# Charles University

# Faculty of Science

Study programme: Biology (N1501)

Branch of study: Immunology (1511T004)



Bc. Tereza Šemberová

**Regulation of IL-17 receptor complex signaling**

**Regulace signalizace receptorového komplexu pro IL-17**

Diploma thesis

Supervisor: Mgr. Peter Dráber, Ph.D.

Prague, 2021

**Prohlášení**

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

V Praze dne    10. 8. 2021

Podpis

## Acknowledgments

I would like to thank my supervisor Mgr. Peter Dráber, Ph.D. for his patience and recommendations provided during writing of my diploma thesis, for his valuable guidance and assistance with experimental work, and primarily for the opportunity to participate in projects and research in the Laboratory of Immunity and Cell Communication, BIOCEV. Further, I thank all of my colleagues for amazing and friendly team, for their help, advices and perfect collaboration within our projects. Many thanks also to the head of the Laboratory of Adaptive Immunity, ASCR, Mgr. Ondřej Štěpánek, Ph.D. and my colleague Mgr. Martina Huranova, Ph.D. for their assistance during my microscopy experiments.

Also, I would like to greatly thank my family and friends for continuous support, encouragement and family background they provided me during my studies.

## Poděkování

Ráda bych na tomto místě poděkovala svému školiteli Mgr. Peteru Dráberovi, Ph.D. za trpělivost a doporučení věnované při zpracovávání mé diplomové práce, za vedení a nápomoc při experimentální práci a především za možnost podílet se na projektech a výzkumu v rámci Laboratoře imunity a buněčné komunikace, BIOCEV. Též bych ráda poděkovala všem svým kolegům v laboratoři za úžasné přátelské prostředí, za pomoc a rady a za skvělou spolupráci při práci na projektech. Děkuji také vedoucímu Laboratoře adaptivní imunity, AV ČR, Mgr. Ondřeji Štěpánkovi, Ph.D. a své kolegyni Mgr. Martině Huranové, Ph.D. za poskytnutou pomoc a rady při mikroskopických experimentech.

V neposlední řadě bych chtěla moc poděkovat své rodině a známým za jejich neustálou podporu, motivaci a zázemí, které mi během mého studia poskytli.

# Abstract

Inflammatory immune response is essential for maintaining the defense against invading pathogens, although its aberrant activation leads to impaired self tolerance and development of autoimmune pathologies. Interleukin-17A (also known as IL-17), is a major proinflammatory cytokine, which contributes to the development and maintenance of inflammation and provides protection against several bacterial and yeast infections. However, extreme activation of IL-17 signaling leads to autoimmune pathologies. Thus, a strict regulation of IL-17 signal transduction is crucial to prevent progression of autoimmunity.

Non-degradative ubiquitination is one of the main mechanisms regulating IL-17 signaling. Main E3 ubiquitin ligase within this signal transduction is TRAF6, which is also participating in several signaling pathways within the immune system. Non-degradative polyubiquitin chains created within inflammatory signaling complexes recruit signaling proteins such as IKK complex and TAK1 kinase, crucial for triggering of NF-κB and MAPKs downstream pathways. However, activation of these pathways upon IL-17 is very weak in comparison with other inflammatory stimuli, indicating a presence of a strong negative feedback loop.

In this thesis, we establish the role of several regulatory molecules in IL-17 signaling. First, we clarified the role of TBK1 and IKKε kinases in IL-17 signaling. We have revealed that these kinases serve as negative regulators of IL-17-mediated signal transduction, thus providing a major negative feedback loop. As the second part, we have found a new component of IL-17 receptor signaling complex, a plasma membrane localized protein CMTM4, which is necessary for assembly of IL-17 receptor signaling complex and subsequent triggering of IL-17-induced signaling. Thus, our findings provide new insights into how IL-17 signaling is activated and reveal new regulatory molecules which might be in future targeted for therapeutic modulation of IL-17-induced signaling in autoimmune disorders.

## Keywords

# Abstrakt

Přestože je zánětlivá imunitní odpověď důležitá pro obranu proti patogenům, přehnaná aktivace imunitního systému může vést k poruše tolerance vlastních tkání a rozvoji autoimunitních onemocnění. Interleukin-17A (též označován jako IL-17), jakožto prozánětlivý cytokin přispívá k rozvoji a udržení obranné imunitní reakce a poskytuje ochranu proti řadě bakteriálních nebo plísňových infekcí. Přehnaná aktivace IL-17 signalizace však může vést k rozvoji autoimunity. Proto je regulace IL-17 signalizace klíčová pro zabránění rozvoje autoimunitních patologií.

Nedegradující ubikvitinace je jedním z hlavních mechanismů regulace IL-17 signalizace, zprostředkována především TRAF6 E3 ubikvitin ligázou sdílenou několika signálními drahami v rámci imunitního systému. Nedegradující polyubikvitinové řetězce v zánětlivých drahách aktivují signální proteiny jako IKK komplex a TAK1 kinázu, které jsou klíčové pro aktivaci NF-κB a MAPK signálních drah. Signalizace přes IL-17 je však v porovnání s ostatními zánětlivými stimuly velmi slabá, což naznačuje přítomnost silné negativní zpětné vazby.

V této práci se nám podařilo objasnit roli některých regulačních molekul v IL-17 signalizaci. Nejprve jsme otestovali roli kináz TBK1 a IKKε v IL-17 signalizaci. Zjistili jsme, že tyto kinázy fungují jako negativní regulátory IL-17 signální transdukce a podílí se tak na hlavní negativní zpětné vazbě. Též jsme objevili nový protein v rámci IL-17 receptorového signálního komplexu, protein CMTM4 lokalizovaný na plazmatické membráně, který slouží jako klíčová molekula pro složení IL-17 receptorového komplexu a následnou aktivaci IL-17 signalizace. Naše zjištění by mohla přispět k porozumění, jak aktivace IL-17 signální transdukce probíhá a odhalit nové potenciální regulátory, které by v budoucnu mohly sloužit jako cíle pro terapii autoimunitních chorob způsobených nadměrnou signalizací přes IL-17.

## Klíčová slova

Interleukin-17, IL-17 receptorový signální komplex, ubikvitinace, TRAF6, TBK1, IKKε, CMTM4

# Contents

# List of abbreviations

| | |
|---|---|
| **Ab** | Antibody |
| **Act1** | NF-κB activator 1 |
| **APC** | Antigen-presenting cell |
| **ATB** | Antibiotics |
| **CD** | Cluster of differentiation |
| **CIKS** | Connection to IκB kinase and Stress-activated protein kinases |
| **CMC(D)** | Chronic mucocutaneous candidiasis (disease) |
| **CMTM** | CKLF-like MARVEL transmembrane domain containing protein |
| **DC** | Dendritic cell |
| **DDM** | n-Dodecyl β-D-maltoside |
| **E1** | Ubiquitin-activating enzyme |
| **E2** | Ubiquitin-conjugating enzyme |
| **E3** | Ubiquitin protein ligase |
| **ER** | Endoplasmic reticulum |
| **G-CSF** | Granulocyte colony-stimulating factor |
| **GFP** | Green fluorescent protein |
| **HOIL-1** | Heme-oxidized IRP2 ubiquitin ligase 1 |
| **HOIP** | HOIL-1-interacting protein |
| **HuR** | Human antigen R |
| **IκB** | Inhibitor of NF-κB |
| **IFN** | Interferon |
| **Ig** | Immunoglobulin |
| **IKK** | IκB kinase |
| **IL** | Interleukin |
| **ILC** | Innate lymphoid cell |
| **IRAK** | Interleukin receptor associated kinase |
| **IRF** | Interferon regulatory factor |
| **JNK** | c-Jun $NH_2$-terminal kinase |
| **KO** | Knock-out |
| **LN** | Lymph node |
| **LPS** | Lipopolysaccharide |
| **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 |
| **MHC** | Major histocompatibility complex |
| **MS** | Mass spectrometry |
| **MyD88** | Myeloid differentiation primary-response protein 88 |
| **NAK** | NF-κB-activating kinase |
| **NAP1** | Nucleosome assembly protein 1 |
| **NF-κB** | Nuclear factor κB |
| **NK** | Natural killer cell |
| **PCR** | Polymerase chain reaction |
| **PD-1** | Programmed death receptor-1 |
| **PD-L1** | Programmed death ligand-1 |
| **PGE-2** | Prostaglandin 2 |
| **RE** | Restriction endonuclease |
| **RING** | Really interesting new gene |
| **RIPK1** | Receptor-interacting protein kinase 1 |
| **RORγt** | Retinoic acid receptor-related orphan receptor γ-T |
| **SEFIR** | Similar expression of fibroblast growth factor and IL-17R |
| **SHARPIN** | SHANK-associated RH-domain-interacting protein |
| **TAK1** | Transforming growth factor β-activated kinase |
| **TBK1** | TANK binding kinase 1 |
| **TGF** | Transforming growth factor |
| **Th** | T helper cell |
| **TIR** | Toll/IL-1 receptor |
| **TLR** | Toll like receptor |
| **TNF** | Tumor necrosis factor |
| **TNFR** | Tumor necrosis factor receptor |
| **TRAF** | Tumor necrosis factor receptor-associated factor |
| **TRIF** | Toll-interleukin receptor domain containing adaptor-inducing interferon-β |
| **Tregs** | Regulatory T cells |
| **VE-cadherin** | Vascular-endothelial cadherin |
| **WT** | Wild type |

# 1. Introduction

Interleukin-17 (IL-17), also known as IL-17A, is an important proinflammatory cytokine which protects our body against several invading pathogens, especially bacteria and yeast. IL-17 belongs to the IL-17 family, consisting of 6 known inflammatory cytokines IL-17A to IL-17F. All family members share a certain sequence and structural homology and bind to receptors from related IL-17 receptor family composed of 5 members IL-17RA to IL-17RE [1, 2]. Within IL-17 family, IL-17A and IL-17F are the most closely related proteins. Moreover, both cytokines can function either as IL-17A or IL-17F homodimers or IL-17A/F heterodimer and bind the same heteromeric receptor compounded of IL-17RA/IL-17RC [2]. Because IL-17A is the most studied member of IL-17 family, this thesis focuses specifically on this protein and its receptors, although our findings can very likely be directly applied to IL-17F.

IL-17 is produced by a variety of cell types, including cells of innate immune system such as myeloid cells, natural killer (NK) cells, γδT cells or type 3 innate lymphoid cells (ILC3) [3]. It was also reported that NKT cells are able to produce quite high amounts of IL-17 [4]. However, the main production of IL-17 is secured by Th17 cells, a special population of CD4$^+$ T helper cells [5]. Th17 differentiate from lymphoid T cell precursor in the presence of TGF-β and IL-6 cytokines. Subsequently, IL-23 is crucial for expansion and maintenance of Th17 population [6, 7]. Major transcriptional factor of Th17 cells is the retinoic acid receptor-related orphan receptor γ-T (RORγt), which promotes formation of Th17 lineage and activates transcription of IL-17 [7, 8].

The main cell types responding to IL-17 stimulation are epithelial, endothelial and fibroblast cells. Their stimulation with IL-17 results in production of various proinflammatory and antimicrobial products. These include cytokine IL-6 as an acute phase protein, chemokines such as IL-8, CXCL1 and CXCL2 which help with neutrophil activation, prostaglandin E2 (PGE-2), defensins and mucins or granulocyte colony stimulating factor (G-CSF) [9-11]. In general, all these products contribute to the activation and maintenance of inflammatory immune response.

Biological activity of IL-17 serves particularly for the elimination of bacterial, fungal and yeast infections. Patients unable to produce or signal via IL-17 suffer from oral or chronic mucocutaneous candidiasis (CMC) caused by *Candida albicans* [12, 13] and infections caused by *Staphylococcus aureus* [14]. By contrast, excessive activation of IL-17 downstream signaling contributes to the development of several autoimmune diseases such as psoriasis, systemic lupus erythematosus, psoriatic arthritis or multiple sclerosis [11, 15]. Currently, neutralizing antibodies targeting primarily IL-17, IL-17RA or IL-23 are used for the treatment of human psoriasis disease [16]. However, further

studies in IL-17 receptor complex signaling might reveal new possible approaches which might help to treat patients suffering from IL-17-mediated pathologies.

Following section describes crucial components of IL-17 proximal receptor complex, a nuclear factor NF-κB activator 1 (Act1, also known as connection to IκB kinase and stress-activated kinase (CIKS)) and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), which are absolutely essential for triggering of IL-17 downstream signaling.

## 1.1. Act1 and TRAF6 are crucial components of IL-17 receptor complex

Non-degradative ubiquitination is one of the main posttranslational modifications influencing signal transduction in cells, including signaling via IL-17. The process of ubiquitination is ensured by ubiquitin-proteasome enzymatic cascade consisting of three enzymes, an ubiquitin-activating (E1), ubiquitin-conjugating (E2) enzyme and ubiquitin-protein ligase (E3). The main function of E3 enzyme is to determine the substrate which will be ubiquitinated [17, 18].

As previously mentioned, both IL-17A homodimer and IL-17A/F heterodimer signal through a receptor complex compounded of two receptor subunits IL-17RA and IL-17RC [19]. Intracellular part of IL-17R family members contains several structural and interacting domains such as similar expression of fibroblast growth factor and IL-17R (SEFIR) domain, which is to a certain extent homologous to Toll/IL-1 receptor (TIR) domain found in IL-1/Toll-like receptor superfamily [20, 21]. SEFIR domain is essential for recruitment of Act1 adaptor [22, 23]. Act1 contains its own SEFIR domain located in the C-terminus, thus forming a homotypic interaction with the SEFIR of IL-17RA and IL-17RC in the receptor complex [23]. Apart from SEFIR domain necessary for binding of Act1 to the receptor complex, Act1 contains a TRAF-interacting motif in its N-terminal part, which enables the interaction with members of the TRAF family, especially TRAF6 [24]. Thus, the main function of Act1 in IL-17 signaling is to recruit TRAF6 to receptor signaling complex (Figure 1). Subsequently, TRAF6 creates a non-degradative polyubiquitin chains recruiting several signaling proteins activating downstream pathways, which will be discussed in following section.
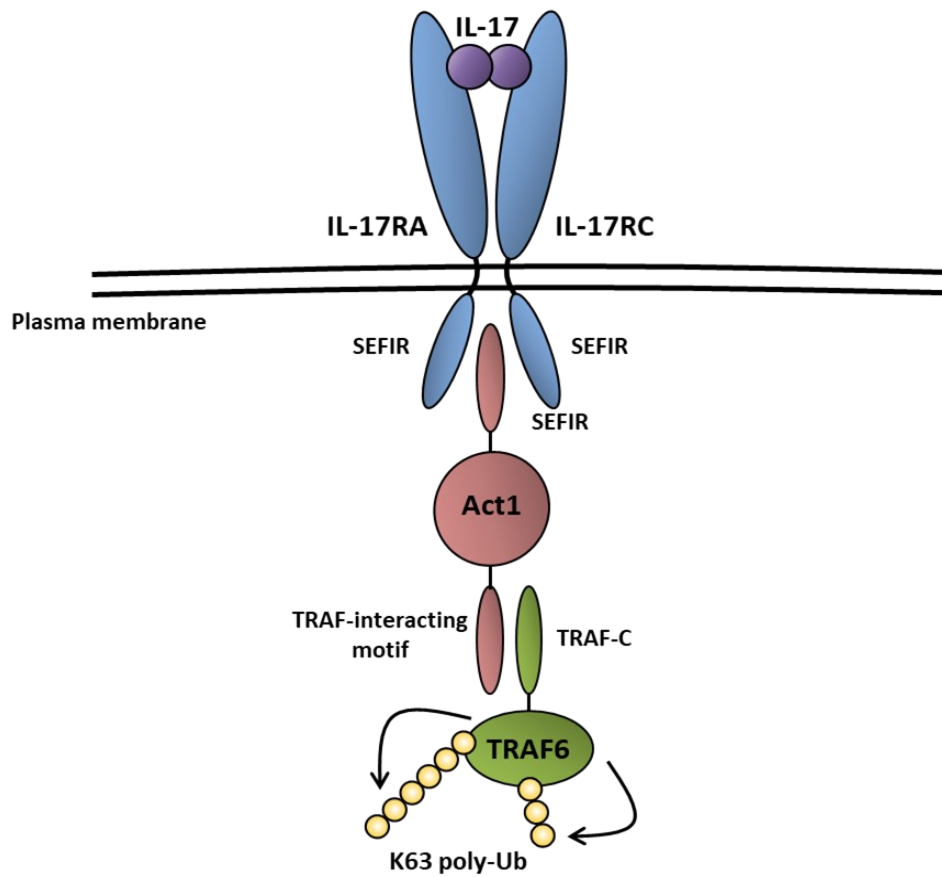
**Fig. 1.: Structure of IL-17 proximal receptor signaling complex.**
IL-17 binds to receptor complex consisting of subunits IL-17RA and IL-17RC. These receptor subunits contain intracellular SEFIR domain, enabling recruitment of SEFIR domain of Act1. Act1 possesses a N-terminal TRAF-interacting motif, through which it interacts with TRAF-C domain of non-degradative E3 ubiquitin ligase TRAF6. Activated TRAF6 then creates a non-degradative polyubiquitin chains which recruit effector proteins to induce downstream signaling.

TRAF6 belongs to TRAF family composed of six classical members TRAF1 to TRAF6 and one atypical protein TRAF7. It is a family of related proteins sharing a similar secondary structure. TRAF6 is highly conserved across species and is expressed in numerous tissues in mammals. It contains several interacting domains and motifs crucial for maintenance of its biological function [25] (Figure 2).

One of these domains represents a really interesting new gene (RING) domain in the N-terminus. Similarly as other family members except TRAF1, TRAF6 has an ubiquitin ligase activity, which is provided by RING domain. Thus, TRAF proteins belong to a family of RING domain E3 ubiquitin ligases which are able to create a non-degradative, lysine-63 (K63)-linked poly-ubiquitin chains [17]. TRAF6 functions in the complex with heterodimeric E2 conjugating enzyme composed of Ubc13 and Uev1A. In this complex, Uev1A contains an ubiquitin-binding site and provides contact between the acceptor and the donor of ubiquitin, while Ubc13 is crucial for auto-ubiquitination of TRAF6. Interaction of TRAF6 with Ubc13 requires the RING domain together with the first zinc finger motif within N-terminus. Moreover, RING domain is crucial for TRAF6 dimerization, which is together with auto-ubiquitination essential for TRAF6 activation and biological function [26].

RING domain is followed by several zinc finger domains and a trimeric TRAF domain located in the C-terminus and divided into two subdomains TRAF-C and TRAF-N (also known as coiled-coil domain). TRAF-C domain is essential for protein-protein interactions, thus enabling TRAF6 and other family members to directly interact with several receptor complexes and downstream adaptors within signaling pathways, such as Act1, CD40, RANK or adaptors within IL-1R and TLR signaling (described below). Moreover, this domain is needed for TRAF6 oligomerization [25]. On the other hand, TRAF-N domain was shown to contribute to TRAF6 autoubiquitination and for its interaction with Ubc13/Uev1A E2 enzymatic complex [27, 28].

In conclusion, Act1 is a crucial molecule within IL-17 signal transduction required for the recruitment of TRAF6 and thus proper assembly of IL-17 proximal receptor signaling complex which is pivotal for activation of downstream signaling pathways.
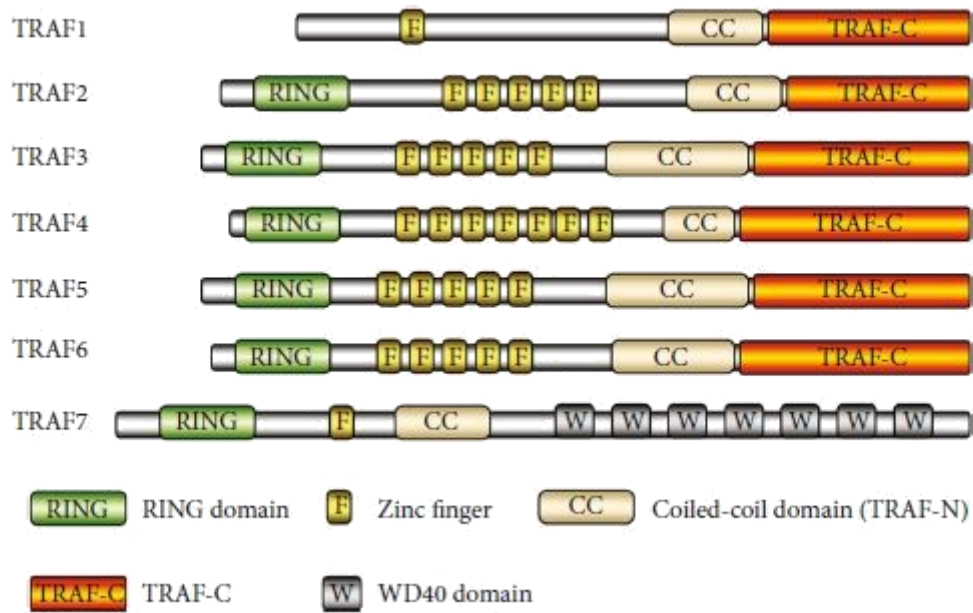
**Fig. 2.: Structure of TRAF family proteins.**
TRAFs contain various structural domains, such as RING domain in the N-terminus, followed by several, frequently five zinc fingers. In the C-terminus a TRAF domain is located and is separated into two distinct motifs TRAF-N, also called a coiled-coil domain, and TRAF-C. Adapted from [29].

## 1.2. TRAF6-mediated ubiquitination is the main activator of IL-17 signal transduction

As already mentioned, TRAF6 belongs to the family of E3 ubiquitin ligases which create a K63-linked polyubiquitin chains (Figure 1) that recruit several important signaling complexes activating downstream signaling pathways.

One of the complexes recruited to K63-polyubiquitin chains is IκB kinase (IKK) complex compounded of 3 subunits IKKα, IKKβ and IKKγ also known as NEMO protein. In this complex, adaptor NEMO serves as a regulatory subunit and binds polyubiquitin linkages, while kinases IKKα and IKKβ act as catalytic subunits mediating phosphorylation of target proteins [30]. The main function of IKK complex is phosphorylation of the inhibitor of NF-κB (IκB), which in unstimulated cells upon normal conditions interacts with nuclear factor κB (NF-κB) transcriptional factor, thus preventing its transport to nucleus and activation of its transcriptional activity [30]. Upon cell stimulation, IKK complex is activated and phosphorylates IκB at two serine sites S32 and S36, leading to its K48-linked ubiquitination and subsequent degradation, which enables release of NF-κB followed by its translocation to nucleus and transcription of target genes [28, 31].

Another protein recruited to synthesized polyubiquitin chains is transforming growth factor β-activated kinase 1 (TAK1) which forms a complex with adaptors TAB2 and TAB3 [32, 33]. Both TAB2 and TAB3 contain a zinc finger motif by which these adaptors bind polyubiquitin chains and thus recruit TAK1 complex to polyubiquitin linkages [33], which induces TAK1 activation and subsequent triggering of downstream mitogen-activated protein kinase (MAPK) pathways. Also, TAK1 complex is required for activation of NF-κB transcriptional factor as it phosphorylates IKKβ subunit at two serine sites S177 and S181 within IKK complex and enables activation of its kinase activity crucial for degradation of IκB [28, 32].

These signaling complexes are recruited to K63-linked polyubiquitin chains upon stimulation of numerous receptors within the immune system. Moreover, this signaling scheme is shared between three main inflammatory signaling pathways activated upon IL-1, TNF-α and IL-17 [34, 35]. However, although being similarly triggered, activation of downstream pathways such as MAPKs or NF-κB is upon IL-17 significantly weaker in comparison to other proinflammatory stimuli IL-1 and TNF-α, suggesting a presence of a strict regulation of IL-17 signaling. The mechanism of IL-17 poor signaling activation was previously unclear.

In conclusion, TRAF6 has a highly pivotal role in triggering of IL-17 signaling by creating the non-degradative polyubiquitin chains, which provide a scaffold for binding of crucial signaling components IKK complex and TAK1 kinase, contributing to the activation of both NF-κB and MAPKs pathways [31, 36].

## 1.3. TRAF6 in other signaling pathways

TRAF6 participates in numerous signaling pathways activated both by innate and adaptive immunity and also in the maintenance of bone homeostasis. TRAF6, similarly as other family members, was termed after its direct association with several receptors from TNFR superfamily, such as CD40 or RANK. However, apart from its role in IL-17 signaling, the role of TRAF6 was mainly characterized in signal transduction upon interleukin-1 receptor (IL-1R) and is also employed by several members of Toll-like receptor (TLR) superfamily [37].

Both TLR and IL-1R signaling contributes to the activation of immune response. TLRs and IL-1R interact with microbial components which results in activation of inflammatory pathways and subsequent production of proinflammatory cytokines and chemokines. Interaction of these receptors with pathogens induces recruitment of adaptor molecules such as myeloid differentiation primary-response protein 88 (MyD88) and Toll-interleukin receptor domain containing adaptor-inducing interferon-β (TRIF), thus activating either MyD88-dependent or TRIF-dependent pathway. In MyD88-dependent pathway, an interleukin receptor associated kinase 4 (IRAK4) associates with MyD88 adaptor and subsequently it binds other kinases from IRAK family, especially IRAK1, 2 and/or 3. These kinases contain a C-terminal extension with incorporated TRAF-interacting motif, which enables binding of TRAF6 and Ubc13/Uev1A E2 enzymatic complex to the TLR/IL-1R receptor signaling complex [35, 37, 38]. Following its recruitment, TRAF6 ubiquitinates itself and also IRAK1 kinase, which is crucial for triggering of downstream pathways [39]. TRAF6 participates also in MyD88-independent/TRIF-dependent pathway activated predominantly upon TLR3 and/or TLR4 stimulation, in which it binds to receptor complex via TRAF3 and ubiquitinates RIP1 kinase which in turn activates NF-κB and interferon regulatory factor (IRF) pathways [35, 38, 40].

As already mentioned, through its TRAF-C interacting domain TRAF6 directly associates with several receptors from TNFR superfamily such as RANK, a receptor involved in bone homeostasis, and CD40. CD40 is a crucial costimulatory molecule on the surface of myeloid cells, especially macrophages and dendritic cells (DCs). In dendritic cells, stimulation via CD40 is required for surface expression of major histocompatibility complex (MHC) class II. and other costimulatory molecules such as B7.2., which is lowered in the absence of TRAF6. This leads to attenuated proliferation of antigen-specific T cells. Similarly, dendritic cells-mediated production of cytokines such as IL-12 or IL-6 is TRAF6-depedent. Thus, TRAF6 is a crucial molecule for the maturation and biological activity of DCs and subsequent effective T cell responses [41]. TRAF6 also participates in the process of CD40-induced vacuole-lysosome fusion through autophagy, which is crucial for elimination of pathogens, such as *Toxoplasma gondii* in macrophages [42]. In addition, TRAF6 is needed for macrophages-dependent cytokine production, such as IL-12, which is dependent on CD40 stimulation and Src

tyrosine kinase activity, leading to activation of MAPKs and triggering of downstream pathways which results in target genes expression [43]. Altogether, TRAF6 is essential for maturation and effector functions of antigen-presenting cells (APCs), which in turn activate adaptive immune responses.

Apart from APCs, CD40 is a crucial molecule for development, proliferation and maintenance of biological activity of B cells. While interacting with CD40, TRAF6 is required for secretion of immunoglobulins (Ig), affinity maturation and class switching. These processes are disrupted in TRAF6 deficient B cells which are able to produce only low-affinity IgM class of Ig. TRAF6 has also a positive regulatory effect on IL-6 production, which in turn helps with Ig secretion. Moreover, deletion of TRAF6 led to reduced numbers of mature B cells both in the bone marrow and spleen. Apart from this, TRAF6 was shown to up-regulate the levels of B-cells surface co-stimulation molecule B7.1., which was significantly decreased in the absence of TRAF6. TRAF6 is also required for the generation of memory B cells and long-lived plasma cells producing antibodies (Ab) [44-46].

In conclusion, TRAF6 ubiquitin ligase is shared by several important pathways within both the innate and adaptive immune system and thus serves as crucial molecule which influences several biological processes in various cell types.

## 1.4. TRAF6 deficiency

Since being broadly shared within signaling pathways of numerous receptors across the immune system, biological activity of TRAF6 has an important regulatory effect on various cell types and processes. TRAF6 has a pivotal role in the embryonic development as its deficiency causes reduced prenatal survival [47, 48]. In case of viable pups, defects in TRAF6 cause substantial weight loss and severe inflammatory phenotype, which results in mortality quickly within 2 weeks after birth. These mice show impaired signaling upon stimulation via lipopolysaccharide (LPS), CD40L or IL-1 and develop disrupted peripheral tolerance because of impaired development of peripheral lymph nodes (LN) accompanied by immune cell infiltrates in various tissues [49]. Moreover, several types of LN such as mesenteric, mandibular or axillary are completely absent in TRAF6 deficient mice and these mice exhibit a splenomegaly, which indicates requirement of TRAF6 for the development of secondary lymphoid organs [47, 48].

Dendritic cells-specific deletion of TRAF6 leads to spontaneous impairment of mucosal tolerance and development of Th2 pathological response in mice, located primarily in the small intestine. These mice show higher levels of infiltrated eosinophils and Th2 CD4^{+} T cells both in small intestine and lungs [50-52] and higher serum levels of IgE [50]. By contrast, levels of forkhead box P3 (FoxP3)[+] Tregs are significantly reduced in these tissues, similarly as the presence of commensal microbiota in gut, which is in accord with and contributes to impaired mucosal immune tolerance [50, 51]. Moreover, levels of IL-2, which is a crucial cytokine for the development of Tregs, were also lowered in TRAF6-deficient mice, suggesting that TRAF6-deficiency-dependent reduction of Tregs levels might be caused by decreased production of IL-2 by DCs [51]. In addition, Tregs-specific deletion of TRAF6 results in progression of allergy and autoimmune phenotype in these mice, because of the preferred polarization to Th2 pathogenic response in the periphery. Also, Tregs-specific TRAF6 deficiency causes loss of FoxP3 and mild increase in the expression of T-bet and GATA-3 transcriptional factors, thus preventing the differentiation of Tregs. Moreover, TRAF6-deficient Tregs have a stronger tendency for Th2-specific cytokine secretion, such as IL-4. Thus, TRAF6 is crucial for proper proliferation, expansion and maintenance of Treg population, which in turn regulates the progress of Th2 immune response and controls the development of Th2-specific pathologies [53].

Apart from a crucial role of TRAF6 in the immune system in which it drives the development of lymphoid tissues, it was revealed to participate also in the bone homeostasis. TRAF6 knockout mice have shortened long bones, possess reduced bone marrow cavities, show an abnormal bone formation and impaired RANK signaling which prevents precursor differentiation into functional osteoclasts and impairs a bone remodeling [47, 48].
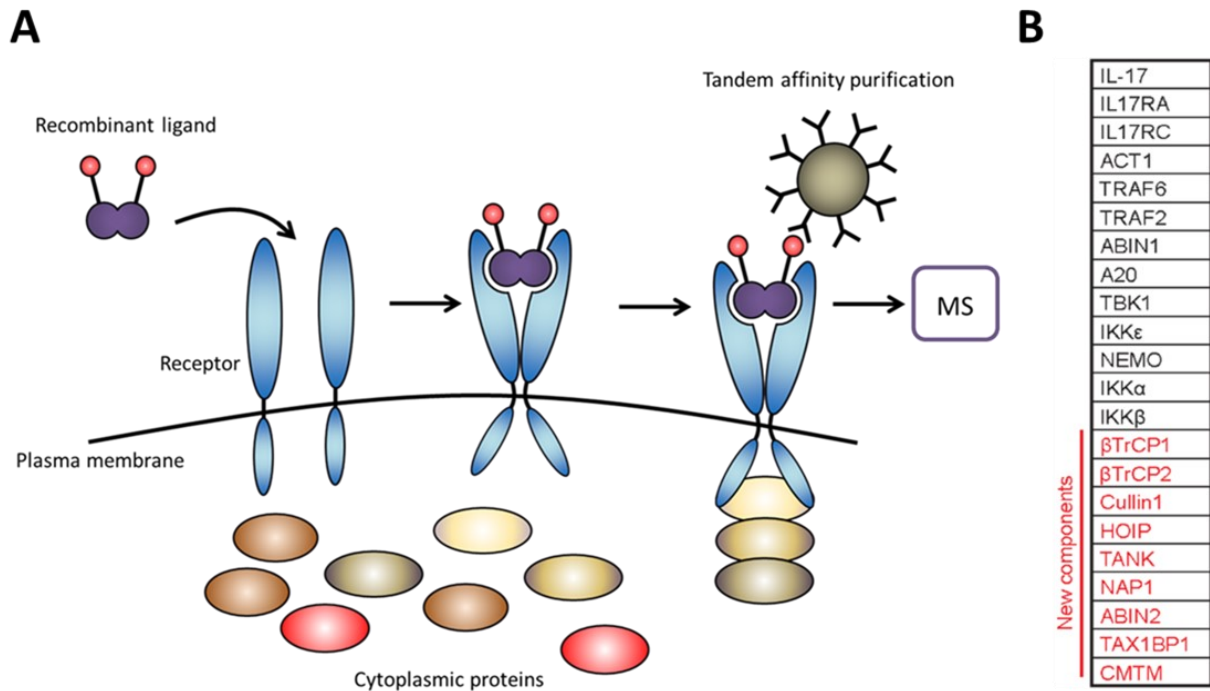
## 2.    Mass spectrometry analysis of IL-17R signaling complex

This thesis is a part of a research project, in which our laboratory studies signal transduction of IL-17 cytokine. Predominantly, we focus on discovery of novel regulators of IL-17 signaling and on establishing the mechanism how these regulators influence activation of downstream pathways. Proper understanding of IL-17 activation might reveal new possible targets for treatment of autoimmune diseases caused by IL-17.

My colleagues from Laboratory of adaptive immunity developed a strategy, how to study signaling outcomes triggered by stimulation of IL-17 receptor signaling complex (IL-17RSC) via IL-17A. In this approach they prepared a recombinant ligand containing tandem affinity purification tag (2xStrep tag, 1xFlag tag) followed by either murine of human IL-17. This ligand was subsequently used for cell stimulation which resulted in assembly and activation of IL-17 receptor signaling complex. Stimulated and control cells (left untreated) were solubilized in 1% n-Dodecyl β-D-maltoside (DDM) containing lysis buffer and IL-17 recombinant ligand was added post lysis to control samples. Solubilized samples were cleared by centrifugation and subjected to tandem affinity purification of assembled proximal receptor signaling complex [54].

Isolated IL-17 receptor complex was subsequently analyzed via mass spectrometry (MS), which revealed a variety of proteins that are recruited to IL-17 receptor signaling complex and very likely directly involved in IL-17 signal transduction. These proteins included a number of proteins already known to be associated with IL-17R. However, the analysis also revealed some new components in IL-17 pathway (Figure 3a, b).

For our next research projects we have chosen a TBK1 and IKKε kinases and a newly discovered protein within IL-17 signaling termed CMTM4, which will be described in following sections.

**Fig. 3.: Mass spectrometry analysis of IL-17RSC**

**A:** Scheme of tandem affinity purification. Cells were stimulated with recombinant ligand containing a SF-purification tag. Cell stimulation resulted in assembly of IL-17 receptor signaling complex, which was subsequently isolated and analyzed by MS.

**B:** List of molecules recruited to IL-17RSC revealed by MS analysis. Newly discovered components are indicated in red.

# 3.    TBK1 and IKKε kinases

TANK-binding kinase-1 (TBK1), also known as NF-κB-activating kinase (NAK), and IκB kinase epsilon (IKKε), also known as the inducible kinase (IKKi), are highly homologous kinases closely related to IKKα and IKKβ kinases [55, 56]. TBK1 was discovered due to its binding to TRAF family member-associated NF-κB activator (TANK) adaptor, while IKKε was identified based on its homology with IKKα and IKKβ. Both IKKα and IKKβ were established as canonical kinases because they are components of IKK kinase complex crucial for the activation of NF-κB pathway and production of target proinflammatory cytokines and chemokines. On the other hand, TBK1 and IKKε kinases are considered as non-canonical as they are not part of this kinase complex [55].

In case of the innate immune system, both TBK1 and IKKε seems to have very similar functions. However, *in vivo* the biological activity of TBK1 seems to be more important as deficiency in TBK1 kinase cause liver apoptosis degeneration resulting in lethality during embryonic development. In comparison, IKKε defects preserve the viability but cause increased susceptibility to viral infections [57, 58].

Both TBK1 and IKKε kinases were shown to be involved in the regulation of interferon (IFN)-β production upon TLR stimulation or viral infection. IFN-β together with IFN-α belongs to type I. IFNs crucial for the anti-viral immune responses. Production of IFN-β is induced predominantly upon viral infection and regulated via several pathways and transcriptional factors such as NF-κB and especially IRF-3 or IRF-7 [59]. Also, TLR3 and TLR4 stimulation with their ligands induces IFN-β expression via TRIF-dependent IRF-3 activation. IRF-3 is considered to be an initiator of IFN-β gene expression, while IRF-7 serves as its amplifier. TBK1 and IKKε were shown to phosphorylate IRF-3 on several serine/threonine sites upon viral infection, followed by its homodimerization and transport to nucleus where it activates transcription of IFN-β gene [60, 61]. Apart from viral infection, TBK1 deficiency also resulted in reduced expression of IFN-β and IFN-inducible genes upon stimulation with LPS, indicating the effect of TBK1 activity in TLR4-mediated IFN-β expression [59, 62]. However, expression of IFN-β was only partially reduced upon poly(I:C) stimulation in the absence of TBK1, while complete reduction was achieved while TBK1/IKKε double deficiency. Thus, both kinases participate in activation of IRF-3 pathway and regulate immune response crucial for elimination of viral infections [59, 62].

Recently, both TBK1 and IKKε kinases were reported to participate in signaling pathway upon TNF stimulation, especially in the regulation of TNF-induced cell death [63, 64]. TNF proinflammatory cytokine signals through two distinct receptor complexes. Complex I is associated with receptor TNFR1 and is crucial for activation of transcriptional factors and target genes expression. In contrast, cell death-inducing complex II is formed upon release of receptor-interacting protein kinase (RIPK)-1

from complex I to cytoplasm. RIPK1 subsequently recruits FADD and caspase-8 to induce apoptosis or RIPK3 which can activate programmed necroptosis [65]. Within TNFR signaling pathways, a linear-ubiquitin chain assembly complex (LUBAC) composed of 3 proteins, a heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), SHANK-associated RH-domain-interacting protein (SHARPIN) and a catalytic subunit HOIL-1-interacting protein (HOIP), and possessing E3 ubiquitin-ligase activity is essential for preventing of TNF-induced cell death [66, 67]. While recruited to TNFR1 signaling complex (TNFR1-SC), LUBAC creates linear (also known as M1) polyubiquitin linkages on target proteins, such as RIPK1 or NEMO protein, a regulatory molecule within IKK complex [34, 68, 69]. RIPK1 is subsequently phosphorylated by activated IKK complex, which prevents the formation of complex II and induction of cell death [70].

LUBAC complex is essential for the recruitment of TBK1 and IKKε kinases to TNFR1 as their activation and association with the receptor complex was reduced in HOIP-deficient cells. As already mentioned, TBK1 was identified according to its ability to associate with TANK adaptor [55] which is recruited to TNFR1 dependently on HOIP. Recruitment of IKKε but not TBK1 was completely abolished in the absence of adaptor TANK, which suggested that there is additional adaptor for TBK1 kinase recruitment. This second adaptor might be represented by nucleosome assembly protein 1 (NAP1) as TBK1 recruitment was not observed in TANK/NAP1 double knock-out cells. Thus, association of TBK1 to TNFR1 relies on mutual presence of both TANK and NAP1 adaptors while TANK alone is sufficient for the recruitment of IKKε [63]. Subsequently, both adaptors are brought to TNFR via NEMO protein, which is recruited to receptor signaling complex via M1-ubiquitin chains created by LUBAC complex. In conclusion, while also being part of IKK complex, NEMO serves as a crucial molecule for recruitment of both canonical and non-canonical IKKs to TNFR1 complex [63].

In contrast to other signaling pathways, TBK1 and IKKε are not involved in the TNF-induced activation of transcriptional factors and subsequent gene expression. Instead, both kinases were shown to protect cells from induction of apoptosis or necroptosis as their chemical inhibition and depletion led to markedly increased sensitivity to cell death. This is caused by TBK1/IKKε-mediated phosphorylation of RIPK1 in TNFR1, which prevents its activation and a formation of complex II. Thus, activity of TBK1 and IKKε kinases provides a crucial mechanism in preventing TNF-induced, RIPK1-dependent cell death and decreases susceptibility to lethal shock *in vivo* [63].

## 3.1. TBK1 and IKKε kinases in IL-17 signaling

TBK1 and IKKε were also described to participate in IL-17-mediated signaling. However, the published data concerning the role of these kinases were inconsistent, as they were described to function both as positive and negative regulators of signaling.

Upon IL-17 stimulation, these kinases were described to associate with Act1 [71, 72], a crucial adaptor within IL-17 proximal receptor signaling complex, of which activity is necessary for triggering of IL-17-induced activation of transcriptional factors and their downstream signaling pathways such as NF-κB or MAPKs (as discussed above). Although IKKε was shown to participate in regulation of MAPKs pathways, its deficiency did not affect the rate of NF-κB activation. Instead, IKKε was shown to promote IL-17-dependent chemokine mRNA stabilization [71].

IL-17-mediated signaling contributes to the expression of proinflammatory cytokines and chemokines both by transcription of target genes and production of corresponding products de novo, and posttranscriptionally by prolonging the half-life of already produced mRNAs. Although IKKε deficiency had no impact on expression of chemokine genes such as CXCL1 de novo and their levels were similar to that in WT, these mRNAs were degraded more quickly in IKKε-deficient cells in comparison to WT, suggesting a crucial role of IKKε in IL-17-dependent mRNA stabilization [71]. Moreover, a biologically inactive IKKε mutant failed to contribute to the stabilization, indicating that this process is dependent on IKKε kinase activity [71].

IL-17 stimulation induces the phosphorylation of adaptor Act1, however, its phosphorylation was affected in the absence of IKKε, indicating the importance of IKKε kinase activity for this process. IKKε-mediated Act1 phosphorylation was described to take place at serine 311, which is situated adjacent to putative TRAF-interacting motif required for association of TRAFs with Act1 and thus IL-17R. Phosphorylation of S311 within Act1 promoted binding of TRAF2/TRAF5, which is involved in regulation of mRNA stability [71, 73].

TBK1 was also shown to contribute to the prolongation of IL-17-induced mRNAs stabilization. One of the mechanisms causing the decay of each mRNA is the process of decapping mediated by a decapping enzymes such as Dcp1. Act1 was reported to form complexes with several proteins involved in the regulation of mRNAs stability, such as Dcp1, SF2 or HuR (human antigen R) [74, 75]. Upon IL-17 stimulation, TBK1 but not IKKε mediates phosphorylation of Dcp1 at S315 which blocks its decapping activity but not its interaction with Act1. Thus, TBK1-mediated Dcp1 phosphorylation prolongs a half-lives of target mRNAs [72]. In conclusion, both TBK1 and IKKε kinases are involved in the regulation of IL-17-induced stabilization of mRNA transcripts encoding main targets of IL-17 signal transduction. However, their role in IL-17-dependent activation of transcriptional factors

leading to de novo production of IL-17 target products, including primarily numerous proinflammatory cytokines and chemokines such as CXCL1, remained poorly understood.

By contrast, another study described TBK1 and IKKε kinases to phosphorylate Act1 adaptor on 3 serine sites which resulted in reduced downstream signaling. This suggests that both kinases act negatively on IL-17 signaling [76]. However, the mechanism of TBK1/IKKε-mediated inhibition was not completely clarified and this article is often misinterpreted or ignored. Thus, the role of TBK1 and IKKε in IL-17 signaling long remained controversial.

# 4.    CMTM4 protein

CKLF-like MARVEL transmembrane domain containing protein 4 (CMTM4) belongs to poorly examined CMTM family consisting of 8 proteins CMTM1-8, each containing a transmembrane MARVEL domain which possesses 4 transmembrane helices and N- and C-terminus both with intracellular orientation. This domain was originally identified in proteins of myelin and lymphocyte (MAL), similarly as in physins, gyrins and occludin family. MAL protein was shown to regulate apical transport of both secretory and transmembrane molecules. Moreover, some of MARVEL-containing proteins are also involved in the vesicular transport or regulation of tight junctions. Thus, MARVEL domain was termed after MAL and related proteins for vesicle trafficking and membrane link [77].

CMTM proteins are divided into two groups according to the chromosome where encoded. The first group contains CMTM1-4 and is encoded on chromosome 16, while the second group consisting of CMTM6-8 is encoded on chromosome 3 in humans [78]. By contrast, murine proteins from the first group are located on chromosome 8, while from the second on chromosome 9. Both murine and human CMTM5 is encoded on chromosome 14. Proteins from CMTM family were shown to regulate a transport and localization of several surface and/or secretory molecules. However, the role of these proteins is still poorly understood.

The most studied member of this family is CMTM6, which was reported to associate with programmed death ligand-1 (PD-L1), a major significant inhibitory molecule within the immune system. PD-L1 is a transmembrane protein expressed predominantly in tumors or immunoprivileged sites such as brain or anterior chamber of the eye. Its receptor programmed death receptor-1 (PD-1) is primarily expressed on activated T-cells. Interaction between PD-L1 with PD-1 on the surface of T-cells results in the reduction of T-cell activation, effector function and expansion. It was reported that depletion of CMTM6 significantly reduced the total PD-L1 levels in melanoma cells or cells of breast and lung cancer [78]. However, CMTM6 failed to promote stabilization of PD-L1 in all tested cancer cell lines, such as HAP1 cells, indicating that there might be another protein involved in this process. Other studies revealed, that biological function of CMTM6 is shared with its most closely related member within CMTM family, a CMTM4 protein which possesses about 55% homology with CMTM6 [79, 80]. Depletion of CMTM6 led to markedly reduced expression of PD-L1, which was more significant in the absence of CMTM4. In addition, biological activity of CMTM4 resulted in complete restoration of PD-L1 expression in CMTM6 knock-out cells, suggesting that CMTM4 might also serve as a crucial regulator of this checkpoint molecule expression [79, 80]. This indicates that both CMTM4 and CMTM6 are crucial molecules regulating the expression of PD-L1 in cancer cell lines. Moreover, mRNA levels of PD-L1 were the same both in the presence and the absence of CMTM4/6, indicating that CMTM4/6 do not serve as transcriptional regulators of *PD-L1* gene expression [78-80].

PD-L1 molecule contains several N-glycans which provide protection against endoglycosidase-H and thus prolong the half-life of PD-L1 mRNA. The process of glycosylation takes place both in endoplasmic reticulum (ER) and Golgi. Although CMTM6 was shown to be unnecessary for PD-L1 trafficking between ER, Golgi and the plasma membrane, it is required for its stabilization on the cell surface and cells lacking CMTM6 are unable to preserve the PD-L1 expression for longer time. This is caused by impaired recycling of PD-L1 in CMTM6 knock-out cells. CMTM6 associates with PD-L1 also in recycling endosomes [78] and the absence of CMTM4/6 causes higher ubiquitination of PD-L1, likely leading to its degradation in proteasome. Thus, CMTM4/6 deficiency results in reduced re-expression of PD-L1 on the cell surface caused by its higher proteasomal degradation and reduction in its protein levels [78-80].

These days, blocking antibodies targeting either PD-L1 or its receptor PD-1 are used for the treatment of several cancers, as blocking of this checkpoint signaling pathway stimulates T-cell specific anti-tumor effector functions. However, cancer cells are able to actively manipulate the activity of PD-L1/PD-1 axis, thus preventing anti-tumor immunity. This ability of cancer cells results in lowered responsiveness of some patients to this type of therapy. Thus, therapeutic approach blocking PD-L1/PD-1 axis cannot be used for the whole spectrum of cancers and the efficacy differs in each patient [81, 82]. Interestingly, CMTM6 deficiency was at the same efficacy in blocking the tumor progression in comparison with anti-PD-L1/PD-1 blocking Ab or PD-L1 deletion. This indicates that CMTM4/6 might represent promising targets for future treatment of human cancers and might help to increase the efficacy of existing therapeutic approaches [78-80].

Besides the regulation of PD-L1 surface expression and stabilization, CMTM4 was also shown to be involved in the process of angiogenesis. Angiogenesis is indispensable during the embryogenesis and is also included in a wound-healing. However, its excessive activation might also contribute to the tumor progression and abnormalities in angiogenesis might cause cardiovascular pathologies. Since the angiogenesis is crucial during embryogenesis, the experiments done with zebrafish larvae model showed a pivotal role of CMTM4 protein as its silencing negatively affected the embryonic development. CMTM4 depletion in endothelial cells blocked a neovascular formation and a significant reduction in the number of junctions, similarly as in the number and length of tubules [83].

As mentioned above, CMTM4 was established to colocalize with and drive the recycling of PD-L1 checkpoint molecule. Similar function was observed during angiogenesis where CMTM4 colocalizes with markers of recycling vesicles such as Rab4, similarly as with vesicles determined to lysosomal degradation through the association with Rab7 marker. Thus, CMTM4 is involved in the process of endocytosis, in which it contributes to both the recycling and the lysosomal degradation of transported contents. Analysis of CMTM4 cell localization revealed that CMTM4 colocalizes with both

internalized and membrane form of vascular-endothelial (VE)-cadherin [83], which is an important constituent of adherent junctions located between endothelial cells and its reduced surface levels result in impaired vascular stability [84]. The colocalization between CMTM4 and VE-cadherin helps to recycle and stabilize VE-cadherin within the adherent junctions by providing its connection to actin cytoskeleton, thus contributing to the endothelial barrier function and stability of these adherent junctions [83].

In conclusion, CMTM4 protein seems to be a crucial molecule in regulation of expression, stability and recycling of cell surface molecules. Our results obtained from MS analysis revealed CMTM4 to be a new component of IL-17-induced signal transduction. Thus, our studies aimed to elucidate the role of CMTM4 in IL-17-mediated signaling because its role in this signal transduction pathway was not described yet.

# 5.    Aims of the thesis

This thesis consists of two parts. The first part aims to elucidate the role of TBK1 and IKKε kinases in IL-17 signaling. Although these kinases are known to influence IL-17 signal transduction, their role still remained poorly understood and controversial. It was published that phosphorylation of Act1 by IKKε is crucial for recruitment of TRAF2/5 complex to IL-17R, resulting in stabilization of mRNA and removal of IKKε kinase decreases activation of this pathway [71]. Another study revealed that depletion of TBK1 kinase also lowered IL-17-mediated mRNA stability and production of IL-17 target genes, such as CXCL1 or TNF-α [72]. These studies suggest that both kinases TBK1 and IKKε serve as positive regulators of IL-17 signaling pathways. Nevertheless, TBK1 and IKKε kinases were also described to phosphorylate Act1 adaptor on 3 serine sites which resulted in reduced downstream signaling, suggesting both kinases to act negatively on IL-17 signaling [76]. Thus, our aim was to resolve the function and mechanism how these two kinases influence IL-17-mediated signal transduction.

The second part focuses on newly discovered component of IL-17 signal transduction, a CMTM4 protein, whose role in IL-17-induced signaling wasn't described so far. The main aim of this part is to elucidate if CMTM4 affects IL-17 signaling and how is this potential regulation mediated.

# 6.    Material and methods

## 6.1.    Cell lines and cell culture

ST2 cells were kindly provided by Jana Balounová (Institute of Molecular Genetics, Prague). HeLa, Hek293, ØNX-Eco and ØNX-Ampho cells were kindly provided by Tomáš Brdička (Institute of Molecular Genetics, Prague). All cells were cultivated on 10-cm dishes at 37°C, 5% $CO_2$ in complete DMEM medium containing 10% fetal bovine serum (FBS, Gibco) and penicillin/streptomycin antibiotics mix (Biosera). Cells from each cell line were frozen in FBS containing 10% DMSO and stored at -80°C for future use. Each cell line was tested for Mycoplasma contamination regularly by PCR reaction.

## 6.2.    Production and testing of recombinant SF-IL-17 ligand

Recombinant SF-IL-17 ligand composed of CD33 leader, 6xHis, 2xStrep tag, 1xFlag tag, and either murine IL-17 (AA 26-158) or human IL-17 (AA 24-155) was prepared using GeneArt Gene Synthesis (Thermo Fisher) and together with pcDNA3.1 vector cleaved via restriction reaction (Table 1). Cleaved samples were analyzed by agarose gel electrophoresis (1% agarose in TAE buffer (2M Tris, 50 mM EDTA, 1M Acetic acid) with 10.000x diluted EliDNA Green Ultra) and DNA was isolated from the gel using Zymoclean Gel DNA Recovery Kit (Zymo Research, #D4001).

Cleaved insert and vector were mixed in ligation reaction (Table 2) and incubated at the room temperature overnight. The next day, constructs were transformed into competent bacteria and cultivated on ampicillin plates overnight. The ligation was tested via PCR reaction (Table 3 and 4). Successfully transformed colonies were inoculated into LB medium, incubated overnight at 37°C on a shaker and used for the isolation of prepared plasmids using Plasmid Mini Kit II (E.Z.N.A., Omega Bio-Tek, #D6945-02). Isolated DNA was eluted in $dH_2O$, tested via sequencing and stored at -80°C.

| Reagent | Volume |
|---------|--------|
| 10x Green Buffer | 2 µl |
| KpnI | 0.5 µl |
| ApaI | 0.5 µl |
| $H_2O$ | 12 µl |
| Insert/vector | 5 µl |
| Total | 20 µl |

Table 1: Restriction reaction

| Reagent | Control | Ligation |
|---|---|---|
| Cleaved vector | 4 µl | 4 µl |
| Cleaved insert | - | 4 µl |
| 10x T4 Ligase buffer | 1 µl | 1 µl |
| T4 Ligase | 1 µl | 1 µl |
| $H_2O$ | 4 µl | - |
| Total | 10 µl | 10 µl |

Table 2: Ligation reaction

Primers used:

| #R178 | pcDNA3.1 Seq FWD | ccactgcttactggcttatcg |
|---|---|---|
| #R179 | pcDNA3.1 Seq REV | caacagatggctggcaacta |

| Reagent | 1 sample | Final concentration |
|---|---|---|
| PPP mix | 10 µl | 1x |
| Primer mix (50 µM each) | 0.2 µl | 0.5 µM |
| $H_2O$ | 8.8 µl | - |
| Bacterial culture | 1 µl | - |
| Total | 20 µl | - |

Table 3: Preparation of PCR master mix

| Number of cycles | Temperature | Duration |
|---|---|---|
| 1 x | 95 °C | 3 min |
| 30 x | 95 °C | 30 s |
| | 60 °C | 30 s |
| | 72 °C | 2 min (1 min/kb) |
| 1 x | 72 °C | 5 minutes |

Table 4: PCR reaction

Prepared recombinant SF-IL-17 was produced in HEK293 cells. Cells were grown on 15-cm dish to 70-90% confluency. At the day of transfection, the medium of HEK293 cells was changed for fresh DMEM containing 0,5% FBS without ATB. Transfection was performed using 75 µg of polyethylenimin (PEI, Polysciences) mixed with 30 µg of DNA (Table 5). The mixture was incubated at the room temperature for 10 min and then added to target cells. The medium of target cells was changed for fresh complete DMEM (+ FBS, + ATB) 3 hours after transfection and cells were incubated 3 days at 37°C. Afterwards, supernatant was collected and purified using His GraviTrap TALON column (GE Healthcare). Samples were washed and eluted with 20 mM and 350 mM of imidazole, respectively. Imidazole was subsequently removed using centrifugal filter (Merck Millipore, 30kDa

molecular weight cutoff) and followed by 3 washes with purification buffer (50 mM sodium phosphate pH 7,4, 300 mM NaCl). Isolated recombinant ligands were mixed with glycerol to obtain 50% glycerol stock, stored at -80 °C and used for cell stimulation. Concentration of purified ligands was measured by NanoDrop (ND-1000, Thermo Fisher).

Production of recombinant ligands was subsequently tested - ligands were mixed with SDS sample buffer (250 mM Tris pH 6.8, 8% SDS, 40% glycerol, 0.2% bromophenol blue), reduced with 50 mM dithiothreitol (DTT), loaded on SDS-PAGE gel and stained with Coomassie InstantBlue (Expedeon).

| Reagent | Required for 1 dish |
|---|---|
| DNA | 30 µg DNA |
| PEI | 75 µl PEI (1 µg/µl) |
| DMEM (- FCS/- ATB) | 1 ml |

Table 5: Preparation of transfection solution

## 6.3. Cell stimulation and Western blot analysis

ST2 and HeLa cells were grown on 12-well plates to 90% confluency, washed and cultivated for 30-60 min in serum-free DMEM medium without ATB. Cells were separated into non-stimulated control group and a second group activated with either murine of human recombinant SF-IL-17 ligand (500 ng/ml) for indicated time points. After stimulation, cells were lysed in 1% n-Dodecyl-b-D-Maltoside (DDM) (Thermo Fisher) containing lysis buffer (30 mM Tris pH 7.4, 120 mM NaCl, 2 mM KCl, 2 mM EDTA, 10% glycerol, 10 mM chloroacetamide, 10 mM Complete protease inhibitor cocktail, and PhosSTOP tablets (Roche)) and incubated for 30 min at 4°C. Afterwards, samples were centrifugated (21,130 g, 30 min, 2°C) and mixed with SDS sample buffer containing 50 mM DTT and boiled (92°C, 3 min).

Prepared samples were separated using SDS-PAGE electrophoresis and subsequently transferred on a nitrocellulose membrane (0,45 µm pore size, GE Healthcare, Amersham Protran). Membranes were blocked in 2,5% milk (P-Lab, 2.5% milk, 0.02% NaN$_3$). Signaling in cells was tested using primary and secondary antibodies listed below (Tables 6 and 7). After staining, membranes were incubated with ECL solution (1M Tris pH 8.5, 1.25 mM Luminol, 0.2 mM p-Coumaric Acid, 0.02% H$_2$O$_2$) and analysis was performed using a ChemiDoc MP Gel Imaging System (Bio-Rad).

## 6.4.   Immunoprecipitation of IL-17R signaling complex

For isolation of IL-17RSC, ST2 or HeLa cells were grown on 15-cm dishes and cultivated for 30-60 min in serum-free DMEM medium without ATB and then stimulated with mixture containing serum-free DMEM medium and either murine or human SF-IL-17 ligand (500 ng/ml) for 15 min. After stimulation, cells were lysed in 1% DDM (Thermo Fisher) containing lysis buffer (30 mM Tris pH 7.4, 120 mM NaCl, 2 mM KCl, 2 mM EDTA, 10% glycerol, 10 mM chloroacetamide, 10 mM Complete protease inhibitor cocktail, and PhosSTOP tablets (Roche)) and incubated 30 min on ice. Into non-activated samples, recombinant SF-IL-17 ligand was added post-lysis as a control. All samples were subsequently cleared by centrifugation (21,130 g, 30 min, 2°C). Clear samples were incubated with anti-Flag M2 affinity agarose gel (Sigma) overnight and the next day washed 3x with 0,1% DDM containing lysis buffer, mixed with SDS sample buffer with 50 mM DTT and boiled (92 °C, 3 min). Samples were analysed via immunoblotting using primary and secondary antibodies listed below (Tables 6 and 7).

## 6.5.   Antibodies

| Antigen | Specificity | Company | Cat. number |
|---------|-------------|---------|-------------|
| p-TBK1 | rabbit | Cell Signaling | #5483S |
| TBK1 | rabbit | Cell Signaling | #3013 |
| p-IKKε | rabbit | Cell Signaling | #8766S |
| murine IKKε | rabbit | Cell Signaling | #3416S |
| human IKKε | rabbit | Cell Signaling | #2905S |
| NEMO | rabbit | Santa Cruz | #sc-8330 |
| TANK | rabbit | Cell Signaling | #2141S |
| NAP1 | rabbit | Abcam | #ab192253 |
| IκBα | rabbit | Cell Signaling | #9242S |
| p-p105 | rabbit | Cell Signaling | #4806S |

| | | | |
|---|---|---|---|
| **p-p38** | rabbit | Cell Signaling | #4511S |
| **p-SAPK/JNK** | rabbit | Cell Signaling | #4671S |
| **TRAF6** | rabbit | Abcam | #ab40675 |
| **IL-17RC** | goat | R&D | #AF2270-SP |
| **GFP** | rabbit | Sigma | #SAB4301138 |
| **CMTM4** | rabbit | Sigma | #HPA014704 |
| **Act1** | mouse IgG1 | Santa Cruz | #sc-398161 |
| **FLAG** | mouse IgG1 | SIGMA | #F3165 |
| **β-Actin** | mouse IgG2b | Cell Signaling | #3700 |

Table 6: List of primary antibodies used for immunoblotting and flow cytometry analysis

| **Specificity** | **Company** | **Cat. number** |
|---|---|---|
| **Donkey Anti-Rabbit IgG (H+L) – HRP** | Jackson Immunoresearch | #147745 |
| **Goat Anti-Mouse IgG1 – HRP** | Jackson Immunoresearch | #148255 |
| **Goat Anti-Mouse IgG2b – HRP** | Jackson Immunoresearch | #149717 |
| **Donkey Anti-Goat IgG (H+L) - AF647** | Jackson Immunoresearch | # 148548 |

Table 7: List of secondary antibodies used for immunoblotting and flow cytometry analysis

## 6.6. Cell reconstitution using retroviral vectors

Coding sequence of CMTM4 protein was fused to mCherry, sequences of IL-17RA and IL-17RC were fused to either C-terminal 2xStrep-3xFlag tag or EGFP and all these sequences were inserted into pBabe retroviral vector expressing GFP under SV40 promoter (vector was kindly provided by Matouš Hrdinka, University Hospital Ostrava, Czech Republic). Prepared constructs were sequenced and used for transfection of Phoenix platinum Eco (for murine ST2 cell line) or Phoenix platinum Ampho cells (for human HeLa cell line). Cells were grown on 6-well dishes to 50-70% confluency and transfected in the presence of Lipofectamin 2000 (Invitrogen) solution (Table 8). The Lipofectamine solution was incubated for 5 min at the room temperature and then mixed with DNA constructs and incubated once again for 20 min. At the day of transfection, the medium of Phoenix cells was changed for fresh DMEM containing 10% FBS without ATB and the DNA/Lipofectamine solution was added to each well. Cells were incubated overnight and the next day the medium was changed again for fresh DMEM containing 10% FBS without ATB. The next day, supernatants with produced viruses were collected, passed through 0.2 µl filter into 15ml falcon tube and in the presence of polybrene (6 µg/ml, Sigma) added to target cell lines. 0,5 ml of viral supernatants were frozen and stored at -80°C for future use. The transfection was performed by centrifugation (spinfection, 2500 rpm, 45 min, 30°C), cells were incubated for 24 hours and sorted for GFP positivity using FACSAriaIIu flow cytometer.

| Reagent | 1 sample |
|---|---|
| Lipofectamine 2000 | 5 µl |
| DMEM | 125 µl |

Table 8: Lipofectamine solution

## 6.7. Flow cytometry

Flow cytometry was performed in order to detect surface expression of IL-17RC. Cell suspensions of WT and CMTM4 KO cells reconstituted with IL-17RC-EGFP were spinned down (400 g, 3 min, 4°C) and resuspended in FACS buffer (PBS, 2% FBS, 0.1% NaN$_3$). Cells were stained for 30 min on ice using an anti-IL-17RC primary antibody (diluted 100x) (goat, #AF2270-SP, R&D, Table 6). After staining, cells were washed with FACS buffer and stained with Donkey Anti-Goat IgG (H+L) AF647 (diluted 500x) (#152199, Jackson Immunoresearch, Table 7) secondary antibody. Surface expression of IL-17RC was measured on Bricyte E6 flow cytometer and data were analyzed using FlowJo software (TreeStar).

## 6.8. Fluorescent microscopy

WT and CMTM4 KO cells expressing either IL-17RC-EGFP alone or expressing both IL-17RC-EGFP and CMTM4-mCherry were used. Cell suspensions were stained with Hoechst33342 (Thermo Fisher) for 5-10 minutes. After incubation, cells were washed with HBSS medium (low $Ca^{2+}$) and fixed for 5 minutes in formaldehyde solution (10x diluted formaldehyde in PBS, Sigma). Fixed cells were resuspended in HBSS medium (low $Ca^{2+}$) and the cell suspensions were added into 8 chambered coverglass system (Cellvis C8-1.5H-N). Fluorescent microscopy was performed using an Olympus Delta Vision Core (inverted microscope version IX-71, Olympus America Inc., Center Valley, PA, USA).

## 6.9. Statistics and data analysis

For the statistical analysis, a Prism (GraphPad Software) was used. Two-tailed nonparametric Mann-Whitney statistical test was used to analyze results from flow cytometry experiments. Western blot results were analyzed using Image Lab software (from Bio-Rad). Analysis of results from fluorescent microscopy was performed using an ImageJ software.

## 6.10. Chemicals and reagents

| Reagent | Company | Cat. number |
|---|---|---|
| Agarose | VWR | #438795A |
| 2-Chloroacetamide | Sigma | #C0267 |
| Ammonium persulfate | ThermoFisher Scientific, VWR | #17874 |
| p-Coumaric acid | Sigma | #C9008 |
| Luminol | Sigma | #A8511 |
| Powdered milk | ROTH | #T145.2 |
| DMSO | Sigma | #D2650-100ML |
| Glycerol | Sigma | #G2025-1L |
| n-Dodecyl-beta-D-Maltoside | ThermoFisher Scientific | #89903 |

| | | |
|---|---|---|
| **DTT** | Sigma | #D9779 |
| **PhosSTOPEASYpack Phosphatase Inhibitor Tablets** | Roche | #04906837001 |
| **cOmplete, EDTA-free Protease Inhibitor Tablets** | Roche | #05056489001 |
| **Trypsin-EDTA** | Biosera | #XC-T1717 |
| **EliDNA Green** | Elisabeth Pharmacon | #ED03 |
| **T4 DNA Ligase** | ThermoFisher Scientific | #EL0016 |
| **KpnI RE** | ThermoFisher Scientific | #FD0524 |
| **ApaI RE** | ThermoFisher Scientific | #FD1414 |
| **Polyethylenimin (PEI)** | Polysciences | #23966-2 |
| **CoomasieInstantBlue** | Expedeon | #ISB1L |
| **MRT67307 inhibitor** | Tocris | #5134 |
| **Lipofectamin 2000** | Invitrogen | #11668-027 |
| **Polybrene** | Sigma | #TR-1003 |
| **Hoechst33342** | ThermoFisher Scientific | #62249 |
| **Formaldehyde** | Sigma | #F8775-25ML |
| **PPP master mix** | Top-Bio | #C208 |
| **Sodium chloride** | Sigma | #S9888 |
| **Trizma base** | Sigma | #T6066 |
| **Sodium dodecyl sulphate** | VWR | #444464T |
| **Glycine** | VWR | #101196X |

| | | |
|---|---|---|
| **EDTA. 2Na dihydrate** | VWR | #33600,267 |
| **Trisma hydrochloride** | Sigma | #T5941 |
| **Ponceau S** | Sigma | #P3504 |
| **Bromophenol Blue** | Sigma | #114391 |
| **Sodium phosphate monobasic dihydrate** | Sigma | #71505 |
| **Imidazole** | VWR | #0527-100g |

Table 9: List of used reagents and chemicals

# 7.   Results

The result section of this thesis has two parts. First part (Sections 7.1 - 7.4) focuses on the role of TBK1 and IKKε kinases in IL-17 signaling. Second part (Sections 7.5 - 7.6) focuses on the analysis of CMTM4 in assembly of IL-17RSC.

## 7.1.   TBK1 and IKKε kinases associate with IL-17RSC upon IL-17 stimulation

TBK1 and IKKε kinases were described to be recruited to IL-17R complex upon IL-17 stimulation and also identified in our mass-spec analysis of IL-17RSC (Figure 3b). Both kinases are strongly activated upon TNF-stimulation and protect cells from TNF-induced cell death [63]. However, their role in IL-17 is still poorly understood and highly controversial, as they were proposed to function both as positive and negative regulators of IL-17 signaling (as discussed above).

TNF is very strong activator of downstream signaling, such as activation of c-Jun $NH_2$-terminal kinase (JNK) or p38 or NF-κB. In contrast, IL-17 induces only very mild activation of these pathways. In the following set of experiments, we aimed to compare the activation of TBK1 and IKKε induced by either IL-17 or TNF. We stimulated cells with either IL-17 or TNF-α recombinant ligands and analyzed the activation of signaling pathways in these cells by immunoblotting. As expected, stimulation of cells with TNF-α, but not IL-17, induced strong activation of MAP kinases JNK and p38. Moreover, stimulation via TNF-α caused much stronger degradation of IκB protein, indicating substantially higher activation of NF-κB pathway. However, our data very surprisingly showed that both TBK1 and IKKε kinases were activated upon IL-17 either equally or at even higher level than upon TNF-α stimuli. These data indicate that although IL-17 is, in general, only a weak activator of proinflammatory responses, the pathway connected to TBK1 and IKKε is activated upon IL-17 with a significant preference (Figure 4).
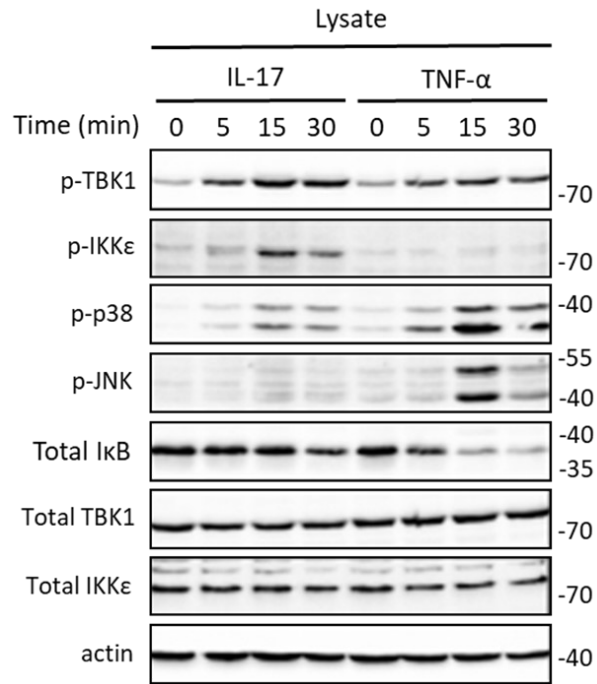
**Fig. 4.: TBK1 and IKKε kinases are recruited to IL-17RSC upon IL-17 stimulation**

Western blot analysis of cells stimulated or not with recombinant SF-IL-17 (500 ng/ml) and TNF-α (500 ng/ml) for indicated time points.

Results were obtained from three independent repetitions.

## 7.2. TBK1 and IKKε kinases serve as negative regulators of IL-17 signaling

To resolve the controversial function of these kinases in IL-17 signaling described by various scientific publications, we analyzed signaling outcomes in cells with impaired function of TBK1 and IKKε kinases. First, cells were incubated with highly specific TBK1/IKKε inhibitor MRT67307 inhibitor or left untreated. Cells were subsequently stimulated with IL-17 and analyzed via immunoblotting for activation of downstream pathways. Our results showed that in cells pretreated with MRT67307 the signaling was upregulated and resulted in stronger activation of NF-κB and MAPKs compared to non-treated cells, indicating that TBK1 and IKKε kinases might serve as negative regulators of IL-17 signaling pathways (Figure 5a).

In order to confirm our results from experiments with MRT67307 inhibitor, we generated single knock-out cells deficient either in TBK1 or IKKε alone, or double knock-out cells deficient in both kinases simultaneously. These knock-out cells were prepared using CRISPR/Cas9 method by my colleagues in laboratory and were kindly provided to me for further experiments. Western blot analysis of downstream signaling in these cells revealed that deficiency in both kinases led to upregulation of IL-17-induced signaling in double knock-out cells (Figure 5b). Surprisingly, removal of single kinase alone only slightly decreased activation of signaling pathways (results not shown). All

together these data indicate, that TBK1 and IKKε kinases negatively regulate IL-17 signaling and their function is reciprocally redundant.
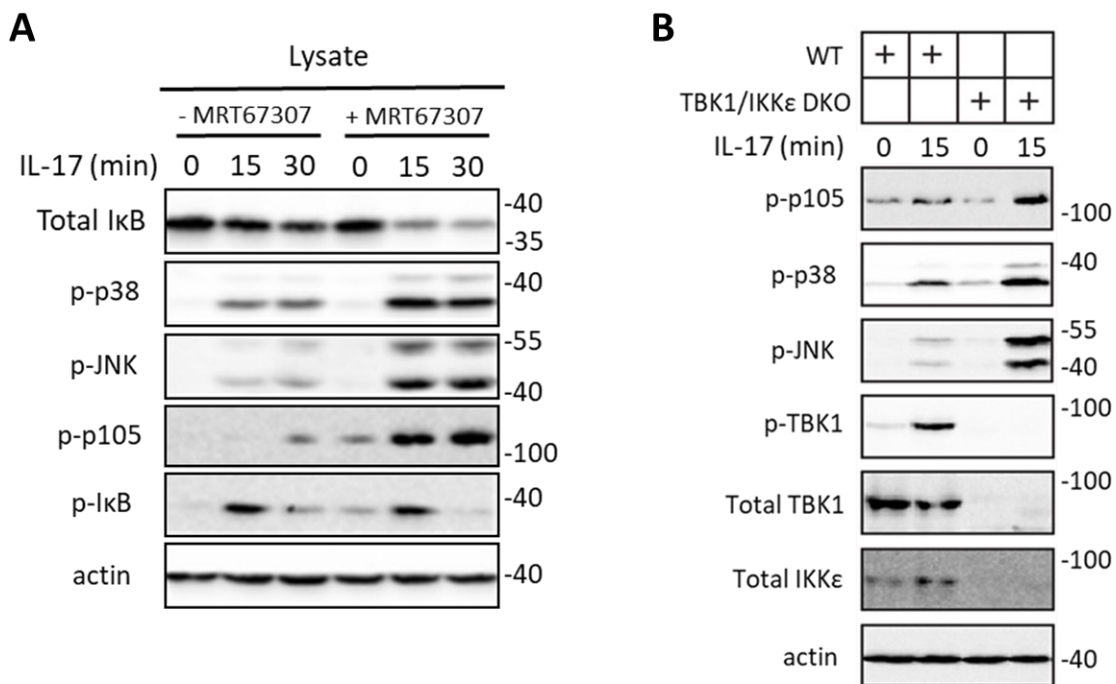


**Fig. 5.: TBK1 and IKKε negatively regulate IL-17-induced signaling**
**A:** Western blot analysis of cells either untreated or incubated for 30 min with MRT67307 inhibitor (2 µM). Cells were subsequently stimulated with recombinant SF-IL-17 (500 ng/ml) for indicated time points.
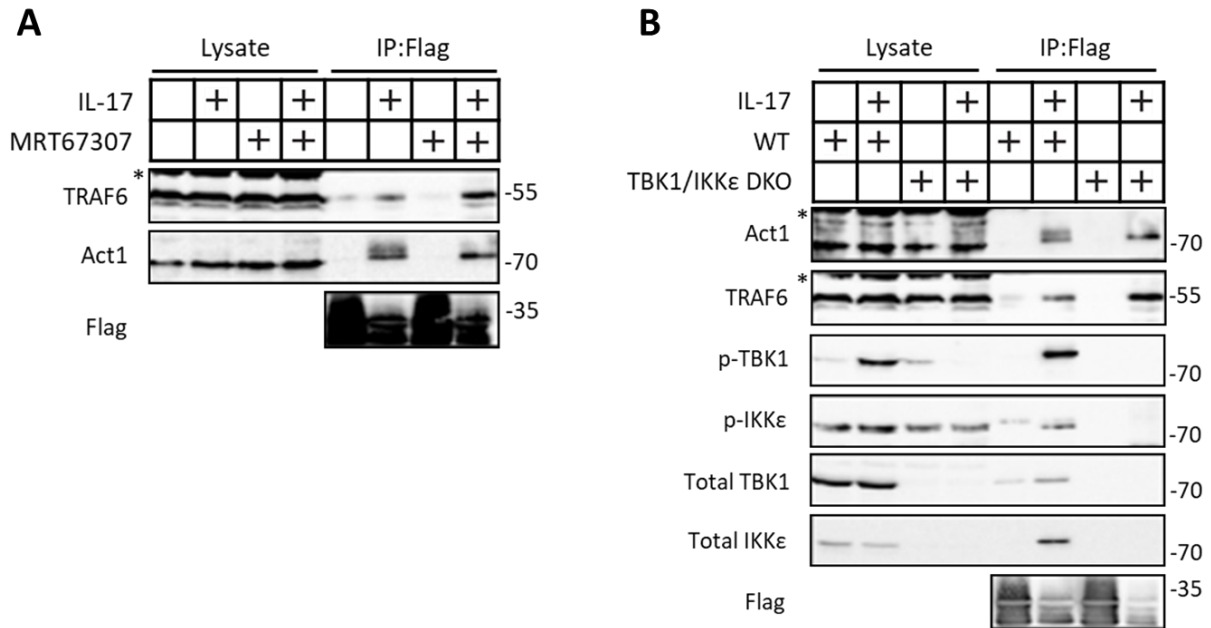**B:** Western blot analysis of WT and TBK1/IKKε DKO cells stimulated or not with SF-IL-17 (500 ng/ml) for 15 min.
Results of each experiment were obtained from three independent repetitions.

## 7.3. Activity of TBK1 and IKKε kinases causes release of TRAF6 from signaling complex

In the next step we wanted to clarify the mechanism how these kinases reduce activation of IL-17 signaling pathways. First, cells were either pretreated or not with MRT67307 inhibitor and then were stimulated or not with IL-17 recombinant ligand for indicated time points. Subsequently cells were solubilized and subjected to anti-Flag immunoprecipitation to isolate ligand and associated IL-17RSC, and molecules recruited to receptor complex were analyzed by Western blot. The analysis showed that chemical inhibition of kinases by MRT67307 inhibitor resulted in augmented recruitment of TRAF6 to the proximal receptor complex via Act1 adaptor, while recruitment of Act1 wasn't affected. However, our results also showed that inhibition of both kinases led to reduced phosphorylation of Act1 (Figure 6a, b), which is in conformity with previous studies describing the ability of TBK1 and IKKε kinases to phosphorylate Act1 at several serine sites [71, 76]. To confirm results with chemical inhibition, we repeated analysis in TBK1/IKKε DKO cells, in which the absence of both kinases also reduced the recruitment of TRAF6 and phosphorylation of Act1. These data indicate that TBK1/IKKε-mediated phosphorylation of Act1 at several sites inhibits recruitment of TRAF6 and thus attenuates activity of signaling pathways such as NF-κB or MAPKs.

**Fig. 6.: TBK1 and IKKε kinases influence the assembly of proximal IL-17RSC**

**A, B:** An anti-Flag immunoprecipitation of IL-17RSC from cells pretreated for 30 min or not with MRT67307 inhibitor (2 μM) **(A)** or WT and TBK1/IKKε DKO cells **(B)**. Cells were stimulated with SF-IL-17 (500 ng/ml) for 15 min or left unstimulated and isolated IL-17RSC was analyzed by immunoblotting. To unstimulated control samples SF-IL-17 ligand was added post-lysis.

Results of each experiment were obtained from three independent repetitions.

(*) unspecific background

## 7.4. NEMO protein recruits TBK1 and IKKε kinases to IL-17RSC

As last we studied how TBK1 and IKKε kinases associate with IL-17R complex. It was reported that NEMO protein, a part of IKK kinase complex, brings TBK1 and IKKε kinases to TNFR signaling complex upon TNF-α stimulation via binding of TANK and NAP1 adaptors [63]. Because TBK1 and IKKε are known to associate with TANK and NAP1 [85, 86] and these adaptors were revealed by MS as new components of IL-17RSC (described earlier, Figure 3), it made us to hypothesize that the recruitment of TBK1 and IKKε kinases might work similarly upon both TNF-α and IL-17 stimulation.

To test this hypothesis, my colleagues generated and provided me with NEMO KO cells, which were prepared using CRISPR/Cas9 method. We stimulated WT and NEMO KO cells with IL-17 recombinant ligand and subjected cellular lysates to anti-Flag immunoprecipitation in order to isolate IL-17RSC. Our results demonstrated that TBK1 and IKKε were less recruited to IL-17RSC upon stimulation in the absence of NEMO protein. The data also showed reduced levels of TANK and NAP1 adaptors recruitment in NEMO KO but not WT cells, indicating that recruitment of both adaptors and kinases TBK1/IKKε to IL-17RSC requires the presence of NEMO. Moreover, TRAF6 ubiquitin ligase recruitment to IL-17RSC was enhanced in NEMO KO cells (Figure 7), which confirms our previous results indicating that activity of TBK1 and IKKε causes phosphorylation-mediated release of TRAF6

from Act1 and proximal signaling complex overall. All these data demonstrate that NEMO seems to be a crucial protein for recruitment of TBK1 and IKKε kinases to IL-17RSC, causing a negative feedback loop. These data are greatly surprising and to a certain extend unexpected, because NEMO, as a component of IKK kinase complex, serves as a positive regulator of downstream signaling pathways activation. However, it is also a major negative regulator of IL-17-induced signaling.
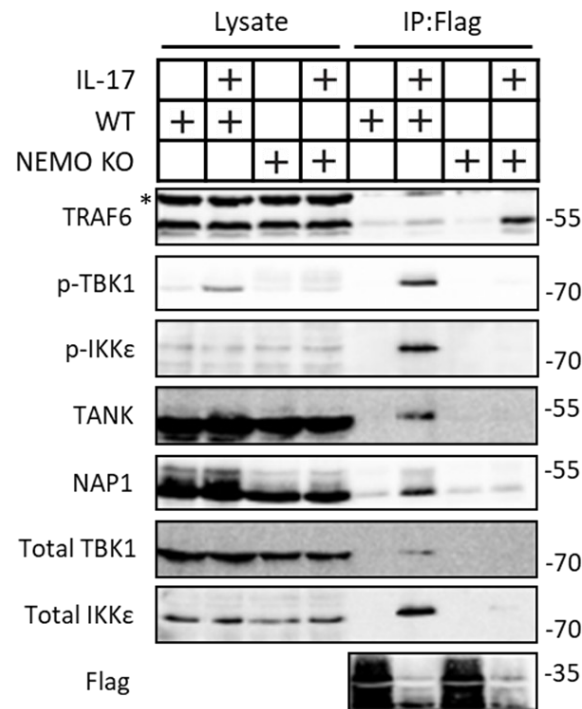


**Fig. 7.: TBK1 and IKKε kinase are recruited to IL-17RSC via NEMO protein**
An anti-Flag immunoprecipitation of IL-17RSC from WT and NEMO KO cells stimulated with SF-IL-17 (500 ng/ml) for 15 min or left unstimulated. To unstimulated control samples SF-IL-17 ligand was added post-lysis. Isolated IL-17RSC was analyzed by immunoblotting.
Results of each experiment were obtained from three independent repetitions.
(*) unspecific background

Altogether, we successfully elucidated the role of TBK1 and IKKε in IL-17-induced signaling and the mechanism of how these kinases associate with IL-17RSC. Our results show that both kinases are recruited via NEMO and serve as negative regulators of signal transduction. This attenuation of signaling is caused by phosphorylation of Act1 which results in release of TRAF6 from signaling complex and premature termination of downstream signaling.

All these data are a part of our recently published paper: "Systematic analysis of the IL-17 receptor signalosome reveals a robust regulatory feedback loop" [54].

## 7.5.    CMTM4 is required for surface expression of IL-17RC in ST2 cells

Another molecule involved in IL-17 signaling and revealed by our MS analysis was protein CMTM4. CMTM4, similarly to its most closely related family member CMTM6, was described to be involved in regulation of surface expression and stability of PD-L1, an important inhibitory molecule in the immune system [78-80]. Therefore, we hypothesized that CMTM4 could also stabilize the IL-17 receptor or at least one of its subunits. IL-17RA is constitutively expressed on plasma membrane of variety of cell types, including hematopoietic cells. On the contrary, surface expression of IL-17RC is bound only to cells of non-hematopoietic origin [87, 88]. However, our data indicated that CMTM4 is not directly regulating IL-17RA (data not shown). Thus, we aimed to analyze whether CMTM4 regulates the surface expression of IL-17RC.

In order to perform this analysis, we reconstituted WT and CMTM4 KO cell lines with IL-17RC, which was fused with EGFP tag, and analyzed its surface localization via fluorescent microscopy. We analyzed cells in suspension fixed in 10% formaldehyde. Microscopy images showed significant difference in plasma membrane localization of IL-17RC between WT and CMTM4 KO cells as IL-17RC was not expressed on the surface of CMTM4 KO cells (Figure 8a). These data indicate that plasma membrane expression of IL-17RC is dependent on CMTM4.

To support our results from fluorescent microscopy analysis, we wanted to confirm differences in IL-17RC surface expression via flow cytometry. For this analysis we used again WT and CMTM4 KO cells reconstituted with IL-17RC-EGFP. Reconstituted cells were stained with IL-17RC-specific primary antibody, which labeled only surface, but not intracellular IL-17RC. The analysis was performed using flow cytometry which revealed a significantly reduced surface localization of IL-17RC in cells lacking CMTM4. Its expression was lowered approximately to 30 % compared to that in WT cells (Figure 8b, c). However, the total GFP signal was comparable to that in WT cells (data not shown), suggesting that while CMTM4 affects the surface expression of IL-17RC, it does not serve as a regulator of its transcription. These data confirm our previous results from fluorescent microscopy analysis, showing that plasma membrane expression of IL-17RC is dependent on CMTM4.

Next, we analyzed whether CMTM4 and IL-17RC co-localize together on the cell surface. We reconstituted IL-17RC-expressing CMTM4 KO cells with CMTM4 protein fused with mCherry tag and analyzed membrane localization of both proteins. Indeed, the microscopy data confirmed that the returning of CMTM4 protein into deficient cells rescued the IL-17RC membrane localization. Moreover, the data showed a mutual overlapping of GFP and mCherry signals (Figure 8d), indicating that IL-17RC and CMTM4 co-localize on plasma membrane.
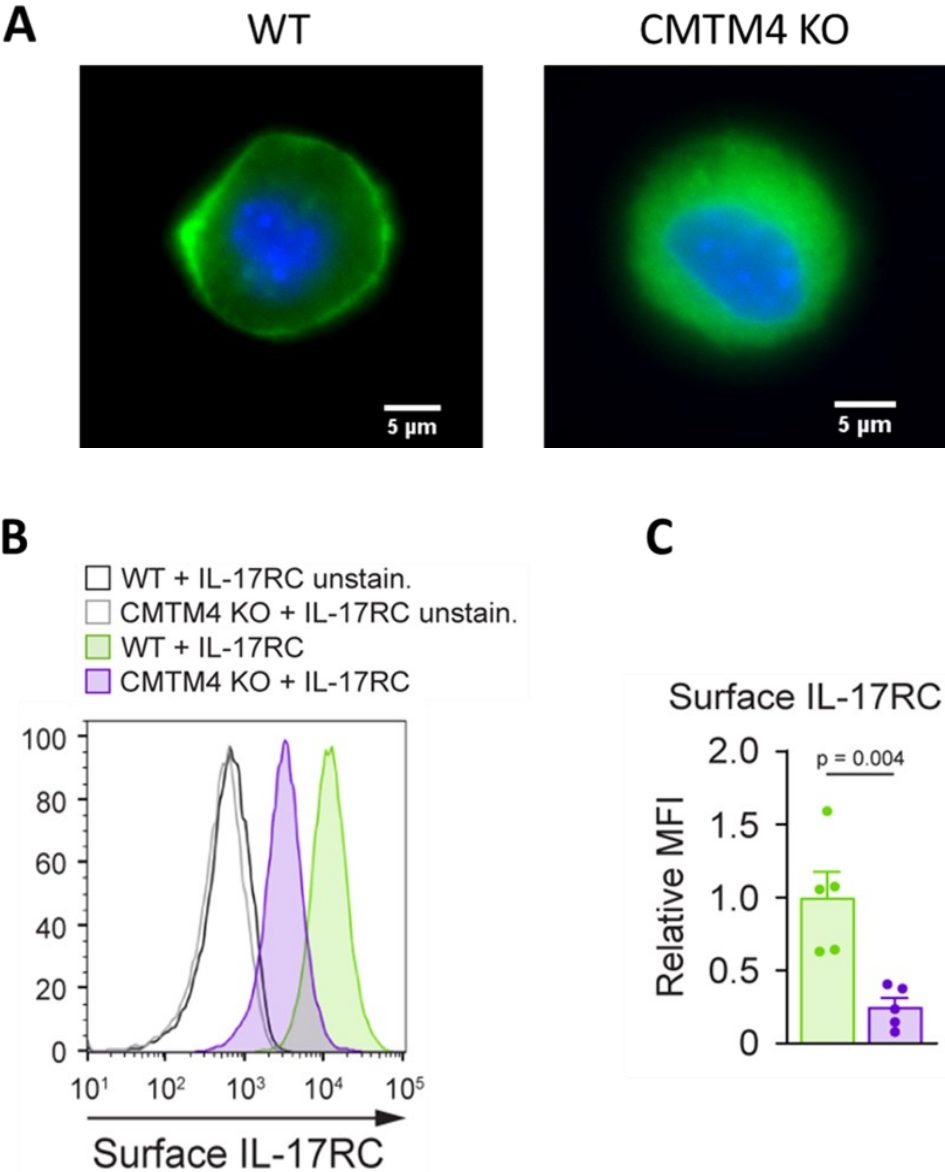
**D**



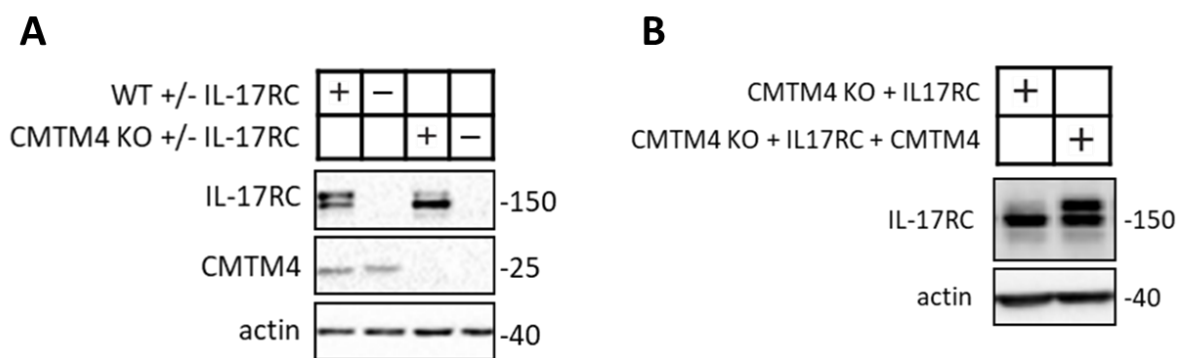CMTM4 KO reconstituted with CMTM4-mCherry
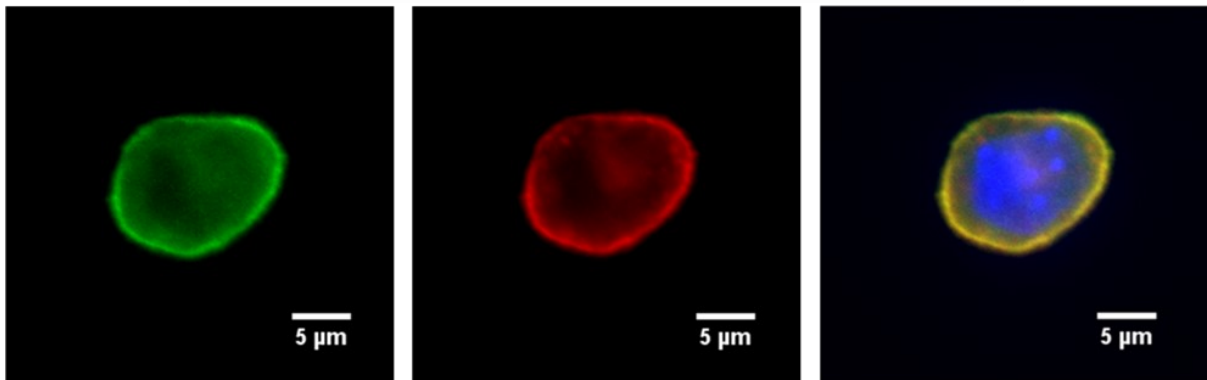
**Fig. 8.: CMTM4 regulates transport and expression of IL-17RC on cell surface**

**A:** Fluorescent microscopy analysis of IL-17RC surface expression in WT and CMTM4 KO cells reconstituted with IL-17RC-EGFP. Cells were stained with Hoechst33342 (DAPI, blue) and measured for GFP intensity (green).

**B, C:** Flow cytometry analysis of surface expression of IL-17RC in WT and CMTM4 KO cells reconstituted with IL-17RC-EGFP. Mean + SEM from 5 independent experiments. Mann-Whitney test. MFI = mean fluorescence intensity.

**D:** Fluorescent microscopy analysis of surface localization of IL-17RC and CMTM4 protein in CMTM4 KO cells reconstituted with IL-17RC-EGFP and CMTM4-mCherry. Cells were stained with Hoechst33342 (DAPI, blue) and measured for GFP (green) and mCherry intensity (red).

Results of microscopy experiments were obtained from three independent repetitions.

Surprisingly, immunoblotting performed on WT and CMTM4 KO cells expressing IL-17RC-EGFP revealed a modification of IL-17RC in WT cells, represented by the band of higher molecular weight, thus forming a double band of IL-17RC which was missing in CMTM4-deficient cells. Similar modification was also observed in CMTM4 KO cells reconstituted with IL-17RC-EGFP and re-expressed with CMTM4-mCherry (Figure 9a, b). It was published that extracellular domain of IL-17RC includes several N-glycosylation sites [89]. Thus, it is likely that this modification might represent glycosylated IL-17RC that is dependent on the presence of CMTM4 protein. Altogether, our data indicate that CMTM4 likely regulates the maturation and posttranslational modification of IL-17RC and its plasma membrane localization.



**Fig. 9.: CMTM4 protein regulates posttranslational modification and maturation of IL-17RC subunit**
**A, B:** Western blot analysis of WT and CMTM4 KO cells expressing or not IL-17RC-EGFP **(A)** or CMTM4 KO cells reconstituted with either only IL-17RC-EGFP alone or together with CMTM4-mCherry **(B)**. Results were obtained from three independent repetitions.

## 7.6.    CMTM4 is essential for activation of IL-17-mediated signaling

Our previous data showed that CMTM4 is required for IL-17RC surface expression. Therefore, we aimed to elucidate the role of CMTM4 in IL-17-mediated signaling. We prepared CMTM4 deficient cells which were stimulated with recombinant SF-IL-17 and subsequently analyzed for signaling pathways activation by immunoblotting. In comparison with WT cells, the analysis revealed a significantly lowered levels of activated MAPKs such as JNK or p38, similarly as reduction in activation of NF-κB pathway in the absence of CMTM4 (Figure 10a). Re-expression of CMTM4 deficient cells with CMTM4 but not with empty vector rescued the signaling (Figure 10b). These data indicate that CMTM4 is a crucial protein for activation of downstream signaling.

Next, we stimulated WT and CMTM4 KO cells with IL-17 recombinant ligand for indicated time points and cellular lysates were subjected to anti-Flag immunoprecipitation to isolate IL-17RSC. Results showed reduced recruitment of both Act1 adaptor and TRAF6 ubiquitin ligase to IL-17RSC in KO cells, molecules necessary for signal transduction (Figure 10c). Recruitment of these molecules was rescued again after re-expression of cells with mCMTM4 (Figure 10d). These results demonstrate that CMTM4 protein is essential for proper assembly of IL-17R proximal signaling complex which is crucial for triggering of IL-17 signaling.
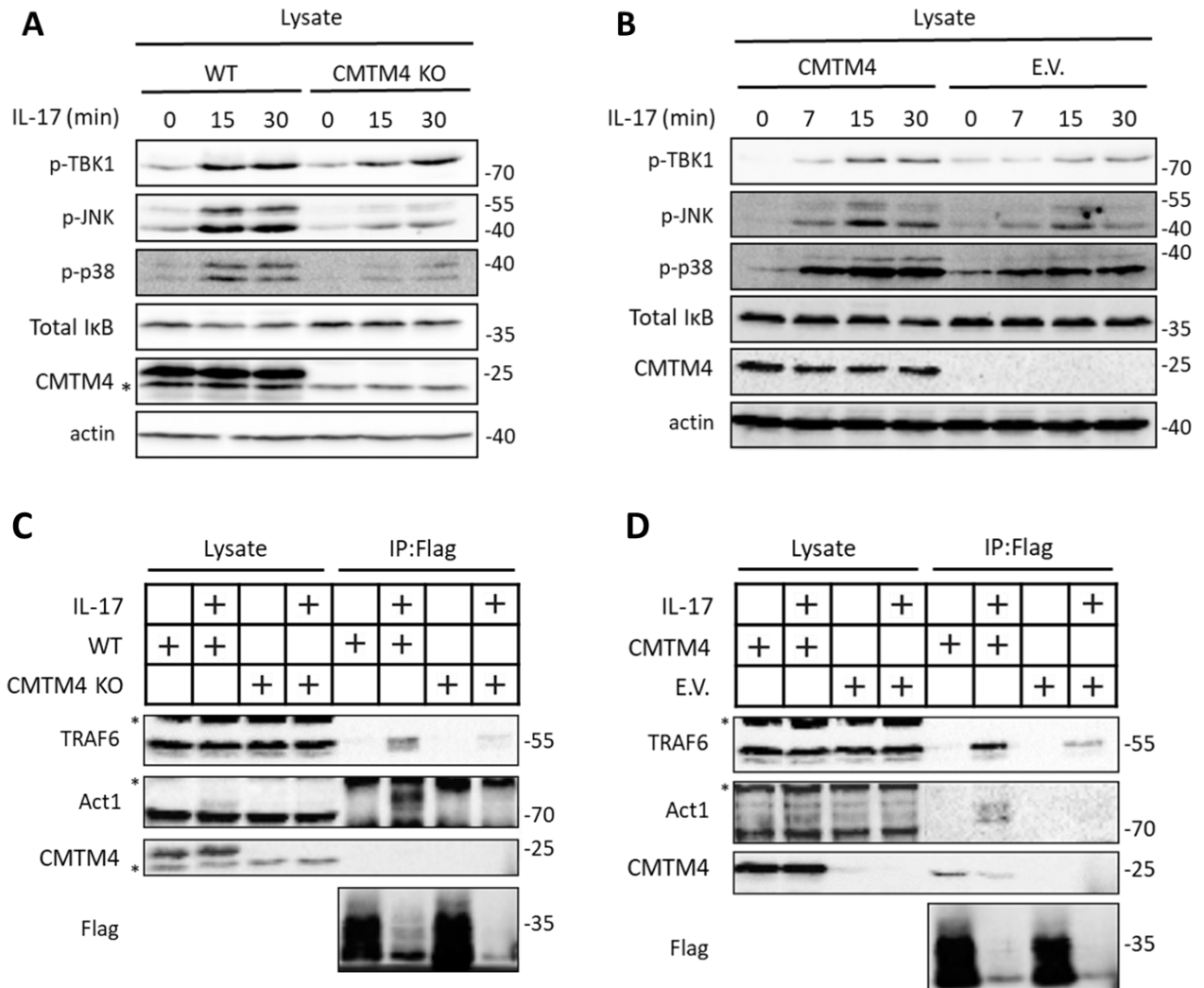
**Fig. 10.: CMTM4 protein has a pivotal role in activation of IL-17-mediated signaling**

**A, B:** Western blot analysis of WT and CMTM4 KO cells **(A)** or CMTM4 KO cells reconstituted with either murine CMTM4 or empty vector (E.V.) **(B)** stimulated or not with SF-IL-17 (500 ng/ml) for indicated time points.

**C, D:** An anti-Flag immunoprecipitation of IL-17RSC from WT and CMTM4 KO cells **(C)** or CMTM4 KO cells reconstituted with either murine CMTM4 or empty vector (E.V.) **(D)**. Cells were stimulated with SF-IL-17 (500 ng/ml) for 15 min or left unstimulated and isolated IL-17RSC was analyzed by immunoblotting. To control samples SF-IL-17 ligand was added post-lysis.

Results of each experiment were obtained from three independent repetitions.

(*) unspecific background

CMTM4 is a newly discovered component of IL-17-mediated signaling, whose function in this signal transduction wasn't yet described. Our data revealed that CMTM4 is crucial for triggering of IL-17 signaling and its absence leads to reduced activation of downstream pathways such as NF-κB and MAPKs. This is caused by its essential role in maturation and subsequent localization of IL-17RC subunit on plasma membrane. Taken together, CMTM4 regulates proper assembly of IL-17RSC which is necessary for subsequent signal transduction and transcription of IL-17 target genes, which contribute to activation and maintenance of inflammatory responses.

All these data are part of project which is currently in preparation for publication.

# 8.  Discussion

IL-17 is a crucial proinflammatory cytokine protecting our body against several bacteria and yeasts such as *Candida albicans*, which causes chronic mucocutaneous candidiasis disease (CMCD) in patients with impaired IL-17 signaling. This protection is achieved by IL-17-induced activation of MAPKs and NF-κB signaling pathways, resulting in production of chemokines and other inflammatory and antimicrobial products, including IL-6, IL-8 or CXCL1. These products stimulate numerous immune cells and subsequently activate their effector functions contributing to elimination of invading pathogens. By contrast, excessive activation of IL-17 signal transduction is involved in the development of several autoimmune pathologies including psoriasis and psoriatic arthritis. Thus, a tight regulation of this signaling is essential in order to prevent IL-17-induced autoimmunity.

IL-17 is not a single cytokine, but belongs to family of several members from IL-17A to IL-17F. Similarly, its receptor compounded of IL-17RA and IL-17RC subunits is a part of related family of IL-17 receptors, composed of 5 members IL-17RA-IL-17RE. However, IL-17A, which is usually referred as IL-17, is the most studied member of this family. Due to this, in our projects we focused on the research of signal transduction and its regulation upon IL-17A cytokine and its receptor IL-17RA/RC.

Interestingly, IL-17RA receptor subunit is shared by majority of IL-17 ligands, thus forming a heteromeric receptor complexes binding different family members. As previously described, several interacting domains are located in the cytoplasmic tail of IL-17RA. One of these highly important domains is a SEFIR motif, which is crucial for recruitment of Act1 to the receptor complex through homotypic interaction and thus triggering the whole downstream signaling [22, 23]. Since IL-17RA serves as a common receptor subunit shared by IL-17 members, it suggests that the signaling mechanism might be common within IL-17 cytokine family.

Apart from IL-17RA, Act1 is recruited via SEFIR-SEFIR interaction to another receptors from IL-17R family, such as IL-17RE which forms a heteromeric complex with IL-17RA and serves as a receptor for IL-17C, which also activates NF-κB and MAPKs signaling pathways similarly as IL-17A [90]. Another receptor recruiting Act1 is IL-17RB, which functions together with IL-17RA as receptor for IL-17E, also known as IL-25 [91].

Altogether, signaling scheme used by IL-17A might be shared within IL-17 cytokines, as several family members was shown to interact with Act1, a crucial adaptor for IL-17 signaling. This indicates that the assembly of proximal receptor signaling complexes and their downstream signaling might be triggered similarly within IL-17 family. However, the detailed mechanisms of downstream signal transduction and complete understanding of how signaling via other IL-17 cytokines works is still poorly explored and requires further research.

Using the specific methodological approach my colleagues studied the assembly of IL-17RSC via mass spectrometry. This analysis revealed a variety of molecules recruited to receptor signaling complex, including kinases TBK1 and IKKε. Both kinases were shown to be strongly recruited and activated upon IL-17 stimulation, which was similar to levels activated upon a strong inflammatory stimulus TNF-α.

The role of TBK1 and IKKε in IL-17-induced signaling long remained controversial and not fully understood. Both kinases were reported by two studies to be positive regulators of IL-17 signaling and the ablation of either TBK1 or IKKε alone reduced triggering of downstream signaling, especially the pathway leading to stabilization of already produced mRNAs [71, 72]. Another study confirmed that defects in both kinases simultaneously led to significantly reduced signaling upon stimulation of cells with IL-17 in combination with TNF-α [92]. However, it was recently demonstrated that the absence of both TBK1/IKKε kinases results in cell death induced by TNF-α stimulation, which causes higher susceptibility of mouse embryonic fibroblast (MEFs) used in this study to TNF-α-dependent apoptosis or necroptosis [63, 64].

Nevertheless, another study using an overexpression system of either TBK1 or IKKε kinase in double deficient cells demonstrated that both kinases negatively regulate IL-17 signaling by phosphorylating of Act1 on three specific serine sites, which in turn prevents recruitment of crucial ubiquitin ligase TRAF6 [76]. By contrast, our study is based on using a knock-out experimental models which properly explains the mechanism and thus resolves the controversy of TBK1/IKKε-mediated IL-17 signaling regulation.

TRAF6 is a crucial molecule within IL-17 proximal receptor complex, which interacts with Act1 adaptor through its TRAF-interacting domain upon IL-17-mediated stimulation, and creates a K63-linked non-degradative polyubiquitin chains essential for binding of several downstream signaling complexes, especially TAK1/TAB2/TAB3 and IKK complex compounded of IKKα, IKKβ and NEMO. These complexes are recruited to K63-polyubiqutin linkages upon various stimuli and contribute to activation of downstream MAPKs and NF-κB transcriptional factor. However, activation of corresponding signaling pathways is significantly weaker upon IL-17 stimulation compared to other proinflammatory stimuli.

NEMO serves especially as a regulatory molecule within IKK complex, thus enabling its recruitment to K63-linkages. Surprisingly, our results revealed that NEMO also brings TBK1 and IKKε to IL-17RSC through their interaction with TANK and NAP1 adaptors, which are known to associate with NEMO [63].

TBK1 and IKKε were shown to be involved in regulation of numerous processes such as autophagy, interferon response and TNF-α-induced cell death. Our data revealed that both kinases serve as negative regulators of IL-17 signal transduction by mediating the phosphorylation of Act1,

which causes reduced recruitment of TRAF6 to IL-17RSC. Moreover, deficiency in both kinases simultaneously markedly enhanced activation of IL-17 signaling pathways. Thus, TBK1 and IKKε provide a significant negative feedback loop, which might explain the weak inflammatory response to IL-17 stimulation in cells. This also reveals a new inhibitory function of NEMO protein mediated through the recruitment of both kinases to IL-17RSC, thus indicating a dual role of NEMO in IL-17 signaling, which is unexpected and was not described so far.

In conclusion, TBK1 and IKKε while brought to IL-17RSC via NEMO protein provide a negative feedback loop, preventing the upregulated activation of IL-17 signal transduction (Figure 11). As the IL-17 signaling is involved in the development of several autoimmune pathologies, this inhibitory axis including TBK1, IKKε and NEMO might contribute to the control of proper IL-17 activation and to the balance between required immune response and protection from autoimmunity.
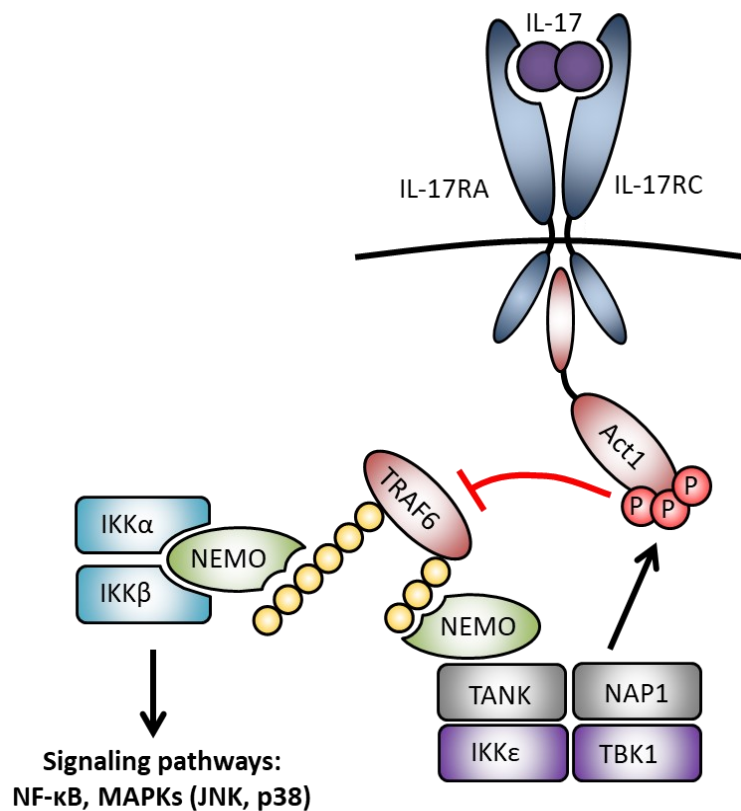


**Fig. 11.: Role of TBK1 and IKKε kinases in IL-17 signal transduction**
TBK1 and IKKε kinases are strongly activated upon IL-17 stimulation, which causes phosphorylation of Act1 adaptor on three serine sites. This phosphorylation prevents binding of TRAF6 ubiquitin ligase and results in attenuated and prematurely terminated downstream signaling. Both TBK1 and IKKε associate with TANK and NAP1 adaptors and in this complex are brought to IL-17RSC via NEMO protein, which is also a component of IKK complex triggering IL-17 downstream pathways.

Proximal receptor signaling complex is composed of two receptor subunits IL-17RA and IL-17RC which bind an adaptor Act1. IL-17RA receptor subunit is expressed continuously on the surface on numerous cell types, including cells of both hematopoietic and non-hematopoietic origin. By contrast, IL-17RC can be found only on non-hematopoietic cells, especially epithelial, endothelial and fibroblast cells [87, 88]. Thus, IL-17RC determines the cell types which will respond to IL-17 stimulation.

In our work we identified a new component of IL-17RSC, a CMTM4 protein, which was not yet described within IL-17 signal transduction. CMTM4 belongs to poorly studied CMTM family and its role in the immune system is poorly defined. It was reported that CMTM4 is a crucial regulator of surface localization and recycling of VE-cadherin [83], and together with its most closely related family member CMTM6 is also involved in regulation of PD-L1 surface expression and transport to cell plasma membrane [79, 80]. Our results suggest that CMTM4 has a pivotal role within IL-17 signaling in which it colocalizes with IL-17RC receptor subunit and regulates its maturation and transport to the cell surface. Absence of CMTM4 prevents assembly of IL-17 proximal receptor complex, especially binding of TRAF6, which reduces triggering of IL-17 signal transduction.

Therefore, CMTM4 is a new component of IL-17RSC and contributes to its proper assembly, which is essential for recruitment of other signaling molecules such as Act1, TRAF6 and IKK and TAK1 complexes and for the activation of downstream pathways (Figure 12).
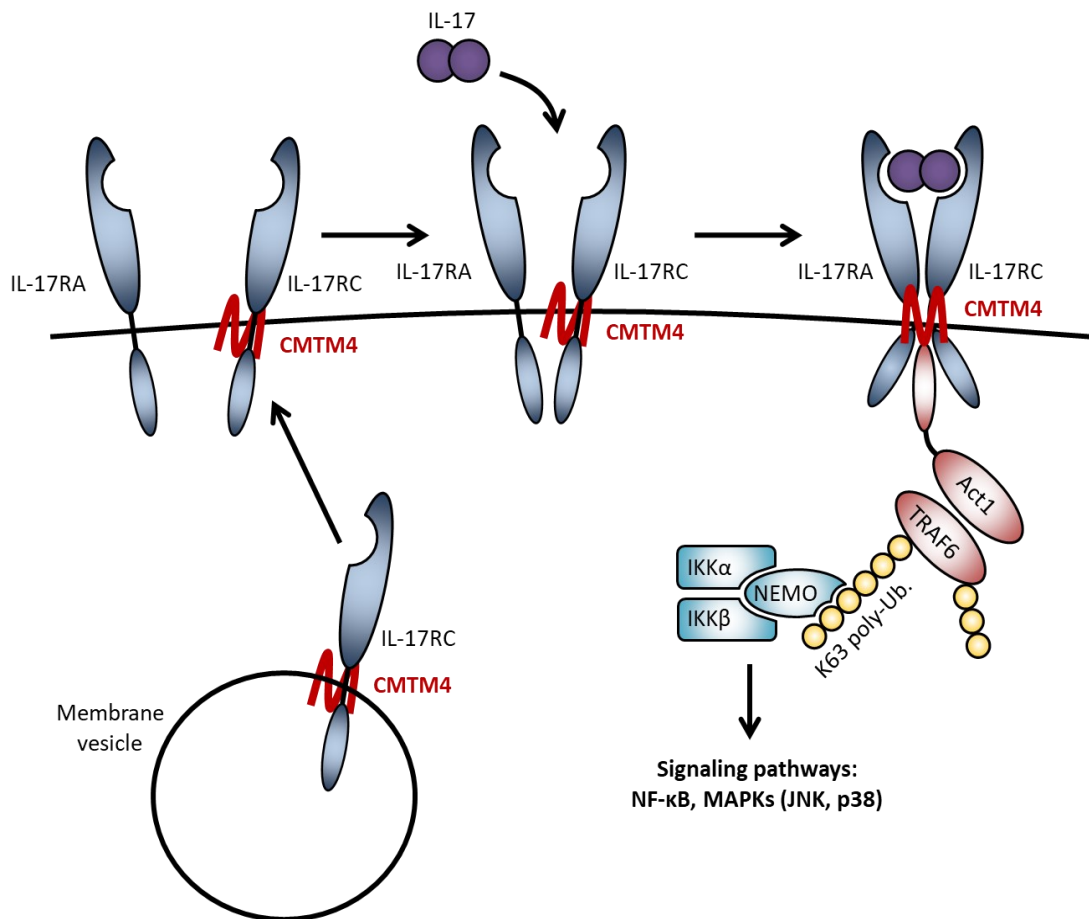
**Fig. 12.: Role of CMTM4 protein in IL-17 signal transduction**

CMTM4 is a new component within IL-17 signal transduction, which is crucial for plasma membrane transport and surface expression of IL-17RC receptor subunit. This enables proper assembly of IL-17RSC, which recruits signaling molecules such as Act1 and TRAF6 E3 ubiquitin ligase. TRAF6 subsequently creates K63-linked non-degradative polyubiquitin chains, which bind signaling proteins, such as IKK complex and contribute to triggering of downstream signaling pathways such as MAPKs and NF-κB.

As already known, upregulated IL-17-induced signaling causes the development of several autoimmune disorders, especially psoriasis. Nowadays, treatment of psoriasis is based on using of neutralizing antibodies targeting predominantly IL-17A, IL-17RA and IL-23/IL-12 cytokines [16]. However, it might be possible to target other components of IL-17 proximal receptor complex, such as IL-17RC and/or Act1. In contrast to IL-17RA, the expression of IL-17RC is restricted to tissues of non-hematopoietic origin. Therefore, it might be reasonable to target this receptor subunit instead of IL-17RA, which is much more ubiquitous [87, 88]. Importantly, as we discovered CMTM4 protein to be a new component of IL-17RSC which participates in signaling activation, it might serve as a new possible target for treatment of IL-17-induced autoimmune pathologies. In addition, since Act1 is a unique molecule in triggering of IL-17 downstream signaling, its blockade might specifically reduce exclusively IL-17 signal transduction, while not affecting signaling pathways upon other stimuli. By contrast, another component of IL-17 proximal receptor complex TRAF6 is extensively shared molecule within the immune system and its targeting might cause numerous pathologies due to impaired signaling from various stimuli and their receptors. Thus, treatment of IL-17-induced autoimmune pathologies might target on molecules upstream from TRAF6 within IL-17 receptor complex.

# 9.   Conclusion

Because precise regulation of IL-17 receptor complex signaling is essential in order to prevent the development of IL-17-mediated autoimmunity, the main aim of this thesis, as well as our projects, was to elucidate the role of several regulatory molecules of IL-17 signal pathway, which included kinases TBK1 and IKKε and a newly discovered protein CMTM4. These molecules were revealed by MS analysis as a part of IL-17RSC, however, their function in signaling wasn't to date fully understood.

In the first part dedicated to TBK1 and IKKε we ascertained that these kinases are extensively activated upon IL-17 stimulation. While recruited to IL-17RSC via NEMO protein, they negatively regulate activation of downstream signaling pathways by phosphorylation of Act1 adaptor molecule within proximal receptor signaling complex. This phosphorylation takes place at several sites of Act1 and prevents binding of TRAF6 ubiquitin ligase, leading to attenuated activation of downstream pathways. In conclusion, recruitment of TBK1 and IKKε kinases to IL-17RSC results in negative feedback loop, which likely contributes to the protection from IL-17-triggered autoimmunity.

In the second part we have established the role of new component of IL-17 signaling, the protein termed CMTM4. Our data indicated a crucial role of this protein in IL-17 signaling activation as it drives maturation and transport of IL-17RC subunit, an essential component of IL-17 receptor complex, to the plasma membrane, thus enabling its subsequent association with the second, IL-17RA subunit and final assembly of IL-17RSC.

In conclusion, we have successfully established the role of several regulatory molecules in IL-17-mediated signal transduction. All these findings provide a new insight into IL-17 signaling regulation and might contribute to proper understanding of how signaling via IL-17 is triggered. Moreover, these obtained findings might be helpful for further research of potential therapeutic targets for treatment of IL-17-induced autoimmune pathologies.

# 10. References

* secondary references

1.      *Iwakura, Y., et al., *Functional specialization of interleukin-17 family members.* Immunity, 2011. **34**(2): p. 149-62.
2.      *Monin, L. and S.L. Gaffen, *Interleukin 17 Family Cytokines: Signaling Mechanisms, Biological Activities, and Therapeutic Implications.* Cold Spring Harb Perspect Biol, 2018. **10**(4).
3.      *Cua, D.J. and C.M. Tato, *Innate IL-17-producing cells: the sentinels of the immune system.* Nat Rev Immunol, 2010. **10**(7): p. 479-89.
4.      Rachitskaya, A.V., et al., *Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion.* J Immunol, 2008. **180**(8): p. 5167-71.
5.      Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.
6.      Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
7.      Manel, N., D. Unutmaz, and D.R. Littman, *The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat.* Nat Immunol, 2008. **9**(6): p. 641-9.
8.      Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
9.      Kao, C.Y., et al., *IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways.* J Immunol, 2004. **173**(5): p. 3482-91.
10.     Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
11.     *Onishi, R.M. and S.L. Gaffen, *Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease.* Immunology, 2010. **129**(3): p. 311-21.
12.     Kisand, K., et al., *Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines.* J Exp Med, 2010. **207**(2): p. 299-308.
13.     Conti, H.R., et al., *Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis.* J Exp Med, 2009. **206**(2): p. 299-311.
14.     Marchitto, M.C., et al., *Clonal Vgamma6(+)Vdelta4(+) T cells promote IL-17-mediated immunity against Staphylococcus aureus skin infection.* Proc Natl Acad Sci U S A, 2019. **116**(22): p. 10917-10926.
15.     *Blauvelt, A. and A. Chiricozzi, *The Immunologic Role of IL-17 in Psoriasis and Psoriatic Arthritis Pathogenesis.* Clin Rev Allergy Immunol, 2018. **55**(3): p. 379-390.
16.     *Bai, F., et al., *Short-Term Efficacy and Safety of IL-17, IL-12/23, and IL-23 Inhibitors Brodalumab, Secukinumab, Ixekizumab, Ustekinumab, Guselkumab, Tildrakizumab, and Risankizumab for the Treatment of Moderate to Severe Plaque Psoriasis: A Systematic Review and Network Meta-Analysis of Randomized Controlled Trials.* J Immunol Res, 2019. **2019**: p. 2546161.
17.     *Deshaies, R.J. and C.A. Joazeiro, *RING domain E3 ubiquitin ligases.* Annu Rev Biochem, 2009. **78**: p. 399-434.
18.     *Hu, H. and S.C. Sun, *Ubiquitin signaling in immune responses.* Cell Res, 2016. **26**(4): p. 457-83.
19.     Wright, J.F., et al., *The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex.* J Immunol, 2008. **181**(4): p. 2799-805.
20.     Ely, L.K., S. Fischer, and K.C. Garcia, *Structural basis of receptor sharing by interleukin 17 cytokines.* Nat Immunol, 2009. **10**(12): p. 1245-51.

21.    Maitra, A., et al., *Distinct functional motifs within the IL-17 receptor regulate signal transduction and target gene expression.* Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7506-11.

22.    Zhang, B., et al., *Structure of the unique SEFIR domain from human interleukin 17 receptor A reveals a composite ligand-binding site containing a conserved alpha-helix for Act1 binding and IL-17 signaling.* Acta Crystallogr D Biol Crystallogr, 2014. **70**(Pt 5): p. 1476-83.

23.    Chang, S.H., H. Park, and C. Dong, *Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor.* J Biol Chem, 2006. **281**(47): p. 35603-7.

24.    Kanamori, M., et al., *NF-kappaB activator Act1 associates with IL-1/Toll pathway adaptor molecule TRAF6.* FEBS Lett, 2002. **532**(1-2): p. 241-6.

25.    *Park, H.H., *Structure of TRAF Family: Current Understanding of Receptor Recognition.* Front Immunol, 2018. **9**: p. 1999.

26.    Yin, Q., et al., *E2 interaction and dimerization in the crystal structure of TRAF6.* Nat Struct Mol Biol, 2009. **16**(6): p. 658-66.

27.    Yang, K., et al., *The coiled-coil domain of TRAF6 is essential for its auto-ubiquitination.* Biochem Biophys Res Commun, 2004. **324**(1): p. 432-9.

28.    Deng, L., et al., *Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain.* Cell, 2000. **103**(2): p. 351-61.

29.    *Wang, J., et al., *Mechanism by which TRAF6 Participates in the Immune Regulation of Autoimmune Diseases and Cancer.* Biomed Res Int, 2020. **2020**: p. 4607197.

30.    *Israel, A., *The IKK complex, a central regulator of NF-kappaB activation.* Cold Spring Harb Perspect Biol, 2010. **2**(3): p. a000158.

31.    Hata, K., et al., *IL-17 stimulates inflammatory responses via NF-kappaB and MAP kinase pathways in human colonic myofibroblasts.* Am J Physiol Gastrointest Liver Physiol, 2002. **282**(6): p. G1035-44.

32.    Wang, C., et al., *TAK1 is a ubiquitin-dependent kinase of MKK and IKK.* Nature, 2001. **412**(6844): p. 346-51.

33.    Kanayama, A., et al., *TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains.* Mol Cell, 2004. **15**(4): p. 535-48.

34.    Rahighi, S., et al., *Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation.* Cell, 2009. **136**(6): p. 1098-109.

35.    *Cohen, P., *The TLR and IL-1 signalling network at a glance.* J Cell Sci, 2014. **127**(Pt 11): p. 2383-90.

36.    Schwandner, R., K. Yamaguchi, and Z. Cao, *Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction.* J Exp Med, 2000. **191**(7): p. 1233-40.

37.    Ye, H., et al., *Distinct molecular mechanism for initiating TRAF6 signalling.* Nature, 2002. **418**(6896): p. 443-7.

38.    *Satoh, T. and S. Akira, *Toll-Like Receptor Signaling and Its Inducible Proteins.* Microbiol Spectr, 2016. **4**(6).

39.    Conze, D.B., et al., *Lys63-linked polyubiquitination of IRAK-1 is required for interleukin-1 receptor- and toll-like receptor-mediated NF-kappaB activation.* Mol Cell Biol, 2008. **28**(10): p. 3538-47.

40.    Cusson-Hermance, N., et al., *Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation.* J Biol Chem, 2005. **280**(44): p. 36560-6.

41.    Kobayashi, T., et al., *TRAF6 is a critical factor for dendritic cell maturation and development.* Immunity, 2003. **19**(3): p. 353-63.

42.    Subauste, C.S., R.M. Andrade, and M. Wessendarp, *CD40-TRAF6 and autophagy-dependent anti-microbial activity in macrophages.* Autophagy, 2007. **3**(3): p. 245-8.

43. Mukundan, L., et al., *TNF receptor-associated factor 6 is an essential mediator of CD40-activated proinflammatory pathways in monocytes and macrophages.* J Immunol, 2005. **174**(2): p. 1081-90.

44. Jalukar, S.V., B.S. Hostager, and G.A. Bishop, *Characterization of the roles of TNF receptor-associated factor 6 in CD40-mediated B lymphocyte effector functions.* J Immunol, 2000. **164**(2): p. 623-30.

45. Ahonen, C., et al., *The CD40-TRAF6 axis controls affinity maturation and the generation of long-lived plasma cells.* Nat Immunol, 2002. **3**(5): p. 451-6.

46. Kobayashi, T., et al., *TRAF6 is required for generation of the B-1a B cell compartment as well as T cell-dependent and -independent humoral immune responses.* PLoS One, 2009. **4**(3): p. e4736.

47. Naito, A., et al., *Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice.* Genes Cells, 1999. **4**(6): p. 353-62.

48. Lomaga, M.A., et al., *TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling.* Genes Dev, 1999. **13**(8): p. 1015-24.

49. Cejas, P.J., et al., *TRAF6 inhibits Th17 differentiation and TGF-beta-mediated suppression of IL-2.* Blood, 2010. **115**(23): p. 4750-7.

50. Han, D., et al., *Dendritic cell expression of the signaling molecule TRAF6 is required for immune tolerance in the lung.* Int Immunol, 2017. **29**(2): p. 71-78.

51. Han, D., et al., *Dendritic cell expression of the signaling molecule TRAF6 is critical for gut microbiota-dependent immune tolerance.* Immunity, 2013. **38**(6): p. 1211-22.

52. Chiffoleau, E., et al., *TNF receptor-associated factor 6 deficiency during hemopoiesis induces Th2-polarized inflammatory disease.* J Immunol, 2003. **171**(11): p. 5751-9.

53. Muto, G., et al., *TRAF6 is essential for maintenance of regulatory T cells that suppress Th2 type autoimmunity.* PLoS One, 2013. **8**(9): p. e74639.

54. Draberova, H., et al., *Systematic analysis of the IL-17 receptor signalosome reveals a robust regulatory feedback loop.* EMBO J, 2020. **39**(17): p. e104202.

55. *Durand, J.K., Q. Zhang, and A.S. Baldwin, *Roles for the IKK-Related Kinases TBK1 and IKKepsilon in Cancer.* Cells, 2018. **7**(9).

56. Peters, R.T., S.M. Liao, and T. Maniatis, *IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex.* Mol Cell, 2000. **5**(3): p. 513-22.

57. Bonnard, M., et al., *Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription.* EMBO J, 2000. **19**(18): p. 4976-85.

58. Tenoever, B.R., et al., *Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity.* Science, 2007. **315**(5816): p. 1274-8.

59. Hemmi, H., et al., *The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection.* J Exp Med, 2004. **199**(12): p. 1641-50.

60. Fitzgerald, K.A., et al., *IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway.* Nat Immunol, 2003. **4**(5): p. 491-6.

61. Sharma, S., et al., *Triggering the interferon antiviral response through an IKK-related pathway.* Science, 2003. **300**(5622): p. 1148-51.

62. Perry, A.K., et al., *Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection.* J Exp Med, 2004. **199**(12): p. 1651-8.

63. Lafont, E., et al., *TBK1 and IKKepsilon prevent TNF-induced cell death by RIPK1 phosphorylation.* Nat Cell Biol, 2018. **20**(12): p. 1389-1399.

64. Xu, D., et al., *TBK1 Suppresses RIPK1-Driven Apoptosis and Inflammation during Development and in Aging.* Cell, 2018. **174**(6): p. 1477-1491 e19.

65. Micheau, O. and J. Tschopp, *Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes.* Cell, 2003. **114**(2): p. 181-90.

66. Tokunaga, F., et al., *SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex.* Nature, 2011. **471**(7340): p. 633-6.

67. Ikeda, F., et al., *SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis.* Nature, 2011. **471**(7340): p. 637-41.

68. Draber, P., et al., *LUBAC-Recruited CYLD and A20 Regulate Gene Activation and Cell Death by Exerting Opposing Effects on Linear Ubiquitin in Signaling Complexes.* Cell Rep, 2015. **13**(10): p. 2258-72.

69. Haas, T.L., et al., *Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction.* Mol Cell, 2009. **36**(5): p. 831-44.

70. Dondelinger, Y., et al., *NF-kappaB-Independent Role of IKKalpha/IKKbeta in Preventing RIPK1 Kinase-Dependent Apoptotic and Necroptotic Cell Death during TNF Signaling.* Mol Cell, 2015. **60**(1): p. 63-76.

71. Bulek, K., et al., *The inducible kinase IKKi is required for IL-17-dependent signaling associated with neutrophilia and pulmonary inflammation.* Nat Immunol, 2011. **12**(9): p. 844-52.

72. Herjan, T., et al., *IL-17-receptor-associated adaptor Act1 directly stabilizes mRNAs to mediate IL-17 inflammatory signaling.* Nat Immunol, 2018. **19**(4): p. 354-365.

73. *Shembade, N. and E.W. Harhaj, *IKKi: a novel regulator of Act1, IL-17 signaling and pulmonary inflammation.* Cell Mol Immunol, 2011. **8**(6): p. 447-9.

74. Sun, D., et al., *Treatment with IL-17 prolongs the half-life of chemokine CXCL1 mRNA via the adaptor TRAF5 and the splicing-regulatory factor SF2 (ASF).* Nat Immunol, 2011. **12**(9): p. 853-60.

75. Herjan, T., et al., *HuR is required for IL-17-induced Act1-mediated CXCL1 and CXCL5 mRNA stabilization.* J Immunol, 2013. **191**(2): p. 640-9.

76. Qu, F., et al., *TRAF6-dependent Act1 phosphorylation by the IkappaB kinase-related kinases suppresses interleukin-17-induced NF-kappaB activation.* Mol Cell Biol, 2012. **32**(19): p. 3925-37.

77. *Sanchez-Pulido, L., et al., *MARVEL: a conserved domain involved in membrane apposition events.* Trends Biochem Sci, 2002. **27**(12): p. 599-601.

78. Burr, M.L., et al., *CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity.* Nature, 2017. **549**(7670): p. 101-105.

79. Mezzadra, R., et al., *Identification of CMTM6 and CMTM4 as PD-L1 protein regulators.* Nature, 2017. **549**(7670): p. 106-110.

80. Imamovic, D. and S. Vranic, *Novel regulators of PD-L1 expression in cancer: CMTM6 and CMTM4-a new avenue to enhance the therapeutic benefits of immune checkpoint inhibitors.* Ann Transl Med, 2017. **5**(23): p. 467.

81. *Wang, Q. and X. Wu, *Primary and acquired resistance to PD-1/PD-L1 blockade in cancer treatment.* Int Immunopharmacol, 2017. **46**: p. 210-219.

82. *Guan, J., et al., *Programmed Death Ligand-1 (PD-L1) Expression in the Programmed Death Receptor-1 (PD-1)/PD-L1 Blockade: A Key Player Against Various Cancers.* Arch Pathol Lab Med, 2017. **141**(6): p. 851-861.

83. Chrifi, I., et al., *CMTM4 regulates angiogenesis by promoting cell surface recycling of VE-cadherin to endothelial adherens junctions.* Angiogenesis, 2019. **22**(1): p. 75-93.

84. *Vestweber, D., *VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation.* Arterioscler Thromb Vasc Biol, 2008. **28**(2): p. 223-32.

85. *Helgason, E., Q.T. Phung, and E.C. Dueber, *Recent insights into the complexity of Tank-binding kinase 1 signaling networks: the emerging role of cellular localization in the activation and substrate specificity of TBK1.* FEBS Lett, 2013. **587**(8): p. 1230-7.

86. *Chau, T.L., et al., *Are the IKKs and IKK-related kinases TBK1 and IKK-epsilon similarly activated?* Trends Biochem Sci, 2008. **33**(4): p. 171-80.

87. Toy, D., et al., *Cutting edge: interleukin 17 signals through a heteromeric receptor complex.* J Immunol, 2006. **177**(1): p. 36-9.

88. Kuestner, R.E., et al., *Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F.* J Immunol, 2007. **179**(8): p. 5462-73.

89. Goepfert, A., et al., *Structural Analysis Reveals that the Cytokine IL-17F Forms a Homodimeric Complex with Receptor IL-17RC to Drive IL-17RA-Independent Signaling.* Immunity, 2020. **52**(3): p. 499-512 e5.

90. *Song, X., et al., *The roles and functional mechanisms of interleukin-17 family cytokines in mucosal immunity.* Cell Mol Immunol, 2016. **13**(4): p. 418-31.

91. Claudio, E., et al., *The adaptor protein CIKS/Act1 is essential for IL-25-mediated allergic airway inflammation.* J Immunol, 2009. **182**(3): p. 1617-30.

92. Tanaka, H., et al., *Phosphorylation-dependent Regnase-1 release from endoplasmic reticulum is critical in IL-17 response.* J Exp Med, 2019. **216**(6): p. 1431-1449.