations (founder effect). The treatment consists of supplementing with recombinant IL-1Ra protein (anakinra) in the patients (Aksentijevich et al., 2009).

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease presenting with skin rash (typically butterfly-shaped face rash), joint swelling, and renal disorders, resulting from pathologic activation of T cells and auto-antibody production by B cells (Tsokos et al., 2016)*. Due to its complex nature, SLE is diagnosed based on meeting a set of classification criteria rather than a diagnostic test (Aringer et al., 2019). Determining whether a patient has SLE is therefore heavily dependent on the diagnosing physician and can lead to many patients going undiagnosed. Elevated IL-18 has been detected in patients with SLE (Italiani et al., 2018; Lee & Song, 2020). IL-18 is a strong inducer of IFNγ and IL-17, which have also been reported to exacerbate SLE (Ebrahimi Chaharom et al., 2023; Jackson et al., 2016). From biologics, the most commonly prescribed are antibodies targeting B cells to prevent autoantibody production (belimumab, rituximab) (Tani et al., 2024; Watson et al., 2015). Inhibition of NLRP3 inflammasome is emerging as a new treatment for SLE on the basis of preventing IL-1β and IL-18 processing and release (Wu et al., 2024)*.

Multiple sclerosis (MS) is a chronic progressive neurodegenerative disease in which the host's cells attack the protective myelin sheath on axons leading to neuronal loss. Clinically, this causes patients to suffer from myalgia, muscle weakness or tremors, gait impairment, and difficulty focusing, among others (Katz Sand, 2015)*. Sustained inflammation of the central nervous system in MS patients damages the blood-brain barrier in the long run and can accelerate the progression of the disease and increase the risk of ischemic stroke (Dziedzic et al., 2024)*. In the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), the activity of the IL-1 pathway has long been established. Mice deficient in IL-1R1, the common receptor for IL-1α/β, fail to develop EAE (Sutton et al., 2006). A more recent study revealed that IL-1β produced by neutrophils and monocyte-derived macrophages is crucial for the development of EAE and genetic deletion of IL-1β prevents its development. Interestingly, ablation of IL-1α has not led to the disease protection (Lévesque et al., 2016). This observation is in accordance with other studies emphasizing the role of the NLRP3 inflammasome in MS and its diagnostic potential as a biomarker of the disease (Keane et al., 2018).

Adult-onset Still's disease (AOSD) and systemic-onset juvenile idiopathic arthritis (sJIA) are autoinflammatory diseases clinically presenting with recurrent fevers, skin rash, and joint pain and swelling. In the past, AOSD and sJIA were classified as two different conditions based mainly on the age of symptom manifestation. Presently, they are perceived as a single condition with a varying disease onset time (Nirmala et al., 2015). The etiology of the disease is still not well understood. However, HLA polymorphisms, activating mutations of TNF receptor 1, or disturbances in the MEFV or NLRP3 inflammasomes have been described in a number of patients. Blockage of the IL-1 pathway shows generally favorable clinical response (Bindoli et al., 2024)*. Similar manifestations are observed in Cryopyrin-associated periodic syndrome (CAPS). Patients also often have neurological symptoms such as hearing loss, meningitis, or mental retardation. Currently, CAPS is split into three categories, based on disease severity (from least to most severe): familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID). The disease is caused by a gain of function mutation in the *NLRP3* gene, causing constitutive activation of the inflammasome and concomitant overproduction of IL-1β (Molina-López et al., 2024; Welzel & Kuemmerle-Deschner, 2021).

The existence of a naturally occurring antagonist, IL-1Ra, is an interesting aspect of the IL-1 pathway. It points to the need for its tight regulation and its importance in maintaining the immune homeostasis. Soon after its discovery and successful cloning, potential use of IL-1Ra as a therapeutic drug was examined. The first trials aimed to treat of RA, where IL-1 is a known contributor. Treating patients with recombinant human IL-1Ra administered subcutaneously led to remarkable improvement in joint swelling and mobility (Bresnihan et al., 1998; Campion et al., 1996). Since then, recombinant IL-1Ra termed anakinra (sold under the name Kineret), has been FDA-approved for the therapy of RA, DIRA, and NOMID. In these diseases, anakinra is rarely used as a monotherapy and is usually prescribed in combination with standard immunosuppressive treatment with methotrexate, corticosteroids, NSAIDs, or other biologics (e.g., anti-TNF drugs) (Arnold et al., 2022)*. The use of anakinra has recently been authorized for the emergency use in COVID-19 patients to treat pneumonia based on the results of SAVE-MORE phase 3 study (Kyriazopoulou et al., 2021). It is prescribed off-label for the treatment of rheumatic conditions besides RA (e.g., systemic sclerosis, osteoarthritis, gout, ankylosing spondylitis), diabetes, or Alzheimer's disease (Batista et al., 2021; Dinarello & Van Der Meer, 2013). Furthermore, anakinra can improve tissue remodeling in patients with post-myocardial infarction and reduce future risk of heart failure (Abbate et al., 2008; Moroni et al., 2024). The possibility of utilizing anakinra for other conditions is presently being explored. What sets Anakinra apart from other biologics is its remarkable safety (Dinarello & Van Der Meer, 2013)*. In RA treatment, the most common adverse effect was site-of-injection reactions, otherwise the drug is very well tolerated across patient groups (Cohen et al., 2004).

The contribution of pro-inflammatory IL-1 family members to such a broad spectrum of diseases comes as no surprise given their ability to rapidly activate and modulate the immune response. Not only can they initiate inflammation, but they also sustain it for extended periods of time, affecting a wide variety of immune and non-immune cells. Among the genes induced by agonistic IL-1 cytokines are prostaglandins which increase blood flow to the affected area, acute phase proteins, chemokines (e.g., IL-8, CCL2, CXCL10) which recruit leukocytes to the inflamed tissue, and cytokines – mainly IL-6 and TNF – which regulate T cell polarization, cell survival, and cell death (Boraschi, 2022)*.

1.3. TNF pathway is a target of many therapies

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine originally discovered as a serum factor released following LPS stimulation. Later it was recognized as the factor responsible for inducing death in tumor cells after infection and was given its name. It is primarily produced by activated immune cells, such as macrophages, T cells, and NK cells, with smaller amounts coming from non-immune cells like fibroblasts and endothelial cells (Carswell et al., 1975; Epstein Shochet et al., 2017). TNF is synthesized as a membrane-bound trimeric protein which is subsequently cleaved from the membrane to release the 17kDa soluble TNF (Black et al., 1997). Upon release, TNF can bind to TNF receptor 1 (TNFR1), leading to the trimerization of the receptors and cellular response activation. Alternatively, membrane-bound TNF can bind to TNFR2. While TNFR1 is expressed ubiquitously, the expression of TNFR2 is limited to immune cells and only a few non-immune cell types. Unlike TNFR1, TNFR2 lacks the intrinsic ability to trigger cell death and its function is more homeostatic (Medler & Wajant, 2019). Consequently, in the context of inflammatory responses TNFR1 plays a dominant role. TNFR1 signaling induces the formation of two distinct complexes: receptor-associated complex I and cytosolic complex II. Complex I forms rapidly after TNF binding and consists of TNFR1-associated death domain protein (TRADD), receptor-interacting protein kinase 1 (RIPK1), and ubiquitin ligases TNF receptor-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis 1 and 2 (cIAP1/2). TRAF2 and cIAP1/2 then decorate the complex with K63- and K11-linked ubiquitin chains and attract LUBAC which adds M1-Ub. The scaffold of mixed ubiquitin chains then serves as a docking site for the TAK1 and IKK complexes to activate the MAPK and NF-κB pathways, thereby initiating the inflammatory response through cytokine production (Haas et al., 2009). Complex II, on the other hand, is formed when receptor-bound RIPK1 phosphorylates itself and dissociates from the receptor. In the cytosol, RIPK1 binds Fas-associated death domain protein (FADD) and caspase 8. Subsequent activation of caspase 8 within complex II initiates apoptosis or cleavage of gasdermin D (GSDMD) to instigate pyroptosis (Micheau & Tschopp, 2003; Orning et al., 2018). Alternatively, RIPK1 can bind and activate RIPK3 and mixed lineage kinase domain-like (MLKL). RIPK3-mediated phosphorylation of MLKL triggers its oligomerization and insertion into the plasmatic membrane. The subsequent influx of ions and water through the pores results in necroptosis (Sun et al., 2012). The ability to induce cell death renders the TNF pathway a potent immune modulator vital for host protection, yet it can have dire consequences if dysregulated.

Uncontrolled TNF production and signaling are implicated in numerous autoinflammatory and autoimmune diseases, such as RA, plaque psoriasis (PPs), psoriatic arthritis (PsA), ankylosing spondylitis (AS), or inflammatory bowel disease (IBD). Blockers of this pathway are therefore widely used in the treatment of such illnesses. By far the most commonly used are antibodies or soluble receptors aimed at binding free or membrane-bound TNF and preventing the association with TNFR1 or TNFR2. Currently there are five approved drugs aiming at the inhibition of the TNF pathway: etanercept, infliximab, adalimumab, certolizumab, and golimumab. Etanercept was the first anti-TNF drug to be approved in 1998 for the treatment of RA. It is a recombinant protein containing the extracellular part of TNFR2 fused to a human IgG1 Fc fragment (Moreland et al., 1997). In terms of structure, etanercept stands out from other anti-TNF biologics because it is distinct as the sole non-antibody in this category. Unlike the other antibody-based biologics, etanercept is capable of binding another TNF superfamily member, lymphotoxin alpha(Neregard et al., 2014). Therefore, it can potentially influence immune processes in a broader range. In addition to RA, etanercept is used in patients with AS, psoriasis, sJIA, and PsA. Infliximab, a chimeric mouse/human IgG1, is prescribed for AS, PPs, PsA, RA, Crohn's disease, and ulcerative colitis. Adalimumab and golimumab, both human IgG1, are approved for AS, PsA, RA, and ulcerative colitis (S. Zhao et al., 2018)*. Certolizumab pegol is a humanized mouse Fab fragment fused to polyethylene glycol (PEG) to enhance the stability of the molecule. The absence of the Fc portion on certolizumab ensures there is no risk of triggering antibody-dependent cytotoxicity or complement activation. It is indicated for AS, PsA, RA, and Crohn's disease treatment (Rivkin, 2009). There are continuing attempts to improve on the current therapeutics. A wide array of biosimilars of etanercept, infliximab, and adalimumab have been developed and tested. In the efficacy and safety assessment, these drugs usually performed similarly compared to their predecessors (Komaki et al., 2017). Despite the advancements, challenges remain, including high production costs, complex administration, and increased susceptibility to opportunistic infections among patients (Feldmann & Maini, 2015). To address these issues, novel drug designs are exploring small molecule inhibitors through *in silico* studies (Kwak et al., 2020; Saddala & Huang, 2019).

The ability to trigger cell death is a significant aspect of TNF in disease pathology. Multiple checkpoints within TNFR1 signaling regulate TNF-induced cell death. Currently, three major

checkpoints are recognized: (*i*) the IKK, (*ii*) NF-κB, and (*iii*) caspase 8 checkpoints. If any of them is removed, TNF signaling is shifted from survival to death. (*i*) The IKK checkpoint, occurring at the receptor level in complex I, depends on IKK kinases: the canonical IKKα and IKKβ, and non-canonical TANK-binding kinase 1 (TBK1) and its homolog IKKε. However, IKKε seems of lesser importance as its deletion doesn't cause any observable phenotype (Hemmi et al., 2004). After recruitment to the complex, the IKK kinases phosphorylate RIPK1 on multiple residues to inactivate it and prohibit its dissociation from the receptor into the cytosol. IKKα/β and TBK1/IKKε act in a non-redundant manner, as the inhibition of either sensitizes cells to TNF-induced cell death (Dondelinger et al., 2015; Lafont et al., 2018). A common phosphorylation site on RIPK1 targeted by the IKK kinases is serine 25 (S25). Genetically mimicking the phosphorylation of S25 protects against RIPK1 kinase-dependent apoptosis and necroptosis while preserving the activation of MAPK and NF-κB pathways (Dondelinger et al., 2019). Failure of the IKK checkpoint leads to the formation of cytoplasmic complex II and cell death. (*ii*) The NF-κB checkpoint is the NF-κB-induced transcription of pro-survival genes. The most prominent being cellular FLICE-like inhibitory protein (cFLIP). Its binding to caspase 8 in complex II prevents caspase 8 cleavage and promotes cell survival (Tsuchiya et al., 2015)*. Knock-down of cFLIP or inhibition of protein synthesis by cycloheximide leads to the induction of apoptosis (Wang et al., 2008). (*iii*) Caspase 8 has a surprising pro-survival function of its own. This property of caspase 8 lies in its ability to cleave RIPK1 in order to prevent the RIPK1-mediated activation of RIPK3 and necroptosis (Newton et al., 2019). There are more regulators of TNF-induced cytotoxicity still being discovered. Recently, autophagy related protein 9A (ATG9A) was described to promote lysosomal degradation of caspase 8-containing complex II through the recognition of M1-Ub chains on RIPK1 (Huyghe et al., 2022). Additionally, the Janus kinase 1 (JAK1) and SRC were reported to be capable of phosphorylating RIPK1 or caspase 8 (Tsang et al., 2016; Tu et al., 2022). These observations suggest a complex interplay of multiple signaling pathways in preventing cell death.

1.4. TBK1 is a kinase with various functions

TBK1 is a serine/threonine protein kinase from the IKK kinase family that plays a crucial role in immune defense, cellular metabolism, autophagy, and cell death. Best known for inducing the transcription of interferon-stimulated genes (ISGs) and production of interferon β (IFNβ), TBK1 is acting downstream of key signaling pathways of antiviral immunity such as TLR3, TLR4, mitochondrial antiviral signaling protein (MAVS), and stimulator of interferon genes (STING). Upon activation by the above-mentioned receptors, TBK1 phosphorylates interferon regulatory factor (IRF) 3 and IRF7, allowing them to translocate into the nucleus and initiate transcription of ISGs (Fitzgerald et al., 2003).

Furthermore, TBK1 can influence autophagy and metabolism through acting on a key regulator of cellular metabolism, mechanistic target of rapamycin (mTOR). However, the effects are often contradicting and most likely cell type specific. One study shows that TBK1 activation by chronic inflammation mediated by STING suppresses mTOR complex 1 (mTORC1). In this model, affected mice had lower body mass compared to wild-type, and mouse-derived cells showed decreased respiration rate and increased glycolysis (Hasan et al., 2017). Others reported that TBK1 has the opposite effect following epidermal growth factor or TLR3 or TLR4 stimulation (Bodur et al., 2018). These contrasting reports may be dependent on whether TBK1 interacts with and phosphorylates Raptor, a regulatory component of mTOR in mTORC1, or with AKT, which then phosphorylates mTOR within mTORC1. Phosphorylation of Raptor then has inhibiting effects, while it has an activating effect on mTOR (Antonia et al., 2019). Nonetheless, regulation of this mechanism remains enigmatic.

The importance of TBK1 in the regulation of autophagy is becoming more evident. For example, activation of the autophagy receptor sequestosome 1 (SQSTM1, or p62) by phosphorylation limits the duration of STING signaling. Activated SQSTM1 can recognize ubiquitinylated STING and navigate it into the phagosome (Prabakaran et al., 2018). Another study reported that IRF3 could not undergo autophagic degradation in the absence of TBK1 (Wu et al., 2021). More of TBK1 targets that play a role in the regulation of autophagy are still being discovered. Generally, the loss or inactivation of TBK1 leads to defects in autophagy and can also contribute to neurodegenerative diseases (Duan et al., 2019; Freischmidt et al., 2015). Owing to the influence it has on metabolism and autophagy, TBK1 is associated with tumorigenesis. Even

though no mutations in TBK1 have been found in tumors, its overactivation or aberrant activation within them was observed and can promote cancer growth (Runde et al., 2022)*.

In order to connect many different pathways, TBK1 interacts with a broad spectrum of adaptor proteins. TLR3 and TLR4, which recognize nucleic acids in endosomes and native viruses in the extracellular space, respectively, recruit TBK1 via an adaptor protein TIR domain-containing adaptor inducing IFN (TRIF) (Sato et al., 2003). The transcription of IRF3 response genes following TLR3 or TLR4 stimulation is dependent on TRIF-recruited TBK1 (McWhirter et al., 2004). Signals from RIG-I and MDA5 receptors, which detect viral RNAs in the cytosol, converge onto MAVS. After its binding with RIG-I/MDA5, MAVS starts to aggregate on the mitochondrial membrane and recruit TRAF family proteins which in turn promote TBK1 binding and activation (Liu et al., 2013). Following the sensing of cytosolic double-stranded DNA, cyclic GMP-AMP synthase (cGAS) converts DNA into the second messenger cGAMP which activates STING to recruit TBK1 in order to phosphorylate IRF3 and IRF7 (Tanaka & Chen, 2012).

Even though TBK1 is involved in numerous processes, its main role is preventing TNF-induced cell death (Fig. 2). Genetic deletion of TBK1 in C57BL/6 mice is embryonically lethal by E14.5 due to extensive liver apoptosis (Bonnard et al., 2000). Mice expressing a catalytically inactive truncated version of TBK1 display immune cell infiltrates in tissues, high levels of circulating blood monocytes, and lower median survival than their wild-type (WT) littermates (Marchlik et al., 2010). Patients with mutated TBK1 develop chronic autoinflammation resulting from aberrant TNF-mediated cell death (Taft et al., 2021). This is underscored by previous observation that TBK1 inhibits cell death by RIPK1 phosphorylation (Lafont et al., 2018). The recruitment of TBK1 to the TNF-RSC was suggested to be mediated by two adaptors: TRAF family member-associated NF-κB activator (TANK) and 5-azacytidine-induced protein 2 (AZI2). The precise mechanism and role of these adaptors in TNFR1 signaling *in vivo* has been elusive. Mice deficient in either TANK or AZI2 were born in normal frequency and appear healthy. However, TANK knockout (KO) mice developed glomerulonephritis over time, stemming from aberrant TLR signaling. This condition was alleviated by MyD88 ablation (Kawagoe et al., 2009). In comparison, AZI2 KO exhibit no observable phenotype (Fukasaka et al., 2013).

Our laboratory developed a mouse model with a combined deletion of TANK and AZI2. Strikingly, these mice were born in a sub-Mendelian ratio, and developed a severe autoinflammatory disorder, which is in part mediated by TNFR1-induced cell death. I participated in the manuscript describing these mice and the role of TANK and AZI2 in the context of TNF-RSC, which is currently waiting for submission.

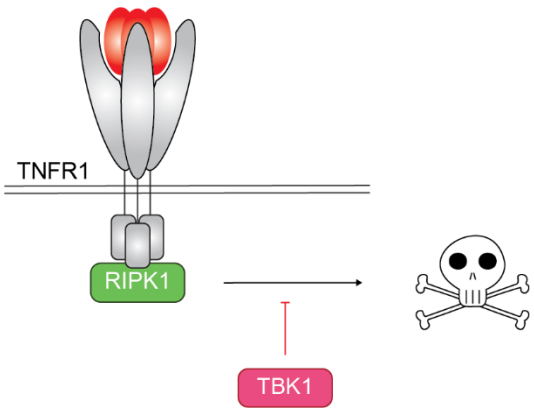


Fig. 2. TBK1 inhibits TNF-mediated cell death

The kinase TBK1 prevents the dissociation of RIPK1 from the receptor-associated complex I and the transition to the death-inducing cytosolic complex II.

1.5. TBK1 in MyD88 signaling complex

Although the role of TBK1 in inducing IFNβ via TRIF following TLR3 and TLR4 stimulation is well understood, the function of TBK1 downstream other TLRs is not so clear. Multiple reports have suggested IFNβ induction by TLR2, which exclusively utilizes MyD88. However, this exclusivity was challenged by a study showing *Listeria* infection triggering IFNβ production in a TLR2 and TRIF-dependent manner. Another possible explanation is a cooperation between TLR2 and TLR4 (Aubry et al., 2012). Additionally, a different study suggests distinct roles for MyD88 in different cellular compartments (Dietrich et al., 2010). Moreover, TBK1 and MyD88 were found to be indispensable for activating inducible nitric oxide synthase (iNOS) and NO production in response to TLR2 stimulation (Kulsantiwong et al., 2017). TBK1 was also shown to influence myeloid development via mTOR and IRF7 activation during TLR2-recognized infection (Bono et al., 2022). Furthermore, TBK1 was identified to increase the rate of glycolysis after TLR challenge and immunoprecipitated with MyD88 following stimulation of murine BMDMs with LPS (TLR4), Pam3CSK4 (TLR1/2), and R848 (TLR7/8) (Tan & Kagan, 2019).

Despite these advances, the role of TBK1 in MyD88 signaling complex remains enigmatic.

2. Aims of the thesis

This thesis aimed to identify new regulators of the MyD88 signaling complex. For that purpose, I first analyzed the composition of the MyD88 signaling complex formed upon IL-1 stimulation by biochemical methods and mass spectrometry. Among the new components of the complex was discovered kinase TBK1.

Next, I aimed to identify the role of TBK1 in this signaling complex and how it is recruited. This led to the identification of TANK and AZI2 as critical regulators of TBK1 activation upon IL-1 stimulation. To verify the importance of these two adaptors in IL-1 signaling, I employed TANK and AZI2 deficient mice available in our laboratory and showed that the combined deficiency of these two adaptors leads to severe autoimmunity, which can be rescued by MyD88 deletion.

Finally, since MyD88 is not a well-druggable target, I tested the possibility of rescuing the severe disease phenotype in TANK/AZI2-deficient mice by ablation of IRAK4 or its enzymatic activity.

Altogether, my data established TANK and AZI2-mediated recruitment of TBK1 to MyD88 signaling complex as a critical checkpoint preventing development of autoimmune diseases and demonstrated that IRAK4 can be targeted in the treatment of autoimmunity caused by aberrant MyD88 signaling.

3. Materials and methods

3.1. Cell lines and cell culture

All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) containing 10% Fetal Bovine Serum (FBS, Biosera) and regularly tested for Mycoplasma contamination using Mycoplasmacheck services (Eurofins Genomics). Mouse embryonic fibroblasts (MEFs) were isolated from embryos between E11.5 and E13 and subsequently immortalized by transduction with SV 40 large T antigen. ØNX-Eco cells were kindly provided by T. Brdicka (Institute of Molecular Genetics, Prague, Czech Republic).

3.2. Cell reconstitution

Coding sequences of MyD88 or IRAK4 amplified from murine ST2 WT cell lines using primers flanking the coding region with the introduction of restriction sites. The reaction was performed using Phusion polymerase (Thermo). Obtained sequences were fused to N- or C-terminal 2xStrep-3xFlag (SF) tag and inserted into pBabe vector containing GFP reporter under the SV40 promoter (empty vector was kindly provided by M. Hrdinka, University Hospital Ostrava, Czech Republic). All constructs were sequenced using LightRun Tube service (Eurofins Genomics). ØNX-Eco cells were transfected with 2 µg of pBabe-GFP containing protein sequences or with empty vector using Lipofectamine 2000 (Thermo). Following day, supernatants were collected and passed through 0.2µm filter. Obtained viruses were then added to target cells with 6 µg/ml of polybrene (Sigma) followed by centrifugation (1 260 g, 30°C, 45 min). After 24h, GFP-positive cells were sorted using BD FACSAria™ Fusion.

PCR primers	
target	sequence
EcoRI-MyD88 FWD	ataGAATTCTCTGCGGGAGACCCCCGCGT
MyD88-BglII REV	tatAGATCTTCAGGGCAGGGACAAAGCCT
MfeI-MyD88 (noSTOP) FWD	ataGAATTCgccaccATGTCTGCGGGAGACCCCCG
MyD88 (noSTOP)-BglII REV	tatAGATCTGGGCAGGGACAAAGCCTTGG
EcoRI-IRAK4 FWD	ataGAATTCAACAAGCCGTTGACACCATC
IRAK4-BglII REV	tatAGATCTTTAAGCAGACATCTCTTGTA
MfeI-IRAK4 (noSTOP) FWD	ataGAATTCgccaccATGAACAAGCCGTTGACACC
IRAK4 (noSTOP)-BglII REV	tatAGATCTAGCAGACATCTCTTGTAGCA

3.3. Analysis of signaling pathways and immunoprecipitation

MEFs were washed and incubated in serum-free DMEM or serum-free DMEM containing 2μM MRT67307 (Sigma) for minimum of 30 minutes. Cells were then stimulated with murine IL-1α (Peprotech) or FSL-1 (InvivoGen) for indicated times. At the end of stimulation, cells were lysed on ice with lysis buffer consisting of 30mM Tris (Sigma), pH 7.4, 120 mM NaCl (Sigma), 2mM KCl (Sigma), 2mM EDTA (VWR), 10% glycerol (Sigma), 10mM chloroacetamide (Sigma), cOmplete protease inhibitor cocktail (Roche), PhosSTOP tablets (Roche) and containing 1% n-Dodecyl-β-D-Maltoside (DDM, Thermo). The lysates were incubated on ice for 30 minutes followed by centrifugation (21 000 g, 2°C, 30 min). Cleared lysates were mixed with sample buffer containing sodium dodecyl sulfate (SDS, VWR) and 50mM dithiothreitol (DTT, Sigma), and heated (92°C, 3 min).

For isolation of the MyD88 signaling complex, cells reconstituted with SF-tagged proteins were stimulated as indicated and lysed as described above. After the centrifugation step, the cleared lysates were subjected to immunoprecipitation via ANTI-FLAG® M2 Affinity Gel (Sigma) overnight at 4°C. The beads were then washed in 0.1% DDM lysis buffer three times, resuspended in sample buffer containing SDS and 50mM DTT, and heated (92°C, 3 min).

Samples were separated on SDS-PAGE and analyzed by immunoblotting. Experiments were performed at least three times.

3.4. Tandem affinity purification and mass spectrometry analysis

Cells reconstituted with SF-tagged proteins and stimulated with 50 ng/ml of IL-1α for indicated times. At the end of stimulation, cells were lysed and subjected to immunoprecipitation as described above. After the overnight incubation, the beads were washed three times with 0.1% DDM lysis buffer and resuspended in 1% DDM lysis buffer containing 5 mg/ml of 3X FLAG® Peptide (Sigma) and incubated overnight at 4°C. The following day, the samples were centrifuged (2 350 g, 2°C, 2 min) to sediment the beads. The supernatant was subjected to immunoprecipitation via Strep-Tactin® Sepharose® resin (IBA Lifesciences) overnight at 4°C. The following day, the samples were centrifuged (2 350 g, 2°C, 2 min), washed two times in 0.1% DDM lysis buffer, then two times with no-detergent lysis buffer.

Mass-spectrometry analysis was performed at the OMICS Proteomics and Metabolomics laboratory, Faculty of Science of the Charles University.

Obtained raw data were analyzed using MaxQuant version 2.4.13.0 where they were searched against the murine UniProt database (https://www.uniprot.org/ downloaded 22.12.2020).

3.5. RT-qPCR

MEFs were stimulated in the presence or absence of 2μM MRT67307 (Tocris) with 50 ng/ml IL-1α for 2 and 4 hours. Total RNA was isolated using Quick-RNA MiniPrep (Zymo Research) followed by DNAse I (Zymo Research) treatment according to manufacturer protocol. 1 μg of RNA was transcribed to cDNA using LunaScript RT SuperMix (New England Biolabs). Quantitative PCR prepared with Luna Universal qPCR Master Mix (New England Biolabs) and detected by LightCycler 480 II (Roche). The PCR reaction was executed as follows: 1. initial denaturation (95°C, 1 minute), 2. amplification (95°C, 15 seconds), 3. annealing/elongation (60°C, 30 seconds), 4. melting curve analysis Data was collected at step 3. Steps 2-3 were repeated 45 times. Raw data were analyzed using second derivation analysis in the LightCycler 480 Software 1.5. The output Ct values were normalized to GAPDH and quantified.

RT-qPCR primers	
target	sequence
Tnf FWD	CCACCACGCTCTTCTGTCTAC
Tnf REV	AGGGTCTGGGCCATAGAACT
Il6 FWD	ATGGATGCTACCAAACTGGAT
Il6 REV	TGAAGGACTCTGGCTTTGTCT

3.6. Mice

All mice were housed in compliance with the laws of the Czech Republic in a specific-pathogen-free facility with a 12h/12h light/dark cycle with a temperature of 22 ± 1°C and a relative humidity of 55 ± 5 % at the Institute of Molecular Genetics of the Academy of Sciences (IMG). The mice were provided with a standard laboratory rodent diet and water. All protocols for the animal experiments were approved by the Resort Professional Commission for Approval of Projects of Experiments on Animals of the Czech Academy of Sciences, Czech Republic. Both males and females were used in all experiments.

The murine strains were generated at the Czech Centre for Phenogenomics (CCP) at IMG. To introduce targeted indel mutations into the *Tank*, *Azi2*, *Myd88*, and *Irak4* genes, a mixture containing Cas9 mRNA (100 ng/ml) and target-specific sgRNA (50 ng/ml) was microinjected into zygotes derived from C57BL6/N mice. For the preparation of the IRAK4 KD allele harboring the

inactivating K213-214A mutation, a single-stranded DNA repair template (10 μM) was included. The acquired embryos were subsequently implanted into foster mothers.

Genomic DNA was isolated from tail biopsies of 3-week-old mice, and the DNA sequence surrounding the CRISPR/Cas9 recognition site was amplified by PCR using Phusion polymerase (Thermo). The amplified DNA was sequenced using the LightRun Tube service (Eurofins Genomics). Founders were backcrossed onto the C57BL/6J background for at least three generations. Primers for genotyping were designed to distinguish between the WT and modified alleles with the use of Combi PPP Master Mix (Top-Bio).

3.7. Histology

Histology samples were processed at the Histopathology unit of CCP as a part of routine phenotyping service. Mice were sacrificed between 20-24 weeks of age. Tissue samples from the skin, liver, kidneys, spleen, white adipose tissue, pancreas, skeletal muscle, and lungs were isolated and fixed in 4% formaldehyde overnight and transferred to 70% ethanol the following day. The samples were processed by the Leica ASP6025 Vacuum Tissue Processor according to the program Standard processing overnight, embedded in Paraplast X-tra (Leica Biosystems) using the Tissue Embedding Station Leica EG1150. The sample blocks were cut into 5 μm thick sections by the Leica Fully Motorized Rotary Microtome RM2255-FU. The obtained slides were stained with Hematoxylin H (Biognost) and Eosin Y (Carl Roth) using the Leica ST5010-CV5030 Stainer Integrated Workstation. Congo red was stained with the Congo red Staining Kit (Roche) in Ventana Staining Machine for Special Stains (Roche) according to manufacturer manuals. Picrosirius red was stained using Weigert's Iron Hematoxylin Set and Direct Red 80 (Sigma-Aldrich) according to manufacturer protocol. The slides were imaged using ZEISS Axio Scan.Z1 Slide Scanner.

3.8. TNF injections

8-10-week-old mice of indicated genotypes were injected with 500 ng of TNF per 1 g of mouse weight and observed closely for a period of 12 hours and sacrificed after 48 hours.

3.9. Antibodies

Primary WB antibodies				
Name	Isotype	Manufacturer	Cat. #	Dilution
TBK1/NAK	Rabbit	Cell Signaling	3013	1000x
TBK1 Antibody (108A429):	Mouse IgG1	Santa Cruz	sc-52957	1000x
NEMO (DA10-12)	Mouse IgG1	Cell signaling	2695	1000x

FLAG (M2)	Mouse IgG1	SIGMA	F3165	10 000x
TBK1	Rabbit	Cell Signaling	3013S	1000x
P-TBK1 (S172) (D52C2)	Rabbit	Cell Signaling	5483S	1000x
TANK	Rabbit	Cell Signaling	2141S	500x
NAP1 (Azi2)	Rabbit	Abcam	ab192253	500x
TRAF6	Rabbit	Abcam	ab40675	1000x
Phospho-SAPK/JNK (Thr183/Tyr185) (98F2)	Rabbit	Cell Signaling	4671S	1000x
Phospho-IKKε (Ser172) (D1B7)	Rabbit	Cell Signaling	8766S	1000x
Phospho-IκBα (Ser32/36) (5A5)	Mouse IgG1	Cell Signaling	9246S	1000x
Phospho-IKKα/B (Ser176/180) (16A6)	Rabbit	Cell Signaling	2697S	1000x
IκBα Antibody	Rabbit	Cell Signaling	9242S	1000x
Phospho-p38 (Thr180/Thr182) (28B10)	Mouse IgG1	Cell Signaling	9216	1000x
β-Actin (8H10D10)	Mouse IgG2b	Cell Signaling	3700	1000x
MyD88 (D80F5)	Rabbit	Cell Signaling	4283S	1000x
MyD88	Goat IgG	R&D	AF3109-SP	1000x
NEMO (FL-419)	Rabbit	Santa Cruz	sc-8330	1000x
A20 (A-12)	Mouse IgG2a	Santa Cruz	sc-166692	1000x
IRAK4	Mouse IgG2a	Santa Cruz	sc-374349	1000x
pIRAK4 (T345)	Mouse IgG1	Thermo Fisher	600-560	1000x

Secondary WB antibodies			
Name	Manufacturer	Cat. #	Dilution
Donkey Anti-Rabbit IgG (H+L) - HRP	Jackson Immunoresearch	711-035-152	10 000x
Goat Anti-Mouse IgG1 - HRP	Jackson Immunoresearch	115-035-205	10 000x
Goat Anti-Mouse IgG2a - HRP	Jackson Immunoresearch	115-035-206	10 000x
Goat Anti-Mouse IgG2b - HRP	Jackson Immunoresearch	115-035-207	10 000x
Goat Anti-Rabbit IgG Fc fragment specific - HRP	Jackson Immunoresearch	111-035-046	10 000x

3.10. Chemicals and reagents

Name	Manufacturer	Cat.#
Dulbecco's Modified Eagle Medium (DMEM) medium	Sigma	
Fetal Bovine Serum (Sounth America), Heat Inactivated	BioSera	FB-1001H
Trypsin-EDTA 10X	BioSera	XC-T1717
Penicillin/Streptomycin	BioSera	XC-A4122

n-Dodecyl-β-D-Maltoside	Thermo Scientific	89903
2-Chloroacetamide	Sigma	C0267
DL-dithiothreitol	Thermo Scientific	R0861
TEMED	VWR	443083G
Ammonium persulfate	Thermo Scientific	17874
p-Coumaric acid	Sigma	C9008
Luminol	Sigma	A8511
Powdered milk	ROTH	T145.2
Trizma base	Sigma	T6066
Glycine	VWR	101196X
EDTA.2Na dihydrate	VWR	33600.267
Tween 20	VWR	28829.296
HCl 37%	Sigma	H1758
Sodium chloride	Sigma	S9888
Glycerol	Sigma	G2025-1L
Potassium chloride	Sigma	P3911
cOmplete, EDTA-free Protease Inhibitor Cocktail	Roche	5056489001
PhosSTOP EASYpack, Phosphatase Inhibitor Cocktail Tablets	Roche	4906837001
Lipofectamine 2000	Thermo Scientific	11668019
Polybrene	Sigma	TR-1003
Combi PPP Master Mix	Top-Bio	C210
Phusion™ High-Fidelity DNA Polymerase	Thermo	F530S
Quick-RNA MiniPrep	Zymo Research	R1054
ANTI-FLAG® M2 Affinity Gel	Sigma	A2220
3X FLAG® Peptide	Sigma	F4799
Strep-Tactin® Sepharose® resin	IBA Lifesciences	2-1201-010
DNAse I	Zymo Research	E1010
LunaScript RT SuperMix	New England Biolabs	E3010
Luna Universal qPCR Master Mix	New England Biolabs	M3003
Precision Plus Protein Dual Color Standards	Bio-Rad	1610374
FSL-1	InvivoGen	tlrl-fsl
mIL-1α	Peprotech	211-11A
MRT67307	Tocris	5134

3.11. Statistics and data analysis

The statistical analyses indicated were performed using GraphPad software. In the case of unpaired t-test, the normality was assessed using the Shapiro-Wilk test.

4. Results

4.1. TBK1 is associated with the IL-1 receptor signaling complex upon stimulation

To investigate the composition of the MyD88 signaling complex, we reconstituted *Myd88* knockout (KO) mouse embryonic fibroblasts (MEF) with either N- or C-terminal Strep-Flag tagged MyD88 (SF-MyD88 or MyD88-SF, respectively) or with control vector expressing GFP-SF. Subsequently, to determine the functionality of exogenously expressed MyD88 in reconstituted cells, we stimulated WT, *Myd88*^{KO}, and SF-MyD88-expressing *Myd88*^{KO} MEFs with IL-1α for 5, 15, and 30 minutes and analyzed the activation of signaling pathways by immunoblotting. As expected, *Myd88*^{KO} MEFs were unable to activate signaling pathways, which was rescued by the re-expression of SF-MyD88 (Fig. 3A). The slight decrease in the strength of signaling in SF-MyD88-expressing cells may be caused by the overexpression of MyD88 which could lead to a slight destabilization of the signaling complex. Next, we stimulated SF-MyD88-expressing MEFs with IL-1α for 15 minutes and isolated IL-1R signaling complex (IL-1R-SC) through tandem-affinity purification via sequential Flag and Strep immunoprecipitation from cellular lysates. Unstimulated SF-MyD88-expressing MEFs were used as a control. The composition of the isolated complex was then analyzed by mass spectrometry (mass-spec). The analysis revealed the association of several known constituents of the complex, such as IRAK4 or IRAK2 and new component, the kinase TBK1, which has so far not been described in the IL-1R-SC (Fig. 3B). We validated these results by stimulating SF-MyD88, MyD88-SF, and GFP-SF-expressing MEF for 15 minutes and subjecting cellular lysates to Flag immunoprecipitation. Immunoblot analysis confirmed the association of TBK1 with the IL-1R-SC and its activation within the complex in cells reconstituted with SF-MyD88 and MyD88-SF, but not with control GFP-SF (Fig. 3C). The apparent weaker association in the MyD88-SF-expressing MEFs may be due to steric interference of the SF tag with the TIR domain of MyD88 and weaker binding of MyD88 to the receptor. To address this problem, we also performed immunoprecipitation of IRAK4, a protein downstream of MyD88. Analogously to MyD88 reconstitutions, we reconstituted *Irak4*^{KO} MEFs with SF-IRAK4, IRAK4-SF, or GFP-SF, stimulated the cells with IL-1α for 15 minutes, and isolated the complex via Flag immunoprecipitation. As anticipated, TBK1 was present and activated in the complex isolated from SF-IRAK4 and IRAK4-SF-expressing cells, while we observed no association or activation in cells expressing GFP-SF. Combined, these experiments establish TBK1 as a newly identified component of the IL-1R-SC.

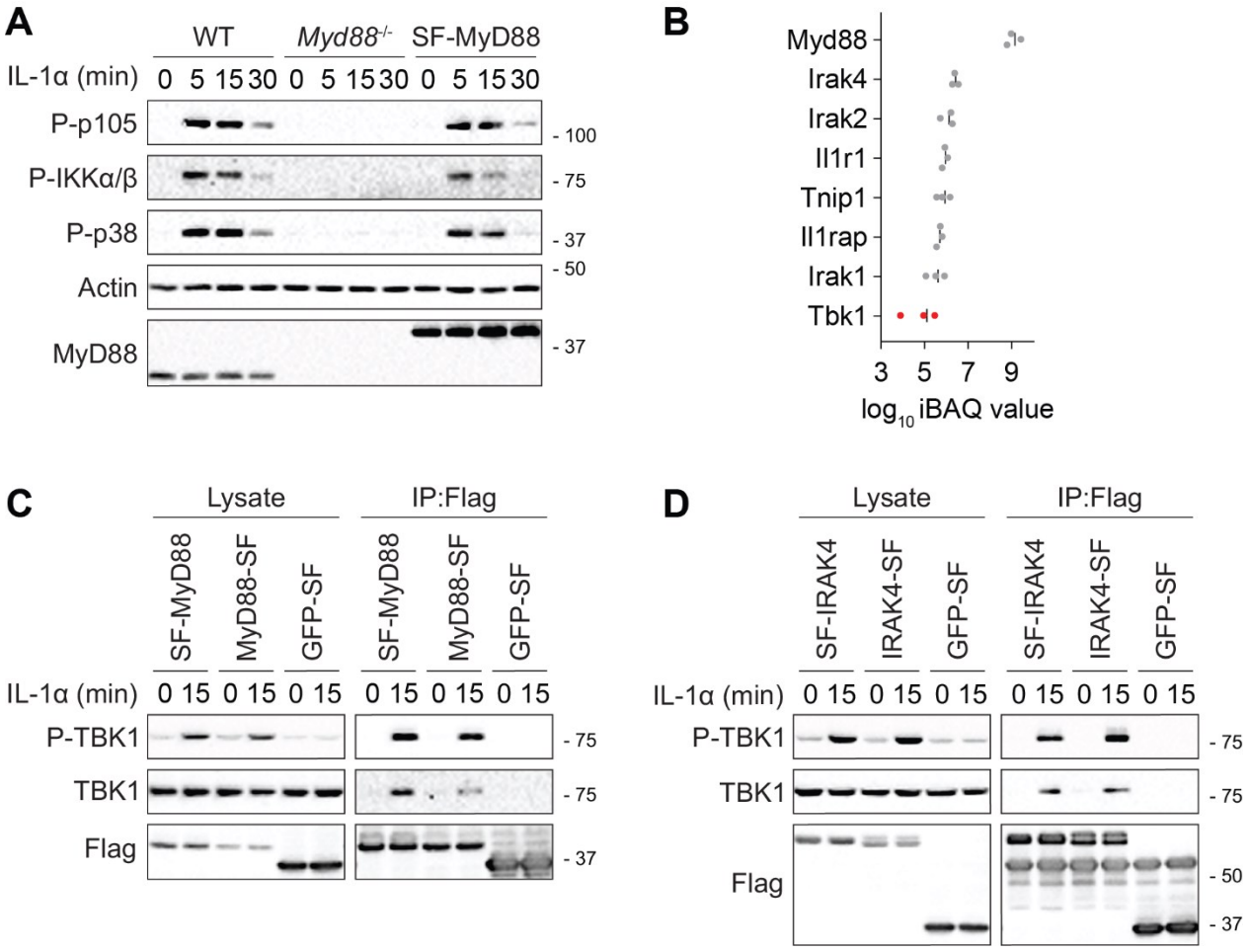


Fig. 3. Kinase TBK1 associates with the IL-1 signaling complex upon stimulation

(A) *Immunoblot analysis of lysates from MEF WT, Myd88$^{-/-}$, and Myd88$^{-/-}$ reconstituted with SF-MyD88 and stimulated with IL-1α (50 ng/ml) for indicated times.* (B) *Mass spectrometry analysis of IL-1-RSC isolated via tandem-affinity purification from lysates from MEF expressing SF-MyD88 after stimulation with IL-1α (50 ng/ml) for 15 min. Listed proteins were detected in the stimulated samples but not in the unstimulated control. Relative quantification is based on iBAQ values from three independent experiments.* (C-D) *Immunoblot analysis of lysates from MEF expressing SF-MyD88, MyD88-SF, or GFP-SF* (C) *and MEF expressing SF-IRAK4, IRAK4-SF, or GFP-SF* (D) *stimulated with IL-1α (50 ng/ml) for 15 min and subjected to Flag immunoprecipitation. Figure* (A) *is representative of three independent experiments.*

4.2. Chemical inhibition of TBK1 potentiates IL-1 signaling

To assess the role of TBK1 within the IL-1R-SC, we treated WT MEFs with the TBK1/IKKε inhibitor MRT67307 and stimulated them with IL-1α. Strikingly, chemical inhibition of TBK1 led to increased activation of MAPK and NF-κB pathways and prolonged duration of signaling (Fig. 4A-B). In line with the increased activation of pro-inflammatory signaling, the transcription of pro-inflammatory cytokines TNF and IL-6 was enhanced in cells treated with MRT67307 prior to stimulation (Fig. 4C). Even though we could not perform statistical analysis due to the small sample size, there is an observable trend that is unlikely to be due to random distribution. More experiments will be conducted in order to substantiate our results. Together, these observations indicated that TBK1 functions as negative regulator of IL-1 signaling.

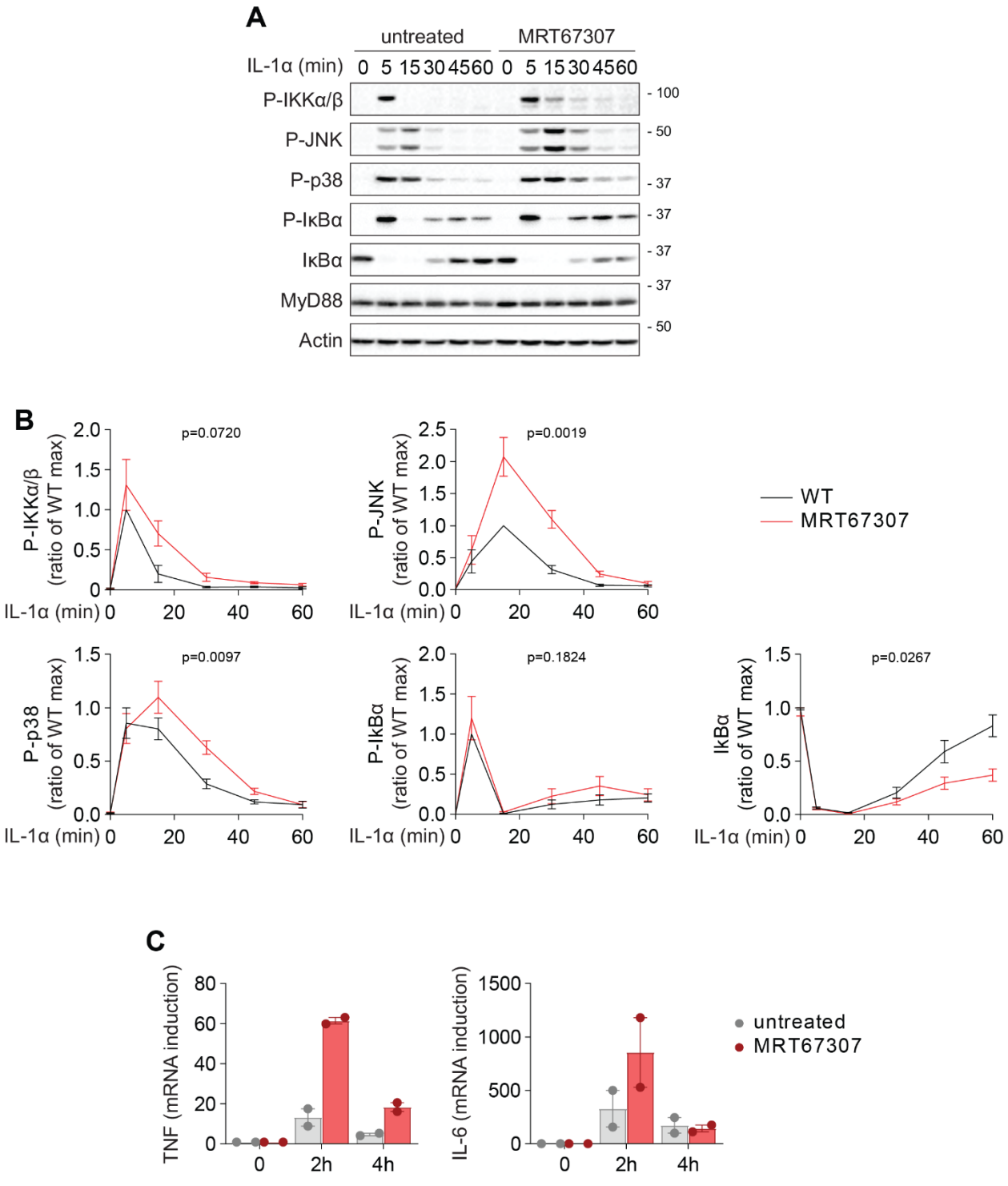


Fig. 4. Chemical inhibition of TBK1 potentiates IL-1 signaling

(A-B) *Immunoblot analysis of lysates from MEF WT treated with MRT67307 inhibitor (2 μM) or left untreated and stimulated with IL-1α (50 ng/ml) as indicated. A representative experiment* (A) *and quantification of four independent experiments normalized to maximum response of untreated*

cells, mean ± SEM (B). *Statistical analysis was based on area under curve (AUC) for each condition per experiment.* (C) *Real-time quantitative PCR analysis of induction of TNF and IL-6 transcripts in MEF WT treated or not with MRT67307 inhibitor (2 μM) and stimulated with IL-1α (50 ng/ml) for indicated times. Unpaired t-test was used for statistical analysis. Figure* (A) *is representative of four independent experiments.*

4.3. Adaptors TANK and AZI2 recruit TBK1 to the myddosome

TBK1 was previously described to bind to adaptors TANK and AZI2 (Fujita et al., 2003; Pomerantz & Baltimore, 1999), and to be recruited by them to TNFR1 and IL-17R signaling complexes (Draberova et al., 2020; Lafont et al., 2018). We wondered whether TBK1 could be recruited to the IL-1R-SC in a similar way. To test this theory, we immunoprecipitated IL-1R-SC from SF-MyD88 MEF and analyzed the composition of the complex by immunoblotting. Remarkably, both adaptors TANK and AZI2 precipitated with the complex, however with different kinetics. While TANK is recruited in the early timepoints and dissociates not long after, AZI2 is delayed in its recruitment, but remains at the receptor for the rest of the observed period (Fig. 5A).

To further investigate the role of the two adaptors in TBK1 activation, we compared the level of TBK1 activating phosphorylation upon IL-1stimulation in *Azi2*^{KO}, *Tank*^{KO}, and *Tank*/*Azi2* double knockout (DKO) MEFs. Indeed, the activation of TBK1, as measured by its phosphorylation, was decreased in *Azi2*^{KO}, *Tank*^{KO} cells and mostly gone in *Tank*/*Azi2*^{DKO} (Fig. 5B).

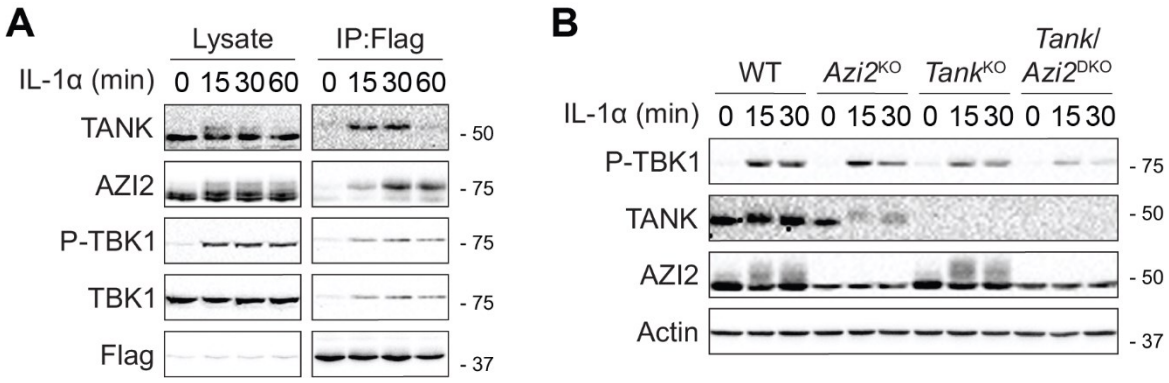


Fig. 5. TBK1 is recruited to the IL-1-RSC by adaptors TANK and AZI2

(A) *Immunoblot analysis of lysates from MEF expressing SF-MyD88 stimulated with IL-1α (50 ng/ml) as indicated and subjected to Flag immunoprecipitation.* (B) *Immunoblot analysis of lysates from MEF WT, Azi2*$^{-/-}$*, Tank*$^{-/-}$*, and Tank/Azi2*^{DKO} *stimulated with IL-1α (50 ng/ml) as indicated. Figures* (A,B) *are representative of three independent experiments*.

Immunoblot analysis of the activation of signaling pathways in *Tank*/*Azi2*^{DKO} cells showed that in the absence of TANK and AZI2, TBK1 phosphorylation is severely impaired. Moreover, there is an observable increase in the duration and strength of MAPK and NF-κB signaling, albeit the data did not reach statistical significance (Fig. 6A-B). In addition, we stimulated *Azi2*^{KO} and *Tank*/*Azi2*^{DKO} MEF with FSL-1, a TLR2/6 agonist, with a similar result (Fig. 6C). The increase in signaling in *Tank*/*Azi2*^{DKO} extent recapitulated our observations from MRT67307-treated cells, underscoring the importance of TANK and AZI2 for activating TBK1 downstream MyD88-employing receptors. Together, these findings provide compelling evidence that TANK and AZI2 recruit TBK1 to the MyD88 signaling complex to inhibit MyD88-dependent signaling.

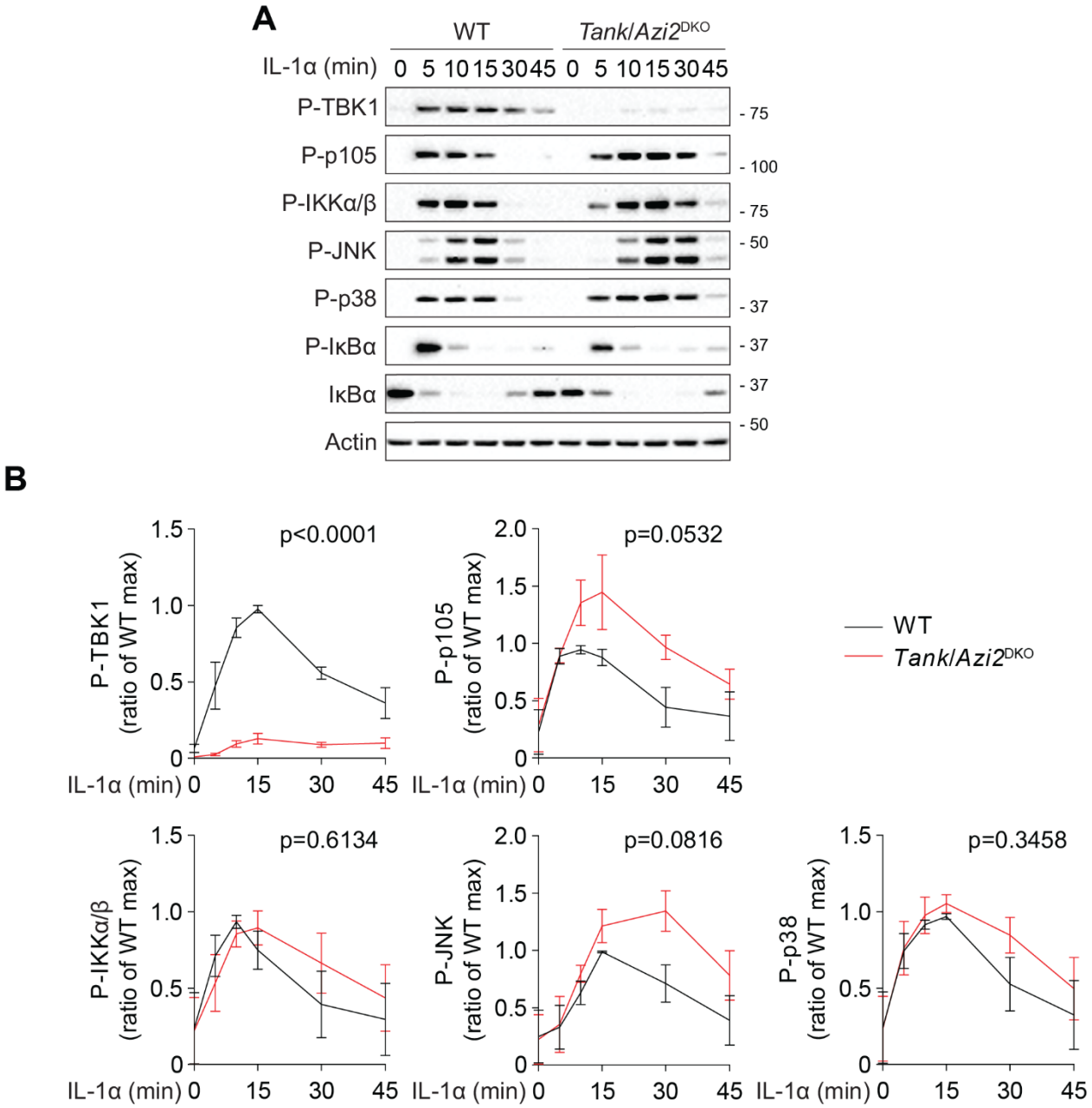

A
WT
Tank/Azi2DKO
IL-1α (min) 0 5 10 15 30 45 0 5 10 15 30 45
P-TBK1
P-p105
P-IKKα/β
P-JNK
P-p38
P-IκBα
IκBα
Actin
- 75
- 100
- 75
- 50
- 37
- 37
- 37
- 50
B
p<0.0001
p=0.0532
p=0.6134
p=0.0816
p=0.3458
P-TBK1 (ratio of WT max)
P-p105 (ratio of WT max)
P-IKKα/β (ratio of WT max)
P-JNK (ratio of WT max)
P-p38 (ratio of WT max)
IL-1α (min) 0 15 30 45
WT
Tank/Azi2DKO
C
Azi2KO
Tank/ Azi2DKO
FSL-1 (min) 0 15 30 0 15 30
P-TBK1
P-105
P-JNK
P-p38
Actin
- 75
- 100
- 50
- 37
- 50

Fig. 6. Combined deletion of TANK and AZI2 promotes IL-1 signaling

(A-B) *Immunoblot analysis of MEF WT and Tank/Azi2DKO stimulated with IL-1α (50 ng/ml) as indicated. A representative experiment* (A) *and quantification of four independent experiments normalized to maximum response of WT cells, mean ± SEM* (B)*. Statistical analysis was based on area under curve (AUC) for each condition per experiment.* (C) *Immunoblot analysis of lysates from Azi2*$^{-/-}$ *and Tank/Azi2*^{DKO} *stimulated with FSL-1 (100 ng/ml). Unpaired t-test was used for statistical analysis.*

4.4. MyD88 ablation rescues inflammation in TANK and AZI2 deficient mice

To further determine the importance of TANK and AZI2 in MyD88 signaling *in vivo*, we utilized a mouse model of double deficiency in TANK and AZI. *Tank*/*Azi2*^{DKO} mice were born in a drastically decreased ratio, suggesting partial embryonic lethality and died prematurely with a median survival of 27 weeks (Fig. 7A-B). To investigate the impact of MyD88 signaling on the phenotype of *Tank*/*Azi2*^{DKO} mice. Since *Azi2* and *Myd88* are located close to each other on the genome, we generated *Azi2*/*Myd88*^{DKO} allele in collaboration with the Czech Centre for Phenogenomics (CCP). *Azi2*/*Myd88*^{DKO} founders were then crossed to *Tank*^{KO} and we obtained *Tank*/*Azi2*/*Myd88* triple knockout (TKO) mice. Remarkably, *Tank*/*Azi2*/*Myd88*^{TKO} mice are born at the Mendelian ratio (Fig. 7A). Furthermore, the premature mortality of *Tank*/*Azi2*^{DKO} mice is significantly improved in *Tank*/*Azi2*/*Myd88*^{TKO} (Fig. 7B). The findings were confirmed on animals derived from two independent founders, D29 and D231. The likely cause of the premature death in *Tank*/*Azi2*^{DKO} mice is multiorgan failure, as suggested by histology analysis of 20-24-week-old animals. H&A staining revealed numerous infiltrations of immune cells in the skin, and severe inflammation in the kidney leading to glomerulonephritis, characterized by swollen glomeruli (Fig. 7C-D). Picrosirius red staining showed complete loss of subdermal fat and massive scarring of the liver (Fig. 7C, E). In addition, we observed extensive immune cell infiltrations in the skeletal muscles, adipose tissue, and pancreas of these animals (Fig. 7F). In contrast, there are no immune infiltrations in the skin and kidneys of *Tank*/*Azi2*/*Myd88*^{TKO}, as well as in skeletal muscle, adipose tissue, and pancreas. In addition, the subdermal fat is completely restored. Surprisingly, the liver damage in *Tank*/*Azi2*^{DKO} is not affected by the deletion of MyD88 (Fig. 7C). Together, our data suggest that the deletion of TANK and AZI2 in mice leads to the development of a severe inflammatory phenotype and dermatitis, which is largely driven by MyD88-dependent signaling.

A

parents: *Tank*$^{-/-}$/*Azi2*$^{+/-}$

Tank$^{-/-}$/*Azi2*$^{+/+}$	116 (39%)
Tank$^{-/-}$/*Azi2*$^{+/-}$	176 (59%)
Tank$^{-/-}$/*Azi2*$^{-/-}$	6 (2%)
Total	298

p<0.001

parents: *Tank*$^{-/-}$/*Azi2*/*Myd88*$^{+/-}$ (D29)

Tank$^{-/-}$/*Azi2*/*Myd88*$^{+/+}$	100 (31%)
Tank$^{-/-}$/*Azi2*/*Myd88*$^{+/-}$	172 (52%)
Tank$^{-/-}$/*Azi2*/*Myd88*$^{-/-}$	56 (17%)
Total	328

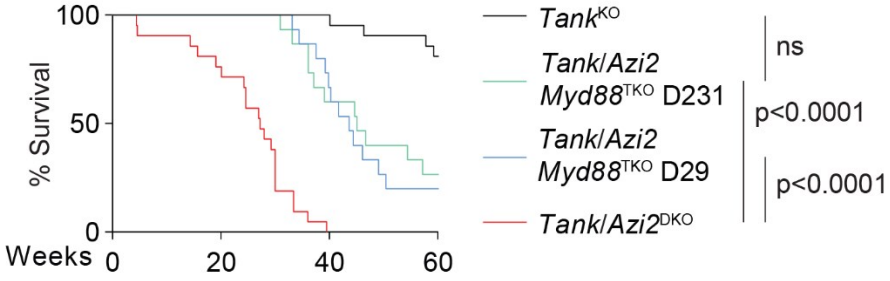
n.s.

parents: *Tank*$^{-/-}$/*Azi2*/*Myd88*$^{+/-}$ (D231)

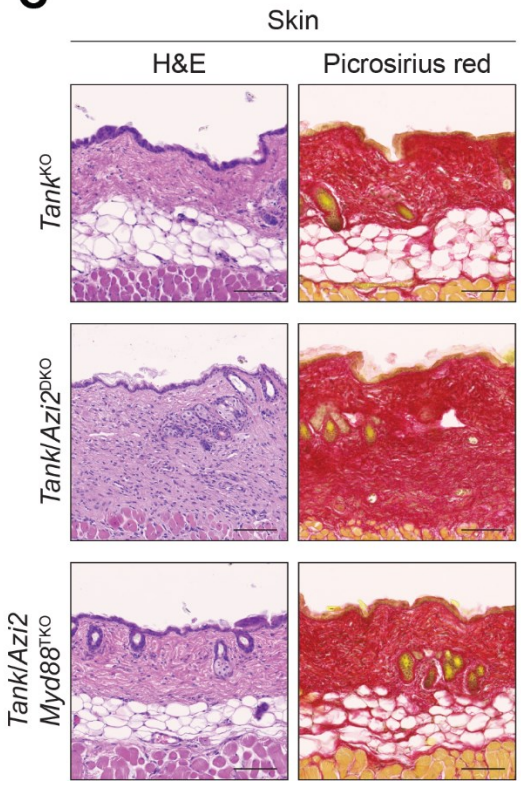
Tank$^{-/-}$/*Azi2*/*Myd88*$^{+/+}$	87 (24%)
Tank$^{-/-}$/*Azi2*/*Myd88*$^{+/-}$	209 (58%)
Tank$^{-/-}$/*Azi2*/*Myd88*$^{-/-}$	63 (18%)
Total	359

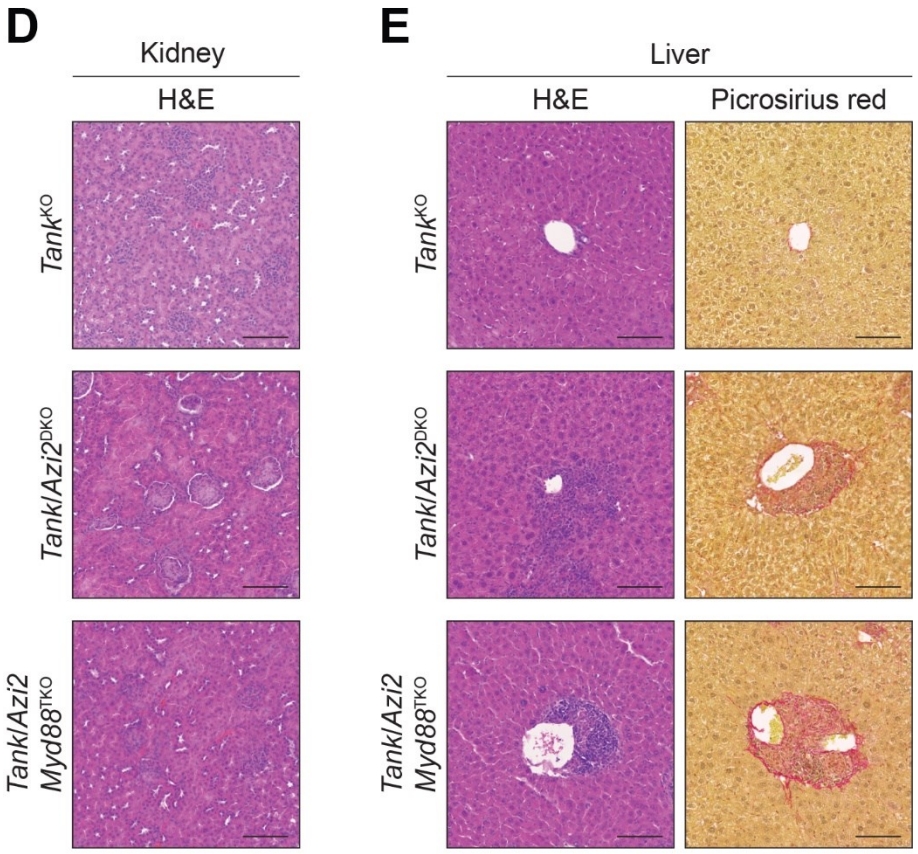
n.s.

B



C




D
Kidney
H&E
Tank^KO
Tank/Azi2^DKO
Tank/Azi2 Myd88^TKO
E
Liver
H&E
Picrosirius red
Tank^KO
Tank/Azi2^DKO
Tank/Azi2 Myd88^TKO

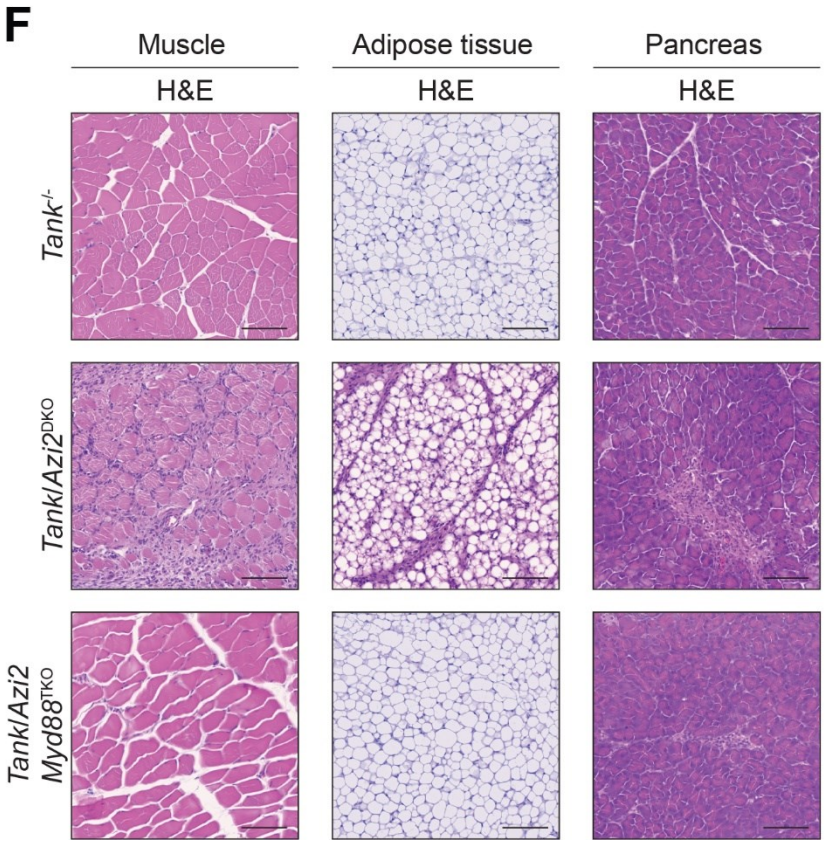

F
Muscle
Adipose tissue
Pancreas
H&E
H&E
H&E
Tank^-/-
Tank/Azi2^DKO
Tank/Azi2 Myd88^TKO

Fig. 7. Mice deficient in TANK and AZI2 develop inflammation that is rescued by MyD88 deletion

(A) *Counts of born pups of indicated genotypes. Chi-square test. ns, not significant.* (B) *Survival proportions of mice with indicated genootype (n=15 per group). Log-rank Mantel-Cox test.* (C-E) *Histology sections collected from 20-24-week-old mice of indicated genotypes stained with hematoxylin and eosin or picrosirius red. Skin* (A)*, kidney* (B)*, liver* (C)*, skeletal muscle, adipose tissue, and pancreas* (E)*. Scale bar 100 μm.*

4.5. IL-1 is a driver of pathology in TANK/AZI2 deficient mice

TBK1 was previously shown to be indispensable in preventing TNF-induced cell death, and TANK and AZI2 are the adaptors that recruit TBK1 to the TNF-RSC (Lafont et al., 2018). In previous work by our laboratory, we discovered that the absence of TANK and AZI2 renders cells more sensitive to TNF-induced cell death. Similarly, *Tank*/*Azi2*^{DKO} mice showed increased cell death in tissues, visualized by TUNEL staining (data not shown). In combination with the observation that MyD88 ablation significantly improves the condition in *Tank*/*Azi2*^{DKO} mice, we hypothesized that it is unrestricted MyD88 signaling that causes overproduction of TNF which then triggers cell death and promotes further inflammation (Fig. 8A-B). Therefore, by the ablation of MyD88 in *Tank*/*Azi2*^{DKO} mice, the production of TNF is decreased, and the inflammatory phenotype alleviated. If this hypothesis were true, *Tank*/*Azi2*/*Myd88*^{TKO} mice should still be sensitive to TNF. To test this, we injected a sublethal dose (0.5 μg/g of mouse weight) of TNF in the tail vein of *Tank*/*Azi2*/*Myd88*^{TKO} and *Tank*^{KO} as a control. In line with our assumption, *Tank*/*Azi2*/*Myd88*^{TKO} succumbed to TNF injection within a few hours, while *Tank*^{KO} remained in good condition (Fig. 8C). This supports our model where the adaptors TANK and AZI2 and the kinase TBK1 restrict inflammation on two levels. Firstly, by limiting MyD88-dependent production of TNF, and secondly by inhibiting subsequent TNF-induced cell death.

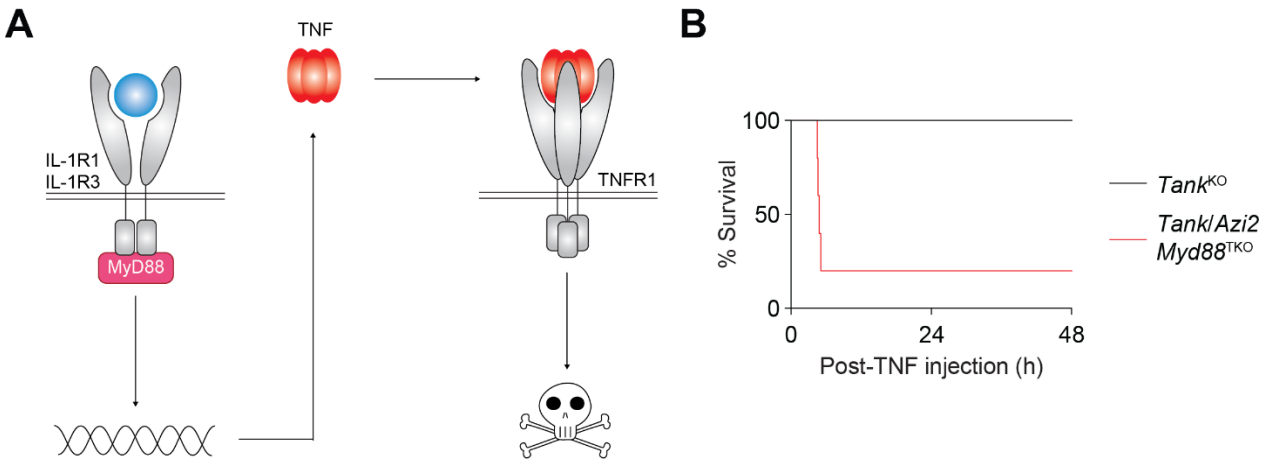


Fig. 8. MyD88 signaling enhances the pathology in Tank.Azi2DKO mice

(A) *Schematic representation of the proposed model of pathology. Uninhibited MyD88-dependent signaling causes overproduction of TNF leading to TNFR1-mediated cell death.* (B) *Survival proportions of mice with indicated genotype injected with 0.5 μg TNF per 1 g of mouse wight (n=6 per group).*

4.6. Downstream kinase IRAK4 is a potential therapeutical target in autoimmunity

Many autoimmune disorders, such as psoriasis or rheumatoid arthritis, are suspected to be triggered by infections or injury (Johnson & Jiang, 2023)*. Such stressors are also often associated with flare-ups. TLRs and IL-1R are crucial in response to invading pathogens and trauma, and they utilize the same signaling complex – the myddosome. Therefore, targeting this pathway can be potentially useful in the treatment and management of autoimmune diseases. Our model proves that limiting MyD88 signaling significantly improves dermatitis and systemic inflammation. However, since MyD88 is not easy to target with pharmaceutics, we explored the possibility of ablating or inactivating a kinase downstream of MyD88, IRAK4. Using the CRISPR/Cas9 approach, in collaboration with CCP, we generated mice lacking IRAK4 (*Irak4*^{KO}) and mice bearing a lysine 213-214 to alanine (K213-214A) mutation in the ATP-binding site of IRAK4 (IRAK4 kinase-dead, *Irak4*^{KD}) (Li et al., 2002), rendering it inactive. We subsequently crossed these mice to *Tank*^{KO} and *Azi2*^{KO} mice and obtained *Tank*/*Azi2*/*Irak4*^{TKO} and *Tank*/*Azi2*^{DKO}/*Irak4*^{KD} mice, respectively. As expected, *Tank*/*Azi2*/*Irak4*^{TKO} were born at the Mendelian ratio and displayed an improved survival rate (Fig. 9A, C). To our surprise, *Tank*/*Azi2*^{DKO}/*Irak4*^{KD} mice were born at a decreased ratio and did not show signs of improvement in their lifespan (Fig.9B, D). It appears that the removal of IRAK4 kinase activity is insufficient to prevent myddosome assembly and the activation of signaling *in vivo*. This is in line with the notion that IRAK4 has two functions in the myddosome, kinase-dependent and a scaffolding kinase-independent function (De Nardo et al., 2018).

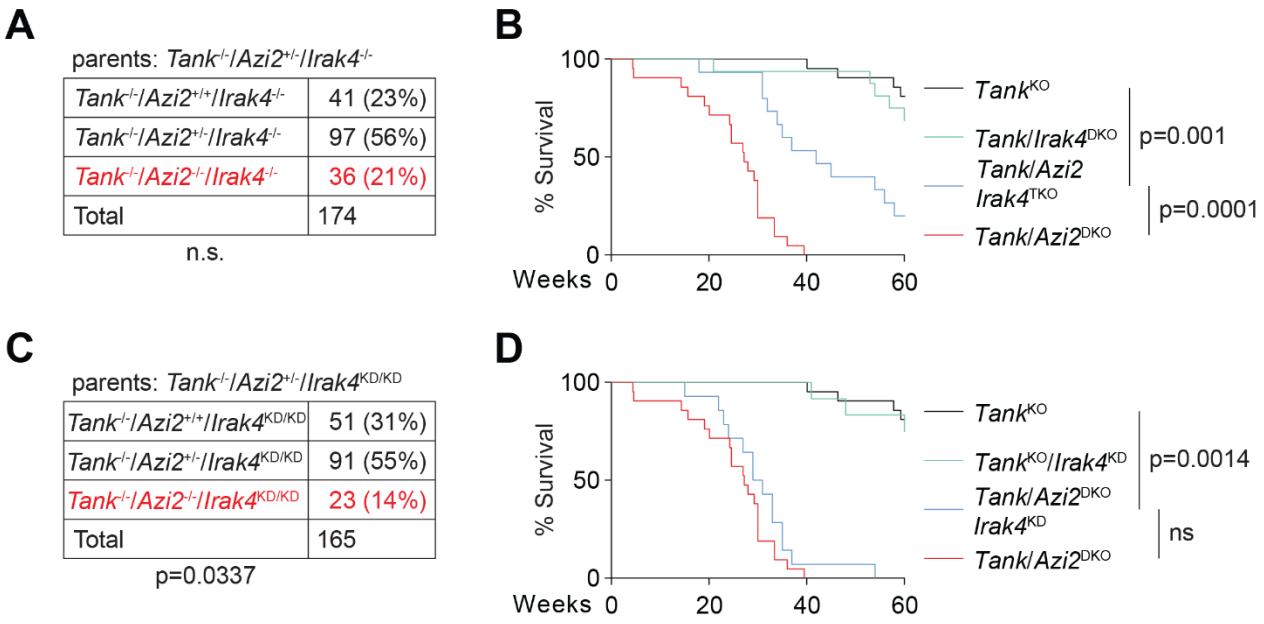


Fig. 9. IRAK4 is a potential therapeutic target in autoimmune diseases

(A-B) *Counts of born pups of indicated genotype. Chi-square test. ns, not significant.* (C-D) *Survival proportions of mice with indicated genotypes. Log-rank Mantel-Cox test.*

5. Discussion

MyD88 is a key molecule in the innate immune system, positioned downstream of infection and cell damage sensors. Despite its importance, many aspects of MyD88 signaling remain elusive. Here, we studied the composition of MyD88 signaling complex following IL-1α stimulation via mass spectrometry. We discovered that kinase TBK1 is recruited to the IL-1R-SC. This expands on recent findings, where TBK1 was shown to immunoprecipitate with MyD88 following TLR stimulation (Tan & Kagan, 2019). The same study also reported that TBK1 is necessary for TLR-induced glycolysis. However, the mechanism of how TBK1 affects the response to the stimulation of MyD88-employing receptors remains elusive. Using a chemical inhibitor of TBK1 kinase activity, we investigated the role of TBK1 in the MyD88 signaling complex. We observed that TBK1 inhibition increased in MAPK and NF-κB activation after stimulation of cells with IL-1α. Additionally, TBK1 inhibition caused enhanced transcription of pro-inflammatory cytokines. We concluded that TBK1 functions as a negative regulator of signaling within the IL-1R-SC. In order to determine whether this is a unique property of IL-1R-SC, or whether these results can be extrapolated on MyD88 signaling in general, stimulations of other MyD88-employing receptors have to be tested. As TBK1 was reported to also activate IRF7 in addition to NF-κB and MAPK after stimulation of TLR2, an exclusively MyD88-employing receptor, the composition of the MyD88 signaling complex could be more intricate (Bono et al., 2022).

Prompted by the notion that TBK1 associates with two adaptor proteins TANK and AZI2 (Draberova et al., 2020; Fujita et al., 2003; Lafont et al., 2018; Pomerantz & Baltimore, 1999), we wondered if TANK and AZI2 could also connect TBK1 to the MyD88 signaling complex. By immunoprecipitating tagged MyD88 from IL-1α-stimulated cells and subsequent immunoblot analysis, we discovered that the adaptors TANK and AZI2 indeed precipitated with the IL-1R-SC upon stimulation. Additionally, we detected TANK and/or AZI2 to a varying degree in individual experiments on mass-spec results, however, not consistently across all measurements. This may be due to the overall lower amount of protein recruited to the receptor, or destabilization of the complex during tandem-affinity purification that led to low peptide counts, insufficient for the identification of the protein. Interestingly, the recruitment kinetics of TANK and AZI2 to the IL-1R-SC were different. TANK binds in the early stages of signaling and starts disappearing from the complex around 30 minutes after stimulation. On the other hand, AZI2 is recruited much later and stays associated with the receptor for over three hours (data not shown). What exactly is behind

the difference in recruitment is yet to be clarified. Other adaptors might be regulating the differing kinetics of TANK and AZI2. It was described that TANK is recruited to the TNF-RSC with NEMO (Lafont et al., 2018). The ubiquitination state of TANK and AZI2 and availability of binding spots on ubiquitin could also influence the recruitment kinetics. Additionally, TANK and AZI2 may not be the only protein that interacts with TBK1, as SINTBAD was also shown to bind TBK1 downstream TLR3 (Ryzhakov & Randow, 2007).

Cells deficient in TANK and AZI2 displayed increased activation of MAPK and NF-κB after IL-1α, while the phosphorylation of TBK1 was largely diminished. The fact that the quantification did not reach statistical significance could be in part caused by the small number of repetitions and variable nature of western blot analysis. The experiments have to be repeated and expanded on in order to substantiate our observations. The slight activation of TBK1 in *Tank*/*Azi2*^{DKO} that is visible on the immunoblot could possibly be accounted for by phosphorylation of free cytosolic TBK1 by activated IKKα/β or other kinases. The signaling outcome of TANK and AZI2-deficient cells stimulated with IL-1α, or TLR2/6 agonist FSL-1, was analogous to that of cells treated with the TBK1 inhibitor. Combined, these experiments confirmed our hypothesis that TANK and AZI2 recruit TBK1 to the IL-1R-SC and likely to the MyD88 signaling complex in general. TANK and AZI2 are also known to bind IKKε, a kinase homologous to TBK1 (Fujita et al., 2003; Nomura et al., 2000). Therefore, the autoimmune disease observed in *Tank*/*Azi2*^{DKO} mice may also be partially caused by an inability to recruit IKKε IL-1R-SC. However, we did not detect IKKε in any run of the mass-spec nor immunoblot analysis of the MyD88 signaling complex, questioning the involvement of IKKε in this pathway. Nevertheless, it may be beneficial to test the role of IKKε in IL-1-induced signaling comparing the signaling outcomes of TBK1 and IKKε-deficient cells or cells lacking both kinases. Combined, our experiments established TBK1 and the adaptors TANK and AZI2 as new components of the IL-1R-SC, and further determined that the two adaptors recruit TBK1 to the IL-1R-SC to limit its signaling.

Double deficiency of TANK and AZI2 in mice causes a severe inflammatory phenotype, characterized by a low median survival, multiorgan inflammation, and dermatitis. Notably, *Tank*/*Azi2*^{DKO} mice display a complete loss of subdermal fat. The involvement of TBK1 in adipose tissue homeostasis has been explored, however, the results vary depending on observed cell type. Specific deletion of TBK1 in adipocytes protects mice fed with high-fat diet from diabetes by increasing energy expenditure, while there was no such effect in mice fed with standard diet (P.

Zhao et al., 2018). In contrast, Deletion of TBK1 in myeloid cells led to adipose tissue hypertrophy (Gao et al., 2022). The phenotype of *Tank*/*Azi2*^{DKO} mice is in large rescued by the ablation of MyD88. To our surprise, the liver damage observed in *Tank*/*Azi2*^{DKO} was still present in *Tank*/*Azi2*/*Myd88*^{TKO}. A possible explanation is that MyD88 deletion itself affects liver function as deletion of MyD88 in hepatocytes causes liver inflammation (Duparc et al., 2017).

TANK and AZI2-deficient cells show high cell death induction when stimulated with TNF. This is in line with the crucial role of these adaptors in the engagement of TBK1 and the protection against TNF-induced cell death. In our model, TANK and AZI2 deficiency leads to insufficient recruitment of the kinase TBK1 to the MyD88 signaling complex, which leads to enhanced signaling and overproduction of TNF. In the absence of TANK and AZI2-recruited TBK1, TNF triggers cell death. TANK and AZI2, therefore, serve as a brake in two consecutive steps in the protection against autoimmunity. Furthermore, MyD88 ablation has been shown to alleviate multiple inflammatory phenotypes with disrupted TNF signaling (Schunke et al., 2021; Sharma et al., 2019; Verboom et al., 2020), which is likely caused by decreased production of TNF. For this reason, MyD88 signaling could potentially serve as a target in therapies. Even though, human deficiency of MyD88 or the downstream kinase IRAK4 causes increased susceptibility to pyogenic bacteria which can lead to life threatening infections in childhood, in adulthood these patients show lower incidence and severity of infections without the need for prophylaxis (Picard et al., 2010). This suggests that blocking of MyD88 pathway could provide substantial improvement in severe inflammatory conditions, without danger of disrupting the host defense system.

Since MyD88 is an adaptor protein that could be hard to pharmaceutically target, we shifted our focus to IRAK4, a kinase that is an important constituent of the myddosome. We generated *Tank*/*Azi2*/*Irak4*^{TKO} and *Tank*/*Azi2*^{DKO}/*Irak4*^{KD} mice. According to our expectations, the deletion of IRAK4 rescued the partial embryonic lethality and survival rate of *Tank*/*Azi2*^{DKO} to a similar extent as MyD88 deficiency. However, the ablation of IRAK4 kinase activity did not provide any protection from the disease. This suggests that the kinase activity of IRAK4 is dispensable and can promote signaling even in its inactive form. Nevertheless, a detailed analysis of these mice is needed. So far, our data suggest IRAK4 might be an attractive therapeutical target, however, the drug design should be focused on either degrading IRAK4 or preventing it from binding to the myddosome rather than inhibiting its activity.

6. Conclusion

We sought to find new regulators of the MyD88-mediated signaling pathway and explore the possibility of their targeting in the treatment of inflammatory diseases. We identified the kinase TBK1 as a previously undescribed component of the IL-1R-SC and demonstrated that TBK1 functions as a negative regulator within this complex, limiting MAPK and NF-κB activation, as well as the transcription of pro-inflammatory cytokines. The recruitment of TBK1 to the receptor is mediated by adaptor proteins TANK and AZI2. Their absence *in vivo* leads to the development of a severe inflammatory phenotype that is rescued by the deletion of MyD88 or its downstream kinase IRAK4. This underscores the importance of TBK1 and MyD88 signaling in the protection from autoimmunity. These insights not only advance our understanding of MyD88 signaling regulation but also identify potential therapeutic targets.

7. References

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