Aus der Klinik für Dermatologie, Venerologie und Allergologie
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DISSERTATION

Frühe mechanische Hautirritation in der TNF-α defizienten Maus
Early physical skin irritation in TNF-α deficient mice

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List of abbreviations

AD	Atopic dermatitis
AU	Arbitrary unites
β-Me	β-mercaptoethanol
bp	Base pair
CCL	Chemokine ligand
DNA	Deoxyribonucleic acid
cDNA	Copy deoxyribonucleic acid
dsDNA	Double-Stranded DNA
CT	Threshold cycle value
CXCL8	CXC ligand 8
DC	Dendritic cell
dDCs	Dermal dendritic cells
ELISA	Enzyme linked immunosorbent assay
g	Acceleration of gravity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
iDECs	Inflammatory dendritic cells
IFNγ	Interferon gamma
IL-	Interleukin
ILC2	Type 2 innate lymphoid cells
IEC-6	Intestinal Epithelioid Cell line No. 6
KCs	Keratinocytes
KO	Knockout
MCs	Mast cells
MDB	Membrane desalting buffer

mIL-	Murine interleukin
NK	Natural killer
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol Myristate Acetate
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
SLS	Sodium lauryl sulphate
TEWL	Transepidermal water loss
TMB	3,3′,5,5′-Tetramethylbenzidin
TNF-α	Tumor necrosis factor-α
TNFR	Tumor necrosis factor receptor
ts	Tape stripping
TSLP	Thymic stromal lymphopoietin
qPCR	quantitative PCR
UTR	Untranslated region
UV	Ultraviolet
ws	wet shaving
WT	Wildtype (C57BL/6)

Abstract

The skin is the largest human organ and forms a protective barrier against the environment. Irritation of the skin may lead to local inflammatory reactions and, once chronified, even to dermatological disorders such as irritative or atopic dermatitis (AD). Atopic dermatitis is one of the most common inflammatory skin disorders worldwide, with a prevalence of up to 20% among children and 3% among adults. Even though our knowledge about AD has increased significantly in recent years, many aspects concerning its molecular pathogenesis still remain elusive. Tumor necrosis factor-α (TNF-α) is one of the best known pro-inflammatory cytokines and a key mediator in several inflammatory diseases, such as inflammatory bowel disease or psoriasis. However, its role in AD still remains unclear. In a mouse model, it has been demonstrated that TNF-KO mice develop an aggravated form of AD compared to wild type (WT) mice. Moreover, AD-like symptoms are a possible side effect in patients after anti-TNF treatment. This raises the question of whether a different cytokine profile may explain an AD predisposition in the absence of TNF-α. The aim of this thesis was to uncover differences in cytokine patterns upon physical skin irritation in WT and TNF-KO mice *ex vivo.*

For this purpose, the skin of TNF-KO and WT mice was irritated by means of either wet shaving only or additional tape stripping. Biopsies were taken and 96 cytokines were measured in skin samples by a multi-array enzyme-linked immunosorbent assay (ELISA). Seven cytokines were selected for further investigation by ELISA and mRNA cytokine expressions were evaluated by qRT-PCR.

Chemokine (C-X-C motif) ligand 1 (CXCL1), Chemokine (C-X-C motif) ligand 2 (CXCL2), interleukin-5 (IL-5), and interleukin-18 (IL-18) expression increased significantly upon wet shaving in TNF-KO and WT mice. When comparing the cytokine levels between the two genotypes, we observed a tendency towards higher levels of IL-5 and significantly higher levels of IL-17BR in TNF-KO mice upon physical skin irritation compared to WT mice. Whereas IL-5 is already associated with AD and irritated skin, it is the first time that IL-17BR was shown to be induced upon physical skin irritation under TNF-deficiency. In

summary, the data suggest deviations of local cytokine responses, which may be responsible for the onset of AD-like symptoms upon TNF-deficiency.

Zusammenfassung

Die Haut stellt das größte Organ des Menschen dar und schützt vor externen Reizen der Umwelt. Hautirritationen können lokale Entzündungen verursachen und wenn sie chronifizieren, gar zu dermatologischen Erkrankungen wie der irritativen oder der atopischen Dermatitis führen. Die atopische Dermatitis stellt mit einer Prävalenz von bis zu 20% in Kindern und bis zu 3% in Erwachsenen, eine der häufigsten dermatologischen Krankheiten dar. Tumor Nekrose Faktor-α (TNF-α) ist bekannt als pro-inflammatorisches Zytokin, welches eine Schlüsselrolle bei verschiedenen entzündlichen Erkrankungen einnimmt. Die Bedeutung von TNF-a im Zusammenhang mit der atopischen Dermatitis ist jedoch noch unklar. In einem Mausmodell wurde gezeigt, dass TNF-KO-Mäuse eine schwerere Form der atopischen Dermatitis entwickelten als WT-Mäuse. Außerdem sind ähnliche Symptome eine mögliche Nebenwirkung von TNF-Antikörper Therapien. Es stellt sich die Frage, ob ein unterschiedliches Zytokinprofil in der TNF-defizienten Maus hierfür verantwortlich ist. Ziel der vorgelegten Promotionsarbeit war es, zu untersuchen, ob Unterschiede in der Zytokinantwort nach mechanischer Hautirritation in der TNF-KO-Maus im Vergleich zur WT-Maus vorliegen. Um dieser Fragestellung nachzugehen, wurde *ex vivo* die Haut der TNF-KO- und WT-Mäuse durch Nassrasur oder zusätzlichem Tape-stripping (Tesafilm-Abriss) irritiert. Biopsien wurden entnommen und mittels eines Multi-Array ELISAs wurden die Konzentrationen von 96 Zytokinen in den Hautproben gemessen. Sieben Zytokine hiervon wurden anschließend mittels eines Einzel-ELISA überprüft. Die mRNA-Expression der Zytokine wurde mittels qRT-PCR gemessen.
Die Expression von Chemokine (C-X-C motif) ligand 1 (CXCL1), Chemokine (C-X-C motif) ligand 2 (CXCL2), Interleukin-5 (IL-5) und Interleukin-18 (IL-18) ist signifikant durch Irritation mittels Nassrasur in WT und TNF-KO Maus gestiegen. In der TNF-KO Maus ist im Vergleich zum WT die Proteinexpression für IL-17BR signifikant höher und für IL-5 tendenziell höher nach Hautirritation angestiegen. Während die vermehrte Expression von IL-5 bei der AD bekannt ist, konnte zum ersten Mal gezeigt werden**,** dass in Folge von mechanischer Hautirritation, IL-17BR in der TNF-defizienten Maus stärker ansteigt als im WT. Zusammenfassend weisen die Daten darauf hin, dass eine Dysregulation der lokalen Zytokinreaktion, den Ausbruch einer AD-ähnlichen Symptomatik unter TNF-Defizienz fördern könnte.

1. Introduction

1.1 Anatomical skin structure

The skin is the largest human organ and forms a protective barrier against external dangers such as microbes or chemicals (2-4). The skin consists of three different layers which are tightly connected: epidermis, dermis and subcutis (Figure 1) (5). The epidermis itself is composed of multiple layers of flattened cells, which are mainly formed by keratinocytes (3). Immune cells such as Langerhans cells but also melanocytes, Merkel cells and stem cells of the skin are located here as well (4, 6).

The epidermis consists of five layers: stratum corneum, stratum granulosum, stratum spinosum and stratum basale. The stratum corneum, the top layer, is composed of anucleated keratinocytes that are loaded with keratin filaments (2, 4). It provides foremost the mechanical protection of the skin (5, 7). Moreover, it plays a crucial role for transepidermal water loss (TEWL), which describes the regulation of water loss from the organism to the atmosphere through the skin (7). The stratum granulosum contains the basophile keratohyalin granules, in which filaggrin is the main component (8). The stratum spinosum is composed of multiple layers of spindle shaped keratinocytes that are connected through desmosomes (8, 9). The stratum basale, which is the lowest layer of the epidermis, contains basal stem cells which are responsible for the continuous regeneration of the epidermis (2, 8, 10).

The dermis consists primarily of connecting tissue and is located between the epidermis and the subcutis (11). The papillary dermis, or dermoepidermal junction, is the uppermost part of the dermis where the basement membrane of the epidermis is connected with the dermis via rate ridges and collagen fibers. Lymphatic and blood vessels are also located in the dermis (5, 8, 11). Constriction or dilatation of these blood vessels provides a form of thermoregulation of the body (11). Additionally, the dermis contains sensory receptors, sweat glands, hair follicles and sebaceous glands (5, 8, 11). The subcutis or hypodermis is the deepest layer of the skin. It consists of connective and adipose tissue and contains blood vessels and nerves. It provides isolation and mechanical protection as well as offering a reserve energy supply. The thickness of the subcutis varies considerably among individuals (5, 8, 11-13)

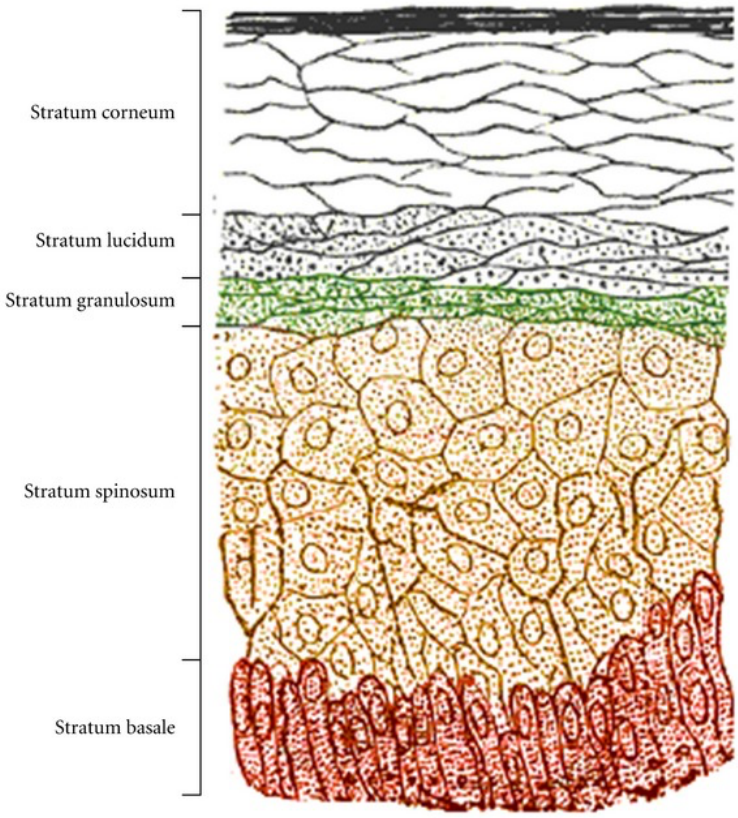


Figure 1: Anatomical skin structure and the epidermal layers

The skin structure is complex and forms a protective barrier against the environment. The epidermis consists of stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The dermis contains mainly collagen, reticular fibres and elastic tissue. Several cell types may be found here such as fibroblasts, dendritic cells, mast cells and T-cell subsets. The subcutis consists of adipose and connective tissue. Taken from Skin Barrier Function and Its Importance at the Start of the Atopic March, Mary Beth Hogan, Kathy Peele, and Nevin W. Wilson, Journal of Allergy (2012) (14).

1.2. Physical skin irritation in skin pathology

The skin is a metabolically active organ with various functions. Passive functions of the skin include protection from thermal hazards, UV radiation, chemicals, dehydration, pressure and friction (1, 5, 15). The most important active functions of the skin are the defense against penetrating pathogens and allergens, the resorption of active substances, the production of sweat and sebum, thermoregulation by means of blood circulation, and sensory perception (3, 5, 13). In order to maintain these functions, several factors are required, such as stable cell to cell adhesion, continuous renewal of skin cells,

a working cell to cell interaction and adequate responses from the immune system (5, 15). In the case of an acute skin barrier disruption, keratinocytes start producing and releasing various pro-inflammatory cytokines such as Interleukin-1α (IL-1α), IL-8, IL-10 or TNF-α (16, 17). These factors can trigger the production of other pro-inflammatory mediators, or attract them to the place of damage in order to repair and to maintain the skin homeostasis (15). Physical skin irritation may lead to an impairment of the skin homeostasis and, once chronified, even to dermatological disorders such as atopic dermatitis (7, 10). Various experimental setups have been designed in order to better understand the molecular mechanisms of acute or chronic skin irritation. However, due to ethical reasons most of this work is performed on mouse skin. Physical and chemical irritants on skin are both common methods used in this context (16-21). Two techniques used for physical skin irritation are shaving and tape stripping (16, 17, 22, 23). Escobar-Chavez *et al.* showed that after 30 times tape stripping with an adhesive tape, the layers of the stratum corneum were removed (18). Such a disruption of the stratum corneum increases the TEWL and induces the production and release of various pro-inflammatory cytokines (15-17). Wood *et al*. observed an increase of IL-1α, IL-1ß, GM-CSF and TNF-α expression upon tape stripping the skin of hairless mice (16, 17). Another cytokine expressed by keratinocytes upon skin irritation and inflammatory cytokine stimulation is thymic stromal lymphopoietin (TSLP), a major initiator of atopic dermatitis (24, 25). Inter alia, TSLP is expressed by epithelial cells of the skin and mast cells (26). Studies have shown that wet shaving as well as tape stripping induces TSLP production in murine skin *in vivo* as well as *ex vivo* (19, 27). Yoo *et al.* reported that TSLP overexpression in murine skin resulted in the development of spontaneous dermatitis, the hallmark of human AD (28). Additionally, Jessup *et al*. showed that a direct administration of TSLP resulted in AD-like skin lesions in mice (29). TSLP has been shown to promote the proliferation and differentiation of Th2 cells, and to induce the expression of its associated cytokines such as IL-4, IL-5 and IL-13 (26, 29). Angelova-Fischer *et al*. investigated the effect of tape stripping on the human skin and showed that tape stripping leads to damage to the stratum corneum and consequently to an increase of TSLP expression in the human epidermis as well (30). Furthermore, TSLP has been described to be highly expressed in keratinocytes from AD patients (26).

1.2.2 Atopic dermatitis (AD)

Atopic dermatitis is a genetically determined, chronic-inflammatory skin disease and is, along with bronchial asthma and allergic rhinitis, considered as an atopic disorder (31, 32). Clinically, it is characterised by erythematous, pruritic, dry and flaky skin as well as papules, seropapules, erosions and lichenification (5). Furthermore, the disease has a typical localisation pattern, depending on the age of the patient (5, 31). AD is a widespread disorder, especially among industrialised countries, with prevalences of up to 20% in children and of 3% in adults which are steadily increasing overall. (31-33).

Pathophysiology of atopic dermatitis

AD is a highly complex disease with a multifactorial pathophysiology and molecular processes that are not completely understood yet (34). Nonetheless, two major risk factors for the development of AD have been identified: mutation of the FLG gene (encoding the epidermal barrier protein filaggrin) and a positive family history of AD (35, 36).

Observation studies showed that a positive parental history is a strong predictor of AD in children (32, 37). Additionally, monozygotic twins showed a higher concordance rate than dizygotic twins (36). Genetic studies highlighted the critical role of the FLG gene in AD (32). The FLG gene codes the filaggrin protein, which is an essential skin barrier protein (38). Various studies have demonstrated that a skin barrier dysfunction promotes skin inflammation in AD patients (39, 40). A weak skin barrier not only promotes the penetration of pathogens and allergens into the skin but also results in an increase of epidermal water loss (31, 41). The majority of children with moderate or severe AD carry FLG mutations (39). However, several other proteins, such as Claudin-based tight junctions, play a crucial role in maintaining the skin barrier as well (42). Additionally, a significant number of AD patients do not show any FLG mutation, while conversely a FLG mutation does not lead to AD in all cases. Moreover, many patients eventually outgrow AD, despite carrying mutations in the FLG gene (31, 43, 44). Consequently, it is likely that a disrupted skin barrier alone is not sufficient for the development of AD (44).

Apart from the skin barrier, studies have shown that immune cells also play a crucial role in the pathogenesis of AD (31, 32). In many AD patients, a predominant systemic Th2 disbalance along with eosinophilia and increased IgE levels can be observed (45). By

producing high levels of TSLP, keratinocytes induce Th2 polarization via activated DCs (26, 31). Th2 mediated pro-inflammatory cytokines such as IL-4, IL-5 and IL-13 are upregulated in lesional as well as non-lesional skin of AD patients in the acute phase of the disease (45, 46). Th2 cytokines have also been shown to decrease the expression of skin barrier proteins. (47, 48). Moreover, transgenic mice that overexpress Th2 cytokines were shown to develop skin barrier defects as well as AD spontaneously (49). As depicted in Figure 2, the pathophysiology of AD is a highly complex process in which genetics, skin barrier dysfunction and pro-inflammatory as well as environmental factors interact with one another (50, 51).

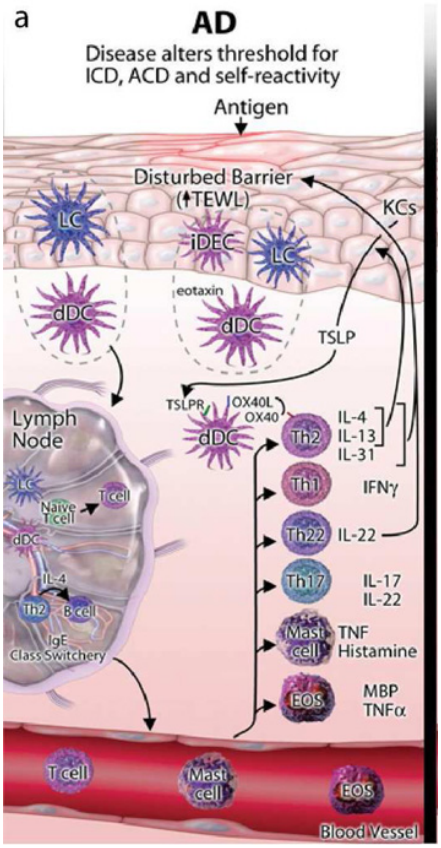


Figure 2: Pathogenesis of atopic dermatitis

In AD, the weak skin barrier allows the penetration of antigens which encounter Langerhans cells (LCs), inflammatory dendritic epidermal cells (iDECs) and dermal dendritic cells, activating Th2 cells to produce IL-4 and IL-13. DCs then migrate to lymph nodes, where they activate effector T-cells and induce IgE class-switching. IL-4 and IL-13 induce TSLP production in KCs. TSLP reinforces Th2 activity by activating OX40 ligand-expressing dDCs. Skin infiltration by DCs,

eosinophils and mast cells is stimulated by cytokines produced by Th2 cells and DCs such as IL-4, IL-5 and IL-13. Th2 and Th22 cells are dominant in AD patients and their cytokines (IL-4, IL-13 and IL-22) inhibit terminal differentiation as well as contributing to the barrier defect in patients with AD. Additionally, Th1 and Th17 cells contribute to its pathogenesis. Reprinted from Journal of Allergy and Clinical Immunology, Vol 131, Issue 2, JK Gittler, JG Krueger and E Guttman-Yassky, 300-313, copyright 2013 with permission from Elsevier (52).

1.3 Tumor Necrosis Factor-α (TNF-α)

1.3.1 TNF-α - a pro-inflammatory cytokine

TNF-α was first described as an endotoxin-induced serum factor that causes necrosis of sarcomas in mice (53). TNF is produced by a broad variety of cell types, including macrophages, T-cells, DCs, MCs and endothelial cells (7, 54, 55). There are two isoforms of TNF-α: one is cell-associated, or membrane bound and the other is secreted or soluble TNF-α. Both isoforms of TNF-α are biologically active (54, 56). Ever since its discovery, TNF-α has been the subject of intense research and is considered as one of the best studied pro-inflammatory cytokines (57). Increased TNF-α is associated with several autoimmune diseases, such as Crohn's disease, psoriasis and rheumatoid arthritis (56, 58, 59). Furthermore, TNF-α has the ability to enhance the expression of other pro-inflammatory cytokines and chemokines, such as IL-1, IL-6 and GM-CSF (60). Consequently, monoclonal antibodies against TNF-α, such as Infliximab, have become part of the standard therapy options in autoimmune disorders like rheumatoid arthritis, Crohn's disease or psoriasis (61-63).

1.3.2 Role of TNF-α in skin irritation

Chemicals, physical stress or irradiation may cause skin irritation and therefore provoke a complex process in the skin that includes skin damage, cell death and activation of keratinocytes, immune cells and other cells (64). In the skin keratinocytes are an important source of pro-inflammatory cytokines such as TNF-α, IL-1α, IL-1ß, IL-18 and TSLP, but also of chemokines such as CXCL1 (15, 30, 65-68). Besides keratinocytes, fibroblasts and vascular endothelial cells also are important sources of TNF-a upon skin irritation. The release of TNF-α leads to an inflammatory phase with local leukocyte

recruitment (69). Additionally, UVB irradiation and chemical irritants such as dimethyl sulfoxide, PMA, formaldehyde, tributyltin and SLS cause an upregulation of TNF-α in the skin (67, 70). Having a pleiotropic effect on human keratinocytes and endothelial cells, TNF-α causes an increased expression of pro-inflammatory mediators such as IL-1, IL-6, GM-CSF, and CXCL8 (66, 71). Moreover, TNF has been shown to weaken the skin barrier by inhibiting the expression of filaggrin and loricrin in human keratinocytes (7, 72). Concordantly, Piguet *et al*. demonstrated that a treatment with a TNF-antibody abrogates the signs of skin inflammation in a mouse model (73).

1.3.3 Role of TNF-α in AD

The role of TNF-α in the pathogenesis of AD is not entirely clear yet. Several authors described lower levels of TNF-α in the skin or in the serum of AD patients compared to healthy controls (74-77). Jeong *et al.* and others measured significantly lower TNF-α mRNA expression in skin biopsies of AD patients compared to healthy controls (76, 77). Moreover, various clinical studies have described the development of eczemas as a common side effect upon anti-TNF therapy in patients with psoriasis, Crohn's disease or rheumatoid arthritis (78-84). In a prospective analysis of patients with rheumatic disease, Lee *et al*. identified the manifestation of eczemas as the most common cutaneous side effect of anti-TNF therapy (79). Additionally, Kumari *et al*. showed in an AD model that TNF-KO mice developed more severe AD-like symptoms compared to WT mice (19). On the other hand, Sumimoto *et al*. measured increased TNF-α levels in 15 children with AD and others reported therapeutical success with anti-TNF therapy in single AD patients (85-89). All in all, there is little evidence in the literature for successful TNF-α directed therapy among AD patients (90). However, TNF-α has also been shown to mediate the apoptosis of auto reactive T-cells as well as to induce local glucocorticoid production (91, 92). Consequently, it can be stated that TNF-α may even exert anti-inflammatory effects.

1.4 Objectives

Although TNF-α has proven to be a crucial pro-inflammatory cytokine in various inflammatory diseases, several authors have also reported anti-inflammatory effects of this cytokine. Eczemas are a common side effect of anti-TNF therapy. The underlying mechanism of this phenomena remains elusive, however. Kumari *et al.* showed in an AD mouse model that TNF-KO mice develop an aggravated form of AD compared to WT mice (19). Furthermore, they were able to demonstrate that these TNF-KO mice had increased levels of TSLP mRNA expression. This raises the question of there being different cytokine patterns in TNF-KO mice upon skin irritation. Several studies have established shaving and tape stripping as forms of physical skin irritation that have an impact on inflammatory processes in the epidermis.

The following questions were addressed in this thesis:

1) Does physical skin irritation (caused by shaving and repeated tape stripping) induce an increase of cytokine concentration in WT and TNF-KO mice?
2) Do TNF-KO mice show different cytokine responses compared to WT mice upon physical skin irritation?

Answering these questions will be helpful for the better understanding of the molecular mechanisms induced by physical skin irritation or acute permeability disruption of the skin of WT as well as TNF-KO mice.

2. MATERIALS AND METHODS

2.1 MATERIALS

Information about reagents, solutions, buffers, chemicals, instruments, software and labwares are listed below:

Table 1: List of reagents

Reagent	Supplier	Catalog Number
Agarose	Biozym	840004
Antibody diluent (Dako REALTM)	DAKO Diagnostika	S0809
Aqua	Braun	2351744
Bovine serum albumin (BSA)	PAA	K45-001
DermaLife K Medium Complete Kit	Lifeline Cell Technology	LL-0007
Deoxyribonucleic acid (DNA) Molecular Weight XIII –50 base pair (bp)ladder	Roche	11721925001
DNA Molecular Weight XIV–100 bp ladder	Roche	11721933001
Ethanol	J.T. Baker	8025
Ethidium Bromide Solution	Invitrogen	15585-011
Hydrogen peroxide (H2O2)	Sigma-Aldrich	216763
Human TSLP ELISA kit	eBioscience	88-7497-88
LightCycler®FastStart DNA Master SYBR Green I	Roche	12239264001

Mouse CCL1 ELISA Kit	BOSTER	EK0566
Mouse Cytokine Antibody Array	RayBio®	
Mouse IL-5 ELISA Kit	RayBio®	ELM-IL5
Mouse IL-17BR ELISA Kit	RayBio®	ELM-IL17BR
Mouse IL-18 ELISA Kit	MBL	7625
Mouse KC ELISA Kit	RayBio®	ELM-KC
Mouse MIP-2 ELISA Kit	RayBio®	ELM-MIP2
Mouse TSLP Duo Set	R&D Systems®	DY555
Nucleo Spin® RNA II	Macherey-Nagel	740955.250
PBS	GE Healthcare	H15-002
Penicillin/Streptomycin	Biochrom	A 2212
Proteinase K	Macherey-Nagel	740506

Table 2: List of materials

Material	**Supplier**	**Catalog Number**
Biosphere Filter Tips 0.5-20 μL 2-100 μL 100-1000μL	Sarstedt	70.1116.20 70.760.212 70.762.211
LightCycler® Capillaries	Roche	04929292001
Micro tube, 0.5mL	Sarstedt	72.699
Micro tube, 1.5 mL	Sarstedt	72.690.001

Micro tube, 2 mL	Sarstedt	72.691
Quality Tips without filter 10 µL 200 µL 1000 µL	Sarstedt	70.1130 70.760.002 70.762
Serological Pipet 5 mL 10 mL 25 mL	BD FalconTM	357543 357551 357525

Table 3: List of instruments

Instrument	Type	Supplier
Centrifuge	Megafuge 1.0R	Thermo Scientific, Schwerte
Electrophoresis System	Sub-Cell®GT	Bio Rad, München
Gel Imager	Gene Genius	Syngene, Cambridge
Light Cycler		Roche, Penzberg
Microplate reader	Dynatech MRX	Dynex Technoloies, Chantilly
Pipette	Eppendorf Reference®/ Research®	Eppendorf, Hamburg

PCR machine	Px2 Thermal Cycler	Thermo Electron Corporation
Thermomixer	Thermomixer comfort	Eppendorf, Hamburg
Tissue homogenizer	Precellys 24	Bertin Technologies, Montigny-le-Bretonneux
Waterbath	MA6	Lauda, Lauda-Königshofen
Vortexer	REAX 2000	Heidolph, Schwabach

2.2 METHODS

2.2.1 Animals

2.2.1.1 Breeding of B6;129S-Tnftm1Gkl/J (TNF-KO) mice

TNF-KO mice were kindly provided by Professor Max Löhning from the DRFZ, Berlin. Originally, the strain was generated by using a targeting vector to replace the TNF gene with MC1neopA cassette (Stratagene), the 438 bp NarI-BglII fragment containing 40 bp of the 5' UTR, and all the coding region, including the ATG translation initiation codon of the first exon and part of the first intron of the mTNF-α gene. The homozygous animals were bred according to animal guidelines at the Charité animal facility (FEM). Control animals (C57B16/JRj) were ordered from a stock breeding at the Charité animal facility (FEM). The animals used for the *ex vivo* skin irritation model were painlessly sacrificed by a qualified member of professor Worm's group and listed accordingly (Tötungsanzeige T0027/19).

2.2.1.2 *Ex Vivo* skin irritation model

Eight- to twelve-week-old female C57B16/JRj (wt) and TNF-KO mice were painlessly sacrificed by a qualified person. Immediately after death, the belly regions were wet shaved and the resulting area was divided into two equal parts as shown in Figure 3. The lower part of the belly was then tape stripped 30 times by using cello tape in order to irritate the skin. The entire shaved area was cut out with scissors and put on a piece of cork with the dermis facing downward. As many biopsies as possible were taken from each area of the skin for ELISAs and mRNA isolation, using a 5.0 mm biopsy punch (Figure 4). Each biopsy was immediately put into a 50ml falcon containing 15ml hydrocorticoid free KBM gold KC medium. At a germ-free bench under airflow, three biopsies from each condition were placed into a well of a 24-well plate filled with 450μl of prewarmed fresh hydrocorticoid free KBM gold KC medium. The biopsies were incubated for four or twelve hours. The supernatant and the biopsies were then snap-frozen separately and stored at -80°C.

Figure 3: Scheme of the skin irritation model ex vivo

Figure 4: Example of the experimental approach

The lower part was wet shaved and tape stripped, whereas the upper part was only wet shaved. Biopsies were taken accordingly.

2.2.2 Multi Array Experiment

The supernatants of the performed *ex vivo* experiment were measured for 96 different cytokine levels (Figure 5). The multi array works similarly to a sandwich based ELISA which is explained below (Figure 8). The fluorescent signal was obtained by using a laser scanner and ImageJ software was used to translate the fluorescent signals into arbitrary units (Figures 6, 7). The analysis was performed based on RayBio®Mouse Cytokine Antibody Array G-Series 1000 according to manufacturer's instructions.

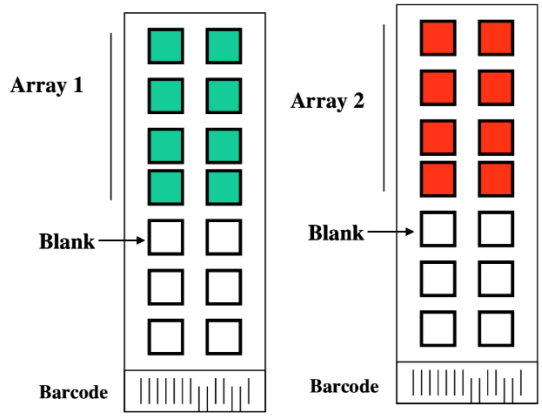


Figure 5: Scheme of the multi array glass slides

Each glass slide has eight subarrays. Array 1 can detect 34 different mouse cytokines in one experiment. Array two can detect 62 different mouse cytokines in one experiment.

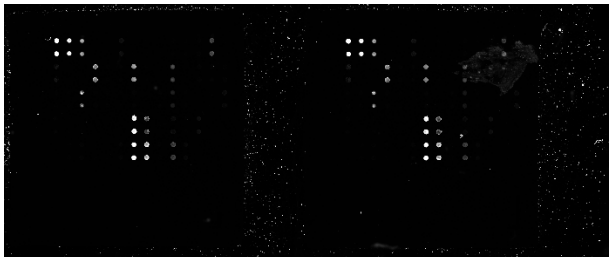


Figure 6: Example of two subarrays

Subarrays on a slide after the fluorescent signal was detected by means of a laser scanner. Each fluorescent circle represents a detected cytokine or a positive control. Negative controls were used for background subtraction.

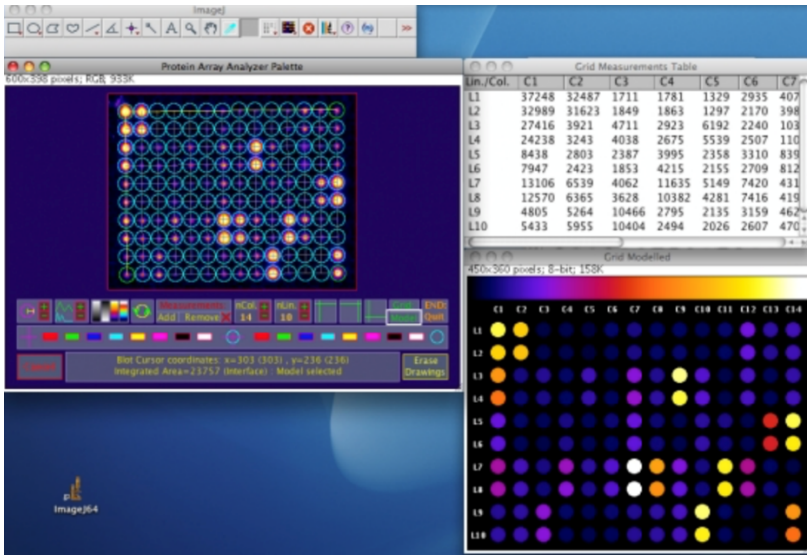


Figure 7: Example of an image of a subarray measured with ImageJ software

The software is able to detect the intensity of the fluorescence and measure it immediately as a table of values.

2.2.3 Mouse Enzyme Linked Immunosorbent Assay (ELISA)

An ELISA is used in order to detect the unknown amount of a protein in a liquid. In this work, several sandwich based ELISAs were used. In this model, specific capture antibodies were coated on the surface of a reaction plate. The supernatant or standard was then added to the plate, followed by an incubation period which allowed the antigen to bind to the primary antibody. In a second step, a biotin-conjugated detection antibody was added to the plate, which was supposed to bind to the capture antibody-antigen complex during another incubation time. Thereafter, the plates were incubated with horseradish peroxidase (HRP), which binds to the biotin-conjugated antibody. Between these steps, the wells were washed with PBS or a specific washing solution depending on the ELISA. Finally, a substrate solution such as TMB was added, causing an enzymatic reaction with a color change in proportion to the amount of the cytokine present in the well (Figure 8). The colored product was measured by a spectrophotometer. The program “Revelation” calculated the concentration of the specific protein in the samples by means of a standard curve for which the blank value was first subtracted. All steps were performed at room temperature.

Based on the results of the multi array experiment, the supernatants of the performed *ex vivo* experiment were then measured for mouse IL-1alpha (IL-1 alpha ELISA kit from R&D systems), mouse CXCL1 (RayBio®Mouse KC ELISA Kit), mouse CXCL2 (RayBio®Mouse-2 ELISA Kit), mouse CCL1 (Mouse CCL1/TCA3 PicoKine™ELISA Kit from BOSTER), mouse IL-5 (RayBio®Mouse IL-5 ELISA Kit) and mouse IL-17BR (RayBio®Mouse IL-17BR ELISA Kit). Additionally, the supernatant was also measured for mouse IL-18 (Mouse IL-18 Platinum ELISA from eBioscience / Mouse IL-18 ELISA Kit from MBL). The analysis was performed according to the manufacturer's instructions.

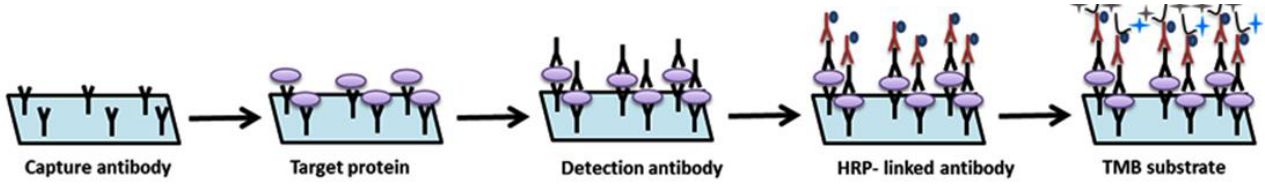


Figure 8: Scheme of sandwich based enzyme linked immunosorbent assay (ELISA)

Adapted from Epitomics - an Abcam Company.

2.2.4 RNA isolation

The frozen skin samples were put into liquid nitrogen and each sample was then transferred into a pre-chilled bead tube (Precellys) containing 250 µL of RA1 buffer (NucleoSpin® RNA isolation kit) and 2.5 µL of β-mercaptoethanol (β-ME). The homogenization was performed by using precellys 24 tissue homogenizer at 55000 rpm for 2*30 sec with a 5 sec pause. The supernatant was then transferred into a NucleoSpin filter and centrifuged for two minutes at 11 000 g at room temperature. Without touching the pellet, the supernatant was taken out and 500 µl of RNase-free water as well as 100 µl of 10% proteinase K were added and mixed and then incubated for 15 minutes at 55°C. Afterwards, the lysate was centrifuged for three minutes at 10 000g. Without touching the pallet, the supernatant was pipetted into a new autoclaved eppy and 500µl of 100% ethanol was added to the lysate and mixed well by pipetting up and down. 750µl of the lysate was then loaded into a NucleoSpin RNA II Column and centrifuged for 15 seconds at 8 000g. The flowthrough was discarded and the same was done to the remaining lysate. 350µl of MDB (membrane desalting buffer) was added to the NucleoSpin RNA II column

and centrifuged for one minute at 8 000 g. 95µl of DNase reaction mixture was pipetted directly onto the center of the silica membrane of the column and incubated for 15 minutes at room temperature. Further RNA isolation was performed according to the manufacturer's instructions. The RNA was eluted with 30 µl of RNase-free water. Using NanoDrop UV-Vis spectrophotometer, the RNA concentration was measured at 260 nm. The eluted samples were stored at -80 °C for further analysis.

2.2.5. Reverse transcription

The RNA was entirely reverse transcribed into single stranded cDNA with TaqMan® reverse transcription reagent according to the manufacturer's instructions. 1 µg of total RNA was used for reverse transcription into cDNA in a thermo cycler with the following protocol. If 1µg was not available, the missing amount was replaced with sterile BRAUN® water.

Table 4: Reverse transcription

Steps	Temperature (°C)	Time (min)
Incubation	25	10
Reverse transcription (RT)	48	40
RT inactivation	95	5

All cDNA samples were stored at -20 °C.

2.2.6 Real-time polymerase chain reaction (qPCR)

Fluorescence-based real-time quantitative polymerase chain reaction (qPCR) was performed for the quantification of gene expression in skin samples on the previously transcribed cDNA. The LightCycler® FastStart DNA Master SYBR Green I (Roche) was used according to the protocol listed below. The cDNA was first diluted 1:3. The specific binding of SYBR green fluorescence dye to the rising amount of double-stranded DNA causes an increased level of fluorescence which may then be measured. Naturally, an unspecific binding of SYBR-green also occurs, which needs to be subtracted. Therefore, the PCR buffer also contains a reference dye in order to normalize the specific binding.

The threshold cycle value (ct) defines the number of cycles that were needed to see a significant increase of the fluorescence. The relative expression of a gene was thus calculated dependent on its CT value and the efficacy of the primer. The expression level of the target gene was normalized to the expression level of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the 2-ΔΔCT method.

Table 5: qPCR

Component	**Final conc.**	***µl*/rxn (rxn=10)**
Sybrgreen	1x	5
Fw-Primer		0.5
Rev. Primer		0.5
Braun water		2

Table 6: Primer sequence

Gene	Primers	Sequence	Size	Product size
mGAPDH	forward	5‘-ctttgtcaagctcatttcctgg-3‘	22	133
	reverse	5’-tcttgctcagtgtccttgc-3’	19	
mIL17-BR	forward	5‘-gcaagcggcagataaagtgg-3‘	20	196
	reverse	5’-tgctcccccaagatagacca-3’	20	
mIL5	forward	5‘-actgtgccatgactgtgcct-3‘	20	114
	reverse	5‘-acggagaagtaaggcccagc-3‘	20	
mIL18	forward	5‘-caactttggccgacttcactg-3‘	21	100
	reverse	5‘-agtcatatcctcgaacacaggc-3‘	22	

2.3 Statistical Analysis

To prove a normal distribution of the values, the D’Agostino-Pearson omnibus normality test, Shapiro-Wilk normality test and Kolmogorov-Smirnov normality test were performed. If one of the above-mentioned tests confirmed a normal distribution of the data, a parametric t-test was performed (paired and unpaired). GraphPad Prism version 5 (GraphPad Software, USA) was used for all statistical analyses. $P < 0.05$ was considered as statistically significant.

3. Results

In a skin irritation model, Kumari *et al.* demonstrated that TNF-KO mice developed a more severe dermatitis in comparison to WT mice upon OVA injections and physical skin irritation within a time span of 71 days (19). Additionally, dermatitis-like symptoms are a possible adverse event of anti-TNF therapy (78, 79, 83) and several studies reported lower TNF levels in AD patients (74-77). This raised the question of whether TNF-KO mice show a different cytokine pattern upon skin irritation, which may eventually lead to a predisposition for AD in the absence of TNF-α. In order to asses this question in detail, 8- to 10-week-old female WT and TNF-KO mice were sacrificed, wet shaved or additionally tape stripped on a defined belly region, and biopsies were taken and incubated in fresh hydrocorticoid free KBM gold KC medium for four or twelve hours. The supernatant was snap frozen and stored at -80°C for protein analysis by ELISA. Skin biopsies were used for qPCR to determine gene expression.

3.1 Multi Array Experiment

In a first step, a multi array experiment was performed detecting 96 different cytokines at once. In this setting, we wanted to determine whether protein levels of these cytokines are different between WT and TNF-KO mice upon physical skin irritation. We chose this panel because, inter alia, it was able to not only detect key cytokines from the IL-1 family, such as IL-1α and IL-1ß, but also Th2 cytokines like IL-4, IL-5, IL-13 and TSLP. IL-18, an important member of the IL-1 family, was not included in the panel. Therefore, we performed IL-18 ELISAs separately.

After four and twelve hours of incubation, IL-1ß levels were unaltered between genotypes (Figure 9A). TSLP levels were similar in both genotypes after four hours of incubation. After twelve hours of incubation, the levels were higher in TNF-KO compared to WT mice (11.500 AU vs. 1390 AU) (Figure 9B). IL-4 levels were only slightly higher in TNF-KO after four (1103 AU vs. 1646 AU) and twelve hours (1509 AU vs. 2063 AU) of incubation compared to WT mice (Figure 9C). The levels of IL-13 were similar in both genotypes after four and twelve hours of incubation (Figure 9D).

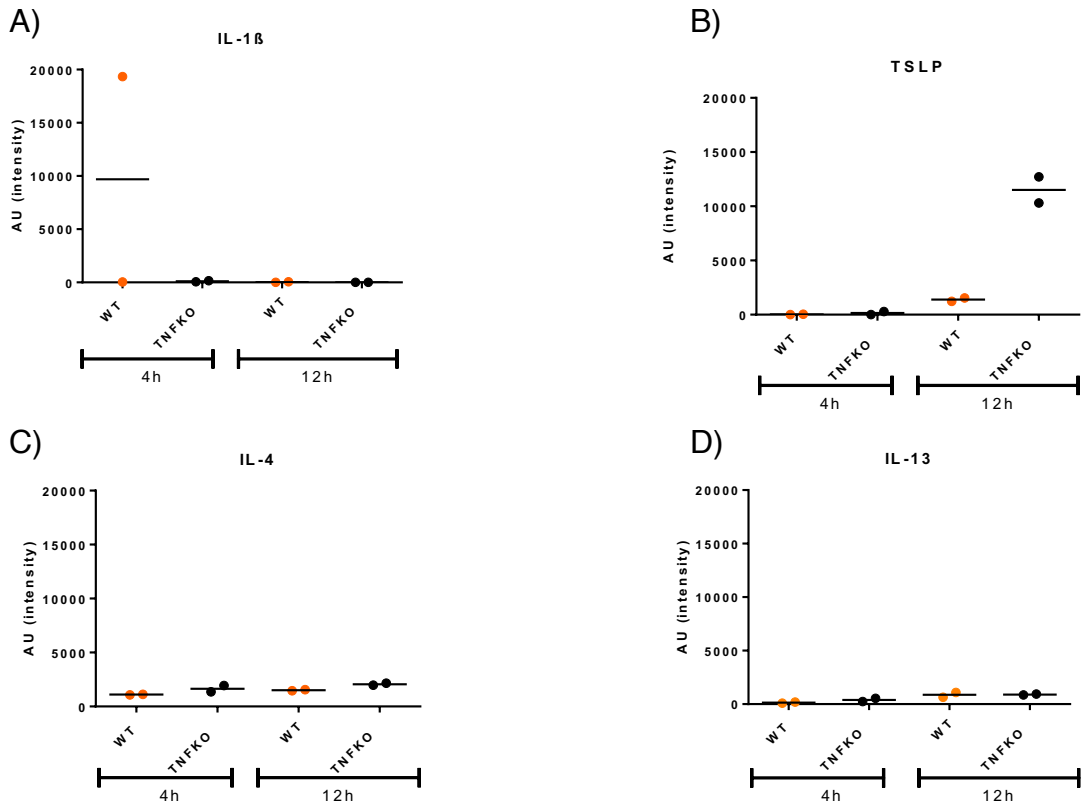


Figure 9: Cytokines of the IL-1 family and Th2 cytokines detected in the multi array experiment in the skin of WT and TNF-KO mice upon irritation

Cytokine levels of IL-1ß (A), TSLP (B), IL-4 (C) and IL-13 (D) after four (left panel) and twelve hours (right panel) post irritation, respectively. Data are shown as mean, each mouse is indicated by a single dot, n = 2 mice/group.

The largest differences of cytokine levels between the genotypes were observed regarding the expression of the proteins IL-1α, CXCL1, CXCL2, CCL1, IL-5 and IL-17BR (Figure 10).

IL-1α is known to play a major role in the skin barrier function and has been shown to play a significant role in the pathogenesis of AD (31, 40, 93). In the multi array experiment, IL-1α levels were similar in both genotypes after four hours of incubation (55.000 AU in WT vs. 52.000 AU in TNF-KO). After twelve hours of incubation IL-1α levels were

approximately two times higher in TNF-KO (106.000 AU) compared to WT mice (50.000 AU) (Figure 10A). CXCL1 and CXCL2 are homologues, and both related to skin injury and inflammatory processes (94-96). In the multi array experiment, CXCL1 levels in WT mice were at 39.000 AU and in TNF-KO mice at 59.000 AU after four hours of incubation (Figure 10B). CXCL2 levels in WT mice were at 73.000 AU and at 208.000 AU in TNF-KO after four hours of incubation (Figure 10C). The levels of both chemokines were similar in both genotypes after twelve hours of incubation (Figure 10B, C). CCL1 is an inflammatory cytokine associated with AD (97, 98). CCL1 levels were twice as high in WT than in TNF-KO mice after four hours of incubation (1200 AU vs. 720 AU). This tendency was less striking after twelve hours but still remained visible (Figure 10D). IL-5 is considered an inflammatory Th2 cytokine and is hence associated with AD (99, 100). After four hours, IL-5 levels were similar in both genotypes. After twelve hours IL-5 levels were two times higher in TNF-KO compared to WT mice (3100 vs 1500 AU) (Figure 10E). IL-17BR is part of the receptor for IL-17B as well as for IL-25 (101, 102). After four hours of incubation, IL-17BR levels were at 450 AU in WT and at 21.500 AU in TNF-KO mice. After twelve hours, the relation was 1250 AU vs. 13.000 AU (Figure 10F). Among the 96 cytokines, these five proteins were the most differentially regulated ones within the experiment.

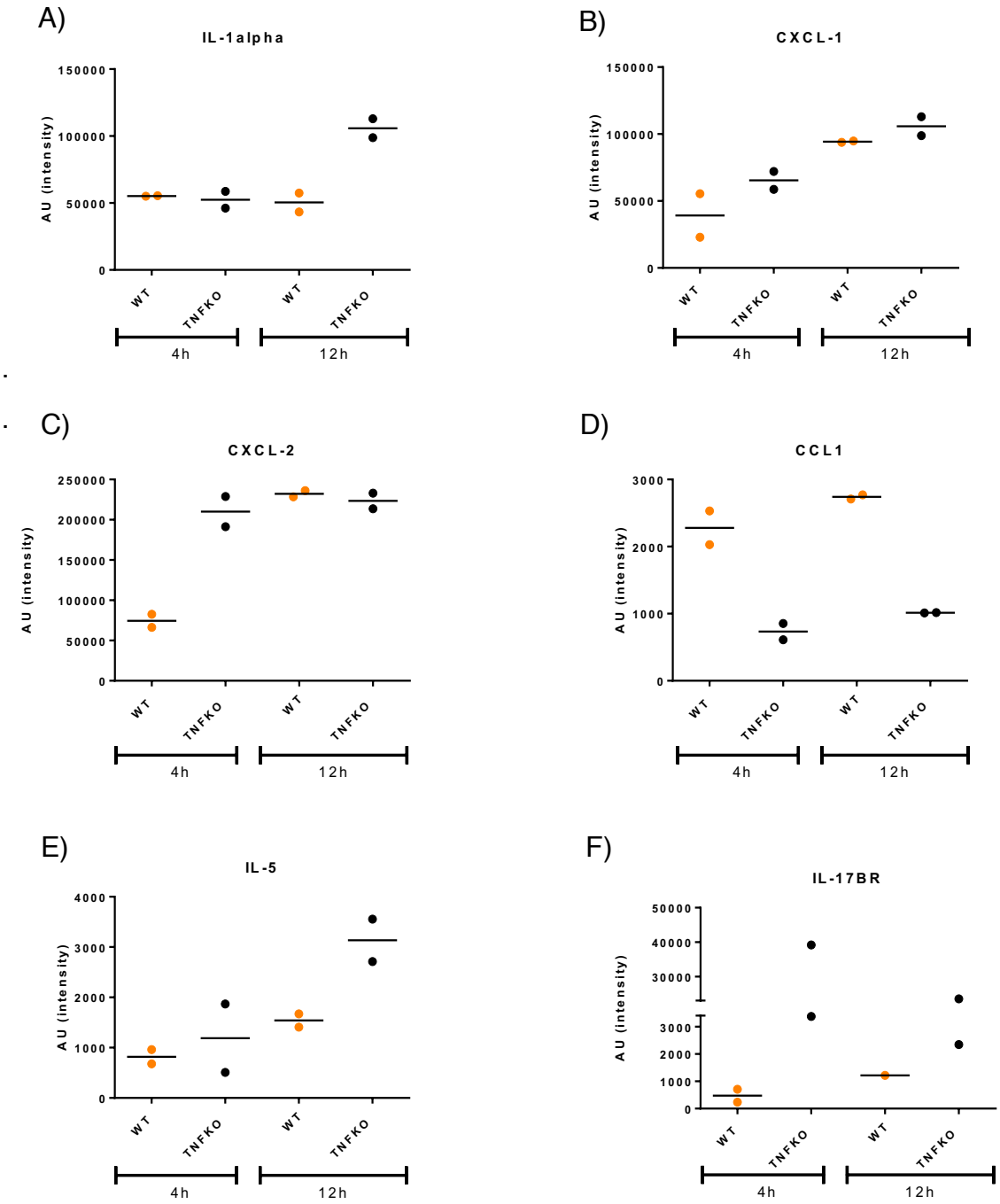


Figure 10: Cytokines detected in the multi array experiment that showed notable differences between the two genotypes ex vivo

Cytokine levels of IL-1α (A), CXCL1 (B), CXCL2 (C), CCL1 (D), IL-5 (E) and IL-17BR (F) after four (left panel) or twelve hours (right panel) post irritation, respectively. Data are shown as mean, each mouse is indicated by a single dot, n = 2 mice/group.

To verify the results of Figure 10, we analyzed the corresponding cytokines by simple protein ELISAs in the next step.

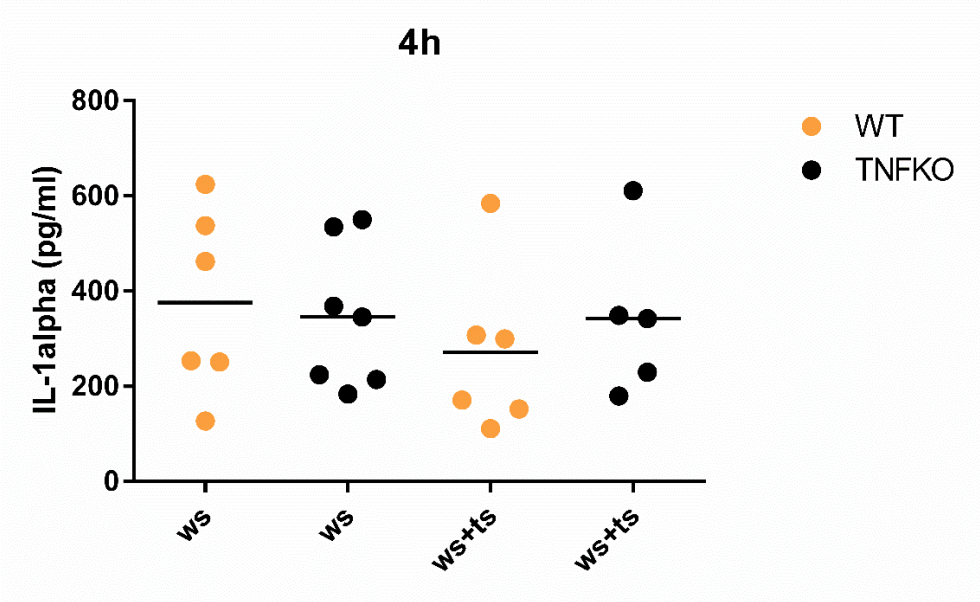
3.2 ELISA

In order to see whether the results from 5.1 were reproducible, specific ELISAs for IL-1α, CXCL1, CXCL2, CCL1, IL-5 and IL-17BR were performed. Based on the literature, we were also interested in IL-18. For this purpose, 8- to 10-week-old female WT and TNF-KO mice were sacrificed and either only wet shaved or additionally tape stripped on the previously defined belly region. Biopsies were taken and incubated in fresh hydrocorticoid free KBM gold KC medium for four or twelve hours. The supernatant was snap frozen and stored at -80°C until ELISAs were performed.

3.2.1 IL-1α

IL-1α is mainly produced by neutrophils, endothelial cells, activated macrophages, epithelial cells as well as by keratinocytes (93, 103). It is known to be a major inflammatory cytokine and is therefore used as an indicator of inflammation or skin impairment in clinical trials (104, 105). In the multi array experiment, we saw higher levels of IL-1α in TNF-KO mice after twelve hours of incubation (Figure 10A). In order to assess reproducibility, we performed an ELISA using skin samples from more mice. After four hours of incubation, the levels of IL-1α were similar in all conditions, while showing a strong variance (Figure 11A). After twelve hours of incubation, the levels of IL-1α were similar in both genotypes upon wet shaving only. Additional tape stripping seemed to decrease the levels of IL-1α in both genotypes. However, statistically there was a tendency of a stronger decrease only in WT mice (Figure 11B, $p=0.058$).

A)



B)

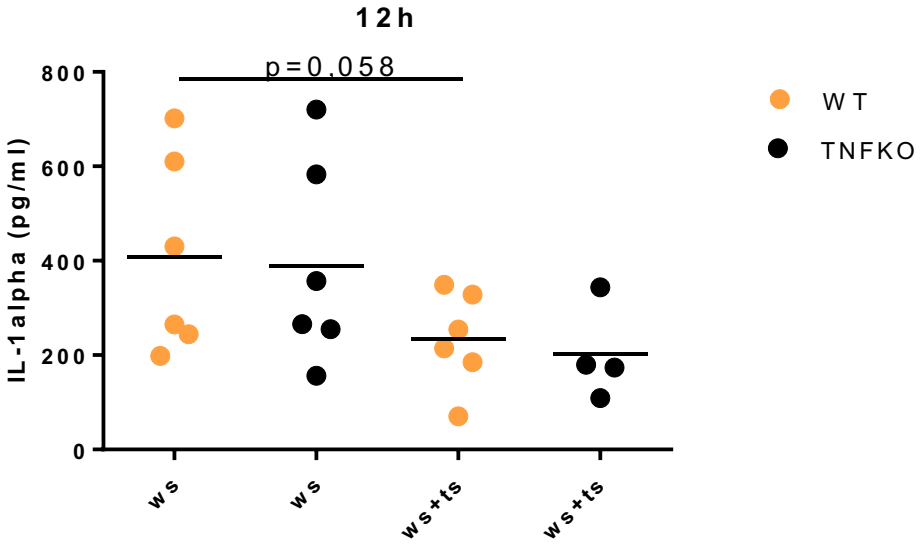


Figure 11: Impact of physical skin irritation on IL-1α production in WT and TNF-KO mice

IL-1α production in the irritated skin after four hours (A) and twelve hours (B) post irritation, respectively. Data are shown as mean, each mouse is indicated by a single dot; n = 4-6 mice/group, ws = wet shaving, ts = tape stripping. Data was analyzed using the paired t-test.

We observed a decrease of cytokine levels upon additional tape stripping compared to only wet shaving in IL-1α. For the following cytokines we performed a first ELISA with less samples to check for this phenomenon. In all cases, a decrease of cytokine levels upon tape stripping was determined. Therefore, we only pursued wet shaving further.

3.2.2 CXCL1

CXCL1 is expressed by macrophages, neutrophils, keratinocytes and epithelial cells, and plays a role in inflammation and wound healing (96, 106, 107). In the multi array experiment, higher levels of CXCL1 were measured, especially after four hours of incubation in TNF-KO compared to WT (Figure 10B). In order to assess reproducibility, we performed a CXCL1 ELISA with samples from more mice. After four hours of incubation, the levels of CXCL1 were similar in both genotypes (Figure 12). Over time, wet shaving led to a significant increase of CXCL1 production in WT and showed the same tendency in TNF-KO. In WT mice, CXCL1 levels increased from 270pg/ml to 1800pg/ml (p=0.02). In TNF-KO mice, the levels increased from 290pg/ml to 2442pg/ml (p=0.067).

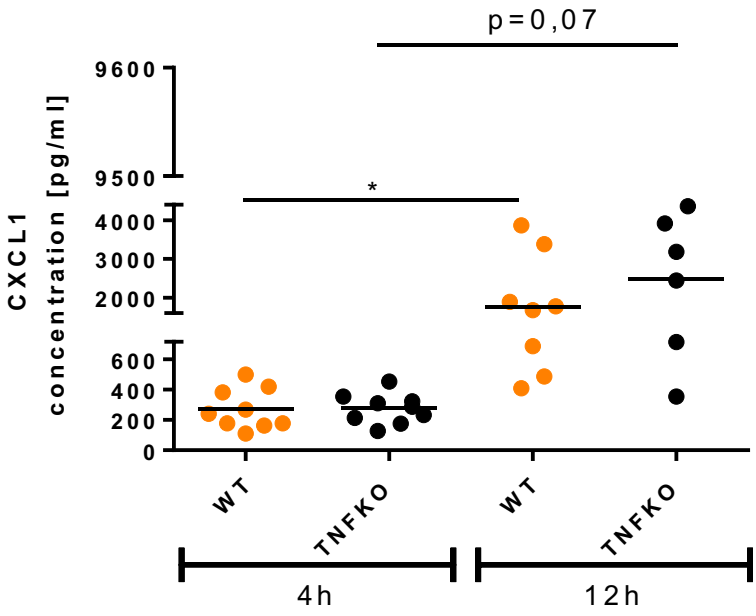


Figure 12: Impact of wet shaving on CXCL1 production in WT and TNF-KO mice

CXCL1 production in the irritated skin after four hours (left panel) and twelve hours (right panel) post irritation, respectively. Data are shown as mean, each mouse is indicated by a single dot; n = 6-9 mice/group. Data was analyzed using the paired t-test (*P ≤ 0.05).

3.2.3 CXCL2

Macrophage inflammatory protein 2-alpha or CXCL2 is secreted by macrophages and monocytes and plays a role in angiogenesis, cancer metastasis and wound healing (108, 109). In the multi array experiment, higher levels of CXCL2 were measured after four hours of incubation in TNF-KO (Figure 10C). In order to investigate whether these results could be reproduced with samples from more mice, a CXCL2 ELISA was performed. Wet shaving induced a significant increase of CXCL2 production in both genotypes time dependently. In WT mice, CXCL2 mean levels increased from 267pg/ml to 3963pg/ml (p=0.002) and in TNF-KO from 318pg/ml to 3802pg/ml (p=0.0056) over time. However, there was no difference between the different genotypes regarding the increase of CXCL2 levels upon irritation (Figure 13).

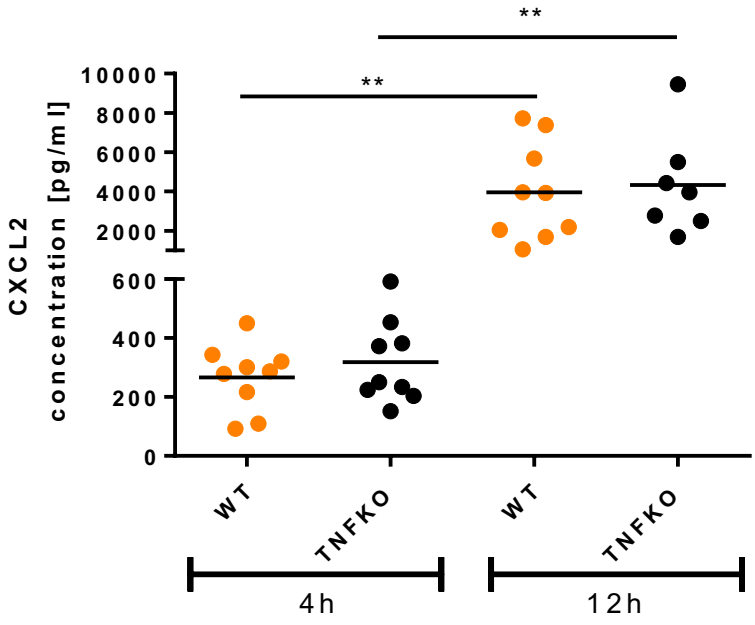


Figure 13: Impact of wet shaving on CXCL2 production in WT and TNF-KO mice

CXCL2 production in the irritated skin after four hours (left panel) and twelve hours (right panel) post irritation, respectively. Data are shown as mean; each mouse is indicated by a single dot; n = 7-9 mice/group. Data was analyzed using the paired t-test. (**P ≤ 0.01).

3.2.4 CCL1

CCL1 is a glycoprotein secreted by activated T-cells and attracts various immune cells such as monocytes, NK-cells and dendritic cells (110, 111). In the multi array experiment, higher levels of CCL1 were measured in WT mice compared to TNF-KO mice upon physical skin irritation (Figure 10D). We performed the experiment on more mice to check for reproducibility. CCL1 levels were similar in both genotypes in all conditions and did not increase or decrease over time (Figure 14).

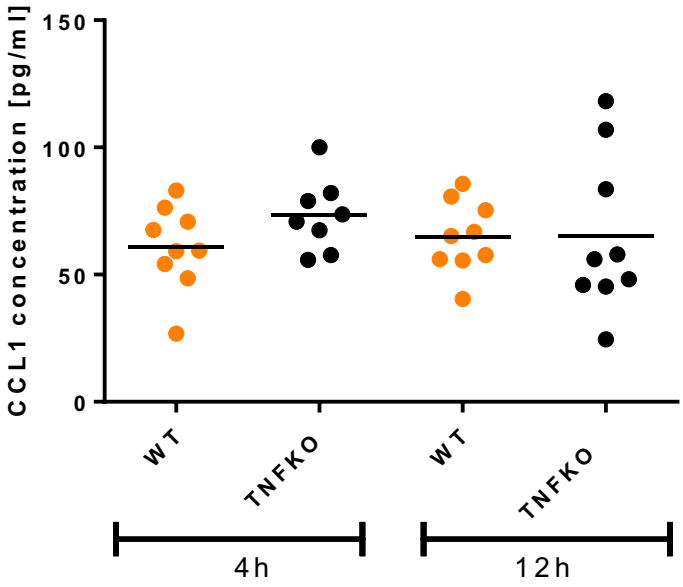


Figure 14: Impact of wet shaving on CCL1 production in WT and TNF-KO mice

CCL1 production in the irritated skin after four hours (left panel) and twelve hours (right panel) post irritation, respectively. Data are shown as mean, each mouse is indicated by a single dot; n = 8-9 mice/group.

3.2.5 IL-5

Interleukin-5 is produced, inter alia, by type-2 T helper cells and mast cells and plays a role in B-cell growth, immunoglobulin secretion and eosinophil activation (45, 112, 113). In the multi array experiment, higher levels of IL-5 were measured in TNF-KO mice compared to WT mice especially, after twelve hours of incubation (Figure 10E). Hence, an IL-5 ELISA with samples from more mice was performed to detect whether this result was reproducible. After four hours of incubation, the levels of IL-5 were similar in all conditions in WT and TNF-KO mice (Figure 15). After twelve hours of incubation, IL-5

levels increased significantly in both genotypes compared to four hours. In WT mice, the mean level of IL-5 increased from 7 pg/ml to 154 pg/ml (p=0.012) and in TNF-KO it increased from 7 pg/ml to 270 pg/ml (p=0.037). Interestingly, IL-5 levels had a tendency of higher levels in TNF-KO (265 pg/ml) after twelve hours compared to WT mice (152 pg/ml) (p=0.072).

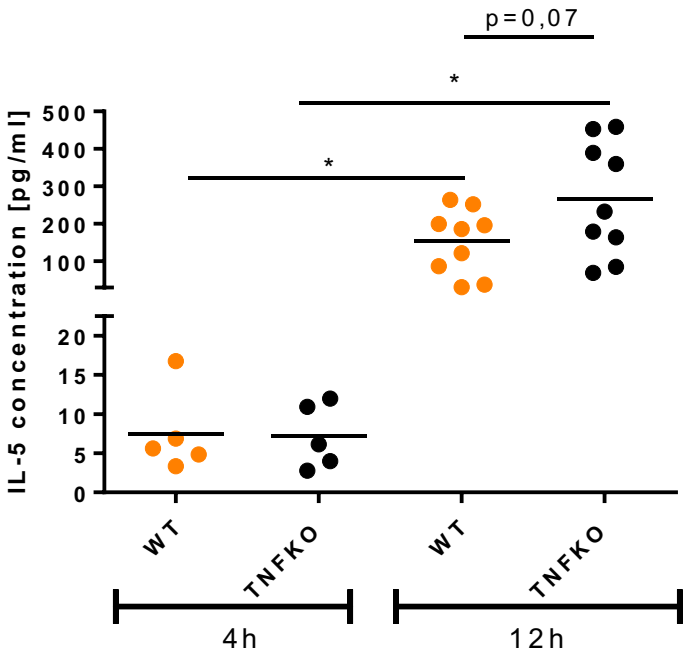


Figure 15: Impact of wet shaving on IL-5 production in WT and TNF-KO mice

IL-5 production in the irritated skin after four hours (left panel) and twelve hours (right panel) post irritation, respectively. Data are shown as mean; each mouse is indicated by a single dot; n = 5-9 mice/group. Data was analyzed using the t-test. (*P ≤ 0.05).

As we observed a tendency for an increased IL-5 production in TNF-KO compared to WT mice upon physical skin irritation, we performed qPCR to check for this finding on a mRNA level after four hours of incubation. However, at the chosen time point, no difference was detected between the genotypes (Figure 16).

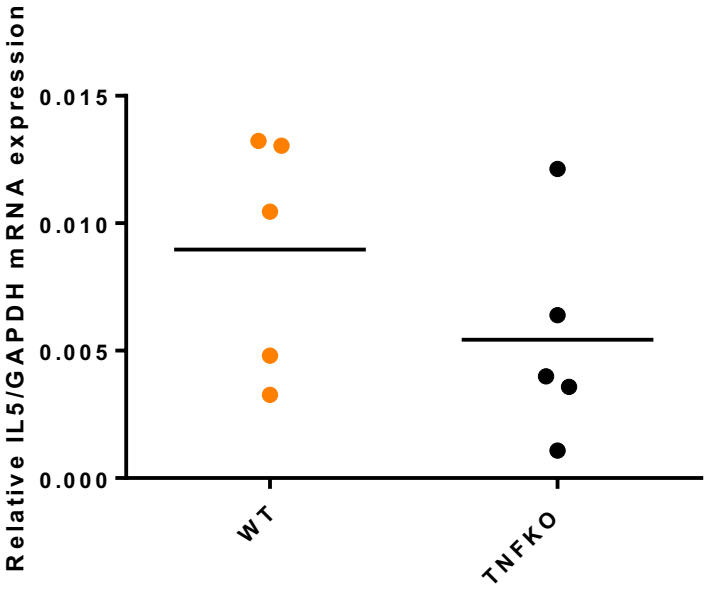


Figure 16: Comparative analysis of IL-5 expression between WT and TNF-KO mice upon wet shaving

IL-5 mRNA expression in the irritated skin four hours post irritation. Data are shown as mean; each mouse is indicated by a single dot; n = 5 mice/group.

3.2.6 IL-17-BR

Interleukin-17B receptor is the receptor to IL-17B and, together with IL17RA, also the receptor to IL17E/IL25. (114-116). It is expressed in various organs such as in the kidney or in the lungs, but also by cells of the immune system such as Th2 memory cells or ILC2 (101, 102). In the multi array experiment, we saw higher levels of IL-17BR in TNF-KO mice compared to WT mice upon physical skin irritation (Figure 10F). We used ELISAs with samples from more mice to scrutinize reproducibility. After four hours of incubation, the levels of IL-17BR were significantly higher in TNF-KO compared to WT (0.7 pg/ml vs. 1.5 pg/ml, p=0.03). After twelve hours of incubation, the levels of IL-17BR were similar in both genotypes (Figure 17). However, the levels of IL-17BR increased significantly over time in WT mice from 0.6 pg/ml after four hours of incubation to 1.5 pg/ml after twelve hours of incubation (p=0.01).

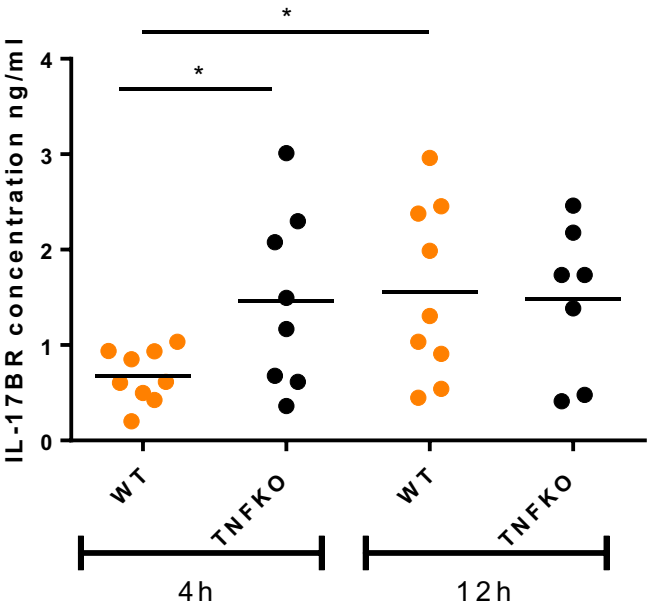


Figure 17: Impact of wet shaving on IL-17BR production in WT and TNF-KO mice

IL-17BR production in the irritated skin four hours (left panel) and twelve hours (right panel) post irritation, respectively. Data are shown as mean, each mouse is indicated by a single dot; n = 7-9 mice/group. The data was analyzed using the t-test. (*P ≤ 0.05).

At the protein level, we determined a significantly higher increase of IL-17BR levels upon wet shaving in TNF-KO compared to WT mice. We performed qPCR to see whether mRNA levels confirm this result. However, mRNA levels of IL-17BR were similar in both genotypes after four hours of incubation.

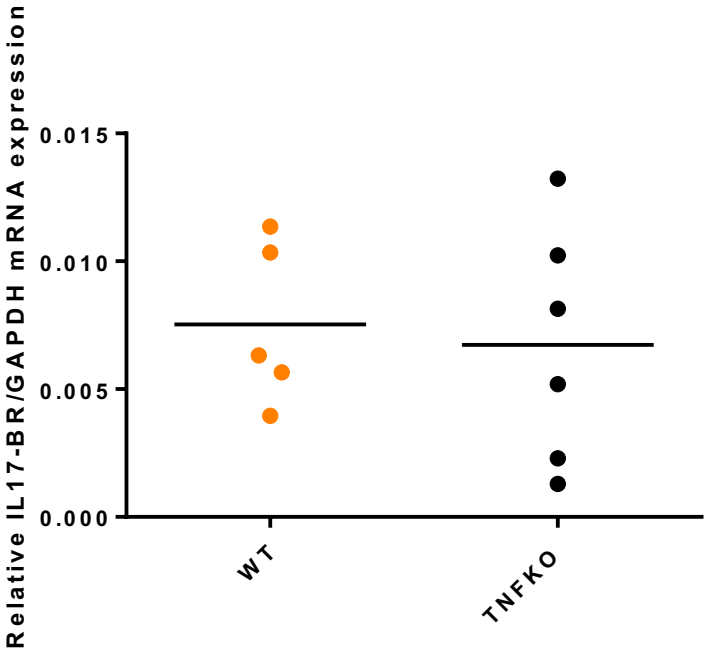


Figure 18: Comparative analysis of IL-17BR expression between WT and TNF-KO mice upon wet shaving

IL-17BR mRNA expression in the irritated skin four hours post irritation. Data are shown as mean; each mouse is indicated by a single dot; n = 5-6 mice/group.

3.2.7 IL-18

IL-18 is produced by various cells, such as dendritic cells or keratinocytes (117, 118). Being able to trigger Th1 as well as Th2 response, it is considered a pleiotropic cytokine (119, 120). Different studies have shown that IL-18 is associated with AD (121-124). Hence, the ELISA was applied to study whether IL-18 is regulated differently in the two genotypes upon physical skin irritation. After four and after twelve hours of incubation, the levels of IL-18 were similar in both genotypes. However, the levels of IL-18 increased in both genotypes significantly over time upon wet shaving. In WT mice the cytokine levels increased from 225 pg/ml to 330 pg/ml (p=0.002) and in TNF-KO, they increased from 259 pg/ml to 359 pg/ml (p=0.028) (Figure 19).

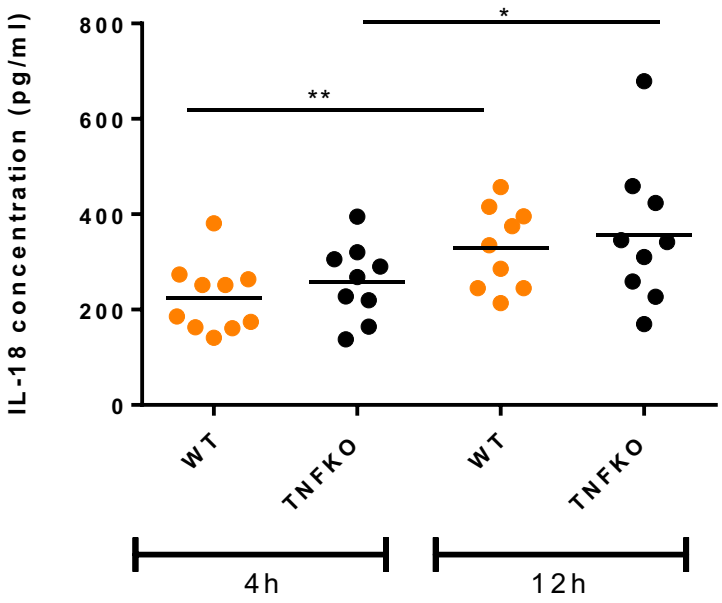


Figure 19: Impact of physical skin irritation on IL-18 production in WT and TNF-KO mice

IL-18 production in the irritated skin four hours (left panel) and twelve hours (right panel) post irritation, respectively. Data are shown as mean; each mouse is indicated by a single dot; n = 9-10 mice/group. Data was analyzed using the paired t-test. (*P ≤ 0.05, **P ≤ 0.01).

After measuring IL-18 production at the protein level, we performed qPCR to evaluate whether mRNA expression of IL-18 is differently regulated in the two genotypes. However, after four hours of incubation, the mRNA levels of IL-18 were similar in the genotypes (Figure 20).

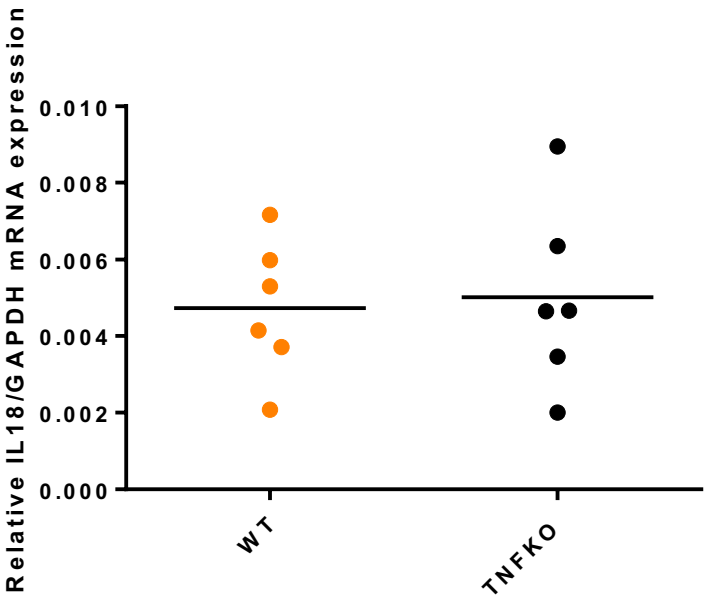


Figure 20: Comparative analysis of IL-18 expression between WT and TNF-KO mice upon wet shaving

IL-18 mRNA expression in the irritated skin four hours post irritation. Data are shown as mean; each mouse is indicated by a single dot; n = 6 mice/group.

4. Discussion

TNF-α is known to be one of the key factors and driving forces in various chronic inflammatory diseases (56, 59, 60). However, the role of endogenous TNF-α in physical skin irritation, skin inflammation and particularly AD remains elusive. Kumari *et al.* reported an adverse interaction between TNF and AD, suggesting that the absence of TNF results in an aggravated AD (19). Kumari et al. showed in an AD mouse model that TNF-KO mice developed an aggravated AD and displayed further increased TSLP expression as well as MCs numbers compared to WT mice (19). However, it is not clear why TNF-KO mice are more prone to develop AD symptoms upon skin irritation compared to WT mice. We speculated that TNF-KO mice show a different cytokine pattern upon skin irritation, which eventually may explain why TNF deficiency predisposes to enhanced manifestation of AD.

This thesis aimed to uncover major differences in cytokine patterns upon physical skin irritation in WT and TNF-KO mice *ex vivo* to pin down the mechanisms underlying AD predisposition in the absence of TNF. For this purpose, the skin of WT and TNF-KO mice was irritated by either wet shaving only or additional tape stripping *ex vivo*. Biopsies were taken and incubated for four and twelve hours. The supernatants were used for measuring the protein levels of cytokines and the biopsies were used to analyze gene expression. First, a multi array experiment detecting 98 different cytokines was performed. Based on the results, six cytokines were chosen for further investigation. This particular array was chosen as it was able to detect Th2 cytokines as well as key cytokines from the IL-1 family. Both groups are known to play a significant role in the pathogenesis of AD (31, 93). As IL-18, another important member of the IL-1 family, was not included in the panel. Therefore, we investigated IL-18 separately.

4.1 IL-1 alpha levels are similar in TNF-KO and WT upon physical skin irritation

Interleukin (IL)-1 alpha (IL-1α) belongs to the IL-1 family and has another isoform named IL1-beta (IL-ß). IL-1α is mainly produced by neutrophils, endothelial cells, epithelial cells as well as by keratinocytes (93, 103). IL-1α is reported to initiate and maintain inflammatory processes and to induce the expression of various other pro-inflammatory cytokines and mediators, such as IL-5 or TNF- α (93, 103, 105). Hence, IL-1α is frequently used as an indicator for inflammation and skin barrier impairment in clinical trials (104, 125, 126). Moreover, IL-1 is produced by keratinocytes upon disruption of the skin barrier and in the acute phase of AD (93). In humans, Machaelidou observed an increase of IL-1α protein levels in healthy individuals upon disrupting the skin barrier via tape stripping (data not published). Additionally, Reilly *et al.* as well as Doege *et al.* described an increase of IL-1α production in human skin upon tape stripping (126, 127). However, Dickel *et al.* did not report an increase of IL-1α mRNA expression in tape stripped human skin (128). The contradicting results may be explained by the different methods that were applied. Doege *et al.* performed 50 tape strips on the skin and measured superficial IL-1α production on Sebutape (126). Reilly *et al*. carried out 10 tape strips and analyzed IL-1α levels in suction blister fluids (127). Dickel *et al.* tape stripped the skin down to the stratum lucidum and measured IL-1α mRNA expression in curettage biopsies (128).
In mice, Wood *et al*. (1992) reported an increase of IL-1α mRNA expression upon tape stripping hairless mice (17). In 1996, these authors confirmed their results by means of immunohistochemistry and ELISA (129). Based on the literature, we expected an increase of IL-1α levels upon tape stripping. Interestingly, we determined a tendency towards a decrease of IL-1α protein levels upon treatment. Again, this may be explained through the different methods used. Wood *et al*. (1992) used male hairless mice, tape stripped them 5-8 times and incubated the skin for 35 minutes. Wood *et al*. (1996) used male hairless mice, tape stripped them four times and incubated the skin for 15 minutes. We shaved sacrificed female mice, tape stripped them 30 times and incubated the biopsies for four or twelve hours. It needs to be pointed out that shaving as well as tape stripping removes layers of the stratum corneum and, as a consequence, it also removes

IL-1α itself (126, 129). Moreover, longer incubation times may result in a decrease of protein levels as cytokine expressions follow certain kinetics. Wood *et al.* have shown that upon skin irritation, IL-1α levels already increase ten minutes upon tape stripping and start to decrease after four hours. Hence, our results are consistent with the literature.
Furthermore, we did not determine any difference between TNF-KO and WT regarding IL-1α protein levels upon physical skin irritation. Kutsch *et al.* showed that human keratinocytes produced significant amounts of IL-1α in response to TNF stimulation (130). Contrary to Kutsch *et al.,* we performed an *ex vivo* and not an *in vitro* experiment and used murine and not human material. Moreover, Kutsch *et al.* worked with exogenous TNF- α. This may explain the differing results. In accordance to our data, Kumari *et al*. from our group showed in an AD model that in lesional skin, TNF-KO mice express similar protein amounts of IL-1α as WT mice (19). In summary, these findings suggest that it is unlikely that IL-1α is a cytokine responsible for AD predisposition in the absence of TNF or that is regulated differently in TNF-KO mice.

4.2 CXCL1 protein concentration increases in TNF-KO and WT mice upon wet shaving and shows higher concentration in TNF-KO mice compared to WT mice

CXCL1, also known as Keratinocyte-derived Chemokine (KC) or growth-related oncogene alpha (GRO-alpha) or cytokine-induced neutrophil chemoattractant-1 (CINC-1), is a homologue to CXCL2 and belongs to the CXC chemokine family that has been reported to attract and activate neutrophils (107, 108, 131, 132). This chemokine family is also known to play a role in directing leukocytes to the sites of injury or inflammation, thus perpetuating the inflammatory process (133). It is expressed by macrophages, neutrophils, epithelial cells, keratinocytes and mast cells (96, 106, 108, 134). Furthermore, CXCL1 is induced in the human epidermis upon skin irritation and its inflammatory processes (96). Even though CXCL1 is considered as an AD related chemokine, its mechanism of action in the pathogenesis of the disease still remains unclear (131, 135-137). Our results show a significant increase of CXCL1 protein expression in both genotypes after twelve hours of incubation compared to four hours of

incubation upon wet shaving. To the best of our knowledge, we were able to show for the first time that wet shaving alone is sufficient to provoke CXCL1 production in murine skin. Using a MC903 irritation mouse model, Welsh *et al.* saw an increase of CXCL1 mRNA expression at a very early time point in their experimental setup before any itch behavior (138). This finding is in accordance with our results and implies an important role of CXCL1 during the early stages of skin irritation/inflammation.

Several authors described an increasing effect of TNF on CXCL1 expression in different experimental settings using human cells. Lo *et al.* showed that the incubation of HUVEC with TNF resulted in a significant increase of CXCL1 mRNA expression and CXCL1 production (139). Additionally, Shieh *et al*. presented similar results using human pulmonary epithelial cells (132). Moreover, Ohta *et al*. reported an increase of CXCL1 mRNA expression in human synovial fibroblasts upon TNF treatment (140). Based on the literature, we expected lower CXCL1 expression in TNF-KO mice. Our results, however, showed that endogenous TNF is dispensable for CXCL1 production, as we saw no difference in protein levels of CXCL1 in TNF-KO compared to WT mice upon physical skin irritation.

Contrary to the other authors mentioned above, we performed *ex vivo* experiments, whereas the other groups performed *in vitro* experiments adding exogenous TNF. Skin irritation is a complex process and its pathomechanism is not completely understood. Different systems and organs are tightly linked with each other. *In vitro* studies are known for being very different from the natural organism, lacking its complexity. *Ex vivo* models, on the other hand, are closer to the *in vivo* setting, mimicking normal skin (141, 142), whereas *In vitro* studies are not able to reproduce the complexity of a living organism. Additionally, the above-mentioned publications show data in human cell lines whereas we performed experiments in mice. The completely different experimental settings are possible explanations for the somewhat contradictory results. However, Segueni *et al.* used an *ex vivo* model and reported that the lungs of TNF-KO mice showed higher protein levels of CXCL1 compared to WT 28 days after a M. tuberculosis infection (143). This observation is in line with our results.

Because CXCL1 has been reported to cause itch and neutrophil infiltration and is released from mast cells as well as keratinocytes, other groups speculate that CXCL1 is

a chemokine that plays a role in AD predisposition in the absence of TNF and/or in physical skin irritation (96, 108, 138). However, in our study, no significant difference between TNF-KO and WT mice regarding CXCL1 expression upon physical skin irritation was determined.

4.3 Wet shaving induces CXCL2 production in WT and TNF-KO mice

Chemokine (C-X-C motif) ligand 2 (CXCL2), also known as macrophage inflammatory protein 2-α (MIP-2), belongs to the CXC chemokine family and is secreted by monocytes, macrophages and mast cells (108, 109). Even though it has been reported that tape stripping increases CXCL-2 production in mouse skin, Takahashi *et al*. observed a decrease in CXCL2 levels when it was tape stripped excessively, probably due to a loss of keratinocytes (94, 95). To the best of our knowledge, we were able to show for the first time that wet shaving alone is sufficient for a significant increase of CXCL2 production in mice over time. Hence, CXCL2 is a chemokine, which may play a role in the early steps of skin irritation/inflammation.

Different authors reported an increase of CXCL2 protein levels and/or mRNA expression upon TNF stimulation *in vivo* or *in vitro* in different organs of rodents. De Plaen *et al.* showed that IEC-6 cells increased CXCL2 protein production when incubated with TNF. This effect was dose dependent and significant at one hour following TNF incubation, and remained present after four hours (144). Li *et al.* showed that a nasal application of 10ng TNF is followed by an increase of CXCL2 mRNA expression in murine lungs of more than 200-fold (145). Additionally, Gong *et al.* saw an increase of CXCL2 mRNA levels in murine kidneys and liver upon intraperitoneal injections of 250ng TNF (4.2 and 6.6-fold increase, respectively). However, the mRNA expression of CXCL2 in the spleen was not affected by TNF injections, indicating that different organs may respond differently to TNF (146). As these authors demonstrated an increasing effect of TNF on CXCL2 expression we expected to see lower CXCL2 levels in TNF-KO mice. However, we did not detect a difference regarding protein levels of CXCL2 between WT and TNF-KO mice upon physical skin irritation. There are several possible factors behind this discrepancy. Contrary to our experimental setup, all the above-mentioned authors performed

experiments adding varying amounts of TNF exogenously. Moreover, De Plaen *et al*. performed an *in vitro* experiment, whereas we performed *ex vivo* experiments. Furthermore, the authors analyzed IEC-6, murine lungs, kidneys and livers for CXCL2 levels upon TNF administration, whereas we performed our experiments on murine skin. Interestingly, Kielian *et al.* performed, just like us, *ex vivo* experiments comparing CXCL2 levels in TNF-KO and WT mice in staphylococcus aureus-induced brain abscesses. The authors did not see any significant difference regarding CXCL2 protein levels, which supports our results (147).

As we detected no differences in CXCL2 production between TNF-KO and WT mice upon physical skin irritation, it is unlikely that CXCL2 represents a cytokine that underlies AD predisposition in the absence of TNF or that is regulated differently in TNF-KO mice.

4.4 TNF-KO and WT mice show similar CCL1 protein concentrations upon physical skin irritation

Chemokine ligand 1 (CCL1) is a small glycoprotein secreted by activated T-cells, MCs, Monocytes and endothelial cells (132, 148). CCL1 is known to attract monocytes and lymphocytes and plays a role in inflammatory processes (132). It has been reported that patients with AD show significantly higher levels of CCL1 mRNA expression and production in their skin lesions compared to nonlesional skin or normal skin (97, 98). Moreover, Gombert *et al.* observed significantly higher CCL1 levels in the serum of AD patients compared to healthy individuals (90). It has been suggested that CCL1 plays a role in the initiation and amplification of AD. However, while being associated with AD the role of CCL1 in the pathogenesis of the disease still remains unclear (98).

N'Diaye *et al*. showed that in human macrophages, CCL1 protein levels as well as mRNA expression increased upon TNF treatment (149). In mice, Heather *et al*. reported an increase of CCL1 protein in cytotoxic T-cells upon TNF treatment as well as in the serum of mice upon TNF injections (150). Therefore, we expected lower levels of CCL1 in TNF-KO compared to WT mice. However, we did not observe any difference in CCL1 protein levels in TNF-KO compared to WT mice upon physical skin irritation. This suggests that TNF plays a rather minor role concerning CCL1 secretion upon physical skin irritation. Contrary to N'Diaye *et al*. and Heather *et al*., we used murine material in an *ex vivo*

setting, which is closer to an *in vivo* setting (142). Moreover, the authors worked with exogenous TNF. Additionally, they did not perform any experiments on the skin as we did. Gombert *et al.* did not see any increase of CCL1 mRNA expression after stimulating human keratinocytes or dermal fibroblasts with TNF/IL-1ß (97). This aligns with our results. Overall, it is unlikely that CCL1 represents a cytokine that underlies AD predisposition in the absence of TNF or that is regulated differently in TNF-KO mice.

4.5 IL-5 protein concentration is significantly increased in TNF-KO and WT mice upon wet shaving

Interleukin (IL)- 5 plays a role in the differentiation of B cells and in the proliferation as well as activation of eosinophiles (112). It is produced by ILC2, granulocytes, T-cells, natural helper cells and MCs (112, 113). IL-5 is also largely produced by Th2 cells, which play a crucial role in the pathogenesis of AD, especially at the early stages of the disease (45). IL-5 mRNA expression was shown to be increased in acute as well as in chronic AD lesions (19). IL-5 is therefore associated with AD and was shown to be a key driver in differentiating AD patients from healthy individuals in their serum (100). Mepolizumab, an anti-IL-5 antibody, has been shown to be an effective drug against asthma, another atopic disease, but has so far failed to achieve significant results against AD (99). In our results, we saw a significant increase of IL-5 protein levels in TNF-KO and WT mice upon physical skin irritation. To the best of our knowledge, we were able to show for the first time that wet shaving as such leads to a significant increase of IL-5 production in the skin of WT and TNF-KO mice.

Moreover, we observed increased IL-5 in TNF-KO compared to WT mice after twelve hours of incubation, although this did not reach statistical significance. However, this result may be viewed as underpowered ($P = 0.07$) due to the low number of samples analyzed. As we performed an explorative study, sample size estimation was not possible beforehand. In agreement with our data, Fei *et al.* demonstrated increased IL-5 mRNA expression in an allergy model in murine lungs when TNF is blocked. Additionally, they observed significantly higher IL-5 protein levels in TNF-KO mice compared to WT mice in their experimental setup (151). These findings are in line with our findings.

The reason for higher levels of IL-5 in TNF-KO mice needs to be evaluated in more detail. IL-5 is known to play a major role in eosinophilia (113), whereas TNF has been described as regulating the neutrophil/eosinophil balance, and to hence have an inhibitory effect on IL-5 production (151). Furthermore, the hypothesis exists that Th1 and Th2 are two opposing immunological pathways and that activation of one pathway leads to the downregulation of the other (83). TNF is reported to be a key mediator in the Th1 pathway, and its absence in TNF-KO mice may lead to a Th2 dominance and its cytokines such as IL-5 (83). This hypothesis offers an explanation of the phenomenon of eczema onset in anti-TNF therapy. Additionally, TNF-KO mice were shown to have higher protein levels of TSLP upon physical skin irritation, and TSLP has been reported to increase the amounts of IL-5 produced per murine T-cell (19, 27, 152). Another possible explanation might be higher numbers of ILC2 or MCs in TNF-KO skin.

The IL-5 mRNA expression did not show any difference between the two genotypes after four hours of incubation. However, this might be due to the chosen time point. Further studies with a higher number of samples could clarify the possibility of underpowered data.

Interestingly, we detected a significant decrease in IL-5 protein levels upon additional tape stripping and twelve hours of incubation in both genotypes. A possible explanation might be the removal of IL-5 producing cells or of IL-5 itself via tape.

4.5 Wet shaving leads to significantly higher protein levels of IL17-BR in TNF-KO mice

Interleukin (IL)- 17BR, IL25R or Evi27 are the receptors to IL-17B, and together with IL17RA, also the receptors to IL17E/IL25, which mediates Th2 immune response, a crucial perpetuator in the pathogenesis of AD (115, 153). IL-17BR is not only expressed in human organs such as kidney, brain, intestines, testis, lung, pancreas and liver, but also in cells of the immune system such as dendritic cells, macrophages, Th2 memory cells and ILC2 (101, 102, 114, 116). The receptor exists in a soluble (sIL-17BR) and a membrane-bound isoform (mIL-17BR) (154). Even though IL-17BR is also the receptor for IL-17B, IL-25 shows a significantly higher binding and is hence considered the main

ligand for IL-17BR (153). IL-17BR is upregulated in the lesional skin of AD patients and is therefore associated with the disorder (115).

In our results, we showed for the first time that wet shaving induces a significantly higher production of IL-17BR in TNF-KO compared to WT mice. As we used supernatants from *ex vivo* skin biopsies for the ELISA, it is likely that we rather measured sIL-17BR. It is somewhat difficult to evaluate and discuss these results, as the role of sIL-17BR is not yet defined and poorly investigated. A possible role for sIL-17BR may be that of a decoy receptor that may decrease the concentration of IL-25. Contrary to that, sIL-17BR could also enhance or/and modify the effects of IL-25 (114). The IL-17BR mRNA expression did not show any difference between the two genotypes after four hours of incubation. It should be noted that on the mRNA level, sIL-17BR as well as mIL-17BR was measured.

4.7 IL-18 protein levels are similar in TNF-KO and WT upon physical skin irritation

Interleukin (IL)- 18 belongs to the IL-1 cytokine family and is not only produced by various cells such as osteoblasts and dendritic cells but also by keratinocytes (117, 118). IL-18 functions as a pleiotropic cytokine, which in the presence of IL-12 triggers the Th1 response, and its absence increases IgE production and induces the Th2 response (119, 120). Th2 cytokines such as IL-4, IL-13 or IL-5 are important inflammatory cytokines in AD. On the other hand, the Th1 response also seems to play a role in the pathogenesis of AD, especially in its chronic phase (31). Indeed, several studies reported higher amounts of IL-18 in the serum of AD patients (122-124). Zedan *et al*. reported a positive correlation between the serum IL-18 protein and AD severity (124). Inoue *et al*. showed that IL-18 is expressed at higher levels in the human epidermis of AD skin lesions compared to healthy individuals, and expression increased with the severity of the disease (155). Additionally, Konishi *et al.* demonstrated that mice producing abundant levels of IL-18 developed AD-like symptoms (121). Moreover, Chen *et al.* demonstrated in an AD mouse model that IL-18-KO mice developed a more mild form of AD, indicating that IL-18 plays crucial role in the development of AD (156). Whereas more and more studies hint at a crucial role of IL-18 in the pathogenesis of AD, its role in skin irritation remains elusive. We saw a significant increase in IL-18 protein levels in WT and TNF-KO

mice upon wet shaving. To the best of our knowledge, we were able to show for the first time that wet shaving as such is sufficient for this effect. In accordance with our results, Michaelidou from our group also detected a tendency of increased IL-18 protein levels in tape stripped human skin (data not published). These results suggest that IL-18 is involved in the initial cytokine response upon physical skin irritation and barrier disruption. In the context of AD, it is known that keratinocytes produce IL-18 in response to pathogens and allergens (155, 157). Our results suggest that scratching might also induce IL-18 production in the skin.

Marotte *et al.* demonstrated that human synovial fibroblasts express higher protein amounts of IL-18 when treated with TNF (158). We did not measure a significant difference of IL-18 protein or mRNA expression between TNF-KO and WT. In contrast to Marotte *et al*. we performed an *ex vivo* experiment using murine material.

Interestingly, IL-18 protein was decreased after additional tape stripping and at a 12 hour incubation time point. One explanation might be that tape stripping removes the upper epidermal cells producing IL-18. Not only Inoue *et al*. but also Lyubchenko *et al.* demonstrated that tape stripping removes significant amounts of IL-18 protein from the stratum corneum (155, 159). In summary, our results do not show a predisposition for increased IL-18 production upon skin irritation in the absence of TNF. However, we detected a deviation of IL-18 protein hinting at increased cytokine levels in TNF-KO mice. As our work was an explorative study, it was not possible to estimate the number of cases beforehand. In summary, IL-18 remains a key cytokine in the pathogenesis of AD and skin irritation. Further studies with a higher number of samples may bring clarity to this matter.

4.8 Reproducibility of results

Regarding our methods, we faced difficulties standardizing our method of physical skin irritation. Each shaving and each tape stripping varies in the pressure that is used or the number of times that the razor has to be ran over the skin, eventually resulting in varying irritation and hence varying cytokine levels. When comparing our results with the literature, it also needs to be considered that other authors, e.g. Doege *et al*., Reilly *et al*.,

Wood *et al*. or Angelova-Fischer *et al*., used different pressures and other kinds of razors or tapes (30, 126, 127).

Comparing the cytokine expression between the two genotypes, we observed a clear deviation of the protein levels of CXCL1, IL-5 and IL-18 twelve hours post irritation. These deviations are however not statistically relevant. We generally used 6-9 samples per group. Being an explorative study, it was not possible to determine the number of cases needed beforehand. Julious demonstrated that a sample size of 12 per group is recommended for pilot studies like ours (160). Thus, some of our results may be underpowered and this therefore limits their statistical relevance.

Another debatable factor in our experimental setup is the effect of the punch biopsy on cytokine production. For investigating further effects of wet shaving on cytokine production, we recommend including samples derived from of non-shaved biopsies as controls.

4.9 Conclusions and recommendations

The epidermis plays a crucial role for the organism as it forms a barrier against external threats such as radiation, heat, chemicals or pathogens (1, 3). Skin irritation may initiate inflammatory processes which, when chronified, may lead to chronic skin diseases such as AD (7, 14). The regulation of cytokines and chemokines as inflammatory mediators can be alternated upon skin irritation (7, 15). Tape stripping is a common technique in dermatological research to perturbate the skin barrier, remove layers of the stratum corneum, increase TEWL and induce an immune reaction via various pro-inflammatory cytokines (17, 95, 129, 161, 162). Hence, tape stripping forms part of AD mouse models, mimicking skin injury inflicted by scratching in patients (163). Whereas a lot of research has been done to understand the effects of tape stripping in murine skin, the consequences of wet shaving at a molecular level are not well defined. In human skin, Dabboue *et al*. showed that wet shaving induces comparable damage and TEWL increase as by 30 times tape stripping (164). To the best of our knowledge, we are the first study group to investigate cytokine production over time upon wet shaving WT and TNF-KO mice. Interestingly, we observed a significant increase of CXCL1, CXCL2, IL-5 and IL-18 upon wet shaving after twelve hours compared to four hours. Others have also

reported an increase of TSLP production in murine skin upon wet shaving (19, 27). Taken together, these results show that wet shaving alone is sufficient to damage the skin and provoke an immune response in mice. This is particularly important for mice models where shaving is required as a preparation step, as it may impact further events and results. In order to avoid this obstacle, we suggest using hairless mice, or to use other hair removal methods such as hair removal cream, or to keep a day in between shaving and further procedures.

Along with the effects of wet shaving, we also investigated differences in cytokine patterns between TNF-KO and WT mice upon physical skin irritation. TNF is known to be a key pro-inflammatory cytokine in different diseases (56). Yet, AD-like symptoms are a known side effect of anti-TNF drugs, and enhanced AD symptoms were reported in TNF-KO mice in an AD mouse model (19, 79, 80). This thesis aimed to pin down differences in cytokine regulation underlying AD predisposition in the absence of TNF. We saw a significant increase of IL-17BR levels in TNF-KO mice compared to WT mice upon wet shaving. However, as long as the role of sIL-17BR remains somewhat elusive, it is difficult to discuss this finding.
Moreover, we saw a tendency ($P = 0.07$) towards higher levels of IL-5 in TNF-KO mice upon wet shaving. It may be argued that the number of samples was too small and therefore this result can be viewed as underpowered. IL-5 is known to be a Th2 cytokine. Th2 cells and its cytokines, such as IL-4, play a crucial part in the pathogenesis of AD. Higher levels of IL-5 in TNF-KO mice could be founded on a stronger Th2 activity in these transgenic mice. Contrary to that, Kumari *et al.* showed in an AD model that in lesional skin, mRNA expressions of other Th2 cytokines (IL-4, IL-10) were equally expressed in WT and TNF-KO mice (19). We did not, however, perform an AD model, but we did investigate the effects of immediate physical skin irritation on cytokine production. Further investigations may answer whether or not TNF-KO mice show a stronger Th2 activity upon physical skin irritation.

Based on the literature, we expected to see an increase of cytokine production upon tape stripping. However, what we saw was rather a decrease in cytokine levels upon tape stripping. Other authors such as Wood *et al*. tape stripped hairless mice, and tape

stripped them only 4-8 times (17, 129, 162). Takahashi *et al.* showed that excessive tape stripping leads to a decrease in cytokine levels (95). Tape stripping removes keratinocytes that not only contain cytokines but also produce them (126, 129). Hence, our recommendation is to not tape strip excessively and to control the damage done to the epidermis by means of TEWL measurement. In this way, better standardization may also be achieved.

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„Ich, Sohrab Elahwiesy, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Frühe mechanische Hautirritation in der TNF-α defizienten Maus, Early physical skin irritation in TNF-α deficient mice“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

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Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Hiermit bescheinige ich, dass Herr Elahwiesy innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: *28.06.2021*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvolle Auswertung und Interpretation der Datenwurden während der Beratung erteilt:

- *Multiples Testproblem*
- *Unterschiede zwischen explorativem und konfirmatorischem Testen besprochen. Bei der Arbeithandelt es sich um explorative Analysen.*
- *Bei kleinen Stichproben Empfehlung nicht-parametrische Tests zu rechnen, da t-Tests in diesen Fällen verfälschte Ergebnisse liefern können*

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

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