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Signální mechanismy zánětlivých odpovědí

Molecular mechanisms of inflammation

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Molecular mechanisms of inflammation

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Doctoral thesis

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V Praze:

Mgr. Daniela Knížková

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List of abbreviations

3'UTR	3' untranslated region
Arid5a	AT-rich interactive domain-containing protein 5A
AZI2	5-azacytidine-induced protein 2
BMDM	bone marrow-derived macrophages
Cas8	caspase 8
CCL	C-C motif ligand
CD93	cluster of differentiation 93
C/EBP	CCAAT/enhancer-binding protein
cFLIP	caspase-8 inhibitor cellular FLICE inhibitory protein
cIAP	cellular inhibitor of apoptosis protein
CKLF	chemokine-like factor
CKLFSF	chemokine-like factor superfamily
CMTM	CKLF-like MARVEL transmembrane domain-containing
CMTM1_v17	CMTM1 splicing variant 17
CMTM1_v5	CMTM1 splicing variant 5
CMTM8_v2	CMTM8 splicing variant 2
CNS	central nervous system
Csf2	Colony stimulating factor 2
CTLA-8	cytotoxic T-lymphocyte-associated protein 8
CXCL	C-X-C motif ligand
CYLD	cylindromatosis
Dcp1	decapping enzyme 1
EAE	experimental autoimmune encephalomyelitis
ERK	extracellular signal-regulated kinase
FADD	FAS-associated death domain-containing protein
HOIL-1	heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1-interacting protein
HuR	human-antigen R
IBD	intestinal bowel disease
IFN-γ	interferon gamma
IKK	IκB kinases
IL-	interleukin
IL-17R	interleukin 17 receptor
IL-17RSC	IL-17 receptor signaling complex
IL-23R	receptor for IL-23

IMQ	imiquimod
iNKT	invariant natural killer T cell
JNK	c-Jun N-terminal kinase
LUBAC	linear-ubiquitin chain assembly complex
MAL	myelin and lymphocyte
MAPK	mitogen-activated protein kinase
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
MEF	mouse embryonic fibroblasts
MLKL	phosphorylates mixed lineage kinase domain-like
MS	multiple sclerosis
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-κB
PD-L1	programmed-death ligand 1
RIPK1	receptor-interacting serine/threonine-protein kinase 1
ROR-γt	retinoic acid receptor-related orphan receptor γt
SF2	splicing factor 2
SHARPIN	shank-associated RH domain-interacting protein
STAT3	signal transducer and activator of transcription 3
TAB1	TAK1-binding protein 1
TAK1	transforming growth factor-β-activated kinase 1
TANK	TRAF family member-associated NF- κB activator
TBK1	TANK-binding kinase 1
T_c17	IL-17-producing cytotoxic CD8+ T cells
TGF-β1	transforming growth factor β1
T_H	T helper cells
TIR	toll-IL-1 receptor
TNF	tumor necrosis factor
TNFR1	TNF receptor 1
TNFR1-SC	TNFR1 signaling complex
TRADD	TNFR1-associated death domain
TRAF	tumor-necrosis factor receptor-associated factor
T_{reg}	regulatory T cells
USP25	ubiquitin-specific protease 25

Abstract

The immune system possesses multiple mechanisms to protect the human body against pathogens. One of the key tools in eliminating the threat is the induction of inflammatory response. Inflammation aims to contain the infection and thus shield the tissue from further damage. Weak inflammatory process allows pathogens to harm the infected tissue. On the other hand, chronic inflammation is the cause of numerous diseases, such as psoriasis or multiple sclerosis. Therefore, it is crucial for the body to regulate the inflammatory process properly. The precise control of the mounted immune response is exerted by cytokines, small proteins secreted into the intercellular space. Cytokines influence various cells throughout the body and spread information about invading pathogens. Among the most important proinflammatory cytokines, interleukin 17 (IL-17) and tumor necrosis factor (TNF) play a crucial role. The presented work discusses the current state of knowledge of these cytokines and the signaling pathways they trigger.

The first part of this doctoral thesis deals with the signaling pathway triggered by IL-17. In our two published studies, we employed a new mass spectrometry-based methodology to study the composition of the IL-17 receptor signaling complex. We provided compelling evidence that IL-17-provoked signaling was negatively regulated by two kinases TBK1 and IKKε via a robust feedback loop. Moreover, we identified a new component of the IL-17 receptor signaling complex – a transmembrane protein CMTM4. We revealed, that this protein was crucial for the mediation of the IL-17 signal in IL-17-dependent autoimmune diseases.

The second part of this doctoral thesis focuses on the signaling pathway triggered by TNF. Although TNF proinflammatory and cell death-inducing signaling has been deeply studied over the past years, some aspects remain elusive. In our study, shich is currently under revisions, we described the role of adaptor proteins TANK and AZI2 in the protection against TNF-induced cell death. We revealed that although these proteins have a redundant role within the TNF signaling, their presence is indispensable for the protection against TNF-induced cell death.

Overall, our studies provide a deeper insight into the regulation of crucial proinflammatory signaling pathways and their role in health and disease.

Abstract (in Czech)

Imunitní system zahrnuje velkou škálu mechanismů, které zajišťují ochranu lidského těla před patogeny. Jedním z takovýchto klíčových nástrojů je spouštění zánětlivé odpovědi. Zánětlivá odpověď má za cíl potlačit infekci, čímž chrání zasaženou tkáň před větším poškozením. Je-li zánětlivý proces nedostatečný, patogeny mohou snadno poškozovat infikovanou tkáň. Na druhou stranu, chronický zánět je často příčinou závážných onemocnění, jako je například psoriasa nebo roztroušená skleróza. Precizní regulace vzniku zánětlivé odpovědi je tudíž zcela nezbytná. Kontrola zánětlivých procesů je zajištěna cytokiny, malými proteiny, které jsou sekretované do mezibuněčného prostoru. Cytokiny ovlivňují různé buňky napříč tělem, čímž dochází k rošíření informace o přítomném patogenu. Mezi nejdůležitější prozánětlivé cytokiny patří interleukin 17 (IL-17) či faktor nádorové nekrózy (TNF). Předkládaná práce se zabývá současnými znalostmi o těchto cytokinech a signálních drahách, které tyto cytokiny spouští.

První část této doktorské práce se zabývá signální dráhou spouštěnou IL-17. V našich dvou publikovaných pracích jsme studovali složení receptorového signalizačního komplexu pro IL-17 pomocí metody založené na hmotnostní spektrometrii. Zjistili jsme, že signalizace spouštěná IL-17 je negativně regulována kinázami TBK1 a IKKε prostřednictvím robustní zpětnovazebné smyčky. Navíc se nám podařilo identifikovat novou součást receptorového komplexu pro IL-17 – transmembránový protein CMTM4. Zjistili jsme, že v kontextu autoimunitních onemocnění závislých na IL-17 byl tento protein zcela nezbytný pro zprostředkování signálu od IL-17 a následný rozvoj onemocnění.

Druhá část této doktorské práce se zaměřuje na signalizační dráhu aktivovanou TNF. Ačkoliv jsou prozánětlivá signalizace a signalizace vedoucí k buněčné smrti závislé na TNF hojně studovány, některé aspekty zůstávají nevyřešené. V naší studii, která je v současné době v recenzním řízení, jsme popsali roli adaptorových proteinů TANK a AZI2 v souvislosti s ochranou proti buněčné smrti indukované TNF. Zjistili jsme, že ač je role těchto proteinů redundantní, jejich přítomnost v signalizačním komplexu je pro ochranu před buněčnou smrtí nepostradatelná.

Naše studie poskytují hlubší vhled do regulace klíčových prozánětlivých signalizačních drah a jejich role ve zdraví a nemoci.

Preface

At the beginning of my graduate studies in the doctoral program "Immunology" at the Faculty of Science of Charles University in Prague, I joined the Laboratory of Adaptive Immunity led by Dr. Ondřej Štěpánek at the Institute of Molecular Genetics of the Czech Academy of Sciences in Krč campus in Prague. I worked under the supervision of Dr. Peter Dráber and my research focused on different aspects of the mechanisms of tissue inflammation.

The first project I worked on was focused on defining the regulation of the IL-17 signaling and the role of kinases TBK1 and IKKε within this signaling cascade. This project was already in its later stages when I joined. Another project slowly crystallized as we studied the composition of the IL-17 receptor signaling complex. This one dug further into the IL-17 signaling and concentrated on the role of the previously unknown part of the IL-17 receptor signaling complex, the CMTM4 protein. I dedicated most of my research work to this project, and it became the main topic of my thesis. Apart from the IL-17 signaling, I also focused on studying the role of adaptor proteins TANK and AZI2 in the TNF signaling cascade.

During the 3rd and 4th years of my Ph.D. studies, I helped to establish the Laboratory of Immunity & Cell Communication of the First Faculty of Medicine of Charles University led by Dr. Peter Dráber. This laboratory is based in the BIOCEV campus and I finished my research there.

My dissertation thesis includes the following chapters: an introduction discussing the current knowledge about the inflammatory signaling triggered by IL-17 and TNF, the aims of my research projects, a brief description of the methods employed, a discussion of the obtained results, conclusions, and reprints of the published works.

1 Introduction

The immune system has a wide range of tools for conquering the threats from pathogens we meet daily. The defense mechanisms of the immune system can be broadly divided into two intertwined parts – innate immunity and adaptive immunity. The innate immunity is based on genome-encoded receptors. It represents the first line of defense against a variety of pathogens. Though it is triggered minutes after the infection, the innate immune response is limited – the system is not flexible enough, and many pathogens can overcome it. In these instances, adaptive or acquired immunity plays a critical role. The adaptive responses are very advantageous – they are specifically tailored against particular invading pathogens, enabling their effective elimination. However, generating an adaptive immune response against newly encountered pathogens can take days. The cooperation between these two branches of the immune system is, therefore, vital.

The immune system employs various mechanisms to fight threats and launch adaptive responses. The core mechanism is the induction of inflammation. Inflammation is a complex tissue response to infection or damage. Its typical manifestations cover redness of the infected area (rubor), higher temperature (calor), pain (dolor), and swelling (tumor). These signs are caused by the combination of signals arising from innate immune cells and material released from injured tissue. If the generated inflammatory process is insufficient, invading pathogens can damage the infected tissue. Despite these positives, persistent (or chronic) inflammation is often connected with numerous diseases like psoriasis or multiple sclerosis[1,2]. Therefore, the very neat regulation of inflammation is necessary.

To ensure the precise control of the mounted immune response, the cells within the body communicate. This communication is maintained through cytokines – small proteins released to the cell environment. They are secreted by cells to the intercellular space, where they affect other cells. Alternatively, the cytokines can be transported by the bloodstream to remote organs and influence cells there. This is the way the information about invading pathogens spreads throughout the body. Among the proteins driving the inflammatory response, cytokines interleukin 17 (IL-17) and tumor necrosis factor (TNF) play a crucial role.

Since its original cloning in 1995, IL-17 has quickly gained the attention of scientific society[3]. Soon, it was described as a pivotal regulator of the inflammatory immune response. It became clear that it is necessary for the host defense against microbial infections, such as candidiasis caused by the yeast *Candida albicans*[4,5], or cutaneous bacterial infection triggered by *Staphylococcus aureus*[6]. However, emerging evidence showed that the dysregulated IL-17 signaling leads to immune disbalance, which results in autoimmune disorders like psoriasis, rheumatoid arthritis, or multiple sclerosis[7–9]. Consequently, blocking the IL-17 signaling pathway has become a potential therapeutic target for these diseases, and several monoclonal antibodies neutralizing the molecules within the IL-17 signaling cascade, such as secukinumab, or brodalumab (targeting IL-17 or IL-17 receptor A, respectively), were designed[10]. Although effective in treating IL-17-dependent autoimmune disorders, their administration leads to diminished IL-17 signaling and patients often suffer from recurrent fungal infections, mainly candidiasis[11]. Therefore, further studies are necessary to reveal new targets in the IL-17 signaling pathway for therapeutical treatment.

TNF is a central proinflammatory cytokine involved in various immune system actions. Originally, in 1975, it was described as a protein highly toxic to numerous malignant tumors causing their necrosis - hence the name “tumor necrosis factor”[12]. The knowledge of TNF has only grown since. TNF can trigger distinct signaling pathways, resulting in very different outcomes: either the production of proinflammatory cytokines, or induction of cell death[13]. Although these actions generally lead to the elimination of the infection, TNF can also become a destructive agent. Aberrant TNF signaling has been shown to promote outbursts of autoinflammation. Several autoinflammatory disorders are caused by genetic mutations in various crucial TNF signaling proteins[14–18]. Since aberrant TNF signaling can result in excessive cell death, several drugs aiming at blocking TNF signaling have already been FDA-approved for medical use. Currently, anti-TNF treatment comprises monoclonal antibodies, such as adalimumab, or soluble receptor proteins like etanercept[19,20]. Although TNF-blockers are widely used to tackle numerous inflammatory diseases (e.g., rheumatoid arthritis, Crohn’s disease, or psoriasis), their usage is often accompanied by many unfavorable adverse effects, like serious infections or even malignancies[21–24]. Therefore, the ongoing research is focused on developing new substances with different inhibition mechanisms of TNF signaling events.

As IL-17 and TNF are the main focus of the presented research, the following chapters will describe the IL-17 and TNF signaling pathways in greater detail.

1.1 Interleukin 17

The pro-inflammatory cytokine interleukin 17, or IL-17, is a crucial component of the immune system. Its effects cover a variety of processes, ranging from host defense through the development of inflammation to cancer. This chapter focuses on a detailed description of the role of IL-17 within the immune system and the pathology arising from its dysregulation.

1.1.1 IL-17 cytokine family

IL-17 was originally described as a human analog of murine cytotoxic T-lymphocyte-associated protein 8 in 1995 (ref.[3]). However, as other cytokines with homological amino acid sequences were soon discovered, it was eventually renamed IL-17A. In 2000, Li et al. cloned and characterized IL-17B and IL-17C[25]; IL-17D, IL-17E (now known as IL-25), and IL-17F quickly followed to complete the whole family[26–28].

Of all IL-17 proteins, IL-17A and IL-17F share the highest homology (about 50 %). The homology among other members is relatively low. For example, IL-17B shows the highest homology with IL-17D, which is, however, only 27 % (ref.[26–28]). All IL-17 proteins have the two invariant disulfide bonds, which form the so-called cysteine-knot fold architecture analogous to nerve growth factor[25,28–31] and they form either homo- or heterodimer[32].

The following text will focus mainly on IL-17A (here called IL-17), as it is the most studied member of the family. However, the pathophysiologic effects of other members will also be discussed.

1.1.2 Cellular sources of IL-17

The main producers of IL-17 are $CD4^+$ T helper type 17 cells (T_H17). However, several other cell types have been reported to produce IL-17 proteins. The following paragraphs will briefly summarize the most important facts about these cells.

T helper type 17 cells

In 2005, two studies showed that IL-17 is produced by T_H17 cells, which differentiated from naïve $CD4^+$ T cells under the influence of IL-23. This differentiation was efficient only in the absence of T helper type 1 and T helper type 2 costimulatory

signals[33,34]. Further investigation revealed, that the induction of T_H17 cells also requires other cytokines. The main driver in T_H17 lineage commitment is transforming growth factor β1 (TGF-β1). Upon the influence of TGF-β1, the naïve T cells start expressing the IL-23 receptor (IL-23R), thus rendering the naïve T cells IL-23-responsive[35]. Intriguingly, TGF-β1 also drives the differentiation of naïve T cells into regulatory T cells (T_{reg})[36,37]. The key regulator of the T_H17/T_{reg} balance is IL-6, which suppresses the development of T_{reg} cells from naïve $CD4^+$ cells under the influence of TGF-β1 (ref.[38–40]). Alternatively, in the absence of IL-6, the differentiation of T_H17 cells can be driven by the cooperation of TGF- β1 with IL-21 (ref.[41]).

Apart from the aforementioned cytokines, the master regulator of the T_H17 lineage commitment is the transcription factor retinoic acid receptor-related orphan receptor gamma t (ROR-γt). It cooperates with other transcription factors, such as signal transducer and activator of transcription 3 (STAT3), which is induced by IL-6 (ref.[42]), to trigger the expression of IL-17 (ref.[43,44]).

Besides IL-17 and IL-17F, T_H17 cells also produce IL-21 (thus creating a feed-forward loop) or IL-22 (ref.[45,46]). Moreover, due to their considerable plasticity, they are capable of secreting granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ, and TNF[47,48].

γδ T cells

γδ T cells are another important source of IL-17 proteins. These cells develop from thymocytes in the thymus, but in contrast to ordinary T cells, γδ T cells bear a less common T cell receptor composed of chains γ and δ. Contrary to T_H17 cells, only TGF-β1 is required for the differentiation of IL-17-producing γδ T cells (also termed γδ17 T cells) in the thymus and secondary lymphoid organs[49,50].

γδ T cells show similar characteristics as T_H17 cells, and upon activation, they can produce a similar range of cytokines (mainly IL-17 and IL-22)[51]. Mouse γδ T cells appear to be „pre-programmed“ already in the thymus - stimulation by IL-1β and IL-23 is sufficient to trigger IL-17 production by γδ T cells even without the engagement of T cell receptor. Accordingly, these cells exhibit a relatively fast, innate-like response to pathogen-associated molecular patterns such as lipopolysaccharide[52]. Their rapid activation and IL-17 production is observed within the first hours after the infection with *Mycobacterium tuberculosis*[53], *Candida albicans*[54], or *Bordetella pertussis*[55]. γδ T cells are, therefore, considered the first line of defense against infection.

On the other hand, the dysregulated activation of γδ17 T cells can result in chronic inflammation or even autoimmune pathologies, such as psoriasis or multiple sclerosis. In the presence of IL-23, dermal γδ T cells proliferate to produce IL-17 and IL-22, which, in turn, drive the development of psoriatic plaques[56,57]. IL-17 produced by dermal γδ T cells was shown to be the main cause of the disease in the imiquimod model of psoriasis, an IL-17-dependent inflammatory condition of the skin[58,59].

Other producers of IL-17 proteins

Throughout the years, other minor producers of IL-17 proteins have been characterized. The development and function of most of them are also dependent on the transcription factor ROR-γt like in the case of T_H17 cells[43,60–62].

As well as γδ T cells, type 3 innate lymphoid cells (ILC3) can produce IL-17 and IL-22 in response to IL-1β, and IL-23, thus underlying the innate-like character of IL-17 (ref.[63,64]). ILC3 cells take part in controlling commensal bacteria and preventing their dissemination[65].

Nevertheless, IL-17-producing ILC3 cells can also promote inflammation in mucosal tissues. They have been connected to the development of intestinal pathology, such as inflammatory bowel disease (IBD)[66,67].

Another type of innate cells capable of secreting IL-17 are invariant natural killer T cells (iNKT). They develop in the thymus and produce IL-17 when activated by glycolipid antigens, IL-1β, or TGF-β1 (ref.[68,69]). Albeit IL-17-producing iNKT cells were connected with some pathologies like asthma or cancer development, their precise function *in vivo* remains enigmatic[70].

Interestingly, a subset of cytotoxic $CD8^+$ T cells secreting IL-17 (T_C17 cells) has also been identified. Regarding activation and cytokine production, mouse T_C17 cells resemble T_H17 cells as TGF-β1, IL-6, and IL-21 also induce them to secrete IL-17 and other cytokines and chemokines, including TNF or GM-CSF[71]. T_C17 cells were present in a broad range of human inflammatory disorders, such as psoriatic plaques[72], active lesions in brain tissue affected by multiple sclerosis[73], or in the liver of patients infected with chronic hepatitis C virus[74].

1.1.3 IL-17 receptor signaling complex

The engagement of the IL-17 receptor with its ligand leads to the production of proinflammatory cytokines and chemokines, like IL-6, TNF, or GM-CSF, through activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. Generating these proteins mobilizes the immune system to fight invading pathogens, especially yeast infections.

The signaling events triggered by IL-17 emanate from the cognate dimeric IL-17 receptor (IL-17R). The family of IL-17R consists of five members named IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE. Various combinations of these proteins result in different receptors binding to different IL-17 dimers. In the steady state, the subunits of IL-17R are separated on the plasma membrane, and their dimerization is induced by the binding of the ligand. This event then triggers the signaling cascade[75].

All the subunits capable of forming the IL-17R are single transmembrane domain-containing proteins bearing a common cytoplasmic signaling motif known as ‚SEF/IL-17R' or SEFIR[76]. The SEFIR domain is, in terms of length and secondary structure, related to the toll-IL-1 receptor domain present in toll-like IL-1 and IL-18 receptors[76,77].

Both IL-17 and IL-17F signal through IL-17RA/IL-17RC heterodimeric receptor[78–81]. In addition, IL-17F signals via the IL-17RC homodimeric receptor and drives IL-17RA-independent signaling[81,82]. IL-17B binds and signals via IL-17RA/IL-17RB heterodimeric receptor[83,84]. The receptor for IL-17C is composed of IL-17RA and IL-17RE subunits and is selectively expressed in epithelial tissues[85,86]. Interestingly, a recent study suggested that the receptor for IL-17D is CD93, which does not belong to the same family as other subunits of IL-17R. Instead, it is a member of the C-type lectin family of transmembrane proteins[87,88]. IL-17E binds strictly to IL-17RB, but for its effector functions, both IL-17RA and IL-17RB are necessary[89,90]. IL-17RD seems to act as an alternate receptor for IL-17 (but not for IL-17F). However, its precise function within IL-17 signaling pathways remains to be determined[91,92].

Since IL-17 and IL-17F are clinically the most important members of the IL-17 family, the following sections will focus on the complexity of the signaling network of the IL-17RA/IL-17RC heterodimeric receptor and the events that are triggered after its ligation to IL-17 and IL-17F.

IL-17 receptor signaling complex assembly

A homodimer of IL-17, IL-17F, or a heterodimer of IL-17/IL-17F signals through a heterodimeric receptor formed of IL-17RA and IL-17RC subunits. Although these ligands stimulate the secretion of the same cytokines, their potency differs. The most potent inducer of cytokine secretion is the IL-17 homodimer, followed by the IL-17/IL-17F heterodimer, and the weakest is the IL-17F homodimer[93].

IL-17 signaling is limited to the cells of non-hematopoietic origin – while IL-17RA is broadly expressed in various cell types, the expression of IL-17RC is restricted to epithelial tissues such as the skin, prostate, liver, thyroid, or adrenal gland[81,94].

The binding of an IL-17 ligand induces the heterodimerization of IL-17RA with IL-17RC, bringing the cytoplasmic SEFIR domains into the vicinity. The SEFIR domains form an interaction platform for the recruitment of Act1 adaptor protein via its SEFIR domain[95,96]. Act1 is the core protein around which the whole signaling cascade is formed. It contains a binding motif for tumor necrosis factor receptor-associated factor (TRAF) and through this motif, it recruits different TRAF proteins to launch distinct signaling events[97]. Rapidly recruited E3 ubiquitin ligase TRAF6 subsequently creates non-degradative K63-polyubiquitin chains. These linkages in turn serve as a docking site for a variety of proteins, triggering the downstream signaling cascades[96,98].

Polyubiquitinated TRAF6 mobilizes transforming growth factor-β-activated kinase 1 (TAK1), via its adaptor proteins TAK1-binding protein 1 (TAB1), TAB2, and TAB3 (ref.[99–101]). The so-called TAK1-TABs complex phosphorylates the inhibitor of κB (IκB) kinases (IKK) within the canonical IKK complex, which is formed by IKKα, IKKβ, and the adaptor protein NF-κB essential modulator (NEMO). Consecutively, the canonical IKK complex phosphorylates IκB leading to its ubiquitination and subsequent degradation. The released transcription factor NF-κB translocates into the nucleus and mediates the transcription of proinflammatory proteins[102]. Alternatively, TAK1 activates MAPK kinases p38, c-Jun N-terminal kinase (JNK), or extracellular signal-regulated kinase (ERK)[103].

Besides known signaling pathways triggered by NF-κB and MAPK, IL-17 also engages other transcription factors, such as CCAAT/enhancer-binding protein family (C/EBP) or IκBζ. Many of the IL-17-target genes have C/EBP binding sites within their promoters, and the stimulation with IL-17 leads to the upregulation of C/EBPβ and C/EBPδ[104,105]. IκBζ also positively regulates the expression of IL-17-triggered genes. Moreover, IκBζ was connected to the development of psoriasis[106–108].

Act1 phosphorylated on serine 311 by non-canonical IκB kinase IKKε also induces the binding and ubiquitination of TRAF2 and TRAF5 (ref.[109–111]). These TRAFs recruit RNA-binding proteins, that affect the half-life of mRNA transcripts arising from IL-17 signaling.

Upon IL-17 stimulation, RNA-binding protein human antigen R (HuR) is recruited to the receptor signaling complex and subsequently interacts with the 3‘ untranslated region (3‘UTR) of *Cxcl1* mRNA and stabilizes it[112]. Next, IL-17 promotes the expression of RNA-binding protein AT-rich interactive domain-containing protein 5A (Arid5a). Arid5a also binds to the 3‘UTR of IL-17-induced mRNAs, such as *Il6, Cxcl1,* or *Cxcl5*, and promotes their stabilization[113]. Surprisingly, Act1 itself can function as an RNA-binding protein. It interacts with 3’UTRs of target mRNA (such as *Cxcl1, Csf2,* or *Tnf*) via its SEFIR domain and preserves otherwise unstable mRNA transcripts. Act1 also improves mRNA stability by inhibiting decapping enzyme 1 (Dcp1) and Dcp2, mRNA-decapping proteins[114].

On the other hand, different RNA-binding proteins can promote mRNA decay to ensure that the ongoing signaling event will not slip out of control. For example, endoribonuclease Regnase-1, an IL-17-induced RNA-binding protein, triggers mRNA degradation and competes with Arid5a for the RNA-binding site[113,115]. A rather similar situation occurs with splicing factor SF2, which also promotes mRNA decay, and competes with HuR[110,114].

Regulation of the signaling complex

Like in other signaling cascades, neat regulation is necessary for the IL-17 signaling pathway to prevent its aberrant activation. The control over IL-17 signaling is provided by deubiquitinases A20 or ubiquitin-specific protease 25 (USP25) recruited to the IL-17RSC upon IL-17 binding. Deubiquitinases cleave polyubiquitin chains within the signaling cascade thus dampening the IL-17-induced activation of the NF-κB-dependent gene transcription[116]. Moreover, USP25 also removes polyubiquitin chains from TRAF5 and therefore restrains IL-17-mediated mRNA stabilization[111]. Additionally, IL-17R also recruits TRAF3. The interaction between IL-17R and TRAF3 disables the formation of the IL-17R-Act1-TRAF6 complex[117]. Furthermore, the signaling events downstream of TRAF6 are also influenced by TRAF4, which competes with TRAF6 for the TRAF-binding site on Act1[118].

The assembly of IL-17RSC and its regulation is depicted in the scheme in the Figure 1.

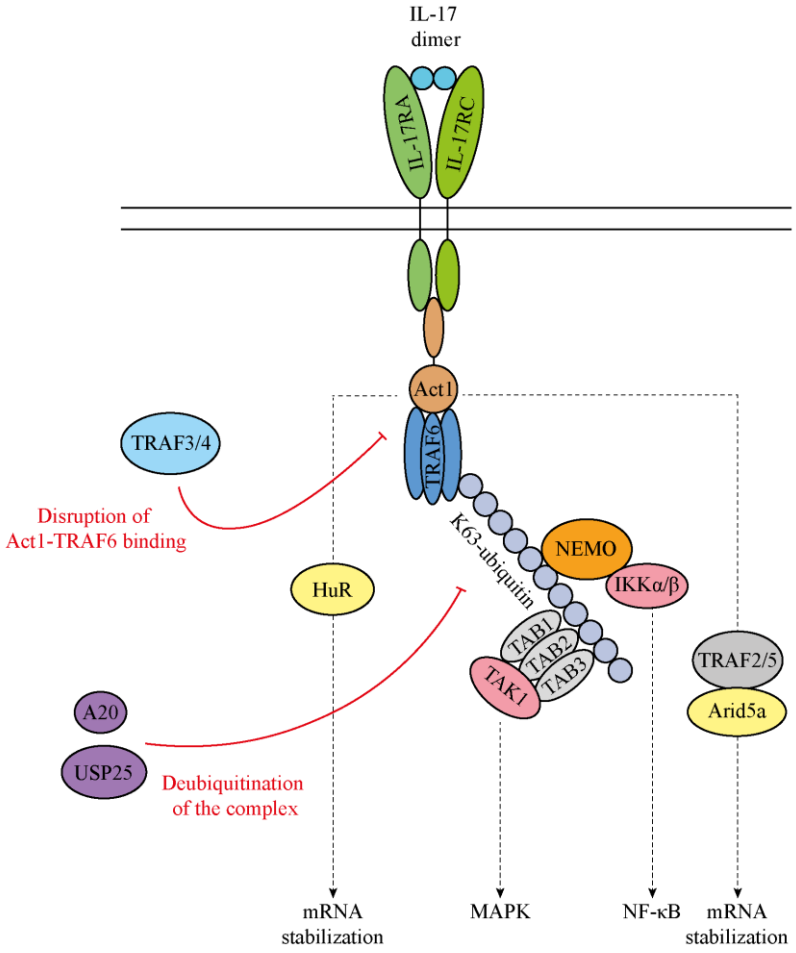

Figure 1. The assembly and regulation of IL-17RSC.

Upon binding of the IL-17 dimer, the receptor subunits dimerize, and recruit intracellular adaptor protein Act1. Act1 binds E3 ubiquitin ligase TRAF6, which in turn forms K63-polyubiquitin chains. These chains form a docking site for TAK1-TABs and IKK complexes triggering the expression of proinflammatory genes. Moreover, RNA-binding proteins HuR and Arid5a are recruited to the IL-17RSC to provide the stabilization of newly transcribed mRNA. Furthermore, the IL-17RSC assembly is regulated on several levels. Proteins TRAF3 and TRAF4 prevent the formation of Act1-TRAF6 complex. Additionally, the disruption of the signaling cascade is also provided by deubiquitinases A20 and USP25.

The following chapters will discuss the physiological and pathological effects of cytokines from the IL-17 family. Although, they will mainly focus on IL-17, since it is

the thesis's spotlight, the effects of other IL-17 proteins will be briefly discussed, and a particular paragraph will be dedicated to IL-17E since its role quite differs.

1.1.4 The role of IL-17 *in vivo*

Over the years, IL-17, together with IL-17F, were implicated in a wide variety of physiological and pathological responses ranging from tissue protection to autoinflammatory setups. This chapter will provide a comprehensible review of the current stage of knowledge about the role of IL-17 in the immune system.

Yeast and bacterial infections

IL-17 is strongly involved in the protection against yeast and bacterial infections. *Candida albicans* is a commensal microorganism colonizing the oral cavity, gastrointestinal tract, and reproductive mucosa. This colonization is generally asymptomatic. However, it can easily get out of control once the host's immune defenses are challenged[119]. When dysregulated, *C. albicans* spreads on the affected tissues causing a condition called *candidiasis.* There are several types of candidiasis. Mucocutaneous candidiasis affects the mucosae, nail, and skin surfaces. It usually manifests as a common thrush in the oral cavity[120]. The colonization of the female reproductive tract by *C. albicans* does not usually exhibit any pathological symptoms. Nevertheless, most women suffer from vulvovaginal candidiasis at least once during reproductive age[121]. The most severe and potentially life-threatening form of *Candida* infection is disseminated candidiasis. This disease form, often caused by improper medical intervention, happens when *Candida* species enter the bloodstream. It is accompanied by relatively high mortality (40 - 60 %)[120].

Many studies described the connection between *C. albicans* and the IL-17 signaling pathway. Oral resident γδ17 T cells are the main driver of IL-17 production in candidiasis and they produce IL-17 within 24 hours after encountering *C. albicans*[122–124]. Chronic mucocutaneous candidiasis is often connected with adaptive immune responses provided by T_H17 cells[125,126]. Secreted IL-17 stimulates keratinocytes and epithelial cells to produce antimicrobial peptides with candidicidal activity, such as β-defensins or histatins, to fight off the infection[124,127.] Besides antimicrobial peptides, IL-17 also stimulates the production of proinflammatory cytokines and chemokines, like CCL20, the ligand for CCR6 (ref.[128]). CCR6 is a chemokine receptor characteristic for T_H17 cells,

and therefore, the enhanced production of CCL20 may recruit more IL-17-producing cells to the infected tissue, thus creating a regulatory feedback loop[129].

The crucial role of IL-17 in conquering infection with *C. albicans* is supported by cumulative studies describing individuals with mutations in the IL-17 signaling pathway. For instance, some patients suffering from autosomal dominant chronic mucocutaneous candidiasis bear mutations in IL-17 itself or proteins of its receptor signaling complex like IL-17RA or Act1 (ref.[130–132]).

Regrettably, the tight relationship between IL-17 and anti-fungal immunity has also its disadvantages. As will be discussed in further chapters, aberrant IL-17 signaling is a causative factor of some autoimmune disorders like psoriasis, multiple sclerosis, or rheumatoid arthritis[133–135]. So far, anti-IL-17 treatment of these diseases has gained positive results in keeping the disease signs at bay. On the other side, these biologics render the patients more susceptible to *Candida* infections[136].

A Gram-positive extracellular bacterium *Staphylococcus aureus* is often the causing agent of skin and soft tissue infections. These usually minor infections can, upon certain conditions, quickly turn into life-threatening illnesses, such as bacteremia or pneumonia[137]. To contain the infection, both IL-17 and IL-17F produced by γδ17 T cells stimulate the production of antimicrobial peptides like β-defensins. Additionally, they recruit the neutrophils to the site of *S. aureus* dissemination[138,139]. According to various studies, the effects of IL-17 and IL-17F seem to be compensatory and redundant[139,140].

Autoimmune disorders

Some autoimmune disorders develop as a consequence of aberrant IL-17 signaling. Although most autoimmune responses are driven by the cooperation of different parts of the immune system, in some cases specific types of immune cells are more prevalent than others. Such examples will be discussed in the next lines.

Psoriasis is a chronic inflammatory skin disease that is connected to the development of erythematous, thickened, scaly plaques of dead skin. These symptoms are the result of altered immune responses leading to keratinocyte activation, and hyperproliferation. Moreover, after a few years of suffering from psoriasis, some patients develop swollen joints (mostly on fingers and toes) – a condition known as psoriatic arthritis[141,142].

The key player in driving psoriatic inflammation is IL-17. The elevated levels of *IL-17* mRNA were reported in both lesional and non-lesional skin of psoriatic patients followed by enhanced production of the IL-17 protein[143–145]. Furthermore, psoriatic lesions also show upregulated production of IL-17C and IL-17F, two other members of the IL-17 cytokine family[146,147]. These IL-17 cytokines are produced by a vast variety of cells, including T_H17 cells, γδ17 T cells, mast cells, or ILC3 cells[148].

The core of psoriatic pathology is the IL-23/IL-17 axis. IL-23 triggers the differentiation, activation, proliferation, and survival of T_H17 cells which, in turn, produce the effector cytokines, like IL-17. Furthermore, the generation of IL-17 can also be IL-23-independent[149,150]. In keratinocytes, IL-17 induces the production of other proinflammatory cytokines (such as TNF, IL-6, IL-1β), chemokines (like CXCL1, CXCL3, or CCL20), and antimicrobial peptides (e.g., lipocalin 2, or β-defensins)[151,152]. The influence of IL-17 on endothelial cells results in the promotion of tissue inflammation (via upregulation of IL-8 and IL-6) and procoagulant activity (through intracellular adhesion molecule-1)[153,154]. IL-17 can also target and induce the expression of proinflammatory proteins in macrophages[155].

Given the central role of the IL-23/IL-17 axis in the pathogenesis of psoriasis and psoriatic arthritis, the current biological therapies for these diseases are focused on these cytokines. To this day, several monoclonal antibodies targeting the IL-23/IL-17 axis have been approved. IL-17-neutralizing antibodies secukinumab and ixekizumab have, so far, shown the highest efficacy, and their usage is associated with (near) complete remission of the disease[156,157]. Brodalumab, which targets the IL-17RA subunit of the IL-17 receptor, is also very potent biologics for the treatment of psoriasis. However, its usage raises concerns, as during phase 3 psoriasis trials several psychiatric adverse effects like depression, anxiety, or suicidal thoughts were reported[158,159]. Ustekinumab is a monoclonal antibody targeting the p40 subunit of IL-23 and the administration of ustekinumab yielded satisfactory results for the treatment of plaque psoriasis and psoriatic arthritis. It is, therefore, considered a first-choice medication for the management of these diseases[160].

Furthermore, during the development of psoriasis, IL-17 effects are strengthened via the synergistic action of TNF. Therefore, anti-TNF therapeutics like etanercept are sometimes used for psoriasis treatment. However, their efficacy does not reach the same level as in the case of secukinumab or ixekizumab[161–163].

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). The main hallmark of MS is the degradation of the myelin sheath or the oligodendrocyte cell body due to the ongoing inflammatory process. Moreover, demyelination is often accompanied by axonal loss and gliosis[164].

Several immune cell types are associated with the development of multiple sclerosis. $CD4^+$ T_H cells specific for the myelin sheath proteins play a central role in the pathogenesis of the disease. The most prominent cells in active MS lesions are IFN-γ-producing T_H1 cells, and IL-17-producing T_H17 (ref.[165]). They are responsible for the activation and maturation of CNS-resident microglia and astrocytes, and monocytes invading the CNS[166]. Several studies implicated, that IL-17 signaling is an important contributor to the development of multiple sclerosis, as mice deficient in IL-17 were partially resistant to experimental autoimmune encephalomyelitis (EAE), a mouse model of MS[167]. However, the EAE model seems strongly dependent on microbial colonization, as disease progression in mice deficient in both IL-17 and IL-17F were partially resistant to EAE primarily due to the differences in microbiota[168]. Additionally, both mouse and human astrocytes bear the receptor for IL-17, and after stimulation by IL-17, they produce proinflammatory cytokines (like IL-6 or TNF) and chemokines (e.g. CXCL1 or CCL20) which promote inflammation of the CNS[169–173].

Furthermore, IL-17 and IL-22 secreted by T_H17 cells weaken the blood-brain barrier by stimulating the production of IL-6, IL-8, and CCL2 to recruit other $CD4^+$ lymphocytes and therefore propagate the neuroinflammation[174,175].

The precise role of IL-17 in MS is, however, controversial. Over the years, some studies of mouse models suggested IL-17 to be important, whereas others claimed that IL-17 has only a minor effect on the development of the disease[167,176,177]. Nevertheless, studies have been launched to investigate the potential of IL-17-neutralizing antibodies in the therapy of the disease. For instance, the treatment with secukinumab, which is used for the treatment of psoriasis with resounding success, resulted in reduced activity of MS lesions[178].

Cancer

IL-17 has also been implicated in tumorigenesis. IL-17 supports the development of skin cancer by upregulation of STAT3 in stromal and tumor cells, thus promoting the infiltration of myeloid cells in the tumor microenvironment[179,180]. Colorectal cancers are also affected by IL-17 according to animal studies. IL-17 is important in the development

of colorectal cancer triggered by enterotoxigenic bacterium *Bacteroides fragilis,* as well as colitis-associated colorectal cancer[181–183]. Besides, IL-17 secreted by γδ17 T cells also promotes the metastasis of breast tumors via expansion and polarization of neutrophils. These tumor-induced neutrophils gain the ability to quench cytotoxic T cells which otherwise restrict the metastasis[184]. Additionally, IL-17 is also associated with lung cancer. Elevated levels of IL-17 cytokine were described in tumor tissues and peripheral blood of patients suffering from non-small cell lung cancer, and its high expression is connected with poor prognosis[185–187].

1.1.5 The role of other IL-17 cytokines

IL-17 is the best-studied member of the IL-17 family of cytokines. Nevertheless, the role of other IL-17 proteins, IL-17E in particular, is also extensively investigated.

IL-17B shares the IL-17RB receptor subunit with IL-17E. Consequently, IL-17B can block IL-17E during mucosal inflammation and therefore exhibits anti-inflammatory effects[84,188]. IL-17B is also implicated in cancerous processes. Its signaling through IL-17RB stimulates cancer cell survival, proliferation, and metastasis, and high levels of IL-17B are associated with poor prognosis in patients with pancreatic, or lung cancer[189,190]. The latest study by Huang et al. suggested, that Schwann-cell-secreted IL-17B takes part in nerve tissue repair by inducing macrophage migration to the site of injury[191].

IL-17C is secreted mainly by epithelial tissues and acts there as a unique autocrine modulator of cellular responses. It stimulates the production of proinflammatory cytokines and antimicrobial peptides in response to epithelial injury[86,192]. Along with IL-17, IL-17C likewise drives psoriatic inflammation in mouse models[86,193] and plays a critical role in the development of inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease[194]. Moreover, IL-17C produced by keratinocytes was connected to the protection of the peripheral nerve system during frequent herpes simplex virus infections[195].

So far, IL-17D is the least known member of the IL-17 protein family. It is expressed in a variety of tissues, including skeletal muscles, the brain, adipose tissue, or the lung, and it induces the expression of a similar range of cytokines as other IL-17 proteins, including IL-6 or GM-CSF[26]. A recent study suggested IL-17D to be part of tumor and viral surveillance since it is upregulated in primary tumor tissues and during

viral infections[196,197]. Nevertheless, further research is necessary to elucidate the role of IL-17D *in vivo* fully.

IL-17E

Interestingly, the physiological responses of IL-17E (sometimes known as IL-25) differ from those promoted by the other members of the IL-17 family. In contrast to other IL-17 cytokines, IL-17E is produced primarily by T helper type 2 cells and, accordingly, it triggers the expression of cytokines connected to type 2 immunity, such as IL-4, IL-5, IL-13, and thymic stromal lymphopoietin[198,199].

Due to its involvement with type 2 immunity, IL-17E is often linked with allergic inflammation and inflammatory diseases. IL-17E mediates the development of allergic airway diseases, a heterogeneous group of diseases including allergic asthma or allergic rhinitis. IL-17E secreted upon meeting stimuli, such as allergens, induces proinflammatory pathways resulting in the expression of type 2 cytokines (e.g., IL-5 or IL-13) which in turn drive the allergic reaction[200,201]. Elevated levels of IL-17E were also connected with skin inflammation such as contact dermatitis, or the development and progression of atopic dermatitis[202–204].

A strong relationship has been implied between IL-17E and cancerogenesis. IL-17E seems to be an important biomarker of tumor progression as it promotes tumor cell proliferation and infiltration[205,206]. Also in some cancers, the disease severity is correlated with IL-17E levels – for example, patients suffering from advanced stages of hepatocellular carcinoma, or colorectal cancer show higher levels of IL-17E[207,208]. On the contrary, in malignant breast and prostate cancer, elevated levels of IL-17E suggest a better prognosis[209,210].

1.2 CMTM family of proteins

Transmembrane protein families are profoundly studied. However, the CMTM transmembrane protein family has remained enigmatic since its first description in 2003 (ref.[211]). At that time, using a computational search strategy, Han et al. identified eight novel genes related to chemokine-like factor (CKLF), which they labeled “chemokine-like factor superfamily members 1-8 (*CKLFSF1-8*). The proteins coded by these genes contain the “MAL (myelin and lymphocyte) and related proteins for vesicle trafficking

and membrane link" (MARVEL) domain. Therefore, they were later renamed "CKLF-like MARVEL transmembrane domain-containing" or CMTM proteins[211–213].

The expression of CMTM genes in the human body varies from member to member. *CMTM1* and *CMTM2* are expressed primarily in testis. The expression of *CMTM5* was detected predominantly in the brain and neural system, and *CMTM7* can be enriched in leukocytes. *CMTM3, CMTM4, CMTM6*, and *CMTM8* are expressed in many tissues throughout the body[211].

The CMTM proteins exist in several splicing isoforms which may impact their function. Like all MARVEL proteins, they have an M-shaped topology – four transmembrane-helix region architecture with cytoplasmic N- and C-terminal regions[213]. Although it is tempting to think of them as tetraspanins with their four α-helical transmembrane domains, the CMTM proteins lack a typical tetraspanin sequence motif – four or more conserved cysteine residues within the larger extracellular loop forming disulfide bonds[214]. Only CMTM8 has about 39% sequence homology with the tetraspanin family member TM4SF11 (transmembrane 4 superfamily 11)). Additionally, CMTM1 shares striking homology with chemokines, such as CCL22 or CXCL1 (up to 47%). The features of other members of the CMTM family lie somewhere between CMTM1 and CMTM8. Therefore, the CMTM family is a novel protein family bearing the characteristics of chemokines and transmembrane 4 superfamily proteins[211].

The following chapters will discuss the current knowledge about the function of individual CMTM proteins. A particular chapter will be focused on CMTM4.

1.2.1 The function of CMTM proteins

Carcinogenesis

Since their discovery, the CMTM proteins have become the research focus of many studies. They were quickly associated with cancer development - they are involved in tumor growth, invasion, metastasis, and even cancer cell death.

CMTM1 is widely expressed in the testis, however, its expression in other tissues is rather low[215]. A splicing variant of *CMTM1*, CMTM1_v17, was found in various human tumor tissues – kidney, ovarian, or breast cancer, where it promoted cell proliferation. Moreover, these cancer cells were also resistant to TNF-induced apoptosis due to the high expression of CMTM1_v17 (ref.[216]). The same splicing variant of *CMTM1* was also detected in tumors from patients suffering from non-small cell lung cancer. In this case,

high expression of CMTM1_v17 was strongly correlated with chemoresistance and poor overall survival prognosis[217]. CMTM1 was also reported to increase the proliferation and invasiveness of glioblastoma cells, and high expression of CMTM1 was connected to the poor overall survival of glioma patients[218]. Interestingly, another splicing variant of *CMTM1*, CMTM1_v5, was reported to decrease tumor growth by inducing apoptosis. Therefore, it is plausible that the function of CMTM1 depends on its specific posttranscriptional modification[219].

The role of CMTM3 and CMTM5 in tumorigenesis is much clearer. Both CMTM3 and CMTM5 expression is downregulated in various cancer cell lines. This silencing is often carried out by methylation of the CpG islands within the promoters of the corresponding genes[220–222]. The restoration of CMTM3 or CMTM5 expression in cancer cells inhibited cell growth and migration[220,222–224]. Some studies even suggested that the overexpression of CMTM3 or CMTM5 in tumor tissues may lead to cell cycle arrest in phase G2 and caspase 3-dependent apoptosis[220,225,226]. CMTM3-driven apoptosis was also reported in hair follicle stem cells[227]. Therefore, CMTM3 and CMTM5 can be considered as tumor suppressors.

CMTM6 is a very interesting and also the most studied member of the CMTM family. The attention of the scientific community turned to CMTM6 in 2017 when two independent studies published in Nature (Burr et al. and Mezzadra et al.) connected CMTM6 with the regulation of programmed-death ligand 1 (PD-L1) in cancer cell lines[228,229]. CMTM6 was shown to control the stability of PD-L1 on the plasma membrane. These studies suggest, that CMTM6 presence protects PD-L1 from degradation. Both studies report that the knockout of CMTM6 in tumor cell lines decreased surface expression of PD-L1 and enhanced T-lymphocyte-mediated anti-tumor immunity[228,229]. Since then, follow-up studies have noted the co-expression of PD-L1 and CMTM6 in various cancer cell lines, like head and neck, liver, or lung[230–232]. A recent study suggested, that apart from regulating PD-L1 in tumors, CMTM6 also binds CD58, an important T-cell costimulatory molecule[233]. The interaction between CMTM6 and CD58 stabilizes CD58 on the tumor cell membrane, thus providing an important stimulation for tumor-specific T cells. The loss of CMTM6 results in decreased CD58 expression on the plasma membrane and consequently increases tumor cell survival. Since PD-L1 and CD58 have opposite effects regarding T-cell activity, it seems that the outcome of CMTM6 downregulation strongly depends on the balance between PD-L1 and CD58 signals[234]. Although studies concerning CMTM6 - PD-L1 interaction indicate,

that CMTM6 is rather an oncogene (contrary to most of the CMTM proteins), it seems that further investigation is needed to precisely define its role in the tumor microenvironment. Nevertheless, as CMTM6 silencing leads to inhibited tumor growth and stimulation of anti-tumor immunity, CMTM6 still might be a promising prognostic marker in cancer development and a potential therapeutic target[232,235].

The information about CMTM7 in tumorigenesis is very few. CMTM7 is downregulated in some carcinoma cell lines, partially by methylation, like its fellow members CMTM3 and CMTM5. The overexpression of CMTM7 inhibited the proliferation of tumor cells, this time by inducing the G1-S phase cell cycle arrest. These rather unsatisfactory data therefore imply that CMTM7 might also be a tumor suppressor. However, this hypothesis requires further investigation[236,237].

CMTM8 might act as a tumor suppressor, too. Its silencing in several cancer cell lines resulted in enhanced proliferation of the cells. Accordingly, the overexpression of CMTM8 or its splicing variant induced both caspase-dependent and caspase-independent apoptosis in well-established cancer cell lines HeLa, HEK293, and PC3 (ref.[238–240]). Furthermore, diminished expression of CMTM8 was connected to the increased aggressivity of bladder cancer tissues. Thus, CMTM8 seems a promising prognostic marker for bladder cancer progression[241,242]. A recent study by Kang et al. identifies CMTM8 in colon cancer and connects its upregulation with poor prognosis. Nevertheless, further studies are necessary to fully understand the role of CMTM8 in tumorigenesis[243].

Although CMTM2 mRNA was upregulated in glioblastomas[218], its precise role in tumorigenesis remains unclear.

In conclusion, it seems, that CMTM proteins are involved in cancer development, although their effects vary. Most of them can be classified as tumor suppressors (CMTM3, CMTM5, CMTM7, and CMTM8), whilst others are rather oncogenes (CMTM1 and CMTM6). However, it is also evident, that our current knowledge concerning these proteins within cancerous processes is still rather insufficient and requires deeper research.

CMTM proteins in non-cancerous tissues

So far, the role of CMTM proteins in non-cancerous tissues is rather enigmatic. CMTM2 was found in human and mouse testis[244,245]. CMTM2A and CMTM2B, which are mouse analogs of human CMTM2, were localized to the membrane of the sperm head and their knockout resulted in impaired sperm function and subsequent male infertility[246].

In 2017, CMTM3 was shown to mediate angiogenesis in developing zebrafish larvae. The most recent study suggests that CMTM3 is also involved in the inflammatory response to the *Helicobacter pylori* infection[247,248].

Besides its significant role in cancer development, CMTM6 has also been identified in Schwann cells, where it is supposed to limit axonal diameters[249].

CMTM7 seems to be connected with B cell function. In 2012, Miyazaki et al. proposed, that CMTM7 links serum immunoglobulin M with the B-cell linker, thus allowing the phosphorylation of the latter and mediating signal transduction[250]. Later, it was revealed that CMTM7 is critical for the development of B-1a cells and for the formation of their B-cell receptor[251–253].

1.2.2 CMTM4

CMTM4 is the most conserved member of the CMTM protein family. It can be found in various tissues throughout the body. Interestingly, its expression in immune cells is very weak[211,254]. Like other members of the CMTM transmembrane proteins family, CMTM4 is also related to cancer development. The first hints about this connection came in 2004 when Kittler et al. studied genes with essential functions in HeLa cell division – one of these genes was also *CKLFSF4* (former designation of *CMTM4*)[255]. Later on, Plate et al. published, that CMTM4 overexpression induced the accumulation of HeLa cells in the G2/M phase of the cell cycle[254]. Further studies demonstrated that CMTM4 is downregulated in several cancer tissues, like renal or brain[218,256]. Intriguingly, high CMTM4 expression was recently detected in hepatocellular and head and neck squamous cell carcinoma samples and associated with poor prognosis. In both cases, the upregulation of CMTM4 was accompanied by high surface expression of PD-L1 (ref.[257–259]). This is in accord with the study by Mezzadra et al. from 2017 showing that CMTM4 can function as a backup regulator of PD-L1 surface expression if CMTM6 is unavailable[229]. Altogether, the role of CMTM4 seems to depend on the type of cancerous tissue. Therefore, it is possible that depending on the tumor, it might function either as a tumor suppressor or as an oncogene.

Besides its ambiguous role in carcinogenesis, CMTM4 was also connected to angiogenesis. A study by Chrifi et al. suggested, that CMTM4, like CMTM3, is necessary for vascular growth in zebrafish larvae and enhances endocytosis in endothelial cells[260].

A strikingly high expression of CMTM4 was detected in the testis. This implies, that CMTM4 may also play an important role in male reproduction. Indeed, a late study by Liu et al. proposes CMTM4 to have a potentially pivotal role in spermatogenesis and sperm quality regulation[261]. Although the role of CMTM4 in angiogenesis or male fertility is interesting, the knowledge in this field is still rather limited and it requires further confirmation.

1.3 TNF signaling

TNF is a ubiquitous proinflammatory cytokine that takes part in various processes involved in immune responses. TNF is a potent player in fighting infections. On the other hand, out-of-control TNF signaling is responsible for numerous autoinflammatory disorders threatening the well-being of the affected individual[262]. Therefore, the neat regulation of these outcomes is vital for keeping the TNF effects under control.

A vast range of cell types secretes TNF. Initially, it is produced as a trimeric type II transmembrane protein which is subsequently cleaved by TNF alpha-converting enzyme into a mature soluble cytokine. Trimerization of a soluble protein renders TNF biologically active[263].

The TNF signaling pathway leading to a strong proinflammatory response is orchestrated mainly via its trimeric receptor TNF receptor 1 (TNFR1), which is expressed on most cell types. TNFR2, expressed mainly on immune and endothelial cells, plays a rather marginal role in TNF-induced proinflammatory and cell death signaling, and therefore will not be discussed in this thesis any further[264].

The ligation of TNF to TNFR1 results in the assembly of a membrane-bound signaling cascade (termed complex I) leading to the activation of proinflammatory genes, and, eventually, to containing the infection. Alternatively, a cytoplasmic complex, or complex II, is formed to trigger cell death. A detailed description of these signaling events is reviewed in the following chapters.

1.3.1 Membrane-bound proinflammatory complex I

The key mechanism necessary for the assembly of gene-activating complex I is ubiquitination. The complex network composed of various polyubiquitin chains allows the fine-tuning and balancing of the signaling events triggered by TNF. Three enzymes

are necessary for ubiquitination: (1) the ubiquitin-activation enzyme (E1), (2) the ubiquitin-conjugating enzyme (E2), and finally (3) the ubiquitin-ligating enzyme (E3). Ubiquitin itself can also be polyubiquitinated on its lysine residues (e.g., K48, or K63) or the amino-terminal methionine (M1), resulting in the formation of polyubiquitin chains. Different ubiquitin linkages have distinct biological properties. For example, K48-linked ubiquitin chains mark proteins for degradation, whereas non-degrading M1- and K63-linkages promote activation of signaling pathways and transcription of proinflammatory genes[265–267].

The formation of the TNFR1 signaling complex (TNFR1-SC) is initiated by the oligomerization of TNFR1 upon binding of trimeric TNF[268]. TNFR1 bears the cytoplasmic death domain and the trimerization brings these domains in proximity, forming a binding site for other signaling proteins[269]. The first proteins to be recruited to TNFR1 are the receptor-interacting serine/threonine-protein kinase 1 (RIPK1), and the adaptor protein TNFR1-associated death domain (TRADD)[270,271]. TRADD subsequently associates with the adaptor TRAF2, which in turn recruits cellular inhibitor of apoptosis proteins (cIAP) 1 and 2 (ref.[272,273]). Proteins cIAP1/2 function as E3 ubiquitin ligases and their role is to ubiquitinate several molecules within TNFR1-SC. They are responsible for the modification of RIPK1 with K63-, K48-, and K11-polyubiquitin chains and they also undergo a self-ubiquitination[274–278]. The K63-polyubiquitination allows the recruitment of another E3 ligase – linear-ubiquitin chain assembly complex (LUBAC). LUBAC is a complex of three proteins: (1) heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), (2) shank-associated RH domain-interacting protein (SHARPIN), and (3) HOIL-1-interacting protein (HOIP), which is the main enzymatically active unit[279,280]. The LUBAC forms additional, linear (or M1-linked) polyubiquitin chains within the TNFR1-SC[281].

Together, the ubiquitination provided by cIAP1/2 and LUBAC creates a scaffold enabling the recruitment of the TAK1-TABs and IKK complexes[282–284]. The association of these two complexes leads to the activation of gene transcription as described in chapter 1.1.3. The expressed genes cover proinflammatory cytokines, as well as pro-survival proteins like the caspase-8 inhibitor cellular FLICE inhibitory protein (cFLIP), thus protecting the cell from TNF-induced cytotoxicity[285].

The visualisation of the complex I formation is shown in the Figure 2 (page 30).

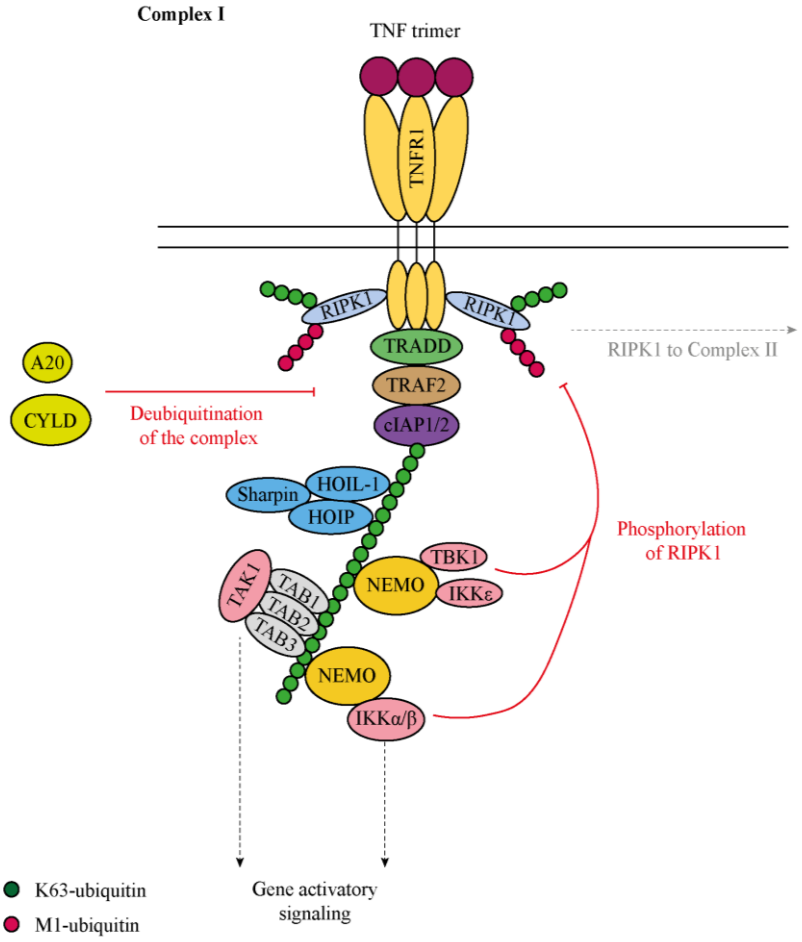

Figure 2. The composition of the complex I.

The TNF trimer binds to the trimeric TNFR1 which leads to the recruitment of kinese RIPK1 and adaptor protein TRADD. TRADD then binds E3 ubiquitin ligases cIAP1/2 through TRAF2. Subsequently, the cIAP1/2 proteins create K63-polyubiquitin chains which serve as a scaffold for diverse proteins promoting the signal from TNF. The complexes TAK1-TABs and IKK are recruited to provide the activation of proinflammatory gene expression. Moreover, the K63-ubiquitin chains bind the LUBAC system (composed of HOIL-1, HOIP and Sharpin subunits), which in turn creates additional M1-ubiquitin chains in the TNFR1-SC. The deubiquinases A20 and CYLD regulate the formation of ubiquitin linkages. Furthermore, kinase TBK1, IKKα/β and IKKε recruited to the TNFR1-SC via NEMO ensures the inactivation of RIPK1 via its phosphorylation.

In the case the complex is not assembled properly, RIPK1 is released from the complex to the cytosol, where it forms cell death-inducing complex II.

1.3.2 Cell death-inducing complex II

Although death is not the primary cell response, under some conditions the TNF stimulation results in the formation of cytosolic cell death-inducing complex II. In case of persistent TNF signal or defective complex I assembly, RIPK1 autophosphorylates itself on serine 166 and is released into the cytoplasm[286], and distinct cell death pathways are activated. RIPK1 recruits the FAS-associated death domain-containing protein (FADD) through its death domain creating a docking site for caspase 8. Caspase 8 then cleaves downstream effector caspases to trigger apoptosis[287,288].

Provided that caspase 8 is unavailable or inactive, the stimulation with TNF triggers necroptosis. In that case, autophosphorylated RIPK1 binds RIPK3 (ref.[286,289,290]). RIPK3 too undergoes autophosphorylation and subsequently phosphorylates mixed lineage kinase domain-like (MLKL) protein[290–294]. This results in a conformational change in MLKL leading to the exposure of the N-terminal domain of MLKL. MLKL then oligomerizes and translocates to the cell membrane. The interference of MLKL with the molecules within the cell membrane induces the formation of pores, which cause membrane disruption and leakage of intracellular content, which is a strong proinflammatory signal[294–296].

The formation of the complex II is depicted in Figure 3 (page 32).

1.3.3 Intracellular control of TNF-induced events

The intracellular control of TNF signaling happens at several levels. The regulation of complex I activity is focused mainly on the regulation of polyubiquitin scaffold resulting in the termination of the signaling cascade. Deubiquitinase A20 creates a negative regulatory feedback loop because, after its upregulation upon TNF stimulation, it is recruited back to TNFR1-SC. There, it binds to M1-ubiquitin chains and removes the K63-linked ubiquitin chains on RIPK1, thus dampening the NF-κB activation[297–300]. The NF-κB signaling can also be reduced by deubiquitinase cylindromatosis (CYLD). CYLD binds to the HOIP subunit of LUBAC and subsequently removes K63- and M1-polyubiquitin chains from the proteins in TNFR1-SC[300–304].

TNF cytotoxicity via activation of complex II can potentially have detrimental consequences. Therefore, it is necessary to keep this arm of TNF signaling under tight control. Several checkpoints regulate the cell death induction[305]. The „IKK checkpoint“

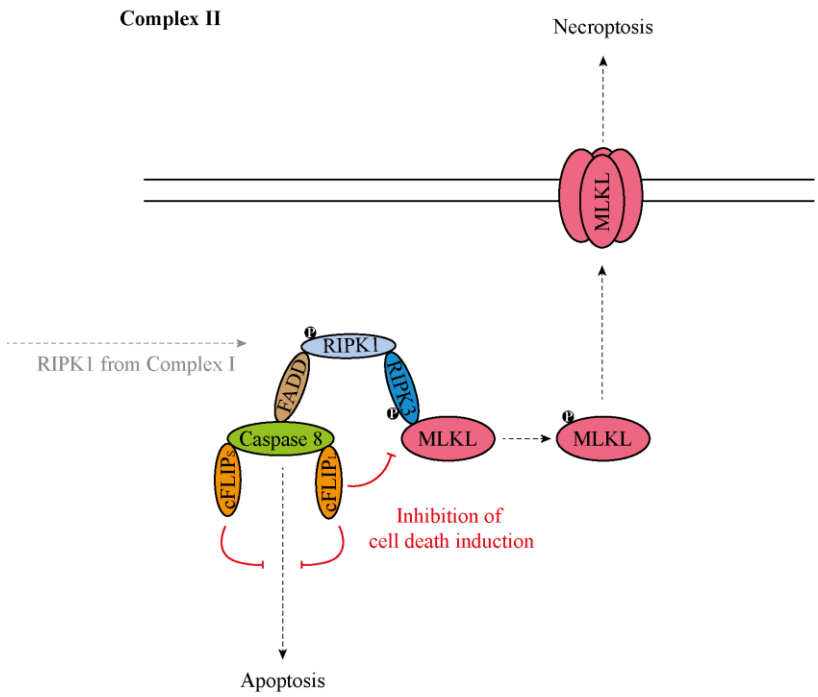

Figure 3. The formation of the complex II.

Under some conditions, RIPK1 autophosphorylates itself on serine 166 and is released from the complex I. In the cytosol, RIPK1 binds caspase 8 via adaptor protein FADD. Caspase 8 subsequently induces the cell death via apoptosis or pyroptosis. Alternatively, RIPK1 phosphorylates RIPK3, which in turn phosphorylates MLKL. Phosphorylated MLKL undergoes a conformational change and oligomerizes. Oligomerized MLKL then creates pores to the plasma membrane and thus executes necroptotic cell death.

is responsible for RIPK1 inactivation, thereby preventing the execution of RIPK-dependent apoptosis and necroptosis. Once RIPK1 is recruited to the TNFR1 and modified by ubiquitination, the ubiquitin chains serve as a docking site for the adaptor protein NEMO. This adaptor is responsible for the recruitment of kinases, which in turn phosphorylate RIPK1 rendering it inactive. These kinases are either IKKα and IKKβ (together with NEMO forming the canonical IKK complex), or non-canonical IKK kinases TRAF family member-associated NF- κB activator (TANK)-binding kinase 1 TBK1 and IKKε, which are recruited via their adaptor proteins TANK and 5-azacytidine-induced protein 2 (AZI2). The two sets of kinases inhibit distinct pools of RIPK1 within the complex I[306–308].

The NF-κB-dependent gene expression is responsible for another level of the TNF cytotoxicity control („NF-κB checkpoint“). Apart from proinflammatory genes, NF-κB

also activates the transcription of cFLIP. Interestingly, the type of cFLIP isoform determines the manner of cell death inhibition. The long isoform, $cFLIP_L$, is recruited to the complex II, where it interacts with caspase 8. Caspase 8 in interaction with $cFLIP_L$ retains its catalytic activity but remains bound to the complex, and, therefore, cannot execute apoptosis. Moreover, caspase 8 can still cleave RIPK1 and RIPK3 and thus inhibit the induction of necroptosis[309–312]. In contrast, if the short isoform of cFLIP, $cFLIP_S$, is recruited to complex II, it inhibits caspase 8 enzymatic activity. Although this results in the inhibition of apoptosis, RIPK1 is not cleaved and therefore is still capable of RIPK3 phosphorylation and execution of necroptosis[293,310,313].

Moreover, a recent study suggests that TRAF6 also prevents the formation of complex II. TRAF6 directly interacts with RIPK1 and therefore restrains it from binding other proteins in complex II such as FADD or RIPK3. For this interaction, the E3 ligase activity of TRAF6 is dispensable[314].

1.3.4 TNF-mediated pathology

Due to its ability to induce the transcription of proinflammatory proteins, TNF is often at the center of mounting immune responses. The gene-activatory pathway is the default effect of TNF stimulation. Nevertheless, when it is compromised by pathogens, the cell switches its response to execute cell death, thus alarming the immune system. From this point of view, cell death is a valuable tool to fight infections, especially from viral pathogens. However, over-excessive TNF-driven cell death can contribute to the development of various pathological conditions. For instance, TNF-induced cell death involving RIPK1 kinase activity lies behind the development of lethal septic shock in mice[315,316].

The dysregulated TNF signaling is associated with the development of various autoimmune diseases. The mutations in crucial proteins within the TNF signaling cascades have been connected to different phenotypes in patients with systemic inflammation.

Homozygous loss-of-function mutation in caspase 8 was found in individuals suffering from autoimmune lymphoproliferative syndrome – an immune disorder that manifests by defective lymphocyte apoptosis, enlargement of lymph nodes and spleen, and autoimmunity[317–320]. Mutations in caspase 8 in mouse keratinocytes also cause severe dermatitis because keratinocytes succumb to excessive necroptosis[321,322]. Likewise, some

patients with severe forms of very early-onset intestinal bowel disease carry mutations in caspase 8. The absence of caspase 8 activity results in elevated levels of RIPK3 which is responsible for the death of intestinal epithelial cells, and subsequent intestinal inflammation[323,324].

Inflammatory bowel diseases, like Crohn's disease, are also associated with other mutations in proteins involved in TNF signaling. Biallelic loss-of-function mutation of RIPK1 in patients results in defective cell response to TNF stimulation which results in severe intestinal inflammation[325,326]. RIPK1 mutations in keratinocytes too are responsible for skin inflammation in mice, again due to keratinocyte necroptosis[327,328]. Intriguingly, recent studies describe several patients to carry pathogenic mutations of RIPK1 which render RIPK1 resistant to cleavage by caspase 8. These patients experience so-called cleavage-resistant RIPK1-induced autoinflammatory syndrome, a disease that manifests by increased susceptibility to apoptosis and necroptosis, and extensive earlyonset autoinflammatory processes[329–331].

Given the indisputable role of polyubiquitination in TNF-induced signaling, the mutations in ubiquitinases are also harmful. Namely, patients bearing loss-of-function mutations in HOIL-1 or HOIP, crucial components of the LUBAC complex, suffer from invasive bacterial infections, amylopectin-like deposits in muscles, severe multi-organ inflammation, and immunodeficiency[332–334]. Accordingly, studies in mice showed, that deficiency in either HOIL-1 or HOIP results in embryonic lethality[335,336]. Moreover, keratinocyte-specific deletion of either HOIL-1 or HOIP in mice leads to the development of severe dermatitis resulting in post-natal lethality due to the aberrant cell death[337]. Finally, the mutation of the third part of LUBAC, SHARPIN, is responsible for chronic proliferative dermatitis (cpdm) in so-called *Cpdm* mice. These mice experience multi-organ inflammation with inflammatory skin lesions which is fully mediated by cell death dependent on RIPK1 kinase activity[281,338–340]. The loss of cIAP1/2 in mice is embryonically lethal due to aberrant TNF-induced signaling[341].

Some autoinflammatory diseases, such as Crohn's disease, rheumatoid arthritis, or type 1 diabetes mellitus, are associated with single-nucleotide polymorphisms in the gene encoding deubiquitinase A20 (ref.[342]). Moreover, uncommon haploinsufficiency in A20 leads to severe autoinflammatory syndrome with recurrent oral, genital, and/or gastrointestinal ulcers as its main characteristics[343,344].

Several damaging mutations of NEMO were also reported. The loss-of-function mutation of NEMO results in an X-linked condition called incontentia pigmenti in

heterozygous females. It manifests as variable abnormalities of skin and skin derivates, or the central nervous system. The mutation is embryonically lethal in male individuals[345]. Severe autoinflammatory syndrome also develops in patients harboring a deletion in exon 5 of the gene encoding NEMO. These patients suffer from, among others, uveitis, or hepatitis[346]. Finally, the deletion of the C-terminal domain of NEMO, which is responsible for the binding of A20, leads to the development of severe inflammation of the skin and intestines accompanied by ectodermal dysplasia, lack of sweating (anhidrosis) and immunodeficiency[347].

Additionally, biallelic loss-of-function mutation of TBK1 in mice is embryonically lethal due to aberrant TNF signaling[308,348,349]. However, in human patients, the loss of TBK1 leads to chronic systemic inflammation which is dependent on TNF-driven cell death via the kinase activity of RIPK1 underlining the particular role of TBK1 within the TNF signaling pathway[350].

The therapy of TNF signaling-associated disorders

Since the disruption of TNF signaling cascades is often connected to strong autoimmune responses, a lot of studies focused on the development of suitable treatment. A soluble TNF receptor fusion protein etanercept is a genetically engineered biologics that mimics TNFR1 and therefore neutralizes TNF in the bloodstream. It was approved for the treatment of psoriasis[351], psoriatic arthritis[352], or rheumatoid arthritis[353].
Several monoclonal antibodies targeting TNF are used to tackle autoimmune diseases such as Crohn's disease, ulcerative colitis (both extreme cases of IBD), or rheumatoid arthritis[354]. Infliximab is the first monoclonal antibody targeting soluble TNF, which has been FDA-approved for clinical use. Together with adalimumab, or certolizumab, these monoclonal antibodies neutralize soluble TNF, thus preventing it from ligating to TNFR1 and initiating the signaling pathway[355].

Interestingly, anti-TNF agents like infliximab or etanercept have also been approved for the treatment of psoriasis or psoriatic arthritis. Nevertheless, as mentioned previously (chapter 1.1.4.), they are less potent in treating these diseases when compared to anti-IL-17 biologics. They are believed to impair the synergistic effect of TNF to IL-17 signaling during the development of these disorders[161,162].

Although usually very effective, the anti-TNF therapy is unfortunately connected with immunosuppression and consequent susceptibility to infections. Therefore, different approaches to treat inflammation are being investigated. A potentially successful

candidate for blunting the TNF-triggered cell death is RIPK1 (ref.[356–358]). Currently, several RIPK1 inhibitors are under development and testing by GlaxoSmithKline and Denali companies. Although some of these compounds have already reached clinical trials for the treatment of Alzheimer's disease or ulcerative colitis, the results are so far inconclusive[359–361].

2 Aims of the study

The presented study is focused on the molecular mechanisms of the inflammatory processes. Although inflammation follows almost every disruption of the host's immune integrity, the precise molecular aspects of it remain elusive. My research concentrated on clarifying the exact regulation of cytokine signaling – specifically, the regulation of IL-17- and TNF-triggered signaling pathways.

In the first part of the presented thesis, I studied the signaling events carried out upon IL-17 stimulation. To this end, I used a unique methodology we established to study the molecular composition of the IL-17 receptor signaling complex (IL-17RSC) via mass spectrometry. Based on the outcome of our initial mass-spec experiments we selected several proteins for further studies. First, we identified non-canonical NF-κB kinases TBK1 and IKKε being highly abundant components of the IL-17 signaling pathway. So far, the role of these kinases in IL-17 signaling has been disputable. Over the past few years, several studies have suggested that TBK1 and IKKε may activate IL-17 signaling[109,114]. On the other hand, it has been shown, that overexpression of either of these kinases may lead to the inhibition of IL-17 signaling[362]. Therefore, to resolve this discrepancy I generated cells deficient in these two kinases and tested the activation of IL-17 signaling pathway and subsequent cytokine production. Additionally, based on our mass-spec data, I also studied the recruitment mode of TBK1 and IKKε to the IL-17RSC.

The next aspect of the IL-17 signaling I wanted to address was the unexpected discovery of the CMTM4 protein as part of IL-17RSC. CMTM4 is a transmembrane protein and therefore we hypothesized, that it might interact with either one or both subunits of the IL-17 receptor. To test this hypothesis, I analyzed the IL-17 signaling in cell lines devoid of CMTM4. Furthermore, to investigate the role of CMTM4 in IL-17-dependent pathology, I also tested the susceptibility of *Cmtm4*-deficient mice generated in the Czech Centre of Phenogenomics at the Institute of Molecular Genetics of the Czech Academy of Sciences (CCP), to, among others, experimental psoriasis.

The third part of the presented thesis focuses on the regulation of the signaling triggered by TNF. Specifically, I aimed to understand the role of adaptor proteins TANK and AZI2. These two adaptors were proposed to recruit TBK1 to the TNF receptor signaling complex to protect from inflammation and cell death. However, their role in protection against cell death *in vivo* remained unexplained. Therefore, to decipher this

issue, I focused on the phenotype characterization of *Tank/Azi2*-double deficient mice generated by CCP and tested their propensity to TNF-driven cell death. Eventually, the *Tank/Azi2*^{DKO} animals were crossed with mice deficient either in TNF receptor 1 or cell death-driving kinase RIPK1 to see whether the absence of either of these proteins would rescue the phenotype.

2.1 Specific aims of the study

1. Clarify the role of kinases TBK1 and IKKε within the IL-17 signaling pathway.
2. Explain the mode of TBK1 and IKKε recruitment to the IL-17 receptor signaling complex.
3. Define the interaction of CMTM4 with the IL-17 receptor.
4. Describe the function of CMTM4 concerning IL-17 receptor subunits.
5. Elucidate the role of CMTM4 in IL-17-mediated inflammation *in vivo*.
6. Characterize the phenotype of *Tank/Azi2*^{DKO} mice and its potential rescue.
7. Describe the role of adaptor proteins TANK and AZI2 within the TNF signaling.
8. Define the recruitment mechanism of TANK and AZI2 adaptor proteins to the TNF receptor signaling complex.

3 Materials and methods

The experiments performed in the studies discussed in my doctoral thesis cover a range of methodologies. I utilized both *in vitro* and *in vivo* analyses.

In general, the approaches in *in vitro* and *ex vivo* experiments used in the published studies combine (among others) (1) immunoprecipitation and immunoblotting, (2) tandem affinity purification and mass-spec analysis, (3) multicolor flow cytometry, (4) cell death analysis, (5) quantitative real-time PCR, and (6) RNA sequencing.

For the *in vitro* experiments, I used both murine and human cell lines. The main murine cell line I worked with was stromal ST2 cells. Additionally, I isolated primary mouse embryonic fibroblasts (MEF) from E11.5 mouse embryos of desired phenotypes and immortalized them by lentiviral infection with the SV40 large T antigen. Moreover, in some experiments, I analysed the response of primary keratinocytes and primary bone marrow-derived macrophages (BMDM). Also, for the presented research I used human cell lines HeLa, HEK 293T, and A549.

Several experiments were focused on the analysis of either knockout or reconstituted cell lines. I generated the knockout cell lines using the CRISPR-Cas9 system with single-guided RNAs targeting selected genes designed by the web tool CHOPCHOP[363]. In some cases, I employed retroviral reconstitution of selected cell lines with proteins of interest. For that purpose, I produced retroviruses in Phoenix-Eco and Phoenix-Ampho cells transfected with a pBabe vector harboring coding sequences of selected genes. The coding sequences of proteins of interest were either amplified from wild-type cells or prepared using the GeneArt Gene Synthesis service from Thermo Fisher Scientific.

The prepared cell lines were used in a range of experiments, including the analysis of activation of signaling pathways via immunoblotting or qPCR analysis, and analysis of signaling complexes via immunoprecipitation. For the proteomic analysis of the IL-17RSC composition, I first stimulated the cells with recombinantly produced Strep-Flag-tagged IL-17. Subsequently, I isolated the formed IL-17RSC via tandem affinity purification. The purified samples were subsequently analyzed via mass spectrometry.

The *in vivo* experiments were carried out on various transgenic models derived from the C57Bl/6J mouse strain in the CCP. Mice were housed in individually ventilated cages in a specific-pathogen-free animal facility of the Institute of Molecular Genetics.

For all experiments, 5 to 24-week-old mice were used. To obtain the *Cmtm4*$^{-/-}$ mice, frozen sperm from mouse strain C57BL/6N-A^{tm1Brd} Cmtm4$^{tm1a(EUCOMM)Wtsi/WtsiBiat}$ was obtained from The European Mouse Mutant Archive repository (EM:06038) and used for in vitro fertilization. I subsequently used *Cmtm4*$^{-/-}$ mice to analyze IL-17 responses. The direct responsiveness of these animals to IL-17 was assessed upon intraperitoneal administration of IL-17, followed by intraperitoneal lavage, which indicated whether IL-17-induced recruitment of immune cells was impacted. I first injected the mice with IL-17 or a control solution. After 4 hours, I analyzed the cells recruited to the mouse peritoneum via flow cytometry. Furthermore, I studied the responsiveness of *Cmtm4*$^{-/-}$ mice to the induction of experimental psoriasis with imiquimod (IMQ). In these experiments, I applied Aldara cream containing 5% IMQ, on the shaved mouse ear and back skin for 5 consecutive days and monitored the development of psoriasis daily. Alternatively, the *Cmtm4*$^{-/-}$ mice were injected with myelin oligodendrocyte protein to induce the EAE.

Tank$^{-/-}$, *Azi2*$^{-/-}$, *Tnfrsf1a*$^{-/-}$, or *Ripk1*$^{KD/KD}$ mice were generated in the CCP. I focused mainly on the phenotypical analysis of these mice via multi-color mass spectrometry. Alternatively, I injected the mice with a sub-lethal dose of TNF and analyzed their susceptibility to TNF-induced cell death.

The precise description of the methods used can be found in respective publications.

4 Results and discussion

Inflammation is the key mechanism of the immune system to tackle the pathogenic processes that develop under certain conditions in our bodies. In reaction to pathogen presence, various signaling pathways are induced to amplify the immune response and contain the infection. In the development of inflammation, the crucial signaling cascades triggered by IL-17 or TNF play a predominant role, as the inability to produce or signal via these two cytokines can lead to severe immunodeficiencies. Although both signaling pathways have been extensively studied over the years, some aspects and the regulation of these pathways remain unexplained. To better understand these signaling pathways, we first studied the composition of the IL-17RSC. Subsequently, we investigated the mode of regulation of IL-17RSC assembly. Additionally, we examined how the loss of a newly identified component of IL-17RSC, the CMTM4 protein, affects the development of IL-17-dependent autoimmunities *in vivo*.

In the second part of our research, we investigated the precise role of adaptor proteins TANK and AZI2 within the TNFR1-SC. Although they have previously been connected to the recruitment of TBK1 to TNFR1-SC[307], their precise role in TNF-induced signaling remained elusive. We, therefore, investigated the function of these proteins *in vivo*. As TBK1 plays a crucial role in protection against TNF-triggered cell death, we focused mainly on the role of TANK and AZI2 in the context of cell death.

4.1 A robust feedback loop regulates IL-17RSC

While IL-17 is a critical part of anti-fungal immunity, overactivation of its signaling leads to the development of autoimmunities. Therefore, precisely regulating IL-17RSC assembly is indispensable to balance these two effects. To study the composition of the IL-17RSC immediately after its formation, we have developed a unique methodological approach based on mass spectrometry (**Publication #1**). Via this method, we identified two kinases, TBK1 and IKKε, as crucial components of the IL-17RSC. The activation of these kinases upon IL-17 stimulation is very strong, up to levels comparable with other proinflammatory cytokines such as TNF or IL-1β. Although these kinases have previously been connected to the IL-17RSC, their role in IL-17-dependent inflammatory signaling has, so far, been very controversial. Several highly cited studies have suggested, that TBK1 and IKKε promote the activation of the IL-17 signaling pathway, as the loss

of either of these kinases prevented the expression of IL-17-triggered proinflammatory genes[109,114,364]. This hypothesis was later supported by a study by Tanaka et al. showing that cells double-deficient in TBK1 and IKKε failed to produce proinflammatory cytokines upon IL-17 stimulation in the presence of TNF[365]. However, TBK1/IKKε double-knockout (DKO) cells have already been shown to be susceptible to TNF-induced cell death[307,308,366]. Therefore, it is possible that TBK1/IKKε DKO cells were unable to trigger IL-17 signaling because they have already succumbed to cell death triggered by TNF.

In contrast, in 2012 a study by Qu et al. demonstrated, that overexpression of TBK1 and IKKε results in attenuation of IL-17 signaling due to impaired recruitment of TRAF6 to Act1 (ref.[362]). Nevertheless, these results depend on the overexpression system and thus provide only a limited insight into the molecular basis behind this phenotype. In **Publication #1** we therefore aimed to resolve this discrepancy.

Our data suggest that within IL-17 signaling, TBK1 and IKKI are part of a robust inhibitory feedback loop. The data from our mass-spec analysis revealed, that both of these kinases were profoundly recruited to the IL-17RSC upon the stimulation with IL-17. The following experiments showed these kinases to work redundantly. Nevertheless, the loss or inactivation of both of them resulted in strong activation of IL-17 signaling. The loss of TBK1/IKKε kinase activity is followed by enhanced recruitment of LUBAC and TAK1-TABs complex. These molecules in turn augment the activation of the NF-κB signaling pathway[367–369].

Furthermore, based on our mass-spec data, we revealed that IL-17RSC recruits six molecules of Act1. Act1 has previously been shown to form homo-oligomers[132,370]. We thus hypothesize, that once recruited to the IL-17RSC, Act1 oligomerizes and forms a binding platform for trimeric K63-polyubiquitin ligase TRAF6. As trimeric TRAF6 is prone to bind oligomeric scaffolds[371], the possibility of Act1 oligomerization provides a suitable option for TRAF6 enrichment within the IL-17RSC. Enzymatically active TRAF6 present at the IL-17RSC appears to be crucial for the propagation of the IL-17 signal within the cell since cells deficient in TRAF6 or expressing its enzymatically inactive version failed to trigger the expression of proinflammatory cytokines.

Canonically, K63-polyubiquitin chains recruit NEMO and kinases IKKα/IKKβ to induce the NF-κB signaling pathway[372]. Unexpectedly, our data revealed, that NEMO plays a dual role within the IL-17RSC. Next to its canonical role in the IKK complex, NEMO also recruits adaptor proteins TANK and AZI2, which, in turn, mobilize kinases

TBK1 and IKKε. An analogous role for NEMO has already been described in connection with the TNF signaling pathway[307]. Kinases TBK1/IKKε can be found in numerous signaling pathways in which they regulate various cellular processes such as interferon signaling[349,373] or TNF-induced cell death[307,308]. The phosphorylation of Act1 by TBK1/IKKε on various residues in a long unstructured region results in the separation of SEFIR and TRAF-binding domains. The spatial segregation of these domains leads to the release of TRAF6 from the signaling complex thus reducing its ability to promote inflammation via polyubiquitination of other molecules recruited to the IL-17RSC.

Together, our data provide compelling evidence that the negative regulation of the IL-17 signaling is provided by TBK1/IKKε kinases. Their kinase activity is responsible for the dissociation of TRAF6 from the complex, thus dampening the induction of the IL-17 signaling (Figure 4).

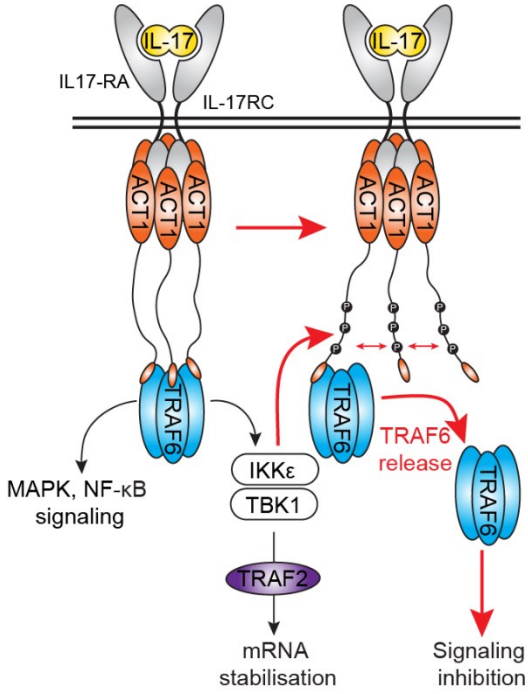

Figure 4. Kinases TBK1 and IKKε negatively regulate IL-17 signaling.

The binding of dimeric IL-17 to its cognate receptor leads to the recruitment of six molecules of Act1. Act1 oligomerization results in the recruitment of trimeric E3-ubiquitin ligase TRAF6 which subsequently enables the activation of MAPK and NF-κB signaling pathways. TRAF6 also recruits kinases TBK1 and IKKε. These kinases have a dual role within the IL-17RSC. First, they provide the mRNA stabilization via TRAF2. Secondly, they phosphorylate Act1 in a long unstructured region of the protein. This phosphorylation leads to the separation of SEFIR and TRAF6-binding domains of Act1, release of TRAF6 from the complex, and subsequent inhibition of the IL-17 signaling (published in ref.[374]).

4.2 CMTM4 is an integral part of IL-17RSC

Publication #2 is a follow-up study arising from **Publication #1** further exploring the composition of the IL-17RSC. The mass-spec-based method we developed in **Publication #1** revealed not only the presence of the TBK1/IKKε kinases at IL-17RSC but also a tetra-transmembrane protein CMTM4 – so far unknown component of the receptor complex. Our experiments implied that CMTM4 was a crucial binding partner of the IL-17RC subunit of the IL-17RSC. The analysis of publicly available RNA sequencing data suggested, that CMTM4 was expressed in various tissues throughout the body except on immune cells, which correlates with the tissue distribution of IL-17RC[81,94].

To further analyze the role of CMTM4 in IL-17 signaling, we produced various cell lines devoid of CMTM4. The cells lacking CMTM4 showed only very weak surface expression of IL-17RC. This expression was restored after reconstitution of *Cmtm4*$^{-/-}$ cells CMTM4. The following experiments showed a strong association between CMTM4 and IL-17RC, which allowed the glycosylation of the latter in the *trans*-Golgi compartment and its subsequent translocation to the plasma membrane. As other transmembrane proteins in *Cmtm4*-depleted cells did not show altered glycosylation and localization, we conclude that this CMTM4 effect is unique for IL-17RC.

The loss of CMTM4 in cell lines is reflected in impaired cellular response to IL-17 stimulation. *Cmtm4*$^{-/-}$ cells were unable to activate signaling pathways and produce proinflammatory cytokines upon stimulation with IL-17. Accordingly, *Cmtm4*-deficient mice were partially resistant to IL-17-induced pathology. IL-17 was described to recruit neutrophils into the peritoneum[375]. However, *Cmtm4*$^{-/-}$ mice were unable to mount this immune response to intraperitoneally injected IL-17. Moreover, *Cmtm4*-deficient mice developed only mild disease symptoms in IMQ-induced psoriasis. Additionally, *Cmtm4*$^{-/-}$ mice showed mild augmentation in EAE progression. Nevertheless, this improvement did not reach statistical significance. Moreover, the role of IL-17 in this animal model of multiple sclerosis is disputable and seems to depend on microbial colonization[167,168,176]. For our EAE experiments, we used *Cmtm4*$^{+/+}$ and *Cmtm4*$^{-/-}$ littermates bred in shared cages. These animal likely had the same distribution of microbiota, which might clarify the relatively similar disease progression in these groups.

Taken together, the data published in **Publication #2** show that CMTM4 is a new subunit of the IL-17RSC, and it is necessary for IL-17-induced cellular responses. CMTM4 works as a crucial binding partner of IL-17RC, facilitating its glycosylation and membrane localization, thus enabling the formation of IL-17RSC. A rather similar role of CMTM4 was described in connection with immunomodulatory protein PD-L1, where CMTM4, together with CMTM6, promoted PD-L1 stabilization[228,229,376].

The function of CMTM4 within IL-17RSC is shown in the scheme in Figure 5.

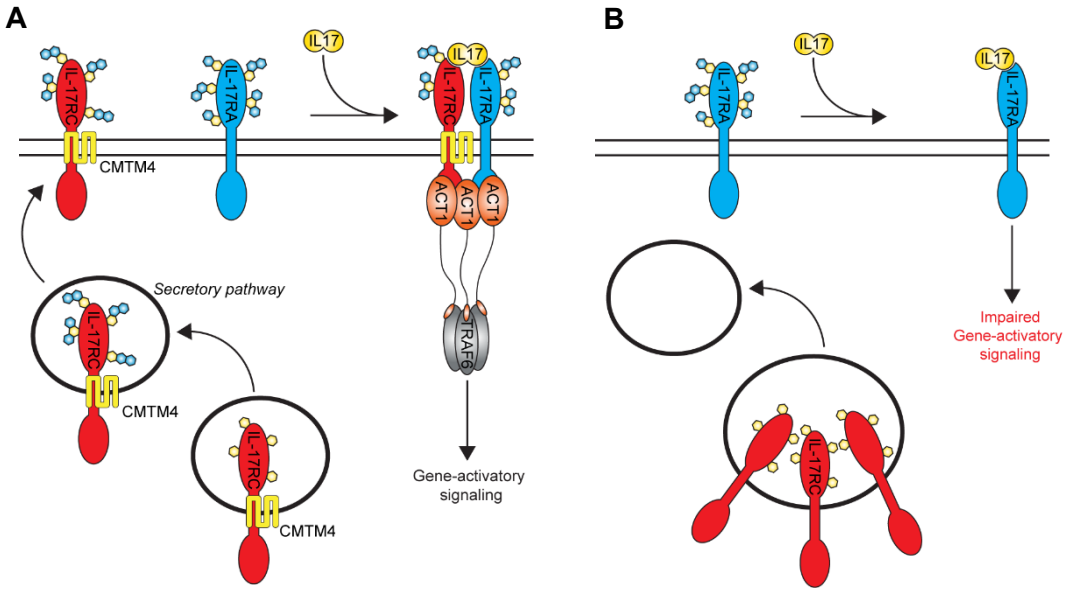

Figure 5. CMTM4 is a crucial part of the IL-17RSC.

(A) CMTM4 is a crucial binding partner of IL-17RC. Once IL-17RC interacts with CMTM4 in the *trans*-Golgi apparatus, IL-17RC is glycosylated and transported to the plasma membrane. Upon IL-17 stimulation, IL-17RC with bound CMTM4 forms a receptor complex with IL-17RA followed by the formation of a signaling complex. This initiates the signaling cascade that results in the activation of proinflammatory genes.

(B) In the absence of CMTM4, IL-17RC remains unglycosylated in the cytoplasm. Since IL-17RC cannot reach the plasma membrane, the formation of the whole IL-17RSC is impaired, and proinflammatory genes cannot be activated (published in ref.[377]).

4.3 Adaptor proteins TANK and AZI2 protect from TNF-induced cell death

The TNF signaling pathway is a crucial player in the immune response to infection. Based on the circumstances, TNF stimulation can either trigger the expression of proinflammatory genes or direct the cell to the cell death pathway with potentially

detrimental effects on the organism. A non-canonical IKK kinase TBK1 plays a dual, but important role within TNF signaling. It partly suppresses the expression of proinflammatory genes triggered by NF-κB and MAPK signaling pathways. On the other hand, it restrains TNF-induced cell death by phosphorylation of RIPK1[307,308]. The recruitment of TBK1 to TNFR1-SC is ensured by two adaptor proteins, TANK and AZI2[307]. However, the precise mode of recruitment of TBK1 via these proteins, as well as their role in TNF-induced cell death *in vivo* remains elusive. **Publication #3** is focused on clearing these issues up.

To elucidate the role of adaptor proteins TANK and AZI2, we first generated various cell lines devoid of either one or both of these proteins. In accordance with previously published studies on *Tbk1*$^{-/-}$ cells[307,308], our cell lines showed slight enhancement in proinflammatory signaling pathways, while being more prone to TNF-induced cell death.

Subsequently, to investigate the role of TANK and AZI2 *in vivo*, we obtained mice lacking either one or both of these adaptors. While neither *Tank*$^{-/-}$ nor *Azi2*$^{-/-}$ show any overt phenotype, the loss of both these proteins results in severe autoinflammatory disorder. *Tank/Azi2*^{DKO} mice are born in sub-mendelian ratio and suffer from multiorgan inflammation, granulonephritis, liver fibrosis, disrupted splenic architecture, and premature death. Moreover, these mice have delayed hair growth, their skin lacks subdermal fat and shows marks of dermatitis. Therefore, it seems that TANK and AZI2 work redundantly. Despite the severity of the phenotype present in *Tank/Azi2*^{DKO} mice, the simultaneous loss of these adaptors results only in partial embryonic lethality. This is in contrast with *Tbk1*$^{-/-}$ mice on the same background, which phenotype is embryonically lethal[348]. This observation is also supported by our results from *in vitro* experiments with *Tank/Azi2*^{DKO} cells, where the activation of TBK1 reflected in its phosphorylation was strongly diminished, but not completely abolished. The activation of TBK1 is provided either by autophosphorylation of TBK1 recruited to the TNFR1-SC, or by phosphorylation by IKKα/β in the cytoplasm[378]. Therefore, although the recruitment of TBK1 to the TNFR1-SC is defective in *Tank/Azi2*^{DKO} cells, it can still be activated in the cytoplasm by IKKα/β to provide partial protection against cell death, thus explaining the diverse phenotype of *Tank/Azi2*^{DKO} and *Tbk1*$^{-/-}$ mice.

To obtain a better insight into TNF-mediated events in *Tank/Azi2*^{DKO} mice, we generated *Tank*/Azi2^{DKO} mice either deficient in TNFR1 (termed *Tank/Azi2/Tnfrsf1a*^{TKO} mice), or bearing catalytically inactive version of RIPK1 (termed

Tank/Azi2^{DKO}*/Ripk1*$^{KD/KD}$ mice). The ablation of TNFR1 resulted in the rescue of the detrimental phenotype of *Tank/Azi2*^{DKO} mice, while the catalytic inactivation of RIPK1 led only to a partial rescue. The *Tank/Azi2*^{DKO}*/Ripk1*$^{KD/KD}$ animals still suffered from liver damage correlated with augmented levels of isotype-switched B cells. Our experiments with bone marrow transfer surprisingly showed that B cells deficient in TANK and AZI2 are intrinsically susceptible to isotype switching. This finding corresponds with a study published in 2012 demonstrating, that TBK1 negatively regulates IgA isotype switching in B cells[379]. The process of isotype switching is strongly enhanced in *Tank/Azi2*^{DKO} mice compared to wild-type mice and it is dependent on TNFR1 as *Tank/Azi2/Tnfrsf1a*^{TKO} mice do not show this phenotype.

Intriguingly, *Tank/Azi2*^{DKO} male mice are sterile. The loss of male sterility was also rescued by TNFR1 ablation or RIPK1 catalytic inactivation. It is therefore possible, that TNF-induced cell death is partially responsible for male infertility. Interestingly, necroptosis along with the elevated levels of TNF seems to be an important regulatory mechanism of the male reproductive system, since mice lacking RIPK3 or MLKL show delayed aging of the testes[380]. TNF-targeted treatment could therefore be a new therapeutical option to tackle the problems with male fertility.

Our data provide compelling evidence, that *in vivo*, TANK and AZI2 work redundantly despite their striking sequential and structural distinction. It is therefore possible, that their role in regulating inflammation might be different. Our study has shown, that both adaptors require NEMO for their recruitment to TNFR1-SC, but the kinetics of their recruitment differ. During the initiation of the TNFR1-SC assembly, TANK interacts with NEMO which is associated with the signaling complex. However, our data show, that the interaction between NEMO and AZI2 is indirect and it relies on deubiquitinase A20. The A20 protein is recruited to TNFR1-SC in a NEMO-dependent manner and subsequently binds to M1-linked polyubiquitin chains. The binding of A20 to the polyubiquitin scaffold ensures that A20 remains connected with TNFR1-SC even in later stages of stimulation when NEMO has already dissociated[381]. Accordingly, individuals bearing a mutated version of NEMO, which is unable to recruit A20 to TNFR1-SC, suffer from autoimmune issues[347]. We, therefore, suggest that the different recruitment kinetics of TANK and AZI2 guarantee TBK1 activity within TNFR1-SC through all phases of the complex assembly, thus protecting the cell against the undesirable execution of cell death (Figure 6, page 46).

Furthermore, the central role of NEMO in recruiting TANK, A20, AZI2, and TBK1 to TNFR1-SC is underlined by the detection of several NEMO mutations in human patients suffering from immunodeficiencies[382]. In our experiments, we demonstrate, that human NEMO bearing the L227P mutation is unable to recruit TANK, A20, AZI2, or TBK1 to TNFR1-SC. According to structural prediction[383], the L227P mutation is located in the alpha-helical segment of NEMO and it might be responsible for the disruption of protein structure.

Altogether, **Publication #3** demonstrates, that adaptor proteins TANK and AZI2 are an indispensable part of the TNF receptor signaling complex ensuring control over the activation of cell death, which has potential detrimental effects on the organism. Although they seem to be highly redundant, presumably, the presence of both these proteins is necessary to properly control TNF-induced signaling and cell death.

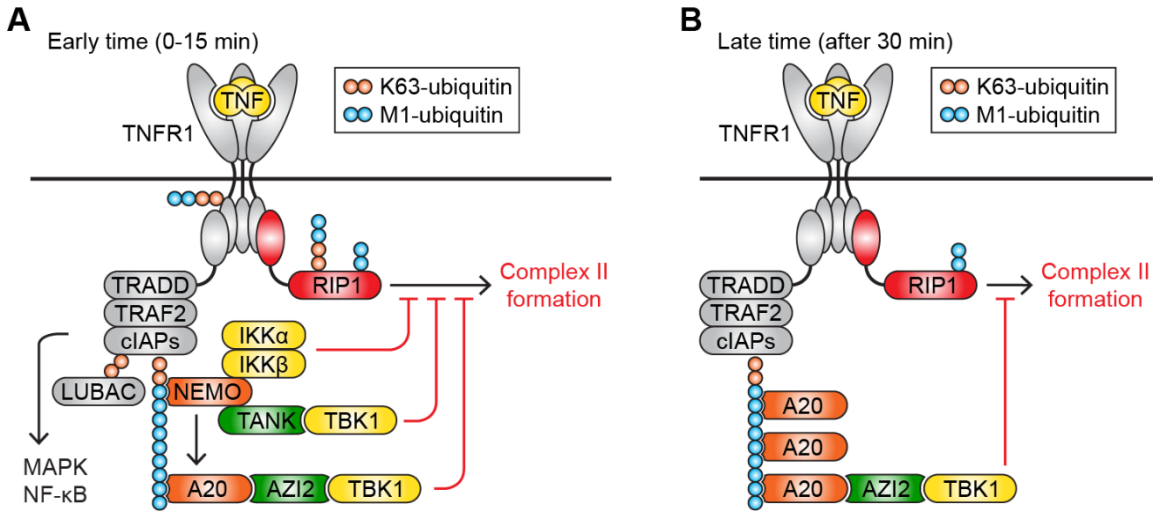

Figure 6. Adaptor proteins TANK and AZI2 protect against TNF-induced cell death.

(A) In the early time points of TNF stimulation a proinflammatory signaling cascade is form and TANK si recruited to the receptor via NEMO. TANK works a an adaptor for TBK1 which in turn phosphorylates RIPK1 thus blocks the formation of cell death-inducing complex II. Eventually, NEMO with bound TANK dissociates from the complex.

(B) AZI2 is recruited to the receptor in the later time upon TNF stimulation and also brings TBK1. However, in contrast to TANK, AZI2 is recruited via deubiquitinase A20 which remains bound to the signaling cascade even in later time points. The different binding kinetics of TANK and AZI2 ensures the activity of TBK1 through all stages of the TNFR1-SC assembly (currently under revisions).

5 Conclusion

Inflammation is a pivotal immune reaction to the pathogenic processes threatening the integrity of the organism. The development of the inflammatory immune response is strongly dependent on intercellular communication via proinflammatory cytokines and chemokines, which are secreted by cells upon activation of various signaling pathways. The presented doctoral thesis discusses three projects concerning the signaling cascades triggered by two crucial proinflammatory cytokines – IL-17 and TNF.

The first and second projects are focused on the composition and assembly of the IL-17 receptor signaling complex. Via unique mass-spectrometry-based methodology, we identified proteins recruited to IL-17RSC immediately after stimulation with IL-17. First, we concentrated on resolving the controversial role of kinases TBK1 and IKKε within IL-17 signaling. In our experiments, we demonstrated, that kinases TBK1 and IKKε are responsible for relatively weak activation of IL-17 signaling when compared to other proinflammatory cytokines like TNF or IL-1β. When recruited to IL-17RSC, TBK1 and IKKε create a robust negative feedback loop through which they keep the activation of the IL-17 signaling cascade under tight control.

Second, we identified a new component of the IL-17 receptor – a transmembrane protein CMTM4. We showed, that this protein is a binding partner for the IL-17RC subunit of IL-17 receptor long before IL-17RC reaches the plasma membrane. The interaction between CMTM4 and IL-17RC allows full maturation of IL-17RC in the *trans*-Golgi compartment and subsequent translocation of IL-17RC onto the cellular surface. Importantly, mice lacking CMTM4 were unable to mount a proper immune response when challenged with IL-17. Therefore, CMTM4 seems to be an indispensable part of the IL-17 signaling pathway, which is completely disrupted in case of CMTM4 unavailability.

The third project discussed in this thesis revolves around the TNF signaling pathway. In this work, we focused on the role of adaptor proteins TANK and AZI2 in the protection against TNF-driven cell death. We show, that TANK and AZI2 are responsible for the recruitment of kinase TBK1 to the TNF receptor signaling complex. Although they are functionally redundant, the simultaneous loss of both adaptors results in severe autoinflammatory disease followed by premature death in mice. The recruitment mode of TANK and AZI2 to TNFR1-SC differs. TANK is recruited via direct interaction with

adaptor protein NEMO, whereas AZI2 is connected to NEMO indirectly via deubiquitinase A20. The distinct recruitment pattern of TANK and AZI2 ensures the presence of TBK1 within TNFR1-SC during all phases of the complex assembly and thus prevents the undesirable activation of cell death.

5.1 Summary of major findings

1) Using a unique methodology based on the mass-spec analysis, we have identified two non-canonical IKK kinases TBK1 and IKKε as major components of the IL-17RSC.
2) We have demonstrated, that six molecules of adaptor protein Act1 are recruited to the IL-17RSC forming a docking site for subsequent signaling cascade.
3) We have revealed, that TBK1 and IKKε recruited to the IL-17RSC create a robust negative regulatory feedback loop by phosphorylating Act1 on multiple residues.
4) We have shown, that CMTM4 is an integral part of IL-17R and strongly associates with the transmembrane domain of IL-17RC.
5) We have demonstrated, that the loss of CMTM4 results in disrupted IL-17 signaling and partial protection against autoinflammation.
6) We have shown that TBK1 is recruited to the TNFR1-SC by adaptor proteins TANK and AZI2 with different kinetics.
7) Adaptor proteins TANK and AZI2 work redundantly, but the simultaneous loss of both these proteins results in strong multiorgan inflammation.
8) The autoinflammatory disease triggered by the loss of TANK and AZI2 is partly driven by TNF-induced cell death.

6 Publications

The reprints of the publications can be found at the end of the thesis in chapter 8.

6.1 List of publications

1. Draberova H[*], Janusova S[*], **Knizkova D**[*], Semberova T, Pribikova M, Ujevic A, Harant K, Knapkova S, Hrdinka M, Fanfani V, Stracquadanio G, Drobek A, Ruppova K, Stepanek O, Draber P. Systematic analysis of the IL-17 receptor signalosome reveals a robust regulatory feedback loop. EMBO J. 2020 Sep 1;39(17):e104202. PMID: 32696476. (IF_{2022} = 11,4)

2. **Knizkova D**[*], Pribikova M[*], Draberova H, Semberova T, Trivic T, Synackova A, Ujevic A, Stefanovic J, Drobek A, Huranova M, Niederlova V, Tsyklauri O, Neuwirth A, Tureckova J, Stepanek O, Draber P. CMTM4 is a subunit of the IL-17 receptor and mediates autoimmune pathology. Nat Immunol. 2022 Nov;23(11):1644-1652. PMID: 36271145. (IF_{2022} = 30,5)

3. Ujevic A, **Knizkova D**, Synackova A, Pribikova M, Trivic T, Dalinskaya A, Drobek A, Niederlova V, Paprckova D, De Guia R, Kasparek P, Prochazka J, Labaj J, Fedosieieva O, Roeck B, Mihola O, Trachtulec Z, Sedlacek R, Stepanek O, Draber P. TBK1-associated adaptors TANK and AZI2 protect against TNF-induced cell death and severe autoinflammatory disease. (under revisions in Nature Communications)

[*] Equal contribution

6.2 Contribution

Ad 1. **Systematic analysis of the IL-17 receptor signalosome reveals a robust regulatory feedback loop**

I joined the project in its later stages. I contributed to biochemical experiments and helped with the preparation of samples for mass spectrometry analysis (Fig. 2H, 4C-E, 4G, 5C-D, EV5B-C). I also performed several mouse experiments with TBK1/IKKε inhibitor MRT67307, which eventually were not used in the study. I am the co-first author of this study.

Ad 2. **CMTM4 is a subunit of the IL-17 receptor and mediates autoimmune pathology**

I am the first co-author of this study. I joined the project in its initial steps and since the beginning, I helped to produce CMTM4-deficient cell lines and analyzed signaling triggered by IL-17A in them (Fig. 2A-B, 3E, 4A-D; extended data Fig. 2A, 5A, 5D, 5F-G). Later on, *in vivo* experiments were my main focus. I performed all experiments with IMQ-induced psoriasis, contributed to the intraperitoneal lavage experiments on *Cmtm4*-deficient mice, and participated in EAE experiments (Fig. 5A, 5C-D; extended data Fig. , 7B-C, 7E, 8A-D, 8F).

Ad 3. **TBK1-associated adaptors TANK and AZI2 protect against TNF-induced cell death and severe autoinflammatory disease**

I am a contributing author of this study. I focused mainly on the phenotypical analysis of *Tank/Azi2*^{DKO} mice via multi-color flow cytometry, and I studied their susceptibility to TNF-induced toxicity (Fig. 2B-F, 3B, 3D-I, 5A-D, 5N-O, supplementary Fig. 2B-E, 3A-F, 5A, 5E-F). I also contributed to biochemical analyses of various cell lines as well as primary cells isolated from mice (4A-B, supplementary Fig. 4C-D).

7 References

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8 Reprints of publications