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1. Goals of study/abstract in English language

The main objective of my diploma thesis was determining the role of S100A8 and S100A9 in the development and progression of inflammatory skin diseases, in particular psoriasis. Both proteins are highly upregulated in the skin of psoriasis patients as well as in the mouse model for psoriasis caused by inducible, epidermal deletion of the two AP-1 family members JunB and c-Jun (JunB^{Δep*} c-Jun^{Δep*} or DKO* mice, (1)). Induction of S100A8 and S100A9 in mice is observed even before appearance of disease symptoms (1). Therefore, analysis of the psoriasis-like skin disease in JunB^{Δep*} c-Jun^{Δep*} mice provides a powerful tool to study S100A8 and S100A9 function. Importantly, loss of S100A9 in the Jun/AP-1 psoriasis mouse model significantly reduces the severity of the inflammatory phenotype (H. Schönthaler, unpublished data).

The two main cell types involved in development of skin inflammations are epidermal keratinocytes and a variety of immune cells. The skin of the Jun/AP-1 psoriasis mouse model displays notable epidermal thickening due to keratinocyte hyperproliferation as well as pronounced immune cell invasion in dermis and epidermis (1). To determine if this is due to the loss of JunB and c-Jun in a cell autonomous manner, we investigated apoptosis and proliferation of keratinocytes in DKO* mice *in vivo* and *in vitro*. The results were compared to JunB^{Δep*} c-Jun^{Δep*} S100A9^{-/-} (TKO*) mice to identify the effect of S100A9 loss. Furthermore, we performed a histological and molecular characterization of the skin of these mice and analyzed the composition of the cutaneous immune cell infiltrate of DKO* and TKO* mice at different stages of the disease. Cytokine production in the dermis and epidermis, systemic mouse serum levels as well as cytokine secretion by cultured keratinocytes of the Jun/AP-1 psoriasis mouse model and TKO* mice was determined. That was done to understand the role of S100A9 in the cross talk between keratinocytes and immune cells in the course of skin inflammation and in order to find S100A9-dependent mediators in this cellular network.

Finally, we hypothesized S100A8 and S100A9 overexpression per se may provoke an inflammatory skin phenotype and analyzed mice carrying a switchable Tet-ON system-controlled transgene for S100A8 and S100A9. Unfortunately, we observed no consistent increase in S100A8 or S100A9 protein levels in the epidermis of mice using induction of the S100A8/A9 transgene by two different transactivator lines.

In summary, S100A9 appears to induce inflammatory, psoriasis disease-like symptoms in multiple ways. Skin architecture, angiogenesis and expression of key inflammatory genes are largely normalized upon loss of S100A9 in the Jun/AP-1 psoriasis mouse model. S100A9 deficiency of TKO* mice leads to a decrease of dermal and epidermal immune cell infiltration, reduced epidermal proliferation and lower inflammatory cytokine levels in mouse sera compared to DKO* mice. Although S100A8 and S100A9 are up-regulated in keratinocytes *in vitro* upon JunB and c-Jun deletion (1), cell proliferation is not affected in a cell autonomous manner. S100A9 does not have a pronounced cell autonomous effect on inflammatory cytokine secretion of keratinocytes, since supernatant levels of cultured DKO* and TKO* keratinocytes are largely similar. Thus, S100A9 appears to play a complex role in the cross talk of keratinocytes and immune cells and seems to have an inducing effect on psoriasis and inflammatory skin reactions.

2. Introduction

The skin is not only the largest organ of the human body and the major sensation facilitator, but also provides protection against dehydration, excessive temperature changes as well as physical and chemical stress. It represents the first line of defense to avoid entrance of possible harmful pathogens from the environment. This is achieved by an acidic pH, a competing surface microbiota and a physical barrier lining the organism (2). The outermost layer of the skin is the epidermis and consists of multiple layers of tightly packed keratinocytes (approximately 95% of the total epidermis), dispersed by Langerhans cells, Merkel cells as well as melanocytes in the basal layer. In order to maintain the integrity of the epidermis, keratinocytes proliferate at the basal membrane and undergo a differentiation program affecting their morphology and cytoskeleton towards the exterior to form a sealed, roof-like surface. Round-shaped keratinocytes form a network of desmosomal cell-to-cell junctions and accumulate lipid- and structural protein-containing intracellular bodies in the spinous layer. Granular keratinocytes release the content of these numerous granules, including Odland bodies as well as lamellar bodies and keratinosomes. Moreover, they initiate degradation of intracellular organelles and the nucleus, flatten out and undergo apoptosis. Depending on their state of differentiation, keratinocytes express distinct types of structural proteins, like Loricrin, Filaggrin

and various keratin proteins. They assemble to disulfide-linked intermediate filaments and form the matrix of the outermost layer of the epidermis. The cornified envelope (stratum corneum) is further supported by large amounts of released lipids, desmosomes and a network of Involucrin molecules cross-linked to membrane proteins (Fig. 1A, (3) and (4)).

The dermis is less densely populated by cells, but rather represents connective tissue and elastic collagen fibers produced by embedded fibroblasts. It contains hair follicles, sebaceous glands and sweat glands, which penetrate through the overlaying epidermis.

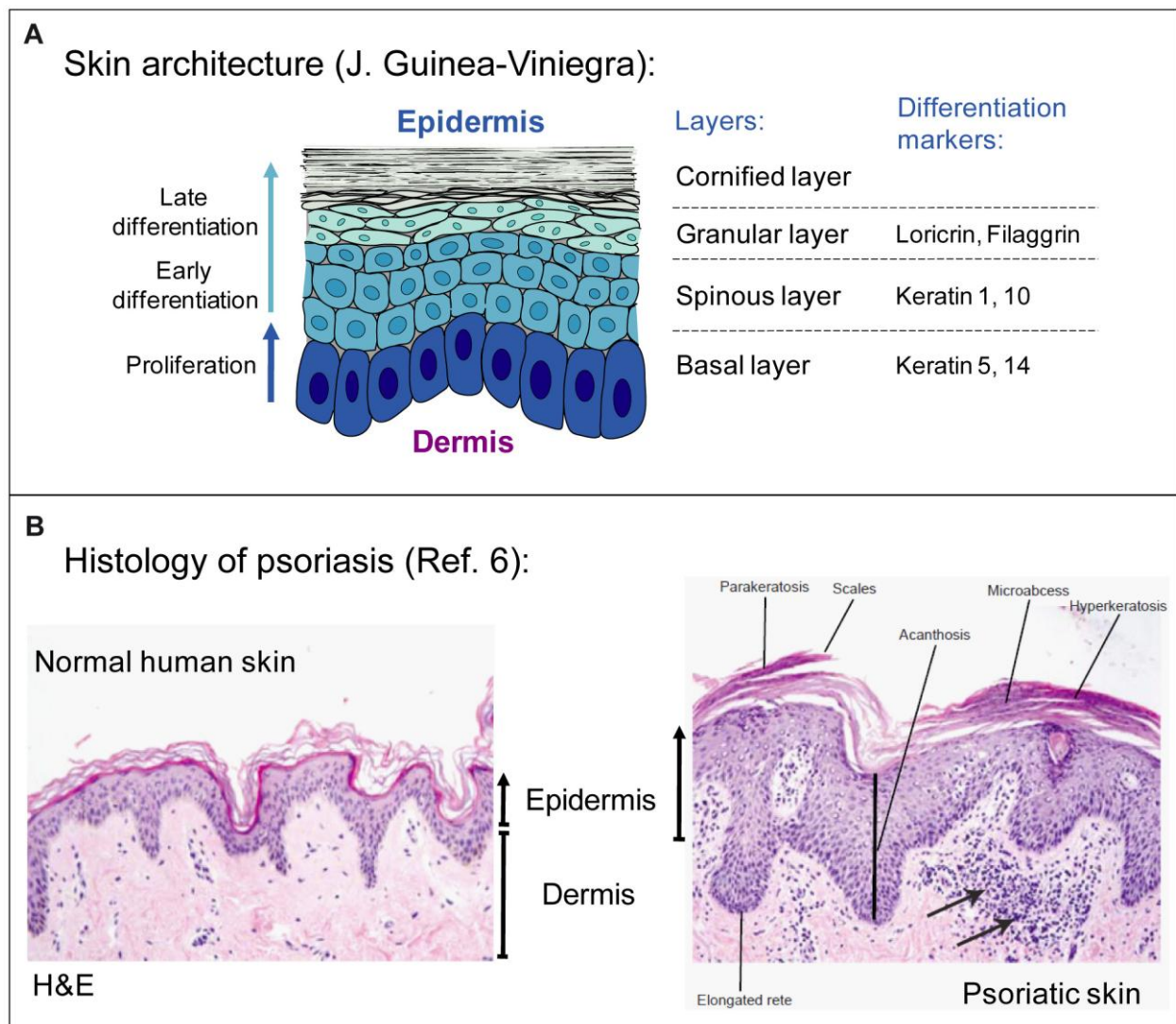


Fig. 1 – Overview of skin architecture, epidermal differentiation and psoriasis histopathology.

(A) Schematic view of the skin with focus on the epidermal layers, keratinocyte differentiation and marker expression (J. Guinea-Viniegra, modified).

(B) Haematoxylin and Eosin (H&E)-stained histology of normal and psoriatic human skin showing hallmarks of psoriasis (modified form (6)).

Networks of nerves, blood and lymph vessels spread throughout the dermis and ensure mechanical stress response, nutrition and moisture supply of the skin. Several immune cell types are resident in the dermis, including macrophages, mast cells, dendritic cells and lymphocytes ((2) and (5)). Due to the dense dermal vasculature and signals originating from skin cells, large numbers of innate and adaptive immunocytes readily infiltrate the dermis and epidermis during inflammatory skin diseases upon deregulation of skin homeostasis.

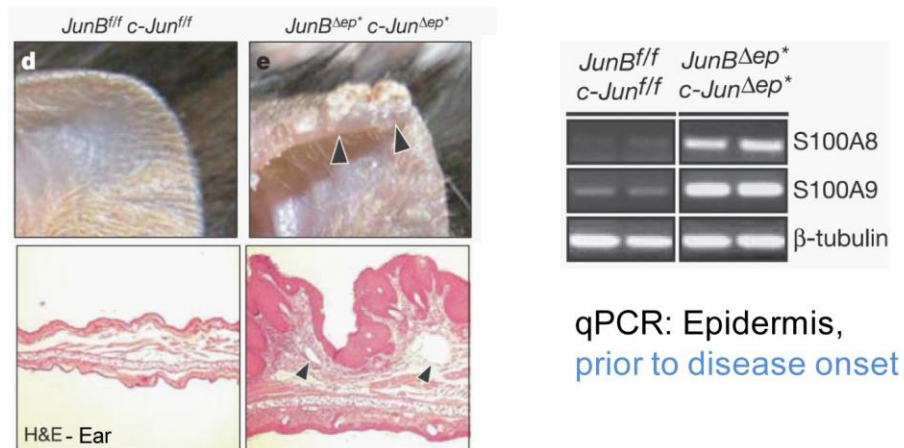
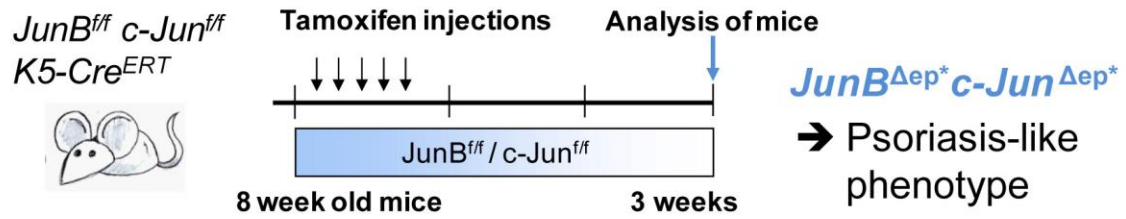
About 2-3 percent of the worldwide population suffer from psoriasis, a very heterogeneous skin disorder of unclear origin. Psoriasis vulgaris is the most frequent variant (around 90%) and numerous patients develop additionally psoriatic arthritis and/or nail psoriasis. The autoimmune-related skin disease has a genetic component, since it is more common among relatives and at least 12 significantly associated chromosomal loci, psoriasis susceptibility regions (PSORS), have been identified. It is characterized by scaly, protuberant demarcated plaques of variable dimension as a cause of epidermal hyperplasia and altered keratinocyte differentiation (Fig. 1B, (6), (7) and (8)). Further hallmarks of psoriasis are increased blood supply by an enlarged network of dilated blood vessels as well as chronic inflammation with pronounced local leukocyte infiltration in the skin and an elevation of various cytokines, locally and systemically. Cytokines and immune cells, primarily TNF α and T lymphocytes, are tackled by most psoriasis targeted-therapies in the clinics, e.g. Infliximab and Efalizumab, respectively ((6), (7) and (8)). However, there is no cure yet and the initial cause of psoriasis as well as the essential steps leading to disease progression remain to be defined.

In this study we employed a mouse model for psoriasis, the Jun/AP-1 psoriasis mouse model (1). Activator protein 1 (AP-1) is a dimeric transcription factor complex integrating several physiological and pathological stimuli to regulate proliferation, differentiation, apoptosis and transformation. Bacterial or viral infections, growth factors, cytokines or oncogenic signals activate MAPK kinase signaling cascades including ERK, JNK and p38-kinases. These, in turn, induce Jun, Fos, activating transcription factor (ATF) and musculoaponeurotic fibrosarcoma (MAF) proteins, which form homo- and heterodimers ((9) and (10)). Knock-out and gain-of-function studies of AP-1 family members mainly in mice, demonstrated their various roles in different organs.

Constitutive mutation of JunB or c-Jun results in lethality (11), epidermal JunB loss (JunB ^{Δ ep}) leads to a multiorgan disease (12) and keratinocyte-restricted c-Jun defi-

A

Jun/AP-1 psoriasis mouse model (Ref.1):



B

Mice used in this study:
(H. Schönthaler)

	genotype
control	<i>JunB^{ff} c-Jun^{ff} S100A9^{+/-}</i>
S100A9-/-	<i>JunB^{ff} c-Jun^{ff} S100A9^{-/-}</i>
DKO*	<i>JunB^{Δep*} c-Jun^{Δep*} S100A9^{+/-}</i>
TKO*	<i>JunB^{Δep*} c-Jun^{Δep*} S100A9^{-/-}</i>

Phenotype:

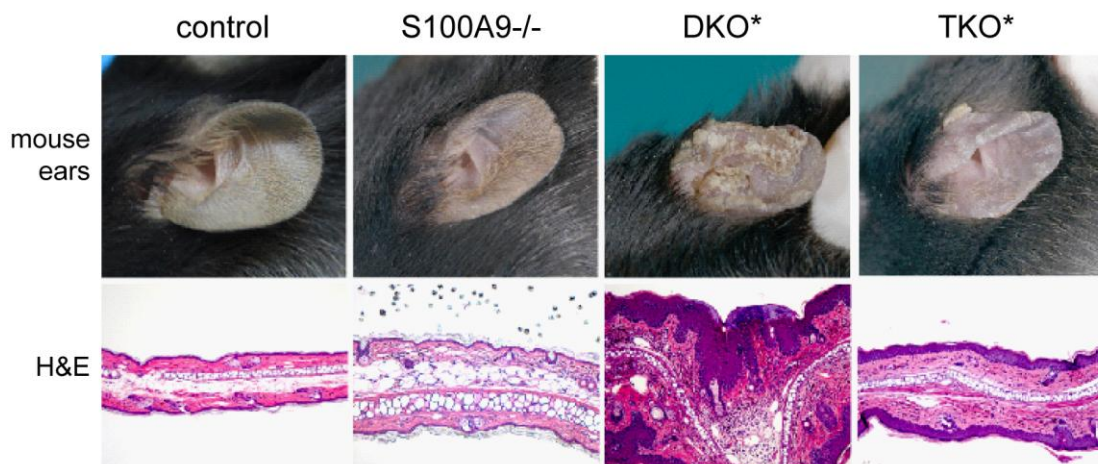


Fig. 2 – Loss of S100A9 in the Jun/AP-1 psoriasis mouse model leads to largely reduced inflammatory symptoms (H. Schönthaler, unpublished observation).

(A) Jun/AP-1 psoriasis mouse model (modified from (1)): Treatment of experimental mice to generate $\text{JunB}^{\Delta\text{ep}*}$ $\text{c-Jun}^{\Delta\text{ep}*}$ mice, the Jun/AP-1 psoriasis mouse model. Five consecutive intraperitoneal injections of 1mg Tamoxifen per day of eight-weeks old mice lead to K5 Cre-mediated deletion of floxed JunB and c-Jun alleles. Mice were sacrificed and analyzed two weeks after the last Tamoxifen injection. Macroscopic images and H&E-stained sections of $\text{JunB}^{\text{f/f}}$ $\text{c-Jun}^{\text{f/f}}$ and $\text{JunB}^{\Delta\text{ep}*}$ $\text{c-Jun}^{\Delta\text{ep}*}$ mouse ears show a psoriasis-like skin disorder. Quantitative PCR amplification of S100A8 and S100A9 mRNA of mouse epidermis with normal appearance 24 hours after tamoxifen injection for 3 consecutive days displays upregulation of these genes in the Jun/AP-1 psoriasis mouse model.

(B) Overview of genotype and terminology of mice used in this study (H. Schönthaler). Control, S100A9^{-/-}, DKO* and TKO* mice received treatment as described in (A). Macroscopic images of mouse ears two weeks after the last tamoxifen injection and microscopic images (magnification 10x) of H&E-stained mouse ear sections reveal reduced symptoms in TKO* mice compared to the pronounced skin inflammation observed in DKO* mice.

ciency ($\text{c-Jun}^{\Delta\text{ep}}$) to open eyes at birth as well as reduced tumor formation (13). The constitutive genetic deletion of both genes in the epidermis ($\text{JunB}^{\Delta\text{ep}}$ $\text{c-Jun}^{\Delta\text{ep}}$) causes death around birth due to massive TNF α shedding, skin inflammation and cachexia (14). Interestingly, the inducible epidermal deletion of JunB and c-Jun in adult mice ($\text{JunB}^{\Delta\text{ep}*}$ $\text{c-Jun}^{\Delta\text{ep}*}$) provokes a skin inflammatory disease highly reminiscent of human psoriasis (1). Eight week old mice carrying JunB and c-Jun floxed alleles as well as the keratin5-Cre^{ERT} transgene and their littermate controls were injected with tamoxifen for five consecutive days to induce JunB and c-Jun gene deletion (Fig. 2A and (1)). Around two weeks after the last injection, mice showed pronounced skin scaling, increased epidermal thickness, retention of nuclei in the cornified layer as well as rete ridges reaching into the dermis and augmented dermal angiogenesis with enlarged, permeable blood vessels (Fig. 2A, (1) and (15)). These histological changes appeared in accordance with a pronounced infiltration of innate immune cells into the dermis, presence of epidermal T lymphocytes and neutrophils and joint inflammation (1). The observed phenotype of $\text{JunB}^{\Delta\text{ep}*}$ $\text{c-Jun}^{\Delta\text{ep}*}$ (DKO*) mice is highly similar to psoriasis (1), making it a very valid model to study the pathogenesis of psoriasis.

On a molecular level, S100A8 and S100A9 protein expression is strongly elevated in the skin of the Jun/AP-1 psoriasis mouse model, even preceding disease symptoms (Fig. 2A and (1)). S100A8 and S100A9 are members of the S100 calcium-binding protein family, contain two EF-hand motifs and form a helix-loop-helix structure (16). They

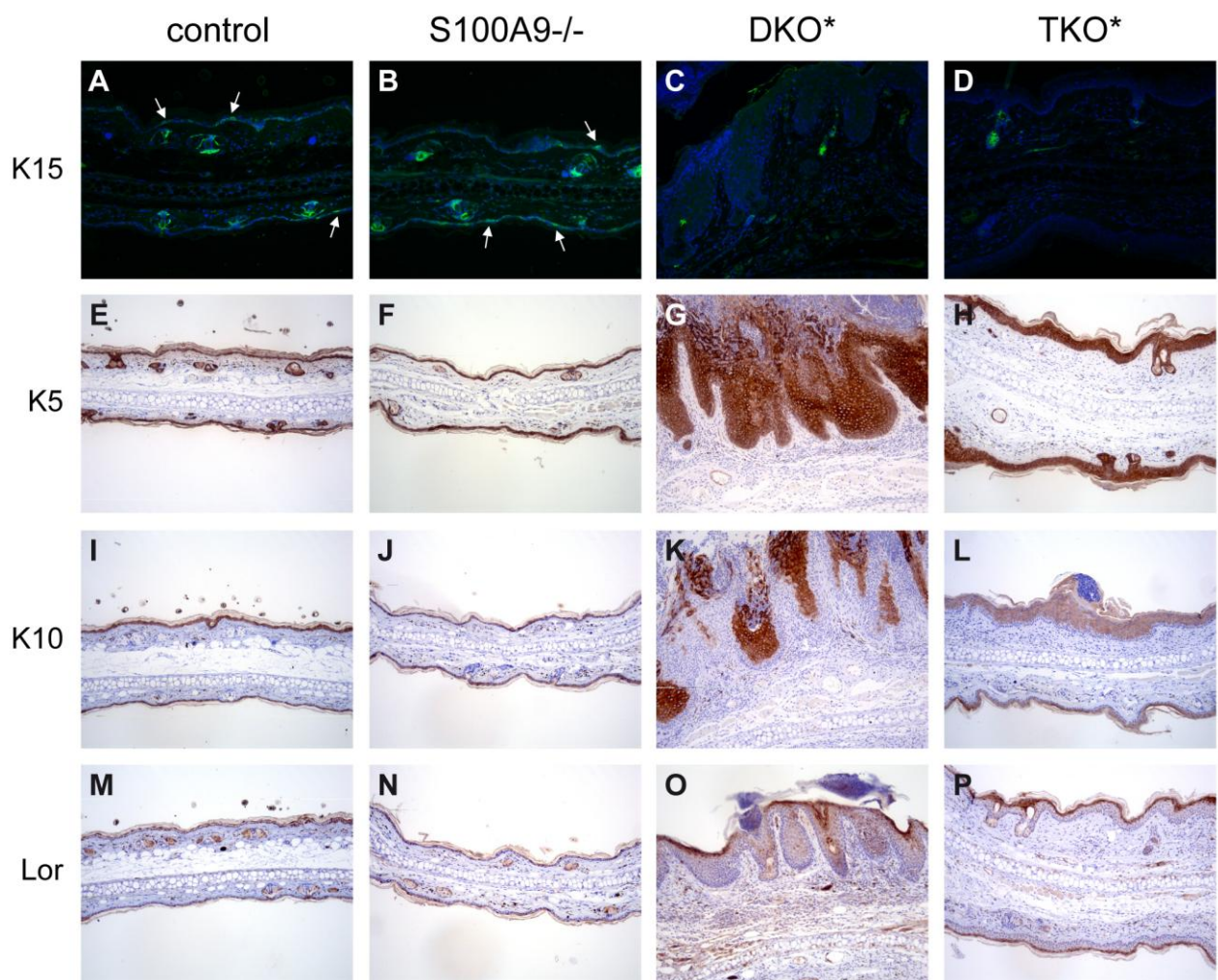
seem to act as both, homo- and heterodimers in mice, while the human S100A8 and S100A9 proteins rather exist as heterodimers (known as Calprotectin) or oligomers (17). S100A8/A9 are remarkably abundant in myeloid cells and constitute 45% of solid proteins in neutrophils and 1-2% in monocytes (18). They are also produced by other cell types, including activated macrophages (19) as well as fibroblasts (20) and their expression is induced in epithelial cells, including keratinocytes, upon infection or injury ((21), (22) and (23)).

Murine and human S100A8 and S100A9 appear to have manifold intracellular and extracellular functions. S100A8/A9 display strong chemotactic activity, especially attracting neutrophils ((24), (25) and (26)), and have several anti-infective properties, e.g. they confer cellular resistance against adherence and invasion of microorganisms (27). S100A8/A9 have been described as damage-associated molecular patterns (DAMPs) secreted by phagocytes (28). They are implicated in migration of tumor cells as well as lung metastasis (29) and vasculature-derived S100A8/A9 influences leukocyte trafficking (30). Additionally, S100A8 seems to be crucial for embryonic development, since its genetic deletion in mice leads to lethality at embryonic day E9.5 (31). In contrast, mice lacking S100A9 are viable, fertile and show absence of S100A8 protein ((32) and (33)). S100A9 deficiency did neither affect chemically induced peritonitis in mice nor myeloid cell functions *in vitro*, although S100A9^{-/-} neutrophils are less dense and show a dose-dependent reduction of chemotactic responses ((32), (33) and (34)). However, fewer granulocytes infiltrate wounded skin of S100A9 null mice due to transendothelial migration defects leading to improved wound healing (35) and the effects of shock and sepsis induction is reduced in mice lacking S100A9, because S100A8/A9 complexes appear to promote endotoxine-mediated inflammation (28).

In humans, the S100A8 and S100A9 genes are located in the psoriasis susceptibility region PSORS4 (1p12) (36), their protein concentrations are profoundly elevated in psoriatic skin ((37) and (38)) and S100A8/A9 serum levels were associated with disease severity (39). Hence, S100A8 and S100A9 are considered to be potential mediators in skin inflammation and, in particular, psoriasis.

We investigated the function of S100A8 and S100A9 proteins in skin inflammation and psoriasis using a gain of function and loss of function approach. Overexpression of S100A8 and S100A9 proteins in the skin of transgenic mice using the switchable Tet-ON system (S100A8/A9-rosa-tetON and S100A8/A9-k5-tetON mice, H. Schönthaler

in collaboration with P. Angel and colleagues) did, however, not result in an inflammatory skin phenotype. In contrast, constitutive S100A9 gene deletion leads to tremendous improvement of psoriasis-like disease symptoms in the Jun/AP-1 psoriasis mouse model (H. Schönthaler). JunB^{Δep*} c-Jun^{Δep*} S100A9^{-/-} (TKO*) mice show less skin scaling, reduced epidermal thickening as well as a diminished inflammatory state of the skin in comparison to DKO* mice. S100A9 mutant mice (JunB^{ff} c-Jun^{ff} S100A9^{-/-}) in a steady state condition seem normal and histologically indistinguishable from littermate JunB^{ff} c-Jun^{ff} S100A9^{+/-} controls (Fig. 2B, H. Schönthaler).



qPCR analysis of tail epidermis:

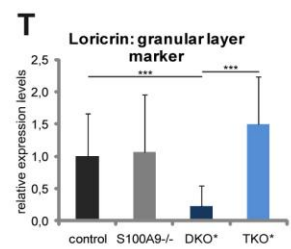
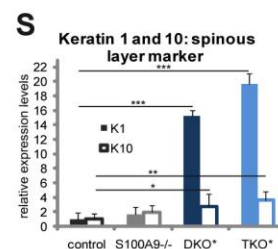
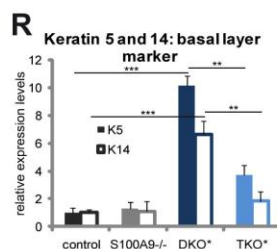
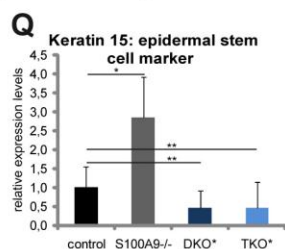


Fig. 3 – S100A9-deficiency causes overall normalization of skin architecture in the Jun/AP-1 psoriasis mouse model.

(A-D) Immunofluorescence for keratin 15 (K15, green channel) of ear sections of indicated mice. White arrows mark some K15-expressing cells in ears of control (A) and S100A9^{-/-} (B) mice and cell nuclei were stained in blue by DAPI. Magnification is 20x, n=3 for control, S100A9^{-/-} and DKO* mice and n=6 for TKO* mice.

(E-P) Representative images of immunohistochemistry for keratin 5 (K5, E-H), keratin 10 (K10, I-L) and Loricrin (Lor, M-P) of control (n=3), S100A9^{-/-} (n=3), DKO* (n=3) and TKO* (n=6) mice. Counterstaining of cell nuclei was performed with haematoxylin (blue) and magnification is 10x.

(Q-T) Quantitative PCR analysis of markers for keratinocyte differentiation of epidermis of mice with specified genotype (n=3, except for TKO* n=6). Data represent mean ± standard deviation and significant alterations between groups were calculated using a student t-test and indicated as follows: (*) P<0.05, (**) P<0.01 and (***) P<0.001. Graphs show mRNA levels of the epidermal stem cell marker K15 (Q), the basal layer-expressed K5 and keratin 14 (K14) (R), markers of the spinous epidermal layer keratin 1 (K1) and K10 (S) and the granular layer-restricted Lor (T).

Here, we determined the effect of S100A9 loss in the Jun/AP-1 psoriasis mouse model on keratinocyte differentiation, apoptosis as well as proliferation *in vivo* and *in vitro* and performed a histological and molecular analysis of the skin. We assessed cutaneous immune cell infiltration of DKO* and TKO* mice as well as systemic cytokine/chemokine levels in mouse sera and keratinocyte-conditioned medium to investigate the role of S100A9 proteins in skin inflammation and psoriasis.

Furthermore, we analyzed endogenous S100A8 and S100A9 protein levels in several tissues and isolated keratinocytes of S100A8/A9-rosa-tetON and S100A8/A9-k5-tetON mice. Unfortunately, no consistent upregulation of S100A8 and S100A9 proteins was found in steady-state condition and upon inflammatory stimulus by TPA application.

3. Results

3.1. Analysis of skin morphology, cell proliferation and apoptosis in the skin of the Jun/AP-1 psoriasis mouse model upon loss of S100A9

Pronounced cellular changes observed in human psoriatic skin are remarkably increased thickness of the epidermis, altered skin architecture, hyperkeratosis and retention of nuclei in the stratum corneum. The skin of the Jun/AP-1 psoriasis mouse model

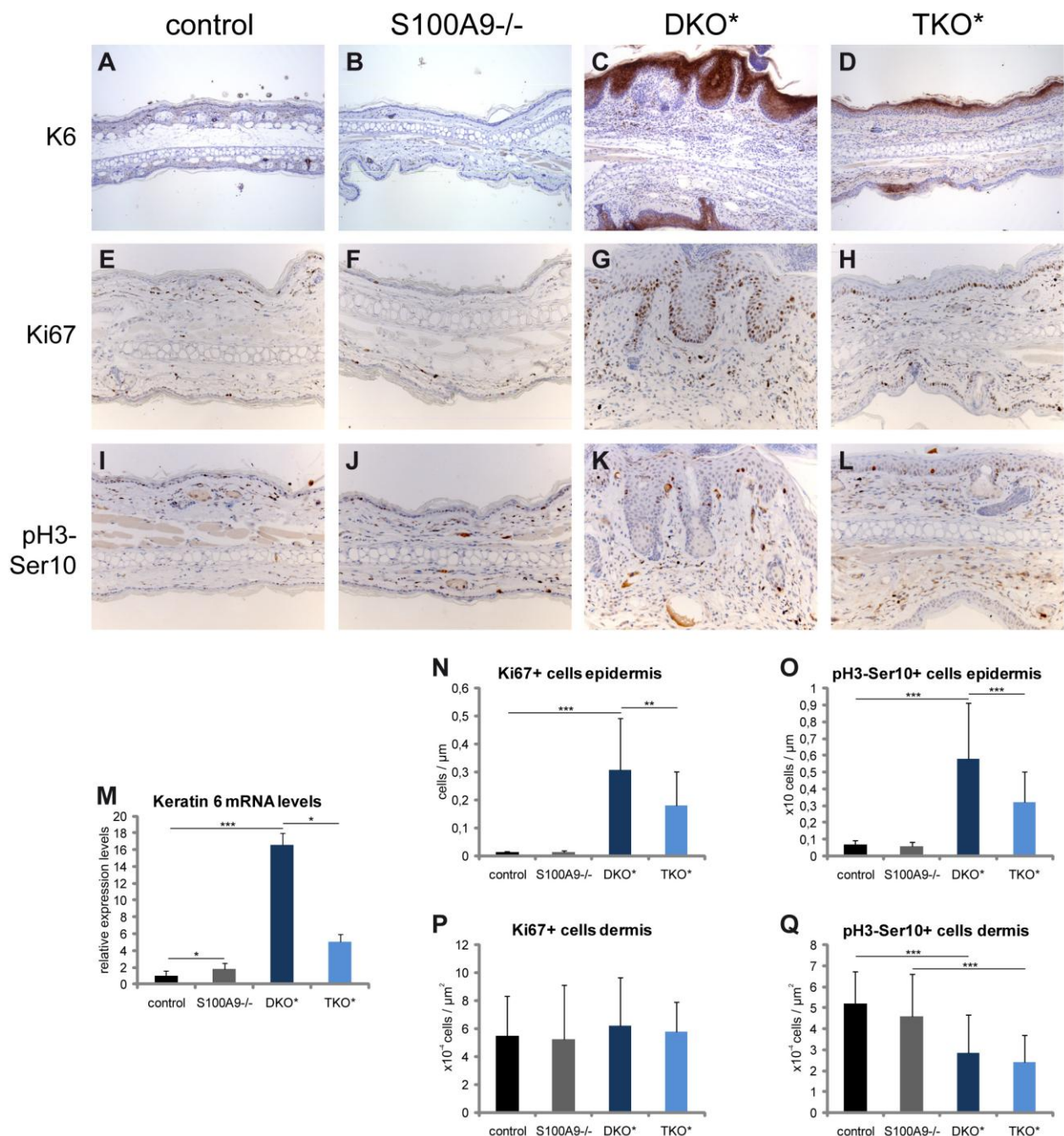


Fig. 4 – Hyperproliferation of psoriatic keratinocytes in DKO* is strongly reduced in TKO* mice.

(A-L) Mouse ear sections were immunohistochemically stained for the keratinocyte-proliferation marker keratin 6 (K6, A-D), the broad proliferation marker Ki67 (E-H) and phosphorylation of Histone 3 on Serine 10 (pH3-Ser10, I-L), a marker for mitotic cells. Nuclei of cells are labeled with haematoxylin in blue and representative 10x (A-D) and 20x magnified (E-L) images of control (n=3), S100A9^{-/-} (n=3), DKO* (n=3) and TKO* (n=6) are shown.

(M) Epidermal mRNA levels of K6 determined by quantitative PCR analysis.

(N-Q) Quantification of mouse ear immunohistochemistry displayed in (E-L). Graphs show numbers of Ki67 positive (N+P) and pH3-S10-labelled cells (O+Q) cells in the epidermis and the dermis.

(M-Q) N=3 per genotype, except for TKO* n=6. Data represent mean ± standard deviation and significant alterations between groups were calculated using a student t-test and indicated as follows: (*) P<0.05, (**) P<0.01 and (***) P<0.001.

(DKO* mice) displays an extremely similar phenotype, including rete ridges projecting into the dermis as well as scaling (Fig. 2A and (1)). These symptoms are mainly caused by abnormal differentiation and uncontrolled hyperproliferation of keratinocytes. Closer analysis of keratinocyte differentiation revealed strongly altered skin architecture in DKO* mice compared to controls (partially observed in (15)). Keratin 15 (K15) is an epidermal stem cell marker and usually expressed in the hair follicle stem cells of the bulge as well as in some keratinocytes of the basal layer ((40) and Fig. 3A). DKO* mice show a pronounced downregulation of K15 mRNA levels and strongly reduced number of K15 expressing cells, especially basal interfollicular keratinocytes, as described for psoriasis (41). No alteration in K15-positive cell number and K15 expression was observed in the Jun/AP-1 psoriasis mouse model upon loss of S100A9 (TKO* mice), although even slightly increased K15 mRNA levels were detected in S100A9^{-/-} versus control animals (Fig. 3A-D+Q). In contrast, markers of the basal epidermal layer, keratin 5 (K5) and 14 (K14), display significant elevation of mRNA levels in DKO* skin compared to controls and expression is spread throughout cell layers. Epidermal K5 mRNA levels as well as its expression pattern are normalized in TKO* mice (Fig. 3E-H+R). mRNA levels of the early differentiation markers keratin 1 (K1) and 10 (K10), which are expressed by spinous layer keratinocytes, are similarly increased in DKO* and TKO* mice versus controls (Fig. 3I-L+S). Remarkably, the granular keratinocyte layer expressing the late differentiation marker Loricrin (Lor) and Lor mRNA levels are not only severely reduced in DKO*, but rescued to control levels in TKO* mice (Fig. 3M-P+T). Thus, S100A9 loss in the Jun/AP-1 psoriasis mouse model leads to normalization of skin architecture and epidermal keratinocyte differentiation.

Next, we assessed the keratinocyte proliferation marker keratin 6 (K6) and found slightly increased mRNA levels in S100A9^{-/-} mice compared to controls, although K6 protein localization appeared unchanged and restricted to the hair follicle. In DKO* mice, K6 mRNA levels were strongly increased and K6 expressed in the interfollicular space throughout the epidermis (described in (15)). Interestingly, TKO* mice display a rather patchy pattern of K6 expression in the epidermis and overall greatly reduced mRNA levels compared to DKO* mice (Fig. 4A-D+M).

Subsequently, we tested Ki67 (antigen identified by monoclonal antibody Ki-67) for proliferation as well as presence of Serine 10-phosphorylation on Histone 3 (pH3-Ser10), an indication for mitosis, by IHC of mouse ears. No alteration was observed in the der-

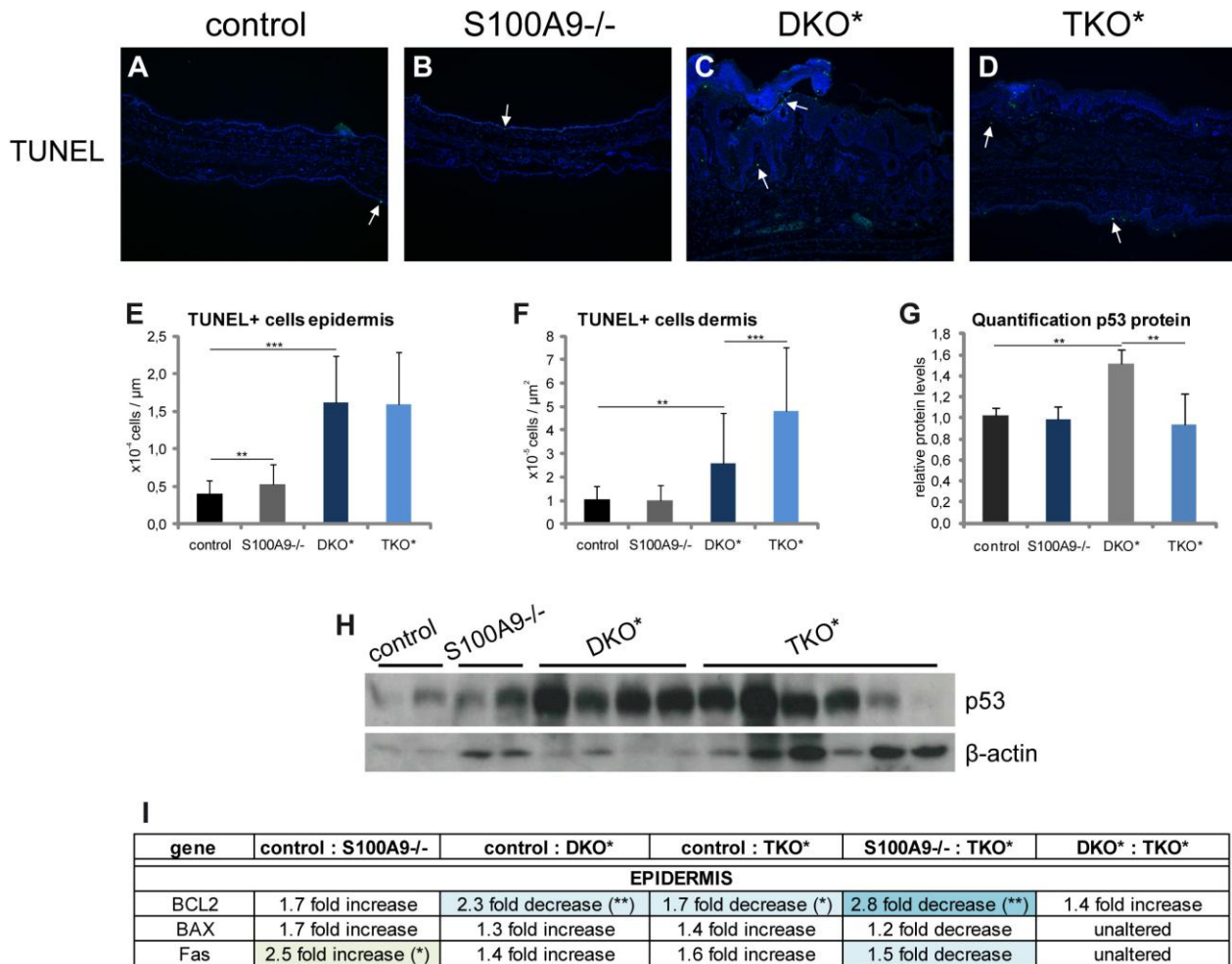


Fig. 5 – Evaluation of apoptosis and cell death in control, S100A9-/-, DKO* and TKO* mice.

(A-D) Fluorescence images of a TUNEL assay, which marked apoptotic cells in green, performed on mouse ear sections. DAPI was used to stain cell nuclei in blue, some TUNEL-positive cells are indicated by white arrows and magnification is 10x.

(E-F) Numbers of apoptotic cells in the epidermis (E) and the dermis (F) of control (n=3), S100A9-/- (n=3), DKO* (n=3) and TKO* (n=6) mice marked by TUNEL as shown in (A-D).

(G-H) Western blot analysis (H) and quantification relative to β -actin loading control (G) of p53 protein levels in epidermal extracts of mice with indicated genotype are shown (n=2 for control and S100A9-/-, n=4 for DKO* and n=7 for TKO* mice).

(E-G) Data represent mean $\pm$ standard deviation.

(I) Table displaying fold-changes of epidermal mRNA expression levels of BCL2, BAX and Fas as-sayed by quantitative PCR. N=3 for control, S100A9-/- and DKO* mice and n=6 for TKO* mice. More than 2-fold increase is indicated in light green, >5-fold increase in intermediate and >15-fold increase in dark green. A decrease is marked in blue, light blue shows a more than 1.5-fold decrease, intermediate blue a >2.5-fold and dark blue a >4-fold decrease.

(E-G+I) Significant alterations between groups were calculated using a student t-test and indicated by stars in brackets; (*) represent $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$.

mal compartment between all 4 tested genotypes for Ki67 positive cell numbers, but pH3-Ser10 positive cells were reduced in DKO* and TKO* mice (Fig. 4E-L+P-Q).

In the epidermis of DKO* mice, Ki67 and pH3-Ser10 positive cells were found significantly elevated and proliferative cells appeared not longer restricted to the basal keratinocyte layer in comparison to controls (partially shown in (15)). In contrast, epidermal Ki67 and pH3-Ser10 positive cell numbers were strongly decreased in TKO* versus DKO* mice, corresponding to the reduction of epidermal thickening (Fig. 4E-L+N-O).

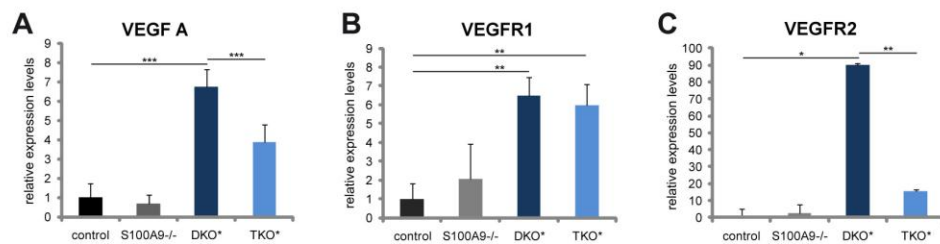
Interestingly, apoptosis assayed by quantification of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-labeled cells is enhanced in the skin of DKO* and TKO* mice compared to control and S100A9^{-/-} mice (Fig. 5A-F). An increase of dermal TUNEL-positive cells in TKO* mice versus the Jun/AP-1 psoriasis mouse model is detected. Unfortunately, quantitative PCR (qPCR) analysis of the cell death suppressor BCL2 (B cell CLL/lymphoma 2) and the apoptosis-inducers BAX (BCL2-associated X protein) and Fas (TNF receptor superfamily, member 6) gene transcription in dermal extracts failed to produce evaluable values. Consistent with the results obtained by the TUNEL assay and previous results in human psoriatic skin (42), epidermal mRNA expression of BCL2 is reduced in DKO* and TKO* mice versus controls, while mRNA levels of BAX and Fas show no alteration (Fig. 5I). Moreover, we tested protein expression of the oncogene and apoptosis promoter p53, which has been shown to be overrepresented in human psoriatic plaques (42) and to control S100A9 expression in oesophageal squamous cell carcinoma (43). Accordingly, we saw overexpression of p53 protein in epidermal extracts of DKO* mice compared to controls and a normalization in TKO* mice (Fig. 5G-H).

In summary, DKO* mice display epidermal hyperproliferation and strongly altered differentiation of keratinocytes as well as increased apoptosis in the skin. Upon loss of S100A9 in the Jun/AP-1 psoriasis mouse model (TKO* mice), the number of apoptotic cells in the dermis is augmented, whereas keratinocyte proliferation and skin architecture are strongly normalized. S100A9 deficiency itself does not affect keratinocyte proliferation or apoptosis comparing S100A9^{-/-} and control mice. Thus, S100A9 is involved in the observed, uncontrolled keratinocyte proliferation and differentiation in psoriasis-like skin disease and its absence in the inflammatory setting lead to reduced symptoms in a mouse model.

3.2. Examination of the blood and lymph vessel network and angiogenic marker expression in skin of DKO* and TKO* mice

An enlarged subepidermal vascular network and dilated blood vessels together with an influx of various types of immune cells in the surrounding tissue are prominent character-

qPCR analysis of tail epidermis:



D

gene	control : S100A9-/-	control : DKO*	control : TKO*	S100A9-/- : TKO*	DKO* : TKO*
EPIDERMIS					
VEGF A	1.5 fold decrease	6.7 fold increase (***)	3.9 fold increase (***)	5.9 fold increase (***)	1.7 fold decrease
VEGFR1	2.1 fold increase	6.5 fold increase (**)	6.0 fold increase (**)	2.9 fold increase	unaltered
VEGFR2	2.7 fold increase	90.2 fold increase (*)	15.3 fold increase (*)	5.7 fold increase	5.9 fold decrease (**)
NRP1	1.4 fold increase	14.5 fold decrease (***)	3.1 fold decrease (**)	4.4 fold decrease (*)	4.7 fold increase (**)
NRP2	2.9 fold increase (**)	1.8 fold decrease	unaltered	3.1 fold decrease (*)	1.7 fold increase
VE-Cadherin	4.1 fold increase (**)	2.4 fold increase (*)	unaltered	3.7 fold decrease (**)	2.1 fold decrease (*)
TGF- α	1.6 fold increase	2.3 fold increase (**)	1.8 fold increase (*)	unaltered	1.3 fold decrease
Tie2	only extremely low amounts detected				
Angiopoetin1	only extremely low amounts detected				
DERMIS					
VEGF A	3.1 fold increase	3.5 fold increase	5.2 fold increase (*)	1.7 fold increase	1.5 fold increase
VEGFR1	1.5 fold increase	3.3 fold increase	1.5 fold increase	unaltered	2.2 fold decrease
VEGFR2	only extremely low amounts detected				
NRP1	1.7 fold increase	unaltered	1.2 fold decrease	2.1 fold decrease (*)	unaltered
NRP2	unaltered	1.5 fold decrease	2.2 fold decrease (*)	2.3 fold decrease (*)	1.4 fold decrease
VE-Cadherin	1.7 fold increase	1.3 fold increase	1.2 fold increase	1.3 fold decrease	unaltered
TGF- α	2.0 fold increase (*)	unaltered	2.7 fold increase (**)	1.4 fold increase	2.7 fold increase (***)
Tie2	unaltered	3.1 fold increase (*)	1.7 fold increase	1.6 fold increase	1.8 fold decrease
Angiopoetin1	2.1 fold increase	3.2 fold increase (**)	unaltered	2.2 fold decrease	3.4 fold decrease (*)

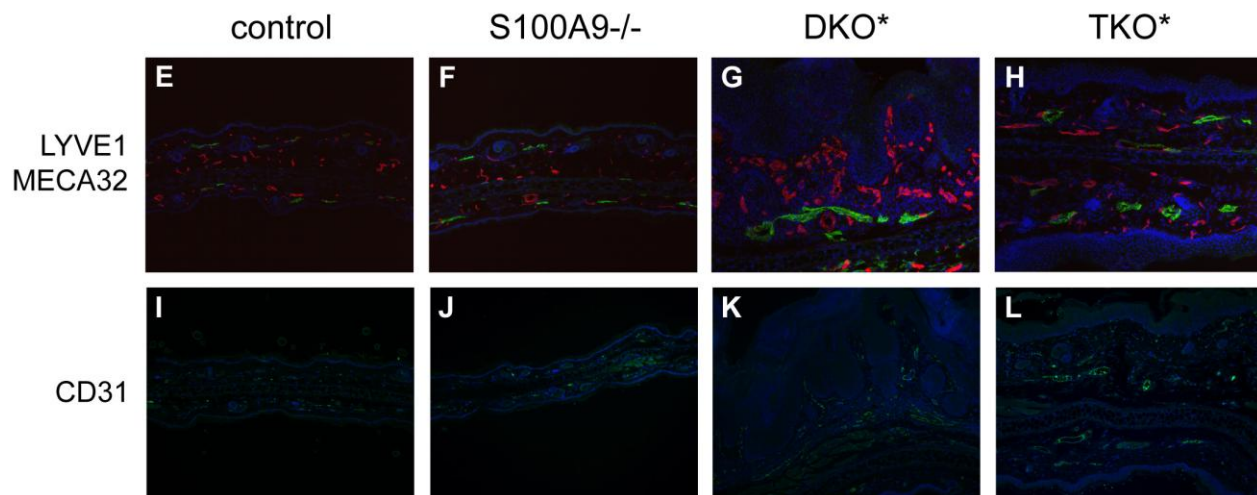


Fig. 6 – Analysis of (lymph-) angiogenesis as well as the vascular network in the Jun/AP-1 psoriasis mouse model and the effect of S100A9 loss.

(A-D) Epidermal and dermal mRNA expression of VEGF A (A), VEGFR1 (B) and VEGFR2 (C) as well as of other factors involved in angiogenesis (D) was measured using quantitative PCR of isolates from the epidermis of indicated mice (n=3 per group, except for TKO* n=6). Data represent mean $\pm$ standard deviation. Significant alterations between groups were calculated using a student t-test and indicated as follows: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$. A more detailed explanation on table interpretation (D) is given in Fig. 5I.

(E-L) Immunofluorescence stainings for LYVE1 (E-H, green channel) and MECA32 (E-H, red channel) as well as CD31 (I-L, green channel) were performed on mouse ear sections from control (n=3), S100A9^{-/-} (n=3), DKO* (n=3) and TKO* (n=6) mice. Cell nuclei are visualized in blue by DAPI, magnification is 10x.

ristics of chronic inflammation and have been observed in DKO* mice (1). Anti-inflammatory therapies as well as anti-angiogenic approaches are either widely used or promising future-therapies for psoriasis ((44), (15) and (45)). Thus, we assessed vascularization and angiogenesis in DKO* mice upon loss of S100A9 using immunofluorescence (IF) and qPCR of mouse ear and tail skin, respectively. A prominent increase of VEGF A (vascular endothelial growth factor A), VEGF receptor (VEGFR) 1 and VEGFR2 mRNA levels in the epidermis of DKO* compared to control mice (previously described in (15)) was reduced in TKO* epidermis (Fig. 6A-D). Then, we analyzed further genes involved in angiogenesis (46), like the VEGF co-receptors NRP1 (neuropilin1) and NRP2, VE (vascular endothelial)-Cadherin and TGF (tumor growth factor)- α . Moreover, expression of the almost exclusively endothelial cell-restricted receptor TIE2 (TEK tyrosine kinase, endothelial), which contributes to psoriasis development (47), as well as Ang-1 (angiopoietin-1), a TIE2-ligand implicated in angiogenic processes apart from that VEGF (48), were tested. By trend, our qPCR results show increased expression of pro-angiogenic factors in the epidermis and dermis of DKO* versus controls and TKO* mice (Fig. 6D). IF for the endothelial cell markers MECA32 (mouse panendothelial cell antigen 32) and CD31 (platelet/endothelial cell adhesion molecule) as well as for the lymphangiogenesis marker LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) of mouse ears was performed (Fig. 6E-L). The outcome also suggest elevated vascularization in the Jun/AP-1 psoriasis mouse model versus controls (previously described in (15)) and a decrease in TKO* mice.

3.3. The innate and adaptive immune cell infiltrate in the dermis and epidermis of TKO* mice determined at different time points

We performed a detailed analysis of changes of the immune cell infiltrate in the skin of TKO* compared to DKO* mice at the full blown disease state, two weeks after the last tamoxifen injection. In general, a dermal innate cell infiltrate mainly consisting of granulocytes, macrophages and mast cells as well as presence of T cells and neutrophils in the epidermis are characteristic for psoriasis and have been observed in the skin of DKO* mice ((1) and (15)). Staining of mouse ears confirmed presence of increased numbers of Gr.1-positive granulocytes, F4/80-positive macrophages and toluidine blue-labeled mast cells in the dermis of DKO* mice compared to controls. Notably, this cell types still infiltrated the dermis of TKO* mice, but to a lesser extend (Fig. 7A-D+I-P+W). Neutrophils were labeled by MPO (myeloperoxidase) using IF and readily infiltrate dermis and epidermis of both, DKO* and TKO* mice, but are significantly reduced in all skin compartments in TKO* versus DKO* mice (Fig. 7E-H+U-V). Further, we assayed presence and activation of epidermal Langerhans cells and dermal dendritic cells in the ear skin, which express Langerin (Lang) and upon activation also MHCII (major histocompatibility complex II). The skin of DKO* mice shows significantly increased number of resting $\text{Lang}^+ \text{MHCII}^-$ and activated $\text{Lang}^+ \text{MHCII}^+$ cells as well as $\text{Lang}^- \text{MHCII}^+$ cells, which represent other antigen presenting (APC) cells like dendritic cell subtypes, macrophages and B cells in the dermis and activated keratinocytes in the epidermis. Despite having similar epidermal $\text{Lang}^+ \text{MHCII}^-$ presence, numbers of activated $\text{Lang}^+ \text{MHCII}^+$ cells and $\text{Lang}^- \text{MHCII}^+$ cells is reduced in the epidermis and dermis of TKO* compared to DKO* mice. No alterations between control and S100A9^{-/-} mice have been observed (Fig. 7Q-T+X-Y). Thus, TKO* mice show a significant decrease of APCs accompanied by diminished Langerhans and dermal dendritic cell numbers.

Concerning the infiltration of adaptive immune cells into the skin, we focused on T cells, because they are important mediators in psoriasis and only a minor role has been described for B cells in the human disease and the Jun/AP-1 psoriasis mouse model ((49), (1) and (15)). IHC and IF showed increased numbers of CD3^+ (general T cell marker), CD4^+ (T helper cell marker) and CD8^+ (cytotoxic T cell marker) T cells in the dermis of DKO* versus control mice and infiltration of those in the epidermis. S100A9-deficiency in healthy skin (S100A9^{-/-} mice) did not lead to an alteration of T cell numbers versus

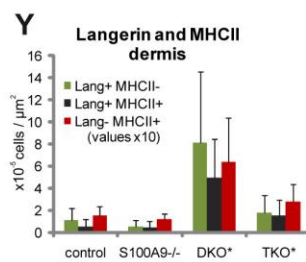
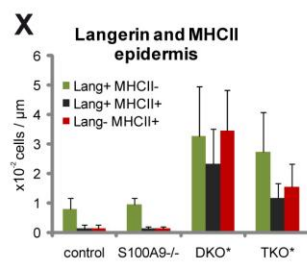
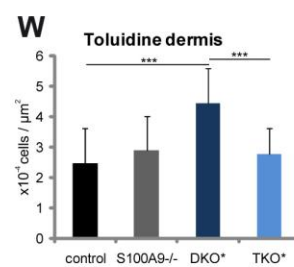
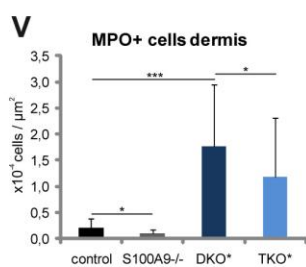
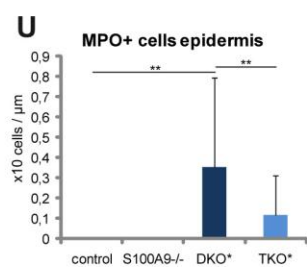
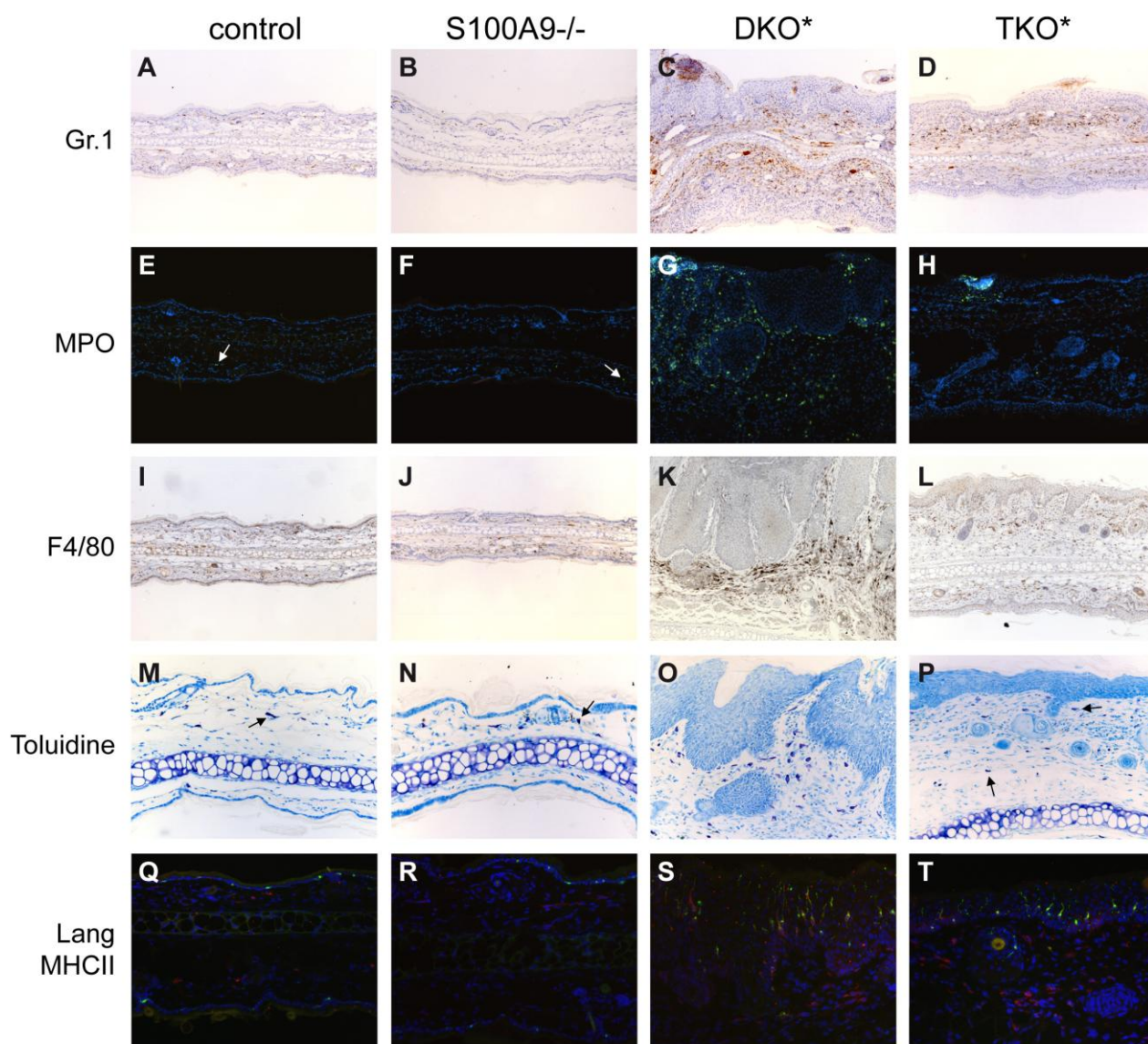


Fig. 7 – The innate immune cell infiltrate observed in the skin of DKO* mice is largely diminished in TKO* mice.

(A-T) Immunohistochemistry on ear sections of control, S100A9^{-/-}, DKO* and TKO* mice was performed for Gr.1(A-D) and F4/80 (I-L), immunofluorescence for MPO (E-H, green channel), Lang (Q-T, green channel) and MHCII (Q-T, red channel) as well as a Toluidine staining (M-P). Some labeled cells are marked by arrows (E-F, M-N+P) for better identification and nuclei are either visualized by haematoxylin (A-D and I-L) or DAPI staining (E-H and Q-T). Magnification is 10x for (A-L) and 20x for (M-T).

(U-Y) Quantification of labeled cells in the dermis and/or epidermis of stainings shown in (A-T) of control (n=3), S100A9^{-/-} (n=3), DKO* (n=3) and TKO* (n=6) mouse ear sections are displayed. Data represent mean $\pm$ standard deviation and significant alterations between groups were calculated using a student t-test and indicated as follows: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$. Significant P-values < 0.05 for quantification of Lang⁺ MHCII⁻ cells in the epidermis: $P = (***)$ for control vs. DKO*, control vs. TKO* and S100A9^{-/-} vs. TKO*; Lang⁺ MHCII⁻ cells in the dermis: $P = (***)$ for control vs. DKO*, S100A9^{-/-} vs. TKO* and DKO* vs. TKO* and $P = (*)$ for control vs. TKO*. Significant P-values < 0.05 for quantification of Lang⁺ MHCII⁺ cells in the epidermis: $P = (***)$ for control vs. DKO*, control vs. TKO*, S100A9^{-/-} vs. TKO* and DKO* vs. TKO*; Lang⁺ MHCII⁺ cells in the dermis: $P = (***)$ for control vs. DKO*, control vs. TKO* and S100A9^{-/-} vs. TKO* and $P = (*)$ for DKO* vs. TKO*. Significant P-values < 0.05 for quantification of Lang⁻ MHCII⁺ cells in the epidermis: $P = (***)$ for control vs. DKO*, control vs. TKO*, S100A9^{-/-} vs. TKO* and DKO* vs. TKO*; Lang⁻ MHCII⁺ cells in the dermis: $P = (***)$ control vs. DKO* control vs. TKO*, S100A9^{-/-} vs. TKO* and DKO* vs. TKO*.

controls, however TKO* mice have notably reduced CD3⁺, CD4⁺ and CD8⁺ T cell infiltrates in epidermis and dermis of the ear compared to DKO* mice (Fig. 8A-P).

Apart from analyzing the cutaneous immune cell presence at the full blown inflammatory state, we were interested in determining immune cell infiltration during disease development, five days after the last tamoxifen injection (Fig. 9A). Contrary to the fully developed disease situation, no macroscopical disease symptoms appeared at this early stage and the skin integrity was similar between control, S100A9^{-/-}, DKO* and TKO* mice (data not shown). We found the total amount of CD3⁺ T cells as well as relative distribution of CD3⁺ CD4⁺ T helper cells and CD3⁺ CD8⁺ cytotoxic T cells unaltered among skin isolates from mice of the four tested genotypes (Fig. 9B-E+N-O). As expected, also B220⁺ B cells numbers remained indistinguishable (Fig. 9B-E). However, an activation of immune cells/keratinocytes in DKO* and TKO* mice could already be observed, as total amount of MHCII⁺ cells in ear and tail skin cell isolates were increased compared to controls (Fig. 9J-M+P). Remarkably, CD11b⁺ Gr.1⁺ granulocyte

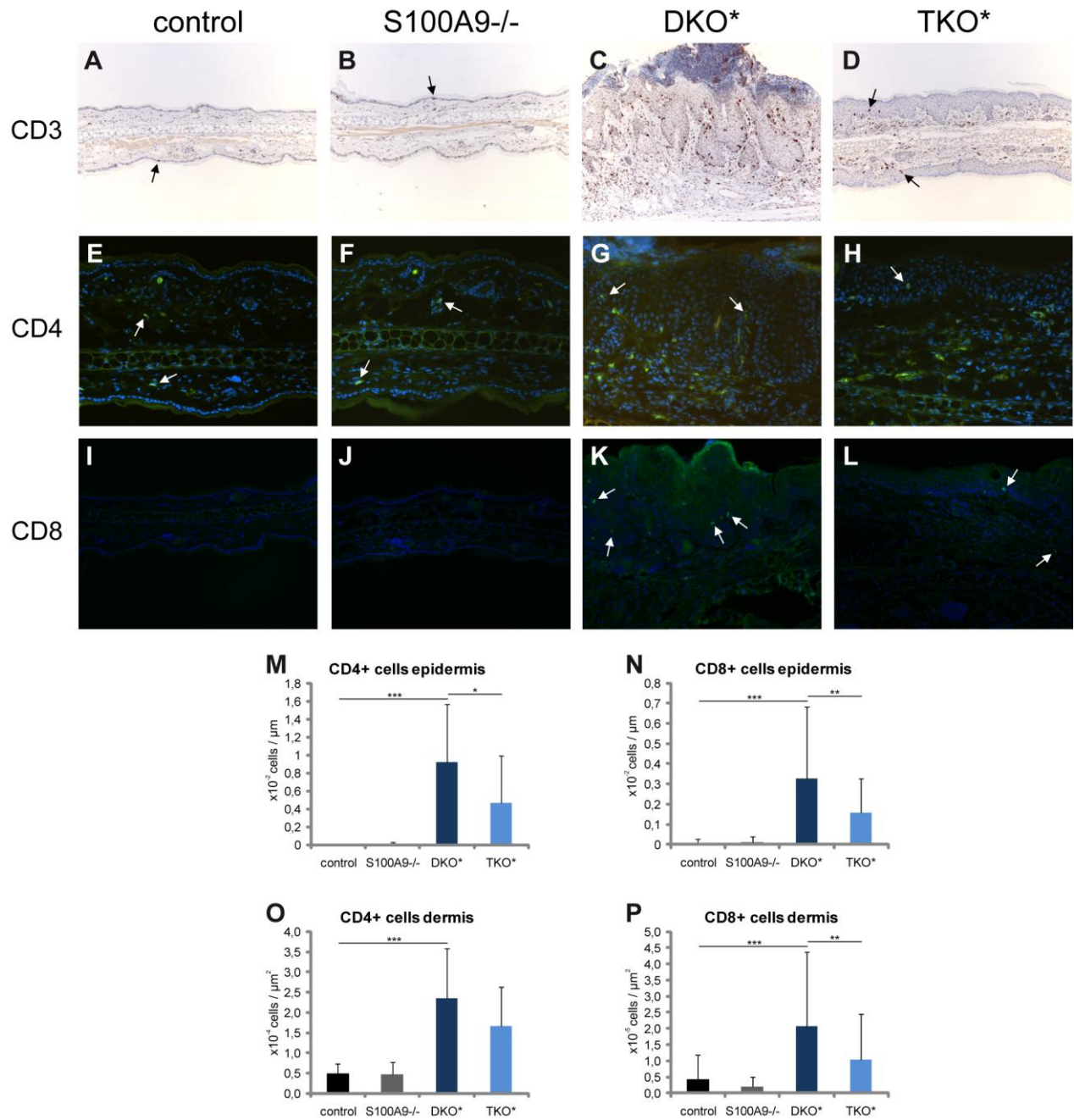


Fig. 8 – T cells are present in the epidermis of TKO* mice, but their numbers are decreased compared to DKO* mice.

(A-L) Ear sections of mice of all four genotypes used in this study (n=3, except for TKO* n=6) were immunohistochemically stained for CD3 (A-D) as well as for CD4 (E-H, green channel) and CD8 (I-L, green channel) using immunofluorescence. Haematoxylin (A-D) or DAPI (E-L) marked cell nuclei in blue and some positively stained cells are indicated by arrows.

(M-R) Graphs display quantified numbers of labeled cells in the epidermis and dermis of control, S100A9-/-, DKO* and TKO* mice from stainings depicted in (E-L). Data represent mean $\pm$ standard deviation and significant alterations between groups were calculated using a student t-test and indicated as follows: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$.

numbers were significantly elevated in DKO* and TKO* mice versus controls, even at this early time point (Fig. 9F-I+Q). Alterations in presence of other CD11b⁺ myeloid cell types, like dendritic cells and macrophages, as well as of activated CD11c⁺ MHCII⁺ dendritic cells were not detected between control, S100A9^{-/-}, TKO* and DKO* mice (Fig. 9F-M+R-S). Hence, CD11b⁺ Gr.1⁺ granulocytes appear to be the first immune cell type to infiltrate the skin during psoriasis development in DKO* mice. This goes along with neutrophils, the most abundant type of granulocytes next to eosinophils and basophils, being in general the earliest detectable leukocytes to appear in an infected area during an immune response. Increased CD11b⁺ Gr.1⁺ cell presence in total ear and tail skin isolates at early time points was similar between DKO* and TKO* mice, although a decreased dose-dependent responsiveness of isolated S100A9^{-/-} neutrophils upon chemoattractant stimuli has been described *in vitro* ((32) and (33)) and S100A8 and A9 proteins are upregulated in the epidermis even 24 hours after three consecutive tamoxifen injections (1). Thus, S100A9 is likely to be dispensable for early leukocyte recruitment to the skin and inflammation initiation, but rather involved in later disease progression and maintenance.

Taken together, we could show a strongly decreased innate and adaptive immune cell infiltration in the skin of TKO* versus DKO* mice at the full blown disease state, while S100A9^{-/-} mice showed no changes compared to controls. Thus, as far as analyzed here, S100A9 deficiency does not affect a specific immune cell type, e.g. neutrophils or macrophages which contain large cytosolic amounts of S100A9. Rather, S100A9 plays a role in the amplification of the inflammation in the skin of DKO* mice.

3.4. Comparison of cytokine/chemokine levels in sera of control, S100A9^{-/-}, DKO* and TKO* mice

Determination of the role of S100A9 on the systemic inflammatory state is crucial, since the local immune cell infiltration in the skin of TKO* mice appears strongly reduced versus DKO* mice. Hence, we examined serum cytokine and chemokine levels by a cytokine array analysis and ELISA (Enzyme-Linked Immunosorbent Assay) and were able to reliably detect 31 of 50 tested factors. Serum levels of 11 cytokines/chemokines

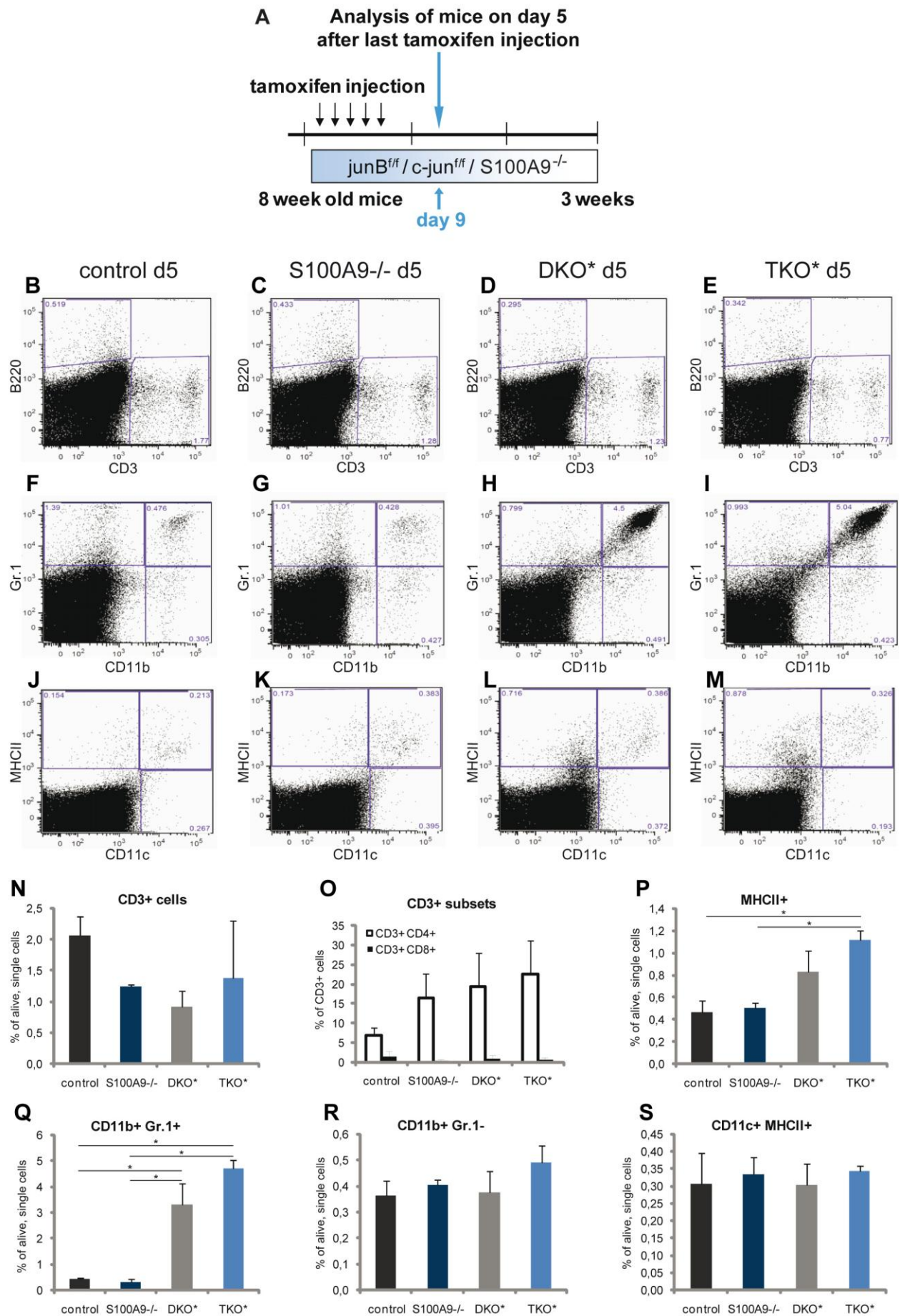


Fig. 9 – Gr.1-positive cells appear to be the first immune cell type to infiltrate the skin of the Jun/AP-1 psoriasis mouse model and TKO* mice during disease development.

(A) Overview of mouse treatment. JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{+/-}, JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{-/-}, JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{+/-} K5-CRE^{ERT} and JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{-/-} K5-CRE^{ERT} eight week-old mice were intraperitoneally injected for 5 consecutive days with 1mg tamoxifen per mouse to induce genomic deletion of JunB and c-Jun. Control d5, S100A9^{-/-} d5, DKO* d5 and TKO* d5 mice were sacrificed and analyzed five days after the last injection.

(B-M) Flow cytometric analysis of cells isolated from total ear and tail skin of indicated experimental mice (n=2-3 per group). Representative dot plots display distribution of alive single cells stained for B220 versus CD3 (B-E); Gr.1 versus CD11b (F-I) and MHCII versus CD11c (J-M). Prior to FACS analysis, isolated cells were stained with DAPI to determine viability.

(N-S) Quantification of FACS data (inter alia) obtained from dot blots displayed in (B-M) of cell isolated from total ear and tail skin from control d5, S100A9^{-/-} d5, DKO* d5 and TKO* d5 mice (n=2-3 per group). Graphs display percentage of (N) CD3 single positive (CD3+), (O) CD3 CD4 double positive (CD3+ CD4+), CD3 CD8 double positive (CD3+ CD8+), (P) MHCII single positive, (Q) CD11b Gr.1 double positive (CD11b+ Gr.1+), (R) CD11b positive Gr.1 negative (CD11b+ Gr.1-) and (S) CD11c MHCII double positive (CD11c+ MHCII+) cells of total isolates. Shown populations were gated on alive single cells (N+P-S) or alive single CD3+ cells (O) and cells were stained with DAPI to determine viability prior to flow cytometric analysis. Data represent mean ± standard deviation and significant alterations between groups were calculated using a student t-test and indicated as follows: (*) P<0.05, (**) P<0.01 and (***) P<0.001.

(IL (interleukin)-5, IL-13, IL-28b, MCP (monocyte chemotactic protein)-5, IP-10 (interferon gamma-induced protein 10)), MIP (macrophage inflammatory protein)-1β, MIP-3α, KC (keratinocyte-derived chemokine), TIMP1 (tissue inhibitor of metalloproteases 1), fractalkine and EPO (erythropoietin)) were unaltered comparing control, S100A9^{-/-}, DKO* and TKO* mice (Fig. 10A). The pro-inflammatory factors IL-7, IL-21, IL-22, IL-25, GM-CSF (granulocyte-macrophage colony stimulating factor), M (macrophage)-CSF, MIP-1α, MIP-3β and MDC (macrophage-derived chemokine) were elevated in the sera of DKO* mice and to an equal extent in TKO* compared to controls (Fig. 10A, F+I). These cytokines/chemokines appear to be unaffected by S100A9 loss in the Jun/AP-1 psoriasis mouse model on a systemic level and, hence, are presumably not involved in the S100A9-dependent reduction of inflammatory symptoms in TKO* mice. Eleven inflammation-mediating factors, which represent one third of total determined factors in mouse sera, were strongly augmented in DKO* sera and significantly reduced to almost control levels in the sera of TKO* mice. They include IL-1α, IL-6, IL-12p40, IL-17A and IL-27, G (granulocyte)-CSF, MCP-1, MIG (monocyte-induced by gamma-interferon),

RANTES (regulated upon activation, normally T-expressed and presumably secreted), VEGF and eotaxin (Fig. 10A-E+G-H). All of these cytokines and chemokines were reported to be involved in psoriasis pathogenesis and/or to be upregulated in psoriatic skin (IL-1 α (50), IL-6 ((51) and (52)), IL-12p40, IL-17A (both (53)), IL-27 (54), G-CSF (55), MCP-1 (56), MIG (57), RANTES (58), VEGF ((46) and (15)) and Eotaxin (1)).

A Cytokine array and ELISA analysis:

mouse sera					
cytokine / chemokine	control : S100A9-/-	control : DKO*	control : TKO*	S100A9-/- : TKO*	DKO* : TKO*
IL-5	unaltered	1.3 fold decrease	1.4 fold decrease	1.6 fold decrease (*)	unaltered
IL-13	unaltered	1.6 fold increase	1.4 fold increase	1.3 fold increase	unaltered
IL-28b	unaltered	1.4 fold increase	2.0 fold increase (*)	2.3 fold increase (**)	1.5 fold increase (*)
MCP-5	1.6 fold decrease (*)	unaltered	unaltered	1.7 fold increase (***)	unaltered
IP-10	unaltered	1.3 fold increase	unaltered	unaltered	unaltered
MIP-1 β	1.5 fold increase	1.7 fold increase	1.8 fold increase (*)	1.3 fold increase	unaltered
MIP-3 α	1.3 fold decrease	1.3 fold increase	1.3 fold increase (*)	1.8 fold increase (***)	unaltered
KC	unaltered	1.5 fold increase	2.0 fold increase	1.8 fold increase (*)	1.3 fold increase
TIMP1	1.5 fold decrease	1.2 fold decrease	1.3 fold decrease	unaltered	unaltered
fractalkine	unaltered	unaltered	unaltered	1.3 fold decrease	unaltered
EPO	unaltered	unaltered	unaltered	unaltered	unaltered
IL-7	1.2 fold increase	2.2 fold increase (*)	1.9 fold increase (*)	1.6 fold increase (*)	unaltered
IL-21	unaltered	1.9 fold increase (**)	1.5 fold increase	1.5 fold increase	1.3 fold decrease
IL-22	unaltered	2.4 fold increase (*)	2.6 fold increase (**)	2.5 fold increase (**)	unaltered
IL-25	1.3 fold increase	1.8 fold increase (**)	1.9 fold increase (*)	1.5 fold increase	unaltered
GM-CSF	unaltered	2.5 fold increase (*)	2.9 fold increase (**)	2.4 fold increase (**)	unaltered
M-CSF	unaltered	2.8 fold increase (***)	2.3 fold increase	2.4 fold increase (*)	unaltered
MIP-1 α	1.7 fold increase (*)	2.3 fold increase (***)	2.8 fold increase (**)	1.6 fold increase (*)	1.2 fold increase
MIP-3 β	1.7 fold decrease	1.8 fold increase (*)	1.5 fold increase (*)	2.6 fold increase (**)	unaltered
MDC	unaltered	2.4 fold increase (*)	2.6 fold increase	2.9 fold increase (*)	unaltered
eotaxin	1.2 fold decrease	1.3 fold increase	unaltered	unaltered	1.5 fold decrease (**)
IL-1 α	unaltered	3.8 fold increase (*)	unaltered	unaltered	3.8 fold decrease (**)
IL-6	1.8 fold increase	5.4 fold increase (**)	3.5 fold increase (**)	1.9 fold increase (*)	1.6 fold decrease (**)
IL-12p40	1.8 fold increase (**)	2.7 fold increase (**)	1.9 fold increase (*)	unaltered	1.4 fold decrease (*)
IL-17 A	1.4 fold increase	23.6 fold increase (*)	11.5 fold increase (*)	8.0 fold increase (*)	2.0 fold decrease (*)
IL-27	unaltered	2.3 fold increase (*)	1.3 fold increase	1.4 fold increase	1.8 fold decrease (*)
G-CSF	5.1 fold decrease (**)	6.3 fold increase (*)	2.0 fold increase (*)	10.4 increase (***)	3.1 fold decrease (**)
MCP-1	1.6 fold increase	5.1 fold increase (**)	1.3 fold increase	1.2 fold decrease	4.0 fold decrease (***)
MIG	2.1 fold increase	2.6 fold increase (**)	1.6 fold increase	1.3 fold decrease	1.6 fold decrease (*)
RANTES	1.2 fold increase	3.7 fold increase (**)	1.8 fold increase	1.5 fold increase	2.0 fold decrease (***)
VEGF	unaltered	1.4 fold increase (**)	unaltered	unaltered	1.4 fold decrease (***)
> 1.8 fold increase		> 4 fold increase	> 1.5 fold decrease	> 2.5 fold decrease	

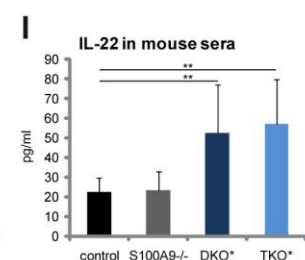
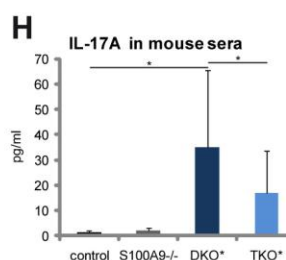
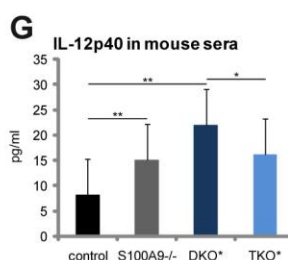
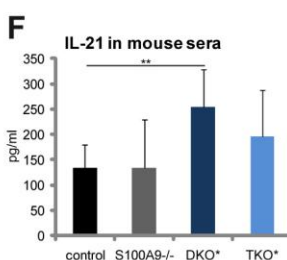
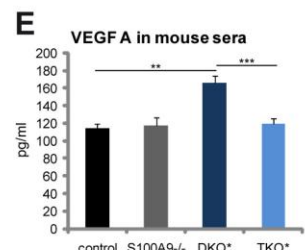
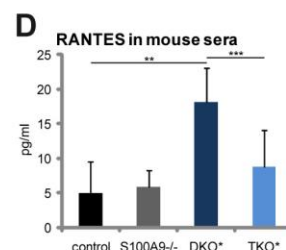
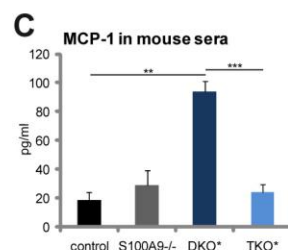
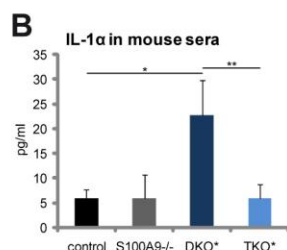


Fig. 10 – Systemic cytokine and chemokine levels in mouse serum are reduced in TKO* versus DKO* mice.

(A) Serum of experimental mice was taken two weeks after the last tamoxifen injection and cytokine array (Millipore) and ELISA analysis was performed to determine cytokine/chemokine levels. Table shows fold-change of detected amounts comparing mice of indicated genotype (control (n=3), S100A9^{-/-} (n=3-4), DKO* (n=5-6) and TKO* (n=6-8)). A more than 1.8-fold increase is indicated in light green and >4-fold increase in dark green. Light blue indicates a more than 1.5-fold decrease and dark blue a >2.5-fold decrease.

(B-I) Graphs show serum levels of the interesting candidates IL-1 α (B), MCP-1 (C), RANTES (D), VEGF A (E), IL-21 (F), IL-12p40 (G), IL-17A (H) and IL-22 (I) in control, S100A9^{-/-}, DKO* and TKO* mice. Data represent mean $\pm$ standard deviation.

(A-I) Significant alterations between groups were calculated using a student t-test and indicated as follows: (*) P<0.05, (**) P<0.01 and (***) P<0.001.

In conclusion, the amount of cytokines and chemokines in sera of S100A9^{-/-} and control mice appears largely similar. However, levels of numerous pro-inflammatory factors are remarkably elevated in sera of DKO* mice and more than half of them were significantly reduced in TKO* mice. This could explain diminished immune cell activation and attraction to skin of these mice (Fig. 7 and Fig. 8). Hence, not only the local immune response at affected areas, but the systemic diseased state of DKO* mice is attenuated upon loss of S100A9.

3.5. Molecular characterization of the skin of the Jun/AP-1 psoriasis mouse model upon loss of S100A9

3.5.1. S100 protein family members and S100A8/A9 receptors

In order to elucidate the role of S100A9 in the context of skin inflammation on a molecular level, we tested the effect of S100A9 deletion in DKO* mice for mRNA levels of factors known to be involved in psoriasis. First, we focused on other members of the S100 family and the receptors of S100A9 and tested their epidermal and dermal mRNA expression using qPCR as well as protein localization by IHC. Human S100A7, also described as psoriasin, belongs to the S100 family of calcium binding proteins and is known to be upregulated in psoriasis (59). Epidermal mRNA expression of S100A7A, the closest mouse homolog to human S100A7, is increased in DKO* mice, but strongly diminished in TKO* mice (Fig. 11A). S100A7A mRNA could not be detected in the der-

A qPCR analysis:

gene	control : S100A9-/-	control : DKO*	control : TKO*	S100A9-/- : TKO*	DKO* : TKO*
EPIDERMIS					
S100A1	2.1 fold increase (*)	1.2 fold increase	1.3 fold decrease	2.8 fold decrease (**)	1.7 fold decrease
S100A3	3.1 fold increase	49.5 fold decrease (***)	2.0 fold decrease	6.2 fold decrease (***)	24.8 fold increase (***)
S100A4	strong variation in results				
S100A7A	1.8 fold increase	5.7 fold increase (*)	1.5 fold decrease	2.8 fold decrease (**)	8.4 fold decrease (**)
S100A10	1.2 fold decrease	2.9 fold decrease (***)	2.3 fold decrease (***)	1.9 fold decrease (**)	1.2 fold increase (*)
S100A16	2.1 fold increase (**)	1.4 fold increase	2.0 fold increase (**)	unaltered	1.4 fold increase
RAGE	1.3 fold increase	unaltered	1.2 fold increase	unaltered	unaltered
Scarb1	1.3 fold increase	unaltered	2.0 fold decrease (**)	2.6 fold decrease (**)	2.1 fold decrease (**)
Scarb2	1.3 fold decrease	1.6 fold decrease (**)	1.8 fold decrease (**)	1.4 fold decrease (*)	unaltered
TLR4	2.3 fold increase (**)	36.4 fold increase (***)	9.0 fold increase (***)	3.8 fold increase (**)	4.1 fold decrease (***)
MyD88	unaltered	3.2 fold increase (**)	2.2 fold increase (**)	2.3 fold increase (**)	1.5 fold decrease
DERMIS					
S100A1	1.7 fold increase	unaltered	1.4 fold increase	1.2 fold decrease	1.3 fold increase
S100A3	1.8 fold increase	18.5 fold decrease (***)	unaltered	2.0 fold decrease	16.7 fold increase (***)
S100A4	1.9 fold decrease	2.2 fold increase (*)	2.1 fold decrease (*)	unaltered	4.7 fold decrease (**)
S100A7A	only extremely low amounts detected				
RAGE	5.3 fold increase (***)	1.2 fold increase	3.6 fold increase (*)	1.7 fold decrease	2.9 fold increase
Scarb1	1.3 fold increase	1.3 fold increase	1.4 fold increase	unaltered	unaltered
Scarb2	1.6 fold increase (*)	1.2 fold increase	unaltered	1.4 fold decrease (*)	unaltered
TLR4	3.0 fold increase (*)	5.0 fold increase (*)	2.5 fold increase (*)	unaltered	2.0 fold decrease

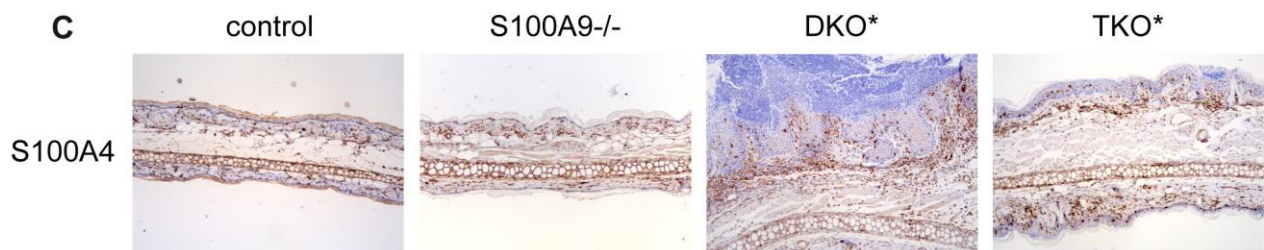
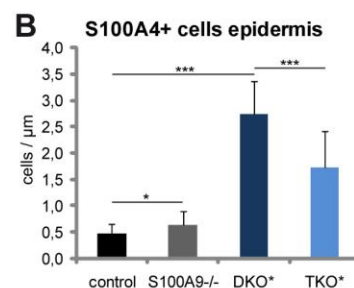


Fig. 11 – Expression levels of S100 family proteins and their receptors in the skin of the Jun/AP-1 psoriasis mouse model and TKO* mice.

(A) MRNA expression of members of the S100 protein family at their receptors. Table showing fold-changes of mRNA expression levels of genes in the epidermis and dermis comparing control, S100A9-/-, DKO* and TKO* mice (n=3-6), tested by quantitative PCR. A more detailed explanation on table interpretation is given in Fig. 5I.

(B) Quantification of epidermal cells staining positive for S100A4 in (C). Data represent mean $\pm$ standard deviation, n=3 for control, S100A9-/- and DKO* mice and n=6 for TKO* mice. Significant alterations between groups were calculated using a student t-test and indicated as follows: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$.

(C) Representative microscopic images of immunohistochemistry for S100A4 of mouse ear sections of indicated genotypes. Cell nuclei are stained in blue with haematoxylin, magnification 10x.

mis of these mice using qPCR (Fig. 11A). The strong metastasis promoter S100A4, mainly known as metastasin (60), has also been associated with psoriasis (61). In accordance with previous findings for human skin, S100A4 mRNA expression in the dermis was increased in the Jun/AP-1 psoriasis mouse model compared to controls and normalized in TKO* mice (Fig. 11A). Also IHC of mouse ears for S100A4 protein showed elevated numbers of labeled cells, which are most likely Langerhans cells, in the epidermis of DKO* mice and a significant reduction upon loss of S100A9 was observed (Fig. 11B-C).

Next, we tested S100A3, which appears to be involved in differentiation of epithelial cells in the hair follicle ((62) and (63)), by qPCR and found its mRNA levels highly reduced in all skin compartments of DKO* mice (Fig. 11A). S100A3 appears to be increased in S100A9^{-/-} mice, showing an antagonistic mRNA expression pattern of S100A3 to S100A8 and S100A9 (Fig. 11A and personal communication P. Angel, DKFZ Heidelberg). Independently, S100A10 and S100A16 proteins have been found to be differentially regulated in the epidermis of control, S100A9^{-/-}, DKO* and TKO* mice (H. Schönthaler, unpublished observation). S100A10 has largely been associated with inflammation (64) and may be involved in membrane remodeling during keratinocyte differentiation (38). Epidermal S100A10 mRNA levels were downregulated to the same extent in DKO* and TKO* mice (Fig. 11A). However, mRNA expression of S100A16, which appears elevated in various human tumors (65), was found to be increased in the epidermis of S100A9^{-/-} and TKO*, but not DKO* mice compared to controls (Fig. 11A). S100A1, which is not directly associated with psoriasis (66), showed no pronounced alteration in mRNA expression in the dermis and epidermis among control, S100A9^{-/-}, DKO* or TKO* mice in a qPCR analysis (Fig. 11A).

No changes in mRNA levels in the epidermis of mice of all four analyzed genotypes were obtained for the S100A8 and S100A9 receptors Scarb1 (Scavenger receptor class B member 1) and Scarb2, also known as CD36 (67), and RAGE (Receptor for Advanced Glycosylation End products), which is required for S100A8/A9 upregulation in an inflammatory setting (68) (Fig. 11A). In the dermis of S100A9-deficient mice, S100A9^{-/-} and TKO*, mRNA levels of RAGE were increased compared to controls, but unaffected in DKO* mice (Fig. 11A). Thus, the reduced inflammatory symptoms of S100A9-loss in the psoriasis mouse model could not be associated with expression changes of the S100A8/A9 receptors Scarb1, Scarb2 and RAGE.

A qPCR analysis:

gene	control : S100A9 ^{-/-}	control : DKO [*]	control : TKO [*]	S100A9 ^{-/-} : TKO [*]	DKO [*] : TKO [*]
EPIDERMIS					
TNF α	1.2 fold decrease	10.6 fold increase (***)	2.1 fold increase (*)	2.6 fold increase (**)	5.0 fold decrease (**)
TNFR1	unaltered	2.6 fold increase (*)	1.3 fold increase	1.6 fold increase	1.9 fold decrease (*)
TNFR2	1.6 fold increase	19.3 fold increase (***)	2.4 fold increase (*)	1.5 fold increase	7.9 fold decrease (**)
p38 α	unaltered	1.5 fold increase	1.5 fold increase	1.3 fold increase	unaltered
TACE	1.8 fold increase (*)	2.6 fold increase (**)	2.3 fold increase (**)	1.3 fold increase	unaltered
TIMP3	2.9 fold increase (*)	5.1 fold decrease (**)	3.6 fold decrease (*)	10.4 fold decrease (***)	1.4 fold increase
DERMIS					
TNF α	1.6 fold decrease	3.0 fold increase	unaltered	1.4 fold increase	3.4 fold decrease (*)
TNFR1	strong variation in results				
TNFR2	1.8 fold decrease (*)	2.1 fold increase (*)	1.2 fold decrease	1.4 fold increase	2.6 fold decrease (**)
TACE	unaltered	1.6 fold decrease	unaltered	unaltered	1.4 fold increase
TIMP3	1.4 fold decrease	3.8 fold decrease (**)	3.4 fold decrease (**)	2.3 fold decrease (**)	unaltered

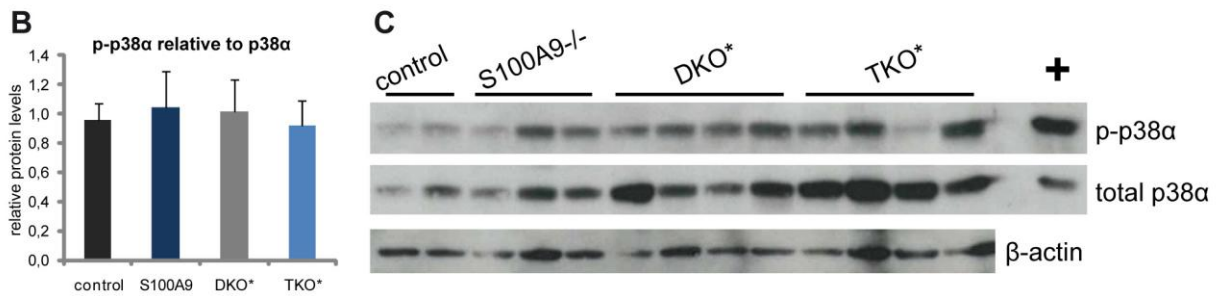


Fig. 12 – Analysis of deregulation of TNF α pathway components in the epidermis and dermis control, S100A9^{-/-}, DKO^{*} and TKO^{*} mice.

(A) mRNA expression of factors involved in TNF α -mediated signaling. Table showing fold-changes of mRNA expression levels of genes in the epidermis and dermis comparing mice of the four genotypes used in this study, tested by quantitative PCR. A more detailed explanation on table interpretation is given in Fig. 51.

(B) Quantification of Western blot analysis shown in (C) of p38 α protein levels in the epidermis of indicated mice. Graph displays the amount of phosphorylated p38 α (p-p38 α) relative to total p38 α . Data represent mean $\pm$ standard deviation, n=2-4 per genotype and P>0.05 among all values. No significant difference was observed comparing total p38 α levels in the epidermis of control, S100A9^{-/-}, DKO^{*} and TKO^{*} mice.

(C) Western blot analysis of p-p38 α and total p38 α levels in the epidermis of mice with indicated genotypes. Protein of JunB ^{Δ ep^{*}} epidermis, which has been described to have elevated p-p38 α levels ((12), provided by Ö. Uluçkan), served as positive control (+) and Ø represents no loaded protein. Loading control was β -actin.

In contrast, qPCR analysis of mRNA expression revealed strong elevation of the Toll-like receptor TLR4, which is also a receptor for S100A8/A9 (17), as well as its adaptor protein MyD88 (myeloid differentiation primary response gene 88) in the epidermis (and dermis) of DKO^{*} mice and normalization in TKO^{*} mice compared to con-

trols (Fig. 11A). This can be explained by upregulation of TLR4 by keratinocytes (69) and the fact that TLR4 is present on the cell surface of many types of immune and inflammation-associated cells, e.g. on neutrophils, which infiltrate the skin of DKO* mice.

3.5.2. *TNF pathway associated genes*

Main mediators of inflammation and psoriasis, such as tumor necrosis factor (TNF) α and its receptors TNFR1 and TNFR2 ((70) and (14)), are notably downregulated in the epidermis and dermis of TKO* compared to DKO* mice on mRNA level, but unaffected upon S100A9 deficiency in normal skin (Fig. 12A). Induced TNF α production in human keratinocytes has been described to be p38 α -dependent (71), however mRNA and p38 α protein phosphorylation levels analyzed by Western blot were unaltered in epidermis of control, S100A9^{-/-}, DKO* and TKO* mice (Fig. 12A-C). The mechanism leading to TNF α membrane shedding is mediated by TACE (TNF α Converting Enzyme, (14)). As previously described in human psoriatic plaques (72), elevated epidermal TACE mRNA levels were detected in the epidermis of the Jun/AP-1 psoriasis mouse model and also TKO* mice (Fig. 12A). Expression of the JunB/c-Jun protein target and

A qPCR analysis:

gene	control : S100A9 ^{-/-}	control : DKO*	control : TKO*	S100A9 ^{-/-} : TKO*	DKO* : TKO*
EPIDERMIS					
IL-23p19	1.3 fold increase	18.7 fold increase (***)	unaltered	1.3 fold decrease	19.4 fold decrease (***)
IL-12p40	1.3 fold decrease	8.7 fold increase (***)	1.3 fold increase	1.7 fold increase	6.5 fold decrease (**)
TGF- β 1	2.5 fold increase	1.8 fold increase	1.7 fold increase	1.5 fold decrease	unaltered
IL-17A	2.3 fold increase	632 fold increase (***)	266 fold increase (***)	115 fold increase (***)	2.4 fold decrease (**)
IL-17F	3.7 fold increase	180 fold increase (***)	30.0 fold increase (***)	8.2 fold increase (*)	6.0 fold decrease (*)
IL-22	3.1 fold increase	3721 fold increase (***)	114 fold increase (***)	37.2 fold increase (***)	32.6 fold decrease (***)
IL-23R	unaltered	6.1 fold increase (*)	2.3 fold increase (*)	2.4 fold increase (*)	2.7 fold decrease (*)
DERMIS					
IL-23p19	10.6 fold increase (***)	10.9 fold increase (***)	9.1 fold increase (***)	unaltered	unaltered
IL-12p40	12.9 fold increase (***)	8.3 fold increase (***)	10.4 fold increase (***)	1.2 fold decrease	1.3 fold increase
TGF- β 1	1.5 fold increase	1.4 fold increase	unaltered	1.6 fold decrease (*)	1.5 fold decrease (*)
IL-17A	unaltered	39.8 fold increase (***)	5.0 fold increase (*)	4.7 fold increase (*)	8.0 fold decrease (**)
IL-17F	3.2 fold increase	9.5 fold increase (*)	3.0 fold increase	unaltered	3.2 fold decrease
IL-22	22.8 fold increase (***)	195 fold increase (***)	28.0 fold increase (***)	1.2 fold increase	7.0 fold decrease (**)
IL-23R	2.5 fold increase (**)	3.7 fold increase (***)	3.7 fold increase (***)	1.5 fold increase	unaltered

Fig. 13 – The Th17 axis in the Jun/AP-1 psoriasis mouse model and altered expression of involved factors upon S100A9 loss.

(A) Table showing fold-changes of mRNA expression levels of components associated with the T helper (Th)-17 cells in the epidermis and dermis of indicated mice, tested by quantitative PCR. A more detailed explanation on table interpretation is given in Fig. 51.

TACE inhibitor TIMP3 (Tissue Inhibitor of Metalloproteases 3, (14)) is significantly downregulated in DKO* as well as in TKO* skin in comparison to controls (Fig. 12A and (15)). Thus, the reduction of TNF α expression in the Jun/AP-1 psoriasis mouse model upon loss of S100A9 appears to be p38 α and TIMP3/TACE independent.

3.5.3. Factors involved in the Th17/IL-23 axis in psoriasis

T helper (Th) cells are considered to play an important role in psoriasis, especially the recently described sub-population of IL-17-producing Th17 cells (73) has been associated with disease pathogenesis (53). Stimulation with several inflammation-mediating cytokines like IL-21 together with TGF- β 1 (tumor growth factor β 1) induces naïve T cells to express IL-23R (IL-23 receptor). Subsequently, binding of IL-23, a heterodimer of the IL-23p19 and IL-12p40 subunits, causes phosphorylation of the transcription factor STAT3 (Signal Transducer and Activator of Transcription 3) and expression of cytokines like IL-17A, IL-17F and IL-22 by Th17 cells (53). We observed mRNA levels of almost all mentioned cytokines involved in the Th17 axis to be significantly elevated in the skin of DKO* mice versus controls (Fig. 13A, (1) and (15)). Transcription of IL-23p19 and IL-12p40, the subunits of the cytokine mediating Th17-differentiation IL-23, were found unaltered in TKO* compared to DKO* dermis on mRNA level. Dermal mRNA expression of IL-23 subunits is already increased in S100A9-/- mice without additional inflammatory stimulus compared to controls (Fig. 13A-E). Nevertheless, TKO* mice display normalized transcription of IL-23R, which is expressed by Th17 cells, in epidermis and dermis compared to DKO* mice (Fig. 13A-E). Also, we detected decreased mRNA levels of cytokines produced by Th17 cells (IL-17A, IL-17F and IL-22) in the skin upon loss of S100A9 in DKO* mice (Fig. 13A). It is necessary to mention at this point, that IL-17A, IL-17F and IL-22 are not exclusively released by Th17 cells. In fact, IL-22 is also produced by NK and NKT cells (74) and $\gamma\delta$ -T-, NK- and NKT cell-derived IL-17A/F appears crucial for several inflammatory responses (75). Moreover, IL-17A might be secreted by keratinocytes upon stimulation (Ö. Uluçkan, unpublished observation).

Elevated mRNA levels of several Th17-associated cytokines and factors were observed in DKO* compared to healthy mouse skin. Moreover, an analysis for Th17/IL-23 related cytokines in the serum showed similar results (Fig. 10A, F-I). These results suggest a high Th17 cell presence in the skin of the Jun/AP-1 psoriasis mouse model, which is at least partially dependent on S100A9.

A qPCR analysis:

gene	control : S100A9 ^{-/-}	control : DKO [*]	control : TKO [*]	S100A9 ^{-/-} : TKO [*]	DKO [*] : TKO [*]
EPIDERMIS					
IL-11	1.8 fold increase	1.8 fold increase (*)	1.2 fold increase	1.5 fold decrease	1.5 fold decrease
IL-20	unaltered	1.3 fold decrease	2.0 fold decrease (*)	2.1 fold decrease (**)	1.6 fold decrease
IL-20R	1.4 fold increase (*)	1.8 fold decrease (*)	unaltered	1.4 fold decrease	1.9 fold increase (**)
IL-1ra	1.4 fold decrease	2.7 fold increase (**)	2.0 fold increase (**)	2.9 fold increase (***)	1.3 fold decrease
IL-9	4.3 fold increase	45.9 fold increase (**)	19.0 fold increase (**)	4.4 fold increase (*)	2.4 fold decrease
IL-13	2.9 fold increase	12.5 fold increase (**)	12.6 fold increase (**)	4.4 fold increase (*)	unaltered
IL-1 β	unaltered	472 fold increase (***)	21.0 fold increase (**)	23.3 fold increase (**)	22.5 fold decrease (**)
IL-6	unaltered	20.9 fold increase (**)	3.9 fold increase (*)	4.4 fold increase (*)	5.3 fold decrease (*)
MIP-1 α	1.3 fold increase	141 fold increase (***)	5.7 fold increase (**)	5.1 fold increase (*)	25.0 fold decrease (***)
MIP-1 β	1.9 fold increase	196 fold increase (***)	12.7 fold increase (***)	6.5 fold increase (*)	15.4 fold decrease (***)
MIP-2	1.6 fold decrease	405 fold increase (***)	6.2 fold increase (*)	9.6 fold increase (*)	65.3 fold decrease (***)
GRO-1	unaltered	57.6 fold increase (***)	7.9 fold increase (***)	8.8 fold increase (***)	7.3 fold decrease (***)
IP-10	unaltered	4.5 fold increase (*)	unaltered	unaltered	4.6 fold decrease (**)
IL-18	1.6 fold increase	3.5 fold decrease (**)	1.5 fold increase	unaltered	5.2 fold increase (***)
DERMIS					
IL-11	8.2 fold increase (*)	1.3 fold increase	4.1 fold increase	2.0 fold decrease	3.0 fold increase
IL-20	2.8 fold increase (**)	1.3 fold increase	2.6 fold increase (**)	unaltered	2 fold increase (*)
IL-1 β	1.3 fold decrease	17.6 fold increase (**)	unaltered	1.4 fold increase	15.6 fold decrease (**)
IL-6	extreme variation in results				
MIP-1 α	11.7 fold decrease (***)	3.0 fold increase (*)	1.9 fold decrease	6.4 fold increase (***)	5.5 fold decrease (**)
MIP-1 β	3.1 fold increase	13.1 fold increase (*)	5.3 fold increase (*)	1.7 fold increase	2.4 fold decrease
MIP-2	2.7 fold increase	4.0 fold increase (*)	3.7 fold increase (**)	1.4 fold increase	unaltered
GRO-1	10.0 fold decrease (*)	5.6 fold increase	1.5 fold decrease	6.6 fold increase (***)	8.5 fold decrease (***)
IP-10	1.7 fold decrease (*)	1.4 fold increase	1.7 fold decrease	unaltered	2.3 fold decrease
IL-18	4.4 fold decrease(*)	3.8 fold decrease (*)	1.6 fold increase	7.1 fold increase (**)	6.1 fold increase (*)

Fig. 14 – Differential expression of cytokines and chemokines in the skin of the Jun/AP-1 psoriasis mouse model upon S100A9 loss.

(A) Table shows fold-change of mRNA expression levels in the epidermis and dermis comparing control, S100A9^{-/-}, DKO^{*} and TKO^{*} mice, tested by quantitative PCR. N=3 for control, S100A9^{-/-} and DKO^{*} mice and n=6 for TKO^{*} mice. Significant alteration of levels between mice of two tested genotypes is indicated by stars in brackets; (*) represent P<0.05, (**) P<0.01 and (***) P<0.001 significance in a student t-test. A more detailed explanation on table interpretation is given in Fig. 5I.

3.5.4. Local cytokine and chemokine expression in mouse skin

The interplay between keratinocytes and immune cells is crucial for skin inflammation and psoriasis pathogenesis. In order to elucidate the impact of S100A9 on the production of cytokines/chemokines involved in inflammation in the skin, we performed a candidate screen of mRNA expression levels in the epidermis and the dermis of control, S100A9^{-/-}, DKO^{*} and TKO^{*} mice.

No significant mRNA expression changes of IL-11 and IL-20 were observed in the skin of mice of all four tested genotypes (Fig. 14A), although IL-11 protein was reported to be released by skin biopsy cultures (76) and IL-20 mRNA levels to be elevated in psoriatic skin ((77) and (78)). IL-1ra (IL-1 receptor antagonist), IL-9 and IL-13 were signifi-

cantly elevated in the epidermis of DKO* and TKO* mice compared to controls (Fig. 14A). Thus, these cytokines are likely to contribute to the psoriasis-like phenotype in a S100A9-independent manner. IL-1 β , IL-6, MIP-1 α , MIP-1 β , MIP-2, GRO-1 (melanoma growth stimulating activity 1) and IP-10 (interferon gamma-induced protein 10) were significantly upregulated as well as IL-18 and IL-20 receptor (IL-20R) downregulated in the epidermis and mostly also in the dermis of DKO* mice, as partly reported previously ((1) and (15)) and similar described for human psoriatic skin ((79), (52) and (78)). Importantly, epidermal (and dermal) mRNA expression of all eight cytokines/chemokines was normalized in TKO* mice (Fig. 14A), suggesting S100A9-dependent regulation of these factors in the skin. Hence, S100A9-deficiency in the setting of skin inflammation appears to cause an overall normalization of epidermal and dermal mRNA expression of tested cytokines and chemokines.

3.5.5. Factors associated with psoriasis

Various other proteins have been implicated in psoriasis pathogenesis and described to be differentially regulated in psoriatic skin. To perform a closer molecular characterization of the skin of TKO* mice compared to the Jun/AP-1 psoriasis mouse model, we tested the cutaneous expression of selected candidate genes by qPCR.

EGF (Epidermal Growth Factor) is a potent inducer of keratinocyte proliferation and its concentration has been shown to be increased in psoriatic patients in the serum (80), while EGFR (EGF receptor) is considered to be a potential target in psoriasis treatment (81). PDGF (Platelet-Derived Growth Factor) was described to be overexpressed in the dermis of psoriasis patients (82) as well as IGF (Insulin-like Growth Factor)-1 to be upregulated in psoriatic epidermis (83). Analysis of epidermal mRNA expression revealed no remarkable changes in EGF, EGFR, IGF-1, PDGF-A and PDGFR α (PDGF receptor α) levels in control, S100A9 $^{-/-}$, DKO* and TKO* mice, while PDGFR β was significantly augmented in DKO* compared to control and TKO* mice (Fig. 15A). However, analysis of protein expression in the skin and receptor tyrosine kinase activation by phosphorylation will be necessary to interpret the relevance of the mentioned growth factors and their receptors in the Jun/AP-1 psoriasis mouse model and TKO* mice.

MMPs (Matrix metalloproteases) are a large family of mediators of inflammation and tissue repair and are controlled by TIMPs (Tissue Inhibitor of Metalloproteases).

A qPCR analysis:

EPIDERMIS					
gene	control : S100A9 ^{-/-}	control : DKO [*]	control : TKO [*]	S100A9 ^{-/-} : TKO [*]	DKO [*] : TKO [*]
growth factors & their receptors					
EGF	unaltered	1.6 decrease	1.5 fold decrease	1.7 fold decrease	unaltered
EGFR	unaltered	unaltered	unaltered	unaltered	unaltered
PDGF A	1.8 fold increase	2.5 fold decrease (*)	1.7 fold decrease	3.2 fold decrease (***)	1.5 fold increase
PDGFR α	1.8 fold increase	2.0 fold increase	1.9 fold increase	unaltered	unaltered
PDGFR β	4.5 fold increase (*)	19.3 fold increase (***)	5.0 fold increase (*)	unaltered	3.9 fold decrease
IGF-1	1.9 fold increase	unaltered	1.5 fold increase	1.3 fold decrease	1.3 fold increase
matrix metalloproteases & their inhibitors					
MMP2	1.3 fold increase	2.0 fold decrease (*)	1.4 fold decrease	1.9 fold decrease	1.4 fold increase
MMP8	unaltered	123 fold increase (***)	7.5 fold increase (**)	6.7 fold increase (**)	16.4 fold decrease (***)
MMP9	2.4 fold increase	30.2 fold increase (***)	2.4 fold increase	unaltered	12.4 fold decrease (***)
TIMP1	5.6 fold decrease (*)	4.1 fold increase (*)	unaltered	6.1 fold increase (*)	3.8 fold decrease (*)
TIMP2	1.6 fold increase (**)	unaltered	1.7 fold increase (***)	unaltered	1.6 fold increase (**)
prostaglandins & their receptors					
PTGS1	2.0 fold increase	1.4 fold decrease	1.6 fold increase	1.2 fold decrease	2.4 fold increase (*)
PTGS2	1.3 fold increase	22.6 fold increase (***)	unaltered	1.2 fold decrease	21.4 fold decrease (***)
PTGER1	2.3 fold increase	1.8 fold increase	5.6 fold increase	2.4 fold increase	3.0 fold increase
PTGER2	1.7 fold increase	1.6 fold increase	4.1 fold increase (*)	2.3 fold increase	2.5 fold increase
PTGER3	1.8 fold decrease	1.8 fold decrease	1.3 fold decrease	1.4 fold increase	1.4 fold increase
PTGER4	1.8 fold increase	2.9 fold decrease (*)	1.4 fold decrease	2.6 fold decrease	2.0 fold increase
other genes associated with psoriasis					
CD44	unaltered	2.3 fold increase (**)	2.0 fold increase (*)	1.7 fold increase (*)	unaltered
Calm1	2.2 fold increase (***)	2.1 fold increase (**)	2.8 fold increase (***)	1.3 fold increase	1.3 fold increase
Slpi	unaltered	42.1 fold increase (***)	7.8 fold increase (***)	6.5 fold increase (***)	5.4 fold decrease (***)
STAT3	1.4 fold decrease	2.8 fold increase (*)	unaltered	1.3 fold increase	2.9 fold decrease (**)
Cav1	unaltered	3.9 fold decrease (***)	1.5 fold decrease (*)	1.7 fold decrease	2.6 fold increase (***)
TGF- β 2	unaltered	10.6 fold decrease (***)	3.0 fold decrease (**)	3.2 fold decrease (***)	3.5 fold increase (**)

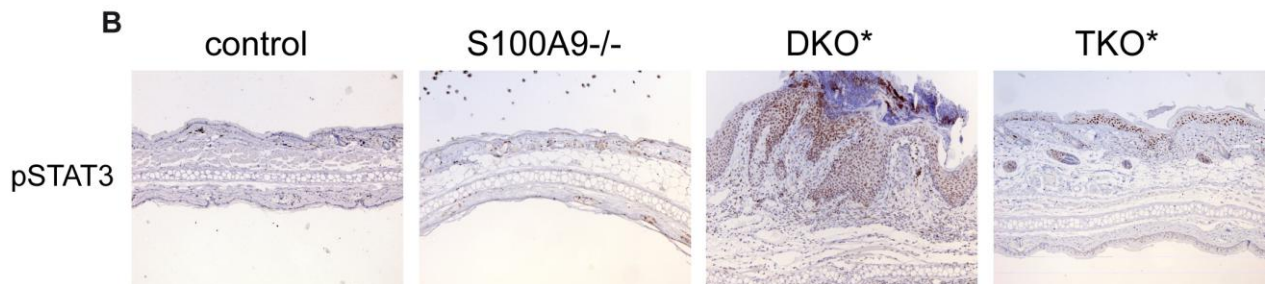


Fig. 15 – Determination of expression levels of factors associated with psoriasis in control, S100A9^{-/-}, DKO^{*} and TKO^{*} mice.

(A) Table showing fold-changes of mRNA expression levels of growth factors and their receptors, matrix metalloproteases (MMPs) and their inhibitors (TIMPs), prostaglandins and their receptors as well as other factors implicated in psoriasis pathogenesis. Epidermal isolates of mice of all four genotypes used in this study were analyzed by quantitative PCR. A more detailed explanation on table interpretation is given in Fig. 51.

(B) Immunohistochemistry for phosphorylated STAT3 (pSTAT3) of cut mouse ear sections of control (n=3), S100A9^{-/-} (n=3), DKO^{*} (n=3) and TKO^{*} mice (n=6). Cell nuclei are stained in blue with haematoxylin, representative images are shown and magnification is 10x.

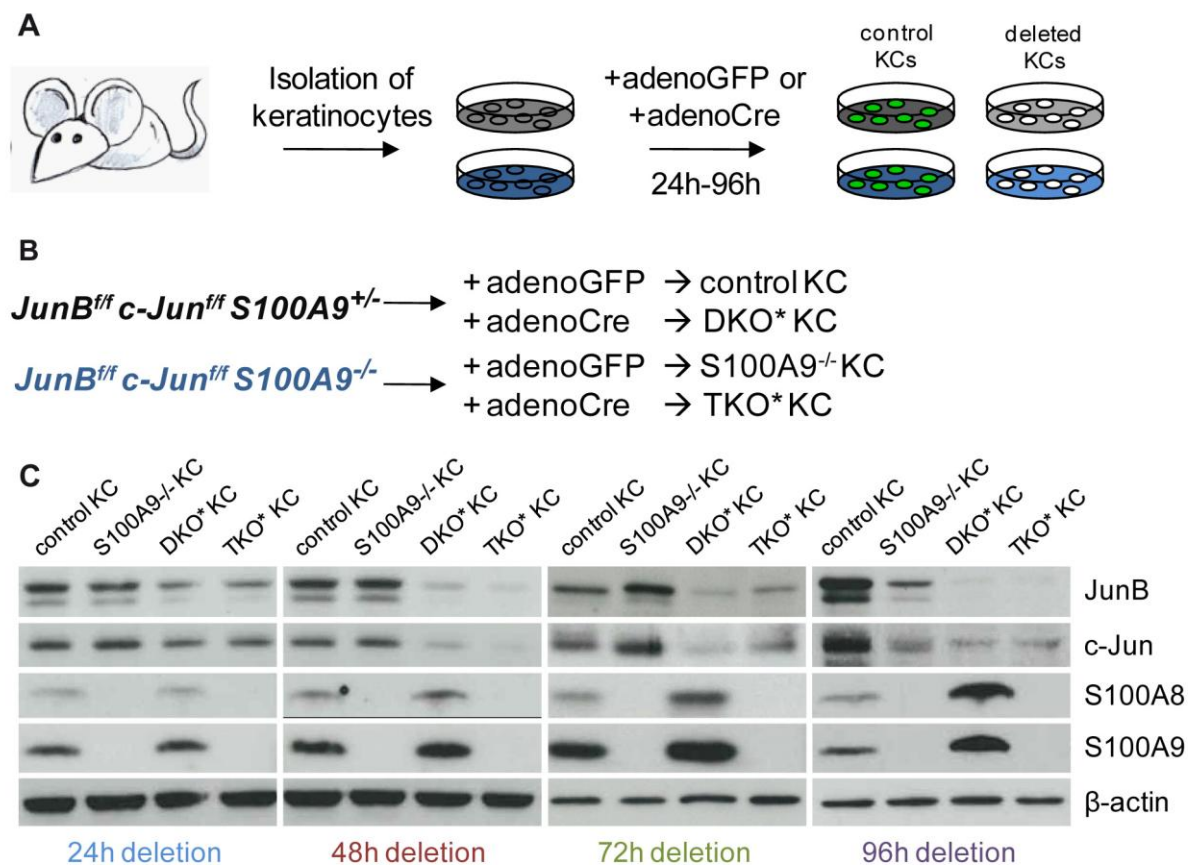
Elevated serum levels of TIMP1 (84) as well as increased expression of MMP9 (85), MMP2 and TIMP2 (86) in the skin of psoriasis patients were reported. MMP2 and TIMP2 epidermal expression remained unaltered comparing the four genotypes used in

this analysis, while MMP8, MMP9 and TIMP1 mRNA levels were significantly augmented in the epidermis of DKO* and normalized in TKO* mice (Fig. 15A). However, TIMP1 protein on a systemic level in mouse sera showed no change between control, S100A9-/-, DKO* and TKO* mice (Fig. 10A).

Finally, we analyzed mRNA expression by qPCR of further factors and markers associated with either psoriasis, skin inflammation or keratinocyte proliferation. PTGS2 (Prostaglandin E2) is overexpressed in human psoriatic skin (87) as well as in the epidermis of DKO* and normalized in TKO* mice (Fig. 15A). Whereas PTGS1 (Prostaglandin E1) as well as PTGER (Prostaglandin E receptor)-1, PTGER2, PTGER3 and PTGER4 epidermal mRNA levels remain unaltered in control S100A9-/-, DKO* and TKO* mice (Fig. 15A).

CD44, the receptor for hyaluronic acid which binds MMPs, is reported to be upregulated in human psoriatic skin (88) as well as Calm1 (Calmodulin 1, (89)), which was also found to be augmented in the Jun/AP-1 psoriasis mouse model (1). Also Slpi (Secretory Leukocyte Protease Inhibitor), whose expression is increased in keratinocytes upon inflammation (90), was described to be upregulated in DKO* mice (1). Moreover, phosphorylation of STAT3 (pSTAT3) in keratinocytes is associated with increased proliferation (91) and mice expressing a constitutively active STAT3 under control of the K5 promoter develop a skin irritation similar to human psoriasis (92). Indeed, qPCR analysis revealed increased mRNA levels of CD44, Calm1, Slpi and STAT3 in the epidermis of DKO* mice compared to controls. While the levels of CD44 and Calm1 remained unaltered between DKO* and TKO* mice, Slpi and STAT3 mRNA was significantly decreased (Fig. 15A). Furthermore, the number of pSTAT3 positive cells appears elevated in the epidermis of the DKO* mice versus controls and reduced in TKO* epidermis (Fig. 15B).

Levels of the scaffolding protein Cav1 (Caveolin1), known to be reduced in psoriatic plaques (93), and the keratinocyte proliferation inhibitor TGF- β 2 were previously observed to be downregulated in the Jun/AP-1 psoriasis mouse model (1). We were able to confirm this finding and, additionally, observed an increase to almost control mRNA levels in TKO* mice (Fig. 15A). These results show that DKO* mice display a highly similar expression pattern of psoriasis-associated genes to the human situation and that these factors are mostly normalized after the deletion of S100A9.



Quantification of protein levels:

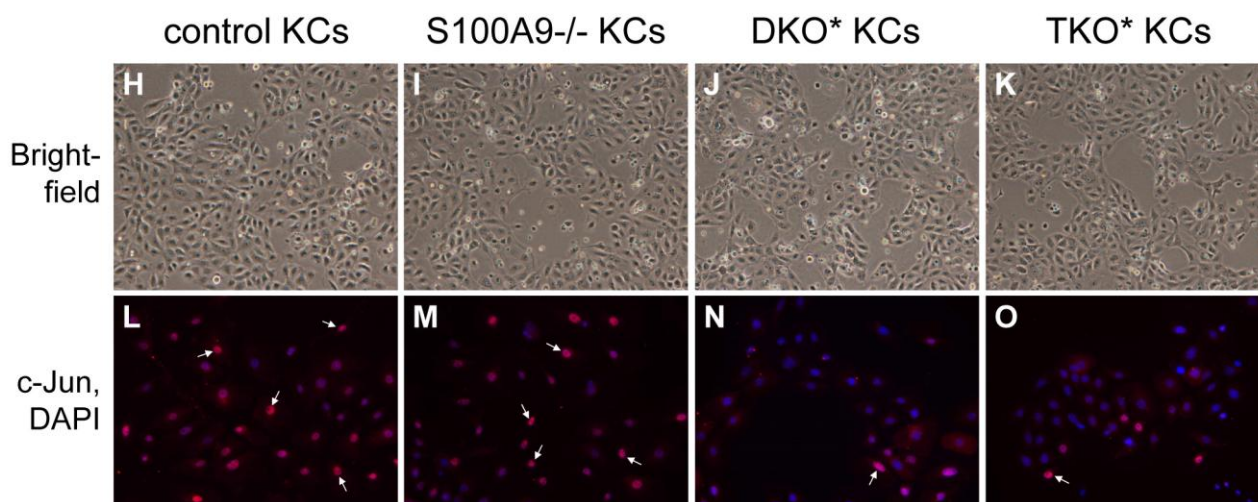
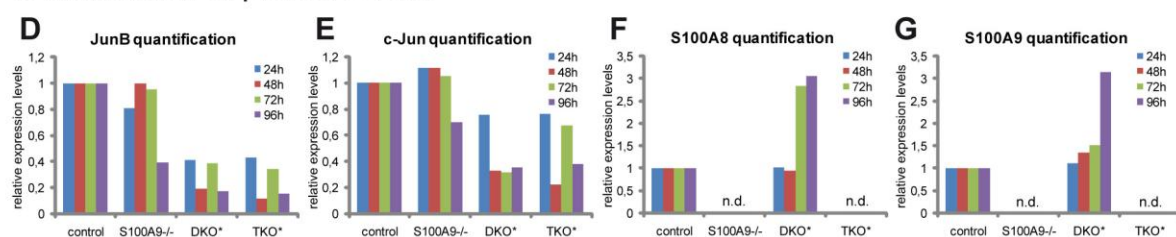


Fig. 16 – *In vitro* system used for adenoCre mediated deletion of JunB and c-Jun in primary keratinocytes.

(A) Schematic view of procedure of adenovirus application to primary keratinocytes for different time periods.

(B) Overview of mice used for keratinocytes isolation. Keratinocytes derived from JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{+/-} and JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{-/-} mice were either treated with an adenovirus carrying GFP (adenoGFP) to obtain control and S100A9^{-/-} keratinocytes (KCs) or an adenovirus carrying Cre recombinase (adenoCre) to produce DKO* and TKO* keratinocytes.

(C-G) Western blot analysis and quantification of JunB (D), c-Jun (E), S100A8 (F) and S100A9 (G) protein levels in keratinocytes of the indicated genotype exposed to adenovirus for 24, 48, 72 or 96 hours. Protein level quantification was performed relative to the loading control β -actin in (D-G). One representative experiment per time period of adenovirus treatment of is displayed.

(H-K) Representative bright-field image of control, S100A9^{-/-}, DKO* and TKO* keratinocytes (KCs) exposed to adenovirus for 96 hours, magnification is 20x.

(L-O) Immunocytofluorescence for c-Jun (red channel) of keratinocytes with indicated genotypes treated with adenovirus for 72 hours is shown. Cell nuclei were stained with DAPI in blue, white arrows mark some c-Jun-positive nuclei and magnification is 20x.

3.6. Determination of cell autonomous proliferation behavior of *in vitro*-deleted JunB ^{Δ ep*} c-Jun ^{Δ ep*} S100A9^{+/-} (DKO*) and JunB ^{Δ ep*} c-Jun ^{Δ ep*} S100A9^{-/-} (TKO*) keratinocytes

In order to determine possible cell autonomous effects of S100A9 deficiency in keratinocytes, we employed a cell culture system and *in vitro* deletion of JunB and c-Jun using an adenovirus (Fig. 16A). Primary keratinocytes were isolated from ear and tail skin of JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{+/-} and JunB^{fl/fl} c-Jun^{fl/fl} S100A9^{-/-} mice and cultured under presence of adenovirus containing Cre recombinase (adenoCre) for Jun protein deletion to obtain DKO* and TKO* keratinocytes or green fluorescent protein (adenoGFP) to obtain control and S100A9^{-/-} keratinocytes (Fig. 16B). When testing for the optimal virus concentrations (150, 300 and 600 particles/cell), we detected highest JunB and c-Jun deletion efficiency using 600 particles/cell of adenoCre (data not shown). Primary keratinocytes were incubated for 24, 48, 72 and 96 hours with the respective adenovirus to increase the extent of Jun gene deletion in keratinocytes (Fig. 16A). More than 3-fold downregulation of JunB and c-Jun protein levels in DKO* and TKO* keratinocytes compared to control and S100A9^{-/-} keratinocytes were already observed after 48 hours and

longer adenoCre exposure by Western blot analysis (Fig. 16C+D-E) and immunocytofluorescence for c-Jun after 72 hours adenoCre infection of keratinocytes (Fig. 16L-O). As expected, DKO* keratinocytes incubated with adenoCre for 72 hours or longer showed a more than 50% increase in S100A8 and S100A9 protein expression versus controls (Fig. 16C+F-G and (1)). Keratinocyte cell shape and density assessed by bright-field microscopy of cultures appear similar between control, S100A9^{-/-}, DKO* and TKO* keratinocytes (Fig. 16H-K).

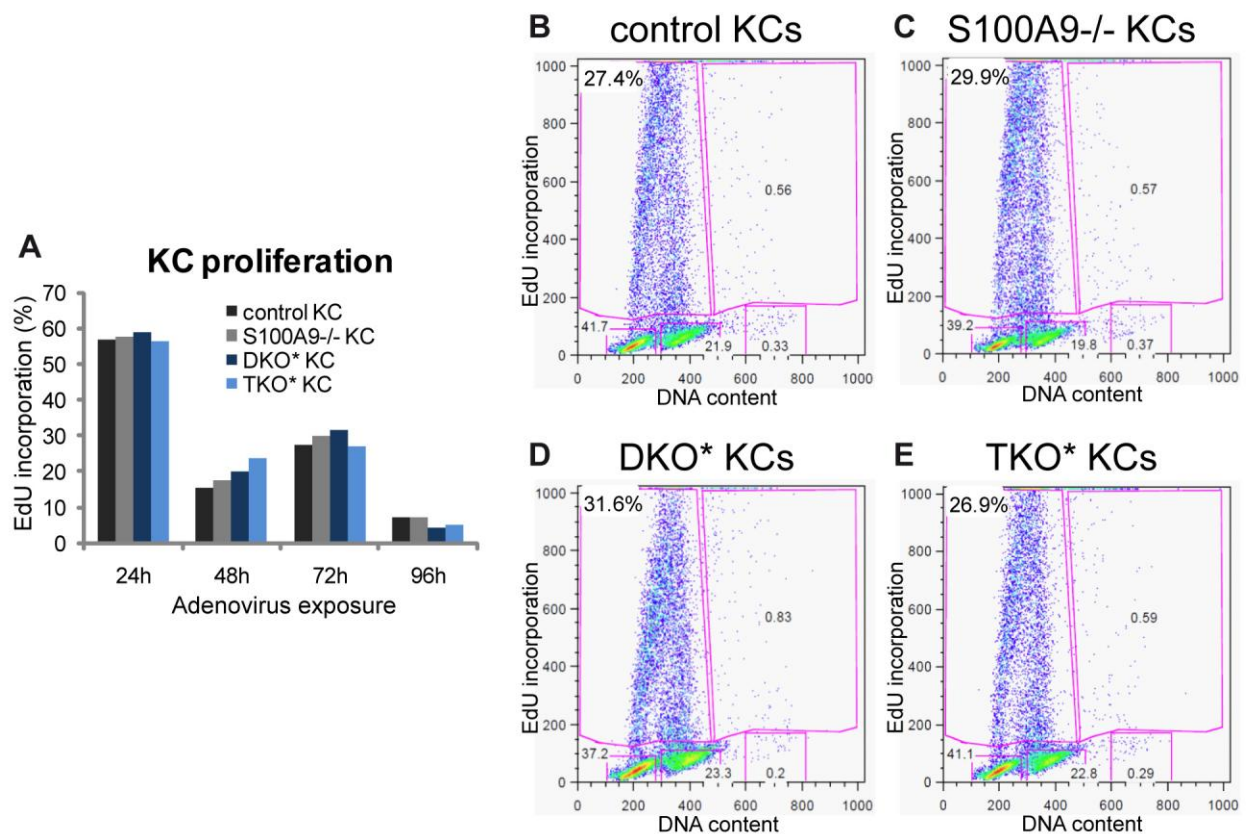


Fig. 17 – Proliferation of primary, *in vitro*-deleted DKO* and TKO* keratinocytes is unaltered compared to controls.

(A) Proliferation of primary keratinocytes was determined by an EdU incorporation assay after an EdU pulse for 1 to 6 hours, depending on the time the cells were cultured. Percentage of EdU positive control, S100A9^{-/-}, DKO* and TKO* keratinocytes (KCs) exposed to adenovirus for the indicated period is depicted. Analysis was performed according to manufacturer's instructions, by staining for EdU and DAPI, to determine DNA content, and subsequent flow cytometric quantification. One representative experiment per time point is shown, data represent mean of duplicates or triplicates.

(B-E) Representative dot blots of flow cytometric evaluation of EdU incorporation versus DNA content (determined by DAPI staining) by keratinocytes of indicated genotypes after 72 hours of adenovirus incubation are shown.

DKO* mice showed a much stronger epidermal hyperproliferation than TKO* mice (Fig. 4), suggesting involvement of S100A9 in keratinocyte proliferation. By *in vitro* studies, we wanted to determine the possible cell autonomous role of S100A9 in keratinocytes. Cell proliferation of *in vitro* deleted primary keratinocytes was determined by an EdU (5-ethynyl-2'-deoxyuridine) incorporation assay. The thymidine nucleoside analog EdU was incorporated in newly synthesized DNA of cultured primary keratinocytes and cells in G1/G0, S and G2 phase could be determined by flow cytometric analysis, (Fig. 17B-E). No difference in EdU-positive cell numbers between control, S100A9-/-, DKO* and TKO* keratinocytes after 24h, 48h, 72h and 96 hours adenovirus or adenogFP exposure was detected (Fig. 17A).

This outcome indicates that increased epidermal proliferation in DKO* mice is no cell autonomous effect of genetic deletion of JunB and c-Jun in keratinocytes and S100A9 appears to be dispensable for keratinocyte proliferation *in vitro*. Most likely, epidermal thickening and elevated keratinocyte proliferation in DKO* mice is a result of the interplay between several cell types and keratinocytes in the skin leading to a psoriasis-like skin disease.

3.7. Identification of S100A9-dependent factors produced by keratinocytes, mediating the cross talk to immune cells in skin

S100A9 loss strongly reduces the overall psoriatic phenotype of DKO* mice (Fig. 2) and we found a diminished amount of inflammatory cytokines on a systemic level in the sera of TKO* compared to DKO* mice (Fig. 10). Thus, we aimed to identify S100A9-dependent, keratinocyte-derived mediators of the immune cell - keratinocyte interplay by evaluating cytokine / chemokine levels in the supernatant of *in vitro* deleted control, S100A9-/-, DKO* and TKO* keratinocytes after 96 hours-adenovirus exposure (Fig. 16) using a cytokine array and ELISA analysis.

We measured reliable levels of 14 cytokines and chemokines in the supernatant of primary control, S100A9-/- keratinocytes and *in vitro*-deleted DKO* and TKO* keratinocytes. Five of these factors (IL-1 α , IL-25, MCP-5, LIX (Lipopolysaccharide-Induced CXC chemokine) and VEGF) were unchanged comparing keratinocytes of all four genotypes (Fig. 18A-B). Elevated levels of more than half of detectable factors in keratinocyte-

A Cytokine array and ELISA analysis:

<i>In vitro</i> : levels in keratinocyte-conditioned supernatant					
cytokine / chemokine	control : S100A9-/-	control : DKO*	control : TKO*	S100A9-/- : TKO*	DKO* : TKO*
IL-1 α	unaltered	1.6 fold increase	1.3 fold increase	unaltered	1.2 fold decrease
IL-25	unaltered	unaltered	unaltered	unaltered	unaltered
MCP-5	unaltered	unaltered	unaltered	unaltered	unaltered
LIX	unaltered	unaltered	unaltered	unaltered	1.3 fold decrease
VEGF	unaltered	unaltered	unaltered	unaltered	1.3 fold decrease
G-CSF	1.2 fold increase	2.5 fold increase (*)	2.3 fold increase (***)	1.9 fold increase (*)	unaltered
M-CSF	1.3 fold increase	2.2 fold increase (*)	2.7 fold increase (*)	1.8 fold increase	1.2 fold increase
MCP-1	unaltered	4.6 fold increase (*)	4.2 fold increase (*)	3.4 fold increase	unaltered
MIP-2	unaltered	2.8 fold increase (*)	2.3 fold increase (*)	2.3 fold increase (*)	unaltered
MIP-3 α	2.2 fold increase	36.8 fold increase (*)	41.3 fold increase (**)	19.2 fold increase (**)	unaltered
KC	1.4 fold increase	2.6 fold increase (***)	2.5 fold increase (***)	1.8 fold increase (**)	unaltered
TIMP1	unaltered	3.3 fold increase (*)	3.3 fold increase (*)	3.3 fold increase (**)	unaltered
LIF	unaltered	3.6 fold increase (***)	2.6 fold increase (***)	2.7 fold increase (***)	1.4 fold decrease (*)
RANTES	1.2 fold decrease	1.2 fold increase	1.2 fold decrease (*)	unaltered	1.4 fold decrease (*)
> 1.8 fold increase > 4 fold increase > 1.5 fold decrease > 2.5 fold decrease					

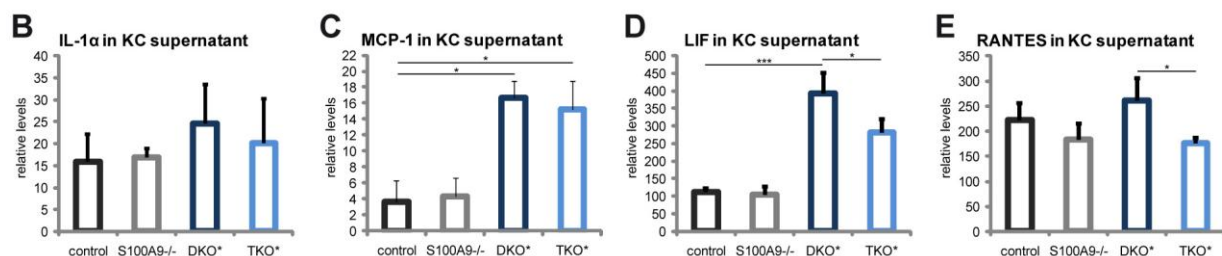


Fig. 18 – Cytokines and chemokines secreted by keratinocytes after deletion of JunB and c-Jun and/or S100A9-deficiency *in vitro*.

(A) Isolated, primary keratinocytes were treated with adenoCre or adenoGFP for 96 hours to obtain control, S100A9-/-, DKO* and TKO* keratinocytes, the medium changed and keratinocyte-conditioned supernatant collected after 24 hours. Multiplex and ELISA analysis was performed to determine cytokine/chemokine levels shown as fold-change of detected amounts comparing keratinocytes of indicated genotype (n=2 per group) in a table. A more detailed explanation on table interpretation is given in Fig. 10A.

(B-E) Selected examples of cytokines with unchanged IL-1 α (B) and increased secreted MCP-1 (C) levels by DKO* and TKO* keratinocytes. The amount of LIF (D) and RANTES (E) in supernatant of TKO* was reduced compared to DKO* keratinocytes. Data represent mean $\pm$ standard deviation.

(A-E) Significant alterations between groups were calculated using a student t-test and indicated as follows: (*) P<0.05, (**) P<0.01 and (***) P<0.001.

conditioned supernatant were observed in DKO* keratinocytes compared to controls, underlining the activation of keratinocytes through JunB/c-Jun deletion. G-CSF, M-CSF, MCP-1, MIP-2, MIP-3 α , KC and TIMP1 exhibited a similarly increased amount in supernatant from TKO* and DKO* keratinocytes (Fig. 18A+C). However, LIF (leukemia inhibi-

tory factor) and RANTES levels were reduced in TKO* keratinocytes compared to DKO* keratinocytes (Fig. 18A+D-E). LIF was below detection threshold in mouse serum, but RANTES also showed significantly elevated serum levels in DKO* mice and a normalization upon loss of S100A9 (Fig. 10A+D). These results indicate that LIF and RANTES production and/or secretion by keratinocytes might be positively regulated by S100A9 in a cell autonomous manner, whereas strong chemotactic proteins like G-CSF, M-CSF, MCP-1 and MIP-2 appear not to be.

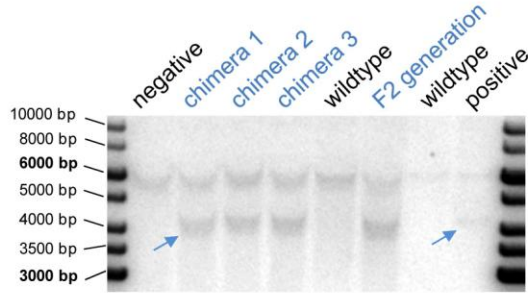
In summary, *in vitro* deletion of JunB and c-Jun in keratinocytes leads to an increase in secretion of several chemotactic and pro-inflammatory factors in a cell-autonomous manner. Although S100A9 is upregulated in keratinocytes upon JunB and c-Jun deletion (1), S100A9 seems to have only a minor cell autonomous effect on secretion of other cytokines/chemokines by keratinocytes, since protein levels in supernatant from DKO* and TKO* keratinocytes appear largely similar. Nevertheless, LIF and RANTES supernatant levels are reduced in TKO* keratinocyte-conditioned medium compared to DKO* keratinocytes, representing interesting candidates for further analysis.

3.8. Transgenic expression of S100A8/A9 to induce skin inflammation

The S100A8 and S100A9 proteins are only weakly expressed in healthy epidermis and strongly upregulated by activated keratinocytes during skin pathologies (38), including inflammation-driven skin carcinogenesis (94), wound healing (23) and psoriasis (37). We aimed to reproduce an inflammatory state of the skin by inducible, transgenic expression of S100A8 and S100A9 proteins using the Tet-ON system in keratinocytes in a mouse model. Mice carrying a S100A8-IRES-S100A9-Myc knock-in allele (tgS100A8/A9) inserted downstream of a tet-Operator (tetO) downstream of the Collagen A1 locus (H. Schönthaler, P. Angel, G. Beranger, L. Bakiri and (95)) and a ROSA- or K5-controlled tetracycline transactivator (rtTA) were tested for proper transgene integration by Southern blot (Fig. 19A). Administration of doxycycline (DOX) renders rtTA protein capable of binding the tetO and activates transcription of S100A8 and S100A9. DOX was given to tgS100A8/A9^{KI/+} ROSA-rtTA^{T/+} (S100A8/A9-rosa-tetON), tgS100A8/A9^{KI/+} and ROSA-rtTA^{T/+} mice (the last two being controls) in drinking water (0.5 gram/liter) from birth, weaning or 7 weeks of age (in the resting phase of the hair

A

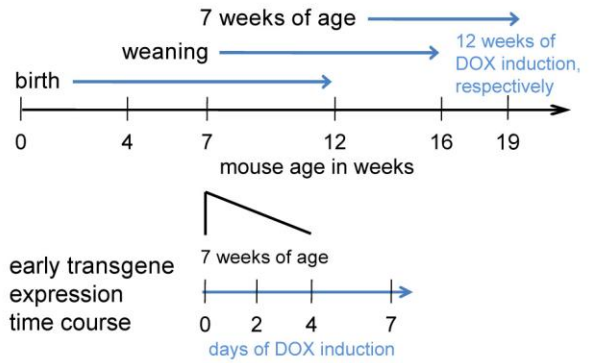
Southern blot of S100A8/A9-tetON mice:



B

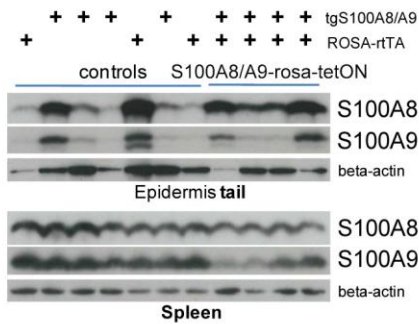
Experimental procedures:

DOX induction at:

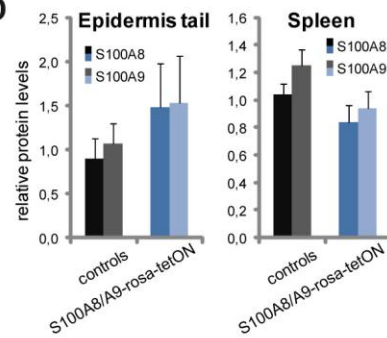


12-week DOX induction, start 7 weeks of age:

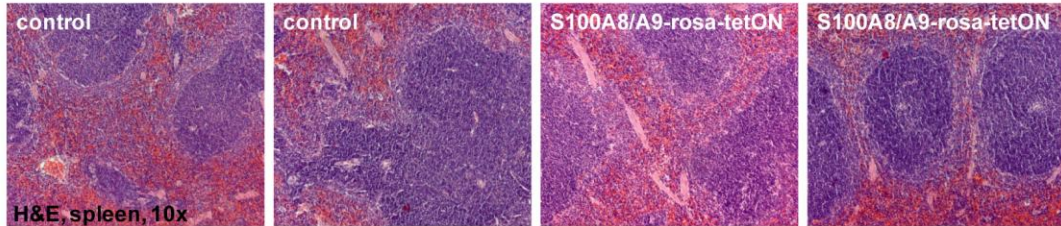
C



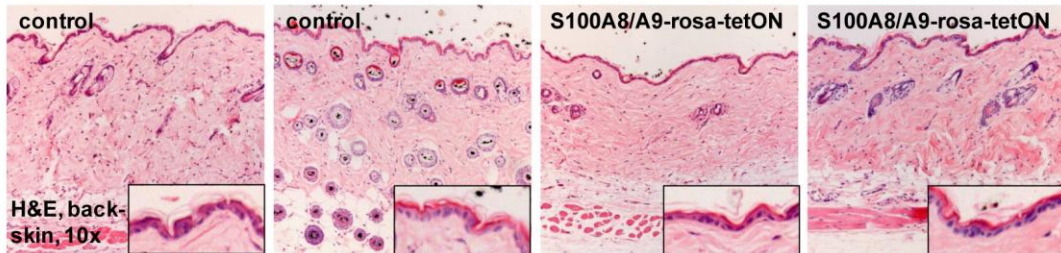
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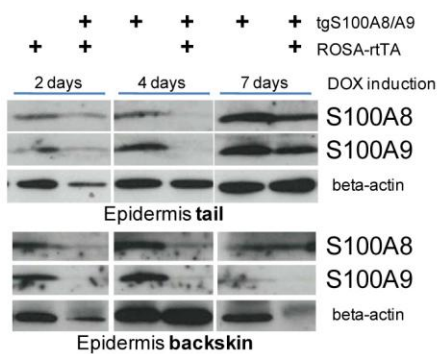


F



G

Early transgene expression time course:



H

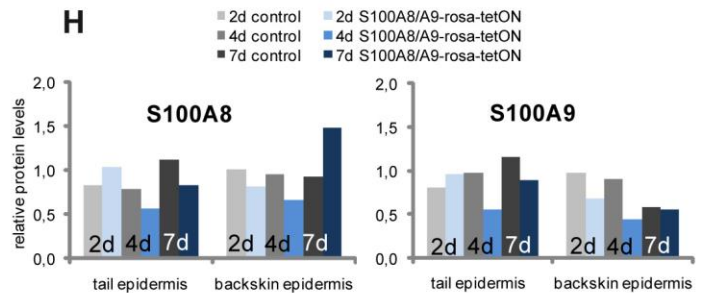


Fig. 19 – S100A8/A9-rosa-tetON mice do not show consistent increase of S100A8 or S100A9 protein levels.

(A) Southern blot showing correct integration of the S100A8-IRES-S100A9-Myc transgene in the CollagenA1 (ColA1) locus. Total DNA from mouse tail was SpeI digested and a 3' probe used for detection of flp-in allele at 4.1kb, wildtype allele at 6.2kb and frt allele at 6.7kb as described in (95). The following mice were used: four mice generated by blastocyst injection with transgenic embryonic stem cells, one of them negative (negative) and three chimeric for the S100A8/A9 transgene (chimera 1-3) as well as one second generation offspring (F2 generation) after germ-line transmission. C57BL/6 mice served as negative control (wildtype) and DNA from a mouse carrying a Fra1 transgene integrated in the ColA1 locus as positive control (positive, provided by S. Hasenfuss).

(B) Treatment of control and S100A8/A9-rosa-tetON mice with 0.5g DOX in drinking water was started at birth, weaning or seven weeks of age and proceeded for 12 weeks before sacrifice and analysis, except for the early transgene expression experiment. Here mice were sacrificed after 2, 4 or 7 days of DOX administration.

(C-D) Western blot (C) of protein isolates from control and S100A8/A9-rosa-tetON mice (12 weeks DOX induction started at 7 weeks of age) for S100A8 and S100A9 in tail epidermis and spleen and quantification (D). Data represent mean $\pm$ standard deviation ($n \geq 6$ per group). No significant alterations were determined between groups using a student t-test.

(E-F) H&E staining of spleen (E) and backskin (F) of mice with indicated genotype (12 weeks DOX induction started at 7 weeks of age), magnification 10x. Inserts in (F) show higher magnification of epidermis.

(G-H) Analysis of S100A8 and S100A9 expression by Western blot (G) in epidermis of tail and backskin of control and S100A8/A9-rosa-tetON mice 2-7 days on DOX, started at 7 weeks of age and quantification (H, $n=1$ per condition).

cycle) for 12 weeks (Fig. 19B). No macroscopic phenotype of mice induced by DOX administration at birth ($n \geq 2$ per group), weaning ($n \geq 2$ per group) or 7 weeks of age ($n \geq 6$ per group) was apparent. We analyzed S100A8 and S100A9 protein levels in mice treated with DOX from 7 weeks of age and no consistent increase in tail dermis, lung, muscle, liver ($n=2$ per group, data not shown), tail epidermis and spleen (Fig. 19C+D) of S100A8/A9-rosa-tetON mice versus control was observed. We obtained similar results testing mRNA levels of S100A8/A9 in several tissues in these mice ($n \geq 6$, data not shown). Analysis of H&E-stained spleen and backskin sections from S100A8/A9-rosa-tetON mice did also not show any histological alterations in (Fig. 19E+F).

Additionally, we checked for transgene induction after 2, 4 and 7 days of DOX treatment of 7-weeks old mice (Fig. 19B). However, upregulation of S100A8 or S100A9 mRNA or protein levels in tail or backskin epidermis of S100A8/A9-rosa-tetON mice compared to controls was not detected (Fig. 19G+H).

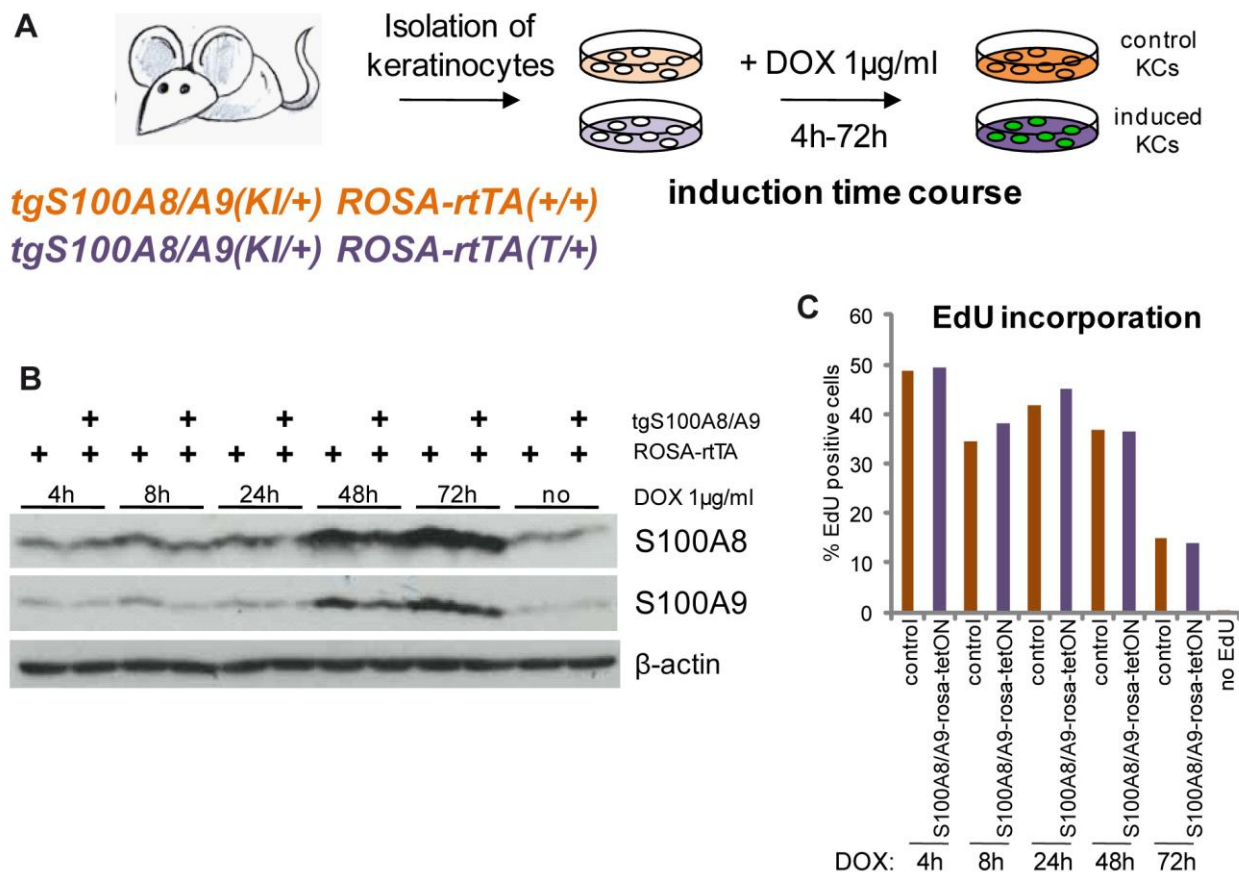


Fig. 20 – Analysis of S100A8/A9-rosa-tetON keratinocytes induced by doxycycline *in vitro* for expression of S100A8 and S100A9.

(A) Overview on treatment of primary keratinocytes isolated from control and S100A8/A9-rosa-tetON mice. DOX (1µg/ml) was added to culture medium for different time periods, as indicated.

(B) Levels of S100A8 and S100A9 protein in control and S100A8/A9-rosa-tetON keratinocytes induced by DOX of the specified period determined by Western blot.

(C) Graph shows percentage of EdU positive cells after EdU incorporation assay for elucidation of proliferation behavior with 1-2 hours EdU pulse.

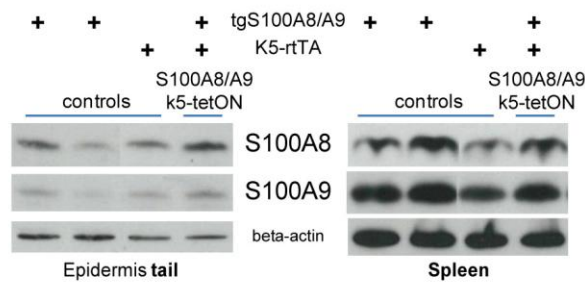
Then, we induced S100A8/A9 expression by DOX administration for 4, 8, 24, 48 and 72 hours *in vitro* in primary keratinocytes isolated from *tgS100A8/A9^{KI/+}* (control) and *tgS100A8/A9^{KI/+} ROSA-rtTA^{T/+}* (S100A8/A9-rosa-tetON) mice (Fig. 20A). No elevation of S100A8 or S100A9 protein levels (Fig. 20B) or altered proliferation behavior due to possible activation of keratinocytes was observed (Fig. 20C).

Next, we analyzed mice carrying an rtTA under the control of the K5 promotor, to test if S100A8/A9 knock-in allele expression can be observed specifically in the skin using a different rtTA mouse line. At 7 weeks of age *tgS100A8/A9^{KI/+} K5-rtTA^{T/+}* (S100A8/A9-k5-tetON), *tgS100A8/A9^{KI/+}* and *K5-rtTA^{T/+}* mice (the last two being controls) were adminis-

tered DOX in drinking water for 12 weeks. However, induced expression of S100A8 and S100A9 on mRNA and protein level was not observed in tail epidermis (n=5 per genotype, data partially shown in Fig. 21A). As a control, the spleen of S100A8/A9-k5-tetON mice was taken and S100A8/A9 protein levels did not show an alteration compared to control mice (n=5 per genotype, Fig. 21A). Also, no reliable increase of S100A8 or S100A9 protein expression could be detected during early transgene induction after 2, 4 or 7 days DOX treatment of 7-weeks old mice in epidermis of tail or backskin (Fig. 21B). Moreover, primary keratinocytes isolated from tgS100A8/A9^{KI/+} k5-rtTA^{T/+} mice did not show an upregulation of S100A8 or S100A9 upon 24 hours stimulation with increasing concentrations of DOX compared to tgS100A8/A9^{KI/+} and k5-rtTA^{T/+} (both controls) or untreated keratinocytes (Fig. 21C).

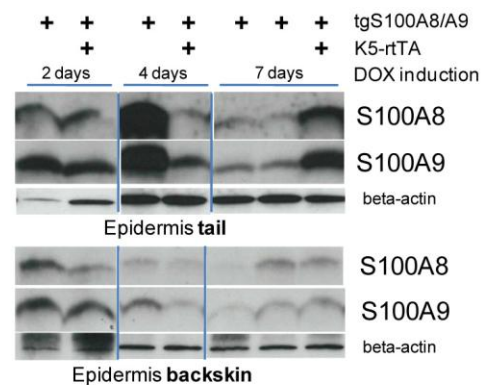
No elevation of S100A8 and S100A9 mRNA (data not shown) and protein amount could be induced by ectopic expression of the S100A8-IRES-S100A9-Myc knock-in allele us-

A 12-week DOX induction, start 7 weeks of age:



B

Early transgene expression time course:



C Primary keratinocytes, 24h DOX induction:

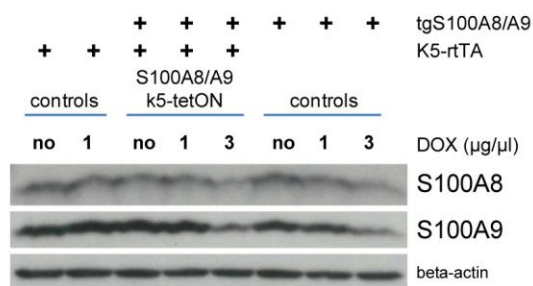


Fig. 21 – No S100A8 and S100A9 protein elevation was detected in epidermis and isolated keratinocytes of DOX-treated S100A8/A9-k5-tetON mice.

(A-C) Western blots analysis of tail epidermis, backskin epidermis and/or spleen of S100A8/A9-k5-tetON mice and littermate controls treated with DOX from 7 week of age for 12 weeks (A) or 2-7 days (B) as well as primary keratinocytes induced with DOX for 24 hours (C) for S100A8 and S100A9 expression.

ing the tetON system at steady-state conditions in mouse skin. Although we were unable to observe changes on total S100A8 or S100A9 levels, we were able to detect presence of transgenic S100A8/A9 mRNA in tail epidermis of S100A8-A9 k5-tetON mice using qPCR with primers detecting only the transgene ($n \geq 5$, data not shown), indicating transcription of tgS100A8/A9.

Hence, we hypothesized S100A8/A9 mRNA and/or protein might be unstable and their levels tightly regulated without additional challenge. Therefore, we decided to stimulate the skin with TPA (12-O-tetradecanoyl-13-phorbolacetate), which not only causes inflammation but also induces S100A8 and S100A9 expression (68), to eventually obtain increased response of S100A8-A9 k5-tetON mice. After 3.5 weeks of DOX administration, which was started at 7 weeks of age, 100 μ l of acetone or 100 μ l of 0.1 μ M TPA dissolved in acetone was topically applied on freshly shaved backskin of S100A8/A9-k5-tetON and control mice (Fig. 22A). Strong elevation of S100A8 and S100A9 protein levels was observed in total backskin 24 and 96 hours after TPA treatment compared to untreated mice. However, no induction or increased stabilization of S100A8 and S100A9 expression was determined in S100A8/A9-k5-tetON versus control mice (Fig. 22B). Also, *in vitro* S100A8 and S100A9 levels in isolated, primary S100A8/A9-k5-tetON or control keratinocytes after DOX and/or TPA treatment remained unaltered (Fig. 22C).

Short time induction of mice with DOX in drinking water for 2 to 7 days, long time induction for 12 weeks, DOX administration start at different stages of mouse development (birth, weaning, in the resting phase of the hair cycle) to overexpress S100A8/A9 proteins stayed unsuccessful as well as additional stimulation with TPA. Additionally, isolated primary S100A8-A9 k5-tetON or S100A8-A9 rosa-tetON keratinocytes did not show upregulation of S100A8 and S100A9 upon DOX and/or TPA addition to culture media compared to controls. Therefore, we were unable to test the effect of ectopic S100A8 and S100A9 expression in mouse skin and keratinocytes.

In summary, transgenic mice carrying a tetO-controlled S100A8-IRES-S100A9-Myc knock-in allele together with a ROSA-rtTA or K5-rtTA did not show a consistent increase of protein levels of S100A8 and S100A9 in the epidermis upon DOX administration. Although, S100A8 and S100A9 expression was inducible by DOX treatment of tgS100A8/A9^{KI/+} ROSA-rtTA^{T/+} mouse embryonic stem cell clones (H. Schönthaler, data not shown) and the transgene was properly integrated (Fig. 19A).

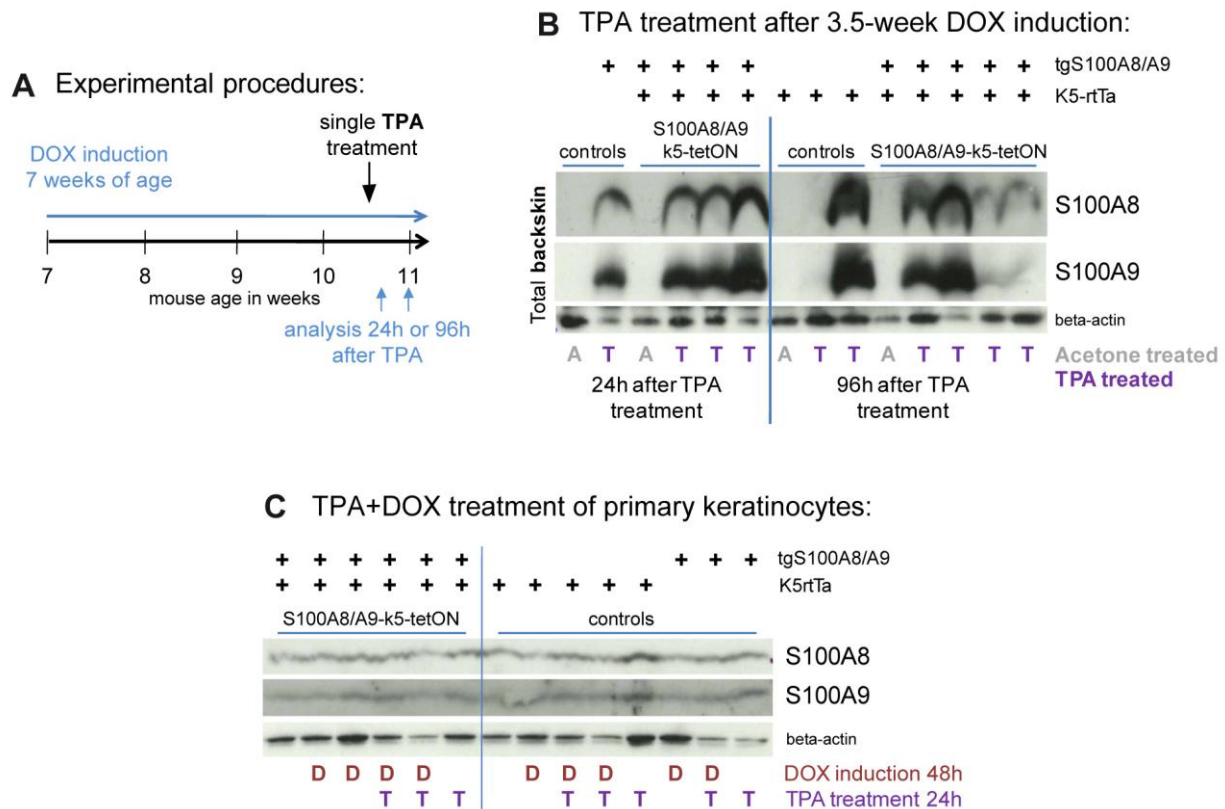


Fig. 22 – Treatment with TPA does induce S100A8 and S100A9 expression to the same extend in the epidermis of DOX-treated control and S100A8/A9-k5-tetON mice.

(A) Schematic representation of mouse treatment. Mice received DOX in drinking water from 7 weeks of age, one local TPA treatment at 10.5 weeks of age and were sacrificed and analyzed 24 or 96 hours later.

(B-C) Western blot of total backskin of control and S100A8/A9-k5-tetON mice (B) or primary keratinocytes *in vitro* (C) treated with DOX and TPA for S100A8 and S100A9.

4. Discussion

In my diploma thesis I made use of the Jun/AP-1 psoriasis mouse model to prove that the genetic deletion of S100A9 leads to a global improvement of all disease symptoms. These include normalization of keratinocyte differentiation and proliferation *in vivo*, in addition to diminished angiogenesis and a reduced immune response at late disease stages. In addition, systemic levels of pro-inflammatory cytokines in serum, as well as the number of skin-invading immune cells were markedly decreased (Fig. 23B). These

results suggest S100A9 to be a key factor in disease progression in the Jun/AP-1 psoriasis mouse model, but possibly also in psoriasis.

Epidermal keratinocytes of DKO* mice become activated upon JunB and c-Jun deletion and exhibit a hyperproliferative state most likely due to an extrinsic feedback loop involving other cell types, causing pronounced thickening of the epidermis. Differentiation of keratinocytes *in vivo* is largely abnormal, S100A8 and S100A9 are upregulated in a cell autonomous manner (1) and they secrete several other pro-inflammatory factors and chemokines. An immune response is induced and initially granulocytes invade the skin, followed by other innate and adaptive immune cells. Approximately three weeks after disease initiation, DKO* mice exhibit a full blown skin inflammation with dermal innate immune cell infiltrates including macrophages, granulocytes and mast cells as well as increased Langerhans/dermal dendritic cell numbers, epidermal neutrophils and T lymphocytes. DKO* mice also display a pronounced inflammation at the molecular level by elevated expression of inflammatory factors including S100A8 and S100A9 (1) and psoriasis associated genes as well as a systemic pathologic state with notably elevated serum cytokine/chemokine levels (Fig. 23A). Overall, our detailed analysis further supports the disease developed in the Jun/AP-1 psoriasis mouse model as being highly reminiscent of psoriasis.

Interestingly, activation of keratinocytes by JunB and c-Jun deletion appears to be independent of S100A9. This is indicated by comparable numbers of MHCII-positive cells in the skin of DKO* and TKO* mice at early time points of disease initiation as well as a highly similar cytokine/chemokine secretion profile of deleted DKO* and TKO* keratinocytes *in vitro*. However, upregulation of LIF and RANTES in DKO* keratinocytes (previously described for human psoriatic lesions ((96) and (58)) appears impaired in TKO* keratinocytes in a cell autonomous, S100A9-dependent manner. RANTES is a known chemoattractant and activating factor for blood monocytes, T lymphocytes as well as granulocytes ((97), (98), (99) and (100)). Hence, S100A9 might not only exhibit a chemotactic, pro-inflammatory role by itself ((26) and (24)), but also directly induce additional factors, like RANTES, in keratinocytes.

Despite S100A9 having immunoattractant properties, the observed attenuation of almost all examined pathologic characteristics of the Jun/AP-1 psoriasis mouse model upon loss of S100A9 can hardly be explained by impaired recruitment of immune cells to the skin. During disease initiation, five days after tamoxifen-mediated epidermal

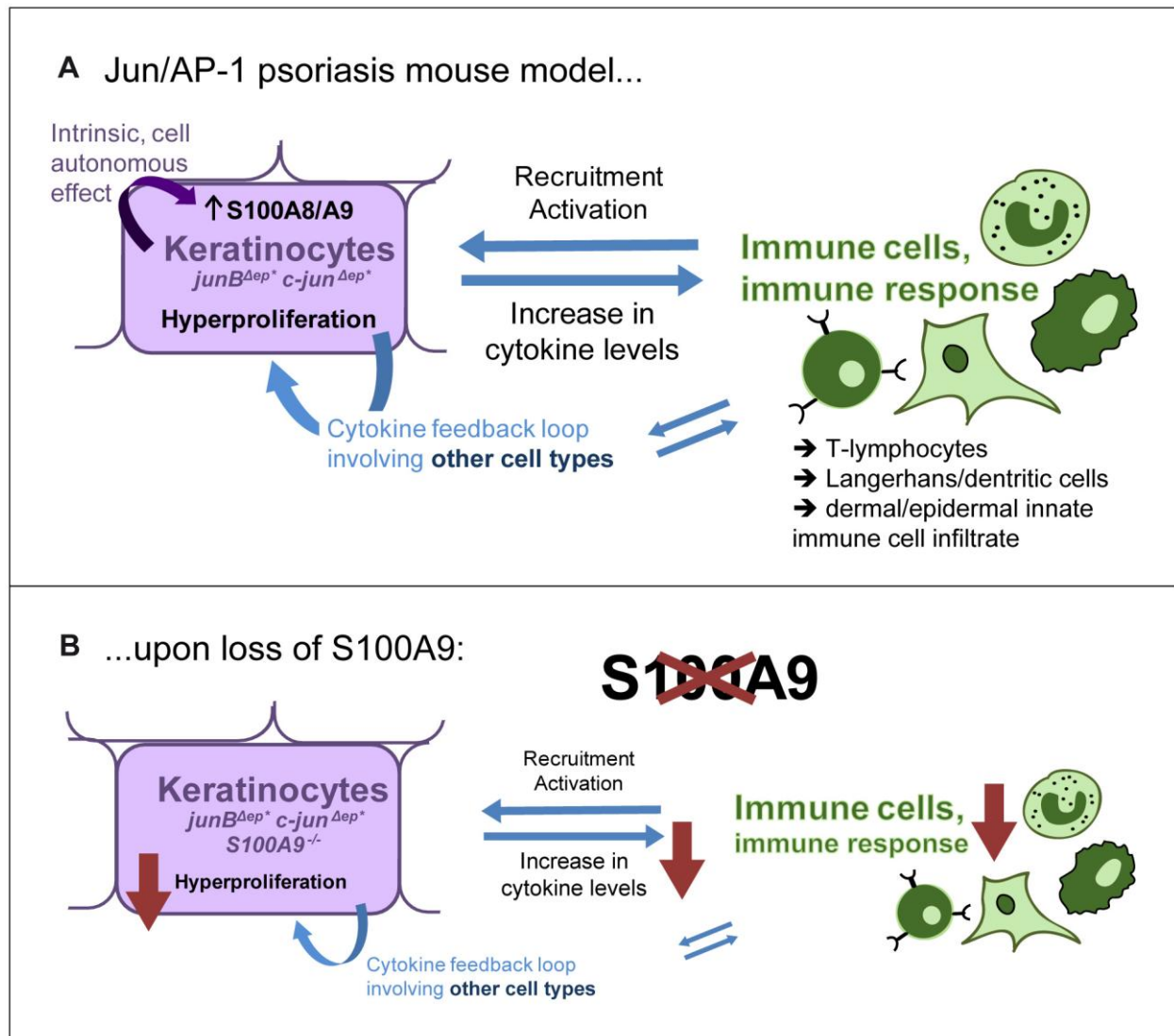


Fig. 23 – Summary of the effects of S100A9 in the Jun/AP-1 psoriasis mouse model.

Pronounced epidermal proliferation and inflammation in DKO* (A) and reduced pathogenesis (B) in TKO* mice.

JunB/c-Jun deletion, granulocytes readily infiltrate the skin of both, DKO* and TKO* mice. Moreover, all tested subsets of immune cells are present in the dermis and epidermis of TKO* mice at later disease stages, albeit to a lesser extent when compared to the Jun/AP-1 psoriasis mouse model. Cellular stress caused by the hyperproliferative state of keratinocytes and cutaneous immune cell infiltration in DKO* and TKO* mice may explain the increase of apoptotic cells in the skin. A partial p53-dependent apoptosis was shown to be induced by upregulated S100A9 in oesophageal squamous cell carcinoma and abrogated by S100A9 knockdown (43). How-

ever, a similar mechanism in our system is unlikely, since the number of apoptotic cells was unaltered in the epidermis of TKO* compared to DKO* mice. We observed a significantly elevated number of apoptotic cells in the dermis of TKO* mice, which we suspect to be immune cells. Thus, S100A9 might prevent their death, what could also explain a reduced immune cell presence in the skin of the Jun/AP-1 psoriasis mouse model upon loss of S100A9.

Detailed molecular characterization of an extended number of selected genes, confirmed that the mRNA expression pattern in the skin of DKO* compared to control mice is highly similar to human psoriatic skin (1). Consistent with the observed skin phenotype (Fig. 2B), loss of S100A9 in DKO* mice leads to a reduced inflammatory state of the skin on a molecular level as well. Additionally, S100A9 homozygous mutation appears to have only minor effects on the molecular phenotype of healthy skin of S100A9^{-/-} versus control mice. Hence, our results suggest a promoting effect of S100A9 on psoriasis severity on a molecular level; therefore, S100A9 represents a promising candidate for regulation of psoriasis pathogenesis.

Contribution of S100A9 to the interplay between epidermal keratinocytes and skin-invading immune cells appears of crucial importance in psoriasis pathogenesis. Deletion of JunB and c-Jun in keratinocytes in mice is sufficient to induce their activation, which is further followed by an immune response and causes a psoriasis-like phenotype *in vivo* (1). In contrast, *in vitro* deleted DKO* keratinocytes are not hyperproliferative and DKO* and TKO* keratinocytes appear surprisingly similar. Also, immune cell infiltration into the skin of DKO* and TKO* mice at initial stages of disease development (5 days after the last tamoxifen injection) is indistinguishable. However, it remains to be shown in which cell type in the network of skin inflammation – keratinocytes, several immune cells or certain subsets – does S100A9 play its crucial role. *In vitro* systems of S100A9 sufficient or deficient cell types or co-cultures of keratinocytes and immune cells provide only limited potential, because disease progression and a systemic contribution are not reproducible. Bone marrow transplantations of DKO* mice receiving TKO* bone marrow and vice versa is an experimental approach to address this question.

Due to the strong upregulation of S100A8 and S100A9 in human psoriatic skin and in the Jun/AP-1 psoriasis mouse model, we aimed to test whether overexpression of both proteins is sufficient to reproduce an inflammatory state in mouse skin. Unfortunately, total S100A8 and S100A9 mRNA and protein levels are not upregulated in the epider-

mis of mice carrying an inducible Tet-ON system-controlled transgene for S100A8 and S100A9 (tgS100A8/A9) upon DOX application. This observation was rather surprising, since proper integration of the tgS100A8/A9 construct in mouse ES cells (H. Schönthaler and G. Beranger, data not shown) and transgenic mice (Fig. 19A) was confirmed by Southern blot. DOX treatment induced ROSA rtTA-driven upregulation of S100A8 and S100A9 mRNA in tgS100A8/A9 mouse ES cells (H. Schönthaler, data not shown) and protein levels in 293 fibroblasts transfected with the same construct (J. Nehmet and P. Angel, data not shown). Moreover, we detected mRNA transcribed from the S100A8/A9 transgene in the epidermis of DOX-induced S100A8/A9-rosa-tetON and S100A8/A9-k5-tetON mice and in *in vitro* cultured keratinocytes, but not in untreated or control mice and cells. Thus, total S100A8 and S100A9 mRNA levels might be tightly controlled by microRNAs or other cellular mechanisms in keratinocytes without an inflammatory stimulus. Additional TPA-mediated stimulation of S100A8 and S100A9 expression did not result in increased mRNA/protein levels in DOX-induced S100A8/A9-rosa-tetON and S100A8/A9-k5-tetON mice and keratinocytes compared to controls. TgS100A8/A9 mRNA could be unstable and may degrade, have a negative effect on endogenous S100A8 and S100A9 mRNA transcription/stability or Tet-ON system-mediated expression of tgS100A8/A9 might not be too inefficient in keratinocytes. Therefore, I was unable to draw any conclusions from these experiments.

Nevertheless, S100A9 loss of function studies in an inflammatory background revealed S100A8 and S100A9 proteins as potent mediators of skin inflammation and psoriasis. S100A9 deficiency leads to remarkably diminished disease pathogenesis in the Jun/AP-1 psoriasis mouse model (H. Schönthaler) on a macroscopic, histological and molecular level. Thus, S100A8/A9 proteins might represent promising targets for future psoriasis treatment with potentially low side effects. Epidermal expression of both proteins is relatively low under steady-state conditions (38) and S100A9 loss in mice appears not to affect healthy skin, as S100A9^{-/-} mice show no apparent skin phenotype. Polyclonal antibodies have been successfully used to block S100A8 and S100A9 in mice ((101), (102) and (103)). Testing the effect of systemic or topical application of neutralizing antibodies against S100A8 and/or S100A9 on disease initiation and progression in psoriasis mouse models represents a suitable approach for development of prospective therapies.

5. Materials and Methods

Mouse strains and treatment of mice (H. Schönthaler)

S100A9 loss of function approach: Mice carrying a disrupted allele of S100A9 (32), loxP-site flanked JunB and c-Jun alleles as well as the tamoxifen-inducible Keratin5-CreER^T transgene (1) and their littermate controls were used in this study. Eight-week old mice were injected intraperitoneally with 1 mg tamoxifen (Sigma) dissolved in 100µl Sunflower seed oil per day for 5 consecutive days to trigger Jun protein deletion and analyzed either 5 days (flow cytometry analysis) or around 2 weeks after the last tamoxifen injection.

S100A8/A9 gain of function approach: Mice with a tet-operator controlled S100A8-IRES-S100A9-Myc transgene (H. Schönthaler in collaboration with P. Angel, DKFZ Heidelberg, G.Beranger and L.Bakiri) as well as ROSA-rtTA or Keratin5-rtTA and littermate controls were given 0.5 gram per liter doxycycline in drinking water for the indicated time period before sacrifice. TPA experiment: Transgenic and control mice at 7 weeks of age were induced with doxycycline for 3.5 weeks followed by one-time application of 100µl of 10⁻⁴M TPA in acetone on the freshly shaved, unwounded backskin. Analysis was done 24 or 96 hours after TPA treatment.

Keratinocyte isolation and treatment, EdU incorporation assay and Immunocytofluorescence

Ear and tail skin was disinfected with Betaisodona®, epidermis and dermis separated by Trypsin incubation and epidermis further digested with DNaseI (Sigma). Suspensions were filtered through a 70µm cell strainer and cells plated on culture dishes coated with Coating Matrix Kit (Invitrogen). Keratinocytes were cultured for 24 hours in normal MEM (minimal essential medium) supplemented with chelated FCS, Glutamine, Gentamycin, Penicilin as well as Streptavidin and subsequently the medium was changed to Keratinocyte Serum-Free Medium (Gibco).

Adenovirus treatment: Primary keratinocytes were incubated with 600 particles per cell of adenovirus containing Cre recombinase (adenoCre) or GFP (adenoGFP, provided by J. Guinea-Viniegra) for the indicated time period (mostly for 96 hours).

Doxycycline and TPA treatment: Doxycycline (1µg/ml) was added to culture medium for 4 to 72 hours depending on experiment. TPA was used in a concentration 50ng/ml for

24 hours. Subsequently, cells were either used for EdU incorporation assay or supernatant was collected for another 24 hours for cytokine array/ELISA analysis and the keratinocytes harvested to isolate mRNA and protein.

EdU incorporation assay: A Click-iT™ EdU Flow Cytometry Assay Kit with Alexa Fluor 647 (Invitrogen Molecular Probes) was used and 10 µM Click-iT EdU (5-ethynyl-2'-deoxyuridine) added to keratinocytes for 60 minutes to 6 hours, depending on the proliferative potential of the cells due to the time they were already cultured. Keratinocytes were harvested and processed according to manufacturers' instructions, Click-iT EdU was detected by click chemistry reaction of an alkyne (EdU) and an azide (Alexa Fluor 647 dye) catalyzed by copper, as described in the Click-iT™ manual from Invitrogen. DAPI (4',6-diamidino-2-phenylindole) was used to determine DNA content and a FACS Canto flow cytometer (BD Pharmingen) and BD FACSDiva software for analysis of labeled cells. Keratinocytes which were not incubated with EdU served as negative staining controls.

Immunocytofluorescence: Keratinocytes were plated on Coating Matrix Kit (Invitrogen) coated glass coverslips, incubated with adenovirus for 72 hours and fixation performed with 4% paraformaldehyde (PFA). Then, cells were treated with Glycine, permeabilized with ice-cold Methanol and unspecific background staining blocked by the blocking agent of the Mouse on Mouse kit (Vector Labs). Incubation with primary c-Jun antibody (mouse, BD Pharmingen) and, afterwards, with Alexa Fluor 647 coupled secondary anti-mouse antibody (Invitrogen) was performed and nuclei stained with DAPI. Cells on coverslips were mounted upside down in ProLong Gold antifade reagent (Invitrogen) and analyzed with a Nikon fluorescence microscope.

Tissue stainings, Immunohistochemistry and Immunofluorescence

After sacrifice, mouse ears and/or backskin and spleen were collected and embedded in paraffin and/or frozen in optimal cutting temperature (OCT) medium (Tissue-Tek). Tissue for paraffin processing was fixed in 3.7% PFA in 1x phosphate buffered saline (PBS) on 4°C overnight. Tissue dehydration was performed from ethanol, xylene to paraffin in a step-wise process using a tissue processor. Subsequently, 5µm sections were cut with a microtome, dried at 50°C overnight and step-wise re-hydrated with xylene and ethanol according to standard procedures before staining. Cut tissue sections were incubated with Haematoxylin (5-10 minutes) and Eosin (1-5 minutes; H&E) stain or

with Toluidine Blue O solution (Sigma, 3 minutes), dehydrated and mounted with a xylene-based medium. TUNEL-labelling of apoptotic cells on paraffin sections employed the *In Situ* Cell Death Detection Kit (Roche) after Proteinase K-mediated antigen retrieval. Cell nuclei were subsequently stained with DAPI (4',6-diamidino-2-phenylindole). Immunohistochemistry (IHC): Antigen retrieval of tissue sections cut from paraffin blocks was performed by heating to 121°C for 10-20 minutes in citrate buffer by a pressure cooker. Hydrogen peroxidase (3%, 5min) and 10% goat serum/1xPBS were used as peroxidase and protein block, respectively. The blocking agent of the Mouse on Mouse kit (Vector Labs) was employed before incubation with primary antibodies raised in mouse. Primary antibody was diluted in 10% goat serum/1xPBS and applied to the tissue for 2 hours at 37°C or overnight at 4°C. Secondary anti-mouse, anti-goat and anti-rabbit antibodies in combination with streptavidin peroxidase and the substrate-chromogen solution of the LSAB+ (Labeled Streptavidin Biotin) System-HRP kit (Dako) were used for immunohistochemical labeling according to manufacturers' instructions. Visualization of cell nuclei was performed with haematoxylin. IHC on mouse ears was performed for Keratin5, Keratin6, Keratin10, Loricrin (all rabbit, Covance Research), Ki67 (rat, Dako), phospho-Histone H3 Serine 10 (rabbit, Upstate Technology), Gr.1 (rat, BD Pharmingen), F4/80 (rat, Santa Cruz), CD3e (goat, Santa Cruz), S100A4 (rabbit, Abcam) and phospho-STAT3 (rabbit, Cell Signaling). Analysis employed a Zeiss light microscope and respective software.

Immunofluorescence (IF): Processing of paraffin sections for IF was performed as described above, without peroxidase blocking. Frozen sections of 5µm were cut using a cryostat, fixed in 4% PFA/1xPBS (10 minutes) and treated with a protein block solution (bovine serum albumin, gelatine, TritonX, goat and donkey serum in 1xPBS) for one hour. Subsequently, tissue was incubated with primary antibodies diluted in protein block solution for 2 hours at 37°C or overnight at 4°C. Alexa Fluor 488 or Alexa Fluor 647 coupled secondary antibodies (mouse, rat or rabbit, Invitrogen) were used for detection of Keratin15 (mouse, Neomarker Antibodies), CD31 (rabbit, Abcam), LYVE-1 (rabbit, Abcam), MECA32 (rat, BD Pharmingen), CD4 (rat, BD Pharmingen), CD8 (rat, Chemicon) and MPO (rabbit, Dako). No secondary antibody was necessary for visualization of PE-coupled MHCII (rat, BD Pharmingen) and Alexa Fluor 488-coupled Langerin (rat, Dextrics) reactive product. DAPI was utilized to label nuclei, ProLong Gold an-

tifade reagent (Invitrogen) for mounting of immunofluorescence stainings and a Nikon fluorescence microscope for analysis.

Quantification of labeled cells by IHC or IF on 6 randomly taken images per mouse (10x or 20x magnification, as indicated) was done manually, supported by the ImageJ software.

Western blotting and quantification

Skin was incubated swimming on 0.8% Trypsin without EDTA in 1xPBS for 45min at 37°C to separate dermis and epidermis. RIPA buffer with added phosphate inhibitor was used to isolate total protein from spleen, total skin, epidermis, dermis or keratinocytes from *in vitro* cultures. Proteins were separated on 10-15% polyacrylamid gels, blotted on nitrocellulose membranes and blocked for 30 min in 5% milk powder in 1xPBS/0.1% Tween20. Primary antibodies against S100A8, S100A9 (both goat, R&D), p53, phospho-p38 MAPK (both rabbit, Cell Signaling), JunB (rabbit, Santa Cruz), c-Jun (mouse, BD Pharmingen) or β -actin (mouse, Sigma) were used. Detection was done with Amersham ECL Plus™-HRP linked secondary antibodies and western blotting detection reagents according to manufacturer's instructions on light-sensitive films. Protein band intensity was quantified with Quantity One 4.6.5 software. Control was set to one and relative values are shown.

Quantitative real-time PCR

TRIzol (Invitrogen) was used for total RNA isolation from tail epidermis after Trypsin-mediated epidermis/dermis separation according to the manufacturer's protocol. cDNA was synthesized following the instructions with the Ready-To-Go™ You-Prime-It First-Strand-Beads (GE Healthcare) and random primers (Invitrogen). Real-time PCR reactions were performed in the presence of SYBR green with 5-PRIME Taq DNA Polymerase and respective reagents with the Mastercycler® ep-realplex (Eppendorf). The Ct (cycle threshold)-value was taken at the narrow range of PCR cycles where PCR product is doubled per cycle and melting curve for every PCR reaction and corresponding product was analyzed. Optimized primers for target genes have been designed using the Primer-BLAST software and quantification of PCR products employed the realplex analysis software and the $2^{-\Delta\Delta C_T}$ analysis method. All data were normalized based on the

mRNA expression levels of β -actin of the sample. Control was set to one and relative values or fold change are shown.

Cytokine array analysis and ELISA

Blood was taken from mice at time of sacrifice by heat puncture and collected in EDTA-containing blood collection tubes. Medium of 96 hours adenovirus-exposed primary keratinocyte cultures was changed and the supernatant collected after another 24 hours. Solid components of blood and 24h-conditioned medium were removed by centrifugation to yield serum and clean keratinocyte supernatant, respectively.

Cytokine array analysis employed the Milliplex MAP Mouse Cytokine/Chemokine Panel I, II and III, was performed according to manufacturers' instructions and evaluated using the Luminex 200 instrument and Luminex/Milliplex analyst software. Colorimetric ELISA kits for IL-1 α , IL-6, MCP-1 or VEGF detection were purchased from R&D systems, utilized according to the manual and resulting optical density measured.

Flow cytometric analysis and skin cell isolation

Cells were isolated from total ear and tailskin by digestion with Liberase (Roche) and DNase I (Sigma) in HANKS buffer (one hour at 37°C) five days after five daily tamoxifen injections (Fig. 9A). Cells were filtered and protein blocking done with 10%FBS/1xPBS (30 minutes on 4°C) as well as with Fc Block antibody anti-CD16/CD32 (5 minutes on 4°C, BD Pharmingen). Per individual staining, 10⁶ cells were incubated (30 minutes on 4°C) with two combinations of fluorochrome-conjugated antibodies CD3e-APC, CD4-PE-Cy7, CD8-FITC, B220-PE, MHCII-Alexa Fluor 700 as well as CD11b-PERCP-Cy5.5, Gr.1-PE-Cy7, CD11c-PE and MHCII-Alexa Fluor 700 (all from BD Pharmingen, except MHCII from eBioscience) diluted in 1%BSA/1xPBS. Stainings were analyzed with a FACS Canto flow cytometer (BD Pharmingen), BD FACSDiva and FlowJo software. Every analysis was gated on single and alive cells, identified by forward and side scatter (FSC and SSC) as well as DAPI-labeling of cell nuclei, except Fig. 9O was gated on alive, single and CD3⁺ cells. Positively labeled cells were determined by comparison with single antibody-stainings and staining-panel combinations missing one antibody.

Southern blot

10µg of genomic tail DNA was digested with SpeI enzyme (Roche), separated on a 1% agarose gel, transferred to a genescreen membrane and cross-linked with UV light. Hybridisation was done with a 3' probe as described in (95) labeled with the Amersham Rediprime™ II DNA Labeling System (GE Healthcare) according to manufacturers' instructions and detection performed with autoradiography films (GE Healthcare).

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7. Annex

7.1. Curriculum vitae

1. Personal data

First names: Stefanie Kristin

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2. Education

Description	Location	Major fields of study	Educational Institution	Years of study
Diploma studies part 2	Austria	Developmental biology, Cell biology, Immunology/Microbiology	University of Vienna	from March 2009
Diploma studies part 1	Austria	Molecular biology	University of Vienna	Oct. 2002 – March 2009

3. Research training

2009/05/15 – 2010/09/30

Position: Diploma student

Institute: Spanish National Cancer Research Centre (CNIO), Madrid, Spain
<http://www.cnio.es>

Research group: Dr. Erwin Wagner; Genes, Development and Disease group of the Cancer Cell Biology Programme

Supervisor: Dr. Helia Schönthaler

Research project: The role of S100A8 and S100A9 proteins in psoriasis-like skin disease.

2008/10/16 – 2009/04/30 and 2010/10/01 – 2011/05/31

Position: Lab assistant, student technician (part-time)

Institute: Institute of Cancer Research of the Medical University of Vienna, Vienna, Austria
<http://www.meduniwien.ac.at>

Research group: Dr. Maria Sibilía, Cellular and Molecular Tumorbiology

Supervisor: M.Sc. Barbara Drobits and Dr. Hanane Lanaya

Research project: Molecular analysis of the anti-tumor immune response of Imiquimod and analysis of the immune cell composition of the bone marrow in AP-1 member-deficient mice.

2008/07/15 – 2008/09/25

Position: Trainee for the special subject Immunology/Microbiology

Institute: Spanish National Cancer Research Centre (CNIO) Madrid, Spain

<http://www.cnio.es>
Research group: Dr. Erwin Wagner; Genes, Development and Disease group of the Cancer Cell Biology Programme
Supervisor: Dr. Helia Schönthaler
Research project: Analyzing the chemoattractant S100A8 and S100A9 proteins in a mouse model for psoriasis skin disease.

2007/11/15 – 2008/06/30

Position: Lab assistant, student technician (part-time)
Institute: Institute of Molecular Pathology (IMP), Vienna, Austria
<http://www.imp.ac.at>
Research group: Dr. Erwin Wagner, Gene Function in Mammalian Development and Disease
Supervisor: Dr. Helia Schönthaler
Research project: The role of VEGF in psoriasis-like skin disease.

2007/03/05 – 2007/09/01

Position: Trainee for the special subject Developmental Biology
Institute: Institute for Research in Immunology and Cancer (IRIC) of the University of Montreal, Montreal, Quebec, Canada
<http://www.irc.ca>
Research group: Dr. Gregory Emery, Vesicular Trafficking and Cell Signaling
Supervisor: Dr. Gregory Emery
Research project: The impact of endocytosis (esp. recycling) on cell migration, using mouse cell culture and the *in vivo* model *Drosophila*.

2006/06/01 – 2007/02/01

Position: Trainee for the special subject Cell Biology
Institute: Institute of Molecular Biotechnology (IMBA) of the Austrian Academy of Sciences, Vienna, Austria
<http://www.imba.oeaw.ac.at>
Research group: Dr. Jürgen Knoblich, Asymmetric Cell Division and Proliferation Control
Supervisor: Dr. Jörg Betschinger
Research project: Proliferation control of *Drosophila* neural stem cell-like cells by several signalling pathways.

2005/10/15 – 2007/02/14

Position: Lab assistant, student technician (part-time)
Institute: Institute of Molecular Biotechnology (IMBA) of the Austrian Academy of Sciences, Vienna, Austria
<http://www.imba.oeaw.ac.at>
Research group: Dr. Jürgen Knoblich, Asymmetric Cell Division and Proliferation Control

2005/09/01 – 2005/10/01

Position: Practical training
Institute: Department of Bioresources - Platform for Integrated Clone Management (PICME) of the Austrian Research Center Seibersdorf, Seibersdorf, Austria
<http://www.bioresources.at/default.asp?id=155&lid=1>
Head of Department: Mag. Silvia Fluch
Supervisor: Dr. Maria Berenyi
Research project: Cloning and amplifying of plant cDNA libraries in E.Coli

4. Publications

Paper in preparation: Schonthaler HB, Ruppen M-I, **Wculek SK**, Guinea-Viniegra J, Ashman K, & Wagner EF "Psoriasis development is triggered by S100A9-dependent regulation of the complement component C3."

Assaker G, Ramel D, **Wculek SK**, González-Gaitán M, & Emery G (2010) "Spatial restriction of receptor tyrosine kinase activity through a polarized endocytic cycle controls border cell migration." *Proceedings of the National Academy of Sciences of the United States of America (PNAS)* 28;107(52).

Schonthaler HB, Huggenberger R, **Wculek SK**, Detmar M, & Wagner EF (2009) "Systemic anti-VEGF treatment strongly reduces skin inflammation in a mouse model of psoriasis." *Proceedings of the National Academy of Sciences of the United States of America (PNAS)* 106(50).

5. Poster presentations & Meeting attendance

1st International PhD Workshop "You Give Me Fever", Current Topics in Inflammation, Vienna, Austria, 14-15 May, 2009

IMP - 20 years anniversary conference, Vienna, Austria, 15-16 May, 2008

Cell, Molecular and Developmental Biology Research Retreat, Ste-Adèle, Quebec, Canada, 11-13 June, 2007

Poster: "A functional recycling endosome is necessary for cell migration *in vivo*."

IMP & IMBA Recess 2006, Vienna, Austria, 4-6 October, 2006

11th Regional Drosophila Meeting, Leipzig, Germany, 2-3 December, 2005

Congress „frauen | gesundheit | männer | krankheit – gendermedizin in theorie und praxis“ ("women | health | men | illness – gender medicine in theory and practice"), Salzburg, Austria, 29-30 October, 2004

6. others

Professional Experience: Practical experience in genetic, molecular biological and biochemical tools to analyze tissues and cells from transgenic mouse models.

Technical Skills (abstract): Experience in working with mice, flies and zebrafish; Cloning; semi-quantitative and quantitative RealTime-PCR reactions; Western blotting; Southern blotting; FACS analysis; cell culture and primary cell culture; isolation of primary cells from tissues; histology; immunofluorescence; production of transgenic flies by microinjection; fluorescence and confocal microscopy.

Languages: Fluent in English and German (mother tongue), basic knowledge of Spanish and French

7.2. Abstract in German language / Deutsche Zusammenfassung

Das Hauptaugenmerk meiner Diplomarbeit lag auf der Untersuchung der Relevanz von S100A8- und S100A9-Proteinen bei Entstehung und Verlauf von entzündlichen Hautkrankheiten, im Besonderen Psoriasis (Schuppenflechte). Beide Proteine sind in der Haut von Psoriasis-Patienten und der Haut des Jun/AP-1-Mausmodells für Psoriasis - hervorgerufen durch die induzierbare, epidermale Deletion der zwei AP-1-Proteine JunB und c-Jun (JunB^{Δep*} c-Jun^{Δep*}- oder DKO*-Mäuse, (1)) - deutlich überexprimiert. Die Induktion von S100A8 und S100A9 in Mäusen wurde bereits vor dem Auftreten von Krankheitssymptomen festgestellt (1). Daher stellt die Analyse der psoriasis-ähnlichen Krankheit bei JunB^{Δep*} c-Jun^{Δep*}-Mäusen eine vielversprechende Möglichkeit zur Untersuchung der Funktion von S100A8 und S100A9 dar. Bemerkenswerterweise reduziert S100A9-Defizienz den Schweregrad des inflammatorischen Phänotyps des Jun/AP-1-Mausmodells wesentlich (H. Schönthaler, unpublizierte Beobachtung).

Bei den zwei Zelltypen, die an der Entstehung von Hautentzündungen hauptsächlich beteiligt sind, handelt es sich um epidermale Keratinozyten und eine Vielzahl an verschiedenen Immunzellen. Die Haut des Jun/AP-1-Mausmodells zeigt sowohl beträchtliche epidermale Verdickung bedingt durch Hyperproliferation der Keratinozyten als auch ausgeprägte Infiltrierung von Immunzellen in die Dermis und Epidermis (1). Um herauszufinden, ob dies durch die Deletion von JunB und c-Jun auf zellautonome Weise hervorgerufen wird, wurde Apoptose und Proliferation von Keratinozyten der DKO*-Mäuse *in vivo* und *in vitro* erforscht. Wir verglichen die Ergebnisse mit JunB^{Δep*} c-Jun^{Δep*} S100A9^{-/-} (TKO*)-Mäusen zur Feststellung des Effekts einer S100A9-Defizienz. Des Weiteren führten wir eine histologische und molekulare Charakterisierung der Haut dieser Mäuse durch und analysierten die Zusammensetzung des kutanen Immunzell-Infiltrates von DKO*- und TKO*-Mäusen in unterschiedlichen Krankheitsstadien. Untersucht wurden die Zytokin-Produktion in der Dermis und Epidermis, der systemische Zytokin-Gehalt im Serum sowie die Zytokin-Sekretion von kultivierten Keratinozyten des Jun/AP-1 Psoriasis-Mausmodells und der TKO*-Mäuse. Dies diente zur Feststellung der Funktion von S100A9 im Zusammenwirken von Keratinozyten und Immunzellen im Verlauf von Hautentzündungen und zur Identifizierung von S100A9-abhängigen Mediatoren dieses zellulären Netzwerks.

Wir vermuteten außerdem, dass Überexpression von S100A8 und S100A9 per se einen inflammatorischen Haut-Phänotyp hervorrufen könnte und analysierten Mäuse, welche ein Tet-ON-systemkontrolliertes Transgen für S100A8 und S100A9 tragen. Unglücklicherweise konnten wir nach Induktion der Expression des S100A8/A9 Transgenes durch zwei verschiedene Transaktivator-Linien keine konsistente Erhöhung der S100A8- oder S100A9-Proteinwerte in der Epidermis der Mäuse feststellen.

S100A9 scheint entzündliche, psoriasis-ähnliche Symptome in vielfältiger Weise zu induzieren. Hautarchitektur, Angiogenese und Expression von entscheidenden entzündungsassoziierten Genen werden großteils durch S100A9-Defizienz des Jun/AP-1 Psoriasis-Mausmodels normalisiert. Darüber hinaus zeigen TKO*-Mäuse verminderte dermale und epidermale Immunzell-Infiltrierung, reduzierte epidermale Proliferation und niedrigere Werte an inflammatorischen Zytokinen im Serum im Vergleich zu DKO*-Mäusen. Obwohl S100A8 und S100A9 in Keratinozyten infolge von JunB- und c-Jun-Deletion *in vitro* hochreguliert werden (1), hat dies keine zellautonome Auswirkung auf deren Proliferation. S100A9 hat auch keinen ausgeprägten Effekt auf die Sekretion von inflammatorischen Zytokinen durch Keratinozyten *in vitro*, nachdem sich die gemessenen Werte in den Kulturmedien von DKO*- und TKO*-Keratinozyten kaum unterscheiden. Daher scheint S100A9 eine komplexe Rolle im Zusammenwirken von Keratinozyten und Immunzellen zu spielen und außerdem eine Induzierung und Amplifikation des Schweregrades von Psoriasis und Hautentzündungsreaktionen zu bewirken.