Charles University
Faculty of Science

Biology
Immunology

Bc. Jan Eliáš

Generation and analysis of mutant mouse model to study roles of KLKs in cutaneous inflammation

Příprava a charakterizace mutantního myšího modelu pro studium úlohy KLK proteáz při zánětlivé reakci kůže

Diploma thesis

Supervisor: Mgr. Petr Kašpárek, Ph.D.

Prague, 2021

Prohlášení

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

V Praze, 10.8.2021 Jan Eliáš

Acknowledgements

Most of all, I would like to thank Petr Kasparek for his unwavering support and for all of his insightful advice, which helped me tremendously during my work. Value of overall counsel that was provided to me by all members of my lab, namely Olga Zbodakova, Silvia Petrezselyova and Marta Slaufova, was also indispensable in the writing of this thesis. Finally, I would like to thank my family and friends for their support.

Abstract

Kallikrein-related peptidases (KLKs) are a subgroup of serine proteases of undisputable importance for a variety of functions, whose dysregulation has been linked to several pathological phenotypes. Among those pathologies, the Netherton syndrome stands out, since it is one of the very few that has its mechanism directly linked to KLK proteases as the main culprit of the disease, namely KLK5, KLK7 and to a lesser degree, KLK14. In this case, a mutation in the *SPINK5* gene leads to uncontrolled hyperactivity of those proteases, which results in epidermal barrier breach due to excessive epidermal desquamation and severe inflammation of the skin. Inflammation mechanisms of NS are still relatively poorly understood, with important roles being attributed to the activities of KLKs in the processing of immune system molecules and also to the dysregulation of the cutaneous microbiome.

TNFα signalling plays a key role in the homeostasis and immune response in the skin. Chronic skin infections may lead to deleterious effects with strong participation of TNFα signalling. To address the degree of its effects on the pathogenesis of NS, we have created a mouse model where the TNFR1 is disrupted by knockout of the *Tnfr1* gene on the background of a previously established mouse model of the Netherton syndrome.

We have successfully created the *Tnfr1*$^{-/-}$ mouse model and subsequently produced the desired *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice. Surprisingly, subsequent analyses suggest that not only *Tnfr1* ablation does not alleviate cutaneous inflammation present in previously created mouse models, but further increases its severity.

Key words: KLK, protease, skin, epidermis, inflammation, mouse model, CRISPR

Abstract (CZ)

Kallikreinové peptidázy (KLKs) jsou podskupinou serinových proteáz se zásadní důležitostí pro řadu funkcí, jejichž dysregulace přispívá k řadě patologických fenotypů. Nethertonův syndrom zaujímá mezi těmito patologiemi zásadní postavení, jelikož deregulace kallikreinových proteáz hraje klíčovou roli, především KLK5, KLK7 a do menší miry také KLK14. V případě této patologie je narušen gen hlavního regulačního proteinu těchto proteáz v kůži, kterým je *SPINK5*. To vede k nekontrolované hyprreaktivitě těchto proteáz a následnému narušení epidermální bariéry díky nadměrné epidermální deskvamaci a vážnému zánětu kůže. Zánětlivé mechanismy Nethertonova syndromu jsou v tuto chvíli relativně málo objasněné a panuje přesvědčení, že hlavní roli hraje zpracování imunitních molekul pomocí těchto proteáz a dysregulace kožního mikrobiomu.

TNFα signalizace hraje klíčovou roli v imunitní odpovědi proti mikrobům a chronické kožní záněty mohou vest k závažným zdravotním stavům, ve kterých je TNFα důležitým komponentem. Abychom adresovali vliv TNFα signalizace v kontextu kallikreinových proteáz a Nethertonova syndromu, tak jsme vytvořili myší model, ve kterém je narušen gen *Tnfr1* kódující protein receptor TNFR1 na pozadí již vytvořených modelů Nethertonova syndromu.

Úspěšně jsme vytvořili Tnfr1-/- myší model a následně jsme vyprodukovali požadovaný *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ model. Dosavadní výsledky naznačují, že narušení funkce tohoto genu tuto patologii nejen nezmírňuje, ale naopak vede ke zhoršení kožního zánětu.

Klíčová slova: KLK, proteáza, kůže, pokožka, zánět, myší model, CRISPR

Table of contents

List of abbreviations

AD	Atopic dermatitis
AMP	antimicrobial peptide
Cas9	CRISPR-associated protein 9
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
cIAP	cellular inhibitor of apoptosis
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CYLD	cylindromatosis
DD	death domain
DETC	dendritic epidermal T cell
ECM	extracellular matrix
FADD	Fas-associated death domain
FLG	filaggrin
FLIP	FLICE-like inhibitory protein
gRNA	guide RNA
H&E	hematoxylin and eosin
ICAM-1	intracellular adhesion molecule 1
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
ILC	innate lymphoid cells
IκB	inhibitor of kappa B

JNK	JUN N-terminal kinase
KLK	Kallikrein-related peptidase
LEKTI	lympho-epithelial Kazal-type-related inhibitor
LUBAC	linear ubiquitin chain complex
MLKL	mixed-lineage kinase domain-like protein
mTNF	membrane TNFα
NASDCL	Naphthol AS-D chloroacetate
NFκB	nuclear factor kappa B
NIK	NFκB-inducing kinase
NMD	nonsense-mediated decay
NS	Netherton syndrome
PAR2	protease-activated receptor 2
PSORS	psoriasis susceptibility region
RIPK1	receptor-interacting serine/threonine-protein kinase 1
RT-qPCR	reverse transcription quantitative PCR
SMAC	second mitochondria-derived activator caspase
SPINK5	serine protease inhibitor Kazal-type 5
sTNF	soluble TNFα
TAB	TGF-β-activated kinase 1 and MAP3K7-binding protein
TACE	TNFα-converting enzyme
TAK1	TGFβ-activated kinase 1
T_H	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor

TNFI	TNFα inhibitor
TNFR	TNF receptor
TNFRSF	TNF receptor superfamily
TNFSF	TNF superfamily
tracrRNA	trans-activating CRISPR RNA
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor-associated factor
TSLP	thymic stromal lymphopoietin

Introduction

Kallikrein-related peptidases (KLKs) are a part of a sub-group of serine proteases, with their family containing 14 members in total (Emami and Diamandis, 2007). These proteases have a variety of physiological activities, among them being the regulation of skin desquamation, and protease-activated receptor 2 (PAR2)-mediated inflammation, which contributes to adequate homeostatic conditions of the skin (Prassas et al., 2015).

The hyperactivity of KLK5, KLK7, and KLK14 has been previously shown as the primary culprit behind the pathological state known as the Netherton syndrome (NS) (Furio and Hovnanian, 2014). In this disease, the serine protease inhibitor Kazal-type (SPINK) 5 gene, which codes for a protein product called lympho-epithelial Kazal-type-related inhibitor (LEKTI), is mutated, which leads to the aforementioned dysregulation (Bitoun et al., 2003). The severity of this dysregulation is dependent on the site of the specific mutation, as the LEKTI protein is composed of 15 inhibitory domains, with different regulatory targets, thus leading to different levels of KLK activities. KLKs themselves then exert deleterious effects in the skin when left unchecked, namely severe inflammation and uncontrolled desquamation of the skin, leading to recurrent infections and the necessity of periodic treatment of the skin in the afflicted. Unfortunately, there is a lack of a reasonably effective treatment for NS patients, since there is still much to be elucidated in regards to the exact molecular mechanisms of this disease. As of now, the treatment options provide a temporal and not entirely effective solution to this problem, although there are several possible treatment options in the making with different levels of persistence (Petrova and Hovnanian, 2020).

Tumour necrosis factor (TNF)α serves a key role in a plethora of immunological conditions and manifests its effects through two different receptors, TNF receptor (TNFR)1 and TNFR2 (Holbrook et al., 2019). These effects are exerted through different mechanisms of action for each receptor and will be elaborated on further later in the thesis. TNFα has been previously linked to several other pathological skin conditions and the TNFα inhibitors (TNFIs) are readily available for the treatment of such conditions (Monaco et al., 2015), making it an interesting option to explore, as it

would greatly speed up possible treatment if there would be a connection between TNFα signalling and NS-related inflammation.

In this work, we aim to test this possibility by creating a *Tnfr1* knock-out mouse to elucidate its effects on the NS-like phenotype of a previously established mouse model.

1. Literature review

This literature review summarizes the immense complexities of underlying mechanisms surrounding KLKs, TNFα signalling and related skin pathologies. Chapter 1 describes KLKs and their physiological and pathophysiological functions in the skin. Chapter 2 provides a brief description of TNFα signalling and exploitation of our knowledge of said signalling in therapeutic applications. And lastly, chapter 3 provides a brief overview of several diseases in which both KLK proteases and TNFα signalling play their role in initiating and maintenance of said pathologies. Lastly, chapter 3 also provides a brief comparison of the most important differences between human and murine skin.

1.1 Overview of kallikrein proteases – roles in health, disease and their regulation

Kallikrein proteases are a family of serine proteases, divided into two sub-groups, plasma kallikreins and tissue kallikreins. The first one contains one member, KLK1B, and is of no interest to the topic of this thesis. The second sub-group is composed of 15 members, of which 14 (KLK2-14) bear the name KLKs. They are characterized by trypsin- and chymotrypsin-like activities and this sub-group specifically is located on the largest protease gene cluster both in humans and in mice, with its location being chromosome 19 and chromosome 4, respectively. Tissue kallikreins retain a high degree of homology across species and varying degrees of homology (35 – 80%) among themselves (Prassas et al., 2015) (Emami and Diamandis, 2007).

Their relevance for a variety of physiological processes is readily visible through a plethora of research done on these proteases, the most important and described being their role in the kinin system (Kayashima et al., 2012), semen liquefaction (Anamthathmakula and Winuthayanon, 2020), tooth enamel formation (Bartlett and Simmer, 2014), cancer progression, neural development (Mella et al., 2020) and skin homeostasis (Nauroy and Nyström, 2020). Albeit the amount of data relating to these proteases is ever-increasing and their effects on health and disease are being elucidated, there is still much to be desired as their exact effects on physiology often remain unclear. Among the main reasons is that studies on these proteases have been oftentimes carried in an *in vitro* setup, which may result in significantly different

activities when compared to the *in vivo* milieu. Nevertheless, the recent advent of genome editing technologies, like clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), and, in extension, highly efficient and fast production of transgenic animals, is enabling scientists to research KLKs *in vivo* effects at a hitherto unattainable pace.

Since this work focuses on the skin, the physiological effects of KLKs will be discussed exclusively in this context and I direct any potential readers to a few reviews that have been cited above that discuss KLKs more comprehensively.

1.1.1 Physiological functions of kallikreins in the skin

KLKs are produced firstly as pre-pro-peptides, where the pre- portion serves as an export signal to the extracellular space and the pro- portion makes the KLKs inactive prior to proteolytic cleavage, after which they become active and exert their given function (Prassas et al., 2015). The proteolysis step that activates KLKs is best characterized as a complex proteolytic cascade, in which mainly KLKs themselves are responsible for pro-peptide processing. The key player in this cascade is KLK5, which activates a variety of KLKs (KLK5, KLK6, KLK7 and KLK14) (Yoon et al., 2007) and also the plasmin protease (Michael et al., 2005), which is capable of processing KLK6 and KLK14 into its active form (Yoon et al., 2008) (Figure 1.1).

Furthermore, their activity is being directly modulated by the pH levels of the extracellular milieu, which serves as a mechanism of spatial regulation for these proteases. As an example, KLK5 activity is restricted to the upper granular layer by the formation of a complex with, primarily, D8-D11 LEKTI-derived peptide fragments, and only after its transport in the extracellular matrix (ECM) along the pH gradient is the KLK5 released and may participate in the processes related to the maintenance of skin homeostasis (Deraison et al., 2007).

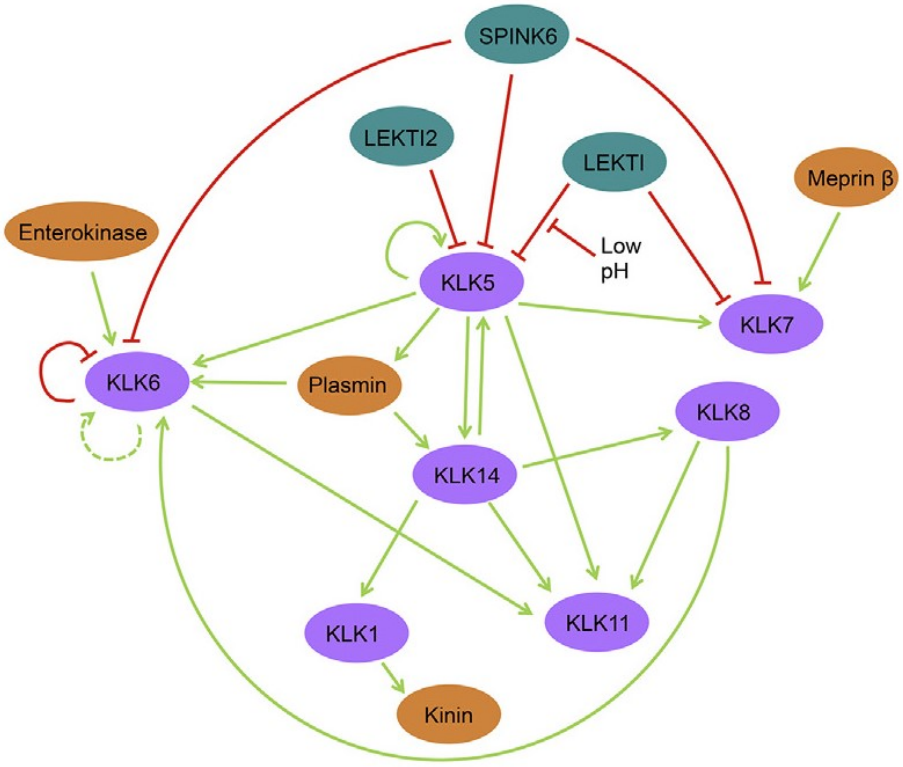


Figure 1.1 KLK proteolytic cascade in the skin. Activities of KLKs are a delicate balance of an array of factors and its focal point is the KLK5 protease. It serves as the main modulator for itself (although to a lesser degree, pro-KLK5 is also processed by mesotrypsin and matriptase; not shown), KLK7 and KLK14 and to a limited degree, for KLK6. KLK6 is primarily activated via enterokinase. Regulation of KLK proteases is mediated by the LEKTI family of serine protease inhibitors. Green arrows are visualizing the activation modality, whereas red lines are showing inhibitory properties of this cascade´s components. Adapted from Nauroy and Nyström, 2020.

The most important roles for KLKs in skin homeostasis are as follows: regulation of skin desquamation, pro-filaggrin (FLG) processing and degradation of mature FLG, keratinocyte proliferation and migration, and their role in the modulation of the innate immune response.

Skin desquamation is a process during which the outermost layer of the epidermis, stratum corneum, periodically sheds its terminally differentiated keratinocytes called the corneocytes. These cells are held together by three proteins, transmembrane proteins desmocollin 1 and desmoglein 1, and by an extracellular protein corneodesmosin which together form a structure called the corneodesmosome. Various KLKs are exerting different amounts of ability to process these proteins and KLK5 again plays the most important part in this process, as it is capable of processing all three of them (Borgoño et al., 2007; Caubet et al., 2004).

The processing of pro-FLG into mature FLG is a key part of the maintenance of skin homeostasis, as FLG is a key contributor to the envelope which surrounds corneocytes and significantly contributes to the faultless workings of the first line of defence, the mechanical barrier of the skin. The final result of FLG activity is its contribution to pH levels maintenance and water retention. FLG can be processed by several KLKs, with KLK5 again being the most important player (Sakabe et al., 2013; Williams et al., 2017).

Keratinocytes are the main cell type of which the skin is composed, and thus are the focal point of skin biology. It comes as no surprise, that plethora of their exerted activities is of paramount importance in a variety of skin biology-related aspects, like wound healing (Pastar et al., 2014) and skin inflammation (Pastore et al., 2006). In the context of wound healing, KLKs regulate this process in as of yet not fully elucidated manner. However, their importance was made obvious in several studies, pointing the research community in the direction that KLKs are at least partially responsible for correct keratinocyte-related wound healing actions. Among those actions are fibrin clot formation (Soley et al., 2016), E-cadherin shedding (Klucky et al., 2007), ECM remodelling via cleavage of a variety of ECM components like collagen I, collagen III and collagen IV, metalloproteinase 9 and others (Wells et al., 2016), and the regulation of surface receptor amounts, which may result in a modulation of a downstream response and impairment of correct ECM structuralization (Loessner et al., 2012).

1.1.1.1 Functions of KLKs in immunity

KLKs are crucial in the processing of several important components in innate immunity. Antimicrobial peptides, namely cathelicidin and its active form, LL-37, are processed by KLKs. KLK5 has been shown as the only KLK protease clearly linked to the LL-37 production (Yamasaki et al., 2006). KLK5, KLK8 and KLK14 have been shown to be able to process LL-37 into smaller fragments, although this has been shown only *in vitro* in the case of KLK8 and KLK14 (Eissa et al., 2011). KLK7 also seems to be able to produce these shorter fragments, although it may serve more as a controlling mechanism of active fragment production, instead of being directly involved in their production on a scale comparable to KLK5 (Yamasaki et al., 2006).

Furthermore, KLK7 is directly participating in innate immune response modulation by pro-interleukin (IL)-1β processing (Nylander-Lundqvist and Egelrud, 1997) and by activation of PAR2, which is mediated mainly by KLK5, but also by KLK4, KLK6, and KLK14 (Heuberger and Schuepbach, 2019).

There is also evidence of proinflammatory effects mediated by KLKs through an as of yet undiscovered means, apart from the effects induced by PAR2 activation (Zhu et al., 2017). It is very likely that this is not the end of the scope by which KLKs are able to exert their modulatory effects, but the available data are insufficient to draw any further conclusions.

1.1.1.2 Regulation of kallikrein activities by protease inhibitors

Without any sort of efficient regulation, unrestrained activities of KLKs would result in serious damage and dysregulation of the homeostatic state. The lympho-epithelial Kazal-type inhibitor (LEKTI) family of serine protease inhibitors fulfils that essential role. There are 3 members of this family which are relevant for KLK regulation in the skin, LEKTI (Deraison et al., 2007), LEKTI-2 (Meyer-Hoffert et al., 2009) and SPINK6 (Fischer et al., 2014), encoded by *SPINK5*, *SPINK9* and *SPINK9* genes respectively. Of these 3, LEKTI is by far the most important according to all available data. LEKTI protein is first synthesized as a protein composed of 15 domains (D1-D15) with serine protease inhibitory properties, that are afterwards cleaved into active peptide fragments with a variety of inhibitory targets.

1.1.2 Pathophysiological functions of kallikreins in the skin

Dysregulation of KLK proteases is clearly linked to several skin disorders and may be possibly linked to even more skin-related pathologies. The ones that have been clearly identified are NS (Petrova and Hovnanian, 2020), Atopic dermatitis (AD) (Morizane, 2019), psoriasis (Komatsu et al., 2007) (Eissa et al., 2013) and acne rosacea (Two and Del Rosso, 2014). In these diseases, the lack of control over their activities results in a state that the very mechanisms by which KLKs normally maintain the skin homeostasis are turned into activities with severe deleterious effects.

Among the most notable of these effects in the context of related pathologies are impaired water retention leading to severe transepidermal water loss, impaired wound healing, excessive skin desquamation, inflammation and periodic infections. Those

conditions and the afflictions in which they play a crucial role will be discussed more comprehensively later in the thesis

1.2 TNFα signalling

TNFα was first fully described in 1975 (Carswell et al., 1975), although indirect hints of its importance were already present (Shear and Perrault, 1944). Nevertheless, prior descriptions of its effects were limited in the scope of the understanding of the actual process. After its initial characterization, it seemed like an ideal candidate for cancer treatment for the world of cancer research, but this enthusiasm proved to be short-lived, as its effects were not fully elucidated and led to widespread inflammation and death of patients treated with TNFα (Tracey et al., 1988). Since that time, the mechanisms and subsequent outcomes of TNFα signalling have been in many ways solved and described. However, there is still much to be desired in terms of the scope of knowledge regarding TNFα mainly due to the immense complexities of its downstream effects, which oftentimes lead to surprising outcomes even after nearly 50 years have passed since its initial characterization.

TNFα belongs to the TNF superfamily (TNFSF) of proteins, which contains 19 members in total. TNFSF proteins have two functionally important forms, both being active as homotrimers. They are first synthesized as type II transmembrane proteins, with the exception of LTα3, and are then converted to their soluble form by a variety of proteases, with metalloproteinase ADAM17, also known as TNFα-converting enzyme (TACE), being the most important in the shedding of membrane TNFα (mTNF) and its conversion to soluble TNFα (sTNF) (Gooz, 2010). Both of these forms are exerting a variety of different outcomes, which may sometimes be overlapping in their downstream effects. The key structure among the TNFSF proteins is their TNF homology domain (THD), which facilitates the trimerization process and also the binding of TNFSF proteins to their cognate receptors (Bodmer et al., 2002).

TNF receptor superfamily (TNFRSF) is the group of proteins that are responsible for the downstream effects of TNFSF proteins. TNFRSF is composed of 29 members, which are predominantly type I transmembrane proteins, but a few are type III transmembrane proteins (f.e. B cell maturation antigen). TNFRSF receptors form either parallel or antiparallel dimers, with their parallel form being the one able to bind its cognate ligand. The key characteristic of their structure is a cysteine-rich

domain, which enables them to bind to their ligands via the THD (Vanamee and Faustman, 2018). TNFRSF receptors are divided into two groups: death receptors and non-death receptors. As the name would suggest, death receptors are usually responsible for the majority of pro-apoptotic effects, which are mediated by a conserved sequence called the death domain (DD) located in their cytoplasmic part. Non-death receptors lack the DD and are often exhibiting pro-survival signals (Dostert et al., 2019). Nevertheless, this presumption is not always the case (Lawlor et al., 2017).

There are two TNFRSF receptors that are responsible for mediating the effects of TNFα, TNFR1 and TNFR2. Whereas TNFR1 belongs to the DD subgroup of TNFRSF receptors, TNFR2 is a non-death receptor. TNFR1 is expressed ubiquitously on all cells and as a death receptor, its effects are mostly pro-apoptotic and pro-inflammatory. TNFR2 expression is much more restricted, mostly to immune cells, but also a few other cell types, like endothelial and neuronal cells and it has mostly regulative and pro-survival functions (Dostert et al., 2019). However, their downstream effects are not straightforward and it is not so uncommon to see counterintuitive effects, further hampering easy prediction of possible outcomes (Siegmund et al., 2016, 2018).

It is important to note, that the processes described below are the bare minimum in the complex labyrinth of TNFα signalling. To try to fully describe the immense variabilities would be a futile attempt far beyond the scope of this thesis and very possibly beyond the scope of our current understanding of TNFα signalling in general.

1.2.1 TNFR1 signalling

TNFR1 is able to be activated by both mTNF and sTNF, but primarily by sTNF, and its activation leads to the recruitment of TNFR1-associated death domain protein (TRADD) and several subsequent possible outcomes. Those outcomes are regulated by the ubiquitination and phosphorylation status of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and result in the formation of 4 possible complexes, dubbed complex I, complex IIa, complex IIb and complex IIc (Brenner et al., 2015).

1.2.1.1 Complex I

Complex I formation begins with the binding of TNFR1 DD to the adaptor protein TRADD, upon TNFR1 activation (Hsu et al., 1995). It is followed by the recruitment of RIPK1 and subsequently TNF receptor-associated factor (TRAF) 2 and TRAF5 (Shi and Sun, 2018). Afterwards, this protein complex then recruits the E3 ubiquitin ligase cellular inhibitor of apoptosis (cIAP) 1 and cIAP2 (Mahoney et al., 2008), which marks the completion of complex I formation. Ubiquitination status of RIPK1 is then modified by cIAP1 and cIAP2 via the addition of K63 polyubiquitin chains, which enables the recruitment of linear ubiquitin chain complex (LUBAC) that additionally modifies RIPK1 by the addition of M1 polyubiquitin chains and finalizes the ubiquitin machinery of complex I (Haas et al., 2009). Upon finalization of complex I ubiquitin scaffold, assembly of two protein complexes is concurrently initiated. First of these two complexes is the inhibitor of kappa B (IκB) kinase (IKK) complex, composed of 3 subunits (Israël, 2010) and the second one is TGFβ-activated kinase 1 (TAK1) - TGF-β-activated kinase 1 and MAP3K7-binding proteins (TABs) complex, also composed of 3 subunits. These complexes interact with each other in a way that facilitates the final output of the complex I signalling. Specifically, TAK1 phosphorylates the IKKβ which then becomes activated, leading to the activation of the canonical nuclear factor kappa B (NFκB) pathway. Additionally, TAK1 mediates the activation of JUN N-terminal kinase (JNK) and p38 pathways (Xu and Lei, 2021). Thus, the final output of complex I activation is as follows: JNK signalling, p38 signalling and canonical NFκB signalling (Figure 1.2).

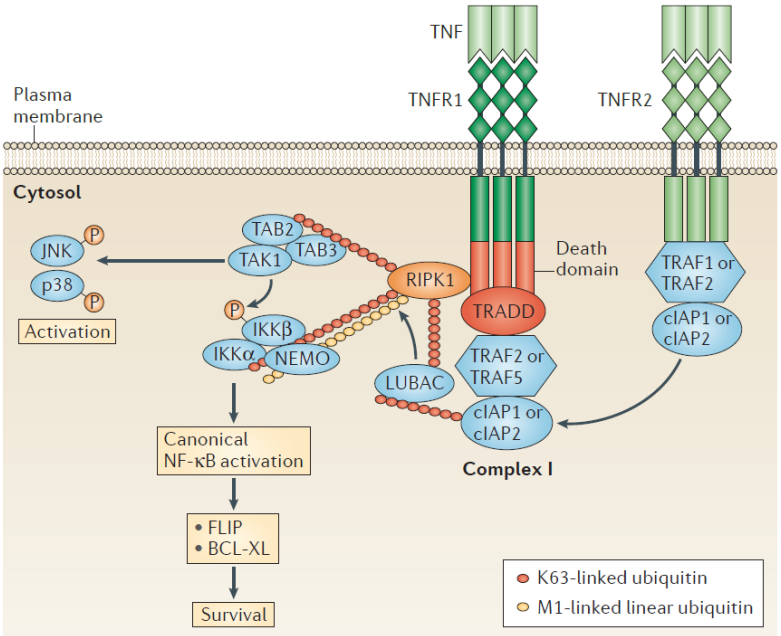


Figure 1.2 Complex I signalling by TNFR1 and TNFR 2 receptors. After TNFα activates TNFR1, complex I formation is initiated. TRADD binds to DD of TNFR1 and serves as a scaffold for other components and recruits RIPK1, TRAF2/5 and cIAP1/2. Polyubiquitination of RIPK1 by cIAP1/2 and LUBAC leads to recruitment of IKK and TAK1-TAB complexes, which are responsible for activation of JNK, p38 and canonical NFκB signalling. TNFR2 is able to assemble

complex 1 without TRADD and with the use of TRAF1/2, instead of TRAF2/5. Activation of complex I-associated pathways via TNFR2 is significantly less efficient than through the TNFR1 receptor. Adapted from Brenner et al., 2015.

1.2.1.2 Complex IIa

Complex IIa assembly occurs after the RIPK1 is deubiquitinated by deubiquitinating enzymes, the most prominent in this process being cylindromatosis (CYLD) (Sun, 2010). After CYLD deubiquitinates RIPK1, it moves to the cytosol and associates with a complex composed of TRADD, Fas-associated death domain (FADD), the long isoform of FLICE-like inhibitory protein (FLIP) and pro-caspase-8 (Micheau and Tschopp, 2003). RIPK1 and RIPK3, which role is central for the apoptosis/survival versus necroptosis direction (Li et al., 2012), are then deactivated by the activity of this complex, specifically by activated caspase-8 homodimer, or by pro-caspase-8 and $FLIP_L$ heterodimer (Oberst et al., 2011). RIPK1 serves a role in the assembly of this complex and plays no further role in the downstream signalling. For the apoptotic events to occur, pro-caspase-8 homodimer has to form and must be activated into its caspase-8 homodimer form via pro-caspase 8 autocleavage, after which it triggers signalling for apoptosis induction (Figure 1.3) (Wang et al., 2008).

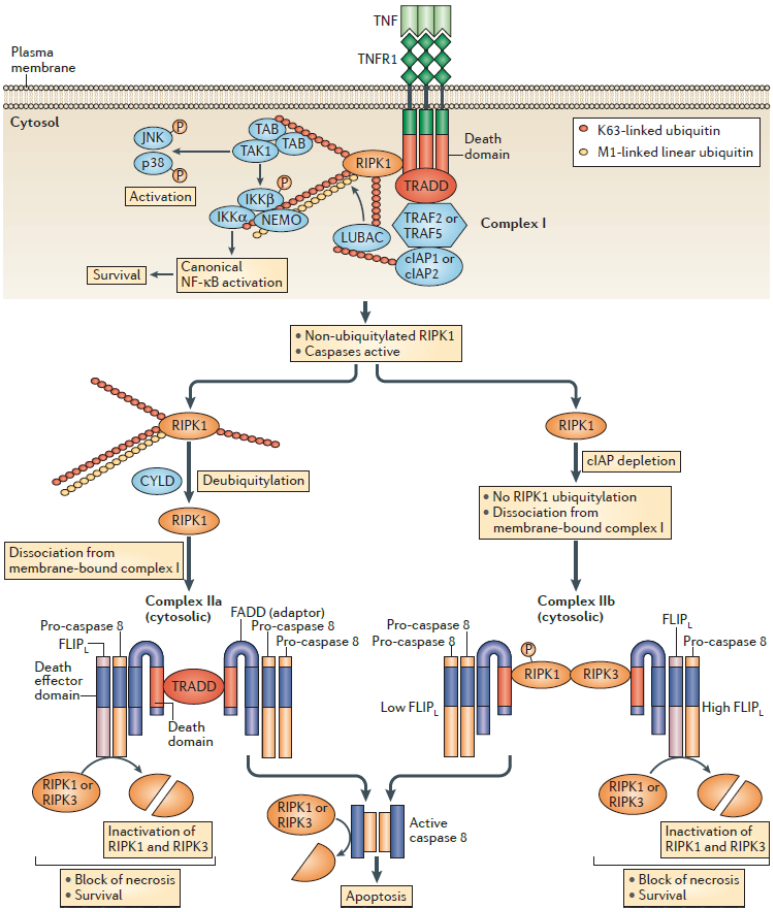


Figure 1.3 TNFR1-mediated complex IIa and complex IIb signalling. If RIPK1 is deubiquitinated by CYLD or other deubiquitinating enzymes, it dissociated from the membrane-bound complex and moves to the cytosol and interacts with TRADD, FADD, $FLIP_L$/pro-caspase 8 heterodimer and pro-caspase 8 homodimer. This complex is dubbed complex IIa and depending on the abundance of $FLIP_L$ may lead either to pro-survival or apoptotic signalling. Complex IIb is formed if the cIAP1/2 of complex I are depleted and RIPK1 dissociates without any ubiquitin chains. Afterwards, phosphorylated RIPK1, RIPK3, FADD pro-caspase 8 homodimer and $FLIP_X$/pro-caspase 8 heterodimer form the complex IIb. Subsequent effects elicited by complex IIb are then comparable with those of complex IIa. Cytosolic RIPK1 and RIPK3 inactivation by complex IIa and complex IIb is a key function for inhibition of the necroptotic pathway. Adapted from Brenner et al., 2015.

1.2.1.3 Complex IIb

As is the case with the complex IIa, RIPK1 is also necessary for the formation of complex IIb. In this case, however, RIPK1 is not or is just barely ubiquitinated and dissociates from pre-complex I assembly. This is because the main mediators of RIPK1 ubiquitination, cIAP1 and cIAP2, are depleted due to K48 autoubiquitination, which leads to their degradation (Wang et al., 2008). This occurs with the help of the second mitochondria-derived activator caspase (SMAC) that seems to bind to the IAP repeat of cIAP1 and cIAP2, which triggers the autoubiquitination and subsequent dissociation of RIPK1 (Du et al., 2000). Phosphorylated RIPK1 assembles with RIPK3, this heterodimer serves a similar role as the TRADD protein in the case of

complex I and from this point forward, signalling of these two complexes merge and similar pathway to that outlined above (Figure 1.3) (Brenner et al., 2015).

1.2.1.3 Complex IIc

Lastly, if RIPK1 and RIPK3 are not degraded via either pro-caspase 8/FLIP$_L$ heterodimer or caspase 8 homodimer, which abrogates the kinase activity of RIPK1 and RIPK3, formation of complex IIc, also known as the necrosome, occurs. In this instance, phosphorylated RIPK1 and many phosphorylated RIPK3 molecules form the necrosome structure. RIPK3 then phosphorylates mixed-lineage kinase domain-like protein (MLKL) that subsequently translocates from the cytosol to the cellular membrane and disrupts its integrity via binding to phosphatidylinositides. This leads to rupture of the cell membrane and release of its contents, finishing the inflammatory process called the necroptosis (Figure 1.4) (Newton and Manning, 2016).

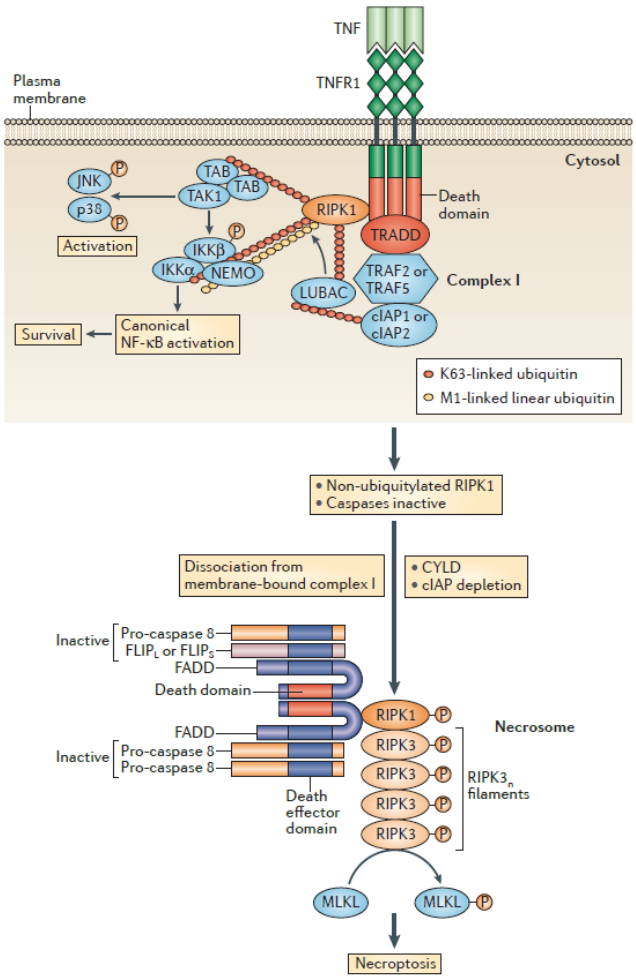


Figure 1.4 Necroptotic pathway of TNFR1 signalling. In the case that RIPK1 and RIPK3 are not inactivated by proteolytic activities of complexes mentioned above, the structure called the necrosome forms. In this case, complex that bears some similarity to complex IIb forms, although in this case, RIPK1 is bound by numerous RIPK3 molecules and kinase activities of these kinases result in MLKL phosphorylation. Phosphorylated MLKL then initiates downstream effects which result in a cell death process called necroptosis, in which the cell membrane ruptures that results in the release of proinflammatory signals. Adapted from Brenner et al., 2015.

1.2.2 TNFR2 signalling

The scope of knowledge available in our understanding of TNFR2 receptor signalling is severely behind that of TNFR1. TNFR2 is incapable of being activated via sTNF and is activated exclusively via mTNF. It is known that TNFR2 downstream effects overlap, to a certain degree, with those of TNFR1, as TNFR2 is also capable of forming complex I, although without TRADD and with the utilization of TRAF1/TRAF2, in contrast to TNFR1 usage of TRAF2/TRAF5 (Figure 1.2) (Dostert et al., 2019). It is worth noting that TRAF affinity for TNFR2 is much lower than that for TRADD (Park et al., 2000).

TNFR2 is capable of activating the non-canonical NFκB pathway. The protein complex that serves a central role in this process is composed of TRAF2, TRAF3, cIAP1, cIAP2 and NFκB-inducing kinase (NIK). Without mTNF signal, cIAP proteins degrade NIK and no signalling occurs. However, after mTNF binds and activates TNFR2, cIAP proteins degrade TRAF3 instead of NIK, leading to the release of NIK. NIK is then capable of phosphorylating IKKα. IKKα phosphorylates the p100 protein in the RelB/p100 heterodimer which leads to p100 processing into p52. RelB/p52 transcription factor then enters the nucleus and activates a plethora of genes, finalizing the non-canonical NFκB pathway activation (Figure 1.5) (Sun, 2017).

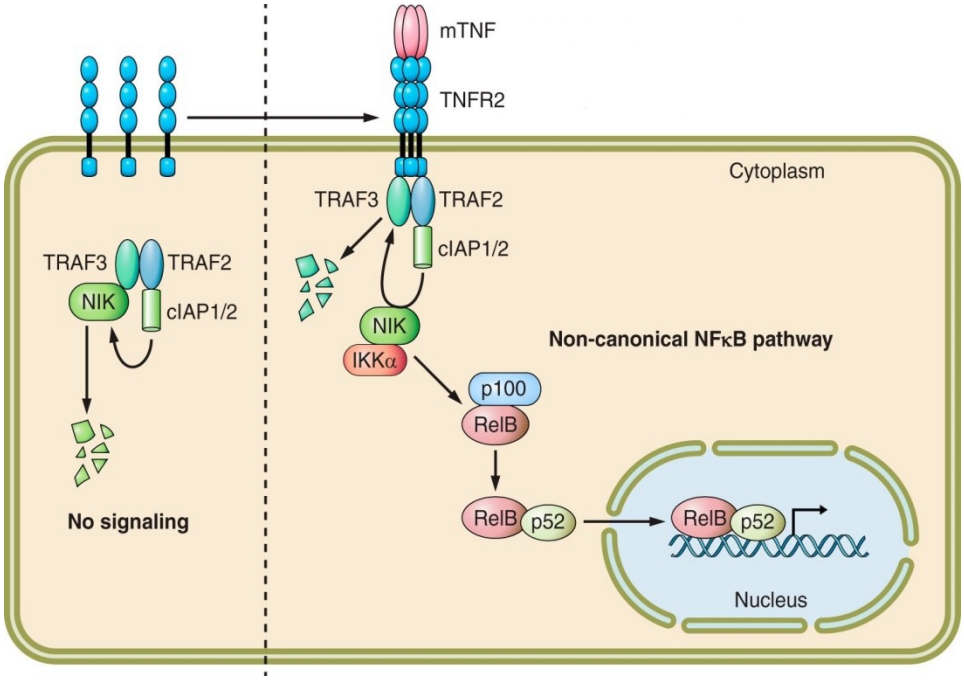


Figure 1.5 TNFR2-mediated non-canonical NFκB pathway activation. Without TNFR2 activation, the cytosolic complex composed of TRAF2/3, NIK and cIAP1/2 is inactive due to cIAP1/2-mediated NIK degradation. Upon TNFR2 engagement, cIAP1/2 mediated the degradation of TRAF3 instead of NIK, leading to the release of NIK. NIK then phosphorylates IKKα which subsequently phosphorylates p100 protein of the p100/RelB heterodimer, leading to p100 proteolytic processing. Subsequently, as the result of said processing, the p52/RelB heterodimer is capable of entering the nucleus and activates transcription of its associated genes. Adapted from Dostert et al., 2019.

1.2.3 Regulation of TNFα signalling

Since the exact mechanisms and subsequent outputs of TNFα signalling are far from being completely elucidated, due to the immense complexities surrounding them, regulation of these mechanisms is to blame to a large degree. In this subchapter, I will try to describe some of the most important mechanisms. For our purposes, TNFα signalling regulation may be divided into three categories: regulation of the state/presence of individual components on a molecular level, regulation of chromatin accessibility and regulation via soluble TNFR (sTNFR). These categories are mutually connected, so it is important to think about them in this manner and not as individual regulatory layers.

1.2.3.1 Component regulation

Regulation of individual components of TNFα signalling is by far the most described of the three categories that are described in this subchapter. As an example, the regulation of two processes will be vaguely described, those being ubiquitination of RIPK1 and the role of FLIP isoforms on complex IIb/complex IIc formation.

In the case of RIPK1, it has been found that its activity is not modulated solely by the addition of K63 and M1 polyubiquitin chains, but that also K48 and K11 polyubiquitin chains are actively added, which result in different outcomes. K48 modification results in RIPK1 degradation via the proteasome, thus hampering RIPK1-mediated signalling. K11 polyubiquitin chains, on the other hand, may result either in positive or negative regulation of RIPK1-mediated signalling (Annibaldi et al., 2018). Difficulties related to the study of ubiquitination processes are hampering the elucidation of the exact relative contributions of various proteins mediating those modifications.

$FLIP_L$ is an important component of complex IIa and complex IIb functions, specifically by directing downstream effects towards pro-survival signalling. Its expression is modulated by both canonical and non-canonical NFκB pathways. $FLIP_L$

is one of three protein isoforms of FLIP, the others being $FLIP_S$ and $FLIP_R$. A delicate balance of expression levels of these components is one of the major regulatory parts in complex IIa formation and even more so in the case of complex IIb since in the case of complex IIa, pro-caspase 8 is the component responsible for the binding of pro-caspase 8/$FLIP_L$ heterodimer to FADD, which serves as a scaffold for their downstream functions. However, in the case of complex IIb, FLIP isoforms mediate the binding of $FLIP_X$/pro-caspase 8 heterodimers to FADD, further complicating any precise predictions related to this pathway as those isoforms have different activities (Brenner et al., 2015).

Overall, it may be summarized that this type of regulation is a delicate balance of expression levels, phosphorylation states, ubiquitination states among other parameters and combination thereof which are intermingled with each other to one very tight and extremely complex knot.

1.2.3.2 Chromatin accessibility

Chromatin accessibility is one of the main reasons for the pleiotropic effects of NFκB signalling. Since NFκB transcription factors are not capable of opening the chromatin to enable their access themselves, they are dependent on a variety of factors to regulate the chromatin landscape to be able to bind to their target sequences and transcribe NFκB inducible genes. NFκB transcription factors are able to recruit chromatin remodelling factors, but this is dependent on, for example, posttranslational modifications, which are in turn modulated by factors like cell type, among others (Bhatt and Ghosh, 2014). Analysis on a single cell level may facilitate the bare possibility of tackling this problem comprehensively.

1.2.3.3 sTNFR-mediated inhibition

Shedding of membrane-bound TNFRs via associated proteases, mainly TACE, is another important aspect in the regulation of this labyrinthine-like signalling pathway. These soluble receptors are thought to balance the TNFα-mediated inflammation levels by binding sTNF and thus hampering their binding to mTNFRs (Van Zee et al., 1992). However, it is important to note the existence of a process called “reverse signalling”, in which the mTNF itself serves as a signal transductor upon sTNFR binding (Juhász et al., 2013).

1.2.4 TNFα-signalling inhibitors in therapeutic applications

TNFI are an attractive and commercially successful approach in the treatment of various inflammatory pathologies, like psoriasis, rheumatoid arthritis, Crohn´s disease, hidradenitis suppurativa and others. Their efficiency for treating various pathologies is without question and a wide array of TNFIs with a variety of mechanisms is currently commercially available or in clinical trials. Different approaches to TNFα signalling regulation have been undertaken with modulatory actions being targeted at TNFα, TNFR1 and TNFR2 in order to achieve specific interrogation and modulation of TNFα signalling effects for each condition that is to be treated with TNFIs. Among notable and the most used TNFIs are the antibodies, or their parts/modifications, targeting the TNFα signalling in general, Infliximab, Etanercept, Certolizumab pegol and Adalimumab (Kontermann et al., 2009) (Fischer et al., 2020).

Nevertheless, their undisputable utility notwithstanding, due to the beforehand mentioned complexities and lack of clarity in TNFα signalling, their application may sometimes lead to either creating new pathological states, or amplification of the current pathology in treated patients. Most notably, in relation to skin disorders, treatment with TNFIs may lead to TNFI-induced psoriasis, and to manifestations of opportunistic infections due to disruption of adequate immune reaction mediated by TNFα (Li et al., 2019) (Garcovich et al., 2019). Great caution and due diligence in the analysis of the state of each patient is thus advised prior to treatment with TNFIs.

1.3 KLK-related diseases of the skin

Skin is the largest organ of the human body, serving a key role in the protection from the external environment as the first line of defence. It serves its role via different mechanisms, with the most important being mechanical barrier of the corneocytes, associated acidic pH levels and upon breach of these two levels of defence, by its residential immune cells and other cell types that are ready to elicit a rapid response to most of the external challenges by possibly harmful substances and organisms (Kabashima et al., 2019).

Due to its immense importance in providing said protection, any dysregulation of related processes may result in effects with varying levels of severity, depending on their nature. Description of these dysregulations and associated pathologies is far

beyond the scope of this thesis and I will focus on describing the basic features of skin-related pathologies and their treatment to which the activity of KLKs has been linked. Those diseases are the NS (Petrova and Hovnanian, 2020), AD (Morizane, 2019), psoriasis (Komatsu et al., 2007) and acne rosacea (Buddenkotte and Steinhoff, 2018).

It is important to say that description of said diseases in this chapter will revolve around the basic characteristics of said pathologies, thus omitting details that may be relevant to get a broader picture of them. However, a detailed description of these afflictions is far beyond the scope of this thesis and because of that will not be undertaken.

1.3.1 Netherton syndrome

NS is a skin pathology belonging to a group of skin diseases called ichthyoses, which are characterized by the presence of scaly, dry skin (Oji et al., 2010). It is quite rare compared to other diseases mentioned in this chapter, with its prevalence estimated to be 1 out of 200000. This estimate may not be accurate due to its relatively high neonatal mortality rate. Typical signs of NS are frequent infections and severe inflammation, various types of skin lesions, itch, abnormalities in the growth of hair known as the "bamboo hair", food allergies, impairment of normal growth, disbalance of the skin microbiota and many more, which either result in or are the cause of these symptoms. However, there is a large degree of variability in the severity of those symptoms, resulting in various levels of danger and reduction in the quality of life for those that are afflicted. The most severe manifestations of its deleterious effects are typically seen in younger patients and during their upbringing, the gravity of related symptoms is usually retracting. As with most pathologies of the skin, symptoms of NS show a certain periodicity and variability in the amount of lesional presence (Petrova and Hovnanian, 2020).

1.3.1.2 Pathophysiology of Netherton syndrome

Deficiency in LEKTI as a result of a variety of mutations in the *SPINK5* has been proved to be the main culprit behind the pathology of NS. This autosomal recessive deficiency leads to severely reduced control over the activities of several KLKs connected to NS pathology, namely KLK5, KLK7, KLK14 (Furio and Hovnanian, 2014; Gouin et al., 2020) and possibly also KLK6 (Zingkou et al., 2019). The main

proteases responsible for the manifestations of this pathology are KLK5 and KLK7, as their ablation proved to be enough to reverse the most severe symptoms of NS in mice (Kasparek et al., 2017). The severity of NS is directly correlated to the location of mutations in the *SPINK5* gene, which may result in a broad scale of severity of this disease (Sprecher et al., 2001).

Dysregulation of NS-associated KLKs leads to deleterious systemic effects that are most pronounced in the skin (Figure 1.6). From a structural standpoint, KLK hyperactivity leads to breach of the epidermal barrier due to premature shedding of the corneocytes that leads to uncontrolled desquamation, which is the result of corneodesmosome destruction (Borgoño et al., 2007; Caubet et al., 2004; Descargues et al., 2006). This enables a variety of pathogens to enter the epidermal milieu and participate in the initiation of severe inflammation and epidermal barrier breach mentioned above, with the leading responsible pathogen being *Staphylococcus aureus* and *Staphylococcus epidermidis* (Williams et al., 2020). Breach of the barrier also results in excessive transepidermal water loss, which is an important contributing factor in the impairment of growth and failure to thrive associated with NS (Erickson et al., 2020).

KLKs are also major players in the immune signalling dysregulation associated with inflammation present in NS patients. KLK5 and to a lesser degree KLK14 are important initiators of PAR2-mediated inflammation in the skin (Stefansson et al., 2008). The role of KLK6-mediated PAR2 activation in the skin is not yet clear. PAR2 activation is connected to the activation of several immune pathways and secretion of a variety of inflammatory cytokines, like thymic stromal lymphopoietin (TSLP), intracellular adhesion molecule 1 (ICAM-1), IL-8 and TNFα, which then participate in the creation and sustainment of inflammation via activation and recruitment of different immune cell types (Rothmeier and Ruf, 2012). KLK7 on the other hand works in PAR2-independent mechanisms that are as of yet not fully elucidated, but its ability to process pro-IL-1β may be of significance for inflammatory progression in NS (Nylander-Lundqvist and Egelrud, 1997). Furthermore, KLKs are promoting infectious events by disrupting the normal processing of cathelicidin, which is an important player in the defence against microbial pathogens (Eissa et al., 2011; Yamasaki et al., 2006).

Neutrophils, eosinophils and mast cells are the most important cell subtypes of innate immunity that are found in NS lesional skin in abnormal amounts. As for the presence and relevance of cell types of adaptive immunity, activated immunoglobulin (Ig) E-producing B cells and T_H17 cells are the most significant T helper cell subset in NS with T_H2 cell subset also playing a major role (Eränkö et al., 2018) (Petrova and Hovnanian, 2020). From the presence of eosinophils and mast cells, one can deduce that IgE is the most important immunoglobulin subset in this pathology and its dysregulation is one of the few stable commonalities present in NS, with other Ig classes apparently not being of major relevance (Petrova and Hovnanian, 2020). It is also worth noting, that T_H2 cytokines are responsible for partial inhibition of expression of genes coding some epidermal structural proteins, further amplifying the barrier defects (Hönzke et al., 2016). The exact knowledge of functions and contributions of various immune subsets is still under debate.

1.3.1.2 Therapeutic options for Netherton syndrome

Therapy of the NS is at this point limited to the effects and not directly targeting the cause of this disease, although this may change in the future with the advent of novel therapeutic approaches. Currently used approaches encompass, but are not limited to, cleansing of the skin with acidic oils, moisturization of the skin (Lodén, 2003) and treatment with antibiotics (Eränkö et al., 2018), corticosteroids (Sarkar, 2018) and calcineurin inhibitors (Oji et al., 2005).

Future therapeutic endeavours focus on a much more targeted approach in the treatment of the NS. Those treatment options may be divided into several categories, namely KLK inhibitors, recombinant serine protease inhibitors and inhibitors of cytokines that are the major players in this pathology (Petrova and Hovnanian, 2020). Lastly, a gene therapy treatment option for NS may prove to be the most effective and permanent causative treatment (Di et al., 2011), since it would remove the prime problem behind this disease and enable normal LEKTI-mediated inhibition.

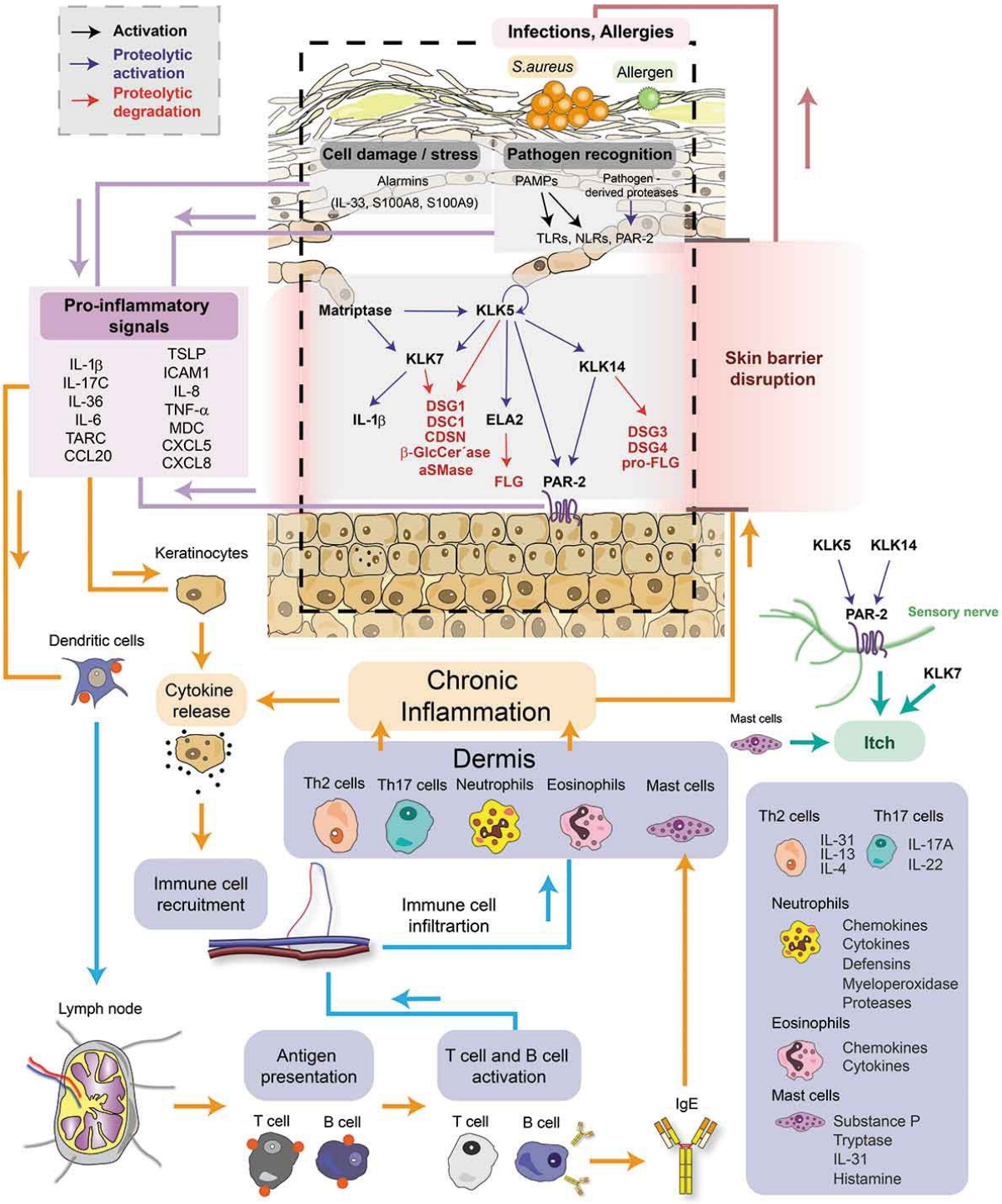


Figure 1.6 Overview of NS mechanisms. Dysregulation of epidermal proteases due to LEKTI deficiency leads to epidermal barrier defects due to proteolysis of structural proteins of the epidermis, inflammation associated with the processing of cathelicidin, PAR2 activation and pro-IL-1β processing and pruritus. This leads to the secretion of pro-inflammatory cytokines and chemokines, primarily by the keratinocytes and innate immune cells, like IL-1β, TSLP, ICAM1, TNFα and others. Downstream outcomes of these effects include but are not limited to, recruitment and activation of T_H17 and T_H2 cells, recruitment of innate immune cells like eosinophils, mast cells and neutrophils, secretion of IgE which increases the severity of pruritus, and further damage to the epidermal barrier. This leads to a feedback loop that results in an increment in the severity of inflammation and epidermal barrier defects and leads to the chronic nature of the progression of this disease. Adapted from Petrova and Hovnanian, 2020.

1.3.2 Atopic dermatitis

AD is a chronic inflammatory disease of the skin with episodes of AD symptoms manifestation and subsequent remission. Intense itch and eczematous lesions are associated with the active phase of AD. It has the largest prevalence among cutaneous inflammatory pathologies with children being the most commonly afflicted, although AD can manifest at any point during life. Depending on the geographical location, prevalence among children may be as high as 25% and among adults, it may be as high as 10%. Its most common symptoms are eczematous lesions and itch that lead to a subsequent decrease in the quality of life for those stricken with the disease by a variety of subsequent effects. It is worth noting, that the severity is highly variable and associated symptoms presence may range anywhere from several places on the skin to its entirety (Weidinger et al., 2018).

1.3.2.1 Pathophysiology of Atopic dermatitis

Unlike the NS, the main causative reasons behind AD remain to be elucidated. Family history of AD is by far the best prognostic tool in AD (Apfelbacher et al., 2011; Wadonda-Kabondo et al., 2004), although several genetic factors have been linked to its manifestation and progression (Paternoster et al., 2015). Among them, the most important are mutations in FLG, an important structural protein of the skin, and its aberrant function very likely leads to higher skin permeability and subsequent inflammation and T cell infiltration (Irvine et al., 2011). However, defective FLG has been shown to not be enough to cause AD on its own and is not necessary at all in the manifestation of AD (Baurecht et al., 2007; Weidinger et al., 2008). To summarize, there is still much to be discovered about the intricacies of AD, but it is thought that AD manifests as a result of a combination of genetic and environmental factors.

Structurally, probably the most important parameter in the severity of epidermal barrier disruption associated with AD is the aforementioned dysfunctional FLG protein. Apart from FLG, T_H2-associated cytokines like IL-4 and IL-13 actively reduce the expression of epidermal structural proteins and contribute to its disruption (Figure 1.7) (Cole et al., 2014; Seltmann et al., 2015). Moreover, the associated itch and subsequent scratching of the skin is responsible for further damage (Correale et al., 1999). As is with the NS, the microbiota of patients stricken with AD is also dysregulated, with the presence of *Staphylococcus aureus* again being the most

significantly increased (Kong et al., 2012). KLK-mediated effects are probably also an important aspect in the severity of AD, although there is nowhere near the degree of clarity as is the case in NS. Several KLKs are dysregulated in AD lesions and are thus thought to participate in the epidermal dysregulations, namely KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13 and KLK14, with KLK7 seemingly playing the most important role (Morizane, 2019). As previously mentioned, KLK7 is likely capable of degrading cathelicidin and its active form, LL-37, and it was reported that the abundance of LL-37 is severely reduced in AD lesions (Ong et al., 2002). LEKTI is also overexpressed in AD lesions, making the interpretation of KLKs overexpression and the effects they may have on the disease progression difficult (Igawa et al., 2017). Lack of data clearly elucidating the degree to which KLKs participate in AD pathogenesis warrants further research of the KLKs/AD relationship.

From the immunological point of view, AD is predominantly associated with T_H2 and T_H22 cluster of differentiation (CD)4+ T cell activity (Gittler et al., 2012). T_H1 and T_H17 contributions are less clear, but genes associated with these subtypes, especially T_H17-associated genes, are also elevated (Gittler et al., 2012; Noda et al., 2015). In the lesional skin, increased presence of various CD4+ T cell subtypes and Langerhans cells (Yoshida et al., 2014) is the mainstay across all AD patients with innate lymphoid cell (ILC) 2 and γδ T cells also present in elevated numbers, although their exact role, apart from productions of cytokines related to AD, is less clear (Chen et al., 2020). Genes that modify the activity of keratinocytes and modulate the infiltration of T cells are upregulated in lesional skin (Gittler et al., 2012). IgE is the main immunoglobulin associated with AD, which sensitizes the loci of its presence to antigens derived from keratinocytes and exogenous antigens of different origins (Gandhi et al., 2016; Tang et al., 2012).

1.3.2.2 Therapeutic options for Atopic dermatitis

Therapeutic options for AD are severely limited by our lack of understanding of mechanisms governing this pathology. Thus, it is limited to the treatment of its manifestations, as causative treatment is not possible at this point. Because of that, treatment options of AD bear many similarities with those used in the case of NS. Among usual strategies of treating AD are skin moisturization (Simpson et al., 2014),

application of topical corticosteroids (Brunner et al., 2016), topical calcineurin inhibitors (Carr, 2013), cAMP-specific 3′, 5′-cyclic phosphodiesterase 4 inhibitors (Zebda and Paller, 2018), phototherapy (Garritsen et al., 2014) and administration of systemic immunosuppressants and immunomodulating agents (Weidinger et al., 2018).

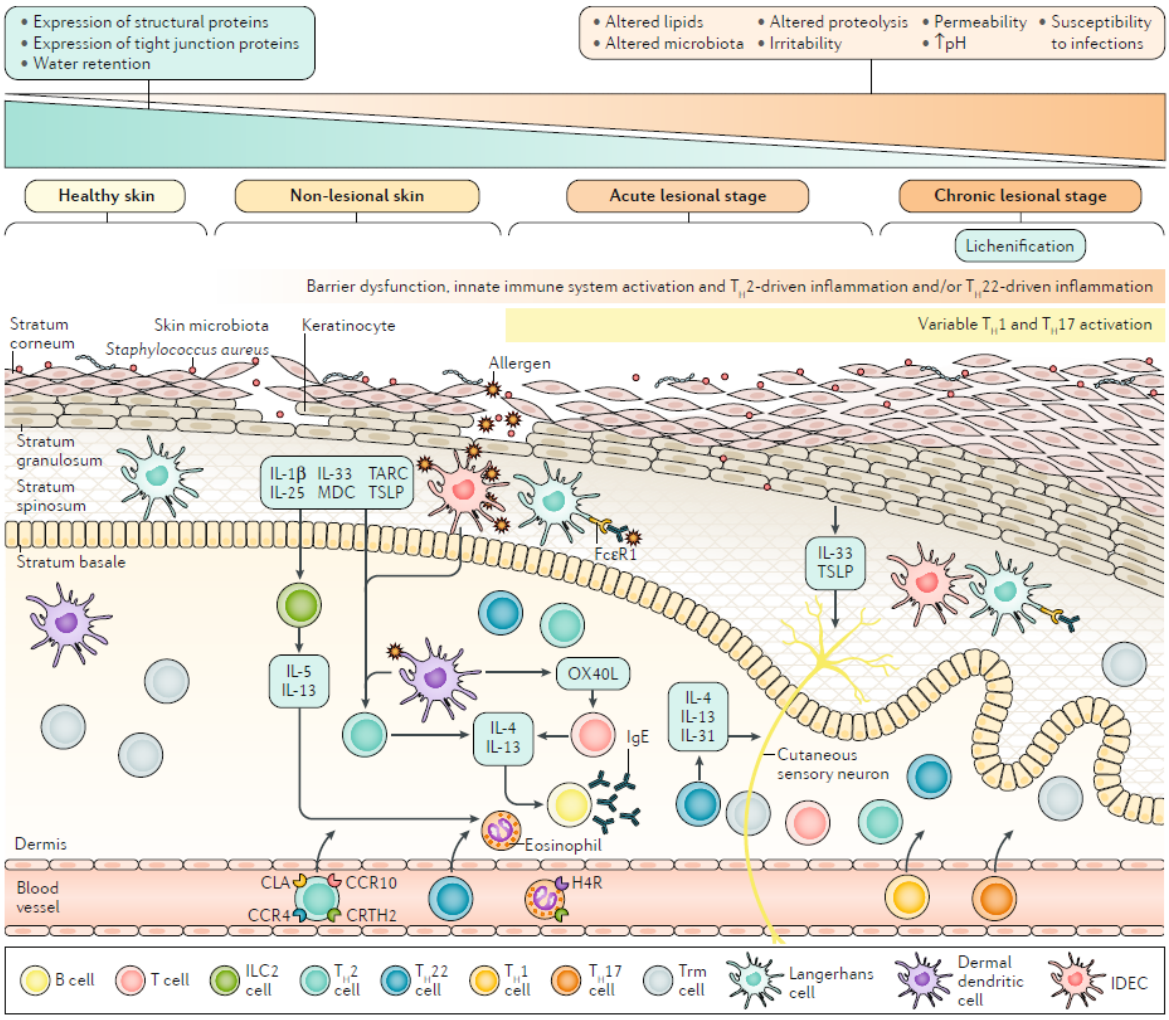


Figure 1.7 Overview of AD mechanism. The damage to the epidermal barrier by external cues leads to the expression of cytokines and chemokines by keratinocytes and innate immune cells, like IL-1β, IL-33, TSLP and others. This leads to T_H2 and ILC2 expression of cytokines like IL-4, IL-5, IL-13 among other mediators of immune response, that subsequently promote inflammation by recruitment of additional immune cell types and secretion of proinflammatory mediators and production of IgE. Activities of T_H22, eosinophils, and activated B-cell- secreted IgE leads to severe pruritus which contributes to epidermal barrier disruption. Immune dysregulations associated with AD lead to different manifestations in the healthy and lesional skin, as is shown in the upper part of the figure. Adapted from Weidinger et al., 2018.

1.3.3 Psoriasis

Psoriasis is an inflammatory disease mediated by dysregulation of the immune system. Since its cutaneous symptoms may be vastly different in its pathophysiology and severity, with psoriasis being separated into several different types, it is hard to generally describe its manifestations and readers are delegated to a review that discusses variants of psoriasis and associated defects more thoroughly (Sarac et al., 2016). The main feature is dysregulation of a variety of primarily immune system components and associated pathways that result in excessive activation of both innate and adaptive immune cell subsets and the release of inflammatory molecules that lead to this disease. Psoriasis is very often associated with various comorbidities, the most common being psoriatic arthritis. As with previously mentioned diseases, the prevalence of all psoriatic variants varies across the globe, with the overall occurrence of all types of psoriasis being estimated at 2-3% worldwide. It is characterised by recurring phases of active severe inflammation and remission that may affect anywhere from a few per cent of body surface area in milder cases to the entirety of the skin in the most severe cases. Manifestation of psoriasis is most common between the ages of 18-39 years and 50-69 years, although it may manifest at any point in life (Greb et al., 2016).

1.3.1.3 Pathophysiology of Psoriasis

All psoriatic types are associated with severe dysregulation of the immune system that behaves aberrantly upon external challenge, and there have been many genes and genetic regions that have been linked to these types. The most important of these regions is a locus containing genes coding for human leukocyte antigens (HLA) called the psoriasis susceptibility region (PSORS) 1 (Veal et al., 2002), although others, like PSORS2 (Tomfohrde et al., 1994), PSORS4 (Capon et al., 1999), PSORS6 (Lee et al., 2000) and PSORS7 (Veal et al., 2001), most of them containing genes coding for components of the immune system, have been linked to this disease with as of yet unknown relative contributions. Overall, the history of psoriasis within any given family is still the best and most rapid prognostic approach available (Solmaz et al., 2020).

From a structural standpoint, the mainstay among all psoriatic variants is acanthosis and epidermal hyperplasia, although this histological feature is further modified and

expanded among said variants and for detailed description, readers are again referred to a much more descriptive review on this subject (Sarac et al., 2016).

The most common immune dysregulations of psoriasis are associated with excessive activation of T_H17, T_H1 and T_H22 cells with important contributions from dendritic cells and keratinocytes (Diani et al., 2016; Li et al., 2020) with additional possible roles of ILC3 and γδ T cells, although their role is less clear (Chen et al., 2020). IL-22 is a signature cytokine associated with psoriatic lesions (Ma et al., 2008; Wawrzycki et al., 2019). Cathelicidin, produced mainly by keratinocytes in this case, along with other AMPs plays a key role in the initiation of the psoriatic inflammatory feedback loop (Figure 1.8) (Dombrowski and Schauber, 2012). This may hint at the possible important role of KLK8, which is severely upregulated in psoriatic lesional skin (Eissa et al., 2013) since it has the potential ability to convert it into its active form and thus may be a contributor to its deleterious activity (Eissa et al., 2011). Additionally, KLK8 has been shown to be capable of inducing the production of IL-36 family members, which are inflammatory cytokines that have been shown to have a role in psoriatic inflammation (Iinuma et al., 2015). KLK6 is another KLK that has been proposed to have a role in the development of psoriatic lesions in a mouse model of psoriasis, although the mechanism behind his role remains to be elucidated (Iinuma et al., 2017).

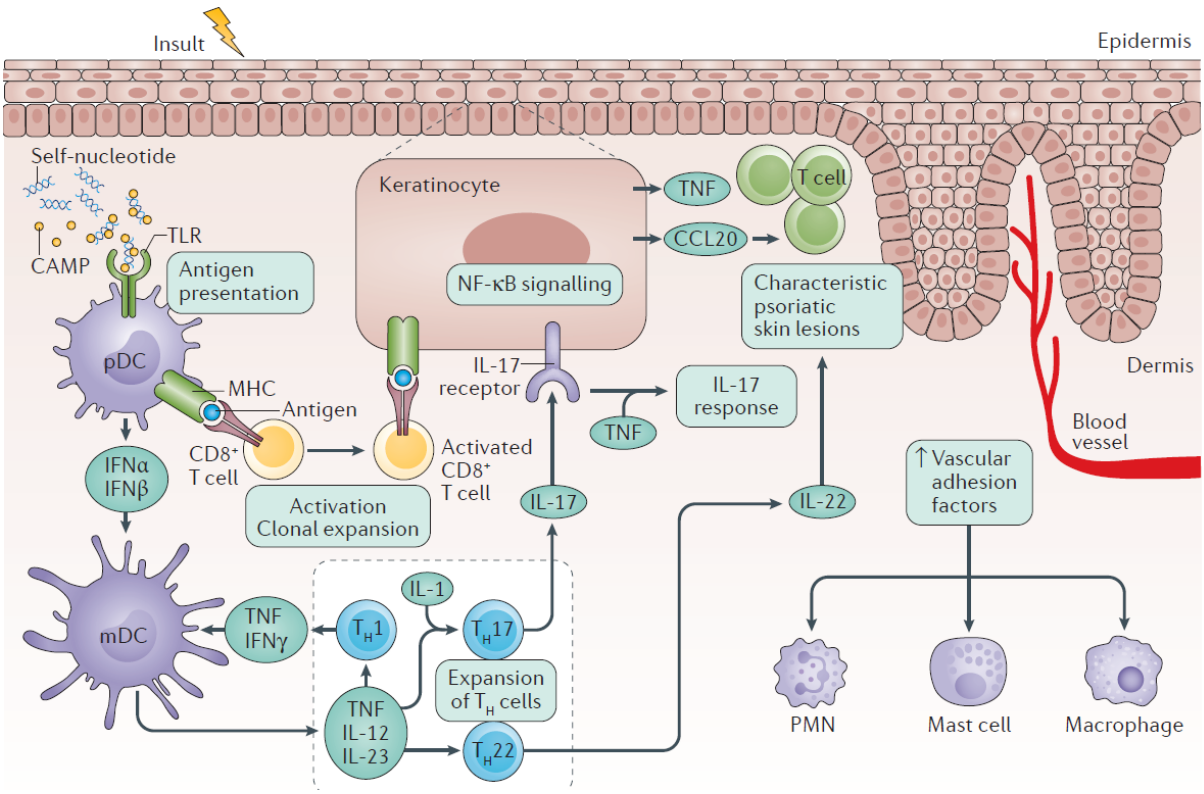


Figure 1.8 Overview of psoriasis mechanisms. Upon an external insult, the release of self-nucleotides that associate with AMPs, like cathelicidin, leads to activation of TLRs on plasmacytoid dendritic cells (pDCs). This leads to activation and subsequent clonal expansion of CD8+ T cells which mediate the expression of proinflammatory cytokines upon their binding to keratinocytes (mainly). Concomitantly, secretion of IFNα and IFNβ by pDCs stimulates myeloid dendritic cells (mDCs) to secrete cytokines responsible for the activation of several subsets of T_H cells. Subsets activated by mDCs include T_H17, T_H1 and T_H22 and their activities lead to further progression of the disease by several activities depicted in the figure. Among the most important of these activities are secretion or induction of further secretion of IL-17, IL-22, TNFα, chemokine (C-C motif) ligand (CCL) 20 and AMPs. An increment in the expression of vascular adhesion factors further contributes to the recruitment of additional immune cells that participate in the manifestations of psoriasis. Adapted from Greb et al., 2016.

1.3.1.3 Therapeutic options for Psoriasis

Depending on the type and severity of the psoriatic phenotype, several treatment options are available to tackle this disease. For less severe manifestations, various topical treatments are being regularly used (Freeman et al., 2003; Lebwohl et al., 1998). If topical treatment becomes less practical due to the scale of psoriatic manifestations, phototherapy is an approach that is used to supplement topical therapies of the most severe lesions (Nolan et al., 2010). In the case of increasing severity of this disease, a systemic approach is undertaken and there is a large inventory of agents used for this purpose. Inhibitor of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (Methotrexate) (Goldminz et al., 2016), inhibitors of T cell activity (Acitretin) (Lebwohl et al., 2001), TNFIs (Etanercept,

Infliximab) (Tobin and Kirby, 2005), inhibitors of phosphodiesterase 4 (Apremilast) (Schafer, 2012), anti-IL-17A inhibitors (Secukinumab) (Canavan et al., 2016) and anti-IL-12/IL-23 (Ustekinumab) (Jeon et al., 2017) are among those commonly used.

1.3.4 Acne rosacea

Acne rosacea also called simply rosacea, is a poorly understood inflammatory disease of the skin, with its effects restricted primarily to the face of the afflicted. The main features of rosacea are the presence of inflammatory pustules and papules, transient erythema, telangiectasia and overall dryness of the skin due to a variety of causative symptoms. Classification of rosacea that was undertaken in recent years has divided this disease into several subtypes with their given characteristics, although this division is not of a permanent nature, since the manifestation of its symptoms may shift from one subtype to the other and oftentimes, characteristics of various subtypes are present and once. Prevalence of rosacea is hard to estimate due to lack of data, but the current estimate is upwards of 5%, with its nature being age- and gender-specific, preferentially manifesting in the younger population in some subtypes and in the older population in other (Buddenkotte and Steinhoff, 2018).

1.3.4.1 Pathophysiology of Acne rosacea

As was stated above, rosacea is still a very poorly understood disease and that definitely entails the details of its pathophysiology. Genetic factors that have been linked to its pathophysiology are for example butyrophilin-like 2 (Chang et al., 2015), glutathione S-transferase (Yazici et al., 2006) and nucleotide-binding oligomerization domain-containing protein 2 (van Steensel et al., 2008), although the degree of relevance from the aforementioned studies must be taken with a grain of salt and additional confirmatory analyses must be undertaken prior to making any conclusions.

Several pathogens are thought to have some effect on the severity and nature of rosacea manifestations, like *Demodex* spp. or *Helicobacter pylori* among others, but again, data for conclusive interpretation of their effects on rosacea are still lacking (Jarmuda et al., 2012; Jørgensen et al., 2017).

Rosacea is strongly associated with T_H1 and T_H17 CD4+ T cells and CD4+ T cells are the main components of the immune cell infiltrates where rosacea-associated symptoms are present. Along with these cell types, cells of innate immunity, mostly

macrophages, neutrophils and plasma cells are present in elevated numbers (Figure 1.9) (Buhl et al., 2015).

Key immunological features linked to rosacea are excessive levels and activation of PAR2 and Toll-like receptor (TLR) 2 (Kim et al., 2014; Yamasaki et al., 2011). Cathelicidin is present in severely elevated amounts (Kim et al., 2014), which along with the aforementioned heightened levels of PAR2 and TLR2, points out the possible importance of microbes in the progression of this skin disease. In previous chapters, I discussed the role of KLKs in the activation of PAR2 and cathelicidin processing. Indeed, KLK5 expression is elevated in rosacea patients, which highlights its likely important role in this disease (Two and Del Rosso, 2014). However, more research is warranted in order to draw any definitive conclusions.

1.3.4.2 Therapeutic options for Acne rosacea

Therapeutic approaches tackling the pathophysiology of rosacea are targeted at symptoms of individual subtypes, apart from general skincare that is recommended for all patients. Therapies may be of topical or systemic nature, depending on the severity of rosacea manifestation (Buddenkotte and Steinhoff, 2018). Common therapy that is shared across all subtypes is laser therapy, but its usage may be limited by the individual to be treated, mainly if they are excessively pain-sensitive (Lonne-Rahm et al., 2004). Lastly, the use of serine protease inhibitors improved the course of rosacea in a small pilot study, which highlights the importance of further research on the role of KLK proteases in rosacea (Two et al., 2014).

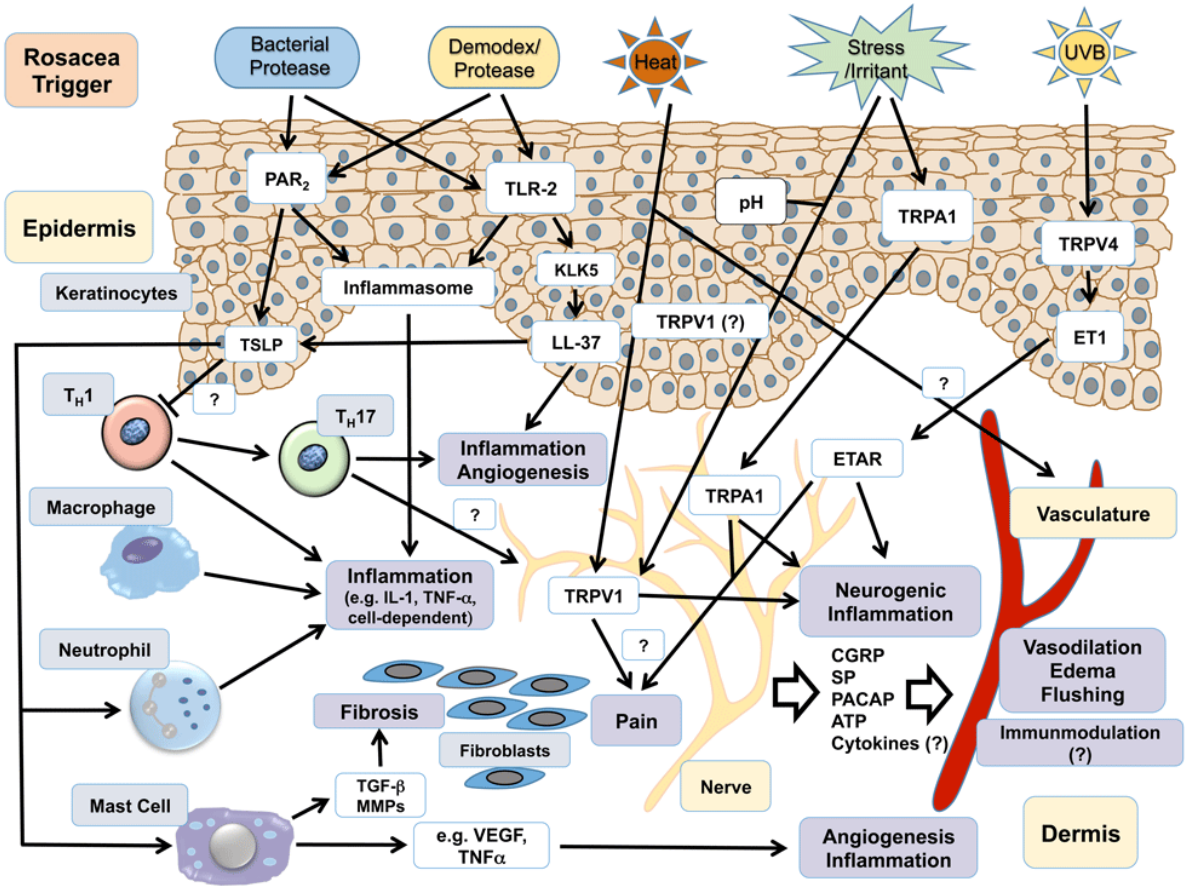


Figure 1.9 Overview of acne rosacea mechanisms. After initiation of this pathology by a variety of triggers depicted in the upper part of the figure, several possible mechanisms have been attributed to the progression of this affliction. Among them, PAR2 and TLR2 activation either by endogenous or pathogen-derived proteases seem to have a key role, leading to the production of inflammatory cytokines, like TSLP, TNFα and IL-1β and subsequent initiation of additional immune cells. T_H17 and T_H1 are thought to play a key role in rosacea, as are several innate immune cell types, like neutrophils, mast cells and macrophages. Several initiators of rosacea also participate in the activation of receptors associated with pruritus, like TRPV1 and TRPV4. Finally, AMPs, and most notably cathelicidin and its active form, LL-37, play a key role in inflammatory processes associated with rosacea. Adapted from Buddenkotte and Steinhoff, 2018.

1.3.5 Mouse models in skin immunology

Animal models are enabling researchers to tackle various questions in an environment that provides them with an unparalleled similarity to the workings of human organisms and indeed, many mechanisms have been elucidated with their utilization. This has been further supported with the "golden age" of genetic manipulation that has been brought by the genome editing technologies like Zinc-finger nucleases, TALENs and most importantly, CRISPR/Cas9, which enabled the production of animal models at speeds vastly superior to previously used approaches based primarily on embryonic stem cells.

Mice are in particular very useful in these endeavours, as they offer rapid generation time, high similarity to humans in their genetic makeup and relatively low cost of maintenance and an array of materials and methods available when compared to other viable alternatives. Many models pertaining to diseases mentioned in the previous chapter have been created, in particular for atopic dermatitis (Gilhar et al., 2021; Nakajima et al., 2019) and psoriasis (Nakajima and Sano, 2018; Schön et al., 2021) and are responsible for a large amount of data and in extension, our knowledge about mechanisms of these pathologies.

Nevertheless, it is important to take the differences between mouse and human skin and their immunological makeup into consideration when one is trying to interpret obtained data and transfer the knowledge to humans in order to elucidate any given mechanism, because incorrect interpretations may possibly lead to devastating effects, mainly in the context of therapeutic interventions.

1.3.5.1 Comparison of mouse and human skin

In this sub-chapter, I would like to briefly depict the main structural and immunological differences between mouse and human skin. Despite their apparent similarities which have been appreciated in research for a long time, the importance of these differences is still incompletely understood which, to a degree, hampers the correct interpretation of obtained data and also poses the question if mice can be thought of as a relevant model in certain research areas.

1.3.5.1.1 Structural comparison

On a basic histological level, mouse and human skin are divided into the same general compartments, dermis and epidermis with hypodermis sometimes also being included (Wong et al., 2016). Epidermis is further divided into *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* (Wickett and Visscher, 2006). However, human skin is significantly thicker than that of mice, and the epidermal layer is usually composed of 5 to 10 cells, compared to that of mice, which is usually 2 to 3 cells, depending on the localization on the body. Additionally, the murine epidermis is much more loosely attached to the dermis underneath than its human counterpart (Zomer and Trentin, 2018). This may prove troublesome in the assessment of skin diseases in which damage of epidermal barrier plays a significant role since thinner and looser epidermis of mice leads to more rapid damaging and thus

may distort our view of the seriousness and adequacy of any given mouse model created for its associated pathology.

Apart from the thickness parameter, mouse skin is nearly ubiquitously covered in hair and contains associated hair follicles, which are an important immune-related and skin repair niche, whereas, in humans, hair follicles are relatively sparse on most sites of the body. There is also a significant difference in the hair cycle between human and murine hair follicles (Zomer and Trentin, 2018). Wound healing is, in general, a rather similar process in humans and mice, although mice rely much more on contraction in healing skin wounds than humans do, which may, among other things, result in problems in wound healing research in mice. Indeed, usage of larger animals with similar wound healing process properties, like pigs, is advisable when the circumstances and available materials are on hand (Masson-Meyers et al., 2020).

Finally, several structures present in human skin are absent in its murine counterpart. Those structures are eccrine sweat glands and so-called Rete ridges which play an important role in skin homeostasis, wound healing and immune response in human skin.

1.3.5.1.2 Immunological comparison

From the perspective of immunology, several notable differences exist that are of interest in the context of correct interpretations of relative contributors to inflammatory phenotypes.

Immune cell populations in the murine epidermis are primarily made of CD8+ T cells, Langerhans cells and dendritic epidermal T cells (DETCs), a γδ T cell subset. Whereas both CD8+ T cells and Langerhans cells are prominent cell types in the human epidermis, DETCs are completely absent. In the underlying dermal layer, the single most striking difference is the substantial presence of γδ T cells to murine cutaneous immunity, whereas, in humans, they represent only a minor fraction of T cells present (Figure 1.10) (Pasparakis et al., 2014). The importance of the contributions these cells make to cutaneous inflammation has been noted and due to the fact that they are absent or present in negligible amounts in human skin, these cell types represent a major issue in the correct interpretation of underlying inflammatory mechanisms of skin diseases based on data obtained from mouse models (Chen et al., 2020; Polese et al., 2020).

Additionally, there are several observed differences in cytokine, chemokine (Zlotnik and Yoshie, 2012) and AMP production in human and murine skin, notably defensins (Gerber et al., 2014; Schittek et al., 2001). Since the skin microbiome plays a large role in skin diseases that were previously briefly discussed, cutaneous immune response to them is of great importance and AMPs are very important contributors to this process (Schauber and Gallo, 2008).

Differences of the aforementioned nature make the creation of an adequate mouse model equivalent to human diseases a rather challenging effort (Gilhar et al., 2021; Nakajima and Sano, 2018; Nakajima et al., 2019; Schön et al., 2021).

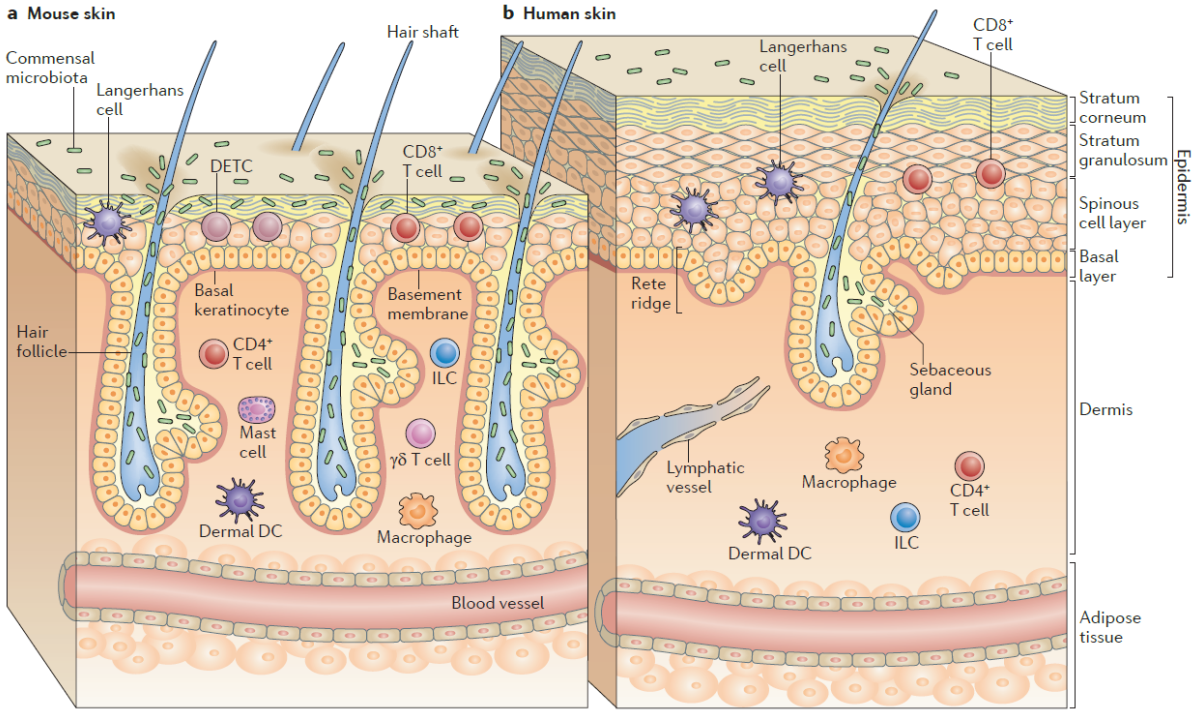


Figure 1.10 Comparison of human and murine skin structure and immune cell populations. Structurally, the main differences between human and murine skin are the thickness of epidermis and dermis, the almost ubiquitous presence of hair follicles in murine skin compared to large interfollicular areas in most parts of human skin and the presence of some additional structures in the human skin, like the Rete ridges. From the immunological point of view, there are indeed several important differences that may affect the mechanisms of immune response. Firstly, populations that are present in both human and murine skin may be represented in different amounts, for example, the neutrophils (not shown). Additionally, hair follicles are important mediators of immune response and their presence or absence may significantly alter the immune process, as does their different structure. Lastly, several cell types are completely or nearly absent from human skin, with those cell types being DETCs in the epidermis and gamma delta T cells in the underlying dermis. Adapted from Pasparakis et al., 2014.

2. Materials and methods

2.1 Materials

2.1.1 Animals

Animals used in this study were of C57BL/6N background and mutant mice were either created *de novo* (*Tnfr1*$^{-/-}$) or produced previously (*Sp5*$^{-/}$ *Klk5*$^{-/-}$) by Mgr. Petr Kasparek, PhD in Czech Centre for Phenogenomics. Breedings were performed in the animal facility of The Institute of Molecular Genetics AS CR, v.v.i. in BIOCEV in individually ventilated cages under specific-pathogen-free conditions. Prior to euthanasia, mice were photographed and weighted.

Animal experiments were performed according to the relevant European directive pertaining to experimentation on animals (2010/63/EU) and were approved by the Czech Central Commission for Animal Welfare.

2.1.2 CRISPR crRNAs

Both guide RNAs were ordered from IDT in 2 nmol concentration and lyophilized format.

ID	Sequence (5´-3´)
gr68	CACTCAGGTAGCGTTGGAAC
gr70	GTAATTCTGGGAAGCCGTAA

2.1.3 Primers

All primers were ordered from Sigma-Aldrich® in lyophilized form and purified by desalting.

2.1.3.1 Genotyping primers

Tnfr1 genotyping primers

ID	Sequence (5´-3´)
Tnfr F2	CGGCTTCTTTTGCTTGTTTC

Tnfr R2	TGTGGGAAAGCGGTTAAGAC
Tnfr1 cDNA F	GGCTCTGCTGATGGGGATAC
Tnfr1 cDNA R	AGATAACCAGGGGCAACAGC

*All primers were designed using Primer3 design tool.

Klk5 genotyping primers

ID	Sequence (5´-3´)
Klk5 KO F1	TGCATGGTTTGGTATGGAGC
Klk5 KO R1	TCCATTCTAGAGCCAATCCTAAATTC
Klk5 KO LacZ	CTCCTGGAGCCCGTCAGTAT

*All primers were designed using Primer3 design tool.

Spink5 genotyping primers

ID	Sequence (5´-3´)
Spink 5-5 F	CCTGTCTCTGCCTTCAGACC
Spink 5-5 R	GGCTGTGGTAACTGTCCAAAA
Sp5 ARMSin F1	TGTGAACTGTGTGCTGAGAATG
Sp5 ARMSin R2	GGGTACTCACGCATTCTAGATCA

*All primers were designed using Primer3 design tool.

2.1.3.2 RT-qPCR primers

Target gene name	Target gene Ensembl ID	Sequence (5´-3´)	
		Forward	Reverse
Il1a	ENSMUSG00000027399	TTCTGAAGAAGAGACGGCTGA	CTGATCTGGGTTGGATGGTC
Il1b	ENSMUST00000028881	AGTTGACGGACCCCAAAAGA	GTGCTGCTGCGAGATTTGAA

Il18	ENSMUSG00000039217	CCAAGTTCTCTTCGTTGACAAAA	CAGTCTGGTCTGGGGTTCAC
Il23a	ENSMUSG00000025383	GTTGTGACCCACAAGGACTCA	CAGGCTCCCCTTTGAAGATGT
Il36a	ENSMUSG00000026984	ACTGGGGGAAATCTTCATCAC	GGGGTGTCTTTGATTGCTTCT
Il36b	ENSMUSG00000026985	CAACAGATGGTATGGGTCCTG	CATCTTGGAATTCCGTGTCTCT
Il36g	ENSMUSG00000044103	GTTCCACGAAGCCACAGAGT	AATGGCAATCCCTTTGTCCT
Ccl20	ENSMUSG00000026166	CTGCTCTTCCTTGCTTTGGC	GTCGTAGTTGCTTGCTGCTT
Ccl22	ENSMUSG00000031779	ACCCTCTGCCATCACGTTTA	TCGGTTCTTGACGGTTATCAA
Cxcl1	ENSMUSG00000029380	AGACCATGGCTGGGATTCAC	CGCGACCATTCTTGAGTGTG
Icam1	ENSMUSG00000037405	GTCACCGTTGTGATCCCTG	AACAGTTCACCTGCACGGAC
Il6	ENSMUSG00000025746	AGCCAGAGTCCTTCAGAGAGAT	TGGAAATTGGGGTAGGAAGGAC
Tslp	ENSMUSG00000024379	AGAAGCCCTCAATGACCACTGC	TCTTGTGCCATTTCCTGAGTACCG
Il33	ENSMUSG00000024810	GCAGGAAAGTACAGCATTCAAGA	GGGGAAATCTTGGAGTTGGAATAC
Tnfr1	ENSMUSG00000030341	TAACTGCCATGCAGGGTTCT	CTGGGGGTTTGTGACATTTG
Actb	ENSMUST00000100497	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA

Tbp	ENSMUSG00000014767	ACAGCCTTCCACCTTATGCTC	TGGAGTAAGTCCTGTGCCGT

* All primers were designed by UCSC Genome Browser qPCR track or by using Primer3 design tool.

2.2 Methods

2.2.1 Zygote electroporation

Mouse carrying a null mutation in the *Tnfrsf1a* gene, designated as *Tnfr1*$^{-/-}$, was generated in a C57BL/6N using a CRISPR/Cas9 genome-editing system. Specific guide RNAs (gRNAs) recognizing exon 3 and exon 4 of the *Tnfrsf1a* gene were designed and off-target analyses were performed using the online software CRISPOR Design Tool (http://crispor.tefor.net/). Sequences of designed gRNAs are noted in section 2.1.2. CRISPR RNAs (crRNAs) (IDT; Alt-R® CRISPR-Cas9 crRNA, 2nmol) and trans-activating CRISPR RNAs (tracrRNA) (IDT; Cat. No. 1072532) were diluted in Nuclease-Free Duplex Buffer (IDT™; Cat. No. 11-05-01-03) to the final concentration of 100 µM. 5 µl of crRNAs were mixed with 5 µl of tracrRNA each and heated at 95°C for 3 minutes to facilitate the crRNA:tracrRNA duplex formation of a 50 µM final concentration. Subsequently, 40 µl of OptiMEM media was added to each crRNA:tracrRNA duplex which achieved a final concentration of 10 µM. SpCas9 protein (made by Cyril Bařinka´s lab) diluted in OptiMEM media (500 ng/µl) (Thermo Scientific™; Cat. No. 31985062) and gRNAs (5 µM each) were used for zygote electroporation, using an electroporation protocol described elsewhere (Jenickova et al., 2021). Correct genome editing events were confirmed by PCR (section 2.2.3) in the founder mice.

2.2.2 DNA isolation

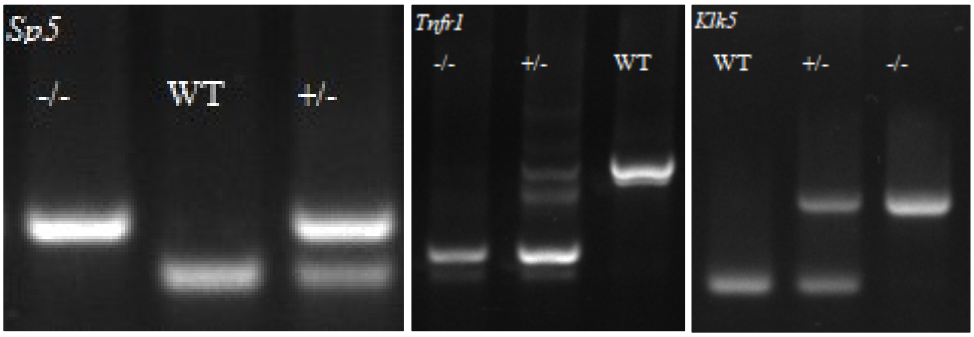
DNA isolation was performed by the neutralized phenol:chloroform:isoamyl alcohol method. Lysis buffer (1M TRIS HCl, 1M NaCl, 0,5M EDTA, 10% SDS) was supplemented with Proteinase K to achieve final concentration of 0,2 mg/ml prior to extraction. 0,5 ml of supplemented lysis buffer was then added to harvested mouse tails and incubated at 55°C overnight. Afterwards, tubes were centrifuged in a microcentrifuge (Eppendorf™ 5424 R) for 20 seconds at 13000 x rpm and 0,5 ml of neutralized phenol:chloroform:isoamyl alcohol (ROTH®; Cat. No. A156.1) was added to each tube and all tubes were shaken vigorously for 1 minute. Subsequently,

samples were centrifuged for 4,5 minutes at 13000 x rpm and 400 µl of the upper aqueous layer was transferred to new microtubes. 0,5 ml of ice-cold 96% EtOH (VWR™; Cat. No. 20823.293) was added to each sample and all samples were inverted 10 times to facilitate DNA precipitation. Following precipitation, samples were centrifuged for 4,5 minutes at 13000 x rpm at 4°C and the supernatant was removed by decanting. 0,5 ml of ice-cold 75% EtOH was added to each sample and samples were again centrifuged for 4,5 minutes at 13000 x rpm at 4°C. The supernatant was then carefully removed by pipetting and the resulting pellets were air-dried for 30 minutes. After drying, 200 µl of ddH_2O was added to each sample to resuspend the DNA pellet and after resuspension, samples were either directly used for PCR or placed in a -20°C freezer until further processing.

2.2.3 Mice genotyping

PCR was performed in order to genotype the mice with the use of primers from Table X and PCR protocols for each locus as denoted under this text. PCR mix used for all PCR reactions had the following composition: 1 µl of each primer (section 2.1.3.1) (10 µM), 2,5 µl of DreamTaq Green Buffer (10X; Thermo Scientific™; Cat. No. EP0714) 0,5 µl of dNTPs (10 mM; Sigma-Aldrich® Cat. No. D7295) mix, 0,2 µl of 0,2 µl DreamTaq DNA Polymerase (5U/µl; Thermo Scientific™; Cat. No. EP0714), 3 µl of DNA and appropriate amount of ddH_2O to achieve the final volume of 25 µl per reaction. PCR reaction was carried out in BIO-RAD T100™ Thermal Cycler and subsequently separated on agarose gel by agarose gel electrophoresis on 2% agarose gel. Electrophoresis was performed at a fixed voltage (90V) for 30 minutes. Agarose gel used for this analysis was prepared from TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and SeaKem® LE Agarose (Lonza ; Cat. No. 50004). 8 µl of each PCR reaction was used in this process, along with 4 µl of GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific™; Cat. No. SM1331) to appropriately measure the DNA size. The resulting gel was then analysed on ChemiDoc™ MP Imaging System (Bio-Rad; Cat. No 1708280).

Example electrophoresis results



Tnfr1 genotyping PCR protocol

Temperature (°C)	**Time** (s)	
95	180	
95	20	34x
60	25	
72	60	
72	180	
12	Inf	

Spink5 genotyping PCR protocol

Temperature (°C)	**Time** (s)	
95	180	
95	20	34x
61	25	
72	25	
72	180	
12	Inf	

Klk5 genotyping PCR protocol

Temperature (°C)	**Time** (s)	
95	180	
95	20	34x
63	25	
72	30	
72	180	
12	Inf	

Tnfr1 cDNA PCR protocol

Temperature (°C)	**Time** (s)	
95	180	
95	20	34x
60	25	
72	40	
72	180	
12	Inf	

2.2.4 Serum preparation

Mice were gently taken by the neck and sedated by injection of 100 µl/25g of 30% Zoletil/Rometar (4:1; diluted in PBS) to each of their thighs. After sedation was confirmed by the lack of responsiveness to pain stimuli, approximately 0,5 mL of blood of each mouse was extracted by inserting a glass capillary into the retro-orbital

plexus and collected into a microtube. Collected blood was left for 10 minutes and then placed on ice for 1 hour. In the meantime, mice were euthanized by cervical dislocation. After 1 hour, blood was centrifuged at 5000 x g for 10 minutes at 4°C in a microcentrifuge (Eppendorf™ 5424 R). Subsequently, serum (upper phase) was transferred to a new microtube and samples were stored at -20°C until further processing.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

Mouse sTNF RI/TNFRSF1A DuoSet ® ELISA (R&D Systems ®; Cat. No. DY425) was used to perform the analysis of serum abundance of sTNFR1 according to a modified manufacturers protocol. The capture antibody was diluted to a working concentration (2 µg/mL) in PBS and an adequate number of wells of a 96-well microplate was coated with this solution, with 100 µl per well. The microplate with capture antibody was then incubated overnight in the fridge. The next day, the capture antibody was removed by aspiration and washed three times with 200 µl of Wash Buffer (0,05% Tween ® 20 in PBS; R&D Systems ®; Cat. No. WA126). When necessary, residual Wash Buffer still present after decanting was carefully removed with a paper towel. Subsequently, the microplate was blocked by adding 200 µl of Reagent Diluent (1% BSA in PBS, 0,2 um filtered, pH 7,2 - 7,4; R&D Systems ®; Cat. No. DY995) and left to incubate for 1 hour at room temperature. After 1 hour, Reagent Diluent was removed and the wash step outlined above was repeated. Next, 50 µl of samples and standards diluted in Reagent Diluent were added in duplicates and left to incubate at room temperature for 2 hours. The washing procedure was then repeated and 100 µl of detection antibody diluted in Reagent Diluent to working concentration of 200 ng/mL was added to each well and incubated at room temperature for 2 hours. The detection antibody was then decanted and the wash procedure was performed again. Afterwards, 100 µl of Streptavidin-HRP solution diluted to a working concentration (40:1 in Reagent Diluent) was added to each well and incubated for 20 minutes at room temperature in total darkness. After 20 minutes, the wash step was again repeated and 100 µl of Substrate Solution (H_2O_2 and Tetramethylbenzidine, 1:1; R&D Systems ® Cat. No. DY999) was added to each well, again incubated at room temperature for 20 minutes in total darkness. After 20 minutes, 50 µl of Stop Solution (2N H_2SO_4; R&D Systems ® Cat. No. DY994) was added to each well. Absorbance was then determined on Epoch™ Microplate

Spectrophotometer set to 450 nm. 540 nm wavelength was also measured and subtracted from 450 nm values to correct for optical imperfections of the plate. The concentration of sTNFR1 was then determined by plotting the concentration of standards against their corrected absorbance values, creation of a linear standard curve sample standard dilution series (500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 0; pg/mL) and calculation of linear regression equation for each sample.

2.2.6 RNA isolation

Dorsal skin RNA was isolated by TRI Reagent ® (Sigma-Aldrich®; Cat. No. T9424) solution following an optimized manufacturers protocol. Dorsal skin samples in microcentrifuge tubes were submerged in liquid nitrogen for 5 seconds and then crushed to fine particles on dry ice. Afterwards, 600 µl of TRI Reagent ® was added and the samples were mixed by pipetting until they seemed to be homogenous by visual inspection and allowed to stand for 5 minutes at room temperature. After 5 minutes, 120 µl of chloroform was added to every sample, vigorously shaken by hand for 15 seconds and incubated for 15 minutes at room temperature. The resulting mixture was centrifuged at 12000 x g for 15 minutes in a microcentrifuge (Eppendorf™ 5424 R) pre-cooled to 4°C. The upper aqueous phase was then transferred to a fresh microcentrifuge tube and 300 µl of 2-propanol was added to each transferred sample to precipitate RNA. After 10 minutes at room temperature, samples were centrifuged at 12000 x g for 10 minutes at 4°C after which there could be seen a formed RNA pellet at the bottom of each microcentrifuge tube. The supernatant was removed by pipetting, 600 µl of 75% EtOH was added and samples were briefly vortexed. Samples were then centrifuged at 12000 x g for 5 minutes at 4°C. The supernatant was then removed and 75% EtOH addition and subsequent centrifugation step were repeated. After the second washing step, the supernatant was removed by pipetting and resulting RNA pellets were allowed to dry, after which they were resuspended in 31 µl RNAse-free H_2O (Thermo Scietific™; Cat No. W4502). Concentrations of the resulting RNA solutions were measured with Implen NanoPhotometer® N50. Analysis of RNA quality was performed by agarose gel electrophoresis on 2% agarose gel and electrophoresis was performed at a fixed voltage (90V) for 30 minutes. Agarose gel used for this analysis was prepared from TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and SeaKem® LE

Agarose (Lonza; Cat. No. 50004). The resulting gel was then analysed on ChemiDoc™ MP Imaging System (Bio-Rad; Cat. No 1708280).

2.2.7 *In vitro* reverse transcription

In vitro reverse transcription was performed with the use of M-MLV Reverse Transcriptase (Promega; Cat. No. M1705) and was performed using an upscaled manufacturers protocol. Firstly, 3 µg of RNA, 6 µl of oligo(dT)^{23} (35 µM) were mixed and adjusted to a final reaction volume of 22,5 µl. Next, samples were incubated at 70°C for 5 minutes for oligo(dT)23 annealing and melting RNA secondary structures. After 5 minutes, samples were immediately placed on ice to avoid secondary structure reformation. 7,5 µl of M-MLV buffer (Cat. No. M5313; Promega), 1,875 µl of deoxynucleotide mix (10 mM, Sigma Aldrich®), 1,5 µl of RNAsin® Ribonuclease Inhibitor (Cat. No. N2515; Promega), 1,5 µl M-MLV Reverse Transcriptase (Cat. No. M1705; Promega) and 2,625 µl of RNAse-free water (Thermo Scientific™; Cat No. W4502) was added to each sample to a total volume of 37,5 µl and samples were incubated in a thermal block (BIOER; Cat No. MB-102) at 42°C for 1 hour. After incubation, samples were diluted by adding 112,5 µl of ddH_2O and were frozen at -20°C until further processing.

2.2.8 Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed with the utilization of LightCycler® 480 SYBR Green I Master (Roche s.r.o.; Cat. No. 4887352001) and were performed as follows. 3 µl of cDNA, 0,25 µl of each primer (10µM), 1,5 µl of ddH_2O and 5 µl of LightCycler® 480 SYBR Green I Master (Roche s.r.o.; Cat. No. 4887352001) were mixed, in duplicates for each sample. The RT-qPCR reaction was then performed according to the protocol below on Light Cycler® 480 (Roche) and relative mRNA levels were calculated with the use of the $2^{-\Delta\Delta Ct}$ method with *Actb* and *Tbp* genes used for normalization.

RT-qPCR protocol

Temperature (°C)	**Time** (s)	**Ramp** (°C/s)	**Mode**
95	120	4,8	Initial denaturation
95	15	4,8	Quantification 50x
60	20	2,5	
72	20	4,8	
95		0,1	Melt Curve Analysis

2.2.9 Dorsal skin dissection and processing

Harvest of dorsal skin was performed on P2 mice. Firstly, mice were euthanized via decapitation and then placed on a polystyrene board covered with aluminium foil, where they were fastened by pins through each of their limbs. About 0,5 cm of tail tissue was taken for DNA extraction and subsequent genotyping. Dorsal skins were then harvested for histological and RT-qPCR analyses. For histological analysis, half of the dorsal skins were placed into embedding cassettes (Leica Biosystems; Cat. No. 39LC-500-1) with a filter paper and fixed in phosphate buffered 4% formaldehyde solution (Sigma-Aldrich®; Cat. No. 1004969011) for 24 hours and then dehydrated and conserved in 70% EtOH at 4°C until further processing. For RT-qPCR, the other half of the dorsal skins were cut into 3 pieces of similar dimensions and snap freezed on dry ice until dissections were finished and then transferred to a -80°C freezer before RNA isolation was performed.

2.2.10 Paraffin block preparation and processing

Tissue samples were automatically processed in Leica ASP6025 Tissue Processor prior to paraffin block preparation. Samples were subjected to a series of ethanol solutions to dehydrate the samples (EtOH, 37°C) (VWR™; Cat. No. 20821.296) and saturate them with paraffin-dissolving solvent (xylene, 45°C) (VWR™; Cat. No. 28973.294). Afterwards, samples were saturated with liquid paraffin (Leica Biosystems; Cat. No. 39603002) at 60°C, which ended the automatic processing. Subsequently, samples were taken to Leica EG1150 Embedding Station, on which

samples were moved from embedding cassettes into metal moulds, liquid paraffin heated to 60°C was poured on individual samples and samples were correctly localized. This process was undertaken on a continuously heated surface to avoid paraffin solidification. Moulds were then moved to a cooling surface on which the paraffin solidified. After solidifying, additional paraffin was added to fill the moulds completely and samples were again left to solidify. Finished paraffin blocks were then processed on Leica RM2255 microtome. Two tissue sections with a thickness of 5 μm were cut from each block and placed on individual glass slides (2 tissue sections/slide) for further processing.

2.2.11 Hematoxylin and eosin (H&E) staining

Prior to the H&E staining itself, dried tissue sections on slides were deparaffinized and rehydrated, during which slides were submerged in a series of solutions in separate cuvettes. These solutions were: xylene (10 minutes) → xylene (5 minutes) → isopropanol (5 minutes) (VWR™; Cat. No. 20842.312) → isopropanol/absolute EtOH (1:1; 5 minutes) → absolute EtOH (5 minutes) → 96% EtOH (5 minutes) → 70% EtOH (5 minutes) → ddH_2O.

Following deparaffinization, slides were placed into a cuvette with Hematoxylin solution (Sigma-Aldrich®; Cat. No. MHS16-500ML) for 5 minutes. Slides were washed under a continuous indirect flow of ddH_2O for 1 minute and then left to stand in ddH_2O for 10 minutes. Afterwards, slides were placed into a cuvette with 0,5% aqueous solution of eosin (Leica Biosystems; Cat. No. 3801590BBE) for 1 minute. Slides were washed under a continuous indirect flow of ddH_2O for 1 minute. After staining, samples were dehydrated and brightened by submerging the slides in a series of solutions in separate cuvettes. These solutions were: 70% EtOH (1 minute) → 96% EtOH (1 minute) → absolute EtOH (1 minute) → isopropanol/absolute EtOH (1:1; 3 minutes) → isopropanol (3 minutes) → xylene (5 minutes) → xylene (10 minutes). After dehydration and brightening, samples were mounted with a drop of Pertex™ (VWR™; Cat. No. 720-2343) mounting medium, covered with a covering glass, left to dry overnight and examined by light microscopy on ZEISS Axio Imager Z.2.

2.2.12 Naphthol AS-D chloroacetate (NASDCL) staining

NASDCL staining was done with Naphthol AS-D Chloroacetate (Specific Esterase) Kit (Sigma-Aldrich®; Cat. No. 91C-1KT). Prior to the staining itself, slides were deparaffinised as described in section 2.2.11. NASDCL staining solution was prepared as follows. 1 mL of Red Violet solution and 1 mL of Sodium Nitrate solution was mixed and left to stand for 2 minutes and added to 40 mL of ddH_2O pre-warmed to 37°C. Afterwards, 5 mL of TRIZMA solution was added, the solution was mixed and 1 mL of NASDCL was added and the solution was again thoroughly mixed. Slides were then placed in a staining cuvette, submerged in the NASDCL staining solution and left to incubate for 1 hour at 37°C. After 1 hour, NASDCL staining solution was removed and slides were washed under a continuous indirect flow of ddH_2O for 2 minutes. After washing, samples were counterstained with Hematoxylin Gill for 20 seconds and washed with a continuous indirect flow of ddH_2O for 1 minute. Slides were then mounted with Aquatex (Sigma-Aldrich®; Cat. No. 1085620057) mounting agent, covered with a covering glass and left to dry overnight. After drying, samples were analysed by light microscopy on ZEISS Axio Imager Z.2. For granulocyte infiltration statistical analysis, four consecutive fields (magnification - 200x) of view were taken from each specimen and NASDCL+ cells were counted in the ImageJ software.

2.2.13 Statistical analysis

All statistical analyses and associated graphs were performed and created in GraphPad Prism software (version 8.4.3 (686), June 17, 2021 release). One-way ANOVA followed by Bonferroni correction was used for all statistical analyses. Column bar graphs represent mean values with error bars depicting the standard deviation. Asterisks above bars are representing p-values of individual comparisons, those values being: $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

3. Aims of this thesis

Previous research performed in our lab revealed that *Sp5*$^{-/-}$ mutant mice, a model for NS, show early postnatal lethality that can be rescued by simultaneous ablation of proteases Klk5 and Klk7. In contrast, a single ablation of Klk5 results in only a partial rescue of NS model lethality, with severe cutaneous inflammation and skin barrier defects at P3-P5. The aim of this work is to elucidate the role of TNFα in the inflammatory phenotype of NS mouse models. Specifically, the aims were set as follows:

- Produce Generation of a *Tnfr1*$^{-/-}$ mice on the C57BL6/N background.
- Combination of *Tnfr1*$^{-/-}$ mice with *Klk5*$^{-/-}$*Spink5*$^{-/-}$ line
- Analysis of the gross, histologic and molecular phenotype of individual mouse models

4. Results

The primary aims of this work were twofold. First, we wanted to create and validate a *Tnfr1*$^{-/-}$ transgenic mouse model on the C57BL/6N genetic background. Second, cross-breeding with *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice on the C57BL/6N genetic background, creation of a *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ triple knockout mode and analyses of said strain for studying the role of TNFα signalling via the TNFR1 receptor on the *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mouse model.

4.1 Transgenic mouse design and production

In order to study KLKs role in inflammation, the *Tnfr1*$^{-/-}$ mouse was created with the utilization of the CRISPR/Cas9 system on the C57BL/6N mouse strain background. Targeting was designed to create a premature stop codon preceding the transmembrane domain that would result in a nonsense product without any functional activity. Typically, this results in rapid degradation of mRNA level via nonsense-mediated decay (NMD), degradation of the truncated protein, or a combination of both.

4.1.1 Cas9-mediated *Tnfr1* knockout design

Two gRNAs were designed in the CRISPOR software to target exon 3 and exon 4 respectively (Figure 4.1) and selected according to the CRISPOR scoring system for *in silico* assessment of on-target and off-target potential of respective gRNA sequences (Concordet and Haeussler, 2018). Zygote electroporation and subsequent implantation were performed according to its description in the 2.2.1 subchapter. Successful deletion of parts of exon 3 and 4 and the intron in between was verified by Sanger sequencing and founder mice were chosen accordingly (Figure 4.2). Created *Tnfr1*$^{-/-}$ mice were viable, with their phenotype being comparable to previously established *Tnfr1*$^{-/-}$ mouse models.

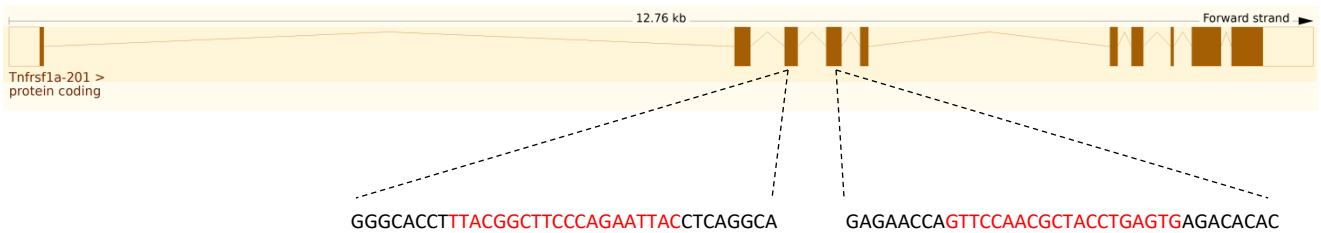


Figure 4.1 Targeting strategy of *Tnfrsf1a* (*Tnfr1*) locus. gRNA sequences are highlighted in red. Gene structure was adapted from Ensembl.org.

WT

GAACCTACTTGGTGAGTGACTGTCCGAGCCCAGGGCGGGATACAGTCTGC
AGGGAGTGTGAAAAGGGCACCTTTACGGCTTCCCAGAATTACCTCAGGCA
GTGTCTCAGTTGCAAGACATGTCGGAAAGGTAAGCCTTGGGATTGGGCCA
GGGCTATAGAAGGTGCATGGTGTGTGAAGACGTGCGAACATGTGTGTGTG
TCCGTGGGTGTTGGCCAGGAGGTCAGGATTTCGAATCTGCTCGTGAGTGT
GGCGGTAGTATGCATGCGTGCACATGCAAGCTCGGGCCTGTGTGCGTAGG
AGGAGTGTCTGTTACAAAGACGAATGCCATGTGGCAGAGCCAGGGGGCG
TCAAGATTTGTGTGGGAAAAGGGATGTGAGACTCACACACCATTTCCTTC
CCTCTTCAGAAATGTCCCAGGTGGAGATCTCTCCTTGCCAAGCTGACAAG
GACACGGTGTGTGGCTGTAAGGAGAACCAGTTCCAACGCTACCTGAGTGA
GACACACTTCCAGTGCGTGGACTGCAGCCCCTGCTTCAACGGCACCGTGA
CAATCCCCT

***Tnfr1*$^{-/-}$**

GAACCTACTTGGTGAGTGACTGTCCGAGCCCAGGGCGGGATACAGTCTGC
AGGGAGTGTGAAAAGGGCACC**TGA**GTGAGACACACTTCCAGTGCGTGGA
CTGCAGCCCCTGCTTCAACGGCACCGTGACAATCCCCT

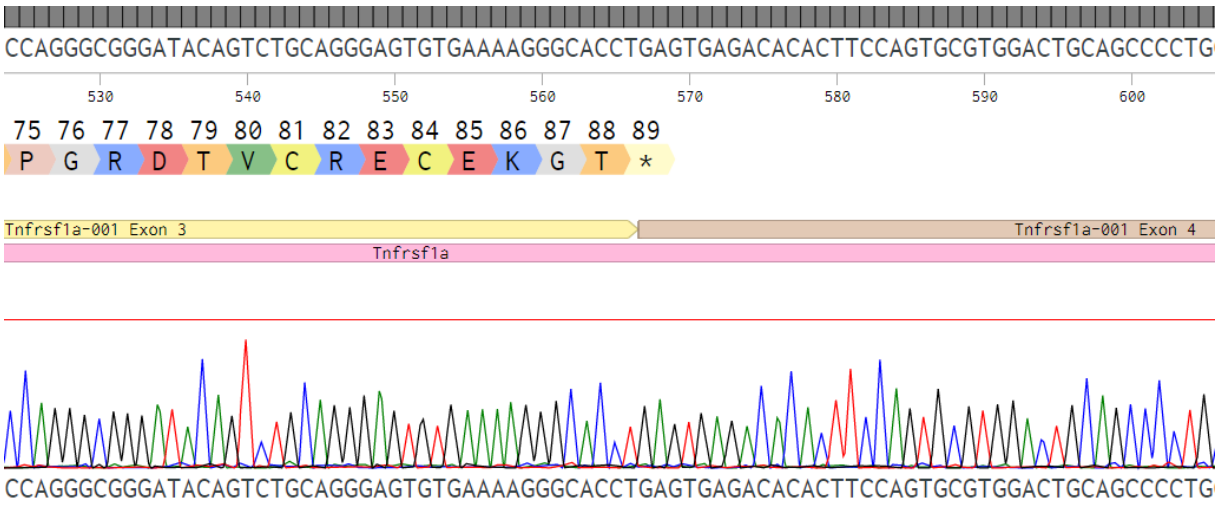


Figure 4.2 Comparison of WT and *Tnfr1*$^{-/-}$ sequence. Exon 3 and 4 sequences are highlighted with green shading and the intron 3 sequence is shaded yellow. Sequences of gRNAs are in red and the nascent stop codon is in bold. Sequencing results of the knockout allele are at the bottom of the picture (Downloaded from Benchling).

4.1.3 *Tnfr1* mRNA level analysis

Tnfr1 relative mRNA level was measured in *Tnfr1*$^{-/-}$ and WT mice by RT-qPCR to assess the effect of NMD on *Tnfr1* expression. This analysis revealed that WT and *Tnfr1*$^{-/-}$ mice exhibit comparable levels of *Tnfr1* mRNA Although the sample size was small (n=1), it was clear that NMD on its own does not significantly degrade *Tnfr1*$^{-/-}$ mRNA (Figure 4.3). RNA was also analysed by Sanger sequencing to exclude the option of exon skipping in *Tnfr1*$^{-/-}$ mice and the results clearly showed that the prevalent mRNA present in mice is the one that results in a premature stop codon (data not shown).

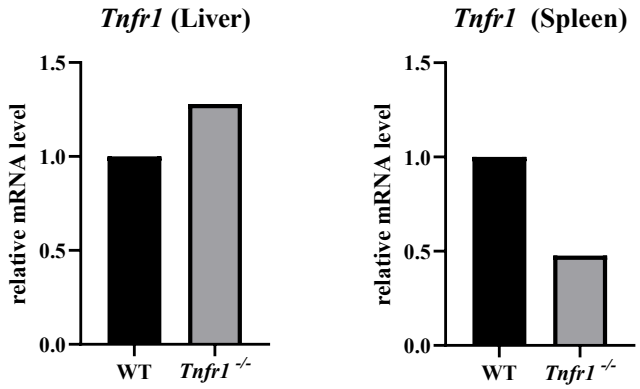


Figure 4.3 RT-qPCR analyses of *Tnfr1* mRNA in WT and knockout mice. NMD does not occur or only to a little degree in *Tnfr1*$^{-/-}$ mice; n = 1; *Actb* gene was used for normalization.

4.1.4 sTNFR1 protein level analysis

To assess the potential presence of TNFR1 protein and functionally verify the knockout, ELISA analysis was performed. Levels of sTNFR1 in serum ranged from 550 pg/mL to 650 pg/mL in WT mice and were undetectable in *Tnfr1-/-* mice (n = 3) (Figure 4.4). ELISA confirmed that generated *Tnfr1-/-* mice are indeed a functional knockout that is viable in further downstream applications. This result finalized analyses of the *Tnfr1*$^{-/-}$ mice validity as a functional knockout.

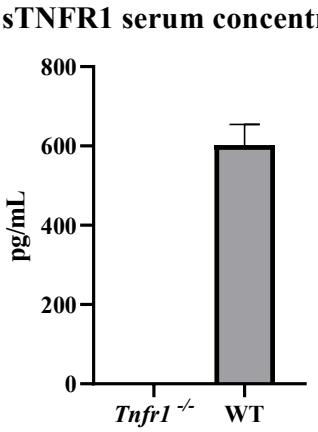


Figure 4.4 ELISA analysis of sTNFR1 levels. ELISA clearly shows the absence of sTNFR1 in the serum of *Tnfr1*$^{-/-}$ mice; n = 3.

4.2 Breeding and Analyses of *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice

Sp5$^{-/-}$ *Klk5*$^{-/-}$ mice exhibit a lethal phenotype due to the *Spink5* deficiency, which increased the difficulty of creating the *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice and the breeding scheme was set up accordingly. *Sp5*$^{+/-}$ *Klk5*$^{-/-}$ mice, which were previously created in our laboratory were bred to *Tnfr1*$^{-/-}$ to obtain the desired triple knockout mouse. *Sp5*$^{+/-}$ *Klk5*$^{-/-}$ mice were bred together to obtain the *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice. Due to apparent complications in breeding, if one was to aim for obtaining WT littermates, control WT mice were bred separately.

It is important to note, that *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice previously died at P5, but now only a negligible amount of this genotype survived to P3 with most dying on P2. This was attributed to probable changes in the environment of the animal facility where these mice were bred. Indeed, this hampers possible direct comparison with previously obtained data, which makes interpretation of the results difficult.

4.2.1 Visual analyses of *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice

Visual inspection of the phenotype of *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice did not reveal any significant features that would stand out when compared to *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice (Figure 4.5). Both genotypes showed a degree of peeling skin and slight growth retardation, probably due to transepidermal water loss as was observed for *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice in a previous analysis of said genotype. The average weight of both *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice was significantly lower than that of WT mice (Figure 4.6). This

was attributed primarily to transepidermal water loss that was previously observed in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice.

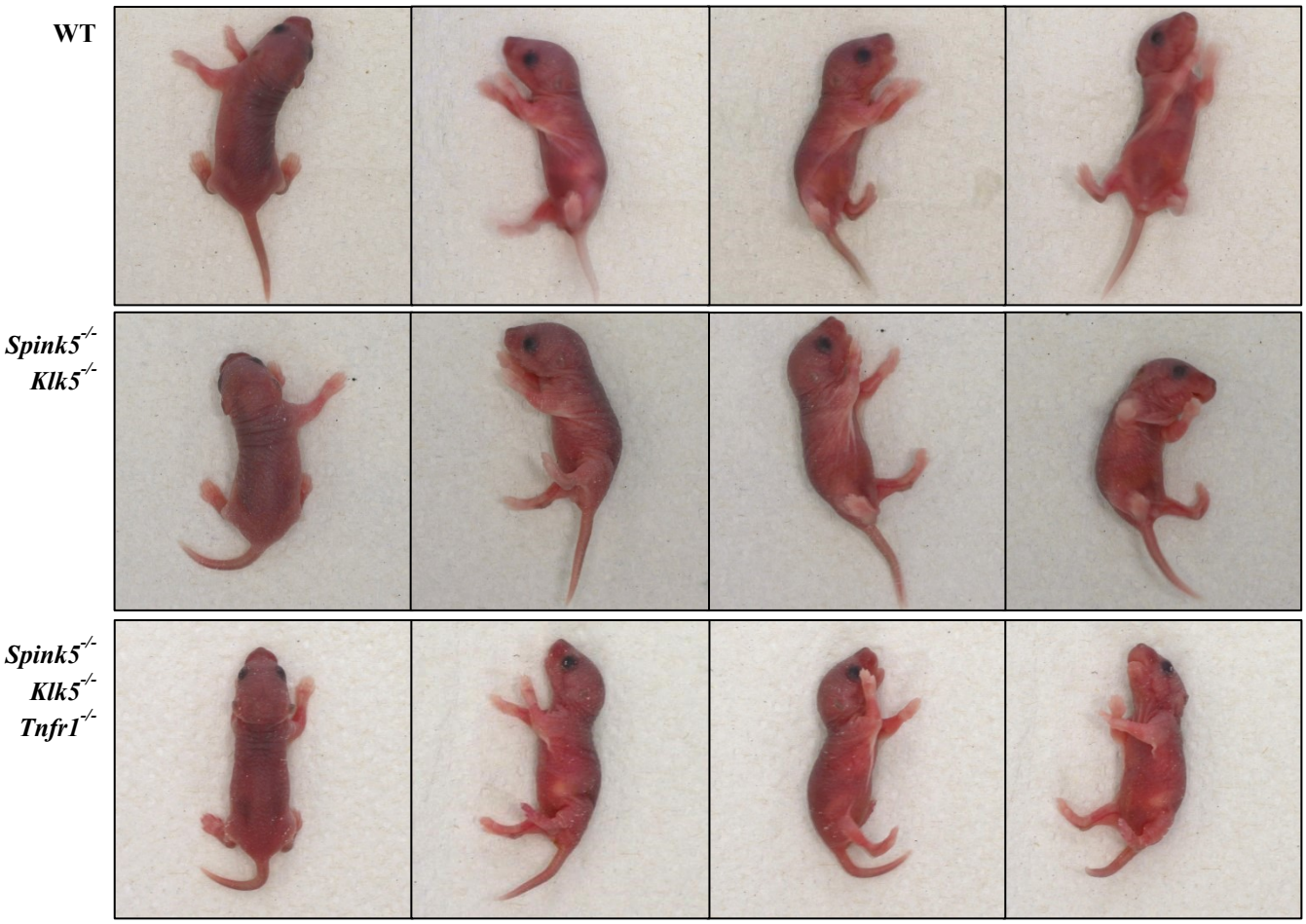


Figure 4.5 Phenotype of P2 pups. Compared to WT mice, peeling skin was visible both in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ and *Spink5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice. Condition of *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice seemed to be more severe, although negligibly.

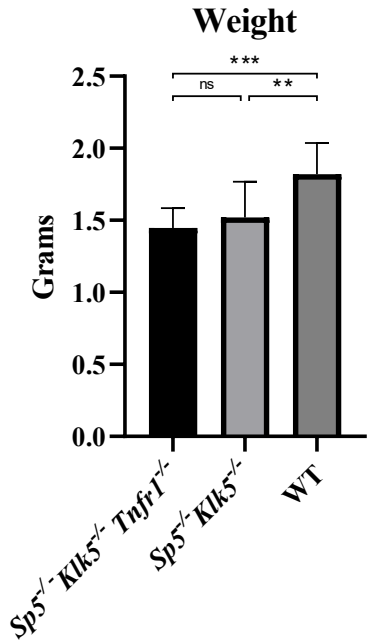


Figure 4.6 Weight of P2 mice. Differences in weight were readily apparent between WT and the *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice; n ≥ 7.

4.2.2 RNA expression analysis

Analysis of expression of inflammatory cytokines in the dorsal skin was done in order to assess the severity of inflammation progression by RT-qPCR analysis. Previously, *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice showed elevated expression levels of mostly T_H17- associated cytokines which were in line with the observation that NS-associated inflammation is predominantly mediated by the T_H17 axis.

Compared to *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice, *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice showed an increase in nearly all proinflammatory cytokines that were measured. Although the significance of these analyses was sometimes not enough, warranting additional confirmation, the trend was nearly ubiquitously present to various levels. The most significantly different cytokines that were expressed in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice compared to the other two genotypes were *Il1a*, *Il36* family members and *Ccl22. Ccl22* stands out in this analysis due to the fact that it had a heightened expression level exclusively in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice. It is likely that the expression of *Ccl20* is also severely heightened in some of the *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice, but the spread of obtained values did not achieve statistical significance. Interestingly, *Il1b* expression was not different between *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice. The only cytokine whose expression level was higher in WT compared to both knockout genotypes was *Il23a*, although the spread of obtained values in the case of WT was too wide to be statistically significant (Figure 4.7).

Surprisingly, it seems that inflammation is more severe *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice than in the *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice, but the enormous spread in the expression levels of some cytokines prevented us to conclusively determine the significance of obtained results for the whole panel. This was attributed to the fact that the epidermal damage was partially done by the mechanical chafing of mice, which is variable.

It is important to note that several additional cytokines were going to be included in this panel but their analysis failed, very likely due to the primers that were used. Unfortunately, these cytokines are extremely important in the studied context, namely *Il4*, *Il12*, *Il13*, *Il17a, Ccl17*, *Il22,* and *Ifng*. Future experiments would encompass additional analysis of these cytokines.

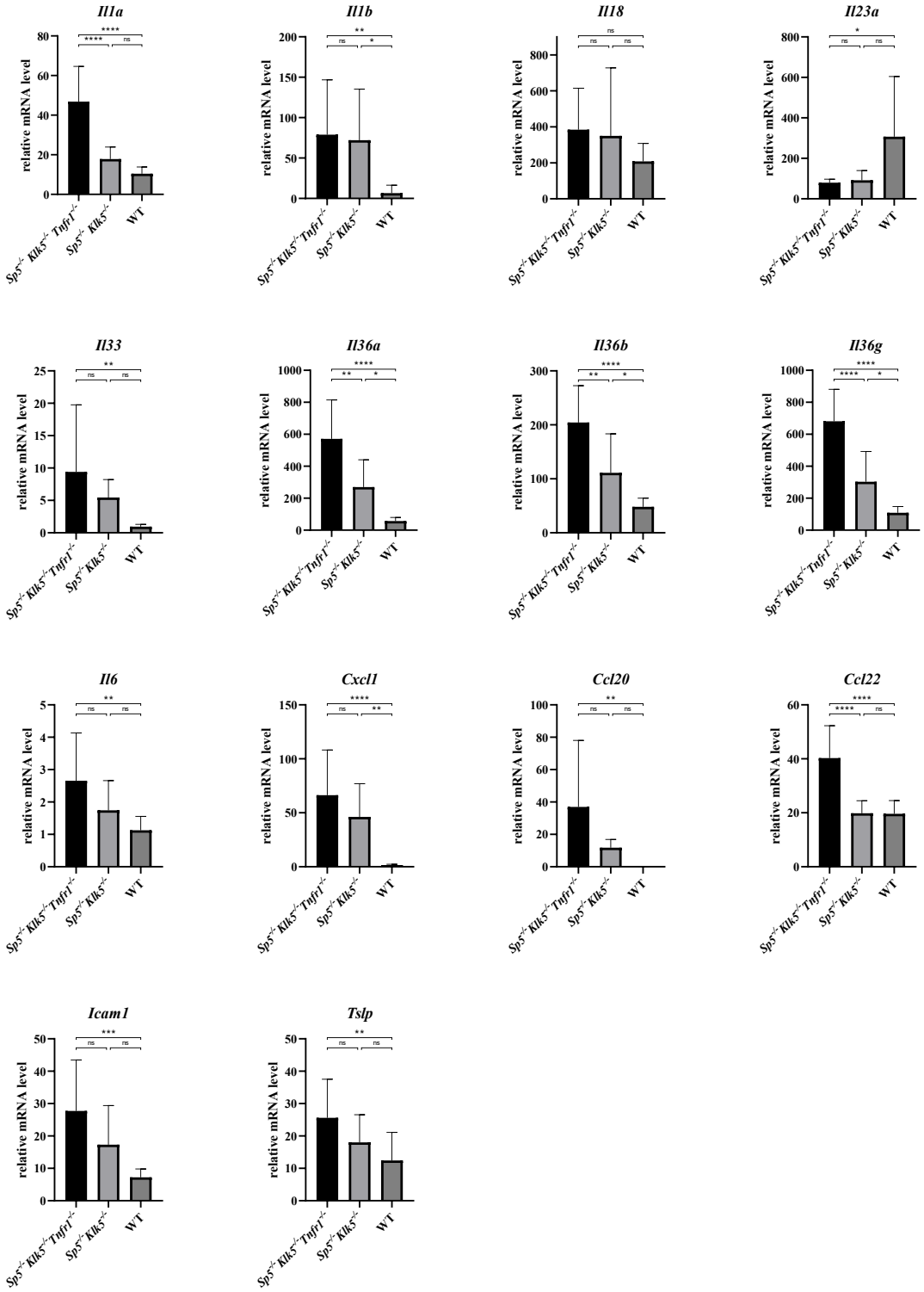


Figure 4.7 RT-qPCR analyses of inflammatory cytokines in the skin. *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice showed an overall increase in the expression of inflammatory cytokines across the whole panel with the expression *Il1a*, *Il36* family members and *Ccl22* being the most markedly upregulated. *Il23a* is the only cytokine that was significantly downregulated compared to WT mice; n ≥ 7; *Tbp* gene was used for normalization.

4.2.3 Histological analyses

To analyse the structural and immunological features of the dorsal skin of studied mice, two histological stainings were performed to assess them. Those staining were H & E staining and NASDCL staining. H & E staining was performed to assess the basic structural features and NASDCL was performed to assess the level of infiltration of granulocytes, predominantly the neutrophils.

4.2.3.1 H & E staining

H & E staining did not show any clear signs of structural differences among the studied genotypes (Figure 4.8). It is hard to determine if the features previously associated with *Sp5*$^{-/-}$ *Klk5*$^{-/-}$, like acanthosis, would manifest later since the time point at which those features were showed was at P5 compared to the P2 time point analysed in this thesis and how would the *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice be affected.

4.2.3.2 NASDCL staining

NASDCL staining did show a slight trend of increased granulocyte infiltration compared to WT mice both in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice (Figure 4.8) and subsequent evaluation of NASDCL positive cells did indeed point in this direction, albeit without statistical significance (Figure 4.9). It is important to note that sample sections showed varying levels of staining intensities, probably owing to the exact spots where the sections were cut.

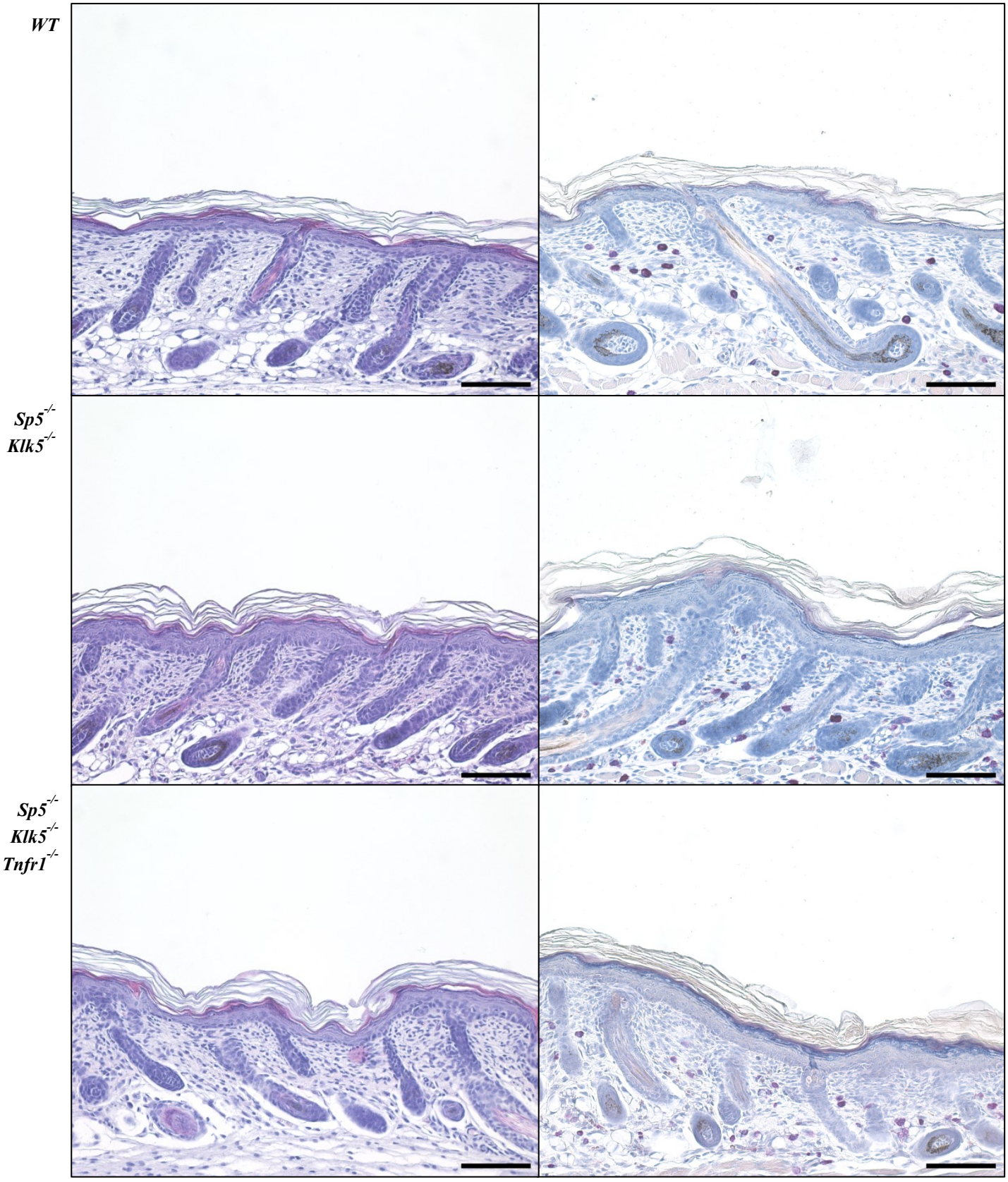


Figure 4.8 H & E staining and NASDCL staining. H & E staining is on the left side of the figure and NASDCL staining is on the right side. Purple cells in the NASDCL-stained sections are the ones showing NASDCL-specific esterase activity. Scale bars represent 100 μm.

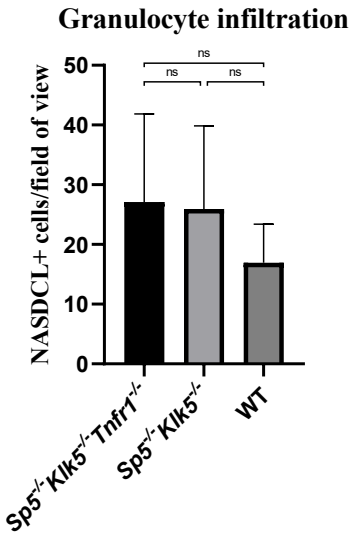


Figure 4.9 Statistical analysis of granulocyte infiltration. The trend of a slight increase in granulocyte infiltration was observed in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice, although without being statistically significant, warranting the need for additional analysis to conclusively confirm its relevance; $n \geq 3$.

5. Discussion

KLKs are increasingly appreciated as important contributors to inflammatory processes, especially in the skin (Nauroy and Nyström, 2020). Two of the most important KLKs in the skin in terms of inflammation, KLK5 and KLK7 have different means of how to elicit their contribution to inflammatory processes. Whereas the mechanism of inflammation induction via KLK5 has been mainly associated with PAR2 receptor signalling (Stefansson et al., 2008) and AMP processing (Yamasaki et al., 2006), the role of KLK7 and its mechanism is much less clear, with the known possible mechanisms being pro-IL-1β processing (Nylander-Lundqvist and Egelrud, 1997) or its contribution to hyperproliferation of keratinocytes (Ny and Egelrud, 2004). In this work, we aimed at addressing this by the production of a *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ transgenic mouse and its subsequent analysis. This would not only enable us to address this question but additionally, explore the possibility of using selective TNFR1 inhibitors in the treatment of cutaneous diseases in which KLK7 contributes to the disease-associated immune dysregulation, with the most emphasis being placed on the NS. Global TNFα knockout proved to be enough to rescue the lethal phenotype of *Sp5*$^{-/-}$*Klk5*$^{-/-}$ mice (Zingkou, 2018). However, targeted inhibition of TNFα signalling is a preferred option when possible, due to risks associated with disruption of such an important pathway in this manner (Fischer et al., 2020).

Successful production of the *Tnfr1*$^{-/-}$ mouse model was a necessary prerequisite to proceed with the main focus of this thesis. CRISPR/Cas9 system was chosen for this effort since its usefulness and flexibility for transgenic mice production is without parallel and it is quicker and cheaper to produce knockout mouse *de novo* by this system than to import already existing models. It was expected that due to the location of the newly created stop codon in exon 4, NMD would play a significant role and destroy nascent transcripts, but strangely, this was not the case even though appropriate design rules were followed (Popp and Maquat, 2016). Although the validity of the knockout was verified on the functional level by the detection of sTNFR1 protein in the serum of *Tnfr1*$^{-/-}$ mice, it has to be noted that the truncated mRNA may still be translated into non-functional truncated protein. Nevertheless, due to the nature of the editing, it is highly unlikely that these effects would result in

functional TNFR1-mediated signalling, which was independently validated by a collaborating laboratory (data not shown).

In the previous work on this topic in our laboratory, it was shown that *Sp5*$^{-/-}$ mice die almost immediately at P0, *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice at P5 and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Klk7*$^{-/-}$ have their lethal phenotype rescued and live up to adulthood, although with slight differences when compared to WT mice (Kasparek et al., 2017). Surprisingly, *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice exhibited a different phenotype upon changing the animal breeding facility, with most of them dying on P2 instead of P5. This may be because the environment somehow changed in regards to the microbiota present in the animal facility in which these mice were bred. The cutaneous microbiome is a key aspect in the homeostasis of the skin and as was discussed in the literature review, plays a key role in the associated diseases. It was previously published that colonization of the skin with various microbial populations and their proportional distribution in the skin of NS patients results in various levels in the severity of disease progression (Williams et al., 2020). This issue brings forth two aspects of interest. Firstly, due to the aforementioned discrepancies, any direct immune comparison with previously published work is impossible. Secondly, it highlights the importance of the microbiome in this particular disease and maybe even warrants periodic screenings of skin microbial populations to ensure the validity and comparability of measured data.

As discussed in the literature review, TNFR1-mediated signalling leads primarily to pro-inflammatory effects, whereas TNFR2 largely elicits regulatory outcomes (Dostert et al., 2019). However, in the cutaneous context, several aspects of TNFα signalling may result in pro-inflammatory effects in the absence of TNFR1 signalling. TNFα signalling is a key aspect of processes like immune response (Kalliolias and Ivashkiv, 2016) and wound healing (Nosenko et al., 2019). Although global TNFα inhibition may be beneficial to reduce its overall deleterious effects, selective inhibition of either receptor is likely to dysregulate normal TNFα signalling that may result in unforeseeable effects that ultimately result in a further increment in the severity of the pathology. Additionally, ablation of TNFR1 would likely result in an increased abundance of mTNF that under normal circumstances engages mTNFR1. This would result in increased potential for signalling via the TNFR2 receptor and also for reverse signalling through the mTNF itself by engagement of TNFR2. Whereas the effect of an increase in TNFR2-mediated signalling is hard to estimate

since it has the potential to ultimately elicit similar responses as TNFR1 under specific circumstances, it has been previously published that reverse signalling by mTNF/TNFR2 (Qu et al., 2017) has one key effect that may significantly alter the cutaneous environment, although not experimentally proven to be relevant in epidermal cells. Keratinocyte differentiation is positively regulated by the presence of calcium and this type of signalling results in an increase of calcium concentration by activation of ERK1/2, thus speeding up this process. Additionally, this signalling directly increases TNFα production which may result in a positive feedback loop that would further dysregulate the regular keratinocyte differentiation process (Rossol et al., 2007).

Several of the cytokines whose expression levels were significantly increased compared both to WT and *Sp5*$^{-/-}$*Klk5*$^{-/-}$ mice are very strongly associated with barrier defence and associated inflammation, namely IL-1α (Di Paolo and Shayakhmetov, 2016) and the IL-36 subfamily (Buhl and Wenzel, 2019), both being a part of the IL-1 superfamily of cytokines. In contrast to IL-1β, IL-1α is produced under homeostatic conditions and has been attributed many roles, especially in epithelial tissues. In the context of our mouse model, its role as an alarmin and local inflammatory driver may be the most important since epidermal damage is one of the central features present in these mice and thus *Il1a* overexpression may signal more severe epidermal damage. IL-36 subfamily has been experimentally proven as being a key player in the epidermal compartment of the skin. In a recent publication, it was shown that IL-36 and especially IL-36α played a crucial role in initiation and sustainment of the inflammatory state upon an epidermal challenge by *S. aureus*, whereas IL-1β was not playing a significant role, only doing so after intradermal challenge (Liu et al., 2017). In the light of our results, one of the possible explanations is that TNFR1 ablation may result in a decreased capacity for microbe removal from the epidermis in a timely manner, which is fueling the inflammation in which IL-36 subfamily cytokines play a significant role. Expression of *Il1b* was similar between *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice, suggesting that the epidermal barrier was not further compromised. Additional experiments are necessary to validate all of these hypotheses. Furthermore, *Ccl22* expression was elevated in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice compared both to *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ and WT mice. This suggests a possible more significant role of T_H2 and T_{reg} cells (Yoshie and Matsushima, 2015) in the phenotype of *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice.

Lastly, *Il23a* expression was lower both in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ and *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice compared to WT mice, although the difference was not enough to be statistically significant in the case of *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ /WT mice comparison. This comes as a surprise since IL-23 is a driver of T_H17 cells expansion and maintenance (Gaffen et al., 2014) and T_H17 cells play a substantial role in the pathology of NS (Petrova and Hovnanian, 2020).

6. Conclusions and prospects

This work aimed to elucidate the role of the TNFα signalling pathway in the inflammatory phenotype of NS mouse models. Our main findings are as follows:

- *Tnfr1*$^{-/-}$ mice were successfully produced by CRISPR based mutagenesis. Ablation of TNFR1 on the protein level was demonstrated by ELISA. The mice are viable and their phenotype is comparable to previously established TNFR1 deficient mouse models.

- Environmental factors play a significant role in the phenotypic manifestations of NS mouse models, as different breeding facilities altered the resulting phenotype of previously studied *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice.

- TNFR1 ablation does not rescue the inflammatory phenotype of *Sp5*$^{-/-}$*Klk5*$^{-/-}$ mice. *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ are not viable and most of them die at P2, comparably to *Sp5*$^{-/-}$ *Klk5*$^{-/-}$. Analysis of cytokine expression suggests that the inflammatory phenotype is even more severe than in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mice.

In conclusion, this work presents a novel mouse model for the study of KLKs in the context of NS and the effects of TNFR1 signalling on the previously established *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ mouse model. Although a thorough assessment of the phenotype of *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice was not done at the time of admission of this thesis, it is clear that the ablation of TNFR1 in *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice does not alleviate the inflammatory phenotype. In extension, *Sp5*$^{-/-}$ *Klk5*$^{-/-}$ *Tnfr1*$^{-/-}$ mice provide a valuable platform for the study of TNFR1 ablation in the context of KLK-associated pathologies like the NS.

7. References

* Anamthathmakula, P., and Winuthayanon, W. (2020). Mechanism of semen liquefaction and its potential for a novel non-hormonal contraception†. Biology of Reproduction *103*, 411–426.

Annibaldi, A., Wicky John, S., Vanden Berghe, T., Swatek, K.N., Ruan, J., Liccardi, G., Bianchi, K., Elliott, P.R., Choi, S.M., Van Coillie, S., et al. (2018). Ubiquitin-Mediated Regulation of RIPK1 Kinase Activity Independent of IKK and MK2. Mol Cell *69*, 566-580.e5.

Apfelbacher, C.J., Diepgen, T.L., and Schmitt, J. (2011). Determinants of eczema: population-based cross-sectional study in Germany. Allergy *66*, 206–213.

Bartlett, J.D., and Simmer, J.P. (2014). Kallikrein-related peptidase-4 (KLK4): role in enamel formation and revelations from ablated mice. Front Physiol *5*, 240.

Baurecht, H., Irvine, A.D., Novak, N., Illig, T., Bühler, B., Ring, J., Wagenpfeil, S., and Weidinger, S. (2007). Toward a major risk factor for atopic eczema: meta-analysis of filaggrin polymorphism data. J Allergy Clin Immunol *120*, 1406–1412.

* Bhatt, D., and Ghosh, S. (2014). Regulation of the NF-κB-Mediated Transcription of Inflammatory Genes. Front. Immunol. *0*.

Bitoun, E., Micheloni, A., Lamant, L., Bonnart, C., Tartaglia-Polcini, A., Cobbold, C., Al Saati, T., Mariotti, F., Mazereeuw-Hautier, J., Boralevi, F., et al. (2003). LEKTI proteolytic processing in human primary keratinocytes, tissue distribution and defective expression in Netherton syndrome. Human Molecular Genetics *12*, 2417–2430.

* Bodmer, J.-L., Schneider, P., and Tschopp, J. (2002). The molecular architecture of the TNF superfamily. Trends in Biochemical Sciences *27*, 19–26.

Borgoño, C.A., Michael, I.P., Komatsu, N., Jayakumar, A., Kapadia, R., Clayman, G.L., Sotiropoulou, G., and Diamandis, E.P. (2007). A Potential Role for Multiple Tissue Kallikrein Serine Proteases in Epidermal Desquamation *. Journal of Biological Chemistry *282*, 3640–3652.

* Brenner, D., Blaser, H., and Mak, T.W. (2015). Regulation of tumour necrosis factor signalling: live or let die. Nat Rev Immunol *15*, 362–374.

Brunner, P.M., Khattri, S., Garcet, S., Finney, R., Oliva, M., Dutt, R., Fuentes-Duculan, J., Zheng, X., Li, X., Bonifacio, K.M., et al. (2016). A mild topical steroid leads to progressive anti-inflammatory effects in the skin of patients with moderate-to-severe atopic dermatitis. J Allergy Clin Immunol *138*, 169–178.

* Buddenkotte, J., and Steinhoff, M. (2018). Recent advances in understanding and managing rosacea. F1000Res *7*, F1000 Faculty Rev-1885.

* Buhl, A.-L., and Wenzel, J. (2019). Interleukin-36 in Infectious and Inflammatory Skin Diseases. Front Immunol *10*, 1162.

Buhl, T., Sulk, M., Nowak, P., Buddenkotte, J., McDonald, I., Aubert, J., Carlavan, I., Déret, S., Reiniche, P., Rivier, M., et al. (2015). Molecular and Morphological Characterization of Inflammatory Infiltrate in Rosacea Reveals Activation of Th1/Th17 Pathways. J Invest Dermatol *135*, 2198–2208.

* Canavan, T.N., Elmets, C.A., Cantrell, W.L., Evans, J.M., and Elewski, B.E. (2016). Anti-IL-17 Medications Used in the Treatment of Plaque Psoriasis and Psoriatic Arthritis: A Comprehensive Review. Am J Clin Dermatol *17*, 33–47.

Capon, F., Semprini, S., Dallapiccola, B., and Novelli, G. (1999). Evidence for Interaction between Psoriasis-Susceptibility Loci on Chromosomes 6p21 and 1q21. Am J Hum Genet *65*, 1798–1800.

* Carr, W.W. (2013). Topical calcineurin inhibitors for atopic dermatitis: review and treatment recommendations. Paediatr Drugs *15*, 303–310.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A *72*, 3666–3670.

Caubet, C., Jonca, N., Brattsand, M., Guerrin, M., Bernard, D., Schmidt, R., Egelrud, T., Simon, M., and Serre, G. (2004). Degradation of Corneodesmosome Proteins by Two Serine Proteases of the Kallikrein Family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J Invest Dermatol *122*, 1235–1244.

Chang, A.L.S., Raber, I., Xu, J., Li, R., Spitale, R., Chen, J., Kiefer, A.K., Tian, C., Eriksson, N.K., Hinds, D.A., et al. (2015). Assessment of the Genetic Basis of Rosacea by Genome-Wide Association Study. J Invest Dermatol *135*, 1548–1555.

* Chen, Y.-L., Hardman, C.S., Yadava, K., and Ogg, G. (2020). Innate Lymphocyte Mechanisms in Skin Diseases. Annu Rev Immunol *38*, 171–202.

Cole, C., Kroboth, K., Schurch, N.J., Sandilands, A., Sherstnev, A., O'Regan, G.M., Watson, R.M., McLean, W.H.I., Barton, G.J., Irvine, A.D., et al. (2014). Filaggrin-stratified transcriptomic analysis of pediatric skin identifies mechanistic pathways in patients with atopic dermatitis. J Allergy Clin Immunol *134*, 82–91.

Concordet, J.-P., and Haeussler, M. (2018). CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. Nucleic Acids Research *46*, W242–W245.

* Correale, C.E., Walker, C., Murphy, L., and Craig, T.J. (1999). Atopic Dermatitis: A Review of Diagnosis and Treatment. AFP *60*, 1191.

Deraison, C., Bonnart, C., Lopez, F., Besson, C., Robinson, R., Jayakumar, A., Wagberg, F., Brattsand, M., Hachem, J.P., Leonardsson, G., et al. (2007). LEKTI Fragments Specifically Inhibit KLK5, KLK7, and KLK14 and Control Desquamation through a pH-dependent Interaction. MBoC *18*, 3607–3619.

Descargues, P., Deraison, C., Prost, C., Fraitag, S., Mazereeuw-Hautier, J., D'Alessio, M., Ishida-Yamamoto, A., Bodemer, C., Zambruno, G., and Hovnanian, A. (2006). Corneodesmosomal cadherins are preferential targets of stratum corneum trypsin- and

chymotrypsin-like hyperactivity in Netherton syndrome. J Invest Dermatol *126*, 1622–1632.

Di, W.-L., Larcher, F., Semenova, E., Talbot, G.E., Harper, J.I., Del Rio, M., Thrasher, A.J., and Qasim, W. (2011). Ex-vivo gene therapy restores LEKTI activity and corrects the architecture of Netherton syndrome-derived skin grafts. Mol Ther *19*, 408–416.

* Di Paolo, N.C., and Shayakhmetov, D.M. (2016). Interleukin 1α and the inflammatory process. Nat Immunol *17*, 906–913.

* Diani, M., Altomare, G., and Reali, E. (2016). T Helper Cell Subsets in Clinical Manifestations of Psoriasis. J Immunol Res *2016*, 7692024.

Dombrowski, Y., and Schauber, J. (2012). Cathelicidin LL-37: a defense molecule with a potential role in psoriasis pathogenesis. Exp Dermatol *21*, 327–330.

* Dostert, C., Grusdat, M., Letellier, E., and Brenner, D. (2019). The TNF Family of Ligands and Receptors: Communication Modules in the Immune System and Beyond. Physiol Rev *99*, 115–160.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell *102*, 33–42.

Eissa, A., Amodeo, V., Smith, C.R., and Diamandis, E.P. (2011). Kallikrein-related peptidase-8 (KLK8) is an active serine protease in human epidermis and sweat and is involved in a skin barrier proteolytic cascade. J Biol Chem *286*, 687–706.

Eissa, A., Cretu, D., Soosaipillai, A., Thavaneswaran, A., Pellett, F., Diamandis, A., Cevikbas, F., Steinhoff, M., Diamandis, E.P., Gladman, D., et al. (2013). Serum kallikrein-8 correlates with skin activity, but not psoriatic arthritis, in patients with psoriatic disease. Clinical Chemistry and Laboratory Medicine *51*, 317–325.

* Emami, N., and Diamandis, E.P. (2007). New insights into the functional mechanisms and clinical applications of the kallikrein-related peptidase family. Molecular Oncology *1*, 269–287.

Eränkö, E., Ilander, M., Tuomiranta, M., Mäkitie, A., Lassila, T., Kreutzman, A., Klemetti, P., Mustjoki, S., Hannula-Jouppi, K., and Ranki, A. (2018). Immune cell phenotype and functional defects in Netherton syndrome. Orphanet Journal of Rare Diseases *13*, 213.

Erickson, T.R., Murphrey, M.B., Abu-Zayed, H., Wu, B., Ibler, E., Rangel, S.M., and Paller, A.S. (2020). Transepidermal water loss in the orphan forms of ichthyosis. Pediatric Dermatology *37*, 771–773.

Fischer, J., Wu, Z., Kantyka, T., Sperrhacke, M., Dimitrieva, O., Koblyakova, Y., Ahrens, K., Graumann, N., Baurecht, H., Reiss, K., et al. (2014). Characterization of Spink6 in Mouse Skin: The Conserved Inhibitor of Kallikrein-Related Peptidases Is Reduced by Barrier Injury. J Invest Dermatol *134*, 1305–1312.

* Fischer, R., Kontermann, R.E., and Pfizenmaier, K. (2020). Selective Targeting of TNF Receptors as a Novel Therapeutic Approach. Front. Cell Dev. Biol. *0*.

Freeman, A.K., Linowski, G.J., Brady, C., Lind, L., Vanveldhuisen, P., Singer, G., and Lebwohl, M. (2003). Tacrolimus ointment for the treatment of psoriasis on the face and intertriginous areas. J Am Acad Dermatol *48*, 564–568.

* Furio, L., and Hovnanian, A. (2014). Netherton syndrome: defective kallikrein inhibition in the skin leads to skin inflammation and allergy. Biological Chemistry *395*, 945–958.

* Gaffen, S.L., Jain, R., Garg, A.V., and Cua, D.J. (2014). The IL-23–IL-17 immune axis: from mechanisms to therapeutic testing. Nat Rev Immunol *14*, 585–600.

* Gandhi, N.A., Bennett, B.L., Graham, N.M.H., Pirozzi, G., Stahl, N., and Yancopoulos, G.D. (2016). Targeting key proximal drivers of type 2 inflammation in disease. Nat Rev Drug Discov *15*, 35–50.

* Garcovich, S., De Simone, C., Genovese, G., Berti, E., Cugno, M., and Marzano, A.V. (2019). Paradoxical Skin Reactions to Biologics in Patients With Rheumatologic Disorders. Front Pharmacol *10*, 282.

* Garritsen, F.M., Brouwer, M.W.D., Limpens, J., and Spuls, P.I. (2014). Photo(chemo)therapy in the management of atopic dermatitis: an updated systematic review with implications for practice and research. Br J Dermatol *170*, 501–513.

* Gerber, P.A., Buhren, B.A., Schrumpf, H., Homey, B., Zlotnik, A., and Hevezi, P. (2014). The top skin-associated genes: a comparative analysis of human and mouse skin transcriptomes. Biological Chemistry *395*, 577–591.

* Gilhar, A., Reich, K., Keren, A., Kabashima, K., Steinhoff, M., and Paus, R. (2021). Mouse models of atopic dermatitis: a critical reappraisal. Experimental Dermatology *30*, 319–336.

Gittler, J.K., Shemer, A., Suárez-Fariñas, M., Fuentes-Duculan, J., Gulewicz, K.J., Wang, C.Q.F., Mitsui, H., Cardinale, I., de Guzman Strong, C., Krueger, J.G., et al. (2012). Progressive activation of Th2/Th22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. J Allergy Clin Immunol *130*, 1344–1354.

Goldminz, A.M., Suárez-Fariñas, M., Wang, A.C., Dumont, N., Krueger, J.G., and Gottlieb, A.B. (2016). Methotrexate improves pro- and anti-atherogenic genomic expression in psoriatic skin. Journal of Dermatological Science *82*, 207–209.

* Gooz, M. (2010). ADAM-17: The Enzyme That Does It All. Crit Rev Biochem Mol Biol *45*, 146–169.

Gouin, O., Barbieux, C., Leturcq, F., Bonnet des Claustres, M., Petrova, E., and Hovnanian, A. (2020). Transgenic Kallikrein 14 Mice Display Major Hair Shaft Defects Associated with Desmoglein 3 and 4 Degradation, Abnormal Epidermal Differentiation, and IL-36 Signature. J Invest Dermatol *140*, 1184–1194.

* Greb, J.E., Goldminz, A.M., Elder, J.T., Lebwohl, M.G., Gladman, D.D., Wu, J.J., Mehta, N.N., Finlay, A.Y., and Gottlieb, A.B. (2016). Psoriasis. Nat Rev Dis Primers *2*, 1–17.

Haas, T.L., Emmerich, C.H., Gerlach, B., Schmukle, A.C., Cordier, S.M., Rieser, E., Feltham, R., Vince, J., Warnken, U., Wenger, T., et al. (2009). Recruitment of the Linear Ubiquitin Chain Assembly Complex Stabilizes the TNF-R1 Signaling Complex and Is Required for TNF-Mediated Gene Induction. Molecular Cell *36*, 831–844.

* Heuberger, D.M., and Schuepbach, R.A. (2019). Protease-activated receptors (PARs): mechanisms of action and potential therapeutic modulators in PAR-driven inflammatory diseases. Thrombosis Journal *17*, 4.

* Holbrook, J., Lara-Reyna, S., Jarosz-Griffiths, H., and McDermott, M.F. (2019). Tumour necrosis factor signalling in health and disease. F1000Res *8*, 111.

Hönzke, S., Wallmeyer, L., Ostrowski, A., Radbruch, M., Mundhenk, L., Schäfer-Korting, M., and Hedtrich, S. (2016). Influence of Th2 Cytokines on the Cornified Envelope, Tight Junction Proteins, and ß-Defensins in Filaggrin-Deficient Skin Equivalents. J Invest Dermatol *136*, 631–639.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495–504.

Igawa, S., Kishibe, M., Minami-Hori, M., Honma, M., Tsujimura, H., Ishikawa, J., Fujimura, T., Murakami, M., and Ishida-Yamamoto, A. (2017). Incomplete KLK7 Secretion and Upregulated LEKTI Expression Underlie Hyperkeratotic Stratum Corneum in Atopic Dermatitis. J Invest Dermatol *137*, 449–456.

Iinuma, S., Kishibe, M., Saito, N., Igawa, S., Honma, M., Takahashi, H., Bando, Y., Yoshida, S., Iizuka, H., and Ishida-Yamamoto, A. (2015). Klk8 is required for microabscess formation in a mouse imiquimod model of psoriasis. Experimental Dermatology *24*, 887–889.

Iinuma, S., Kishibe, M., Saito, N., Igawa, S., Honma, M., Bando, Y., Yoshida, S., and Ishida-Yamamoto, A. (2017). Kallikrein-related peptidase 6 promotes psoriasiform skin inflammation through a protease-activated receptor 2-independent mechanism. Experimental Dermatology *26*, 289–291.

* Irvine, A.D., McLean, W.H.I., and Leung, D.Y.M. (2011). Filaggrin mutations associated with skin and allergic diseases. N Engl J Med *365*, 1315–1327.

* Israël, A. (2010). The IKK Complex, a Central Regulator of NF-κB Activation. Cold Spring Harb Perspect Biol *2*, a000158.

* Jarmuda, S., O'Reilly, N., Żaba, R., Jakubowicz, O., Szkaradkiewicz, A., and Kavanagh, K. (2012). Potential role of Demodex mites and bacteria in the induction of rosacea. J Med Microbiol *61*, 1504–1510.

Jenickova, I., Kasparek, P., Petrezselyova, S., Elias, J., Prochazka, J., Kopkanova, J., Navratil, M., Barinka, C., and Sedlacek, R. (2021). Efficient allele conversion in

mouse zygotes and primary cells based on electroporation of Cre protein. Methods *191*, 87–94.

* Jeon, C., Sekhon, S., Yan, D., Afifi, L., Nakamura, M., and Bhutani, T. (2017). Monoclonal antibodies inhibiting IL-12, -23, and -17 for the treatment of psoriasis. Hum Vaccin Immunother *13*, 2247–2259.

* Jørgensen, A.-H.R., Egeberg, A., Gideonsson, R., Weinstock, L.B., Thyssen, E.P., and Thyssen, J.P. (2017). Rosacea is associated with Helicobacter pylori: a systematic review and meta-analysis. J Eur Acad Dermatol Venereol *31*, 2010–2015.

* Juhász, K., Buzás, K., and Duda, E. (2013). Importance of reverse signaling of the TNF superfamily in immune regulation. Expert Rev Clin Immunol *9*, 335–348.

* Kabashima, K., Honda, T., Ginhoux, F., and Egawa, G. (2019). The immunological anatomy of the skin. Nat Rev Immunol *19*, 19–30.

* Kalliolias, G.D., and Ivashkiv, L.B. (2016). TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nat Rev Rheumatol *12*, 49–62.

Kasparek, P., Ileninova, Z., Zbodakova, O., Kanchev, I., Benada, O., Chalupsky, K., Brattsand, M., Beck, I.M., and Sedlacek, R. (2017). KLK5 and KLK7 Ablation Fully Rescues Lethality of Netherton Syndrome-Like Phenotype. PLoS Genet *13*, e1006566.

* Kayashima, Y., Smithies, O., and Kakoki, M. (2012). The kallikrein–kinin system and oxidative stress. Current Opinion in Nephrology and Hypertension *21*, 92–96.

Kim, J.Y., Kim, Y.J., Lim, B.J., Sohn, H.J., Shin, D., and Oh, S.H. (2014). Increased expression of cathelicidin by direct activation of protease-activated receptor 2: possible implications on the pathogenesis of rosacea. Yonsei Med J *55*, 1648–1655.

Klucky, B., Mueller, R., Vogt, I., Teurich, S., Hartenstein, B., Breuhahn, K., Flechtenmacher, C., Angel, P., and Hess, J. (2007). Kallikrein 6 Induces E-Cadherin Shedding and Promotes Cell Proliferation, Migration, and Invasion. Cancer Res *67*, 8198–8206.

Komatsu, N., Saijoh, K., Kuk, C., Shirasaki, F., Takehara, K., and Diamandis, E.P. (2007). Aberrant human tissue kallikrein levels in the stratum corneum and serum of patients with psoriasis: dependence on phenotype, severity and therapy. British Journal of Dermatology *156*, 875–883.

Kong, H.H., Oh, J., Deming, C., Conlan, S., Grice, E.A., Beatson, M.A., Nomicos, E., Polley, E.C., Komarow, H.D., Murray, P.R., et al. (2012). Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res *22*, 850–859.

* Kontermann, R.E., Scheurich, P., and Pfizenmaier, K. (2009). Antagonists of TNF action: clinical experience and new developments. Expert Opin Drug Discov *4*, 279–292.

Lawlor, K.E., Feltham, R., Yabal, M., Conos, S.A., Chen, K.W., Ziehe, S., Graß, C., Zhan, Y., Nguyen, T.A., Hall, C., et al. (2017). XIAP Loss Triggers RIPK3- and Caspase-8-Driven IL-1β Activation and Cell Death as a Consequence of TLR-MyD88-Induced cIAP1-TRAF2 Degradation. Cell Rep *20*, 668–682.

* Lebwohl, M., Drake, L., Menter, A., Koo, J., Gottlieb, A.B., Zanolli, M., Young, M., and McClelland, P. (2001). Consensus conference: acitretin in combination with UVB or PUVA in the treatment of psoriasis. J Am Acad Dermatol *45*, 544–553.

Lebwohl, M.G., Breneman, D.L., Goffe, B.S., Grossman, J.R., Ling, M.R., Milbauer, J., Pincus, S.H., Sibbald, R.G., Swinyer, L.J., Weinstein, G.D., et al. (1998). Tazarotene 0.1% gel plus corticosteroid cream in the treatment of plaque psoriasis. J Am Acad Dermatol *39*, 590–596.

Lee, Y.A., Rüschendorf, F., Windemuth, C., Schmitt-Egenolf, M., Stadelmann, A., Nürnberg, G., Ständer, M., Wienker, T.F., Reis, A., and Traupe, H. (2000). Genomewide scan in german families reveals evidence for a novel psoriasis-susceptibility locus on chromosome 19p13. Am J Hum Genet *67*, 1020–1024.

* Li, B., Huang, L., Lv, P., Li, X., Liu, G., Chen, Y., Wang, Z., Qian, X., Shen, Y., Li, Y., et al. (2020). The role of Th17 cells in psoriasis. Immunol Res *68*, 296–309.

Li, J., McQuade, T., Siemer, A.B., Napetschnig, J., Moriwaki, K., Hsiao, Y.-S., Damko, E., Moquin, D., Walz, T., McDermott, A., et al. (2012). The RIP1/RIP3 Necrosome Forms a Functional Amyloid Signaling Complex Required for Programmed Necrosis. Cell *150*, 339–350.

Li, S.J., Perez-Chada, L.M., and Merola, J.F. (2019). TNF Inhibitor-Induced Psoriasis: Proposed Algorithm for Treatment and Management. J Psoriasis Psoriatic Arthritis *4*, 70–80.

Liu, H., Archer, N.K., Dillen, C.A., Wang, Y., Ashbaugh, A.G., Ortines, R.V., Kao, T., Lee, S.K., Cai, S.S., Miller, R.J., et al. (2017). Staphylococcus aureus Epicutaneous Exposure Drives Skin Inflammation via IL-36-Mediated T Cell Responses. Cell Host Microbe *22*, 653-666.e5.

* Lodén, M. (2003). Role of topical emollients and moisturizers in the treatment of dry skin barrier disorders. Am J Clin Dermatol *4*, 771–788.

Loessner, D., Quent, V.M.C., Kraemer, J., Weber, E.C., Hutmacher, D.W., Magdolen, V., and Clements, J.A. (2012). Combined expression of KLK4, KLK5, KLK6, and KLK7 by ovarian cancer cells leads to decreased adhesion and paclitaxel-induced chemoresistance. Gynecologic Oncology *127*, 569–578.

Lonne-Rahm, S., Nordlind, K., Edström, D.W., Ros, A.-M., and Berg, M. (2004). Laser treatment of rosacea: a pathoetiological study. Arch Dermatol *140*, 1345–1349.

Ma, H.-L., Liang, S., Li, J., Napierata, L., Brown, T., Benoit, S., Senices, M., Gill, D., Dunussi-Joannopoulos, K., Collins, M., et al. (2008). IL-22 is required for Th17 cell–mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin Invest *118*, 597–607.

Mahoney, D.J., Cheung, H.H., Mrad, R.L., Plenchette, S., Simard, C., Enwere, E., Arora, V., Mak, T.W., Lacasse, E.C., Waring, J., et al. (2008). Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc Natl Acad Sci U S A *105*, 11778–11783.

* Masson-Meyers, D.S., Andrade, T.A.M., Caetano, G.F., Guimaraes, F.R., Leite, M.N., Leite, S.N., and Frade, M.A.C. (2020). Experimental models and methods for cutaneous wound healing assessment. International Journal of Experimental Pathology *101*, 21–37.

* Mella, C., Figueroa, C.D., Otth, C., and Ehrenfeld, P. (2020). Involvement of Kallikrein-Related Peptidases in Nervous System Disorders. Front. Cell. Neurosci. *14*.

Meyer-Hoffert, U., Wu, Z., and Schröder, J.-M. (2009). Identification of Lympho-Epithelial Kazal-Type Inhibitor 2 in Human Skin as a Kallikrein-Related Peptidase 5-Specific Protease Inhibitor. PLOS ONE *4*, e4372.

Michael, I.P., Sotiropoulou, G., Pampalakis, G., Magklara, A., Ghosh, M., Wasney, G., and Diamandis, E.P. (2005). Biochemical and Enzymatic Characterization of Human Kallikrein 5 (hK5), a Novel Serine Protease Potentially Involved in Cancer Progression *. Journal of Biological Chemistry *280*, 14628–14635.

Micheau, O., and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell *114*, 181–190.

* Monaco, C., Nanchahal, J., Taylor, P., and Feldmann, M. (2015). Anti-TNF therapy: past, present and future. International Immunology *27*, 55–62.

* Morizane, S. (2019). The Role of Kallikrein-Related Peptidases in Atopic Dermatitis. Acta Medica Okayama *73*, 1–6.

* Nakajima, K., and Sano, S. (2018). Mouse models of psoriasis and their relevance. J Dermatol *45*, 252–263.

* Nakajima, S., Nomura, T., Common, J., and Kabashima, K. (2019). Insights into atopic dermatitis gained from genetically defined mouse models. Journal of Allergy and Clinical Immunology *143*, 13–25.

* Nauroy, P., and Nyström, A. (2020). Kallikreins: Essential epidermal messengers for regulation of the skin microenvironment during homeostasis, repair and disease. Matrix Biology Plus *6–7*, 100019.

* Newton, K., and Manning, G. (2016). Necroptosis and Inflammation. Annu Rev Biochem *85*, 743–763.

Noda, S., Suárez-Fariñas, M., Ungar, B., Kim, S.J., de Guzman Strong, C., Xu, H., Peng, X., Estrada, Y.D., Nakajima, S., Honda, T., et al. (2015). The Asian atopic dermatitis phenotype combines features of atopic dermatitis and psoriasis with increased TH17 polarization. J Allergy Clin Immunol *136*, 1254–1264.

* Nolan, B.V., Yentzer, B.A., and Feldman, S.R. (2010). A review of home phototherapy for psoriasis. Dermatol Online J *16*, 1.

* Nosenko, M.A., Ambaryan, S.G., and Drutskaya, M.S. (2019). Proinflammatory Cytokines and Skin Wound Healing in Mice. Mol Biol *53*, 653–664.

Ny, A., and Egelrud, T. (2004). Epidermal hyperproliferation and decreased skin barrier function in mice overexpressing stratum corneum chymotryptic enzyme. Acta Derm Venereol *84*, 18–22.

Nylander-Lundqvist, E., and Egelrud, T. (1997). Formation of active IL-1 beta from pro-IL-1 beta catalyzed by stratum corneum chymotryptic enzyme in vitro. Acta Derm Venereol *77*, 203–206.

Oberst, A., Dillon, C.P., Weinlich, R., McCormick, L.L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G.S., and Green, D.R. (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature *471*, 363–367.

Oji, V., Beljan, G., Beier, K., Traupe, H., and Luger, T.A. (2005). Topical pimecrolimus: a novel therapeutic option for Netherton syndrome. Br J Dermatol *153*, 1067–1068.

* Oji, V., Tadini, G., Akiyama, M., Blanchet Bardon, C., Bodemer, C., Bourrat, E., Coudiere, P., DiGiovanna, J.J., Elias, P., Fischer, J., et al. (2010). Revised nomenclature and classification of inherited ichthyoses: results of the First Ichthyosis Consensus Conference in Sorèze 2009. J Am Acad Dermatol *63*, 607–641.

Ong, P.Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R.L., and Leung, D.Y.M. (2002). Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med *347*, 1151–1160.

Park, Y.C., Ye, H., Hsia, C., Segal, D., Rich, R.L., Liou, H.-C., Myszka, D.G., and Wu, H. (2000). A Novel Mechanism of TRAF Signaling Revealed by Structural and Functional Analyses of the TRADD–TRAF2 Interaction. Cell *101*, 777–787.

* Pasparakis, M., Haase, I., and Nestle, F.O. (2014). Mechanisms regulating skin immunity and inflammation. Nat Rev Immunol *14*, 289–301.

* Pastar, I., Stojadinovic, O., Yin, N.C., Ramirez, H., Nusbaum, A.G., Sawaya, A., Patel, S.B., Khalid, L., Isseroff, R.R., and Tomic-Canic, M. (2014). Epithelialization in Wound Healing: A Comprehensive Review. Advances in Wound Care *3*, 445–464.

* Pastore, S., Mascia, F., Mariani, V., and Girolomoni, G. (2006). Keratinocytes in skin inflammation. Expert Review of Dermatology *1*, 279–291.

Paternoster, L., Standl, M., Waage, J., Baurecht, H., Hotze, M., Strachan, D.P., Curtin, J.A., Bønnelykke, K., Tian, C., Takahashi, A., et al. (2015). Multi-ancestry genome-wide association study of 21,000 cases and 95,000 controls identifies new risk loci for atopic dermatitis. Nat Genet *47*, 1449–1456.

* Petrova, E., and Hovnanian, A. (2020). Advances in understanding of Netherton syndrome and therapeutic implications. Expert Opinion on Orphan Drugs *8*, 455–487.

* Polese, B., Zhang, H., Thurairajah, B., and King, I.L. (2020). Innate Lymphocytes in Psoriasis. Front. Immunol. *0*.

* Popp, M.W., and Maquat, L.E. (2016). Leveraging Rules of Nonsense-Mediated mRNA Decay for Genome Engineering and Personalized Medicine. Cell *165*, 1319–1322.

* Prassas, I., Eissa, A., Poda, G., and Diamandis, E.P. (2015). Unleashing the therapeutic potential of human kallikrein-related serine proteases. Nat Rev Drug Discov *14*, 183–202.

* Qu, Y., Zhao, G., and Li, H. (2017). Forward and Reverse Signaling Mediated by Transmembrane Tumor Necrosis Factor-Alpha and TNF Receptor 2: Potential Roles in an Immunosuppressive Tumor Microenvironment. Front Immunol *8*, 1675.

Rossol, M., Meusch, U., Pierer, M., Kaltenhäuser, S., Häntzschel, H., Hauschildt, S., and Wagner, U. (2007). Interaction between transmembrane TNF and TNFR1/2 mediates the activation of monocytes by contact with T cells. J Immunol *179*, 4239–4248.

* Rothmeier, A.S., and Ruf, W. (2012). Protease-activated receptor 2 signaling in inflammation. Semin Immunopathol *34*, 133–149.

Sakabe, J., Yamamoto, M., Hirakawa, S., Motoyama, A., Ohta, I., Tatsuno, K., Ito, T., Kabashima, K., Hibino, T., and Tokura, Y. (2013). Kallikrein-related Peptidase 5 Functions in Proteolytic Processing of Profilaggrin in Cultured Human Keratinocytes *. Journal of Biological Chemistry *288*, 17179–17189.

* Sarac, G., Koca, T.T., and Baglan, T. (2016). A brief summary of clinical types of psoriasis. North Clin Istanb *3*, 79–82.

Sarkar, S. (2018). A Treatise on Topical Corticosteroid in Dermatology. Indian J Dermatol *63*, 530–531.

Schafer, P. (2012). Apremilast mechanism of action and application to psoriasis and psoriatic arthritis. Biochem Pharmacol *83*, 1583–1590.

* Schauber, J., and Gallo, R.L. (2008). Antimicrobial peptides and the skin immune defense system. J Allergy Clin Immunol *122*, 261–266.

Schittek, B., Hipfel, R., Sauer, B., Bauer, J., Kalbacher, H., Stevanovic, S., Schirle, M., Schroeder, K., Blin, N., Meier, F., et al. (2001). Dermcidin: a novel human antibiotic peptide secreted by sweat glands. Nat Immunol *2*, 1133–1137.

* Schön, M.P., Manzke, V., and Erpenbeck, L. (2021). Animal models of psoriasis—highlights and drawbacks. Journal of Allergy and Clinical Immunology *147*, 439–455.

Seltmann, J., Roesner, L.M., von Hesler, F.-W., Wittmann, M., and Werfel, T. (2015). IL-33 impacts on the skin barrier by downregulating the expression of filaggrin. J Allergy Clin Immunol *135*, 1659-1661.e4.

Shear, M.J., and Perrault, A. (1944). Chemical Treatment of Tumors. IX. Reactions of Mice with Primary Subcutaneous Tumors to Injection of a Hemorrhage-Producing Bacterial Polysaccharide1. JNCI: Journal of the National Cancer Institute *4*, 461–476.

* Shi, J.-H., and Sun, S.-C. (2018). Tumor Necrosis Factor Receptor-Associated Factor Regulation of Nuclear Factor κB and Mitogen-Activated Protein Kinase Pathways. Front. Immunol. *0*.

Siegmund, D., Kums, J., Ehrenschwender, M., and Wajant, H. (2016). Activation of TNFR2 sensitizes macrophages for TNFR1-mediated necroptosis. Cell Death Dis *7*, e2375.

Siegmund, D., Ehrenschwender, M., and Wajant, H. (2018). TNFR2 unlocks a RIPK1 kinase activity-dependent mode of proinflammatory TNFR1 signaling. Cell Death Dis *9*, 921.

Simpson, E.L., Chalmers, J.R., Hanifin, J.M., Thomas, K.S., Cork, M.J., McLean, W.H.I., Brown, S.J., Chen, Z., Chen, Y., and Williams, H.C. (2014). Emollient enhancement of the skin barrier from birth offers effective atopic dermatitis prevention. J Allergy Clin Immunol *134*, 818–823.

Soley, B. da S., Morais, R.L.T. de, Pesquero, J.B., Bader, M., Otuki, M.F., and Cabrini, D.A. (2016). Kinin receptors in skin wound healing. Journal of Dermatological Science *82*, 95–105.

Solmaz, D., Bakirci, S., Kimyon, G., Gunal, E.K., Dogru, A., Bayindir, O., Dalkilic, E., Ozisler, C., Can, M., Akar, S., et al. (2020). Impact of Having Family History of Psoriasis or Psoriatic Arthritis on Psoriatic Disease. Arthritis Care Res (Hoboken) *72*, 63–68.

Sprecher, E., Chavanas, S., DiGiovanna, J.J., Amin, S., Nielsen, K., Prendiville, J.S., Silverman, R., Esterly, N.B., Spraker, M.K., Guelig, E., et al. (2001). The spectrum of pathogenic mutations in SPINK5 in 19 families with Netherton syndrome: implications for mutation detection and first case of prenatal diagnosis. J Invest Dermatol *117*, 179–187.

van Steensel, M. a. M., Badeloe, S., Winnepenninckx, V., Vreeburg, M., Steijlen, P.M., and van Geel, M. (2008). Granulomatous rosacea and Crohn's disease in a patient homozygous for the Crohn-associated NOD2/CARD15 polymorphism R702W. Exp Dermatol *17*, 1057–1058.

Stefansson, K., Brattsand, M., Roosterman, D., Kempkes, C., Bocheva, G., Steinhoff, M., and Egelrud, T. (2008). Activation of proteinase-activated receptor-2 by human kallikrein-related peptidases. J Invest Dermatol *128*, 18–25.

* Sun, S.-C. (2010). CYLD: a tumor suppressor deubiquitinase regulating NF- κ B activation and diverse biological processes. Cell Death Differ *17*, 25–34.

* Sun, S.-C. (2017). The non-canonical NF-κB pathway in immunity and inflammation. Nat Rev Immunol *17*, 545–558.

* Tang, T.S., Bieber, T., and Williams, H.C. (2012). Does "autoreactivity" play a role in atopic dermatitis? J Allergy Clin Immunol *129*, 1209-1215.e2.

* Tobin, A.-M., and Kirby, B. (2005). TNFα Inhibitors in the Treatment of Psoriasis and Psoriatic Arthritis. BioDrugs *19*, 47–57.

Tomfohrde, J., Silverman, A., Barnes, R., Fernandez-Vina, M.A., Young, M., Lory, D., Morris, L., Wuepper, K.D., Stastny, P., and Menter, A. (1994). Gene for familial psoriasis susceptibility mapped to the distal end of human chromosome 17q. Science *264*, 1141–1145.

* Tracey, K.J., Lowry, S.F., and Cerami, A. (1988). Cachetin/TNF-α in Septic Shock and Septic Adult Respiratory Distress Syndrome. Am Rev Respir Dis *138*, 1377–1379.

* Two, A.M., and Del Rosso, J.Q. (2014). Kallikrein 5-mediated inflammation in rosacea: clinically relevant correlations with acute and chronic manifestations in rosacea and how individual treatments may provide therapeutic benefit. J Clin Aesthet Dermatol *7*, 20–25.

Two, A.M., Hata, T.R., Nakatsuji, T., Coda, A.B., Kotol, P.F., Wu, W., Shafiq, F., Huang, E.Y., and Gallo, R.L. (2014). Reduction in Serine Protease Activity Correlates with Improved Rosacea Severity in a Small, Randomized Pilot Study of a Topical Serine Protease Inhibitor. J Invest Dermatol *134*, 1143–1145.

Van Zee, K.J., Kohno, T., Fischer, E., Rock, C.S., Moldawer, L.L., and Lowry, S.F. (1992). Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor alpha in vitro and in vivo. Proc Natl Acad Sci U S A *89*, 4845–4849.

* Vanamee, É.S., and Faustman, D.L. (2018). Structural principles of tumor necrosis factor superfamily signaling. Sci. Signal. *11*.

Veal, C.D., Clough, R.L., Barber, R.C., Mason, S., Tillman, D., Ferry, B., Jones, A.B., Ameen, M., Balendran, N., Powis, S.H., et al. (2001). Identification of a novel psoriasis susceptibility locus at 1p and evidence of epistasis between PSORS1 and candidate loci. J Med Genet *38*, 7–13.

Veal, C.D., Capon, F., Allen, M.H., Heath, E.K., Evans, J.C., Jones, A., Patel, S., Burden, D., Tillman, D., Barker, J.N.W.N., et al. (2002). Family-based analysis using a dense single-nucleotide polymorphism-based map defines genetic variation at PSORS1, the major psoriasis-susceptibility locus. Am J Hum Genet *71*, 554–564.

Wadonda-Kabondo, N., Sterne, J. a. C., Golding, J., Kennedy, C.T.C., Archer, C.B., Dunnill, M.G.S., and ALSPAC Study Team (2004). Association of parental eczema, hayfever, and asthma with atopic dermatitis in infancy: birth cohort study. Arch Dis Child *89*, 917–921.

Wang, L., Du, F., and Wang, X. (2008). TNF-alpha induces two distinct caspase-8 activation pathways. Cell *133*, 693–703.

Wawrzycki, B., Pietrzak, A., Grywalska, E., Krasowska, D., Chodorowska, G., and Roliński, J. (2019). Interleukin-22 and Its Correlation with Disease Activity in Plaque Psoriasis. Arch Immunol Ther Exp (Warsz) *67*, 103–108.

Weidinger, S., O'Sullivan, M., Illig, T., Baurecht, H., Depner, M., Rodriguez, E., Ruether, A., Klopp, N., Vogelberg, C., Weiland, S.K., et al. (2008). Filaggrin

mutations, atopic eczema, hay fever, and asthma in children. J Allergy Clin Immunol *121*, 1203-1209.e1.

* Weidinger, S., Beck, L.A., Bieber, T., Kabashima, K., and Irvine, A.D. (2018). Atopic dermatitis. Nat Rev Dis Primers *4*, 1–20.

* Wells, A., Nuschke, A., and Yates, C.C. (2016). Skin tissue repair: Matrix microenvironmental influences. Matrix Biology *49*, 25–36.

* Wickett, R.R., and Visscher, M.O. (2006). Structure and function of the epidermal barrier. American Journal of Infection Control *34*, S98–S110.

Williams, M.R., Nakatsuji, T., Sanford, J.A., Vrbanac, A.F., and Gallo, R.L. (2017). Staphylococcus aureus Induces Increased Serine Protease Activity in Keratinocytes. Journal of Investigative Dermatology *137*, 377–384.

Williams, M.R., Cau, L., Wang, Y., Kaul, D., Sanford, J.A., Zaramela, L.S., Khalil, S., Butcher, A.M., Zengler, K., Horswill, A.R., et al. (2020). Interplay of Staphylococcal and Host Proteases Promotes Skin Barrier Disruption in Netherton Syndrome. Cell Rep *30*, 2923-2933.e7.

Wong, R., Geyer, S., Weninger, W., Guimberteau, J.-C., and Wong, J.K. (2016). The dynamic anatomy and patterning of skin. Experimental Dermatology *25*, 92–98.

* Xu, Y.-R., and Lei, C.-Q. (2021). TAK1-TABs Complex: A Central Signalosome in Inflammatory Responses. Front. Immunol. *0*.

Yamasaki, K., Schauber, J., Coda, A., Lin, H., Dorschner, R.A., Schechter, N.M., Bonnart, C., Descargues, P., Hovnanian, A., and Gallo, R.L. (2006). Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. FASEB J *20*, 2068–2080.

Yamasaki, K., Kanada, K., Macleod, D.T., Borkowski, A.W., Morizane, S., Nakatsuji, T., Cogen, A.L., and Gallo, R.L. (2011). TLR2 Expression Is Increased in Rosacea and Stimulates Enhanced Serine Protease Production by Keratinocytes. J Invest Dermatol *131*, 688–697.

Yazici, A.C., Tamer, L., Ikizoglu, G., Kaya, T.I., Api, H., Yildirim, H., and Adiguzel, A. (2006). GSTM1 and GSTT1 null genotypes as possible heritable factors of rosacea. Photodermatol Photoimmunol Photomed *22*, 208–210.

Yoon, H., Laxmikanthan, G., Lee, J., Blaber, S.I., Rodriguez, A., Kogot, J.M., Scarisbrick, I.A., and Blaber, M. (2007). Activation Profiles and Regulatory Cascades of the Human Kallikrein-related Peptidases *. Journal of Biological Chemistry *282*, 31852–31864.

Yoon, H., Blaber, S.I., Evans, D.M., Trim, J., Juliano, M.A., Scarisbrick, I.A., and Blaber, M. (2008). Activation profiles of human kallikrein-related peptidases by proteases of the thrombostasis axis. Protein Science *17*, 1998–2007.

Yoshida, K., Kubo, A., Fujita, H., Yokouchi, M., Ishii, K., Kawasaki, H., Nomura, T., Shimizu, H., Kouyama, K., Ebihara, T., et al. (2014). Distinct behavior of human

Langerhans cells and inflammatory dendritic epidermal cells at tight junctions in patients with atopic dermatitis. J Allergy Clin Immunol *134*, 856–864.

* Yoshie, O., and Matsushima, K. (2015). CCR4 and its ligands: from bench to bedside. Int Immunol *27*, 11–20.

* Zebda, R., and Paller, A.S. (2018). Phosphodiesterase 4 inhibitors. J Am Acad Dermatol *78*, S43–S52.

Zhu, Y., Underwood, J., Macmillan, D., Shariff, L., O'Shaughnessy, R., Harper, J.I., Pickard, C., Friedmann, P.S., Healy, E., and Di, W.-L. (2017). Persistent kallikrein 5 activation induces atopic dermatitis-like skin architecture independent of PAR2 activity. Journal of Allergy and Clinical Immunology *140*, 1310-1322.e5.

Zingkou, E. (2018). Generation and characterization of novel mouse models to validate the role of KLK5 protease in inflammation for pharmacological applications. University of Patras; Πανεπιστήμιο Πατρών.

Zingkou, E., Pampalakis, G., Charla, E., Nauroy, P., Kiritsi, D., and Sotiropoulou, G. (2019). A proinflammatory role of KLK6 protease in Netherton syndrome. J Dermatol Sci *95*, 28–35.

* Zlotnik, A., and Yoshie, O. (2012). The Chemokine Superfamily Revisited. Immunity *36*, 705–716.

* Zomer, H.D., and Trentin, A.G. (2018). Skin wound healing in humans and mice: Challenges in translational research. J Dermatol Sci *90*, 3–12.