

Function of Matrix Metalloproteinase 19  
in Cutaneous Immunity

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in Cutaneous Immunity

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MEINEN ELTERN

## EINGANG

WER DU AUCH SEIST, AM ABEND TRITT HINAUS  
AUS DEINER STUBE, DRIN DU ALLES WEISST;  
ALS LETZTES VOR DER FERNE LIEGT DEIN HAUS:  
WER DU AUCH SEIST.

MIT DEINEN AUGEN, WELCHE MÜDE KAUM  
VON DER VERBRAUCHTEN SCHWELLE SICH BEFREIN,  
HEBST DU GANZ LANGSAM EINEN SCHWARZEN BAUM  
UND STELLST IHN VOR DEN HIMMEL SCHLANK, ALLEIN.  
UND HAST DIE WELT GEMACHT. UND SIE IST GROSS  
UND WIE EIN WORT, DAS NOCH IM SCHWEIGEN REIFT.  
UND WIE DEIN WILLE IHREN SINN BEGREIFT,  
LASSEN SIE DEINE AUGEN ZÄRTLICH LOS...

RAINER MARIA RILKE

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# List of Abbreviations

<i>g</i>	unit of gravity
A	adenine
ADAM	a disintegrin and metalloproteinase
AEC	3-amino-9-ethylcarbazole
AP	activator protein
APC	antigen presenting cell, allophycocyanine
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumine
C	cytosine
CD	cluster of differentiation
CHS	contact hypersensitivity
CTLA	cytotoxic T lymphocyte antigen
Cy3	indocarbocyanine
DC	dendritic cell
dH <sub>2</sub> O	distilled water
DLN	draining lymph nodes
DN	double-negative (CD4 <sup>-</sup> /CD8 <sup>-</sup> ) T cells
DN	double-positive (CD4 <sup>+</sup> /CD8 <sup>+</sup> ) T cells
dNTP	2-deoxynucleotid-5-triphosphate
DTH	delayed-type hypersensitivity
ECL	enhanced chemiluminescence
ECM	extracellular matrix

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FCS	fetal calf serum
G	guanine
GAPDH	glyceraldehyde-3-phosphat dehydrogenase
GM-CSF	granulocyte-macrophage-colony stimulating factor
GPI	glycosylphosphatidylinositol
HLA	human leukocyte antigen
HRP	horse-radish peroxidase
I-TAC	IFN-inducible T cell $\alpha$ chemoattractant
i.p.	intraperitoneal
ICAM	intercellular adhesion molecule
ICD	irritant contact dermatitis
IFN	interferon
Ig	immune globulin
IGF	insulin-like growth factor
IGF-R	IGF receptor
IGFBP	insulin-like growth factor binding protein
IL	interleukin
kD	kilodalton
LC	Langerhans cell
LFA	lymphocyte function associated antigen
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIF	migration inhibitory factor
MMP	matrix metalloproteinase
MT-MMP	membrane-type of matrix metalloproteinases
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol myristate acetate

RANTES	regulated on activation normal T cell expressed and secreted
RT-PCR	reverse transcription PCR
s.c.	subcutaneous
SDF	stromal cell-derived factor
SDS	sodium dodecyl sulfate
T	thymine
TBE	Tris-borate-EDTA
TBS	Tris buffered saline
TCA	T cell activation gene
TCR	T lymphocyte receptor
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
vol.	volumes
WT	wild-type

# Chapter 1

## Objective

The human matrix metalloproteinase 19 (MMP19) is abundantly expressed in epithelial tissues. Keratinocytes, skin epithelial cells, constitutively express MMP19 in the basal layer where it contributes to IGF-signaling, cell proliferation and migration. MMP19 is able to cleave components of the extracellular matrix and several soluble proteins and is upregulated in skin diseases such as cutaneous cancer, psoriasis, bacterial and fungal infections. Studies towards the elucidation of MMP19 function were performed mainly *in vitro*, but its role *in vivo* is largely unknown.

Mice models are used to establish functions of molecules in a living organism and enables to draw conclusions to the human system. Mice with deletion of a specific gene, so called knockout mice, are widely used to determine the role of this gene by loss of its function. Thus, the phenotype of such mice can mirror the function of the targeted molecule.

To study the function of MMP19 *in vivo* mice with targeted disruption of the *MMP19* gene were generated. To reveal the impact of MMP19-deletion on cutaneous functions is the main goal of the work presented here. Since little is known about the role of MMPs in inflammation and MMP19 shows constitutive epidermal expression, the aim of this work was focused on the characterization of the role of MMP19 in inflammatory reactions of the skin. In order to delineate the role of MMP19 in innate and adaptive (T cell-dependent) immunity of the skin following aims were defined: 1. To elucidate MMP19 functions in acute skin inflammation. 2. To analyze influences of MMP19 on T cell-mediated inflammation and to study activation of T lymphocytes and dendritic cells. 3. To determine effects of MMP19 on cell transmigration.

Analyzing the function of MMP19 in the skin using MMP19-deficient mice can provide insight into the pathophysiological role of this protease in maintaining the epidermal barrier, especially in cutaneous inflammatory processes and immune responses. Furthermore, these studies can support the understanding of MMP activity in the skin.

# Chapter 2

## Introduction

### 2.1 The skin – structure and barrier function

The skin is the largest organ of the body, and its principal physical function is that of a barrier. The skin serves to maintain the homeostasis of the inner milieu by preventing desiccation. It regulates body temperature, produces endocrine mediators, and contains peripheral neural receptors and nerve endings. The skin, composed of epidermis, dermis, and hypodermal fat layer, functions as a barrier against potentially harmful exogenous influences. The barrier function is mainly achieved by the outermost layer of the epidermis, the *stratum corneum*. The physical stability is largely dependent on the underlying dermis, a dynamic connective tissue for the most part consisted of collagen and elastin fibers. The epidermis is a selfrenewable multilayered cornified epithelium build up by keratinocytes. In the basal epidermal layer, the *stratum basale*, keratinocytes are undifferentiated and connected to the subepidermal basal membrane. Generally, keratinocytes are characterized by expression of cytokeratins. Only cells of the *stratum basale* are able to proliferate and are characterized by expression of cytokeratin 5 and 14 (K5 and K14). Upon differentiation the cells loose their proliferative ability and the expression of K5 and K14 is replaced by that of K1 and K10. Additional marker for later differentiation stages are involucrin, fillagrin, and loricrin. After cell replication, a part of basal keratinocytes slowly moves up towards the surface of the epidermis and generates different cell layers. The basal layer is followed by the *stratum spinosum*, the *stratum granulosum*, and finally the *stratum corneum*. Last one consists of dead, anucleated, keratinized cells, which are removed by shedding. The epidermis contains also several other cell types. Melanocytes lie within the basal layer and are important for pigment synthesis and protection against ultra violet light. Langerhans cells, epidermal dendritic cells (DCs), are also located in the epidermis and involved in initiation of immune responses.

The basement membrane separating epidermal and dermal compartment is a highly organized network of extracellular matrix (ECM) that acts as a barrier and support for cell attachment. The latter is critical for the integrity and stable association of epidermis and dermis. The basement membrane is a dynamic structure mainly consisting of collagen type IV, laminins, nidogen, fibronectin, and heparan sulfate proteoglycans. Connection of epithelial cells to this matrix is coordinated by two cell surface receptors, integrins and dystroglycan, that bind to laminins and fibronectins [251]. Laminins are disulfate-linked heterotrimeric glycoproteins assembled from  $\alpha$ -,  $\beta$ -, and  $\gamma$  chains. Integrins are dimeric proteins containing  $\alpha$  and  $\beta$  subunits. Most abundant laminin of the cutaneous basement membrane is laminin5 ( $\alpha3\beta3\gamma2$ ) that functions in keratinocyte adhesion via  $\alpha4\beta6$  and  $\alpha3\beta1$  integrins [65, 72]. Integrins are connected to the cytoskeleton via actin molecules to support a certain flexible stability of cell-matrix interactions. Other subtypes of laminins for instance laminin6 ( $\alpha3\beta1\gamma1$ ) and laminin10 ( $\alpha5\beta1\gamma1$ ) and other ECM molecules contribute to this matrix network necessary for keratinocyte cohesion and skin barrier function [19].

### 2.1.1 The skin immune system

The skin is continuously exposed to a vast number of antigens, either in form of infectious or environmental stimuli such as from plants or chemicals. The skin has established a complicated defense system that is described as immunological. Since different cell types of the skin are associated within this complex cutaneous immune responses, the term skin immune system (SIS) was proposed [35]. Keratinocytes, Langerhans cells, T lymphocytes, vascular and lymphatic endothelial cells, macrophages, monocytes, and mast cells are involved in the skin's immune response, whereas other cell types are not (e.g. melanocytes, fibroblasts, glandular cells, smooth muscle cells). Of great importance for the SIS is the presence of Langerhans cells (LCs), epidermal dendritic cells (DCs) which are professional antigen presenting cells (APCs). LCs are located in the basal and suprabasal layers, forming a network within the epidermis. Like all nucleated cells, LCs express MHC class I molecules and also show constitutively high expression of MHC class II molecules (MHCII), that have important functions in the activation of T lymphocytes (T cells) [262]. LCs are potent to recognize foreign antigenic substances exposed to the skin. This might occur directly by MHCII molecules, but MHC I-restricted reactions also exist [36]. Expression of MHCII molecules on the surface of DCs as well as the MHCII half life increases after contact to exogenous agents [218]. Prior to the peptide uptake, LCs resemble immature DCs and are considered to be

precursors of DCs [250]. After antigen-recognition and -loading, LCs undergo maturation and like DCs express adhesion molecules such as ICAM-1 (intercellular cell adhesion molecule), LFA-3 (lymphocyte function associated antigen), CD80, CD86, and CD40, all of which contribute to antigen-presenting functions. Before presentation, the antigen undergoes intracellular proteolysis and is presented by MHC I and MHC II molecules as a peptide, harboring an antigenic determinant. This antigen-MHC complex can interact with T cells to induce immune reactions. A small part of epidermal DCs constantly migrate into and out of the skin, go into circulation and travel between the skin and lymphoid tissues. After antigen recognition the emigration of DCs out of the skin is strongly increased. Mature DCs also express chemokine receptors (CCR2, CCR7, CCR8, and CXCR4) that facilitate the migration into lymph nodes [87, 176, 205, 224, 241].

To develop adaptive immunity to a certain foreign component, activation of T lymphocytes ( $CD4^+$ ,  $CD8^+$  T cells) is needed that takes place in the lymph nodes by interaction of peptide-loaded DCs and naive T cells. T cells are equipped with antigen receptors (T cell receptors, TCRs) that can recognize antigens only in complex with MHC molecules. To gain optimal stimulation the combination of two defined signals is essential: 1) interaction of the TCR and MHC-peptide complex and 2) expression of costimulatory molecules [266]. The interaction between TCR and MHC-peptide molecules varies and might be dependent on the antigen's nature and the intracellular processing of the antigen. Endosomally modified peptides are loaded on MHC class II molecules and might be detected by MHCII-restricted  $CD4^+$  T cells. Other substances are processed in the cytoplasm and presented on MHC I molecules to  $CD8^+$  T cells. Alternatively, peptides can bind directly to MHC molecules and are recognised by T cells without intracellular processing. Costimulatory cell surface molecules on APCs such as CD80 and CD86 interact with their counterparts on T cells, for instance CD28 and CTLA-4 [266]. Furthermore, adhesion molecules expressed on T cells (LFA-1, CD2, ICAM-3, ICAM-2) serve as ligands for dendritic cell-surface receptors. These receptor/ligand interactions are fundamental for accurate T cell activation: ICAM-1/LFA-1, ICAM-3/LFA-1, LFA-3/CD2, CD80/CD28, CD80/CTLA-4, CD86/CD28, CD86/CTLA-4, and CD40/CD40L [190]. Moreover, cytokines play a supporting role in T cell activation. Upon binding of CD28, IL-2 production is highly upregulated that stimulates T cell proliferation and mediates antiapoptotic effects to protect T cells [32]. Other interleukins (IL-1, IL-6, IL-12, and IL-15) secreted by APCs also serve as costimulatory signals [129, 184, 259, 285]. Fractalkine (CX3CL1), a membrane bound chemokine constitutively expressed by DCs and LCs, is upregulated upon DC maturation and CD40-CD40L interaction to support firm adhesion of T cells [127, 209].

Activated (primed) T cells show altered repertoire of cell surface molecules. Naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells are positive for L-selectin (CD62) that is replaced by activation molecules during stimulation [260]. CD69 occurs very early and is temporary coexpressed with CD62. In addition to continuously expressed CD132 (IL-2 receptor  $\gamma$ -chain, IL-2R $\gamma$ ), the upregulation of CD25 (IL-2R $\alpha$ ) and CD122 (IL-2R $\beta$ ) leads to establishment of a full IL-2R, thereby CD4<sup>+</sup> and CD8<sup>+</sup> T cells exhibit different amounts of CD25 and CD122 [216]. Presence of TFG- $\beta$ , IL-1, and IL-6 enhances expression of IL-2R molecules [285]. Activated IL-2R functions via IL-2-mediated autocrine loop ensuring downregulation of CD62L, T cell proliferation, and activation of memory T cells and remains an activation status for other signaling pathways [29, 32]. The adhesion receptor CD44 expressed on circulating naive T cells is upregulated during stimulation. Subsets of memory T cells are generated that are mainly characterized by expression of both CD122 and high levels of CD44 [39]. Further, expression of CD95 (Fas) on activated T cells seems to support their survival, proliferation, and activation, since expression of CD95 can enhance TCR stimulation and deletion of CD95 on T cells causes lymphopenia [11, 101]. Both regulatory CD4<sup>+</sup> helper T cells and activated cytotoxic CD8<sup>+</sup> T cells are CD25/CD95-positive.

### 2.1.2 Inflammatory reactions of the skin

Inflammation occurs in all tissues of the body as response to different stimuli (pathogens/allergens, toxics, trauma, autoimmune disorders). To generate and resolve inflammatory reactions, immune functions are necessary that involve complex interactions between cells (DCs, T and B lymphocytes, macrophages) and soluble factors, including antibodies, cytokines, and the complement system. The skin immune system includes a large variety of such cellular and humoral factors. A dysregulation of this specific immunophysiology of the skin results in a large number of inflammatory and immunodermatological diseases.

To study inflammation of the skin models for hypersensitivity are widely used and established. Originally, in 1963, Gell and Coombs [90] classified hypersensitivity reactions in four groups: immediate, anaphylactic reactions (type I), antibody-mediated cytotoxic reactions (type II), immune-complex-mediated reactions (type III), and delayed-type hypersensitivity (DTH, type IV). But this classification cannot be used strictly to describe forms of hypersensitivity, since it is limited to specific pathogens, since more than one mechanism can be simultaneously and subsequently involved in a certain reaction, and species-specific differences exist [66, 212, 315]. Moreover, one single allergen or drug often induces a set of hypersensitivity reactions. For example,

a hypersensitivity model in mice to picryl chloride involves both a classical DTH response and IgE-mediated reaction [222] and penicillin in humans can show induction of anaphylaxis, type II allergic anemia, and contact dermatitis (type IV). Further, most cutaneous hypersensitivities to drugs do not fit into the Gell and Coombs's categories and recently defined mechanisms are not included in the classification. Thus, inflammatory hypersensitivity reactions are not simple definable, but can be described more adequately in three groups: 1) pseudo-allergic reactions, 2) primarily antibody-mediated reactions, and 3) cell-mediated reactions [66]. Nevertheless, the former classification is valid for some reactions including also T lymphocyte-mediated contact dermatitis.

Contact dermatitis (contact eczema) is a common cutaneous inflammation; in its acute phase characterized by erythema, pustule formation, pruritus, and infiltration of polymorph nuclear cells [153]. Inflammation occurs after single or multiple exposure to contact allergens, non protein and non immunogenic chemicals with a low molecular weight, also termed haptens. Upon contact to the skin haptens bind to epidermal proteins to form a hapten-carrier complex that is recognized as an antigen. Regarding the pathophysiological processes involved in contact dermatitis, two general forms can be distinguished: 1) irritant contact dermatitis (ICD) that is mediated by activation of the skin innate immunity via proinflammatory and toxic effects of the xenobiotic hapten (irritant), 2) allergic contact dermatitis (ACD) that needs acquired immunity and generation of hapten-specific effector T cells [95]. Insights into the pathophysiological mechanisms underlying ACD can be achieved via animal models, in which dermatitis is initiated by painting the hapten onto the skin's surface. This model, termed contact hypersensitivity (CHS) is used synonymously to ACD and determines a hapten-specific T cell-mediated skin inflammation.

The following parts will refer more on results generated from mouse models, since the inflammatory response and underlying mechanisms observed in the models could differ from the situation in man and should be better viewed separately. Limitation to transfer these two inflammation models is given by the fact that ICD and ACD are induced by haptens that exhibit both sensitizing and irritant capacities [324]. Thus, the irritation of a certain hapten contributes to the strength of inflammation even in a T cell-mediated response, mainly involving the hapten-specific immunity. In contrast to adaptive immunity, the innate immune system comprises non-specific defense mechanisms that recognize different exogenous agents, but do not confer long-lasting or protective immunity [10]. This is attained from cells of the innate system like mast cells, eosinophils, and natural killer cells and phagocytotic cells such as neutrophils, macrophages, and DCs. The last-named act directly on the clearance

of pathogens, whereas the others react with release of toxic substances (hydrogenperoxid, hypochloride, free oxygen radicals) to attack pathogens. Chemokines secreted from injured tissue cells and cells mentioned above induce the recruitment of further inflammatory cells to the site of contact. Due to recruitment of DCs and the process of antigen presentation, the activity of the innate immune system is also necessary to initiate specific immunity [82, 148, 183, 311, 324]. Thus, in regard to contact dermatitis it is important to understand that the inflammatory outcome is influenced by an interplay of irritancy and antigenicity and that innate and adaptive immunity are tightly connected.

### **Acute local inflammatory response – irritant contact dermatitis**

The stimulation of an acute local inflammation is initiated upon tissue injury by an irritant, whereby the severity of irritant contact dermatitis can be dose- and irritant-dependent [288]. Keratinocytes are central in inflammatory responses by producing several cytokines including tumor necrosis factors (TNF), interleukins (IL), interferons (IFN), chemokines, and other growth factors (GF) [80, 194, 269]. Early after stimulation keratinocytes start to produce and release TNF- $\alpha$  and IL-1 $\beta$ , which promote further secretion of other mediators that stimulate recruitment of inflammatory cells [80, 219]. Following IL-1 activation, proinflammatory IL-6, IL-8, and IL-18 as well as MCP-1 (macrophage chemoattractant protein), GM-CSF (granulocyte macrophage-colony stimulating factor), MIP (macrophage inflammatory protein), and complement factors are produced by keratinocytes, contributing to the inflammatory reaction [69, 71, 103, 131, 199, 201, 223, 289, 316]. Therein, IL-1 and IL-18 initiate expression of cell adhesion molecules like ICAM1 and VCAM1 that determine infiltration of inflammatory cells (basophils, mast cells, eosinophils, neutrophils, macrophages) and immune competent cells from blood vessels to the extravascular space to the site of hapten contact [71, 131]. IL-1 $\beta$ -mediated production of MIP-3 $\alpha$  and TNF- $\alpha$ -induced secretion of MCP-1 are important for recruitment of LCs and DCs to enable generation of adaptive immune responses [69, 201, 264]. Presence of IL-1 potentiates the expression of IL-6 and IL-8 also in fibroblasts and endothelial cells that further secrete cytokines like MIF (migration inhibitory factor) helping to induce cell infiltration [38, 93]. Secretion of eotaxin-1 (CCL11) by keratinocytes and fibroblasts shows dominant chemoattractant activity for eosinophils [281]. Additionally, chemokines such as CXCL9, CXCL10, and CXCL11 as well as CCL17 and CCL27 contribute to late inflammatory stages and exhibit involvement in cutaneous inflammatory reactions like atopic dermatitis and psoriasis [128, 264, 298]. The latter mentioned chemokines are predominantly chemoattractant for effector and memory T cells and thus additionally act on T cell-mediated local inflammatory reactions,

for instance ACD [85, 117, 128, 228].

### **T cell-mediated response – contact hypersensitivity**

A T cell-mediated immune response is described by the reaction of contact hypersensitivity (CHS), a model for allergic contact dermatitis, typically seen in reactions to urushiol (poison ivy), various drugs, cosmetics, and industrial chemicals [30, 48, 97, 152, 238]. In its acute phase the reaction is characterized by erythema and edema, followed by appearance of papules, vesicles, oozing, and crusting. Usually the reaction is accompanied by pruritus. In chronic stages the skin is lichenified and fissured. Systemically induced contact dermatitis can be induced by oral or parental application of a certain hapten [296]. Cutaneous CHS requires the machinery of the SIS (2.1.1) and the generation of hapten-specific T cells. The underlying mechanism to generate CHS responses comprises two critical events: sensitization and elicitation (Fig. 2.1).

**The sensitization phase (also afferent phase or induction phase)** occurs upon first hapten contact to the skin of an unsensitized individual and normally passes without symptoms. In humans the sensitization phase takes 10 to 15 days, whereas in mice it lasts only 5 to 7 days. As mentioned above (2.1.1), after crossing the corneal barrier and complex formation with carrier proteins, the haptens are captured by epidermal DCs, mainly LCs, undergo intracellular modifications, and are expressed as haptened peptides on the cell surface via MHCI and MHCII molecules. Hapten-loaded DCs migrate out of the skin into regional lymph nodes where they interact with naive CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the paracortical area [172]. This hapten-specific T cell priming (T cell activation) leads to clonal expansion of specific T cells and emigration of memory T cells into the periphery where they travel between blood, lymphoid organs, and the skin (2.1.1).

**The elicitation phase (also efferent phase or challenge)** of CHS occurs in sensitized individuals after renewed contact to the same hapten and is apparent as skin inflammation after 1 to 3 days and is persistent for another few days. Similar to mechanisms described for acute irritant inflammation (2.1.2), hapten application first induces secretion of cytokines (IL-1, TNF, and MCP-1) by keratinocytes, LCs, and other epidermal cells, which initiate LC activation, expression of surface molecules necessary for antigen presentation, and migration of antigen-transporting cells to regional lymph nodes [190, 198, 201, 219, 275]. Specific T cells resident in the skin and cytokines secreted by these T cells and epithelial cells promote recruitment of neutrophils, eosinophils, DCs, and activated T cells to the affected site. Therein,

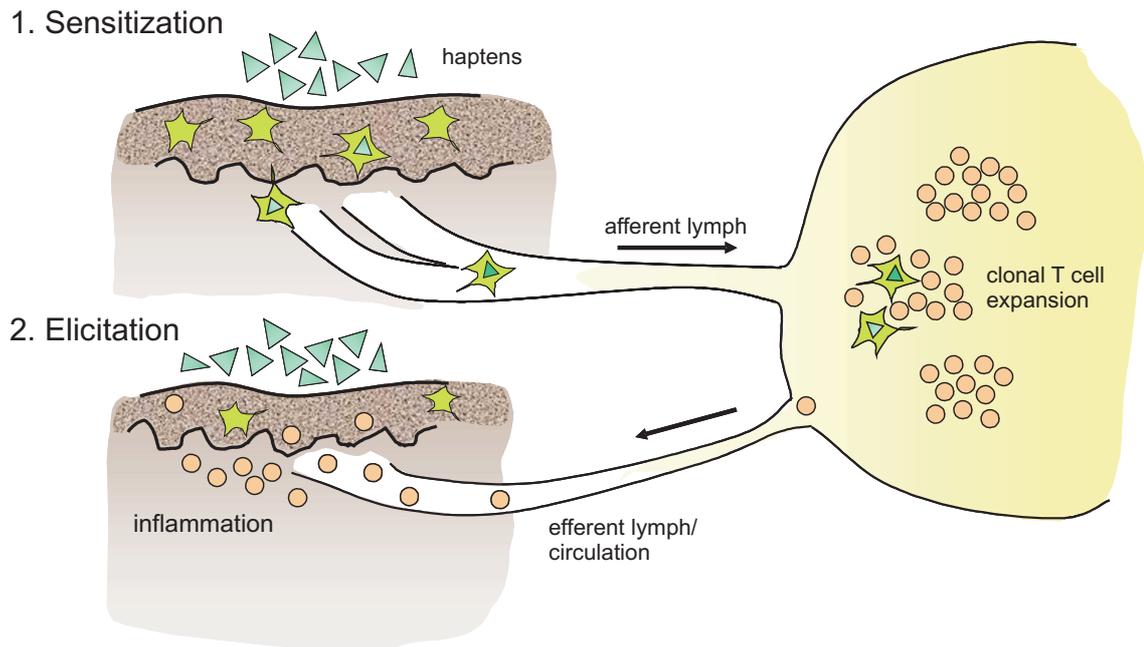


Figure 2.1: Development of contact dermatitis. For the generation of inflammatory reactions two critical steps are necessary: 1. sensitization and 2. elicitation. Sensitization is induced after first hapten contact to the skin. Langerhans cells take up haptens and migrate into lymph nodes, where they encounter with naive T cells leading to clonal expansion of hapten-specific T cells. Elicitation and inflammation occurs after second contact with the same hapten resulting in recruitment of specific T cells to site of renewed contact. Other cells (keratinocytes, neutrophils, and macrophages) and soluble factors (cytokines, chemokines) contribute to the inflammatory reaction.

the amount of recruited T cells is mainly controlled by the intensity of neutrophil infiltration [79]. Further, local APCs present the known hapten to memory T cells, supporting the recruitment of inflammatory cells. Hapten-specific  $CD8^+$  and later on  $CD4^+$  T cells are further attracted into the skin.  $CD8^+$  T cells appears to be the major effectors in CHS expression, since depletion of  $CD8^+$  T cells reduced CHS reaction, while MHC class II-deficiency leads to enhanced response [8,36,37,91,305,320]. Interestingly,  $CD4^{-/-}$  mice also fail to mount a CHS reaction, as these animals lack hapten-specific  $CD8^+$  T cells [239]. Antigen-induced activation of helper T cells (Th cells) results in the production of several cytokines that have inducing and regulatory functions in almost all kinds of immune responses. Production of type 1 (Th1) cytokines ( $IFN-\gamma$ , IL-2,  $TNF-\alpha$ ) or type 2 (Th2) cytokines (IL-4, IL-5, IL-10, IL-13) by both  $CD4^+$  and  $CD8^+$  T cells determine further initiation and resolution of the inflammation, whereby subsets of  $CD4^+$  T cells act prominently in the resolution and downregulation of CHS, partly due to IL-4 and IL-10 secretion [48,96,141,167,230,320]. Accordingly, deficiency of IL-10 results in enhanced LC migration [306]. Secretion of IL-21 leads further to reduction of LC migration,

inhibition of T cell activation, and reduced CHS reaction [40]. IL-16 produced by keratinocytes is chemoattractant for CD4<sup>+</sup> T cells and supports downregulating properties in CHS reactions [177].

In contrast to terminating signals, cytotoxicity of CD8<sup>+</sup> T cells is required for elicitation of a CHS response, as Fas/FasLigand and perforin double-deficient mice show no development of CHS reaction [133]. Proinflammatory capacity of IL-17 produced by CD8<sup>+</sup> cells is also necessary for CHS elicitation [106]. In addition to MCP-1, other chemokines like Gro $\alpha$ , MIF, RANTES (CCL5), and CXCL10 are essential in CHS responses, since mice deficient for these molecules exhibit impaired inflammatory reactions or those with chemokine overexpression showed enhanced responses [70, 75, 92, 186, 199, 255]. Furthermore, presence of eotaxin is important to promote eosinophil influx [280]. It is supposed that the sequential production of chemokines and differential expression of chemokine receptors on T cells determine the T cell infiltration at different times after hapten challenge [92, 238]. Thus, T cell-mediated inflammatory responses are dependent on the combination and interplay of several factors, including keratinocytes, APCs, T cells, and cytokines. In addition, recent studies revealed that NK cells can also mediate CHS responses independently of T and B cells [206].

## 2.2 The family of matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that function extracellularly at neutral pH. Since the discovery of MMPs, first described by Gross and Lapiere in 1962 [98] who observed collagenolytic activity in tadpoles during amphibian metamorphosis, broad investigations in the field of MMPs led to description of the MMP family. Currently, the human MMP family consists of 24 members. Analysis of genomic sequences show also presence of MMPs in a wealth of, if not in all mammals but also in other vertebrates (fishes, amphibians, birds). With greatly minor sequence homology MMP-related sequences can be found also in primitive species (for example tunicates and insects), indicating that this class of proteins developed early in evolution and presume involvement in essential functions of the organisms developed since then [161, 162].

Because of their common structural and functional similarities MMPs form a separate family, the matrixines [123, 317]. A systematical numbering of all MMP members was arranged by H. Nagase in 1997 (Gordon Research Conference, Andover, NH). Depending on the substrate repertoire and structural features, the MMP family are subdivided in collagenases (MMP-1, -8, -13), gelatinases (MMP2, -9), stromelysins

(MMP-3, -10, -11), membrane-type MMPs (MMP14, -15, -16, -17, -24, -25), matrilysins (MMP7, -26) and other MMPs not showing group-specific similarities. An overview is given in figure 2.2 [207] and in table 8.3 (see appendix). Looking on the modular structure, mammalian MMPs are multidomain proteins and characterized by two conserved domains, the propeptide-domain (pro-domain) and catalytic domain. The pro-domain contains a highly conserved sequence (PRCXXPD, X denotes any amino acid) with an unpaired cysteine residue, which interacts with a zinc ion ( $\text{Zn}^{2+}$ ), complexed by three conserved histidine residues in the catalytic domain (HEXXHXXGXXH). The designation of metalloproteinases were made due to this typically ligated  $\text{Zn}^{2+}$ . Binding of the cysteine to the catalytic domain blocks the active site and retains the protease in an inactive form. Presence of a conserved methionine (Met-turn) downstream of the zinc-binding site shows similarity to other metalloproteinases, like serralysines, adamalysines (also known as reprolysines or ADAMs), and astacines [225, 317, 318]. This methionine is part of the  $\beta$ -turn of the peptide chain that forms a hydrophobic basis for the zinc-binding site. Thus, together with the other protease families, astacines, serralysines, and adamalysins, MMPs belong to the metzincin group of proteases, named after the zinc ion and conserved Met-turn [31, 268]. A unified peptidase nomenclature was generated (MEROPS) in which MMPs belong to the M10A subfamily, the M10 family and the MA clan of metalloproteinases [226].

Most MMPs, except of MMP7, -23, and -26, contain a carboxy (C)-terminal hemopexin-like domain that mediates substrate recognition and localization to the cell membrane indirectly via adaptor proteins [42]. As linker, a flexible proline-rich hinge region connects the hemopexin-like with the catalytic domain. Subgroups of MMPs bound directly to the cell membrane exhibit further C-terminal domains. Membrane-type MMPs (MT-MMPs) have a transmembrane and a cytosolic domain, while MMP17 and MMP25 show GPI (glycosylphosphatidylinositol)-anchoring signals. Fibronectin-like domains in MMP2 and MMP9 (gelatinase A and B) might promote interactions with gelatin or gelatin-like substrates.

### 2.2.1 Regulation of MMP activity

Generally, the activity and regulation of MMP activation can not be described by single standardized mechanisms, rather specific circumstances such as cell type and tissue, environment of the cells, availability and amount of substrates, stimulation and activation of surrounding cells and proteins call for the necessity to defined processes, which guarantee optimal activity of each MMP member at a certain place and

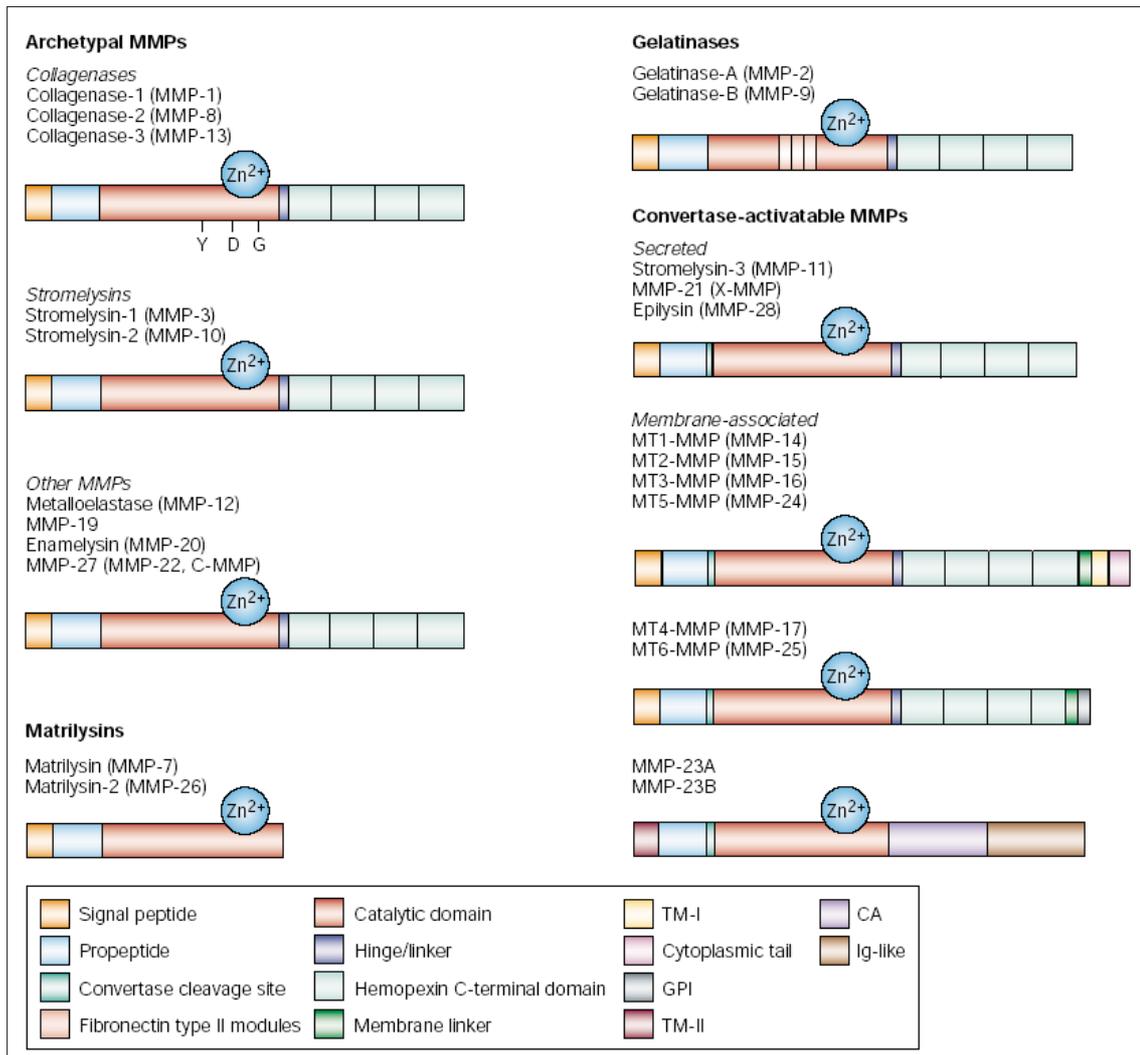


Figure 2.2: Overview of subclasses of the MMP family. Typical structural features of subgroups of the human MMP family. The zinc atom necessary for the catalytic activity of MMPs is pictured as blue circle. (The picture is taken from Overall and Lopez-Otin [207]).

time. Under pathological conditions balance between MMPs and their inhibitors is dysregulated. This suggests a tight regulation of MMP activity in physiological tissue homeostasis, which includes gene expression, proenzyme activation, and MMP inactivation.

Regarding the transcriptional regulation of MMP gene expression, cytokines and growth factors (e.g.  $IL-1\beta$ ,  $TNF-\alpha$ ,  $TGF-\alpha$ , EGF, and bFGF) were referred to stimulate gene expression of many MMPs [165, 229]. Pathways downstream of these stimulators converge often at AP-1 (activator protein-1) binding sites in the MMP promoter regions. Putative AP-1/TRE binding sites are located in nearly all MMP

promoters and AP-1 transcription factors are suggested to be central in gene expression of some MMPs. At least one part of the AP-1 heterodimer complex, consisting of jun and fos proteins, induces MMP promoter activity. Presence of PEA3 elements in almost all MMP promoters implicates further functions of ETS factors in MMP gene expression, which can also interact with AP-1 (reviewed in [49]).

Most MMPs are secreted as latent proenzymes (zymogen) and are activated by different mechanisms, mostly dependent on structural features. The cysteine switch model [267, 294] describes a mechanism, in which the conserved cysteine of the pro-domain, bound to the  $Zn^{2+}$ , dissociates upon stimulation. This switch then makes the active site accessible and leads to activation of the enzyme. Between the pro- and catalytic domain, some MMPs (MT-MMPs, MMP11, and MMP28) contain a recognition site for Golgi-associated furin-like enzymes, whose activity leads already intracellularly to cleavage and removal of the pro-domain and MMP activation. Serine proteases are also involved in the regulation of MMP activation. A potential role for urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), and plasmin in an activation cascade for MMPs has been suggested, whereby a plasmin-mediated stromelysin activation plays central regulation [142]. Few other serin proteases, Trypsin, kallikrein, and chymase were also found to cleave within the pro-domain, activate MMPs, and facilitates autoactivation. This autocatalytic cleavage can further lead to removal of the propeptide and confer permanent activity. The mechanism of spontaneous autoactivation is mostly unclear. In addition, MMPs could be activated by other MMP members. A well described mechanism of activation is that of proMMP2 by active MT1-MMP. Thereby, TIMP2 (tissue inhibitor of metalloproteinases-2) is bound to MT1-MMP and together both molecules serve as a kind of receptor for proMMP2 [45, 109, 272, 305]. Activation of proMMP13 was discussed to go off similarly by active MT1-MMP [143]. Alternative proMMP2 activation independently of TIMP2 is mediated by MT2-MMP [191]. Moreover, presence of reactive oxygen species, mainly generated by leukocytes under inflammatory conditions, may have stimulating properties on MMP activation [89, 215, 312].

On the other hand, to maintain homeostasis activity of enzymes have to be inactivated. MMP activity is blocked by natural, endogenous inhibitors, TIMPs (tissue inhibitors of metalloproteinases). The four existing TIMPs (TIMP1-4) inhibit MMP activity by binding noncovalently to the active site [299], in which they show different affinity to specific MMPs. Due to structural similarities of the catalytic sites of MMPs and ADAMs, some TIMPs, especially TIMP1 and TIMP3, also inhibit specific ADAMs [12, 13].  $\alpha$ 2-macroglobulin, another endogenous inhibitor influences a broad spectrum of proteases, including MMPs [263]. A membrane-bound glycoprotein, called RECK (reversion including cystein-rich protein with kazal motifs) is

potent to inhibit MMPs as shown for MMP2, MMP9, and MT1-MMP [204, 277].

In early studies MMPs were thought to have their only function in the degradation of extracellular matrix (ECM) proteins and accordingly, all MMPs isolated up to day are characterized by their ability to degrade various connective-tissue proteins. In its entirety the MMP family degrades all components of the extracellular matrix (ECM) whereby single family members show a broad overlap in the substrate spectrum. Generally, gelatin, collagens, laminins, fibronectin, and aggrecan are substrates of several MMPs. Besides matrix molecules other non-matrix substrates such as growth factors and cytokines, cell surface molecules, and also other MMPs have been identified as MMP targets. Even here MMPs have an overlapping function. For instance, IGFbps (insulin-like growth factor binding proteins), MCPs (monocytes chemoattractant proteins), SDF (stromal cell-derived factor), plasminogen, and pro-TNF $\alpha$  could be cleaved by several MMPs. Because of this great variety of substrates, MMPs are involved in many different physiological and pathological processes (reviewed in [180, 211, 301]). MMPs have defined influences on cell proliferation, migration, and apoptosis. On the pathological site, MMP-mediated matrix degradation is critical for tumor progression, cell invasion as well as in tumor angiogenesis. Physiological roles of MMPs include development of several tissues, modulation of reproductive functions, vascularization and wound healing mechanisms. Furthermore, they influence inflammatory reactions and the outcome of immune responses [211].

### 2.2.2 Function of MMPs in healthy skin

To understand the function of MMPs in cutaneous disorders it is helpful to look on their role and expression under physiological conditions, which involves both the quiescent epidermis and also tissue repair processes after skin wounding. Skin- or keratinocyte-specific MMPs do not exist and in quiescent, non-wounded situation expression of most MMPs is absent [170, 210], except of constitutively expressed MMP19 and ubiquitously present MMP2. Despite, several MMPs are important for the maintenance of the cellular integrity and the barrier function of the skin and various types of stimulation like wounding lead to induction of MMP expression. The sequence of normal wound healing events comprises hemostasis, inflammation, cell proliferation, and tissue remodeling [54, 68]. These stages show smooth transitions and are influenced by the action of different cell types (fibroblasts, keratinocytes, inflammatory cells, and endothelial cells) to gain ordered wound closure. Balanced interactions between cells and matrix components and the activity

of MMPs, TIMPs and cytokines are fundamental for homeostatic wound healing progression. Upon injury, contact between platelets and matrix components triggers the release of clotting factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF- $\beta$ ). PDGF acts on the chemotaxis of inflammatory cells and subsequently induces strong upregulation of keratinocyte growth factor (KGF), a member of the FGF (fibroblast growth factor)-family that interacts with the KGF-receptors on epithelial cells and is involved in keratinocytes proliferation, migration, and responsible for normal skin morphogenesis [41,286,314]. During the inflammatory stage of wound healing infiltrated polymorphonuclear leukocytes and later on macrophages secrete more PDGF and TGF- $\beta$  and further proinflammatory cytokines, such as IL-1, IL-6, and TNF- $\alpha$ , which contribute to persistent upregulation of KGF in fibroblasts [50,159,168,279,313]. After phagocytotic action of macrophages, fibroblasts attached to the deposited fibrin fibers start to proliferate and produce new matrix elements, mainly collagen type I, to reestablish tissue structure and function. Reepithelialization is stimulated by KGF, EGF (epidermal growth factor), and TGF and requires keratinocyte migration with constant supply from proliferating keratinocytes. Finally, the remodeling phase is characterized by synthesis and degradation of ECM components. ECM processing is mainly mediated by collagenase-cleaving enzymes such as MMP1, MMP3, MMP10, and gelatinases. Studies with broad-spectrum MMP inhibitors show general requirement for MMP activity in reepithelialization of cutaneous wounds [1,321].

To provide insights into proteinase function and regulation, cutaneous wound healing was studied in MMP-deficient and MMP-overexpressing (transgenic) mice, since *in vivo* models combine several healing processes: deposition of provisory fibrin matrix, migration of fibroblasts and keratinocytes, wound contraction, and remodeling of the skin's tissues. MMP1, -2, -3, -7, -8, -9, -10, -12, -26 and MT1-MMP are described to be involved during wound healing [3,18,67,99,151,173,220]. MMP1 is expressed in presence of collagen type I and is suggested to be involved in the detachment of keratinocytes from the basal membrane and in the promotion of cell migration [220]. Accordingly, transgenic mice with increased MMP1 expression in the skin exhibit retarded cell migration and delayed wound healing [67]. At the leading edge of migrating epithelium MMP1 shows colocalization with MMP10 that is needed for correct matrix deposition and further enhances keratinocytes migration probably due to processing of laminin5, an abundant protein of the basement membrane [151]. In contrast, MMP3- and MMP8-deficient mice also exhibit a delay in wound healing, since MMP3 expression is important for wound contraction and MMP8 for resolution of the inflammatory response [43,99]. Similar to MMP10,

MMP9 removes the temporary fibrin matrix and facilitates epithelial regeneration, since absence of MMP9 results in a delayed inflammatory response [188]. During wound healing process some MMPs (MMP3, -9, -10) are upregulated early and downregulated with time, whereas expression of others increases only in later stages (MMP2, -12, MT1-MMP), indicating distinct roles in remodeling processes. Interestingly, MMP12 secreted by macrophages show increased expression in deeper layers of the dermis only after complete reepithelialization [173,174]. Hieta et al. reported, that expression of MMP19 at the margin border of the wound, in dermal fibroblasts and macrophages seems to be involved in matrix remodeling [111]. Upregulation of MMP-26 and MMP-28 in the migrating area contributes further to wound closure [3,234].

Further, TIMP activities are also needed to regulate wound healing and to limit MMP activity. In contrast to MMPs, TIMPs are expressed also in quiescent epidermis and upregulated during normal wound healing processes. TIMP1 and TIMP3 are mainly found in proliferating keratinocytes and highly expressed at the wound margins [126,174,291]. Both, TIMP1 and -3 are also expressed in surrounding cells, like fibroblasts, macrophages, and endothelial cells. TIMP2 is more expressed under the epithelial migrating front [291], while TIMP4 is generally very rarely expressed and not detected in normal wound healing events.

### 2.2.3 Function of MMPs in diseased skin

Under pathological conditions epidermal expression of many MMPs increases dramatically. In neoplastic differentiation, MMPs are the main degrading enzymes and tissue destruction facilitates the penetration and migration of tumor cells. Their upregulation is strongly associated with the development of different skin cancers such as melanoma, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC) [126,136]. Dysregulated MMP expression is further evident in chronic skin inflammations like psoriasis and non-healing wounds and also involved in acute inflammatory reactions like ACD [126,307].

#### **Involvement of MMPs in cancer progression**

Dysregulated expression of MMPs is tightly connected with tumor progression, invasion, and metastasis formation as well as with angiogenic processes. During tumorigenesis, beside degradation of ECM molecules, MMPs cleave cell surface molecules, cytokines and growth factors and thus, act on alterations of cell adhesion and activation of various cell signaling pathways, which impact tumor cell proliferation and migration. In general, attachment of epidermal tumor cells to the basement

membrane and degradation of the surrounding tissue is essential for the process of invasion and metastasis formation. During cancer progression, tumor cells must cross the epidermal-dermal junction to invade into the dermis. Expression of MMPs is not restricted to tumor cells as MMPs are also abundantly produced in surrounding cells of the tumor stroma such as fibroblasts, vascular endothelial cells, macrophages and other inflammatory cells. MMP expression in three types of malignant skin cancers which are most common in humans is described in following paragraphs.

**The basal cell carcinoma (BCC)** arises from multipotential cells within the *stratum basale* and shows generally slow proliferation and low invasive and metastatic potential. In BCC, MMP1 is highly expressed in the surrounding stroma as well as MMP-2, -3, -9, -12, and MT1-MMP [6, 59, 76, 137, 323]. Expression of MMP7-, -10, -13, and -21 is more restricted to epithelial tumor cells while MMP7 appears at the tumor-stromal interface [2, 51, 130]. Extensive expression of MMP12 in macrophages and presence of MMP7 and MMP21 in tumor cells are in line with high invasive forms of BCC [2, 130, 138]. The activity of MMP13 and presence of chemokine receptor CXCR4 on tumor cells significantly contributes to invasion of BCC cells via an SDF-1-directed mechanism, since MMP13 is able to cleave SDF-1 [51, 181]. TIMPs as counterregulatory proteins are potent to decrease tumor growth. They are also located in lesions of BCC, where their expression is more limited to stromal cells. Inhibition of MMP activity might mainly be mediated by TIMP1 that is highly expressed [295]. Presence of TIMP2 is detected more strongly in low invasive forms and TIMP3 is located at the invasive tumor-stromal border, indicating inhibitory functions in aggressive stages [303].

**The squamous cell carcinoma (SCC)** derives from keratinocytes of the *stratum spinosum* and can arise also in other tissues such as airways, esophagus, and vulva. In difference to BCC, SCC shows high proliferative and invasive capacity. Abundant upregulation of several MMPs in stromal and tumor cells are in parallel with the aggressive phenotype of SSC [9, 61, 81, 119, 136, 156]. MMP1, -2, -3, -9, -11, -12, and MT1-MMP are expressed in the tumor stroma, while their presence can be found in primary tumor cells and tumor islets as well, except of MMP11. Therein, MT1-MMP is located at the invasive edge of the tumor epithelium [137]. MMP7, -10, -13, -21, and -26 are mostly expressed in cancer cells, while MMP26 is present only in early stages of SCC and downregulated in later highly invasive grades [3]. The disappearance of MMP19 in chronic wounds is associated with malignant transformation to SCC [121]. MMP19 is rather expressed in hyperproliferative epithelium covering or bordering SCC [235]. In contrast, MMP21 seems upregulated only in invasive stages of SSC [2]. Expression of TIMP1, -2, and -3 in stromal cells in the vicinity

to tumor edges is generally increased, maybe as result of host defense [5, 61, 136].

**The malignant melanoma** arises from neoplastic melanocytes and is characterized by a high invasive and metastatic potential of tumor cells. Increased expression of MMP2 and upregulation of MMP1 and MT1-MMP was reported to contribute to melanoma progression [7, 115, 122, 163, 200]. Thereby MT1-MMP acts at two sites; it directly degrades components of the basement membrane and contributes to the activation of MMP2, that is mainly active in cells of the tumor stroma in close vicinity to invasive melanoma cells [114]. Low expressed levels of MMP1 and MMP2 could be present in early melanoma stages, but strong upregulation and activation of these MMPs promote melanoma cell invasion and migration [257]. Presence of MMP3 and MMP13 correlates with a more invasive and aggressive phenotype [57, 154, 304], while activity of MMP9 seems to play more essential role in early events of melanoma formation and invasion, although it persists also in later stages [113, 292]. Similar to MMP9, MMP21 seems to be expressed only during early melanoma progression and is downregulated in invasive stages showing micrometastasis [154]. TIMPs are essential for inhibiting melanoma cell growth, since overexpression of TIMP1 and TIMP2 results in reduced tumor growth [139, 189]. Presence of TIMP3 induces apoptosis and inhibits melanoma cell invasion [4]. Accordingly, TIMP3-deficiency leads to enhanced metastatic potential of melanoma cells in mice and it is proposed that TIMP3 inhibits MMP2 activity and limits tumor cell invasion [60]. The expression of MMPs is frequently altered in melanoma growth, resulting in uncontrolled tissue remodeling and increased invasive potential of melanoma cells.

### **Function of MMPs in inflammatory reactions**

Although MMPs have important roles in the turnover, processing, catabolism, and destruction of the ECM, they decisively function further in processes like protease activation and inactivation, cleavage and release of cell surface molecules, growth factors, and cytokines and thus, modulate the bioactivity of this molecules and influence also cellular functions.

Apart from neoplastic skin disorders, MMPs are dysregulated in other skin diseases such as psoriasis, chronic wounds, and contact dermatitis, which all go along with inflammation. MMPs were reported to play important roles in inflammatory reactions but little is known about their role in inflammatory reactions of the skin.

**Psoriasis** is a chronic skin disorder that develops on a certain genetic background and suggested as a T cell-mediated immune dermatosis associated with inflammation and epidermal hyperplasia. In severe forms of psoriasis systemic symptoms like

fever, weakness, arthralgia and generalized skin pustulation occur. Psoriatic lesions show erythematous and scaly changes as well as chronic (vasodilation, edema) and acute (neutrophils, macrophages) inflammatory signs [261]. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are present in psoriatic lesions that secrete Th1 (IL-2, IFN- $\gamma$ ) and Th2 (IL-4, IL-10) [20, 247]. High amounts of GM-CSF (granulocyte macrophage-colony stimulating factor) and IFN- $\gamma$  produced by lymphocytes, accompanied with low levels of IL-3 and TNF- $\alpha$  promotes hyperproliferation of keratinocytes [23]. Additionally, IFN- $\gamma$ -mediated activation of complement factors like C5a and C3 and expression of IL-1 and IL-8 contribute to inflammation [27, 248]. These cytokines are capable of regulating MMP transcription and psoriatic lesions show upregulation of MMP2, MMP9, MMP12, and MT1-MMP. MMP9 and MMP12 are produced by macrophages and neutrophils, while MMP2 and MT1-MMP mainly are upregulated in suprabasal keratinocytes [84, 258, 273]. Increased MMP1 expression occurs in mild forms of psoriasis and is reduced again in more severe stages [86]. Further, altered expression of MMP19 in hyperproliferative keratinocytes is reported, whereas MMP28 expression appears not to be upregulated [274]. Increased levels of TIMP1 and TIMP3 occurs in inflammatory cell infiltrates, while TIMP1 and TIMP2 in keratinocytes are downregulated in contrast to healthy skin [258]. It is supposed that changes in both MMP and TIMP expression contribute at least in part to hyperproliferation and matrix remodeling in psoriasis.

**Chronic wounds** are associated with inflammation and ulcerative processes, and excessive activity of MMP8, -12, and -13 as well as reduced levels of TIMP1 dampen wound closure due to increased turnover of matrix components. These MMPs together with gelatinases, MMP2 and MMP9, as well as MT1-MMP appear to be overexpressed exclusively in surrounding cells (neutrophils, macrophages, fibroblasts) in comparison to normal wound healing situation [17, 121, 126, 185, 221, 233, 290]. Increased MMP1 activity in chronic wound fluids occurs probably due to elevated levels of IL-1 $\alpha$  that is able to stimulate MMP1 activity [22]. Notably, expression of MMP3 in keratinocytes of chronic wounds are not different from healing wounds [233]. Appearance of MMP7 and MMP13 in keratinocytes might contribute to malignant transformation in chronic wounds [121]. General downregulation of TIMPs at the wound edges promote MMP expression and especially the lack of TIMP2 may contribute to MMP2 overexpression [291]. Similar situations are obvious in other skin inflammatory diseases like stasis dermatitis that is a consequence of impaired venous drainage. In affected skin production of MMP1, -2, and -13 is upregulated with simultaneous decrease of TIMP1 and TIMP2 [110].

Generally, involvement of MMP activity in inflammatory diseases of the skin is rarely examined. It seems that mere overexpression of MMPs is not the patho-

logical factor in generating chronic skin diseases, rather MMP expression appears mostly upregulated due to inflammatory processes accompanied by downregulation of their inhibitors that contribute to pathogenesis of cutaneous malfunctions.

Contribution of MMP activity in generation of allergic contact dermatitis (ACD) is less analyzed, although Wang et al. [307] clearly demonstrate that mice deficient for MMP3 or MMP9 show altered contact hypersensitivity. In MMP3-deficient mice CHS response was impaired, whereas loss of gelatinase B (MMP9) leads to prolonged response. Lymphocytes of sensitized MMP3  $-/-$  mice do not proliferate upon stimulation with the specific hapten, showing that MMP3 is critical for the initiation of the response. MMP9-deficient mice failed to produce IL-10 demonstrating an essential role of MMP9 for the resolution of the CHS response. Reduced migration of Langerhans cells (LCs) in these MMP-deficient mice may further contribute to skewed CHS responses, since MMP3 and MMP9 are secreted by LCs to facilitate their migration through their dermo-epithelial barrier [144]. Furthermore, activity of both MMPs in fibroblasts and epithelial cells is directly inducible by proinflammatory mediators [310]. MMP12 and MMP13 can exhibit additional involvement in the outcome of CHS reactions, since gene expression analysis show prominent increase of these MMPs in the inflamed tissue [104]. Thus, activity of different MMPs is involved in chronic and acute inflammatory reactions.

#### **2.2.4 The matrix metalloproteinase 19 (MMP19)**

The human matrix metalloproteinase 19 was first discovered by Sedlacek et al. as autoantigen in rheumatoid arthritis and cloned from a cDNA library, generated from the synovium of a rheumatoid arthritis patient [253]. Thereafter, the trivial name RASI (rheumatoid arthritis sign of inflammation) indicates this origin of MMP19 [252]. Independently and in parallel, MMP19 was described by two other groups that cloned MMP19 from cDNA of the liver and mammary gland [58, 214]. The proenzyme consists of 508 amino acids and has a molecular weight of 57.4 kD. MMP19 shows highest sequence homology to epilysin, known as MMP28 [164, 175], and exhibits further similarities to the group of stromelysins and thus it is also defined as stromelysin-4 (Gordon Research Conference, 2001). The proenzyme of MMP19 retains the common domain organization of non-membrane bound MMP members, but also show several unique structural features. The autoinhibitory sequence, usually PRCGVPD, in the propeptide domain is atypical, since valine is replaced by leucine and proline by glutamic acid (PRCGLED). The catalytic domain of MMP19 contains an additional cysteine that is also present in MMP28 and MMPs

from chicken (CMMP) and xenopus (XMMP) [175,322]. Within the hinge region, a unique oligoglutamic acid stretch followed by a proline-rich sequence is located (NH<sub>2</sub>-EEEEETELPTVPPVPTEPSPM). Human (hMMP19) and murine (mMMP19) contain a threonine-rich carboxy-terminal tail of 36 and 56 amino acids, respectively. In the murine MMP19 this tail consists of 40 largely uncharged residues showing hydrophobic characteristics. hMMP19 contains two putative N-glycosylation sites and mMMP19 has four [192, 193, 252].

The hMMP19 gene is located on chromosome 12 mapping to the 14q region [214], while several other MMP genes are found on chromosome 11. Whereas most other MMPs are encoded by 10 exons human and murine MMP19 are encoded by 9 exons. Therein, exon 1-3 determine the prodomain, exon 4 and 5 mainly encode for the catalytic domain, and the hemopexin-like domain is distributed over 4 exons [192,193]. The hemopexin-like domain of MMP19 is responsible for its association to the cell surface [179]. If MMP19 interacts with the cell surface via integrins, similarly as described for MMP2 that binds to  $\alpha_V\beta_3$  [42], is unclear. As analyzed by kinetic studies TIMP2, -3, and -4 are potent to inhibit MMP19 activity, while TIMP1 shows less ability for MMP19 inhibition [271].

### **MMP19 – unique expression pattern and proteolytic activity**

At the mRNA level, MMP19 seems to be expressed in various tissues except of the brain [58, 214], while at the protein level its expression is more restricted to certain cell types. Vascular smooth muscle cells, myoepithelial cells, basal keratinocytes and microglia express MMP19 constitutively whereas endothelial cells and epithelial cells of the mammary gland show differential regulation of this enzyme [73, 146, 179, 236, 237, 293]. Additionally, PBMCs (peripheral blood mononuclear cells) positive for CD14 appears to produce most of the MMP19 in healthy blood [179]. This expression pattern of MMP19 points towards a specific role in epithelial cells and the basal membrane and is closely associated with the processing of components of the basement membrane.

Deregulated MMP19 expression in suprabasal layers of the epidermis was observed in different skin diseases associated with hyperproliferation like psoriasis, tinea, and eczema [237]. Differential expression of MMP19 was observed in endothelial cells, in epithelial cells within neoplastic differentiation of the mammary gland, and in squamous cell carcinoma (SCC). Benign lesions of mammary gland tumors and the surrounding vasculature show strong signals for MMP19 that is lost during malignant transformation and tumor cell invasion [73, 121, 147, 235]. Moreover, MMP19 expression is increased during inflammation such as multiple sclerosis and lyme arthri-

tis [26,293]. MMP19 is upregulated in TIMP1-negative capillary endothelial cells of acute inflamed synovial membranes and constitutively expressed in rheumatoid and traumatic synovial membranes. In capillary endothelial cells MMP19 is coexpressed with vascular endothelial growth factor receptor-2 (VEGF-R2) and the integrins  $\alpha_V\beta_3$  [146,149]. Association of MMP19 to the cell surface seems reasonable, since this allows targeted proteolytical activity in temporal and spatial manner.

MMP19 is capable to cleave several components of the ECM like collagen IV, nidogen, the large tenascin C isoform, laminin5 $\gamma$ 2, fibronectin, cartilage oligomeric protein (COMP), and aggrecan [235,270,271,284]. Also non-matrix proteins are processed by MMP19 like IGFBP3 (insulin-like growth factor binding protein-3) and a variant of VEGF (vascular endothelial growth factor) [158,236]. It is shown that MMP19 is also able to cleave MMP9, but this processing do not affect MMP9 activity [271]. The processing of nidogen-1 by endothelial-derived MMP19 leads to inhibited capillary-like formation of endothelial cells due to disruption of cross-linking sites for ECM molecules like laminin1 and collagen IV [284]. Thus, it is supposed that MMP19 functions to stabilize nascent blood vessel formation, as remodeling and destabilization of matrix proteins are essential for this process.

Studies by Jost et al. [125] regarding tumor growth revealed MMP19 as negative regulator of angiogenesis and invasion, since mice deficient for MMP19 exhibit early onset of angiogenesis after transplantation of malignant keratinocytes. Furthermore, induction of fibrosarcomas by chemical carcinogens seems to be reduced in absence of MMP19 [213].

### **Function of MMP19 in the skin and its impact on IGF-signaling**

Unlike other MMPs that are upregulated first under various stimuli MMP19 is expressed constitutively in basal, undifferentiated keratinocytes of healthy epidermis. In the group of R. Sedlacek it was find out that Tst-1 and Skn-1a, transcription factors important for keratinocyte differentiation, decrease MMP19 promoter activity and protein expression. Transcription factor-induced MMP19 inhibition results further in reduced keratinocyte proliferation and upregulation of terminal differentiation marker involucrin [25].

Processing of basal membrane components such as collagen IV and laminin5 by MMP19 influences epithelial cell migration. Transmigration of HaCaT cells, a human keratinocytes cell line, through collagen type IV and fibrin matrices is promoted by MMP19 [235]. Increased processing of laminin5 $\gamma$ 2 was observed in extracellular matrix and supernatants generated from a human keratinocyte cell line (HaCaT) that was transfected with wild-type MMP19 (WT). Cells producing an

inactive mutant of MMP19 (EA), in which a sequence of the zinc-binding site of MMP19 coding for glutamate was altered to alanin, are not able to process the  $\gamma 2$ -chain [235]. Laminin5 $\gamma 2$  mainly interacts with the integrins  $\alpha 3\beta 1$  and  $\alpha 4\beta 6$  present on keratinocytes, whereby  $\alpha 4\beta 6$  is expressed on quiescent keratinocytes and  $\alpha 3\beta 1$  produced by migrating cells. Migration of untransfected keratinocytes is stimulated by presence of MMP19-induced  $\alpha 3\beta 1$  integrins and inhibited via  $\alpha 4\beta 6$  [235]. This indicates that processing of laminin5 $\gamma 2$  alters the integrin-mediated adhesion and promotes cell migration.

As seen from *in vitro* studies with HaCaT cells transfected with constructs encoding for functionally active MMP19 (WT) and inactive MMP19 (EA), MMP19 plays essential role in proliferation of epidermal cells [236]. Keratinocytes transfected with the EA-construct showed strongly reduced proliferation compared to WT-expressing cells. In the same study Sadowski et al. [236] also describes that MMP19 expressed by keratinocytes cleaves IGFBP-3, the main binding molecule for IGF-1. Thus, upon release, IGF-1 stimulates IGF-signaling by binding to its receptor (IGF-1R). Activation of IGF-signaling promotes cell proliferation that is essential for effective wound closure. Accordingly, MMP19 is found in basal keratinocytes, outer root sheets of hair follicles, and proliferating epithelium near to wound edges (R. Sedlacek, personal communication). Further, MMP19-mediated IGF-1R activation induces dephosphorylation of focal adhesion kinase (FAK) that has central functions in controlling cell adhesion and migration [108]. MMP19 producing cells showed simultaneously increased cell adhesion and migration on collagen I that is no substrate for MMP19. It is suggested that MMP19-dependent activation of the IGF pathway increases FAK turnover, i.e. higher frequency of FAK dephosphorylation and phosphorylation that enables increased cell adhesion and deadhesion and thereby cell migration. Moreover, IGF-1R activation mediates synthesis of a collagenase via MAP-kinase pathway, which reduces cell adhesion via processing of matrix proteins like collagen type I. Integrins appear not to be responsible for this migration process, since MMP19-stimulated IGF-1R activation yields no alterations in the integrin expression of keratinocytes [236]. In accordance, inhibition of MMP activity results in increased cell adhesion through stabilization of focal adhesion contacts [112]. In summary, MMP19 controls epithelial cell migration directly via processing of laminin5 $\gamma 2$  and indirectly by cleavage of IGFBP-3 and activation of IGF-1R. Functions of MMP19 can be described as proproliferative and promigratory.

### Generation of MMP19-deficient mice

The murine ortholog of MMP19 was amplified by PCR from liver cDNA [192] and from a mouse myoblast cell line [47]. Murine *MMP19* gene is located on chromosome 10 and shows 79% similarity to its human orthologue. In the catalytic domain sequence homology increases up to 84%, while the hemopexin-like domain with 65% is less identical to human MMP19 and also lacks one glutamic acid residue in the oligoglutamic acid stretch [47, 192]. mMMP19 protein consists of 527 amino acids and has a molecular mass of 59.1 kD.

As described previously [195], in the group of R. Sedlacek the generation of MMP19-deficient mice were performed by targeted disruption of the *MMP19* gene, depicted in figure 2.3. A targeting construct was generated in which a part of exon 3 and whole exon 4 encoding for the catalytic domain were replaced by a neomycin resistance cassette orientated in the opposite direction to the endogenous *MMP19* gene. For construction of the 5'-arm homology of the targeting construct, a 2.8 kb fragment spanning a region including exon 2 and most of exon 3 was amplified from genomic DNA using PCR. Similarly, the 3'-arm homology was created spanning a region of 2.8 kb including exon 5 and part of exon 8. The complete construct (pPNT/mmp19del) was injected into mouse embryonic stem cells and resulting neomycin-resistant colonies were screened for homologous recombination by Southern blotting [118]. Two selected clones (G5 and F6) were introduced into blastocysts via microinjection and chimeras were generated [116]. Mice harboring the disrupted MMP19 allele were backcrossed with C57Bl/6 mice.

Mice homozygous for the targeted gene develop without obvious alteration in morphology, weight, size, and fecundity compared to wild-type mice, which is consistent with the work of Pendas et al., who generated MMP19-deficient mice by replacing the *MMP19* promoter region and exons 1 and 2 [213]. Breeding of mice heterozygous for the targeted gene for MMP19 produces the expected Mendelian ratio of knockout animals of 25%. Thus, MMP19 does not seem to be essential for blastocyst implantation or embryonic development. No apparent changes in the histology of brain, bones, heart, kidney, liver, lung, reproductive organs, pancreas, skin, and spleen of MMP19-deficient mice were seen at 1, 3, 6, and 12 months.

Previous studies show that MMP19 has promoting roles in cell proliferation and migration and thus, may effect skin development and wound healing. MMP19 is abundantly expressed in healthy epidermis and dysregulated in chronic skin diseases and skin malignancies. Its role in acute inflammatory reactions of the skin is not known.

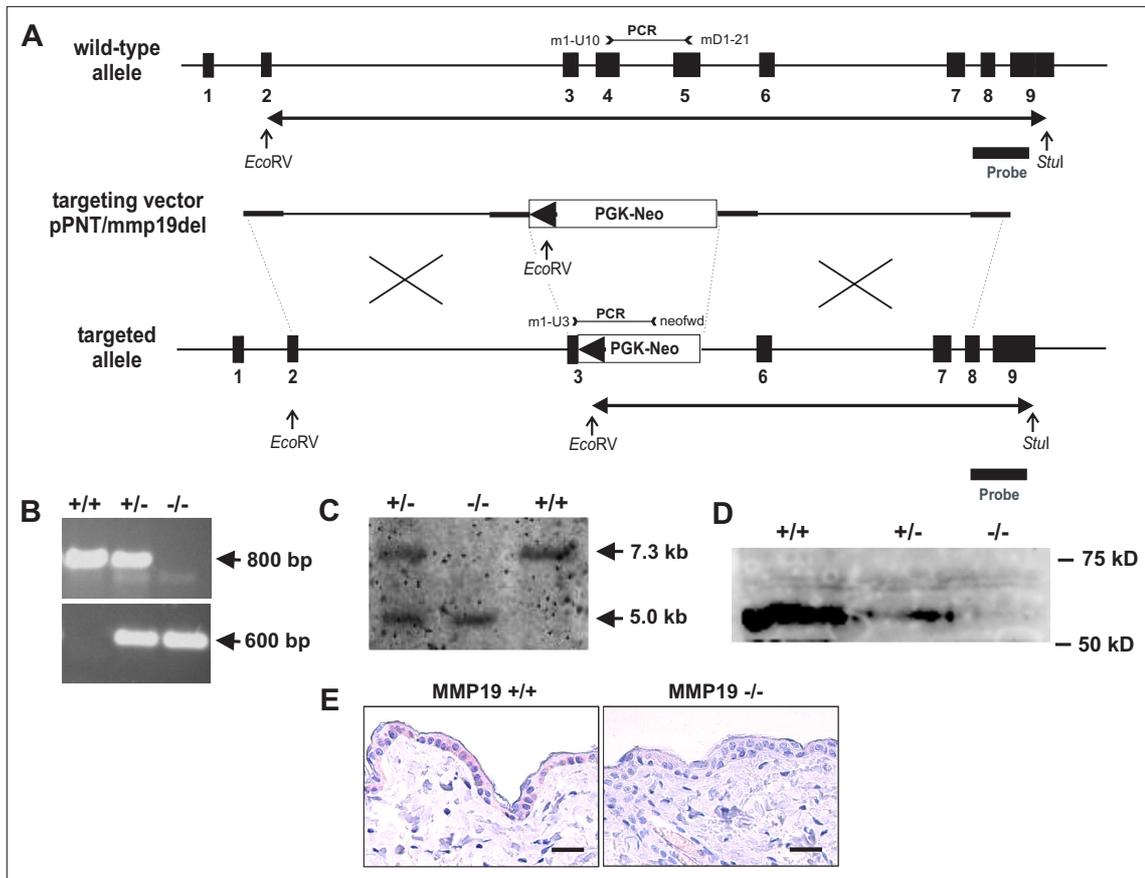


Figure 2.3: (A) Generation of MMP19-deficient mice was done by targeted disruption of the MMP19 gene. Schematic representation of the murine *MMP19* gene and its exon/intron organization as described before [192]. The pPNT targeting vector construct, and the resulting deleted locus of the mouse *MMP19* gene are depicted. The targeting construct (pPNT/mmp19del) was generated by replacing a region of 1088 bp spanning the end of exon 3 and complete exon 4 encoding for the catalytic domain by a neomycin resistance cassette. Homologous recombination leads to introduction of the PGK-Neo cassette and allows selection of homologous recombinants. The 3'-probe used for detection is indicated by thick bars. Also shown are restriction sites used for southern hybridization screening and primer binding sites used for PCR. Screening for replacement events employed restriction of genomic DNA with *StuI* and *EcoRV* and southern hybridization with a non-overlapping 3'-probe amplified with primers derived from exon 8 and exon 9. (B) Genotyping of targeted alleles using PCR. Wild-type alleles are detected by a 800 bp band, while PCR for the MMP19  $-/-$  allele results in a 600 bp product. Primers used for genotyping are indicated in A and described in section 3.1.2. (C) For screening of MMP19-deficient mice southern blot analysis were performed: mouse DNA digested with *StuI* and *EcoRV* was probed with the diagnostic 3'-probe. Probing with the 3'-probe led to identification of either a 7.3 kb band for the wild-type (+/+) or a 5 kb band for the targeted allele (-/-). (D) Primary keratinocytes isolated from wild-type mice, heterozygous (+/-), and homozygous MMP19-deficient mice were analyzed for MMP19 expression by western blotting. MMP19 is detected in size of 59 kD. (E) Immunohistochemical skin analysis. Skin sections from MMP19  $-/-$  and wild-type (+/+) mice were analyzed by anti-MMP19 antibodies purified against a peptide derived from the hinge region of murine MMP19. Gene-deleted mice exhibit no MMP19 expression in keratinocytes where it is highly expressed in wild-type animals. Scale bars: 50  $\mu\text{m}$ . Pictures courtesy of R. Sedlacek.

# Chapter 3

## Materials and Methods

### 3.1 Mice and *in vivo* models

#### 3.1.1 Housing and breeding of the mice

Mice used for the studies were housed individually at 21°C temperature in a 12 h dark/12 h light cycle in the local animal facility of the University of Kiel (Viktor-Hensen-Haus). All studies with animals were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols used were approved by the Institutional Animal Care and Use Committee and the government of Land Schleswig Holstein (Ministerium für Umwelt, Naturschutz und Landwirtschaft).

Mice with targeted disruption of the *MMP19* gene were generated as described above (2.2.4). For colony maintenance *MMP19*  $-/-$  mice were backcrossed to the C57BL/6NCrL background (Charles Rivers Laboratories, Sulzfeld, Germany). All assays were performed with *MMP19*  $-/-$  mice that have been backcrossed at least to the F10 generation. Studies focussing on the role of *MMP19* were performed using both lines generated, the *MMP19* F6 and G5 line. *MMP12*-deficient mice were kindly provided by Wee Yong (University of Calgary, Canada). The generation of *MMP12*  $-/-$  mice was published previously by J.M. Shipley [256]. Experiments to study the role of *MMP12* were carried out with *MMP12*-deficient mice backcrossed to the 129Sv background more than 6 times. In order to obtain aged-matched *MMP*-deficient mice for the experiments, backcrossed mice heterozygous for *MMP19* or *MMP12* were crossed. After genotyping of newborn mice, those with homozygous deletion of the *MMP* locus were crossed further.

### 3.1.2 Genotyping of mice

#### Isolation of genomic DNA from mouse tail

To identify the genotype of a mouse, genomic DNA was isolated according to a protocol described [116]. After cutting off a short part (0.5 cm) of the tail from newborn mice (2-3 weeks old), the tail biopsy was further chopped and incubated in 1 ml lysis buffer (200 mM NaCl, 100 mM Tris, 5 mM EDTA, 0.2% SDS, pH 8.0) with proteinase K (7.2 mU) at 55°C overnight in a rotator. 1 ml phenol: chloroform (1:1) was added to the digested tissue and shaken for 15 s and then centrifuged (900g, 2 min) to separate the phases. The upper phase was transferred to a new tube and mixed with 1 ml chloroform, shaken vigorously, and centrifuged again (see above). The aqueous supernatant was transferred and mixed with 1 ml ethanol (100%) and 100  $\mu$ l sodium acetate (3 M, pH 6.0). To precipitate the DNA the mixture was shaken carefully. After a further centrifugation step the pellet was washed with ethanol (75%). After discarding the ethanol the pellet was dried at room temperature and dissolved in 100  $\mu$ l dH<sub>2</sub>O overnight. The quality of the DNA preparation was measured photometrically at 260 and 280 nm.

#### PCR with genomic DNA

Genotyping of mice was done by PCR routinely according to the method originally developed by Mullis and Falona [197]. After isolation of genomic DNA from newborn mice two separate PCR setups for detecting the targeted and the wild-type allele of MMP19 were performed. Components for the reactions such as PCR buffers, dNTPs, MgCl<sub>2</sub>, and Taq-polymerase were purchased from Fermentas (St. Leon-Rot, Germany). For the WT allele a forward primer derived from exon 4 (m1-U10, CCGCATCTTCAATGTGCCC) and a reverse primer from exon 5 (mD1-21, GATCGCCAGGTTCACTCC) lead to amplification of a 800 bp fragment. For the targeted allele a forward primer from exon 3 (m1-U3, AGATGGATGACGC-CACAAG) and a reverse primer based on the sequence of the PGK-Neo cassette (neofwd, CTTCTATCGCCTTCTTGACG) amplified a product of 600 bp. Control setups with DNA from MMP19 *-/-*, MMP19 *+/-*, and WT mice as well as without DNA template were run additionally. One reaction consisted of 1,5 mM MgCl<sub>2</sub>, 2 mM dNTPs, 0.4  $\mu$ M of each primer, 1.25U Taq-polymerase, and 40-80 ng genomic DNA in PCR buffer (10 mM Tris, 50 mM KCl, 0.8% Nonidet P40, pH 8.8).

For detection of MMP12 wild-type or mutated allele PCR was performed with following primers: The wild-type allele was amplified with a forward (exon1-for, CTGCCTGTGGGGCTGCTCCCAT) and a reverse primer (in1-2rev, GCATTGCACACGGTTGTATGCA). The mutated MMP12 allele was detected by a forward primer

(exon 1, CTGCCTGTGGGGGCTGCTCCCAT) and the same reverse primer (neo-fwd) as used for analysis of the MMP19 knockout allele. PCR fragments for the WT and the disrupted allele of MMP12 showed a size of 1050 bp. Setup of one reaction and the controls were set up analogously to that performed for detecting MMP19 alleles.

PCR for amplification of all wild-type and targeted alleles was run in a Master Gradient Cycler (Eppendorf, Hamburg, Germany) with a program of 1 min at 95°C for initial denaturation, followed by 30 cycles of denaturation at 95°C (30 s), annealing at 60°C (30 s), and extension at 72°C (1 min). Finally, an extension step at 72°C for 7 min were made. Afterwards PCR products were analyzed by gel electrophoresis. PCR products were mixed with 0.2 vol. loading buffer (40% glycerol, 0.1% (w/v) bromophenole blue, 0.1% (w/v) xylene cyanole, 40 mM Tris, pH 8.0) and loaded into slots of agarose gels (1% in TBE buffer) that were run with 9 V/cm<sup>2</sup> in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0). A DNA standard (Fermentas) was used to compare the size of amplified DNA. PCR fragments in the gel were visualized by ethidium bromide (Roth, Karlsruhe, Germany) staining and UV trans-illumination at  $\lambda=312$  nm (BioRad, München, Germany).

### 3.1.3 *In vivo* inflammation models

#### Irritant dermatitis

To examine the impact of MMP19-deficiency in local skin inflammation the model of irritant dermatitis, also termed irritant contact dermatitis was used. Croton oil, prepared from seeds of the euphorbiaceae *Croton tiglium* was used as irritant. Applied to the skin, croton oil acts as strong irritant, inducing inflammation with formation of pustules and edema. The model of croton oil-induced dermatitis is commonly used and was performed with modifications to the protocols described formerly [93, 276, 288]. Irritant dermatitis was induced as follows. At the first day 50  $\mu$ l croton oil solution (0,8% in acetone) was applied epicutaneously on both sites of one ear. Before and 24, 48, and 72 h after application ear thickness was measured with a micrometer (Mitutoyo, Elk Grove Village, IL) to follow up edema formation, i.e. the inflammatory reaction. For each mouse, the ear swelling response was expressed as mean difference in thickness above the basal control value. Each experiment was carried out at least 4 times, each with 4 WT and 4 MMP-deficient mice. Figure 3.1 shows the course of events for this model.

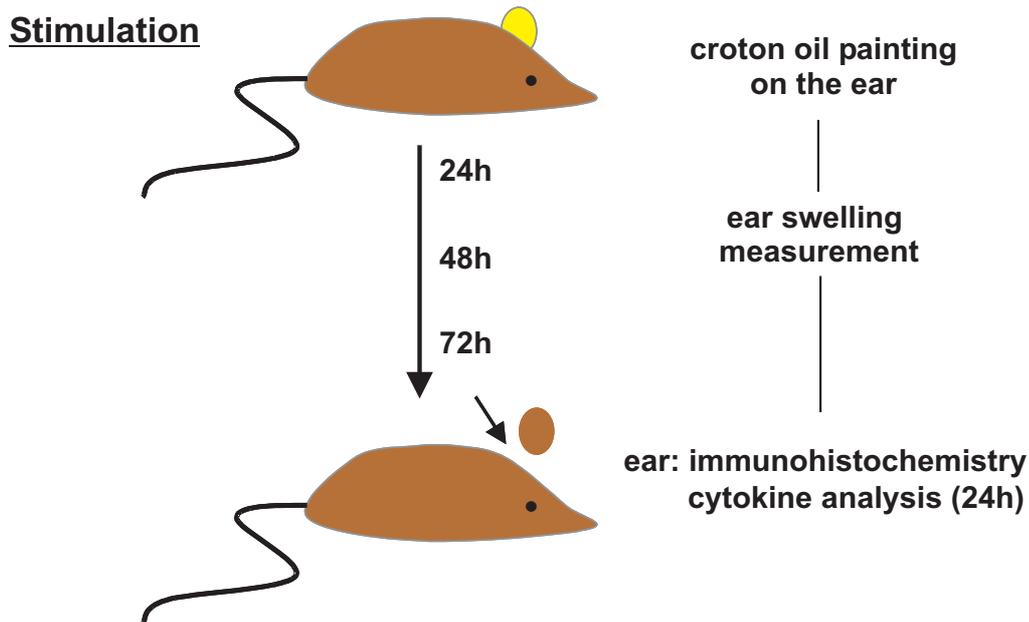


Figure 3.1: Inflammation model of irritant contact dermatitis.

### Contact hypersensitivity (CHS)

To analyze T cell-mediated inflammation of the skin the model of CHS was chosen, which shows a local inflammatory response of the skin following the challenge of hapten-sensitized animals. Studying CHS, normally artificial strong haptens are applied, which do not exist in the normal environment of the animal. After hapten application to the ear, the inflammation intensity can be quantified by ear swelling, the classical manifestation of CHS. In the model used here, FITC (Sigma, St. Louis, MO) was used as hapten, since epidermal DCs and Langerhans cells as well as their activation state can be easily determined by flow cytometry after they took up the fluorescent hapten and migrated into the lymph nodes.

Induction and measurement of CHS was carried out as described before [40, 172]. Briefly, for the sensitization, on day 0 FITC (3.5 mg/ml) were dissolved in 1:1 acetone:dibutylphthalat (Sigma) and 200  $\mu$ l were painted on the shaved abdomen of WT, MMP19  $-/-$ , and MMP12  $-/-$  mice. At day 5, after memory T cells were generated, mice were challenged with 50  $\mu$ l of FITC solution on the dorsal and ventral side of one ear. Ear thickness was measured shortly before (0 h) and 24, 48, and 72 h after challenge using a micrometer (Mitutoyo, Elk Grove Village, USA). CHS was carried out in four independent experiments each with 4 WT and 4 MMP-deficient mice. Mean values are given in mm as a difference to time point 0 h. To analyze DCs and T cells in draining lymph nodes (DLN), cells from the lymph nodes were isolated 48 and 72 h after challenge (3.2.3) and measured via FACS (3.2.3).

DCs were analyzed with antibodies against CD11c (BD Biosciences, San Jose, CA), CD80, CD86, and MHCII molecules (Caltag Laboratories, Burlingame, CA). For analyzing T cell activation cells were stained with antibodies against CD8, CD62L, CD69, CD44, CD122, and CD25 (BD Biosciences). DCs and T cell populations in inguillary lymph nodes were analyzed 24 h after FITC-sensitization with abdominal painting. The course of this model is illustrated in following figure 3.2.

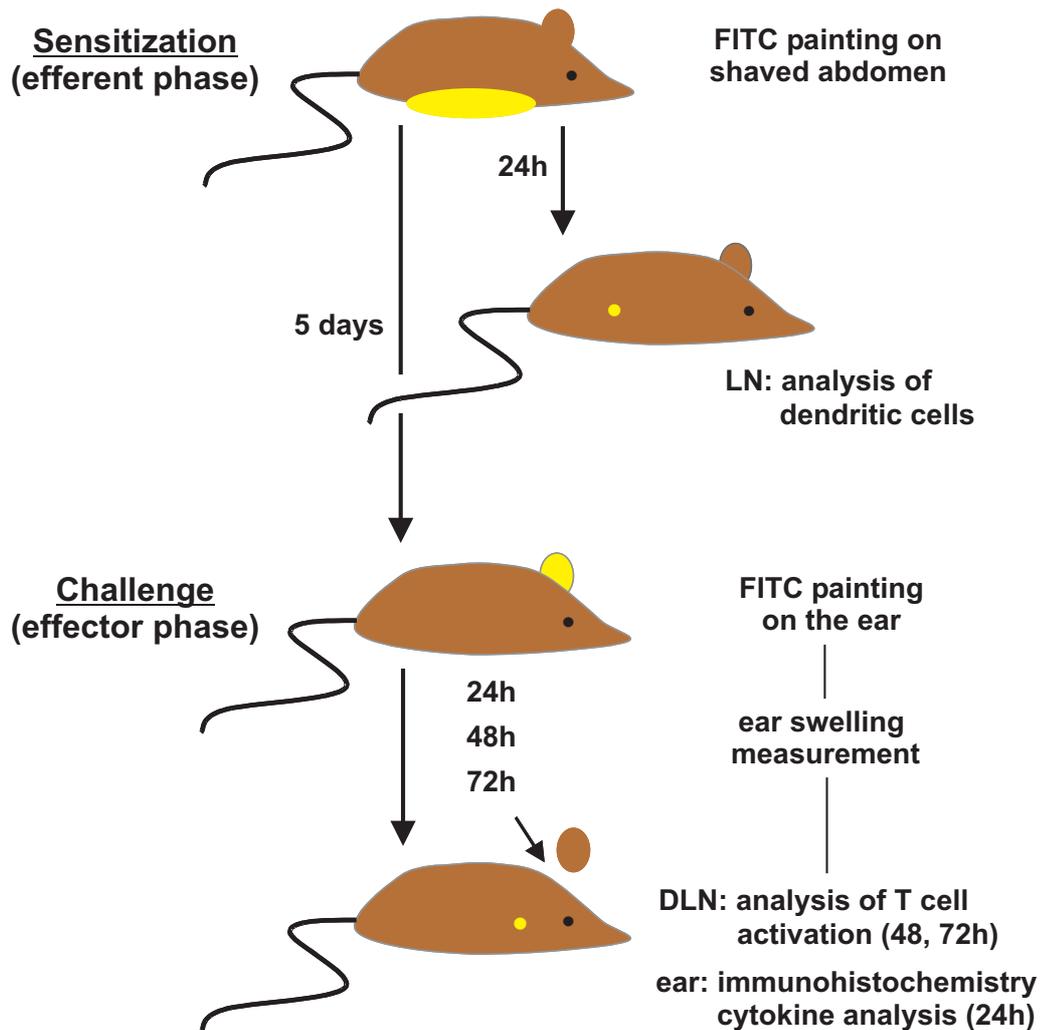


Figure 3.2: T cell-mediated inflammation model of contact hypersensitivity.

### Air pouch model

To investigate transmigration of macrophages *in vivo* an inflammation model, called air pouch model, was used. Therefore an artificial cavity ("pouch") was formed by injection of sterile air s.c. into the dorsal site of the mice. Inducing inflammation by injection of zymosan (Sigma, St. Louis, MO) into the pouch leads to infiltration

of inflammatory cells, which were analyzed by flow cytometry. All injections were done with anaesthetized animals. Zymosan is an insoluble carbohydrate from the cell wall of yeast (*Saccharomyces cerevisiae*) and is recognized and internalized by macrophages. This type of inflammation model was carried out as described [62,63,78,232]. On day 0 the pouch was formed by injection of 6 ml sterile air s.c. into the back of MMP19-deficient and WT control mice. To stabilize the pouch, on day 4 sterile air (2 ml) was again injected into the preformed pouch. Subsequently 1 ml of zymosan (1% (w/v) in PBS) was injected additionally. At different time points after zymosan injection (6, 20, and 40 h) mice were killed and the pouch was lanced as followed. After carefully removal of the skin without penetrating the pouch a small incision into the pouch membrane was made to isolate the exudate out of the cavity. Pouch lavage was performed 3 times with 1 ml PBS. Cells were collected, counted, centrifuged, and resuspended in FACS solution (PBS, 1% BSA, 0.1% NaN<sub>3</sub>). Cells of the pouch lavage were analyzed for CD3, B220, CD11b, CD14, F4/80, and Ly-6. At least 3 WT and 3 MMP19<sup>-/-</sup> mice were taken for each time point. Experiments were performed 3 times for each time point. Figure 3.3 explains the action of this model.

## 3.2 Tissue and cell analysis

### 3.2.1 Tissue preparation and immunohistochemistry

After performing the inflammation models of irritant dermatitis and CHS ears of mice were cut off and analyzed by immunohistochemistry. Tissues of mice were either fixed in 4% formaldehyde and embedded in paraffin (Walter, Kiel, Germany) or directly frozen in tissue freezing medium (Leica Instruments, Nussloch, Germany). Both cryosamples and tissue samples embedded in paraffin were cut into sections (3-5  $\mu\text{m}$ ) and transferred onto glass slides. Before embedding and storing tissues in paraffin they needed to be dehydrated by ethanol and xylene. Paraffin samples were stored at room temperature. For staining procedures sections were deparaffinized with xylene and rehydrated by descending alcohol concentrations again. Frozen tissue samples were stored at -20°C and fixed with ice-cold methanol (5 min at -20°C) shortly before performing the staining procedure. Generally, to get an overview of the tissue structure and the amount of inflammatory cells recruited to the site of inflammation staining with hematoxylin and eosin was performed.

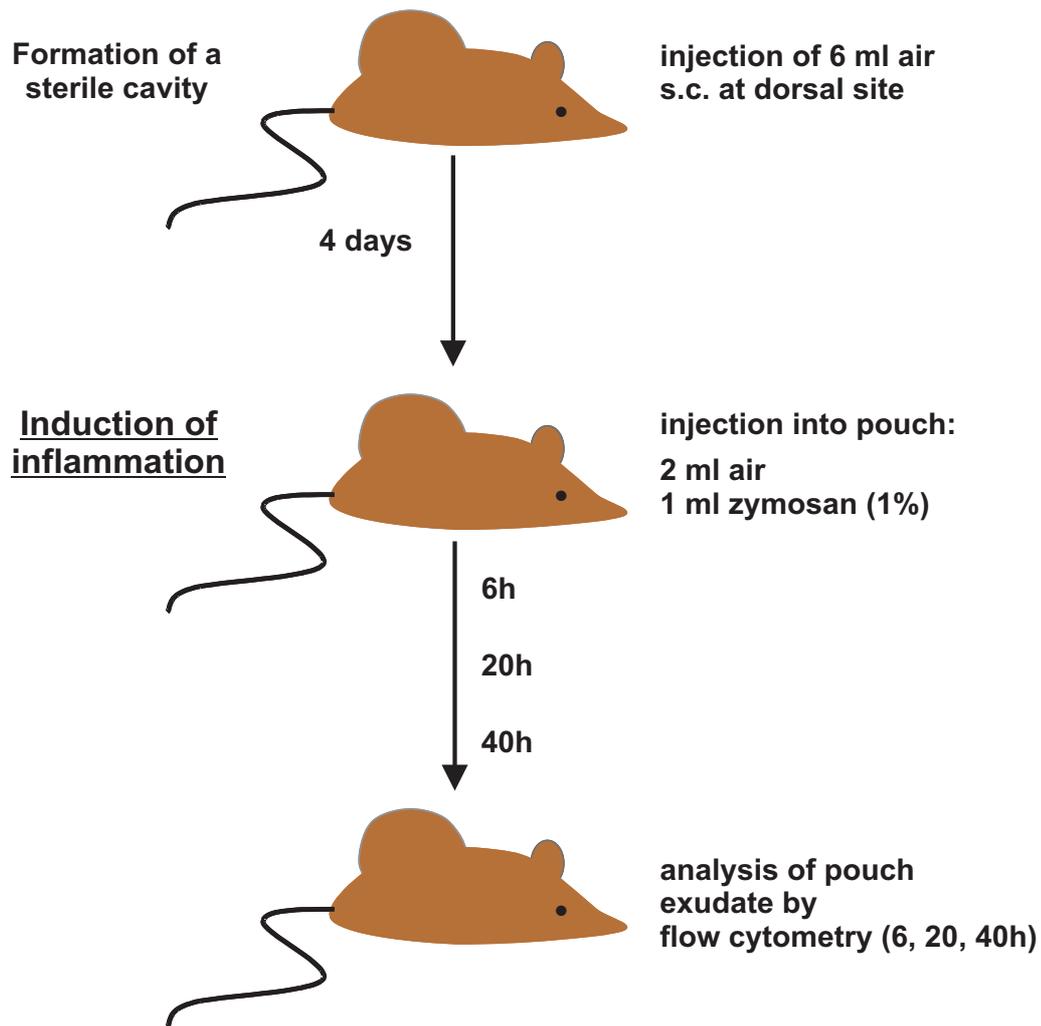


Figure 3.3: Air pouch model to study transmigration of inflammatory cells.

### Hematoxylin-eosin staining

To picture a structural overview tissues were stained with hematoxylin and eosin. This procedure stains nuclei of all cells in deep blue and the surrounding tissue in light purple, thus, enabling also the identification of infiltrated inflammatory cells. The general technique was described first by P. Mayer in 1896 [208] and also by others [160,171]. In detail, after washing in dest. water deparaffinized sections were incubated in hematoxylin (Gill III, Thermo Shandon, Pittsburgh, PA) for 6 min and further rinsed in acetic acid (0.5%) and dH<sub>2</sub>O, each step for 10 s. Afterwards slides were washed in tap water for 6 min. Counterstaining was done with solution containing eosin (Merck, Darmstadt, Germany) (0.1% eosin, 1% acetic acid in 96% ethanol) for 8 s. Slides were incubated in alcohol (ethanol) stepwise with ascending concentrations and finally in xylene (100%). Each washing step was carried out for

8 s. Sections were coverslipped with permanent mounting medium (Eukitt, Kindler, Freiburg, Germany) and examined by microscopy.

### **Peroxidase staining**

To analyze proliferation of keratinocytes and thymocytes sections of mice ears and thymi were stained against the proliferation marker Ki-67. Before antibody treatment sections were partly digested with 0,05% Trypsin (Roth, Karlsruhe, Germany). Monoclonal anti-Ki-67 antibody (clone Tec3) was diluted 1:50 in Antibody Diluent (both Dako Cytomation, Glostrup, Denmark) and incubated on the slide at 4°C overnight. After washing bound antibodies were detected using the Vectastain ABC Elite kit and peroxidase activity was monitored using AEC as a chromogenic substrate (both Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin (Gill III). Washing steps before and after trypsin treatment and antibody incubation were done in TBS (50 mM Tris, 0.9% NaCl, pH 7.6).

### **Immunofluorescent staining**

For immunofluorescent detection of proteins cryosections of inflamed mice ears and of mice thymi were used. After fixation in methanol sections were incubated in protein blocking solution (Dako) for 20 min at room temperature. Without washing sections were directly incubated with first antibodies. Monoclonal anti-CD8 (Chemicon, Temecula, CA), diluted 1:70, anti-Ki-67 (1:30), anti-CD144 (1:50), and polyclonal anti-laminin5 $\gamma$ 2 antibodies (Santa Cruz Biotechnologies, CA), diluted 1:50, were used. Antibodies diluted in Antibody Diluent (Dako) were incubated at 4°C overnight. After washing bound antibodies were detected with Cy3-conjugated goat anti-rat IgG antibodies (Jackson ImmunoResearch, West Grove, PA), diluted 1:1000, and with polyclonal FITC-conjugated donkey anti-goat IgG antibodies (Molecular Probes, Eugene, OR), diluted 1:500. Incubation of fluorochrome-coupled antibodies and following washing steps with TBS were carried out in the dark. Sections serving as negative controls were incubated with a universal negative control cocktail (Dako) that contains various subtypes of mouse immune globulins. Finally, sections were covered with Mounting Medium (Dako) and coverslips. Fluorescent signals were visualized by a fluorescent microscope (BX-50, Olympus, Tokyo, Japan). Images were captured by CCD video camera (Sony, Tokyo, Japan) and processed with the Analysis Soft Imaging System (Soft Imaging System, Lakewood, CA).

Molecule	Host and Species Specificity	Distributor
Ki-67	monoclonal rat anti-mouse	Dako Cytomation, Glostrup, Denmark
CD8	monoclonal rat anti-mouse	Chemicon, Temecula, CA
CD144	monoclonal rat anti-mouse	BD Biosciences
Laminin $\gamma$ 2	polyclonal goat anti-human (identical to corresponding mouse sequence)	Santa Cruz Biotechnologies, CA

Table 3.1: List of antibodies used for immunohistochemistry.

### 3.2.2 Analysis of cytokine expression

Since cytokines play essential roles in the development and resolution of inflammatory reactions, after performing the models of irritant dermatitis and CHS ears of mice were analyzed for cytokine expression. Analysis were done using a mouse cytokine antibody array according to the manufacturer's instructions (RayBiotech, Norcross, GA). List of 40 cytokines provided on this array is attached in the appendix (8.1). Briefly, ears were crushed in liquid nitrogen and resuspended in lysis buffer (provided) supplemented with proteinase inhibitors (Complete-EDTA, Roche, Basel, Switzerland). After incubation on ice for 1.5 h and centrifugation (950g, 15 min, 4°C) protein concentrations in supernatants were analyzed using a BCA protein analysis kit (Pierce, Rockford, IL). 500  $\mu$ g of total ear lysate was taken for each analysis. Bound cytokines were further incubated with a cocktail of biotinylated antibodies and afterwards with HRP-conjugated streptavidin. For signal development the ECL-plus Western blotting detection system (GE Healthcare, Little Chalfout, UK) was used and signals were recorded with a luminescent image analyzer (FujiFilm, Tokyo, Japan). Signal intensity was evaluated via AIDA Image Analyzer Software (Raytest, Straubenhardt, Germany).

### 3.2.3 Isolation and analysis of murine cells

To analyze the impact of MMP19 on specific cell populations different types of cells were isolated from wild-type and MMP19  $-/-$  mice. Experiments were carried out with peripheral blood mononuclear cells (PBMCs), thymocytes, peritoneal macrophages and cells out from inguinal and draining lymph nodes.

Isolation of blood cells were performed by intracardial punctation of anaesthetized mice. Blood was directly mixed with 60  $\mu$ l 0.5M EDTA to avoid clotting. Cells were washed in PBS and centrifuged (1500 rpm). To get rid of erythrocytes cells were

treated with sterile filtered lysis buffer (155 mM  $\text{NH}_4\text{Cl}$ , 16 mM  $\text{Na}_2\text{CO}_3$ , 1 mM EDTA, pH 7.2) for 10 min at room temperature. After centrifugation and washing with PBS cells were resuspended in PBS containing 1% BSA and 0.1%  $\text{NaN}_3$  and used for FACS analysis.

For preparation of DCs and T cells in the model of CHS lymph nodes from mice were isolated, washed in PBS, and incubated in medium (RPMI1640, PAA, Linz, Austria) until flow cytometry measurement. Lymph nodes were mechanically homogenized and cells were resuspended in FACS solution (PBS, 1% BSA, 0.1%  $\text{NaN}_3$ ). Similar procedure with excised thymi of mice was performed to analyze murine thymocytes. To isolate peritoneal macrophages mice were killed and injected with 7 ml PBS (i.p.). After carefully rolling of the mice the abdominal skin was removed without disturbing the peritoneum. Afterwards injected PBS containing peritoneal cells was obtained by punctation of the peritoneal cavity. To separate monocytic cells (macrophages) and lymphocytes peritoneal cells were resuspended in RPMI medium and incubated on petri dishes for 2 h. Monocytes and macrophages adhere onto the plate while lymphocytes remain in suspension and could be easily removed together with the medium. Macrophages were carefully removed mechanically and resuspended in FACS solution.

### **Flow cytometry**

Analysis by flow cytometry was performed with cells isolated from lymph nodes, PBMCs and thymocytes. Cells isolated from the pouch (air pouch model, see 3.1.3) were also analyzed via flow cytometry. Once the cells were resuspended in FACS solution following steps were performed on ice or at 4°C. For each analysis 250000 cells were taken and centrifugation steps were run with 250g for 5 min at 4°C. For detection of surface molecules only antibodies directly conjugated with fluorochromes were used. Staining with antibodies were performed in 100  $\mu\text{l}$  FACS solution for 1 h on ice at a dark place. Antibodies used for flow cytometry are listed in following table (Tab. 3.2). For PBMC analysis, before incubation with fluorochrome-conjugated antibodies cells were pretreated with rat anti-mouse CD16/CD32 antibodies to block Fc-receptors. Additionally, in each experiment unstained cells were prepared to generate the settings of the FACS (FACScanto, BD, CA) before starting the measurement.

### **Quantitative real time RT-PCR (qRT-PCR)**

To perform quantitative RT-PCR RNA from murine thymus and peritoneal macrophages, RNA was isolated with TRIZOL reagent (Invitrogen, Carlsbad, CA) and digested with DNase at 65°C for 10 min (Promega, Madison, WI). cDNA was syn-

Molecule	Host and Species Specificity	Fluorochrome	Distributor
CD3 $\epsilon$	monoclonal hamster anti-mouse	FITC PE-Cy7	BD Biosciences, Burlingame, CA
CD4	monoclonal rat anti-mouse	PE	BD Biosciences
CD8	monoclonal rat anti-mouse	APC FITC	BD Biosciences
CD11b	monoclonal rat anti-mouse	FITC	BD Biosciences
CD11c	monoclonal hamster anti-mouse	FITC APC	BD Biosciences
CD14	monoclonal rat anti-mouse	FITC	Caltag, Burlingame, CA
CD25	monoclonal rat anti-mouse	APC	BD Biosciences
CD40	monoclonal rat anti-mouse	PE	BD Biosciences
CD44	monoclonal rat anti-mouse	APC	BD Biosciences
CD62L	monoclonal rat anti-mouse	APC	BD Biosciences
CD69	monoclonal hamster anti-mouse	PE	BD Biosciences
CD80	monoclonal rat anti-mouse	PE	BD Biosciences
CD86	monoclonal rat anti-mouse	PE	Southern Biotech, Birmingham, AL
CD95	monoclonal hamster anti-mouse	PE	BD Biosciences
CD122	monoclonal rat anti-mouse	PE	BD Biosciences
MHCII	monoclonal mouse anti-mouse	PE	BD Biosciences
F4/80	monoclonal rat anti-mouse	APC	Caltag
Ly-6G/C	monoclonal rat anti-mouse	FITC	BD Biosciences
CD16/CD32	monoclonal rat anti-mouse	—	BD Biosciences
CD45R/B220	monoclonal rat anti-mouse	PE	BD Biosciences
BrdU	mouse anti-BrdU	FITC	Santa Cruz Biotechnologies, CA

Table 3.2: List of antibodies used for flow cytometry.

thesized from 1  $\mu\text{g}$  RNA by reverse transcription (RevertAid<sup>TM</sup> M-muLV Reverse Transcriptase, Fermentas). qPCR was performed using the ABI 7700 Prism Sequence Detection System and TaqMan primer probes specific for murine MMP19 (Mm01159954-g1; Applied Biosystems, Foster City, CA) and glyceraldehyd-3-phosphate dehydrogenase (GAPDH, Hs99999905-m1) as described [107]. RT-PCR was run in 3 replicates of each sample with a total sample volume of 20  $\mu\text{l}$ , containing 1  $\mu\text{l}$  TaqMan primer probe (assay on demand), 10  $\mu\text{l}$  TaqMan Universal Master Mix, and 10 ng cDNA template (diluted in RNase-free water). PCR was run with initial polymerase activation (95 $^{\circ}\text{C}$ , 10min) and 40 cycles of 15 s at 95 $^{\circ}\text{C}$  (denaturation) and 1 min at 60 $^{\circ}\text{C}$  (annealing and extension). GAPDH was used to normalize the values and as positive control. Analysis and calculation of  $C_T$ -values (cycle of threshold) were done as followed.  $C_T$  of each sample was averaged and  $\Delta C_T$ -values

were obtained by subtracting the  $C_T$ -value of MMP19 from that of GAPDH ( $\Delta C_T = C_T\text{MMP19} - C_T\text{GAPDH}$ ). Thus, the lower the MMP19  $\Delta C_T$ -value the higher the mRNA expression in this sample.

### **Transmigration assay of macrophages**

To study transmigration of macrophages *in vitro* the trans-well system (BD Falcon, Bedford, MA) was applied. After counting peritoneal cells isolated as mentioned before (3.2.3) cells were labeled with  $0.75 \mu\text{g}/10^5$  cells Calcein (Molecular Probes, Eugene, OR) in serum-free medium to follow up the transmigrated cells. Transmigration was tested through collagen type I and fibrin matrices. Collagen type I matrix was prepared by coating trans well inserts with  $5 \mu\text{g}/\text{cm}^2$  collagen I (BD Biosciences) in 0.02 N acetic acid. After incubation the inserts were washed with PBS. For the preparation of fibrin matrix ( $10 \text{ mg}/\text{cm}^2$ ) fibrinogen (Calbiochem, San Diego, CA) was dissolved (20% in RPMI medium with 2% FCS) on ice, supplemented with 0.5 U thrombin (Sigma) and incubated on trans well inserts. The matrix, polymerized for 30 min at room temperature, was overlaid with  $500 \mu\text{l}$  RPMI (10% FCS), and incubated for further 12 h at  $37^\circ\text{C}$ . Afterwards inserts were washed with PBS and  $10^5$  cells per well were seeded onto matrices. Cells transmigrated upon stimulation with supernatant of cultured 3T3 fibroblasts given in the lower chamber of the trans-well system. Controls were performed using RPMI medium only. Before and 12 h after incubation at  $37^\circ\text{C}$  the fluorescence in the upper and lower chamber was measured at 485 and 530 nm in a microplate reader (Lambda Fluoro 320, Bio-Tek Instruments, Winooski, VT). Transmigration of macrophages were analyzed in three independent experiments.

### **Proliferation assay of peritoneal monocytes and macrophages**

Isolated peritoneal cells from WT and MMP19<sup>-/-</sup> mice, mainly consisting of monocytes and macrophages were counted and cultured in RPMI. Initially, cells were seeded at 15000 cells/well in a 96-well plate and the medium was supplemented with 10% of alamar Blue (Biosource, Nivelles, Belgium), a dye that converts its color in presence of reducing molecules. During cell proliferation the environment is more reduced, since ratios of NADPH/NADP and FADH/FAD are increased. Additionally, the dye is also reduced by mitochondrial activity, thus indicating further cell viability including differentiation processes. Using alamar Blue reducing processes can also be measured by alteration of fluorescent properties. Directly after addition of the dye (0 h) and at different time points during cell culture ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ) the fluorescence was measured with a fluorescent reader at 530 and 590 nm (Lambda Fluoro 320). For each mice (3 WT and 3 MMP19<sup>-/-</sup> mice) and time point

5 wells were analyzed.

### 3.2.4 Analysis of thymocyte proliferation

To analyze proliferation by immunohistochemistry, cryosections of thymi were stained with anti-Ki-67 antibodies as described above (3.2.1). Evaluation of Ki-67-positive cells was done microscopically. To analyze T cell proliferation in more detail BrdU incorporation was performed. To quantify the proliferation incorporated BrdU in thymocytes was measured using flow cytometry. Mice were injected i.p. with 1 mg BrdU (Sigma) in 1 ml PBS. After 14 h thymi were excised and cells were stained with antibodies against CD3, CD4, and CD8 (mouse lymphocyte cocktail, BD Biosciences). After fixation and permeabilization with ice-cold methanol cells were incubated with FITC-conjugated anti-BrdU antibodies (Santa Cruz Biotechnologies, CA) and analyzed by flow cytometry. Measurement of BrdU incorporation was carried out in three independent experiments, each with 3 WT and MMP19-deficient mice.

### 3.2.5 Immunoblotting (Western blotting)

To analyze expression of laminin5 $\gamma$ 2 in the thymus immunoblotting was performed. Thymi from immature WT and MMP19-deficient mice were excised, crushed with liquid nitrogen and lysed for 45 min on ice in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, and proteinase inhibitor (Complete, Roche, Germany). After centrifugation (950g, 12 min, 4°C) supernatant was determined for protein content using the BCA protein assay (Pierce, Rockford, IL). After addition of reducing loading buffer (250 mM Tris, 40% glycerol, 20%  $\beta$ -mercaptoethanol, 8% SDS, 0,05% bromophenol blue) equal amounts of proteins were separated on gels (10% acrylamide) by SDS-PAGE, followed by electrophoretic transfer onto a PVDF membrane (Macherey-Nagel, Düren, Germany). Membranes were blocked with 3% non-fat milk in TBST buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20, pH 7.5 (3% TBSTM buffer). For detection, polyclonal goat anti-laminin- $\gamma$ 2 antibodies (Santa Cruz, CA) were diluted in 2% TBSTM buffer and incubated on the membrane overnight. After washing steps, bound antibodies were detected using peroxidase-conjugated rabbit anti-goat antibodies (Jackson Immuno Research, West Grove, PA) and the ECL plus Western Blotting Detection System (GE Healthcare, Little Chalfout, UK). Signals were recorded with a Luminescent Image Analyzer (FujiFilm, Tokyo, Japan).

# Chapter 4

## Results

### 4.1 MMP19 $-/-$ mice show altered responses in irritant dermatitis

The *in vivo* inflammation model of irritant dermatitis, also known as irritant contact dermatitis (ICD), shows acute inflammatory response of the skin that is primarily mediated by keratinocytes and other skin resident cells releasing proinflammatory factors. The ICD model was employed to address the role of matrix metalloproteinase 19 in keratinocytes and inflammatory cells during cutaneous inflammation.

#### 4.1.1 MMP19 $-/-$ mice exhibit reduced inflammation

Since matrix metalloproteinase 19 is constitutively expressed in basal keratinocytes of healthy epidermis and is upregulated in hyperproliferative diseases of the skin, possible involvement of MMP19 in skin inflammation was analyzed using MMP19-deficient mice and a model of irritant dermatitis. As irritant, oil of the plant *Croton tiglium* was used. Single painting of croton oil solution on both sides of one mice ear resulted in a local inflammatory reaction measured by an increase in ear thickness that have its peak around 24 h after painting. In contrast to strong ear swelling in wild-type (WT) control mice MMP19  $-/-$  mice showed significant reduced ear swelling (Fig. 4.1). Immunohistochemical analysis of the affected area revealed clear differences in the amount of inflammatory cells and in keratinocyte proliferation in MMP19  $-/-$  and wild-type mice. MMP19  $-/-$  mice exhibit low numbers of granulocytes, mainly neutrophils and eosinophils, in the treated ears whereas wild-type animals have massive influx of cells (Fig. 4.2).

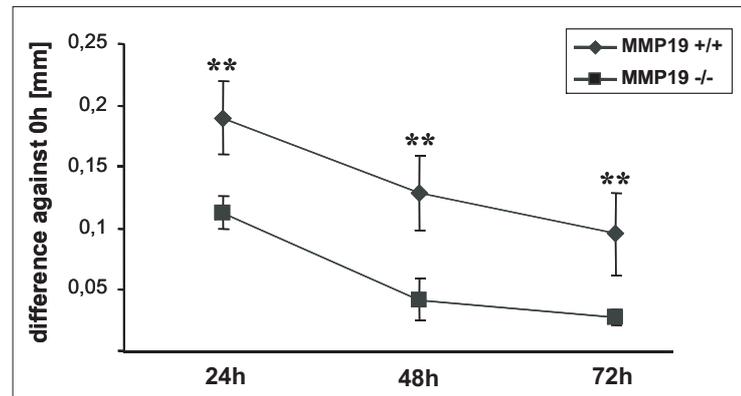


Figure 4.1: MMP19-deficient mice show reduced ear swelling in irritant dermatitis. After single application of croton oil on ears of wild-type and MMP19-deficient mice ear thickness was measured at times indicated. Mean values of ear thickness are given in mm as difference to time point 0 h. Significant differences are marked by asterisks (\*\* $p < 0.01$ ).

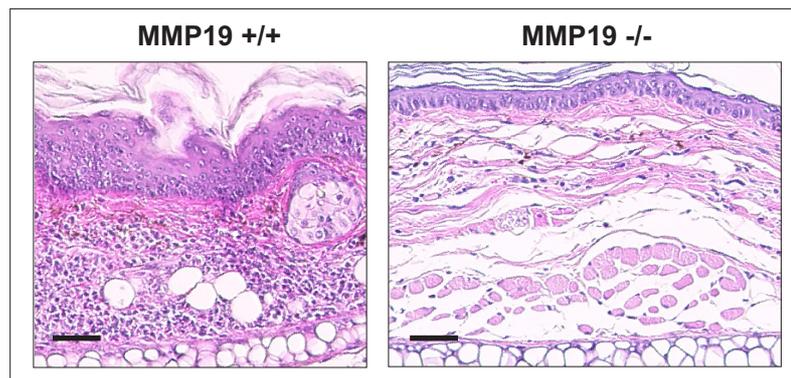


Figure 4.2: MMP19-deficient mice exhibit reduced numbers of inflammatory cells in the inflamed tissue. 24 h after croton oil treatment ear section of wild-type and MMP19  $-/-$  mice were analyzed by hematoxylin-eosin staining. Wild-type mice exhibit strong influx of granulocytes and high proliferation of keratinocytes, both of which are minimal in MMP19  $-/-$  mice. In contrast, MMP19-deficient mice show a thin epidermal layer in comparison to wild-type mice. Scale bars: 50  $\mu\text{m}$

#### 4.1.2 Proliferation of keratinocytes is reduced in MMP19-deficient mice

Analysing immunohistochemical stainings (hematoxylin-eosin) of inflamed ears of wild-type mice showed a thick epidermal layer in WT mice while MMP19  $-/-$  mice did not. Thus, indicating involvement of keratinocytes in ICD reaction. To examine differences in cell proliferation, ear sections were stained against the proliferation marker Ki-67. Evaluating the number of Ki-67-positive cells, wild-type mice showed 40% of proliferating keratinocytes while MMP19-deficient mice exhibit only 10% cell

proliferation. Decreased cell proliferation in MMP19  $-/-$  mice was measured on both basal and total amount of keratinocytes (Fig. 4.3). Although it is not possible to distinguish if the irritant or inflammatory cells caused the increase of keratinocyte proliferation, the keratinocytes of MMP19  $-/-$  mice show certain inability to be stimulated.

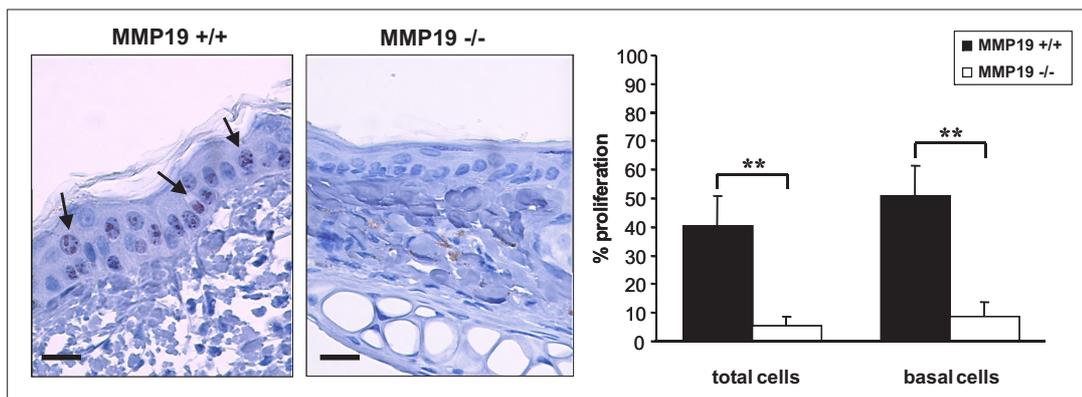


Figure 4.3: MMP19-deficient mice show reduced proliferation of keratinocytes in irritant dermatitis. 24 h after croton oil treatment sections of ear tissues were stained against the proliferation marker Ki-67 and analyzed for proliferating keratinocytes. Ki-67-positive cells show dark red signals in their nuclei (arrows). Determining the number of positive cells, compared to wild-type mice MMP19-deficient mice show significant low proliferation in both basal and suprabasal layers, calculated as percentage of basal and total keratinocytes. Scale bars: 20  $\mu$ m, significance  $**p < 0.01$ .

In the model of irritant contact dermatitis MMP19  $-/-$  mice show strongly reduced inflammatory response, apparent in minimal ear swelling, reduced proliferation of keratinocytes, and decreased influx of inflammatory cells important for inflammatory reaction. Thus, MMP19 is involved in stimulating acute inflammation in irritant dermatitis.

## 4.2 MMP19 impacts cell migration

Migration of cells is essential for inflammatory processes, since granulocytes, macrophages and other blood cells have to cross the basement membrane of vascular endothelium to act at the site of affected tissues. Collagen IV is the main component of basement membranes that has to be disrupted upon transmigration. Migration through connective tissue containing high amounts of collagen type I requires activity of collagen I-cleaving enzymes. MMP19 is potent to cleave collagen IV but not type I collagen. To examine why MMP19-deficiency results in reduced recruitment of inflammatory cells in ICD, transmigration of MMP19-expressing cells was determined.

### 4.2.1 MMP19-deficient macrophages show inhibited transmigration

Analysis of human myeloid cells showed MMP19 expression in CD14-positive cells, consisting of monocytes and macrophages [179]. Concerning the mouse, monocytes and macrophages can easily be isolated from the peritoneal cavity [302]. To examine the influence of MMP19 on cell transmigration, peritoneal monocytes and macrophages from WT and MMP19  $-/-$  mice were isolated and tested for MMP19 expression by quantitative RT-PCR as shown in figure 4.4. Isolated cells express MMP19 mRNA and were taken for transmigration assays. Using the trans-well system, transmigration through fibrin and collagen type IV matrices towards a stimulus showed strongly reduced migratory capacity of macrophages derived from MMP19-deficient mice (Fig. 4.4).

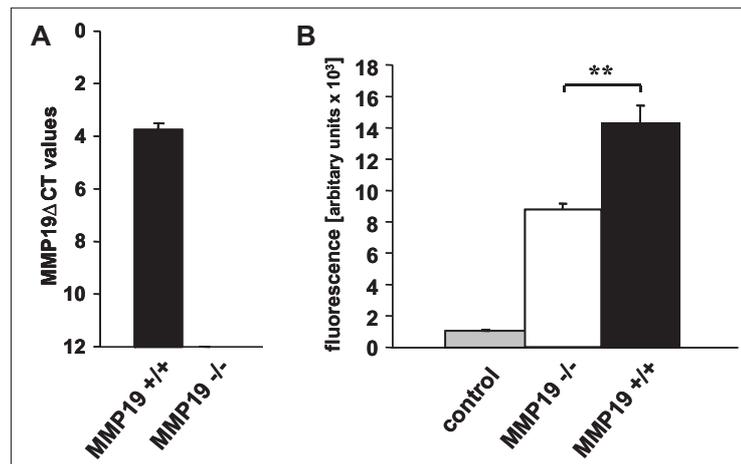


Figure 4.4: MMP19  $-/-$  macrophages show reduced transmigration *in vitro*. Peritoneal monocytic cells including macrophages from WT and MMP19  $-/-$  mice were isolated. (A) Quantitative RT-PCR show MMP19 mRNA expression in WT cells and confirmed the lack in MMP19  $-/-$  mice. (B) Macrophages were stained with calcein and examined for their trans migratory capacity through collagen type IV matrix using trans-well systems. Before and 10 h after seeding cells onto matrices fluorescence was measured in upper and lower chambers of the system. As promigratory stimulus supernatant of 3T3 fibroblasts was given to the lower chamber. Controls were done with unconditioned media. Similar results were obtained for transmigration through fibrin matrix. Significance is marked ( $p^{**} > 0.01$ ).

As human MMP19 influences proliferative behavior of cells, at least of epithelial cells, fraction of peritoneal monocytes and macrophages of wild-type and MMP19-deficient mice were also studied for proliferative capacity. The assay used enables measurement of cell proliferation by means of detecting activity of mitochondria. Thus, changes in cell metabolism or differentiation are determined. Monocytic cells deficient for MMP19 exhibited decreased proliferation and metabolic activity in con-

trast to wild-type cells (Fig. 4.5). Since monocytes differentiate into macrophages that tend to activation *in vitro*, results mirror more cell differentiation and activation processes than mere proliferation. Nevertheless, the cause of the altered proliferation is unknown.

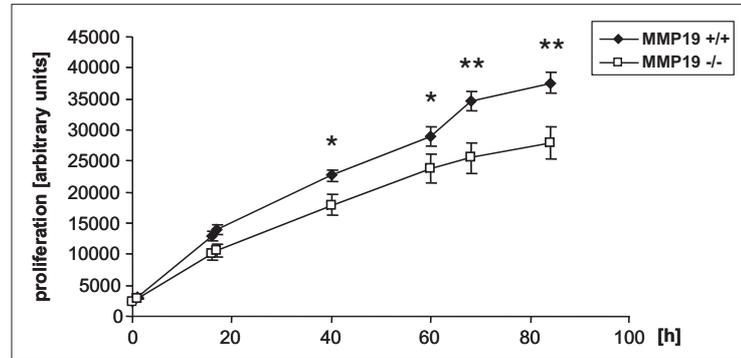


Figure 4.5: MMP19  $-/-$  monocytes show reduced differentiation and activation *in vitro*. Population of peritoneal monocytes and macrophages from WT and MMP19  $-/-$  mice were analyzed for their proliferative and metabolic capacity. Low values of MMP19  $-/-$  cells point to reduced monocyte differentiation and macrophage activation. Significant differences are indicated with \* $p < 0.05$  and \*\* $p < 0.01$

#### 4.2.2 MMP19-deficient mice exhibit reduced migration of macrophages to sites of inflammation

To find out if MMP19-deficiency also affect transmigration of cells *in vivo*, a mouse model with inducible inflammation was performed. In this so called air pouch model a steril cavity is preformed by injecting air s.c. in the back of the mice. Injection of zymosan, a yeast component (*Saccharomyces cerevisiae*), induces a proinflammatory stimulus leading to recruitment, i.e. transmigration of granulocytes and macrophages into the cavity. At different time points after stimulating inflammation, cell populations that migrated into the pouch were analyzed by flow cytometry. At early time points, 6 and 20 h after zymosan treatment, populations of granulocytes (Ly-6<sup>+</sup> cells) and monocytes (CD11b<sup>+</sup> and CD14<sup>+</sup> cells) are comparable between WT and MMP19  $-/-$  mice (Fig. 4.6). Expression of CD14 is not completely restricted to monocytic cells, as small subtypes of granulocytes are also positive for CD14. While cells positive for F4/80, a specific marker for macrophages, are not present after 20 h, analysis of pouch exudates after 40 h revealed presence of macrophages, which show reduced numbers in MMP19  $-/-$  mice compared to WT animals (Fig. 4.7). Presence of T (CD3<sup>+</sup>) and B lymphocytes (B220<sup>+</sup>) were not detectable at any time point. From these experiments the typical course of cells recruited to

certain sites is visible. Granulocytes coming first arise up to 90% followed later by predominance of monocytes (80%) including macrophages. At this time point number of granulocytes decreases.

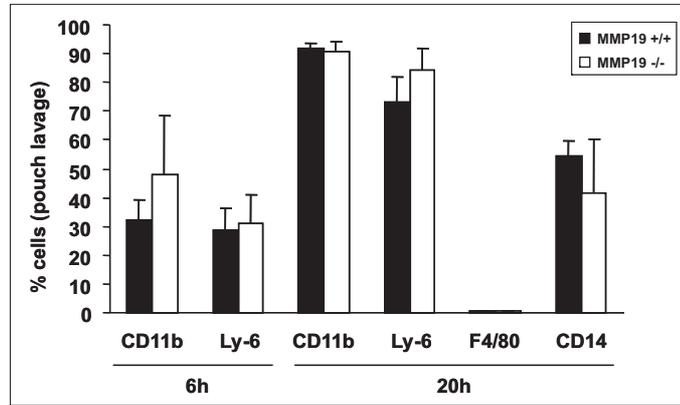


Figure 4.6: Analysis of cell transmigration *in vivo* using the air pouch inflammation model. Injection of zymosan s.c. into a preformed air pouch induces recruitment of granulocytes (Ly-6) and monocytes (CD11b, CD14) that show comparable numbers in pouch exudates from WT and MMP19<sup>-/-</sup> mice after 6 and 20 h. Macrophages (F4/80) are not detected after 20 h.

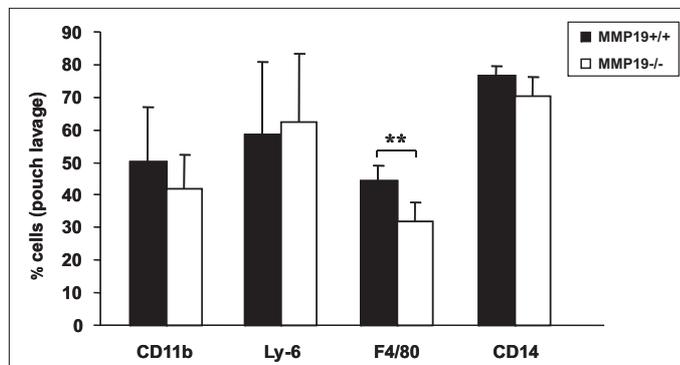


Figure 4.7: MMP19-deficient macrophages show reduced transmigration *in vivo*. Zymosan-induced inflammation shows reduced numbers of F4/80<sup>+</sup> cells in MMP19-deficient mice after 40 h while other monocytes (CD14) and granulocytes (Ly-6) are not altered. Significant differences are indicated (\*\*p < 0.01).

Altogether, MMP19 impacts macrophage transmigration to sites of inflammation as well as the activation status of macrophages, which are influenced most likely due to altered proteolytic capacity of MMP19.

## 4.3 MMP19 $-/-$ mice exhibit impaired contact hypersensitivity (CHS)

### 4.3.1 MMP19 $-/-$ mice show reduced inflammatory response to the hapten FITC

To investigate the role of MMP19 in adaptive immune responses MMP19  $-/-$  mice and wild-type control mice were studied in a model of cutaneous contact hypersensitivity (CHS) that is mainly mediated by T cells. In order to follow up the activation and migration of DCs during the sensitization phase of CHS, FITC was used as a fluorogenic hapten that facilitates the detection of activated dendritic cells (DCs), i.e. FITC-positive DCs, in lymph nodes by flow cytometry. Hapten uptake by epidermal DCs is a critical step during sensitization. Analyzing the amount of FITC-positive mature DCs in lymph nodes 24 h after sensitization no differences between WT and MMP19  $-/-$  mice were seen, showing that antigen presenting cells in WT and MMP19  $-/-$  mice act similar in their ability to take up antigens and migrate into regional lymph nodes, although DCs from MMP19  $-/-$  mice show weak tendency for lower numbers of FITC<sup>+</sup> cells (Fig. 4.8). Since the activation state of DCs is fundamental for antigen presentation to T cells in the lymph nodes, DCs isolated from inguinal lymph nodes were analyzed for overall expression of activation markers. 24 h after abdominal FITC sensitization CD11c<sup>+</sup> cells positive for CD80, CD86, MHC class II, and CD40 were measured by flow cytometry, however, no significant differences in the number or activation of DCs could be detected between MMP19-deficient and MMP19<sup>+/+</sup> mice (Fig. 4.9). Figure 4.9 depicts activation status of all DCs in lymph nodes, while figure 4.8 shows numbers of DCs only carrying the hapten (FITC<sup>+</sup> cells).

After cutaneous sensitization and challenge of the mice by painting FITC on both sides of one ear, wild-type mice developed a local inflammatory response that was measured as an increase of ear thickness with a peak around 24 h after challenge. This swelling declined slowly during the period of three days. In contrast, ear swelling was abrogated in MMP19-deficient mice, also in the peak of the reaction, i.e. after 24 h (Fig. 4.10).

Histology of ears 24 h post FITC challenge revealed strong infiltration of inflammatory cells in MMP19<sup>+/+</sup> mice that was not observed in ear tissue of MMP19  $-/-$  mice (Fig. 4.11). The inflammatory reaction in wild-type mice was accompanied by a strongly increased proliferation of keratinocytes resulting in a thickened epidermis. This thickening of the epidermis was absent in MMP19  $-/-$  mice (Fig. 4.10 and Fig. 4.11). To analyze proliferation of keratinocytes, ear sections were stained against the

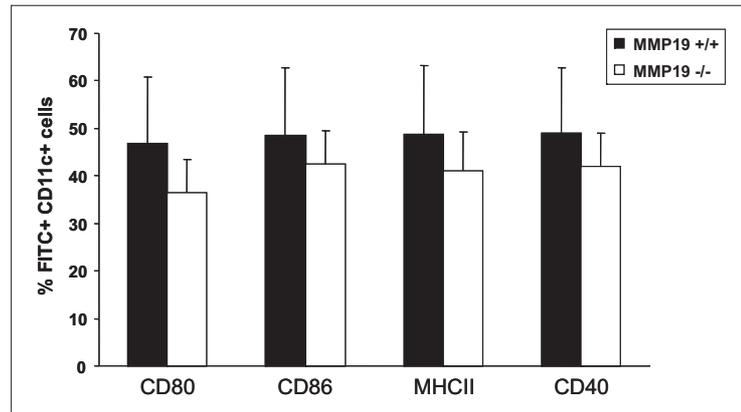


Figure 4.8: Cutaneous dendritic cells of WT and MMP19-deficient mice are able to take up haptens and migrate into regional lymph nodes. 24 h after abdominal FITC sensitization dendritic cells from WT and MMP19  $-/-$  mice were isolated from inguinal lymph nodes. Mature DCs expressing activation markers CD80, CD86, MHCII, and CD40 were analyzed by flow cytometry. CD11c-positive cells of both WT and MMP19  $-/-$  mice are similarly loaded with the hapten (FITC).

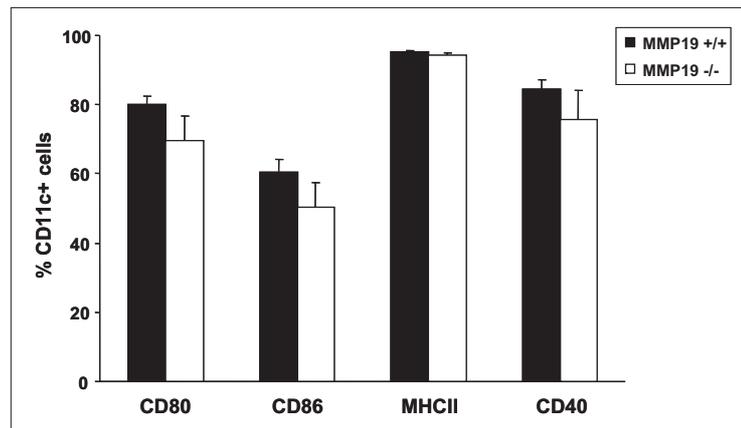


Figure 4.9: Activation of dendritic cells show no differences in wild-type and MMP19  $-/-$  mice. 24 h after FITC sensitization DCs out of regional lymph nodes were analyzed for their activation status. DCs of WT and MMP19  $-/-$  mice show identical expression of activation markers CD80, CD86, MHCII, and CD40. Numbers of DCs include hapten-carrying and unloaded CD11c $^+$  cells.

proliferation marker Ki-67 and evaluation of keratinocytes positive for Ki-67 showed that 60% of total and basal keratinocytes were proliferating in MMP19 $+/+$  mice while in MMP19-deficient mice only 20% of keratinocytes proliferated (Fig. 4.12). Since FITC-stimulated CHS response is mainly mediated by CD8 $^+$  T cells [240,278], the presence of CD8 $^+$  cells in ears of wild-type and MMP19  $-/-$  mice 24 h after FITC challenge as well as in unsensitized mice were determined. In contrast to wild-type mice with abundantly dispersed CD8 $^+$  T cells in the challenged ear MMP19 $-/-$  mice

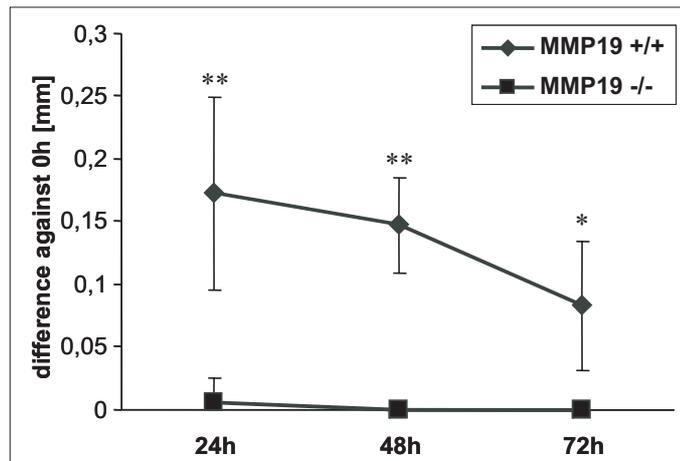


Figure 4.10: MMP19-deficient mice exhibit impaired ear swelling in contact hypersensitivity to FITC. Five days after abdominal FITC sensitization, mouse ears were challenged with FITC and ear thickness was measured directly before and 24, 48, and 72 h after challenge. Mean values are shown in mm as difference to time point 0 h. Significant values are marked with  $**p < 0.01$  and  $*p < 0.05$ .

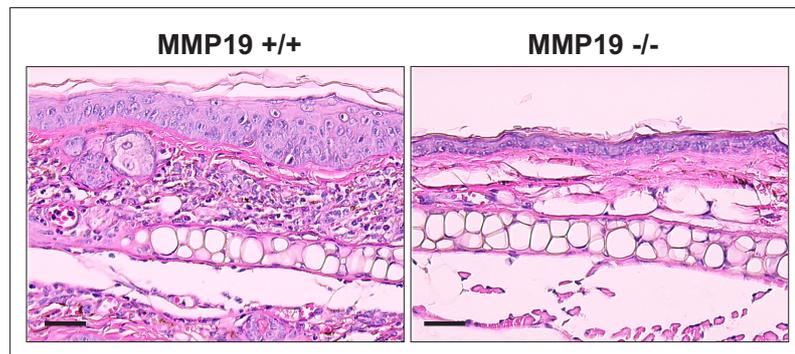


Figure 4.11: MMP19-deficient mice show low numbers of inflammatory cells infiltrating the affected area. Further, different thickness of the epidermal layer is observable. Ear tissue of wild-type and MMP19  $-/-$  mice was analyzed by hematoxylin-eosin staining 24 h after FITC challenge. Scale bars: 50  $\mu\text{m}$ .

showed low numbers of T cells that were comparable to the unsensitized situation (Fig. 4.13). To test whether the distribution of  $\text{CD8}^+$  T cells in MMP19  $-/-$  mice differs from that of wild-type mice also under non-T cell-dependent inflammation, like in irritant contact dermatitis (ICD), mouse ears were treated by a single painting of croton oil as unspecific, inflammatory stimulus. After 24 h, abundance of  $\text{CD8}^+$  T cells in MMP19-deficient mice was very low compared to wild-type mice that exhibit presence of  $\text{CD8}^+$  T cells as high as in CHS reactions (Fig. 4.13). Thus, MMP19-deficient mice show reduced influx of  $\text{CD8}^+$  T cells in inflammatory reactions of CHS and ICD, in T cell-mediated and T cell-independent situations, re-

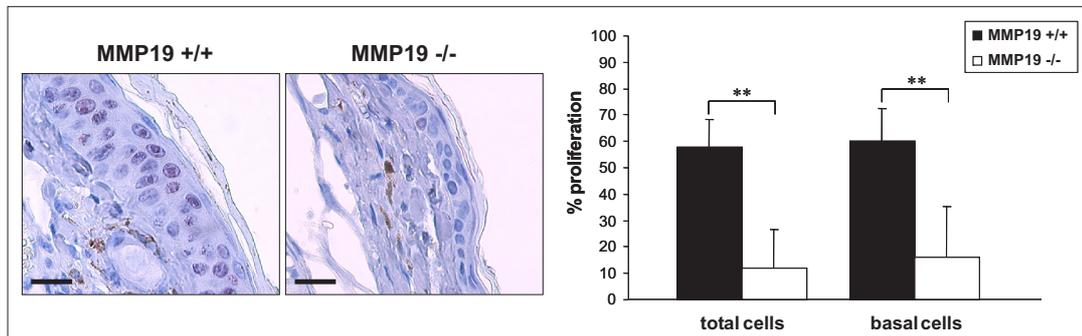


Figure 4.12: Decreased proliferation of keratinocytes in MMP19-deficient mice in CHS. After FITC challenge the affected ear tissue was stained against proliferation marker Ki-67 and analyzed for proliferating cells in basal and suprabasal layers. Ki-67 positive cells were counted and calculated as percentage of basal and total keratinocytes. Scale bars: 20  $\mu\text{m}$ . Significant values are indicated by asterisc (\*\* $p < 0.01$ ).

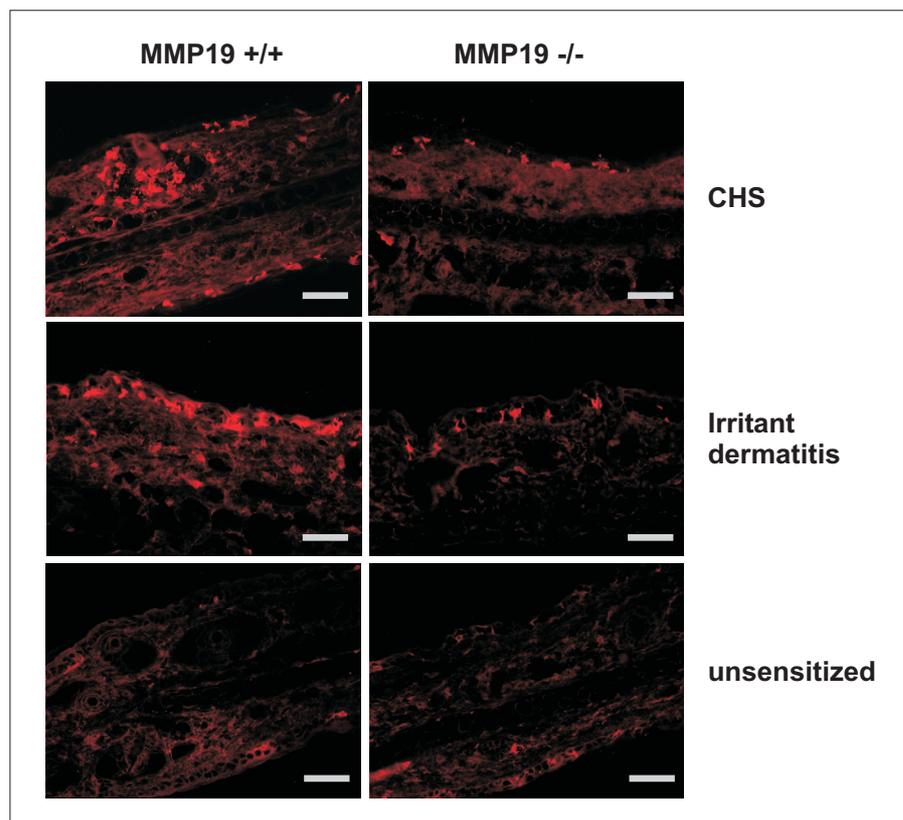


Figure 4.13: Reduced number of CD8<sup>+</sup> T cells in the skin of MMP19  $-/-$  mice in CHS reactions. 24 h after FITC challenge ear sections of WT and MMP19  $-/-$  mice were stained against CD8 (upper panel). Additionally, mice ears stimulated with croton oil (middle panel) as well as unsensitized ears (lower panel) were analyzed for CD8<sup>+</sup> T cells. During inflammation, WT mice show increased amounts of CD8<sup>+</sup> T cells, while MMP19  $-/-$  mice exhibit only low numbers in both CHS and irritant dermatitis. Scale bars: 50  $\mu\text{m}$ .

spectively. Together, these findings indicate that MMP19  $-/-$  mice have a defect in T cell-mediated inflammation, keratinocyte proliferation, and CD8<sup>+</sup> T cell circulation.

### 4.3.2 MMP19 $-/-$ mice exhibit reduced production of inflammatory cytokines at the site of inflammation

Within inflamed tissues chemokines and other cytokines serve as main attractants for granulocytes, monocytes and T lymphocytes. Using a cytokine antibody array ear lysates of wild-type and MMP19  $-/-$  mice were compared for cytokine expression 24 h after FITC challenge. Generally, MMP19  $-/-$  mice showed a reduction of all detectable cytokines when compared to MMP19 $+/+$  mice. Fig. 4.14 shows a representative evaluation of these analyses with highest reduction in lymphotactin (5.1-fold) and IL-12 expression (4.3-fold). Other cytokines such as CD30L, eotaxin, fraktalkine, LIX, MCP-1, and MCSF were moderately reduced. IFN- $\gamma$ , IL-4, and IL-5 were not detectable in these assays. Interestingly, lymphotactin, I-TAC, and TCA-3 all responsible for activation and recruitment of T cells, showed continuously low expression in MMP19  $-/-$  mice. Levels of I-TAC were decreased up to 18-fold and of lymphotactin up to 24-fold (Fig. 4.14). These results correspond with the previous analyses showing low inflammatory response in MMP19 $-/-$  mice and point to a biased distribution of T cells at the inflammatory site.

### 4.3.3 MMP19-deficiency results in reduced activation of T cells

Development of CHS depends on the stimulation and activation of antigen-specific T cells. Thus, CD8<sup>+</sup> T cells isolated from lymph nodes were analyzed in unsensitized and FITC-sensitized mice (i.e. in the afferent phase of CHS) as well as in FITC-challenged mice with an active CHS response in the efferent phase, 48 and 72 h after treatment with the hapten on mice ears. Naive T cells are positive for CD62L (L-selectin) that is downregulated upon activation and displaced by different cell surface molecules. Expression of CD69 (very early activation marker) occurs first, followed by CD122 and CD25. During sensitization memory T cells are generated and determined by expression of CD122 and high levels of CD44.

Analyzing T cells of unsensitized mice, CD8<sup>+</sup> cells are mainly CD62L-positive (85%) and only small proportions are positive for CD69, CD25, or CD122 (each 5%). Comparing CD8<sup>+</sup> T cells from WT and MMP19 $-/-$  mice, the latter exhibited slightly reduced amounts of naive (CD62L<sup>+</sup>) and activated cytotoxic (CD25<sup>+</sup>/CD95<sup>+</sup>) T cells in inguinal lymph nodes. Cells positive for CD69, CD44, and CD122 showed no differences under unsensitized conditions (Fig. 4.15).

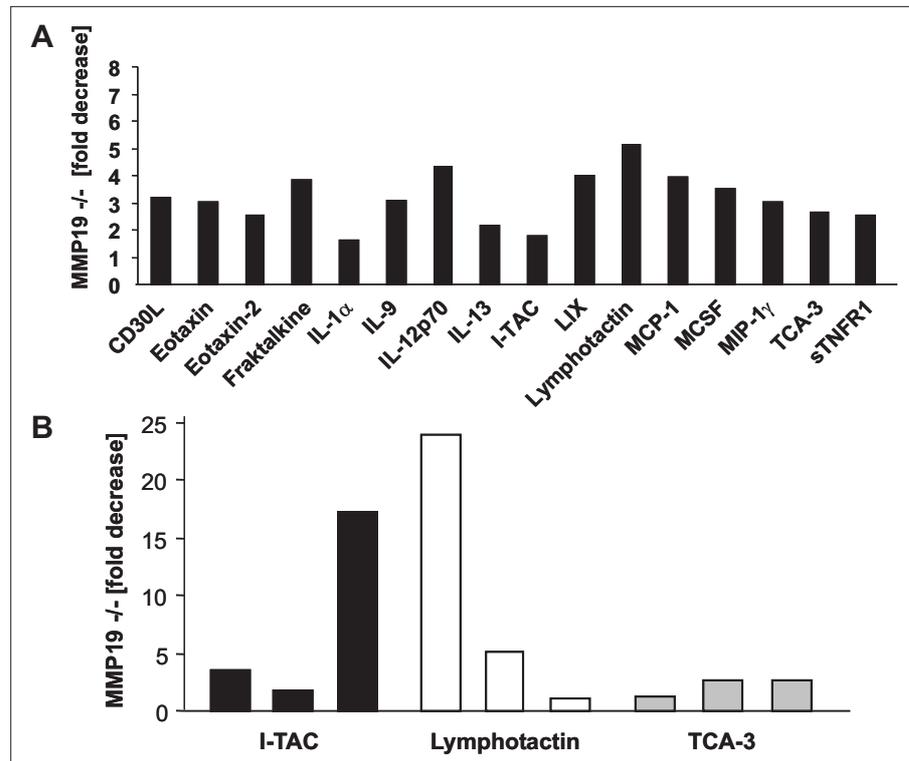


Figure 4.14: MMP19  $-/-$  mice show decreased cytokine levels in the inflamed tissue of FITC challenged ears. 24 h after challenge ear lysates were analyzed for cytokine expression employing an antibody array. (A) In MMP19-deficient mice detected cytokines are generally reduced in comparison to WT mice. Values of cytokines from MMP19  $-/-$  mice are given as decrease to cytokine levels of WT mice. Values from one experiment are shown. (B) Levels of I-TAC, lymphotoctin, and TCA-3, cytokines specifically affecting T cells, from three independent experiments are depicted (fold decrease to WT mice).

24 h after FITC-sensitization, i.e. abdominal hapten application, wild-type mice showed activation of CD8<sup>+</sup> T cells with increased amount of CD69<sup>+</sup> and reduction of CD62L<sup>+</sup> cells. Further, numbers of CD8<sup>+</sup> cells double-positive for CD44/CD69 or CD44/CD122, which represent activated and memory T cells, increased about 10% in WT mice in comparison to the unsensitized situation. In contrast, MMP19-deficient mice exhibited generally reduced activation of CD8<sup>+</sup> T cells in inguinal lymph nodes after sensitization (Fig. 4.16).

Numbers of activated CD8<sup>+</sup> T cells positive for CD62L/CD69, CD44/CD69, and CD44/CD122 were significantly reduced compared to wild-type mice, whereas the number of naive, CD62L<sup>+</sup> cells was not reduced but remained similar to the unsensitized situation (80%), indicating a lack of early T cell activation and memory T cell generation in MMP19-deficient mice (Fig. 4.15 and Fig. 4.16). This might be due to failed downregulation of CD62L and inhibited upregulation of CD44, since CD69 and CD122 expression do not differ between WT and MMP19  $-/-$  mice.

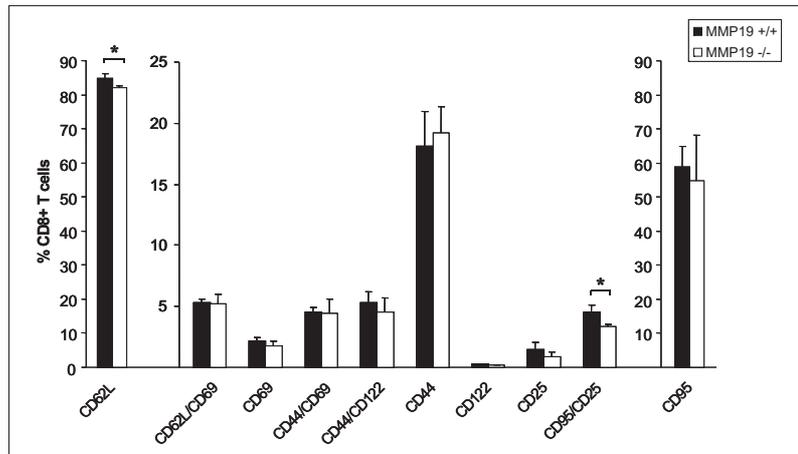


Figure 4.15: Unsensitized MMP19  $-/-$  mice do not show gross alterations in T cell populations in lymph nodes. Lymph nodes of unsensitized WT and MMP19  $-/-$  mice were isolated and CD8 $^+$  T cells analyzed for their activation status by flow cytometry. In MMP19-deficient mice numbers of cells positive for CD62L and CD25/CD95 are slightly different from WT mice, while other subgroups show no deviation. Two scales are used to match relevant data from an identical experiment. Significant differences are marked (\* $p < 0.05$ ).

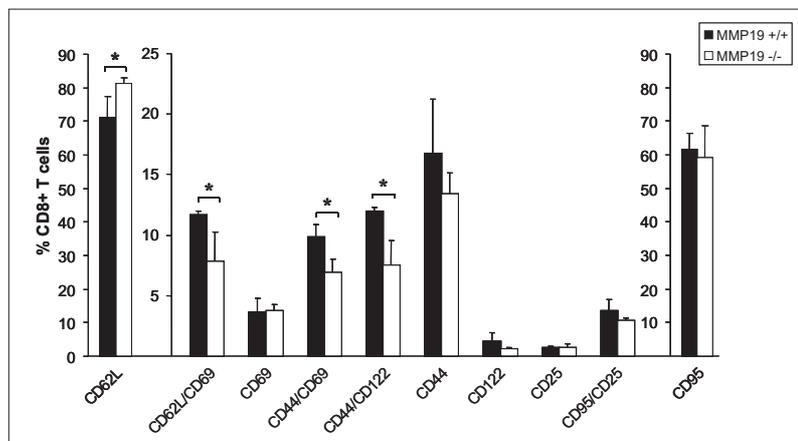


Figure 4.16: MMP19-deficient mice exhibit reduced T cell activation after sensitization. 24 h after abdominal FITC sensitization inguinal lymph nodes were excised and CD8 $^+$  T cells analyzed for activation markers. While numbers of naive T cells (CD62L $^+$ ) remain identical to unsensitized situation (see Fig. 4.15) MMP19  $-/-$  mice have decreased numbers of cells positive for CD62L/CD69, CD44/CD69, and CD44/CD122 in comparison to wild-type mice. Two scales are used to match relevant data from one experiment. Significant differences are indicated (\* $p < 0.05$ ).

Five days after FITC sensitization, i.e. after memory T cells were generated, mice ears were challenged with FITC to induce CHS reaction. CD8 $^+$  T cells in draining lymph nodes exhibit general activation, while only 20% of the population was repre-

sented by naive cells. In correlation to strong inflammatory ear swelling response, the population of activated CD8<sup>+</sup> T cells in the draining lymph nodes of MMP19  $-/-$  mice 48 h after challenge was strikingly diminished. Significant differences between MMP19  $-/-$  and wild-type mice were observed showing a two-fold reduction in the number of CD8<sup>+</sup> T cells expressing CD44<sup>high</sup>, CD44/CD69, and CD25, markers for early activated and cytotoxic T cells (Fig. 4.17).

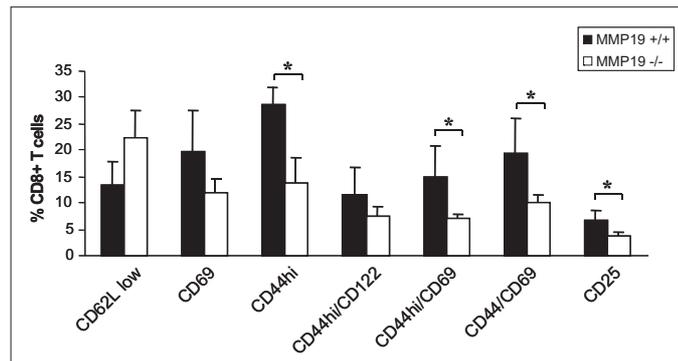


Figure 4.17: MMP19  $-/-$  mice have reduced numbers of activated CD8<sup>+</sup> T cells after FITC challenge. 48 h after challenge draining lymph nodes were excised and CD8<sup>+</sup> T cells analyzed for activation markers. MMP19-deficient mice exhibit low numbers of cells positive for CD44<sup>high</sup> and CD25 as well as double-positive for CD44<sup>high</sup>/CD69 and CD44/CD96. Significance is shown with \* $p < 0.05$ .

Although 72 h after FITC challenge wild-type mice showed abated inflammation in affected ears, differences in CD8<sup>+</sup> T cell activation in draining lymph nodes between WT and MMP19  $-/-$  mice persisted: the number of CD62L<sup>+</sup> cells was increased and CD25<sup>+</sup> cells diminished (Fig. 4.18). Activation status of DCs in draining lymph nodes were also analyzed, but no significant differences in CD80, CD86, CD40, and MHC class II positive cells between MMP19  $-/-$  and wild-type mice were observed (not shown).

Together, MMP19  $-/-$  mice show no changes in activation of DCs in lymph nodes, but exhibit reduced activation of CD8<sup>+</sup> T cells including altered expression of CD62L and CD25 which were also present after CHS reaction. Based on this results shown above, it is hypothesized that MMP19 has a significant role in T cell activation as well as in T cell distribution in peripheral tissues, not only during inflammatory reactions but also during T cell circulation or development.

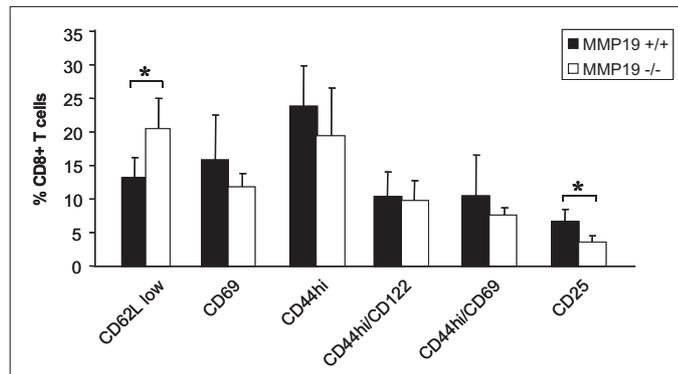


Figure 4.18: Differences in T cell activation in MMP19  $-/-$  mice persist also after subsiding inflammation. Analysis of CD8<sup>+</sup> T cells isolated from draining lymph nodes 72h after challenge still show reduced numbers of CD25<sup>+</sup> and high numbers of CD62L<sup>+</sup> cells while other activation markers are comparable to those of MMP19<sup>+/+</sup> mice. Significant differences are marked (\* $p < 0.05$ ).

#### 4.3.4 MMP19 $-/-$ mice show altered T cell distribution in blood

Since differences in T cell populations between MMP19 $-/-$  and wild-type mice were observed in the skin and in lymph nodes, question arose if the T cell population is generally influenced in the circulation even without stimulation of a T cell-mediated response. Blood of immature and mature unsensitized MMP19 $-/-$  and wild-type mice were analyzed for the basic cell types such as monocytes, granulocytes, B and T lymphocytes. The blood mainly contains lymphocytes and smaller proportions are made up of granulocytes and monocytes (Fig. 4.19).

Analyzing T cell subpopulations in the blood reduced numbers of CD8<sup>+</sup> and CD4<sup>+</sup> T cells in immature (3 weeks old) MMP19 $-/-$  mice were observed (Fig. 4.19), whereas B lymphocytes (B220), monocytes (CD11b), and granulocytes (Ly-6) showed numbers comparable to MMP19<sup>+/+</sup> mice. Interestingly, the population of CD3<sup>+</sup> cells negative for both CD4 and CD8 was increased in the blood of MMP19  $-/-$  mice indicating higher numbers of NK cells (Fig. 4.19).

T cell populations in blood from adult mice were further analyzed. Analysis of 3 months old mice, i.e. adult mice, did not reveal any difference in T cell populations between MMP19<sup>+/+</sup> and wild-type mice although the latter showed a tendency to increased amounts of CD4<sup>+</sup> cells (Fig. 4.20). Hence, the loss of MMP19 influenced T cell populations circulating in the blood of immature mice. This bias was, however, corrected with increased age and may thus mirror impact of MMP19 on mice development, especially on development of the immune system; at least in part.

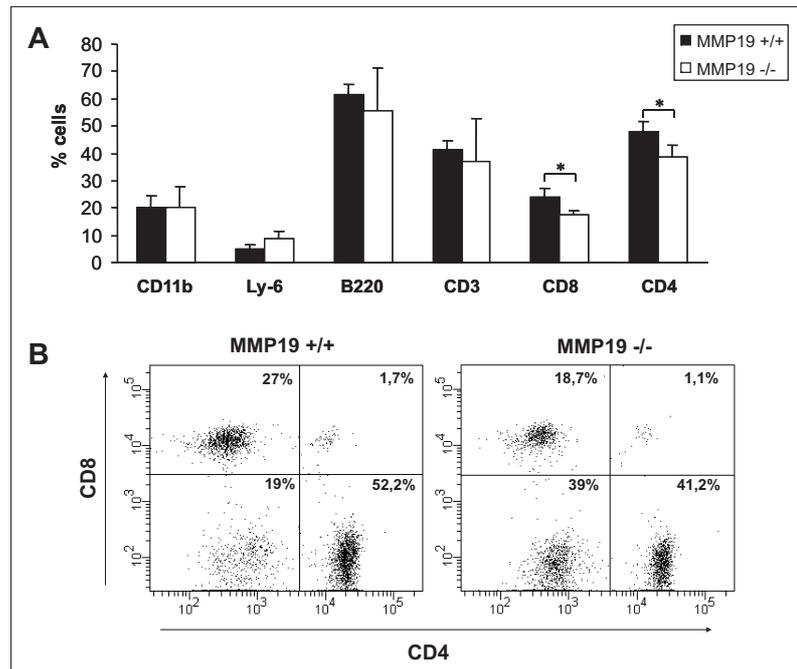


Figure 4.19: T cell populations in blood of immature MMP19 $-/-$  mice is biased. (A) Blood from 3 weeks old WT and MMP19  $-/-$  mice was analyzed for monocytes, granulocytes (CD11b and Ly-6), B cells (B220), and T cell populations by flow cytometry. MMP19-deficient mice show reduced numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, while other cell populations are similar to WT mice. (B) A typical dot plot analysis of CD4<sup>+</sup> and CD8<sup>+</sup> populations gated for CD3<sup>+</sup> cells in individual mice is shown. Notably, amount of CD3<sup>+</sup> cells negative for CD4 and CD8 is higher in MMP19  $-/-$  mice (here 19% (WT) vs. 39%). Significant differences are marked (\* $p < 0.05$ ).

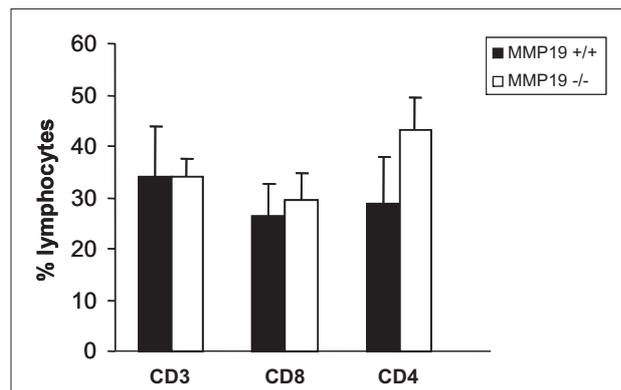


Figure 4.20: T cell population in blood of adult wild-type and MMP19  $-/-$  mice show no differences in thymocyte numbers. Blood from 12 weeks old WT and MMP19  $-/-$  mice was analyzed for T cell populations that show similar amount of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells as wild-type animals.

### 4.3.5 Thymocytes of MMP19 <sup>-/-</sup> mice exhibit decreased proliferation and reduced maturation

Searching for the origin of biased T cell population in blood, lymph nodes, and in peripheral tissues of MMP19 <sup>-/-</sup> mice, structure and subset distribution of T cells in the thymus were examined. In the thymus T cell precursors develop into mature T cells expressing CD3 and either CD4 or CD8, which then emigrate into circulation. The largest fraction of thymocytes (80-90%) is formed by cells double positive for CD4 and CD8 (DP cells). Establishment of T cell receptors (CD3) essential for negative and positive selection of thymocytes occurs after generation of DP cells.

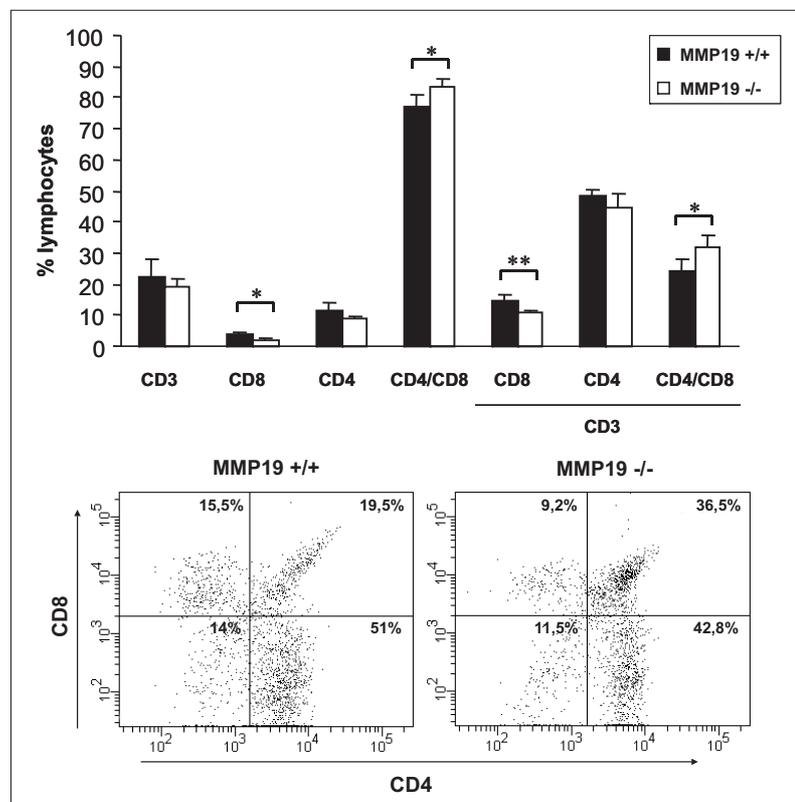


Figure 4.21: Development of single positive lymphocytes in the thymus is impeded. Thymocytes of immature WT and MMP19 <sup>-/-</sup> mice were analyzed for subpopulations by flow cytometry. (A) MMP19 <sup>-/-</sup> mice show lower number of CD8 SP T cells and higher amount of CD4/CD8 DP cells as WT mice. Significance increases in presence of CD3. (B) A typical dot plot analysis of CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations gated on CD3<sup>+</sup> cells shows a decrease of SP and accumulation of DP T cells in thymi of MMP19 <sup>-/-</sup> mice. Significant differences are marked (\*\*p<0.01 and \*p<0.05).

Analyzing thymocyte subpopulations, fractions of CD3<sup>+</sup> cells from immature MMP19-deficient mice at the age of 3-4 weeks exhibit higher numbers of CD4/CD8 DP cells and reduced levels of CD4 and CD8 SP cells compared to wild-type mice (Fig. 4.21).

Developing CD8 SP cells seem to be more influenced than CD4 SP cells. In contrast, analysis of adult animals show similar amounts of DP and SP cells in the thymus (Fig. 4.22). This observation is in concordance with low amounts of SP T cells in blood of immature MMP19  $-/-$  mice (Fig. 4.19 and 4.20).

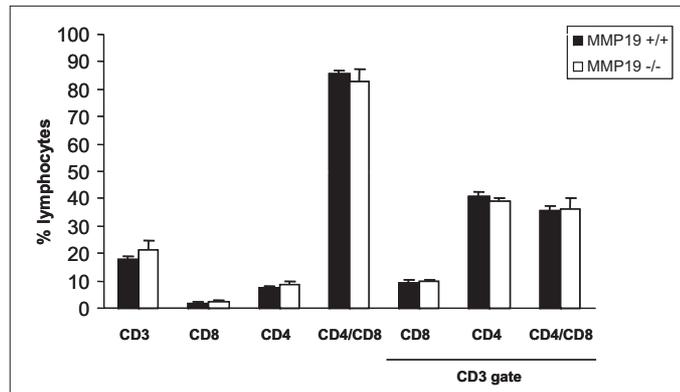


Figure 4.22: Adult MMP19-deficient mice exhibit similar amounts of thymocytes as in WT mice. Thymocytes of 12 weeks old wild-type and MMP19  $-/-$  mice were analyzed by flow cytometry for T cell subsets that show comparable amounts of CD4/CD8 DP and SP T cells.

Lower populations of SP T cells and simultaneously high levels of DP cells indicates dysregulated development of thymocytes in immature MMP19  $-/-$  mice. Due to increased number of DP cells and reduced SP populations in the thymus, it appears that MMP19 affects the transition from CD4<sup>+</sup>/CD8<sup>+</sup> into SP cells. Therefore, thymi from WT and MMP19  $-/-$  mice were studied in more detail. Analyzing the morphology of thymi from MMP19<sup>+/+</sup> and MMP19  $-/-$  mice no differences in the cortical and medullar structure was observed (Fig. 4.24). Also, formation of the

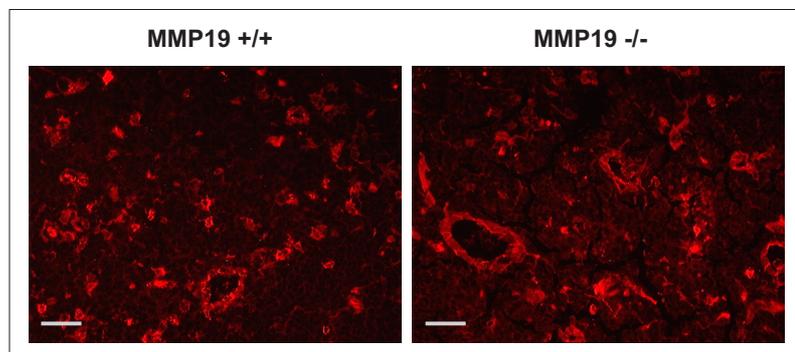


Figure 4.23: Expression of VE-cadherin in thymi of WT and MMP19  $-/-$  mice. Thymi of young WT and MMP19-deficient mice (3 weeks old) were stained against VE-cadherin (CD144), an endothelial marker that indicates no differences in the vascular structure of the mice. Scale bars: 50  $\mu$ m.

thymic vasculature was not altered, as the expression pattern of VE-cadherin (vascular endothelial cadherin, CD144), an endothelial marker, was comparable between wild-type and MMP19  $-/-$  mice (Fig. 4.23).

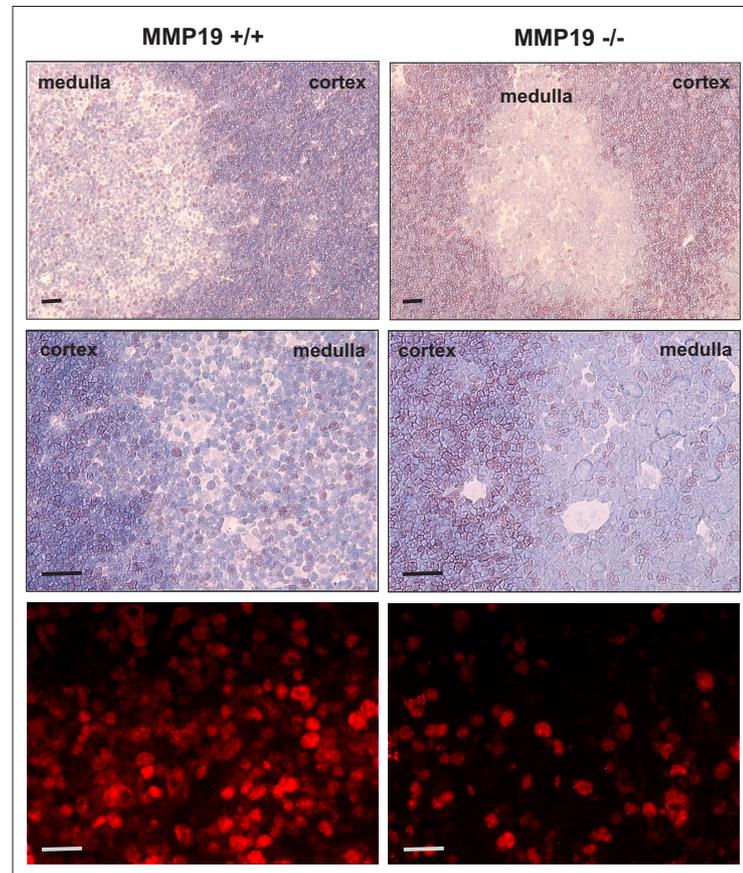


Figure 4.24: Proliferation of thymocytes is reduced in MMP19  $-/-$  mice. Sections of thymi from 3 weeks old WT and MMP19  $-/-$  mice were stained against proliferation marker Ki-67. Positive cells show a dark violet staining that is less frequent in medulla of MMP19  $-/-$  mice. In the cortex differences in Ki-67-positive cells are difficult to determine. Fluorescent immunohistochemical staining of the medulla detects Ki-67-positive signals more easily (lower panel). Scale bars: 50  $\mu\text{m}$  (upper and middle panel), 20  $\mu\text{m}$  (lower panel).

Furthermore, thymocytes of young WT and MMP19  $-/-$  mice were analyzed for their proliferative capacity by immunohistochemical techniques. Staining of thymi sections from 3-weeks old animals for the proliferation marker Ki-67 revealed a lower amount of proliferating cells in the medulla (Fig. 4.24). Since the thymus cortex contains high numbers of mainly proliferating thymocytes, it is difficult to determine altered proliferation in this part only by immunohistochemistry.

To achieve deeper insight into this observation thymocyte proliferation was examined via BrdU incorporation *in vivo*. Mice were injected with BrdU and its incorporation in thymocytes measured by flow cytometry that confirmed the results obtained by

immunohistochemistry. The incorporation of BrdU in thymocytes of MMP19  $-/-$  mice was up to 5-fold reduced in comparison to MMP19 $+/+$  mice (Fig. 4.25).

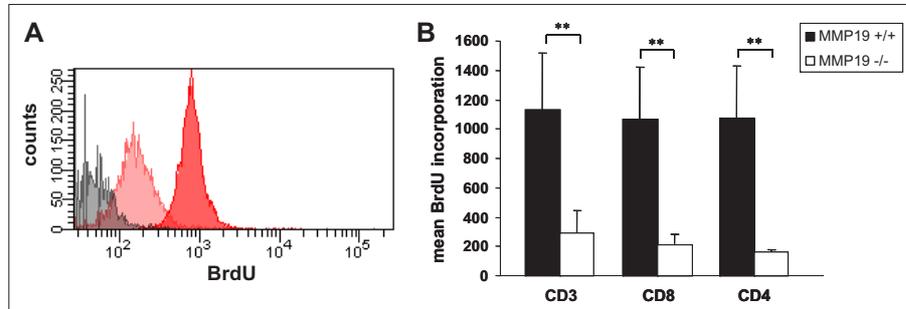


Figure 4.25: Analysis of thymocyte proliferation in WT and MMP19-deficient mice by BrdU incorporation. 14 h after injection of BrdU i.p. thymocytes were analyzed for BrdU incorporation by flow cytometry. (A) Amount of BrdU-positive cells in immature MMP19  $-/-$  mice (light red) is strongly reduced compared to WT mice (red). Isotype control is shown in grey. (B) Reduced proliferation is detected in CD3-, CD4-, and CD8-positive T cell populations. Significant differences are indicated (\*\* $p < 0.01$ ).

This altered proliferation in MMP19  $-/-$  mice was not restricted to particular T cell subsets, since CD3 $^+$ , CD8 $^+$ , and CD4 $^+$  cells showed a similar degree of BrdU incorporation (Fig. 4.25). To get a clue whether these changes could be mediated by the lack of MMP19 expression in the thymus, the expression of MMP19 in mouse thymi was measured using quantitative RT-PCR. Wild-type mice of different age exhibited a low but defined expression of MMP19 in the thymus, which was 10-fold lower than expression in the liver (Fig. 4.26).

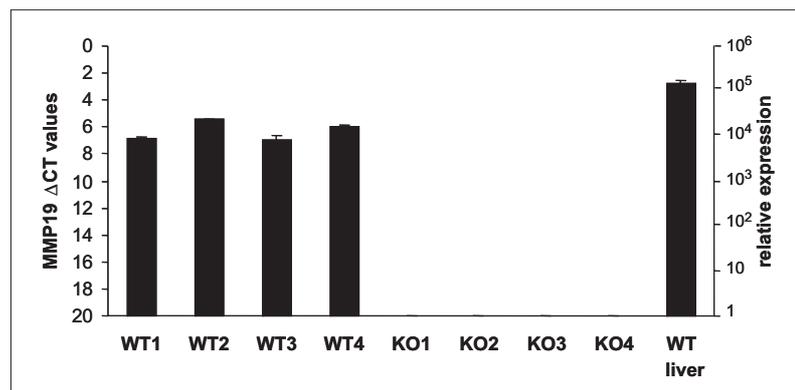


Figure 4.26: Analysis of MMP19 expression in the thymus using quantitative RT-PCR. Thymi from wild-type and MMP19-deficient mice of different age (3 to 10 weeks old) were examined for MMP19 mRNA expression. Analysis show MMP19 expression in WT mice and confirmed the absence in MMP19  $-/-$  mice. Expression of MMP19 in the thymus is lower compared to that in the liver.  $C_T$ , cycle of threshold.

How MMP19 affect proliferation of thymocytes is difficult to figure out, however, MMP19 might be important for defining the thymic microenvironment or for migration of thymocytes among various niches [243]. Thus, MMP19 may influence the transition of DP thymocytes to SP mature  $CD4^+$  and  $CD8^+$  T cells.

In the thymus, development of T cells is a tightly regulated process. Throughout this maturation process thymocytes have to migrate through cortical and medullary regions along ECM components such as laminins, fibronectin, and collagen IV, which in part is mediated by the action of MMPs [243,300].

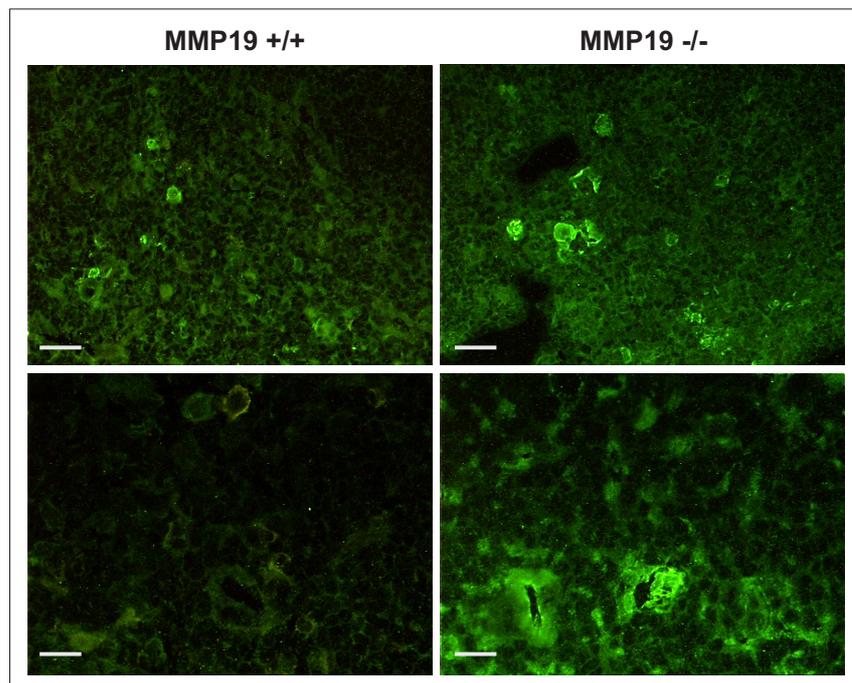


Figure 4.27: Expression of laminin5 $\gamma$ 2 in murine thymus of immature mice analyzed by immunohistochemistry. Staining of thymi sections from WT and MMP19-deficient mice revealed expression of laminin  $\gamma$ 2 chain in the medulla, where MMP19-deficient mice exhibit more intense signals for  $\gamma$ 2 chain. Scale bars 50  $\mu$ m (upper panel), 20  $\mu$ m (power panel).

Human MMP19 is able to cleave several components of the extracellular matrix (ECM), for instance the  $\gamma$ 2 chain of laminin5 [235]. Processing of  $\gamma$ 2 chain was reported to influence T cell development and proliferation [300]. To find out why immature MMP19-deficient mice show reduced thymocyte proliferation, laminin5 $\gamma$ 2 as potential substrate of MMP19 was analyzed in the murine thymus. Immunohistochemical staining against laminin5 $\gamma$ 2 showed expression in subcortical structures and in the medulla (Fig. 4.27). Since the signal intensity was rather low, it was difficult to quantify laminin5 $\gamma$ 2 expression, but it appears that the staining in WT mice is less intense than in MMP19  $-/-$  mice and thus, expression might be reduced. Western blotting analysis of thymi lysates revealed processing of  $\gamma$ 2 chain in imma-

ture mice, which appears to be slightly reduced in MMP19-deficient mice. Although the  $\gamma 2$  chain signals in thymi are weak, it appears that 140 kD- $\gamma 2$  chain in WT mice is more efficiently processed to the 100 kD-form ( $\gamma 2'$ ) than in MMP19  $-/-$  mice (Fig. 4.28). To analyze laminin5 $\gamma 2$  processing in detail and quantitatively it is helpful to employ proteomics technology.

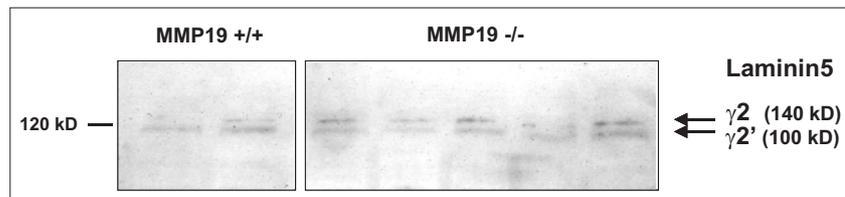


Figure 4.28: Western blotting analysis of laminin5 $\gamma 2$  expression in murine thymus. Lysates of thymi from immature (3 weeks old) WT and MMP19 $-/-$  mice were analyzed for laminin5 $\gamma 2$ . Signals for the full length form of  $\gamma 2$  chain (140 kD) and the 100 kD processed form are detected. In MMP19  $-/-$  mice and in comparison to wild-type mice, the 140 kD form is more intense than the processed 100 kD fragment.

Although it is not clear, why MMP19  $-/-$  mice exhibit reduced proliferation of thymocytes it is supposed that missing MMP19 activity in the thymus contributes to decreased proliferation due to reduced laminin5 $\gamma 2$  chain processing that is important for T cell migration and proliferation and thus for proper development of these cells. Beside altered thymocyte numbers in the thymus of MMP19  $-/-$  mice T cell populations in the blood are also decreased, thus, MMP19 seems to affect also the emigration of mature SP thymocytes into the circulation.

## 4.4 Deficiency of MMP12 affects CHS

Since MMP19-deficiency showed strong impact on irritant and T cell-mediated skin inflammation and since deficiency of stromelysin-1 (MMP3) and gelatinase B (MMP9) also influenced contact hypersensitivity [307], the question arises if also other MMPs are involved in CHS. For that, MMP12 (macrophage elastase, metalloelastase) was selected because of its expression in macrophages, which might cooperate with that of MMP19. Thus, mice deficient for MMP12 were examined for the role of MMP12 in inflammatory reactions of the skin. As described above, the models of irritant dermatitis and contact hypersensitivity were performed with MMP12  $-/-$  and wild-type mice. Although it was reported that MMP12-deficiency does not impact CHS responses [220], in studies presented here, MMP12-deficient mice show reduced ear swelling response in CHS to FITC (Fig. 4.30). In contrast to MMP19-deficient mice, stimulation of ICD by croton oil application exhibited

no alterations in the ear swelling response between wild-type and MMP12  $-/-$  mice (Fig.4.29). However, it is important to note that the used MMP12  $-/-$  mice feature the 129Sv genetic background. Thus, the comparison between MMP19- and MMP12-deficient mice should be done with caution.

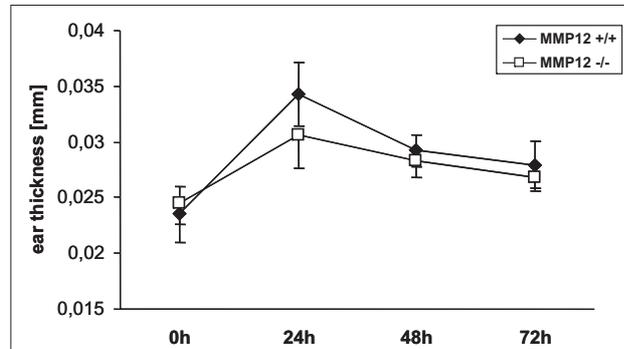


Figure 4.29: MMP12-deficiency has no impact on irritant dermatitis. After croton oil application on ears of WT and MMP12  $-/-$  mice local ear swelling was measured after time points indicated. Treatment with croton oil shows strong increase in ear thickness in both WT and MMP12-deficient mice.

Analysis of inflamed ear tissue by immunohistochemistry 24 h after FITC challenge revealed strong accumulation of inflammatory cells (neutrophils and eosinophils) in wild-type mice and lower presence of these cells in MMP12-deficient mice. Differences in the thickness of epidermal layers, i.e. keratinocyte proliferation like in MMP19-deficient mice were not observed (Fig. 4.31).

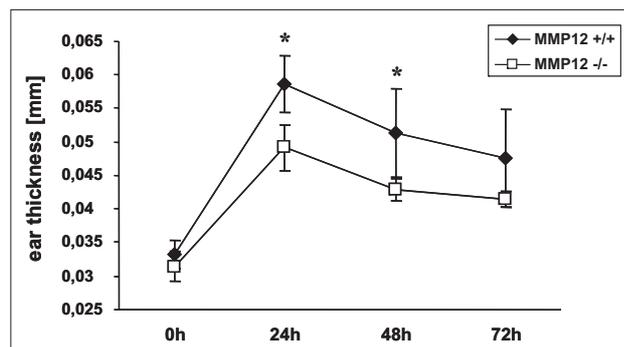


Figure 4.30: MMP12-deficient mice show reduced ear swelling in CHS reaction. Five days after abdominal FITC sensitization mice ears were challenged with FITC and ear swelling measured after 24, 48, and 72 h. Both WT and MMP12  $-/-$  mice show an increase in ear thickness that is less prominent in MMP12-deficient animals. Significant differences are indicated (\* $p < 0.05$ ).

Summarized, MMP12-deficient mice exhibit reduced response in CHS but no alterations in ICD. Whether MMP12-deficiency influences activation of DCs or T cells in

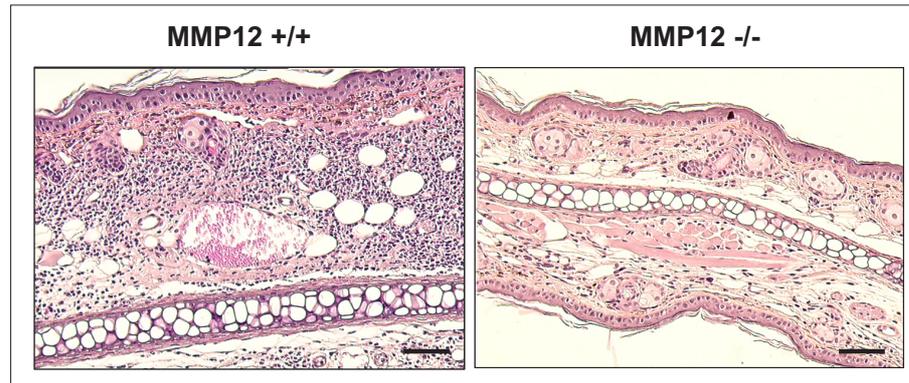


Figure 4.31: MMP12-deficient mice show altered inflammatory reaction in CHS to FITC. Hematoxylin-eosin staining of ear sections 24 h after FITC challenge revealed reduced presence of inflammatory cells in MMP12  $-/-$  mice compared to wild-type mice. Scale bars, 100  $\mu\text{m}$ .

FITC-induced CHS has to be examined in future. MMP12 is able to cleave matrix components such as elastin, fibronectin, laminin, and fibrinogen. Former analysis of MMP12-deficient mice revealed an essential role of MMP12 in macrophage-mediated proteolysis and matrix invasion [256]. Several reports point towards the role of MMP12 in leukocyte recruitment in inflammatory reactions. In studies of lung inflammation, MMP12 activity leads to neutrophil accumulation, increased cytokine production, and macrophage recruitment [202], whereas its absence rescued inflammation in a model of chronic obstructive pulmonary disease [105]. Thus, in the model of CHS used here MMP12-deficiency might reduce the amount of macrophages and neutrophils at the site of inflammation due to inhibited transmigration and reduced production of proinflammatory cytokines.

# Chapter 5

## Discussion

### 5.1 MMP19 is involved in T cell development

The present results on MMP19-deficient mice show that MMP19 plays a pivotal role during T cell-mediated cutaneous immune response and has an impact on the development of T lymphocytes, as the mice show skewed T cell populations in thymus and blood. Thymic T cell maturation is a tightly regulated process that comprises cell proliferation, differential protein expression, gene rearrangements, programmed cell death, and cell migration. Different maturation stages occur within two compartments: While the cortex contains only immature thymocytes, the medulla harbors cells of a more mature phenotype. Thymic T cell progenitors derived from the bone marrow are CD34-positive and do not express T cell receptors (CD34<sup>+</sup>/TCR<sup>-</sup>). Since they lack also surface expression of CD4 and CD8 coreceptors, they are referred as double-negative (DN) cells [35,265]. Different DN stages are defined by up- and downregulation of cell surface-expressed CD25 and CD44. In this stages TCR genes ( $\beta$ ,  $\gamma$ , and  $\delta$ ) rearrange and only cells with in-frame TCR- $\beta$  genes express the pre-TCR, proliferate, and mature further, a process called  $\beta$ -selection [265]. During transition to a double positive (DP) state they acquire CD4 and CD8 expression. In the thymic medulla, after rearrangement of TCR- $\alpha$  locus intact T cell receptors are established and DP cells undergo positive or negative selection that involves interaction of TCRs with MHC molecules. DP thymocytes that fail the selection process or are negatively selected die at this stage. Positive selection leads to transition into CD4<sup>+</sup> or CD8<sup>+</sup> thymocytes and emigration of mature T cells into periphery. Once outside the thymus, they colonize specific regions of peripheral lymphoid organs, where they regulate cell-mediated immune responses.

Analysis of T cells in thymi from wild-type and MMP19-deficient mice revealed reduced amounts of CD4 and CD8 SP cells and higher number of CD4/CD8 DP T cells in immature mice, while adult animals showed balanced T cell subpopulations in the

thymus. These observations indicate a delay in T cell development, especially in the transition from DP to SP cells. MMP19 was previously reported to be abundant in human thymus [214], which is in line with its expression in mouse thymus. Although the underlying mechanism of MMP19 function in the thymus is not known, recent studies with metalloproteinase (MP) inhibitors indicated possible functions of metalloproteinases in thymocyte differentiation. Treatment of thymocytes in DN stages with broad spectrum MP inhibitors significantly reduced the numbers of CD4/CD8 DP and mature SP T cells, inhibited thymocyte proliferation, and led to incomplete upregulation of CD25 (IL-2 receptor  $\alpha$ -chain) [100]. This study shows impact of MPs on  $\beta$ -selection, but do not clarify, if the transition of DP into SP cells is also affected by MPs. Since DP cells are generated in MMP19-deficient mice, it is unlikely that MMP19 plays an essential role in  $\beta$ -selection. However, it might have an impact on the selection of SP cells and thymocyte migration. MMP19  $-/-$  mice show reduced thymocyte proliferation and thus, as a consequence may result in lowering the amount of SP cells. Here, differentiation into CD8<sup>+</sup> cells was more affected than into CD4<sup>+</sup> cells. It is difficult to find an explanation why SP thymocytes of MMP19-deficient mice are reduced, however, one reason might be an alteration of thymic ECM composition. A second possible reason could be changes in cell signaling responses that may further contribute to the skewed cell proliferation.

Although several MMPs are expressed in the thymus their specific role is only sparsely known [14, 203, 300]. It was suggested that MT1-MMP that is upregulated in thymocytes upon contact to laminin 5 contributes to thymocyte migration via releasing CD44 [300]. MMP19 might also be involved in adhesion, detachment, and migration of thymocytes since it processes several ECM proteins including type IV collagen, nidogen-1, and laminin5 $\gamma$ 2 that were reported to be relevant for the migration and development of thymocytes [235, 249, 284, 300].

In human thymus laminin5 ( $\alpha$ 3  $\beta$ 3  $\gamma$ 2) is predominantly present in the medulla, where it is produced by primary epithelial cells [74, 157]. Laminin5 is mainly found in bimembranous structures of the thymus, named conduits, since they resemble similar formations in other lymphoid organs like lymph nodes [74]. These conduits, proposed to function as a transport system for small molecules within the thymus, are associated with tenascin-C, nidogen, and type IV collagen, which are all substrates of MMP19. If chemokines, necessary for migratory processes, are transported via the conduits is unclear. However, ECM molecules and cytokines trigger thymocyte migration and, thus, they impact maturation and emigration of T cells into the periphery [244]. For instance, SDF-1 (CXCL12) in combination with fibronectin or laminin as well as CCL19 in combination with fibronectin promote T cell migration

in a higher degree than the chemokines alone [244, 245]. The proteolytic activity of MMPs towards both chemokines and ECM components is strongly suggested to influence a chemokine-ECM interplay in the thymus [244]. For instance, studies with mice deficient for cathepsin-L, a lysosomal cysteine peptidase, show impairment of positive selection of CD4<sup>+</sup> T cells. Interestingly and unexpected, these mice exhibit reduced amounts of laminin, fibronectin, and collagen in the thymus and augmented transfer of CD8<sup>+</sup> cells into circulation [166]. Moreover, laminin5 serves as adhesive substrate for mature CD8<sup>+</sup> T cells and mediates promigratory stimulus for mature thymocytes *in vitro* [157, 300]. Lack of MMP19 in the murine thymus might contribute to inefficient ECM processing and, thus, to reduced CD8<sup>+</sup> thymocyte proliferation and migration. Reduced processing of laminin5 $\gamma$ 2 protein in the thymus of MMP19-deficient mice may confirm this idea.

In contrast to former publication showing laminin5 expression only in outer cortex of murine thymi [140], in the work presented here, expression of laminin5 in thymi from wild-type and MMP19-deficient mice is found in subcortical structures but also in the medulla and, according to this, correlates with its localization in human thymus. Although it is not clear, which cells in the murine thymus express MMP19, from analysis of human thymus it can be proposed that MMP19 is abundantly present in medullar epithelial cells and most likely in close proximity to laminin5 (G. Klein, Tübingen, personal communication, see Fig. 5.1).

Alteration of growth factor signaling pathways in the thymus may be a second reason why thymocyte proliferation is decreased in MMP19-deficient mice. Insulin-like growth factor-1 (IGF-1) plays a direct and essential role in regulating hematopoiesis, especially lymphopoiesis and immune functions [52, 134]. It signals via binding to IGF-1 receptor (IGF-1R), a tyrosine kinase receptor that is abundantly expressed on rodent and human thymocytes [150, 297]. IGF-1R activation involves phosphorylation of insulin-receptor substrate-1 (IRS-1) resulting in differentiating and promitogenic stimulation [124]. In the thymus, IGF-1 is mainly produced by epithelial and macrophage-like cells [16, 283], where it promotes thymus growth, cell proliferation, and ECM production by epithelial cells [53, 64, 287]. Accordingly, blockade of IGF-1R signaling inhibits T cell proliferation and differentiation [132]. MMP19 is described to impact IGF-signaling due to its ability to regulate IGF-1 availability via cleavage of IGFBP-3, the main binding protein of IGF-1 [236]. Thus, thymi of MMP19  $-/-$  mice might have reduced levels of unbound IGF and lowered IGF-1R activation that reduces thymocyte proliferation.

One can further speculate that due to reduced proliferation and migration of developing thymocytes, interactions of TCRs with MHC-complexes in the thymus are

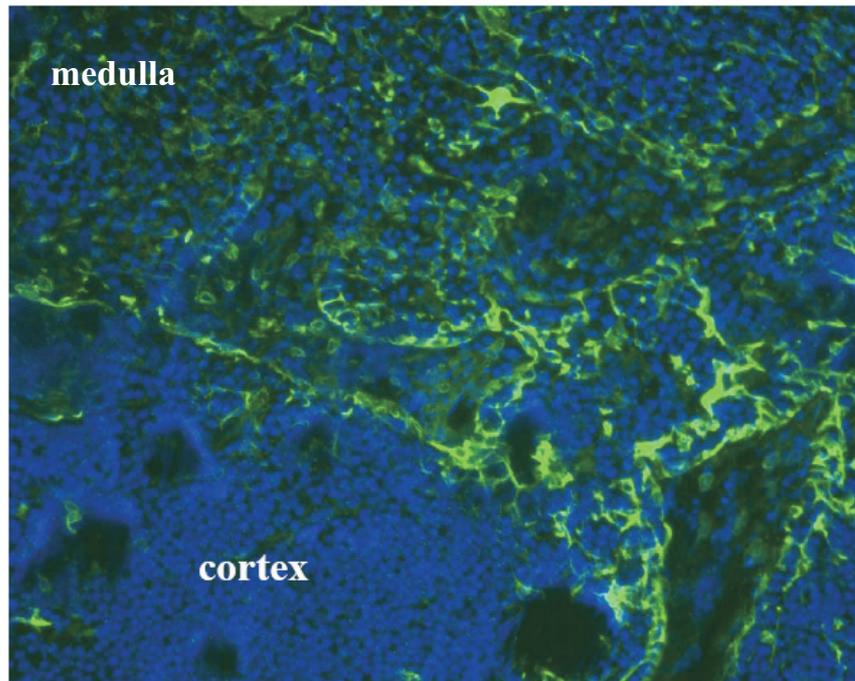


Figure 5.1: Expression of MMP19 in human thymus. MMP19 expression is restricted to the thymus medulla. Cryosections of juvenile thymus were stained against human MMP19 using monoclonal CK8/4 antibodies (green signals). Counterstaining of nuclei were performed with DAPI. Picture courtesy of G. Klein, University of Tübingen.

less frequent resulting in reduced positive selection and lowered number of SP cells in the blood.

In line with the situation in the thymus, studies on T cell populations in blood of immature WT and MMP19  $-/-$  mice disclosed diminished numbers of CD4<sup>+</sup> and CD8<sup>+</sup> cells, indicating a delay in establishment of T cell subsets also in the circulation. It seems reasonable to assume that altered T cell populations in the blood is a direct consequence of reduced SP T cells in the immature thymus, especially since older mice show compensated T cell subsets in both blood and thymus. Influence of MMP19 on blood cell maturation seems limited to the lymphoid cell lineage developed in the thymus, as B cells, granulocytes, and monocytes show equal levels in wild-type and MMP19  $-/-$  mice.

## 5.2 MMP19 affects inflammatory responses in the skin

Since MMP19 shows a unique expression in the skin [236,237] and is upregulated under various inflammatory conditions [26,147,179,274,293] the studies were focused on inflammatory responses in the skin. Regarding the role of MMP19 in innate immune responses of the skin the model of irritant dermatitis was performed showing decreased inflammatory reaction in MMP19  $-/-$  mice. Also in T cell-mediated contact hypersensitivity MMP19-deficiency impairs overall inflammatory response, suggesting a requirement of this MMP in cutaneous inflammation. The distorted T cell development and biased T cell subpopulation in the blood are most likely not responsible for abrogation of the cutaneous inflammatory reaction, since adult mice that were used for experiments did not exhibit these differences. Thus, MMP19 affects the local inflammation independently from developmental alterations of T cells.

In both inflammation models MMP19  $-/-$  mice exhibit reduced recruitment of neutrophils and show low keratinocyte proliferation. Like in the thymus, the latter may be influenced by reduced levels of available active IGF-1 due to impaired IGFBP-3 cleavage by MMP19 [236], since generally, IGF-1 signaling positively influences mitogenic functions of keratinocytes [34]. In the skin, IGF-1 is produced by melanocytes and fibroblasts, whereas basal keratinocytes are positive for IGF-1R and secrete IGFBP-3 [77,231]. In the group of R. Sedlacek, reduced processing of IGFBP-3 was confirmed in keratinocytes from MMP19-deficient mice (see figure 5.2). Via activation of pathways involving MAPK phosphorylation IGF-1R activation can further stimulate expression of other MMPs and growth factors that push up keratinocyte proliferation [229]. IGF-1 is reported to induce IL-6 production that support further cell proliferation. Thus, reduced keratinocyte proliferation in MMP19  $-/-$  mice might be caused by reduced levels of both IGF-1 and MMPs.

In the model of CHS MMP19-deficient mice are not able to mount adaptive immune responses. A similar reduction of the inflammatory reaction in CHS was described also in stromelysin-1 (MMP3)-deficient mice while the lack of MMP9 resulted in a prolongation of the response [307]. The latter was caused by impaired production of IL-10 that acts as antiinflammatory cytokine and contribute to resolution of CHS [83]. However, how this mechanism might works was not explained by the authors.

Interestingly, deficiency of MMP3 and MMP9 weakened also the immune reaction in the lung during acute injury although only MMP3 affected the influx of neu-

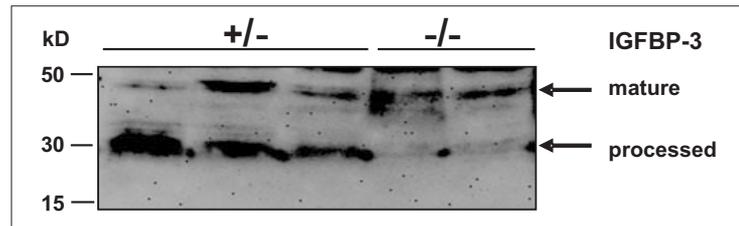


Figure 5.2: Decreased processing of IGFBP-3 in MMP19-deficient mice. After culture of primary keratinocytes from MMP19<sup>+/-</sup> and MMP19<sup>-/-</sup> mice conditioned media were concentrated and analyzed for IGFBP-3 proteolysis by western blotting. Arrows indicate the position of intact IGFBP-3 (mature, 45 kD) and processed 30 kD fragment of IGFBP-3. Similarly reduced processing of IGFBP-3 was found also in the liver of MMP19<sup>-/-</sup> mice (R. Sedlacek, personal communication). Picture courtesy of R. Sedlacek.

trophils [309]. Neutrophils are also controlled by MMP12 as MMP12-deficient mice exhibit a markedly reduced influx of neutrophils into the alveolar space during acute lung injury [308]. In a similar way to MMP3, the lack of MMP19 not only dramatically reduced the inflammation characterized by low influx of inflammatory cells and reduced proliferation of keratinocytes but also strikingly diminished activation of CD8<sup>+</sup> T cells which are responsible for this immune reaction. Based on studies towards the role of MMP3 and MMP19 in CHS one can suggest that these two MMPs might cooperate during the development of this type of immune reaction. Accordingly, both MMPs show similar repertoire of substrates like collagen IV, fibronectin, entactin, and IGFBP-3 [88,236]. However, lack of an MMP does not lead necessarily to altered CHS response, as MMP7-deficient mice show comparable CHS reaction as wild-type mice [307].

To find out if the reduced activation of CD8<sup>+</sup> T cells in MMP19<sup>-/-</sup> mice was due to an inability of DCs to migrate into lymph nodes and to present the hapten to T cells FITC-labeled DCs in inguinal draining lymph nodes were analyzed but the numbers and the activation state of DCs in MMP19<sup>-/-</sup> and wild-type mice were comparable. In contrast, activation of CD8<sup>+</sup> T cells and formation of memory T cells was significantly decreased, especially the transition from naive (CD62L-positive) to early activated (CD69-positive) T cells was inhibited. Upon T cell activation CD62L (L-selectin) is cleaved by the metalloproteinase TNF- $\alpha$  converting enzyme (TACE, also called ADAM17) but other MMPs are speculated to contribute to constitutive processing of CD62L [217,260]. On circulating lymphocytes and leukocytes CD62L is responsible for transmigration of these cells from blood vessels into tissues, especially in initial stages to arrest cells on endothelium [15]. MMP19-deficient mice present both minimal recruitment of lymphocytes under inflammatory conditions and reduced activation of T cells. The rising question, if MMP19 is able to process

CD62L was analyzed with recombinant proteins *in vitro*, but CD62L does not seem to be a substrate for this MMP (data not shown). In addition to reduced T cell activation that probably results in limited homing of primed T cells to the skin, it is further supposed that proinflammatory factors produced by the inflamed tissue itself contribute to T cell distribution and the skewed inflammatory reaction in MMP19-deficient mice.

With regard to the impaired inflammation it is possible that the amount of cytokines and chemokines produced from the epidermis are not sufficient for the attraction and migration of T cells. The reduced proliferation of keratinocytes in MMP19  $-/-$  mice points towards this possibility. This is also supported by markedly reduced concentrations of lymphotactin, I-TAC, and TCA-3. Lymphotactin is a powerful attractant for T cells and is expressed by activated CD8<sup>+</sup> T cells as well as by epidermal dendritic T cells affecting migration of CD8<sup>+</sup> T cells [33,135,196]. I-TAC, interferon-inducible T cell  $\alpha$  chemoattractant (also known as CXCL11 or IP-9), is produced by keratinocytes and exhibits potent chemoattractant activity for interleukin-2-activated T cells [56,282] and binds to the chemokine receptors CXCR7 and CXCR3. CXCR7 provides a survival advantage, increased adhesiveness of cells, and is involved in the migration of T cells [21,44]. CXCR3 plays a non-redundant role in T cell migration and is critical for recruitment of activated T lymphocytes to dermal inflammatory sites [44,187]. Binding of I-TAC to CXCR3 causes its internalization that is thought to play a role in transendothelial cell migration [242]. TCA-3 (I-309 or CCL1) is secreted by dermal endothelial cells, DCs, and mast cells and signals via its selective chemokine receptor CCR8 that is mainly expressed on cutaneous T cells and upregulated upon stimulation [55,246]. Due to continuous expression of TCA-3 by dermal microvessels and epidermal APCs and due to maximal responsiveness of CD25<sup>+</sup> and CLA (cutaneous lymphocyte-associated antigen)-positive T cells to this chemokine, it is proposed that TCA-3 regulates trafficking of cutaneous regulatory T cells and memory T cells into the skin [55,246]. This chemokine not only attracts T cells but also specifically mediates eosinophil recruitment as shown in a model of allergic airway inflammation [28]. Additionally, signaling via CCL1/CCR8 complexes also induces MMP secretion, as shown for MMP2 and thus, reduced expression of TCA-3 can result in reduction of MMP expression leading into a cycle of anti-inflammatory signals [102].

Reduced fractalkine (CX3CL1) expression detected in affected skin of MMP19-deficient mice might contribute to limited cell recruitment. Fractalkine is predominantly expressed by epithelial cells, including keratinocytes and exists either membrane anchored or as soluble form [169]. The soluble form is a potent chemo-

tactic factor for T cells, monocytes, NK (natural killer) cells, and mast cells, all expressing the receptor CX3CR1, whereas membrane-bound fractalkine acts as adhesion molecule [24,120]. Upregulation of fractalkine in cutaneous DCs is observed in inflammatory situations [227]. Several MMPs are described not only to process ECM components but also chemokines like SDF-1 and MCP molecules [181,182] and directly initiate the generation of chemoattractant activity. Both, fractalkine and MMP19 are abundantly expressed by basal keratinocytes and evidence exists that MMP19 is able to process this chemokine (R. Sedlacek, personal communication). Thus, lack of MMP19 may result in reduced CX3CL1 levels that again limits cell trafficking at sites of inflammation.

Together, chemokines are potent molecules controlling the key regulatory processes of cell recruitment into inflamed tissue. Since CD8<sup>+</sup> T cells were less abundant at the inflammatory sites of MMP19 <sup>-/-</sup> mice than in control mice, it is suggested that the MMP19-dependent dysregulation of chemokines, such as lymphotactin, I-TAC, TCA-3, and fractalkine is responsible for the skewed distribution and circulation of T cells in the periphery and in lymph nodes. In addition, single application of an irritant also led to recruitment of CD8<sup>+</sup> T cells in wild-type mice, which was diminished in MMP19 <sup>-/-</sup> mice. All these alterations of the immune response in MMP19-deficient mice point towards the role of MMP19 in controlling attraction and circulation of inflammatory cells.

### 5.3 MMP19 promotes cell migration

During inflammation, the basement membrane, mainly composed of laminins, collagen IV, and fibronectin, represents the major barrier for migrating cells that require expression of proteases, including MMPs to penetrate through this barrier and arrive to inflamed tissues. Macrophages secrete several MMPs such as MMP1, -2, -3, -7, -9, and -12 that can directly be upregulated upon ECM contact [94]. Additionally, macrophages express also MMP19 that was studied in its ability to effect macrophage transmigration.

*In vitro* and *in vivo* MMP19-deficient cells exhibit strongly inhibited capacity to transmigrate through ECM matrices. Generally, lack of MMP activity leads to inefficient cleavage of matrices that reduces passing of cells to sites of inflammation [254]. Analysis of MMP9 revealed essential role of this MMP in cell transmigration. In a model of zymosan-induced peritonitis MMP9-deficient mice responded with strongly reduced accumulation of leukocytes in the peritoneal cavity [145]. Role of MMP12 in recruitment of macrophages was demonstrated in MMP12-deficient mice using a

model of COPD (chronic obstructive pulmonary disease). Lack of MMP12 impairs macrophage influx into the lung and protects mice from pulmonary inflammation and emphysema formation [105]. Furthermore, MT1-MMP was shown to stimulate monocyte migration on fibronectin and endothelial cells in a manner dependent on  $\alpha4\beta1$  and  $\alpha5\beta1$  integrins. Contact of monocytes to fibronectin, VCAM-1, or ICAM-1 increases MT1-MMP expression and the fibrinolytic activity [178]. In addition to these MMPs, the work presented here shows influence of MMP19 activity in macrophage migration. Other cell types such as granulocytes and monocytes are not affected, as populations of CD11b<sup>+</sup>, Ly-6<sup>+</sup>, and CD14<sup>+</sup> cells show similar transmigration rate in WT and MMP19 <sup>-/-</sup> mice. As reported for MT1-MMP, upregulation of MMP19 expression in macrophages under inflammatory conditions or upon contact to ECM components can be proposed, since increase of MMP19 expression was shown in keratinocytes upon contact to collagen type I and also in macrophages after adhesion [179, 237].

MMP expression is also induced in T cells and necessary for their transmigration, shown in studies mainly using MMP inhibitors. In a model of allergen-airway inflammation it was shown that MMP2 and MMP9 are needed for T cell migration into the lung [155]. The plant flavonoid astilbin, which has immune suppressive activity and reduces TNF- $\alpha$  and IFN- $\gamma$  production in lymphocytes, is proposed to inhibit MMP activity and lymphocyte migration resulting in impaired hypersensitivity reactions [46].

Reduced recruitment of MMP19 <sup>-/-</sup> macrophages is likely to be a direct cause of impaired matrix processing, since macrophages abundantly express MMP19. Reduced presence of CD8<sup>+</sup> T cells in inflamed skin of MMP19-deficient mice may be influenced more indirectly due to impaired expression of other MMPs responsible for transmigration through collagen type I matrix. Studies regarding migration of effector T cells through collagen clearly show involvement of Wnt-signaling in MMP expression, which was mediated by  $\beta$ -catenin binding to TCF (T cell factor)-binding sites on MMP2 and MMP9 promoters [319]. In respect to the idea that also MMP19 expression might be induced in T cells upon inflammation, promoters of human and murine MMP19 were analyzed for putative TCF-binding sites. Using promoter sequences described before [192, 193] consensus elements for TCFs were identified (Fig. 5.3), which turn MMP19 into a possible target of Wnt-signaling.

It is suggested here, that MMP19 expression is upregulated in lymphocytes during extravasation and contributes to subendothelial basal membrane degradation. Analysis of inflamed tissue of human skin tumors point towards MMP19 expression in lymphocytes (unpublished data).

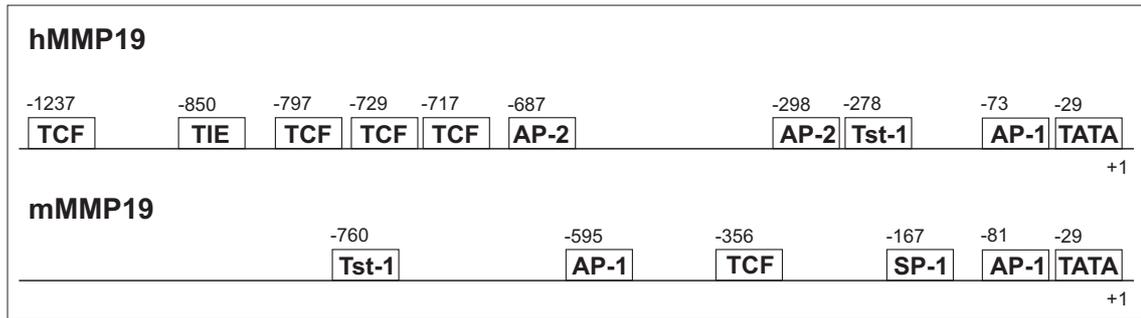


Figure 5.3: Promoters of human and murine *MMP19* gene contain putative TCF-binding sites. Transcription start (+1), TATA-box, and positions of regulatory elements like AP-1, AP-2, and SP-1 are indicated. Promoter analysis was done using programs available online (MatInspector, [www.genomatix.de/products/MatInspector/index.html](http://www.genomatix.de/products/MatInspector/index.html) and TF Search, [www.cbrc.jp/research/db/TFSEARCH.html](http://www.cbrc.jp/research/db/TFSEARCH.html)).

MMP19-deficiency further influences transmigration of macrophages. MMPs are widely reported to affect leukocyte transmigration due to processing of ECM components. Macrophages express several MMPs such as MMP-1, -3, -7, -9, -12, and MT1-MMP, which degrade interstitial collagens and basement membrane proteins [254]. Collectively, the findings indicate that MMP19 participate in macrophage transmigration, especially since it is located at the cell surface. However, its function might be redundant and partially compensated by other MMPs.

Altogether, analysis of MMP19-deficient mice revealed that MMP19 plays an important role in the development of the cutaneous immune response, especially in CHS, and is involved in development of T lymphocytes, their distribution, and activation. However, future investigations has to be performed in order to elucidate the function of MMP19 in distinct cell types, tissues, and cell signaling processes.

# Chapter 6

## Summary

In the work presented here, the role of matrix metalloproteinase 19 (MMP19) was studied in inflammatory reactions of the skin. Mice deficient for MMP19 were used to perform two cutaneous inflammation models. In the first model of irritant dermatitis (ICD) inflammatory reactions are independent of T lymphocytes (T cells), whereas the second model of contact hypersensitivity (CHS) requires hapten-specific T cells and thus adaptive immunity.

In both models, ICD and CHS, MMP19-deficient mice (MMP19  $-/-$ ) exhibit strongly reduced skin inflammation, indicated by minimal edema formation, limited proliferation of keratinocytes, and reduced recruitment of inflammatory cells to the affected site. In the inflamed tissue, MMP19  $-/-$  mice exhibit decreased numbers of T lymphocytes. During CHS, MMP19  $-/-$  mice respond with reduced secretion of proinflammatory cytokines such as I-TAC, lymphotactin, and TCA-3, which act mainly as chemoattractant for T lymphocytes and thus contribute to reduced cellular influx. Moreover, MMP19-deficient mice show diminished activation of CD8-positive T cells, however, accurate T cell activation is essential for the elicitation of hypersensitivity inflammation.

Under non-inflammatory conditions MMP19  $-/-$  mice exhibit defective development of T cells that becomes apparent in reduced proliferation and maturation of thymocytes. MMP19-deficient mice show also reduced T cell populations in the blood, pointing towards limited emigration of mature T cells from thymus into the blood. Migration of cells to sites of inflammation requires proteolytic processing of connective tissue components. Since MMP19 is able to cleave several components of extracellular matrix (ECM) proteins such as collagen type IV, fibronectin, laminin, and nidogen, it is proposed that the lack of MMP19 activity contributes to inhibited cell migration. Employing MMP19  $-/-$  macrophages, reduced trans migratory capacity was confirmed *in vitro* and *in vivo*.

Altogether, MMP19 plays a critical role in inflammatory reactions of the skin, where

MMP19 stimulate cytokine production, cell proliferation and migration. Additionally, MMP19 impacts thymocyte development and probably T cell emigration processes into periphery.

# Chapter 7

## Zusammenfassung

In der vorliegenden Arbeit wurde die Rolle der Matrixmetalloproteinase 19 (MMP19) in entzündlichen Reaktionen der Haut untersucht. Für diese Studien wurden zwei Entzündungsmodelle an MMP19-defizienten Mäusen durchgeführt. Im Modell der irritanten Dermatitis (ICD) laufen Entzündungsprozesse ohne direkte Beteiligung von T-Lymphozyten (T-Zellen) ab, dagegen erfordert das Modell der Kontakthypersensitivität (CHS) die Beteiligung von haptenspezifischen T-Zellen innerhalb einer adaptiven Immunantwort.

In beiden Modellen zeigen MMP19-defiziente (MMP19  $-/-$ ) Mäuse eine stark reduzierte Entzündung, die mit Verringerung der Gewebeschwellung, reduzierter Proliferation von Keratinozyten und verminderter Rekrutierung von Neutrophilen und anderen Entzündungszellen einher geht. In beiden Reaktionen liegt zudem eine verminderte Anzahl von T-Lymphozyten im entzündeten Gewebe vor. Im Modell der CHS zeigen MMP19-defiziente Mäuse weiterhin eine verminderte Sekretion von proinflammatorischen Zytokinen am Ort der Entzündung. Eine verminderte Ausschüttung der Chemokine I-TAC, Lymphotactin and TCA-3, die die Anlockung von T-Lymphozyten (T-Zellen) vermitteln, trägt zur verringerten Einwanderung von Zellen bei. Weiterhin zeigen MMP19-defiziente Mäuse eine verminderte Aktivierung von CD8-positiven T-Zellen, die jedoch für die Entstehung von akuten Entzündungsreaktionen entscheidend ist.

Unabhängig von entzündlichen Prozessen wurden Defekte in der Entwicklung von T-Lymphozyten in MMP19  $-/-$  Mäusen festgestellt, die sich in reduzierter Proliferation und verzögerter Reifung von Thymozyten äußern. Zudem zeigen diese Tiere eine veränderte Population von T-Zellen im Blut, was auf Einschränkungen in der Migration von reifen T-Zellen aus dem Thymus ins Blut hindeutet. Um an den Ort der Entzündung zu gelangen, müssen Zellen mittels Proteolyse Bindegewebsbestandteile prozessieren. Da MMP19 in der Lage ist, eine Vielzahl von Proteinen der extrazellulären Matrix (ECM) wie z. B. Kollagen Typ IV, Fibronectin, Laminin

und Nidogen zu spalten, ist die verminderte Einwanderung von Zellen ins entzündete Gewebe mit dem Fehlen der proteolytischen Aktivität von MMP19 zu verstehen. Dies wurde an MMP19-defizienten Macrophagen bestätigt, die *in vitro* und *in vivo* eingeschränkte Transmigration zeigen.

Zusammengefasst spielt die Matrixmetalloproteinase 19 eine wichtige Rolle in entzündlichen Reaktionen der Haut, wo sie die Proliferation, Zytokinsekretion und Migration von Zellen stimuliert. Ferner ist MMP19 an der Thymozytenentwicklung und möglicherweise an der Auswanderung von reifen T-Zellen aus dem Thymus beteiligt.

# Chapter 8

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# Appendix

BLC	CD30L	Eotaxin	Eotaxin-2	FasLigand
Fractalkine	GCSF	GM-CSF	IFN $\gamma$	IL-1 $\alpha$
IL-1 $\beta$	IL-2	IL-3	IL-4	IL-6
IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13
IL-17	I-TAC	KC	Leptin	LIX
Lymphotactin	MCP-1	MCSF	MIG	MIP-1 $\alpha$
MIP-1 $\gamma$	RANTES	SDF-1	TCA-3	TECK
TIMP-1	TIMP-2	TNF $\alpha$	sTNFRI	sTNFRII

Table 8.1: List of 40 cytokines, available in the mouse antibody array provided by RayBio Tech.

<b>Systematic name</b>	<b>Trivial description (human)</b>	<b>Trivial description (mouse)</b>
CXCL1	Gro- $\alpha$	Gro/KC
CXCL2	Gro- $\beta$	Gro/KC
CXCL3	Gro- $\gamma$	Gro/KC
CXCL5	ENA-78	LIX
CXCL8	IL-8	unknown
CXCL9	Mig	Mig
CXCL10	IP-10	IP-10
CXCL11	I-TAC/IP-9	I-TAC
CXCL12	SDF1 $\alpha/\beta$	SDF1
CXCL13	BLC	BLC
XCL1	Lymphotactin/ATAC	Lymphotactin
CX3CL1	Fractalkine	Fractalkine/neurotactin
CCL1	I-309	TCA-3
CCL2	MCP-1	MCP-1
CCL3	MIP-1 $\alpha$	MIP-1 $\alpha$
CCL4	MIP-1 $\beta$	MIP-1 $\beta$
CCL5	RANTES	RANTES
CCL9	unknown	MIP-1 $\gamma$
CCL11	Eotaxin	Eotaxin
CCL17	TARC	TARC
CCL19	MIP-3 $\beta$	MIP-3 $\beta$
CCL20	MIP-3 $\alpha$	MIP-3 $\alpha$
CCL24	Eotaxin-2	Eotaxin-2
CCL25	TECK	TECK
CCL27	CTACK	CTACK

Table 8.2: Chemokines, systematically listed with their trivial description. Families of chemokines classified by arrangement of cysteines (C) in their sequence are grouped (CXC, C, CX3C, and CC chemokines). Regarding this work, only relevant chemokines and those related to these are mentioned.

Table 8.3: List of MMPs and their substrates

Systematic name	Trivial description	Matrix substrates	Non-matrix substrates
<b>Collagenases</b>			
MMP1	Collagenase-1	collagen type I-III, VII, X gelatin, aggrecan nidogen, tenascin perlecan	MCP-1, -3, -4, proMMP2 SDF-1, IL-1 $\beta$ , proTNF- $\alpha$ IGFBP-2, IGFBP-3 $\alpha$ 2-macrogobulin $\alpha$ 1-proteinase inhibitor
MMP8	Collagenase-2	collagen type I-III, VII, X gelatin, aggrecan nidogen, tenascin	MCP-1, L-selectin proTNF $\alpha$ , proMMP8 $\alpha$ 2-macrogobulin $\alpha$ 1-proteinase inhibitor
MMP13	Collagenase-3	collagen type I-III, VII, X gelatin, aggrecan nidogen, tenascin	MCP-3, SDF-1, proMMP9, proTNF- $\alpha$ $\alpha$ 1-antichymotrypsin
MMP18	Collagenase-4, xCol4 (only in Xenopus)	collagen type I-III gelatin	
<b>Gelatinases</b>			
MMP2	Gelatinase A 72 kD-gelatinase	collagen I, IV, V, VII, X, XI gelatin, elastin laminin5, fibronectin brevican, decorin vitronectin, aggrecan	MCP-3, SDF, proIL-1 $\beta$ proTGF- $\alpha$ 1, proTNF- $\alpha$ IGFBP-3, -5, FGFR-1 endothelin-1, galectin-3 proMMP1, proMMP13 $\alpha$ 2-macrogobulin $\alpha$ 1-proteinase inhibitor
MMP9	Gelatinase B 92 kD-gelatinase	collagen I, IV, V, VII, X, XI elastin, fibronectin laminin, aggrecan vitronectin	SDF-1, GRO $\alpha$ , proTNF- $\alpha$ proTGF- $\beta$ , IL-2R $\alpha$ plasminogen, FGFR-1 galectin-3, proMMP9 and -13 $\alpha$ 1-proteinase inhibitor $\alpha$ 2-macrogobulin
<b>MT-MMPs</b>			
MMP14	MT1-MMP	collagen I-III gelatin, fibronectin vitronectin, aggrecan	MCP-3, SDF1, CD44 proTNF- $\alpha$ , proMMP2 $\alpha$ 2-macrogobulin $\alpha$ 1-proteinase inhibitor

Table 8.3: MMP nomenclature – continued

Systematic name	Trivial description	Matrix substrates	Non-matrix substrates
MMP14 (cont.)			tissue transglutaminase
MMP15	MT2-MMP	proteoglycan	proMMP2, proTNF- $\alpha$ tissue transglutaminase
MMP16	MT3-MMP	collagen type III fibronectin	proMMP2, proTNF- $\alpha$ tissue transglutaminase
MMP17	MT4-MMP	gelatin fibrin/fibronectin	proMMP2, proTNF- $\alpha$
MMP24	MT5-MMP	fibronectin, gelatin proteoglycans	proMMP2
MMP25	MT6-MMP	collagen IV, gelatin laminin, fibronectin proteoglycans (chondroitin- sulfate), fibrinogen	proMMP2, proMMP9 $\alpha$ 1-proteinase inhibitor
<b>Stromelysins</b>			
MMP3	Stromelysin-1 Proteoglycanase	aggrecan, fibronectin laminin, gelatin, nidogen non-triple collagen II-V, IX, X perlecan, tenascin, elastin vitronectin fibrin/fibrinogen	MCP-1,-2, -3, -4, SDF-1 proTNF- $\alpha$ , L-selectin hbEGF, plasminogen pro-IL-1 $\beta$ E-cadherin, IGFBP-3 proMMP1, -7, -8, -9, -13 $\alpha$ 1-proteinase inhibitor $\alpha$ 1-antichymotrypsin $\alpha$ 2-macrogobulin
MMP10	Stromelysin-2	gelatin type I, III, IV, V fibronectin, proteoglycan	proMMP1 and -8
MMP11	Stromelysin-3	fibronectin laminin, aggrecan	IGFBP-1 $\alpha$ 1-proteinase inhibitor $\alpha$ 2-macrogobulin
<b>Matrilysins</b>			
MMP7	Matrilysin PUMP	laminin, gelatin, fibronectin non-helical coll. IV, V, IX-XI aggrecan, nidogen tenascin, vitronectin fibrin/fibrinogen	$\alpha$ -defensin, proTNF- $\alpha$ FasL, E-cadherin $\beta$ 4 integrin, plasminogen proMMP1, -2, -9 $\alpha$ 1-proteinase inhibitor $\alpha$ 2-macrogobulin

Table 8.3: MMP nomenclature – continued

Systematic name	Trivial description	Matrix substrates	Non-matrix substrates
MMP26	Matrilysin-2	collagen type IV, fibronectin	IGFBP-1, proMMP9
	Endometase	gelatin, fibrin/fibronectin	$\alpha$ 1-proteinase inhibitor
<b>Other MMPs</b>			
MMP12	Metalloelastase	elastin, fibronectin	plasminogen, proTNF- $\alpha$
	Macrophage elastase	laminin, proteoglycan	$\alpha$ 1-proteinase inhibitor
	Metalloelastase	fibrin/fibronectin	
MMP19	RASI	collagen IV, fibronectin	IGFBP-3, VEGF
	Stromelysin-4	gelatin, laminin, nidogen aggrecan, COMP fibrin/fibrinogen	
MMP20	Enamelysin	amelogenin	
	amelogenin	COMP, aggrecan	
MMP21	XMMP (xenopus)	not determined	
MMP22	CMMP (chicken)	not determined	
MMP27	(human homolog)		
MMP23	CA-MMP	gelatin	
	(cysteine-array-MMP)		
MMP28	Epilysin	not determined	

Table 8.3: Matrix metalloproteinases, systematically listed with their trivial description and substrates. Families of MMPs are listed by their proteolytic activity: collagenases, gelatinases, MT-MMPs, stromelysins, matrilysins, and other MMPs. Adapted after C. Overall's web page: <http://www.clip.ubc.ca/>.

## **Erklärung**

Hiermit erkläre ich, dass die vorliegende Arbeit

„The Function of Matrix Metalloproteinase 19 in Cutaneous Immunity“

abgesehen von der Beratung durch meinen Betreuer nach Inhalt und Form meine eigene Arbeit ist und unter Einhaltung der Regeln guter wissenschaftlicher Praxis entstanden ist. Diese Arbeit hat in dieser Form zuvor keinem anderen Prüfungsverfahren vorgelegen.

Teile dieser Arbeit sind zur Veröffentlichung eingereicht worden.

Kiel, im Mai 2007

Inken Maria Beck