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at the University Medical Center Schleswig-Holstein, Campus Kiel
at Christian-Albrechts-Universität zu Kiel - Kiel University

**Analysis of oxidative stress induction in fibroblasts and
fibroblast and keratinocyte crosstalk in a 2-dimensional
Atopic Dermatitis model**

Dissertation
to acquire the doctoral degree (Dr. med.)
at the Faculty of Medicine
at Christian-Albrechts-Universität zu Kiel – Kiel University

presented by
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Kiel (2022)

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Date of oral examination: 18.04.2023

Approved for printing, Kiel, 23.01.2023

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Abbreviations

| | |
|-------------------------------|---|
| a. u. | arbitrary units |
| AD | Atopic Dermatitis |
| CaCl ₂ | calciumchloride |
| cm | centimetre |
| CO ₂ | carbon dioxide |
| DABCO | 1,4-diazabicyclooctane |
| DAPI | 4',6'-diamidin-2-phenyleindol |
| DCF/DCFDA | 2',7'-dichlorofluorescin diacetate |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | dimethylsulfoxid |
| DNA | desoxyribonucleic acid |
| DPI | diphenyliodonium |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol bis(β-aminoethylether) tetraacetic acid |
| ERK | extracellular signal-regulated kinase |
| g | gravity of earth |
| Hsp | heat shock protein |
| HO ⁻ | hydroxide |
| H ₂ O | water |
| H ₂ O ₂ | hydrogen peroxide |
| IgG | immunoglobulin G |
| IL | interleukin |
| JNK/SAPK | s-jun N-terminal or stress-regulated protein kinase |
| KGM | keratinocyte growth medium |
| MAPK | mitogen-activated protein kinase |
| ml | milliliter |
| mm | millimetre |
| mM | millimolar |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| ng | nanograms |
| nm | nanometre |
| NOX | nicotinamide adenine dinucleotide phosphate oxidase |

| | |
|-----------------------------|---------------------------------|
| O ₂ ⁻ | superoxide ion |
| p | phosphorylated (used as prefix) |
| PBS | phosphated buffered saline |
| pH | potential of hydrogen |
| RIPA | radioimmunoprecipitation |
| ROS | reactive oxygen species |
| rpm | rounds per minute |
| TH | T-helper cell |
| TNF | tumor necrosis factor |
| w/o | without |
| μl | microliter |
| μM | micromolar |
| °C | degree Celsius |
| % | percent |

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1. Introduction

1.1. The human skin

The human skin is the most visible part of the human body and is at the front to get in touch with the environment. It is needed as a protection towards physical, mechanical or microbial influences from the outside but also to protect from loss of body water and other substances (Fritsch and Schwarz 2018). It functions as an interface between inside and outside. The skin is a place of perception as temperature gradients, pressure and physical damage can be felt (Gould 2018). It also works in the other direction as the skin is able to give information to other humans for example through physical signs as flush, sweating or pallor (Wong et al. 2016).

There are three different layers of the human skin and each has different functions. The epidermis is the most superficial layer, followed by dermis and subcutis (Gould 2018).

The epidermis consists mainly of keratinocytes which differentiate from stem cells to cornified cells. The physical barrier between the organism and the environment is primarily localized in the *stratum corneum* as the outmost layer of the epidermis and consists of cornified keratinocytes and lipid enriched intercellular domains (Jensen and Proksch 2009). Additionally, the keratinocytes are producing factors by which they play a role in promoting inflammatory reactions and by which they communicate with other cells like cells of the dermis and cells of the immune system. There is a wide variety of factors they can produce like interleukins and chemokines, growth factors, hormones, enzymes and antimicrobial peptides (Fritsch and Schwarz 2018).

The dermis consists of collagen and elastic fibres produced by fibroblasts and the space between these fibres is filled with glycosaminoglycans and proteoglycans (Fritsch and Schwarz 2018). The dermis can be divided into two parts which are *stratum papillare* and *stratum reticulare*. The *stratum papillare* is the outer one and consists of loosened collagen fibres and more elastic fibres whereas the *stratum reticulare* consists of more tightened collagen fibres and less elastic fibres. The extracellular matrix causes the skin to be tearproof and elastic (Lüllmann-Rauch and Asan 2019). The dermis is the place where nerves and blood vessels run along which provide a continuous exposure of immune cells and lymphocytes to the skin.

The subcutis consists of adipocytes and is supported by connective tissue. The adipocytes function as storage for fat and therefore energy but are also needed as a mechanical buffer, a

thermal isolator and for the release of endocrine mediators. In this part of the skin run nerves and blood vessels as well (Fritsch and Schwarz 2018).

1.2. Atopic Dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease. Its symptoms include intense itching, eczematous lesions and skin dryness which can affect any part of the body. There is a typical age-related distribution of the lesions. It affects up to a fifth of the population in developed countries and is the most common inflammatory skin disorder (Weidinger and Novak 2016). It mostly starts in childhood but is still existent in adults and is associated with an increased risk of immune-mediated inflammatory diseases like asthma or allergic rhinitis. Morphologic features are hyperlinearity of the palms and soles, Dennie-Morgan lines and Herthoge's sign (Weidinger and Novak 2016). Because of its visibility, AD has psychological as well as physical effects (Gould 2018) and can diminish quality of life (Fivenson et al. 2002). It is a disease with complex and not yet fully understood pathophysiological mechanisms, but it is clear that environmental and genetic factors, the microbiome, an impaired barrier integrity and immune dysregulation contribute to the development of AD (Malik et al. 2017). Known risk factors of developing AD are mutations leading to epidermal barrier defects, for example mutations of filaggrin which is an important structural protein in the epidermis and is the most significant genetic risk factor for the development of AD (McAleer and Irvine 2013). Filaggrin downregulation can lead to a disturbed corneocyte cellular matrix, reduced amounts of natural moisturizing factor components, increased permeability and impaired keratinocyte differentiation (Pendaries et al. 2014). Because of the barrier defects patients also have an increased susceptibility to allergens (Kim et al. 2016). Additionally, the skin of AD patients can be deficient of ceramides and antimicrobial proteins as cathelicidins (Kapur et al. 2018). Alterations of structural proteins, lipids of the *stratum corneum* and epidermal tight junctions lead to a diminished skin barrier function and are a major factor for transepidermal water loss, a decreased hydration and therefore an increased skin pH (Kezic et al. 2014). Another factor can be a deficient immune system, especially the innate immune system. In the lesions there is a predominantly T-helper-1 (TH-1) and T-helper-2 (TH-2) response and therefore a pro-inflammatory cytokine expression which leads to systemic inflammatory responses (Kapur et al. 2018). In acute lesions AD is associated with TH-2, T-helper-22 (TH-22) and also T-helper-17 (TH-17) cytokine activation while in chronic lesions TH-2 and TH-22 activation intensifies

with the appearance of a significant TH-1 component but not a complete switch (Leung and Guttman-Yassky 2014).

There are some known environmental risk factors besides genetic risk factors as well, for instance a western diet, living in urban regions or broad-spectrum antibiotic exposure, whereas an increased exposure to non-pathogenic microorganisms was reported to be protective (Flohr and Mann 2014). AD is also characterized by a frequent *Staphylococcus aureus* colonization (McAleer and Irvine 2013). Active atopic lesions show impairments of epidermal function with disturbed keratinocyte differentiation and a weakened barrier function with impaired keratinocyte cohesion and increased permeability for water and low weight molecules (Weidinger and Novak 2016).

In AD lesions there are higher levels of specific cytokines which function as mediators between the different cells. It could be shown that a set of Interleukin-22 (IL-22), Interleukin-4 (IL-4), Interleukin-13 (IL-13) and Tumor-necrosis factor α (TNF α), which are elevated in the lesions, are able to develop an AD like state of normal human epidermal keratinocytes in an in-vitro-model (Bernard et al. 2012).

It is known that fibroblasts are able to alter proliferation and differentiation of keratinocytes. Normal fibroblasts were shown to be able to lead to a higher proliferation and reduced stratification of AD keratinocytes. In contrast, fibroblasts from AD skin promote normal keratinocytes towards hyperproliferation and reduced expression of differentiation markers leading towards an atopic-like phenotype (Berroth et al. 2013).

Treatment of AD is still in need for more sophisticated and safe therapeutics. Before beginning systemic treatment the topical treatment including skin care should always be the first-line treatment option (Simon and Bieber 2014). Current standard therapy for AD includes moisturizing lotions and ointments, as well as topical corticosteroids and calcineurin inhibitors (Weidinger and Novak 2016). For severe cases the clinical efficiency of T-cell targeting therapeutics is proven while oral glucocorticoids and phototherapy still remain in use. However, these therapies last mentioned are not suitable for long term use due to multiple side effects (Brunner et al. 2017). In the german AWMF (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften e.V.) guidelines for the treatment of AD four different levels of therapy were proposed, depending on the state of the skin. In the first level there is supposed to be a basic topical therapy and skin care, in the second level topical glucocorticoids and topical calcineurin inhibitors are added. In the third level more potent glucocorticoids and calcineurin inhibitors are used and in the fourth level systemic immunomodulating therapies are used additionally (Werfel et al. 2009). Systemic immunomodulating therapies include oral

glucocorticoids as well as antibodies such as dupilumab, which is an IL-4 and IL-4/13 receptor antibody which blocks two key cytokines of the inflammation and helps to achieve healing or at least symptom relief of the eczematous lesions (Werfel et al. 2021). The efficacy of dupilumab is an example for the importance of the knowledge of signaling cascades participating in the development of AD as well as the knowledge of the complex interplay between different cell types and mediators. If more signaling cascades were known these could lead to the identification of new drug targets and therefore a more efficient AD therapy.

1.3. Oxidative stress

Oxidative stress arises from an imbalance between pro- and antioxidant species which results in molecular and cellular damage (Tan et al. 2018). Oxygen and reactive oxygen species (ROS) are able to oxidize nearly all biomolecules and damage their function (Okayama 2005). ROS are formed by the partial reduction of oxygen. Examples for ROS are O_2^- , H_2O_2 , and HO^\cdot (Bedard and Krause 2007). When there are ROS in a cell it is possible that chemical chain reactions develop and the modifications may spread (Reuter et al. 2010). The consequence of the elevated levels of ROS and therefore increased oxidative stress is cellular damage. This damage might picture itself in desoxyribonucleic acid (DNA) strand breaks or base changes as well as the modification of cytosolic proteins and their spatial structure and therefore a loss of function of organelles. Furthermore, ROS can lead to lipid peroxidation which results in loss of function of membrane lipids (Heinrich et al. 2014). As a guard against these consequences of oxidative stress cells have enzymatic and non-enzymatic protection mechanisms. Enzymes that remove reactive oxygen species are for example superoxidedismutase, catalase and glutathionperoxidase, whereas vitamin C and vitamin E are non-enzymatic molecules which protect the cell against oxidative damage (He et al. 2017). If the capacity of these mechanisms is exhausted, cells try to repair the damage of organelles or break down the affected proteins and lipids and synthesize new ones (Marnett 2000). ROS are continuously generated in low levels as a result of metabolism or can be actively formed by ROS-producing enzymes at sites of inflammation (Forrester et al. 2018). ROS work as messengers and take part in signaling systems as for example growth factor signaling, tyrosine kinase and other specific phosphatase signaling as well as they modify enzymatic activity (Finkel 2011). ROS can be produced on purpose by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, those NADPH oxidase derived ROS serve to ease or enable signal transduction and to maintain homeostasis (Schröder 2019). NADPH oxidases serve diverse functions such as mediation of inflammation

or host defense in case of infections, they modulate vascular reactivity and cellular signaling pathways as well as they help to maintain cellular homeostasis (Schröder 2019). Other enzymes such as the mitochondrial electron chain, cytochrome P450, monoamino oxidases and some more produce ROS as a byproduct (Andreyev et al. 2005, Bedard and Krause 2007). The human microbiome is also able to produce or induce ROS, whereas it is also a target of them in case of antimicrobial defense systems of granulocytes and macrophages (Egea et al. 2017). Another source of ROS is the environment. There, these can be generated by ultraviolet radiation, environmental pollutants and other hazardous chemicals (Ndiaye et al. 2014).

As an answer towards oxidative stress cells activate different intracellular pathways. One of them is the mitogen-activated protein kinase (MAPK) pathway (Xu et al. 2019). The mammalian family of MAP kinases includes the extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK) and the p38 group of protein kinases (Kim and Choi 2010). All of them play roles in cellular response to signals reaching the cell and activate distinct intracellular pathways resulting in the activation of transcription factors and gene expression (Ray et al. 2012). ERK is activated mostly through phosphorylation by growth factors and proinflammatory stimuli like ROS (Bickers and Athar 2006). Activation of ERK is associated with cell proliferation, differentiation, migration, senescence and apoptosis (Sun et al. 2015). P38 is activated by extracellular stimuli like ultraviolet light, heat, osmotic shock, inflammatory cytokines and growth factors. pP38 describes the phosphorylated and therefore activated form. P38 has various functions and promotes gene transcription as well as activation of other kinases. It leads to promotion of inflammation and has a role in apoptosis, slowing of the cell cycle and results in a senescent phenotype (Zarubin and Han 2005). Heat shock proteins (Hsp) function as molecular chaperones and are important for correct folding of proteins. Their expression is induced by environmental stress and they provide protection against the negative effect of cellular stress (Beck et al. 2000). Phosphorylated Hsp27 is an activated heat shock protein and its activation is mediated by pP38 signaling (Liu et al. 2016). The JNK cascade is activated by several forms of cell stress such as heat shock, radiation and oxidation. JNK also phosphorylates transcription factors such as P53 which is another point of convergence of ERK, P38 and JNK pathways (Khan et al. 2004).

Previous studies showed that AD is associated with elevated levels of reactive oxygen species and there have been efforts to identify biomarkers of this process. It is shown that levels of urinary 8-hydroxy-2'-deoxyguanosine, which is a marker of DNA oxidation and therefore oxidative stress, are significantly higher in AD patients than in a control (Omata et al. 2001). AD patients also have increased blood levels of malondialdehyde which is a marker of lipid

peroxidation, and decreased levels of enzymatic and non-enzymatic antioxidants which also suggests that oxidative stress plays a role in the pathophysiology of AD and that the patients are more susceptible to damage caused by ROS (Sivaranjani et al. 2013). Additionally, the levels of urinary nitrate and malondialdehyde which are both marker for oxidative stress, are not only higher in AD patients than in a control but also correlate with the severity of atopic dermatitis (Nakai et al. 2009).

In vitro models of AD demonstrated that AD-model keratinocytes exhibit elevated levels of ROS and accumulate more DNA damage than control cells after induced oxidative stress. These elevated levels of oxidative stress could be reduced by inhibiting NADPH oxidases which inhibits elevated stress signaling and promotes survival (Emmert et al. 2020).

1.4. Aims

Although many studies characterized the role of keratinocytes in AD, there is still little known about skin fibroblasts and their active role in AD. Especially the fibroblasts' reaction towards oxidative stress in AD and their stake in the disease is almost unexplored, so it is important to learn more about their participation in the development of AD. Thus, the reaction of fibroblasts towards oxidative stress shall be explored as well as the extent of this reaction after fibroblasts have been treated with an AD-specific cytokine mix before. Additionally, the consequences of oxidative stress towards fibroblasts shall be characterized.

Another point of interest is the interaction of fibroblasts and keratinocytes in AD in relation with their response towards oxidative stress. Furthermore, it would be interesting whether their reaction towards oxidative stress changes when they interact with the other cell type.

2. Materials

2.1. Chemicals, kits and consumables

| Chemicals | Manufacturer |
|--|--|
| Bovine serum albumin | Roth, Karlsruhe |
| Cryo SFM | Promocell, Heidelberg |
| DABCO (1,4-diazabicyclooctane) | Roth, Karlsruhe |
| DAPI (4',6'-diamidin-2-phenyleindol) | Sigma-Aldrich, USA |
| Dimethyl sulfoxide (DMSO) | Roth, Karlsruhe |
| Diphenyleneiodonium (DPI) | Santa Cruz Biotechnology, USA |
| Dulbecco's Modified Eagle Medium (DMEM) | Life Technologies, USA |
| Dulbecco's phosphated buffered saline (PBS) | Life Technologies, USA |
| EDTA (ethylenediaminetetraacetic acid) | Calbiochem, USA |
| EGTA (ethylene glycol bis(β -aminoethylether) tetraacetic acid) | Roth, Karlsruhe |
| Ethanol 70% | Roth, Karlsruhe |
| Fetal Calf Serum (FCS) | Capricorn, USA |
| Formaldehyde (37 %) | Roth, Karlsruhe |
| Goat serum | Sigma-Aldrich, USA |
| Hydrogen peroxide (H ₂ O ₂) 30% | Sigma-Aldrich, USA |
| Interleukin 4 | PeproTech, Hamburg, Lot # 041814I1018 |
| Interleukin 13 | PeproTech, Hamburg, Lot # 101723D1818 |
| Interleukin 17 | PeproTech, Hamburg, Lot # 041284D1218 |
| Interleukin 22 | PeproTech, Hamburg, Lot # 0102246A2816 |
| Keratinocyte Growth Medium Kit | Promocell, Heidelberg |
| Nonidet P-40 | US Biochemical Corporation, USA |
| Moviol | Merck, Darmstadt |
| Pen/Strep (Penicillin, Streptomycin) | Life Technologies, USA |
| Pierce ® 660 nm Protein Assay Reagent, 750 nm | Thermo Scientific, USA |
| Sodium dodecyl sulfate | Roth, Karlsruhe |
| Sodium deoxycholate | Sigma-Aldrich, USA |
| Sodium chloride | Roth, Karlsruhe |

| | |
|--|-------------------------------|
| Temed (N,N,N',N'-Tetramethylethylenediamine, 1,2-bis-(dimethylamino)-ethan) | Roth, Karlsruhe |
| Tris | Thermo Fisher Scientific, USA |
| Triton X 100 | Roth, Karlsruhe |
| Trypan Blue Stain (0.4%) | Life Technologies, USA |
| Trypsin EDTA Solution | Life Technologies, USA |
| Tumor necrosis factor alpha (TNF α) | PeproTech, Hamburg |
| Tween 20 | Sigma-Aldrich, USA |
| Water, distilled | Life Technologies, USA |

| Kits | Manufacturer |
|---|-----------------------|
| Anti-Goat Detection Module | ProteinSimple, USA |
| Anti-Mouse Detection Module | ProteinSimple, USA |
| Anti-Rabbit Detection Module | ProteinSimple, USA |
| DCFDA/H2DCFDA – Cellular Reactive Oxygen Species Detection Assay Kit | abcam, Great Britain |
| EZ Standard Pack 1 | ProteinSimple, USA |
| Fluorometric Cell Viability Kit I (Resazurin) | Promocell, Heidelberg |

| Consumables | Manufacturer |
|--|---|
| Biolite 100mm Tissue Culture Dish | Thermo Fisher Scientific, USA |
| Biosphere ® Filter tips (0.5-20µl/2-100µl/100-1000µl) | Sarstedt, Nümbrecht |
| Biosphere ® Safe Seal Tube (2ml/1.5ml/0.5ml) | Sarstedt, Nümbrecht |
| Cell scraper 16cm, 2-position blade, sterile | Sarstedt, Nümbrecht |
| Corning ® 175/75 cm ² Cell Culture Flask | Corning Incorporated, USA |
| Microscope Cover Glasses 13 mm Ø No 1 | Paul Marienfeld GmbH&Co.KG, Lauda- Königshofen |
| CryoPure-Tube | Sarstedt, Nümbrecht |
| Falcon®-tube 15ml/50ml | Sarstedt, Nümbrecht |
| Menzel-glasses Superfrost ® Plus | Thermoscientific, Schwerte |
| Multiply ®-Pro 0.2ml Biosphere | Sarstedt, Nümbrecht |
| Parafilm ‘M’ | Pechiney Plastic Packaging, USA |
| Serological pipettes, sterile 5ml/10ml/25ml | Sarstedt, Nümbrecht |

TC-Plate 96 Well Standard, F

Sarstedt, Nümbrecht

12 Well Cell Culture Tissue Cluster Costar ® 3513 Corning Incorporated, USA

2.2. Solutions and buffer

| Solutions and buffer | Manufacturer/Composition |
|--|--|
| Blocking solution for immunofluorescence | 1 % bovine serum albumin 10 % goat serum Solved in PBS |
| Cytokine mix | 40 ng/ml IL-4 40 ng/ml IL-13 40 ng/ml IL-22 10 ng/ml TNF α Solved in DMEM culture medium or KGM only with 0.06 mM CaCl ₂ |
| DMEM culture medium | 450 ml DMEM 50 ml fetal calf serum 5 ml penicillin/streptomycin |
| DPI solution | 25 mg diphenyleneiodonium 7947.86 μ l dimethyl sulfoxide |
| H ₂ O ₂ | H ₂ O ₂ solved 1:20000 in DMEM culture medium for fibroblasts H ₂ O ₂ solved 1:40000 in KGM culture medium only with 0.06 mM CaCl ₂ for keratinocytes |
| Interleukins 4/13/22 and TNF α | 100 ng/ μ l, 0.01% bovine serum albumin, in H ₂ O |
| KGM culture medium | 500 ml KGM 5 ml Pen/Strep 0.06 mM CaCl ₂ Supplements (Ingredients from kit) |
| Moviol/DABCO/DAPI | 1 ml Moviol 100 μ l DABCO 1 μ l DAPI |

| | |
|-------------------|------------------------------|
| RIPA-Lysis buffer | 10 mM Tris pH8 |
| | 1 mM EDTA |
| | 0.5 mM EGTA |
| | 1 % Triton-X-100 |
| | 0.1 % sodium deoxycholate |
| | 0.1 % sodium dodecyl sulfate |
| | 140 mM sodium chloride |
| | 50 µl Nonidet P-40 |

2.3. Cells

| Cells | Manufacturer |
|--------------------------------------|-------------------------------|
| Normal Human Epidermal Keratinocytes | Promocell, Lot number 407Z001 |
| Human Dermal Fibroblasts, neonatal | Gibco, Lot number 1998537 |

2.4. Antibodies

| Antibodies | Manufacturer |
|--|----------------------------------|
| Alexa Fluor 488 donkey-anti-rabbit | Life Technologies, USA |
| Anti-Actin Rabbit Antibody | Cell Signaling Technologies, USA |
| Goat-anti-rabbit IgG | Jackson ImmunoResearch, USA |
| Mouse-anti-goat IgG | Jackson ImmunoResearch, USA |
| P38 MAPK Rabbit Antibody | Cell Signaling Technologies, USA |
| P-Histone H2A.X (S139)(20E3) Rabbit monoclonal Antibody | Cell Signaling Technologies, USA |
| p-HSP27 (S82)II Rabbit Antibody | Cell Signaling Technologies, USA |
| p-P38 MAPK (T180/Y182)(28B10) Mouse Antibody | Cell Signaling Technologies, USA |
| p-P44/42 MAPK (Erk1/2) Rabbit Antibody | Cell Signaling Technologies, USA |
| p-P44/42 MAPK (T202/Y204) Rabbit Antibody | Cell Signaling Technologies, USA |

2.5. Further equipment and software

| Further equipment | Manufacturer |
|---|-------------------------------------|
| Eppendorf centrifuge 5810 R | Eppendorf, Hamburg |
| Eppendorf centrifuge 5417 R | Eppendorf, Hamburg |
| Eppendorf Reference (0.5-10µl/10-100µl/100-1000µl) | Eppendorf, Hamburg |
| Mr. Frosty™ Freezing Container | ThermoFisher Scientific, USA |
| Infinite M-Plex | Tecan, Switzerland |
| Incubator | Binder, Tuttlingen |
| Jess/Wes Separation 12-230 kDa 8x25 Capillary Cartridges | ProteinSimple, USA, Lot 04667 |
| Neubauer counting chamber | Th. Geyer GmbH & Co KG, Renningen |
| Sterile Bench | Köttermann, Uetze |
| Vortex Genius 3 | IKA-Werke GmbH & Co KG, Staufen |
| Wes™ | ProteinSimple, USA |
| Zen-Software 2.5 Blue | Zeiss, Oberkochen |
| Zeiss Axio Vert.A1 | Zeiss, Oberkochen |
| 96 Universal Gradient peqStar | peqLab Biotechnology GmbH, Erlangen |
| Software | Manufacturer |
| Compass | ProteinSimple, USA |
| Fiji | ImageJ, USA |
| GraphPad Prism | GraphPad Software, USA |
| R-Studio | R-Studio, USA |

3. Methods

3.1. Cell culture

All cell cultural work was done under a sterile bench.

Normal human epidermal keratinocytes as well as fibroblasts were cultivated at 37 °C and 5 % CO₂ in an incubator. Keratinocytes were cultivated in KGM culture medium and fibroblasts in DMEM culture medium. At least every third day medium was changed. When cells reached a confluence of 90 % they were passaged. They were washed with PBS and incubated with Trypsin EDTA solution at 37 °C, fibroblasts for four minutes and keratinocytes for 10 minutes. The reaction was stopped through resuspension of cells with PBS with 10 % fetal calf serum for keratinocytes or DMEM culture medium which contains 10 % fetal calf serum for fibroblasts. Then the suspension was centrifuged at 300 rpm for three minutes. The supernatant was discarded, and the pellet was resuspended in culture medium. 25 µl of the suspension were taken for cell counting in a Neubauer counting chamber. The rest of the cell suspension was used for experiments or further cultivation. In both cases cells were seeded in the required density into new cell culture flasks or onto new plates and were then placed in the incubator again. Fibroblasts from passage 5 to 30 and keratinocytes from passage 5 to 10 have been used. For long time storage of cells, stocks were frozen in liquid nitrogen and defrosted when needed. For freezing cells were washed with PBS and incubated with Trypsin EDTA solution at 37 °C, fibroblasts for four minutes and keratinocytes for ten minutes. The reaction was stopped through resuspension of cells with PBS with 10 % fetal calf serum for keratinocytes or DMEM culture medium which contains 10 % fetal calf serum for fibroblasts. Then the suspension was centrifuged at 300 rpm for three minutes. The supernatant was discarded, the pellet resuspended in Cryo SFM and put into CryoPure Tubes. These were then taken into the freezer in a freezing container, taken out after three days and placed in a nitrogen tank.

In order to induce an AD cell culture model cells were treated with an AD-specific cytokine mix for 24 hours to induce a disease specific environment. Cells were stimulated with IL-4, IL-13 and IL-22 in a final dilution of 40 ng/ml and TNF α in a dilution of 10 ng/ml as described previously (Emmert et al. 2020). For fibroblasts the cytokines were diluted in DMEM culture medium and for keratinocytes in KGM only with 0.06 mM CaCl₂.

To induce oxidative stress cells were treated with H₂O₂. For keratinocytes a dilution of 250 µM was chosen as described previously (Emmert et al. 2020). For fibroblasts the required

concentration of H₂O₂ was determined in a series of dilutions of 250 µM, 500 µM, 1 mM and 2 mM. 500 µM was the concentration where an effect on the fibroblasts could be seen without a reduction of cell numbers and therefore this dilution was chosen.

3.2. DCFDA-assay

For DCF-assay fibroblasts were seeded in a 96-well plate with 8000 cells per well in 100 µl DMEM culture medium each. The next day cells were stimulated with 10 ng/µl IL-4, IL-13 and IL-22 in a final dilution of 40 ng/ml and TNFα in a dilution of 10 ng/ml in normal culture medium. In various controls, DPI solution or DMSO were added at the same time as the cytokines in a final dilution of 1:1000 each as well. After 24 hours the DCF reagent was prepared as per manufacturers instruction, therefore the buffer was solved 1:10 in H₂O and DCFDA was added in a dilution of 1:1000, then 50 µl were poured into each well. Cells were incubated in the incubator for 45 minutes and after that washed with PBS once. Then DMEM culture medium was added to some cells while other cells were treated with 500 µM H₂O₂. Therefore the 30 % H₂O₂ solution was diluted 1:20000 in DMEM culture medium. Cells were placed for ten minutes in the incubator and then washed two times with PBS. After this fluorescence was measured with the Infinite M-Plex. Here a top measurement was used while the excitation was 485 nm and the emission 535 nm. Each assay had three biological replicates.

3.3. Resazurin-assay

For Resazurin-assay fibroblasts were seeded in a 96-well plate with 6000 cells per well in 100 µl culture medium each. The next day cells were stimulated with IL-4, IL-13 and IL-22 in a dilution of 40 ng/ml and TNFα in a dilution of 10 ng/ml in DMEM culture medium. After 24 hours cells were stressed for ten minutes in a final dilution of 500 µM H₂O₂, for which 30 % H₂O₂ solution was diluted 1:20000 in DMEM culture medium. For control wells, cells were not treated with H₂O₂ but instead fresh medium without H₂O₂ was added. Then the medium was extracted and cells were washed with PBS one time. After that 100 µl medium plus 10 µl resazurin solution were added into each well. After waiting for two and a half hours fluorescence was measured with the Infinite M-Plex. Here the excitation was 545 nm and the emission 590 nm while each assay had three biological replicates

3.4. Immunofluorescence

For immunofluorescence, cells were seeded in a 12 Well Cell Culture Tissue plate. A cover slip was placed in each well, was sterilized with 70 % ethanol for five minutes and washed with PBS three times. After that fibroblasts were seeded with 20000 cells per well in 1 ml medium and the plate was placed in the incubator. After 24 hours, cells were stimulated with IL-4, IL-13 and IL-22 in a dilution of 40 ng/ml and TNF α in a dilution of 10 ng/ml in DMEM culture medium. Besides, DPI solution or DMSO were added in a dilution of 1:1000 each. After 24 hours in the incubator some cells were stressed with 500 μ M H₂O₂ for one hour, for which 30 % H₂O₂ solution was diluted 1:20000 in DMEM culture medium. Then the liquid was removed, cells were washed with PBS and fixed with 3-4 % paraformaldehyde diluted in PBS. After 30 minutes at room temperature the cover slips were washed with PBS again and then 0.1 % Triton-X 100 diluted in PBS was added. After 15 minutes the liquid was removed and the blocking solution was added for half an hour. After washing with PBS three times the primary antibody P-Histone H2A.X was diluted 1:200 in blocking solution and given on the cover plates for one hour. Cells were washed with PBS three times and the second antibody Alexa Fluor 488 diluted 1:400 in blocking solution was added for another hour. Again, cells were washed with PBS three times. Then the cover glasses were fixed on the microscope slides with 5 μ l Moviol/DABCO/DAPI each and sealed with nail varnish and later on examined under the microscope where images were taken. In each biological replicate 3 images were taken with round about 6 cells per image.

For statistical analysis with ImageJ cell nuclei were manually selected by the DAPI staining to create a mask. The mask was used to measure the pH2aX signal and the DAPI signal in the 488 nm and the DAPI 463 nm channel. After that the fluorescence intensity of the total pH2aX channel signal was normalized to the fluorescence intensity of the total DAPI channel signal for each image. From the results of the different images of one biological replicate the mean was calculated.

3.5. Western Blot

For the Western Blot fibroblasts were seeded in 10 cm cell culture plates. When cells reached 90-100 % confluence, they were stimulated with IL-4, IL-13 and IL-22 in a dilution of 40 ng/ml and TNF α in a dilution of 10 ng/ml in DMEM culture medium. Besides, DPI solution or DMSO were added in a dilution of 1:1000 each. After 24 hours cells were stressed for one hour with

500 μM H_2O_2 , for which 30 % H_2O_2 solution was diluted 1:20000 in DMEM culture medium. Then dishes were placed on ice and washed with PBS one time. After that 300 μl RIPA-lysis buffer was given onto each dish, cells were collected with cell scrapers and in the liquid given into tubes. These were cooled on ice for 20 minutes and then centrifuged with 13000 g at 4 $^\circ\text{C}$ for 20 minutes. Afterwards the supernatant was collected in other tubes and from these the protein concentration was measured. Therefore 100 μl 660 nm Protein Assay Reagent were poured into the needed wells of a 96 well-plate, 6 μl of the protein solution were added each and the fluorescence was measured with the Infinite M-Plex in absorption mode and with a wavelength of 665 nm as per manufacturers instruction. Protein concentrations of samples were determined using a standard curve from known protein standards. Protein standards were generated using dilutions of bovine serum albumin in PBS and processed in the same manner. The measured absorbances of the standards were used to plot and calculate a standard curve using excel. The rest of the protein solution was stored in the freezer at -20 $^\circ\text{C}$ until it was used. Western Blots were made with ProteinSimple's WesTM as written in the user manual and the suiting kits were used, which were EZ Standard Pack 1 and Anti-Goat/ Anti-Mouse/ Anti-Rabbit Detection Modules. Primary antibodies were diluted 1:30 in Antibody Diluent 2 and secondary antibodies were used as delivered.

For the statistical analysis each protein is referred to another protein with either a constant concentration like Actin or a dependent concentration as for example pP38 as a subform of P38.

3.6. Crosstalk experiment

The crosstalk experiment was carried out with keratinocytes and fibroblasts. Both were seeded in 96-well plates each. 7000 fibroblasts were seeded per well in DMEM culture medium and 10000 keratinocytes per well in KGM culture medium with supplements, CaCl_2 and penicillin/streptomycin. When cells had grown sufficiently some of the wells were treated with IL-4, IL-13 and IL-22 in a dilution of 40 ng/ml and TNF α in a dilution of 10 ng/ml in DMEM culture medium for fibroblasts and in KGM only with 0.06 mM CaCl_2 for keratinocytes. These cells should produce the supernatant. After 24 hours cells for supernatant production were washed with PBS and then KGM only with 0.06 mM CaCl_2 was added onto fibroblasts and DMEM culture medium onto keratinocytes for one hour. After that the supernatant was collected in a tube and DPI solution or DMSO added in a dilution of 1:1000. This was added onto the other type of cells what meant that DMEM culture medium from keratinocytes was given onto fibroblasts and vice versa. Control cells were treated with IL-4, IL-13 and IL-22 in

a dilution of 40 ng/ml, TNF α in a dilution of 10 ng/ml and DPI solution or DMSO in a dilution of 1:1000. In negative control wells, media was switched to media containing only DPI solution or DMSO in a dilution of 1:1000. For fibroblasts DMEM culture medium was used and for keratinocytes KGM only with 0.06 mM CaCl₂. After another 24 hours in the incubator the medium was removed, and cells were treated with DCFDA reagent which was prepared as described in the kit. After that, cells were washed with PBS and some then stimulated with H₂O₂ for ten minutes. Fibroblasts were stimulated with 500 μ M H₂O₂ for which 30 % H₂O₂ solution was diluted 1:20000 in DMEM culture medium and keratinocytes were stimulated with 250 μ M H₂O₂ for which 30 % H₂O₂ solution was diluted 1:40000 in KGM only with 0.06 mM CaCl₂.

Then they were washed with PBS two times and the fluorescence was measured with the Infinite M-Plex. Here a top measurement was used while the excitation was 485 nm and the emission 535 nm. Each assay had three biological replicates. The experimental setup of the crosstalk experiments can be seen in figure 1 and figure 2.

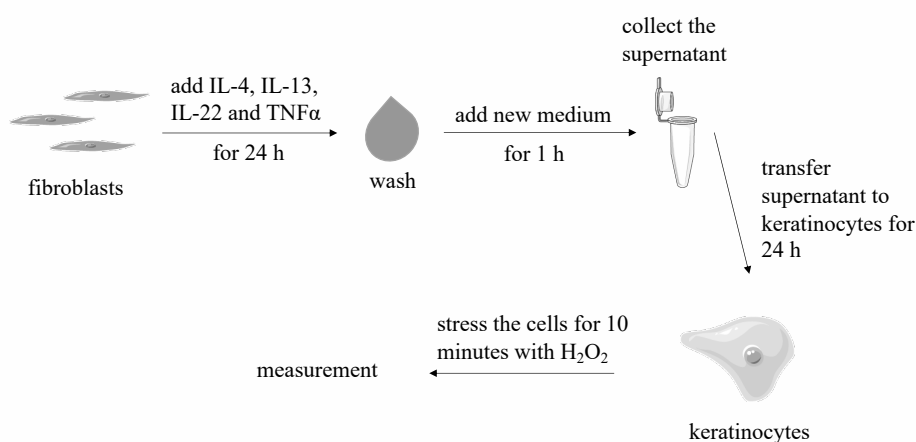


Figure 1: Experimental setup of the crosstalk experiments with keratinocytes. Fibroblasts were stimulated with IL-4, IL-13, IL-22 and TNF α for 24 hours, then they were washed and new medium was added. After one hour the supernatant was collected and transferred to the keratinocytes. After 24 hours keratinocytes were stressed with H₂O₂ for 10 minutes and the level of oxidative stress was measured.

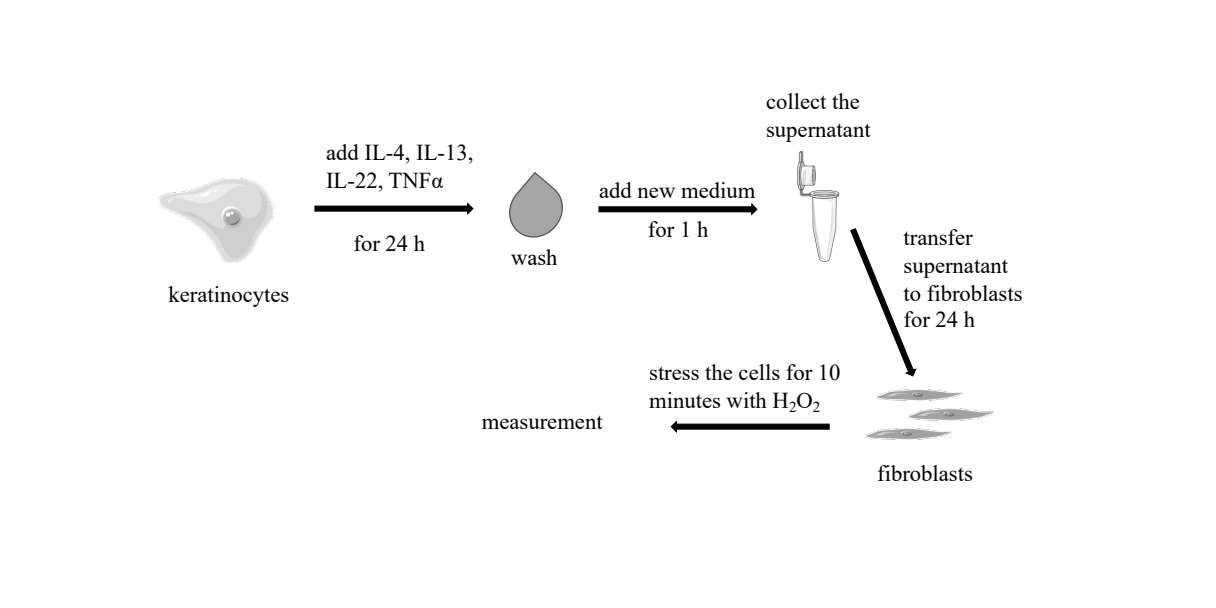


Figure 2: Experimental setup of the crosstalk experiments with fibroblasts. Keratinocytes were stimulated with IL-4, IL-13, IL-22 and TNF α for 24 hours, then they were washed and new medium was added. After one hour the supernatant was collected and transferred to the fibroblasts. After 24 hours fibroblasts were stressed with H₂O₂ for 10 minutes and the level of oxidative stress was measured.

3.7. Statistical analysis

The results have been analyzed with Shapiro-Wilk-test for normality by using the program R-Studio. The further graphical and statistical analysis was generated with GraphPad Prism. If data was normally distributed results have been analyzed with unpaired student's t-test and otherwise a Wilcoxon-Rank-test was used. In the figures the mean is shown which consists each of at least three measurements with an error bar that depicts the standard error of the mean.

4. Results

4.1. AD-model fibroblasts show elevated levels of oxidative stress

Previous experiments of our study group with models of AD keratinocytes revealed that AD-model keratinocytes have increased oxidative stress levels. Additionally, experiments showed that the induction of oxidative stress via H_2O_2 led to increased levels of ROS and that this could be inhibited by treatment with diphenyleneiodonium chloride (DPI), which is a pan-NADPH oxidase inhibitor (Emmert et al. 2020). To find out whether fibroblasts elicit similar high levels of oxidative stress the experiments were adapted and repeated with fibroblasts.

First the sensitivity of fibroblasts towards oxidative stress was tested with a DCFDA assay which measures intracellular ROS levels, results are shown in Figure 1. Treatment with H_2O_2 led to higher ROS levels both in AD-model and control fibroblasts. Compared to control fibroblasts, in AD-model fibroblasts the basal level of ROS was 1.6 times higher. The induced oxidative state of AD-model fibroblasts was 1.2 times higher than in control cells when treated with H_2O_2 . Treatment with H_2O_2 led to 3.9 times increased ROS in AD-model fibroblasts and 4.9 times increased ROS in control fibroblasts compared to not with H_2O_2 treated AD-model and control fibroblasts. That indicates that AD-model fibroblasts are characterized by an increased oxidative state.

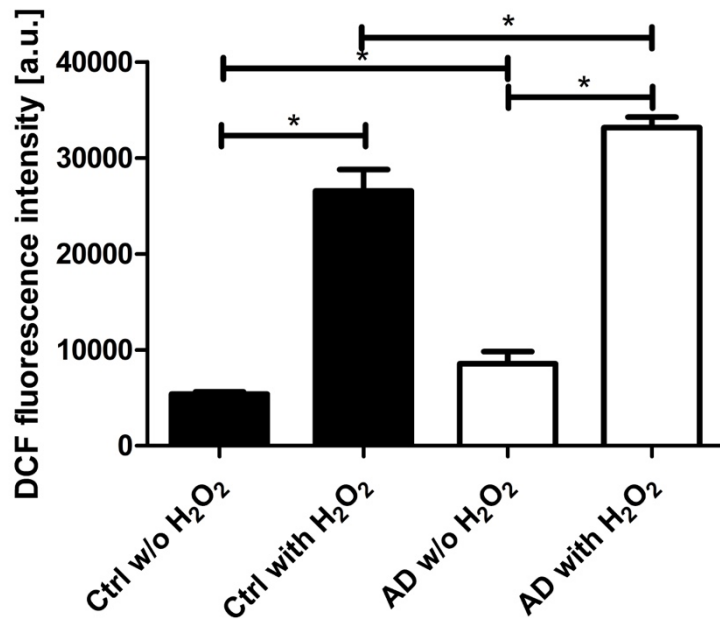


Figure 3: Reaction of control and AD-model fibroblasts towards oxidative stress. Atopic Dermatitis-model (AD) and control (Ctrl) fibroblasts were treated with 500 μ M H₂O₂. Reactive oxygen species production was measured via DCFDA assay. Atopic Dermatitis model fibroblasts reacted more sensitive to oxidative stress than control fibroblasts and showed a high basal level of reactive oxygen species. Results represent the means and standard error of the mean from 6 independent experiments. (a.u.=arbitrary units, w/o=without, * p <0.05)

It was shown that the in AD-model keratinocytes elevated oxidative state is due to an increased ROS production (Emmert et al. 2020). In order to elucidate whether elevated oxidative stress in fibroblasts is also due to increased ROS production, DCFDA assay was repeated with additional use of the pan-NADPH oxidase inhibitor DPI which suppresses intracellular production of ROS by inhibiting NADPH oxidase activity. Results can be seen in Figure 2. The effects of stress induction via H₂O₂ were the same as described above. DPI addition lowered the level of ROS significantly in control as well as in AD-model fibroblasts when they were stressed with H₂O₂. In unstimulated fibroblasts addition of DPI lowered the level of ROS by 75 % while in AD-model fibroblasts DPI lowered the level of ROS by 83 % after stress induction with H₂O₂. DPI also lowered the basal level of ROS in unstressed cells, in AD-model fibroblasts by 62 % and in control fibroblasts by 33 %, which was not significant. In conclusion, the higher oxidative stress level in AD-model fibroblasts is due to an increased ROS production.

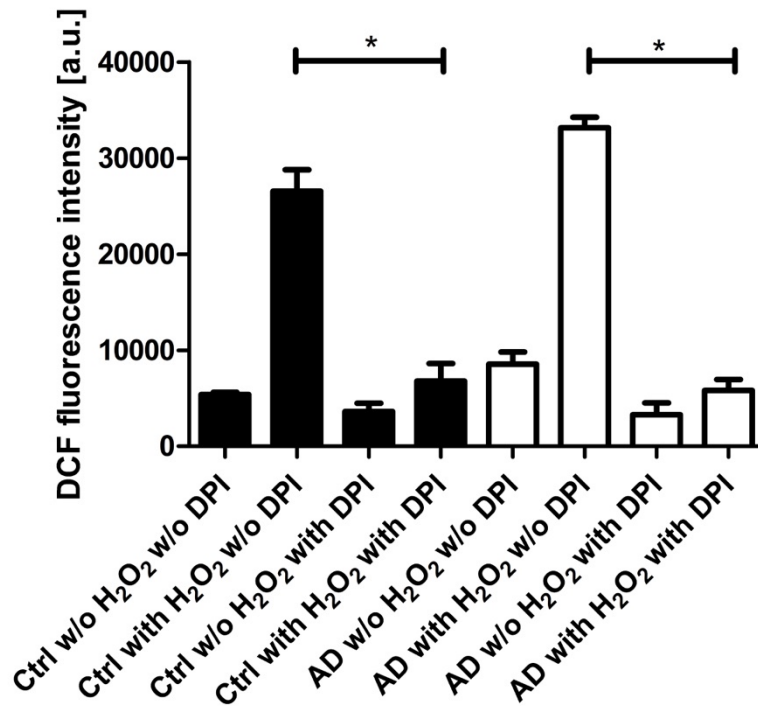


Figure 4: Inhibition of the reaction of control and AD-model fibroblasts towards oxidative stress with DPI. Atopic Dermatitis-model (AD) and control (Ctrl) fibroblasts were treated with 500 μ M H₂O₂ and with diphenyleneiodonium chloride (DPI) which is a pan-NADPH-oxidase inhibitor. Reactive oxygen species (ROS) production was measured via DCFDA assay. Treatment with DPI reduced H₂O₂-induced elevated ROS-levels. Results represent the means and standard error of the mean from 6 independent experiments. (a.u.=arbitrary units, w/o=without, * $p < 0.05$)

Another interesting point was whether the higher level of oxidative stress had an influence on the viability of AD-model fibroblasts. Results are pictured in Figure 3. The cell viability of fibroblasts was slightly reduced by treatment with H₂O₂ but there was no significant difference between AD-model and control fibroblasts. The viability of AD-model fibroblasts was reduced by 12.9 % and viability of control fibroblasts was reduced by 10.4 % by treatment with H₂O₂.

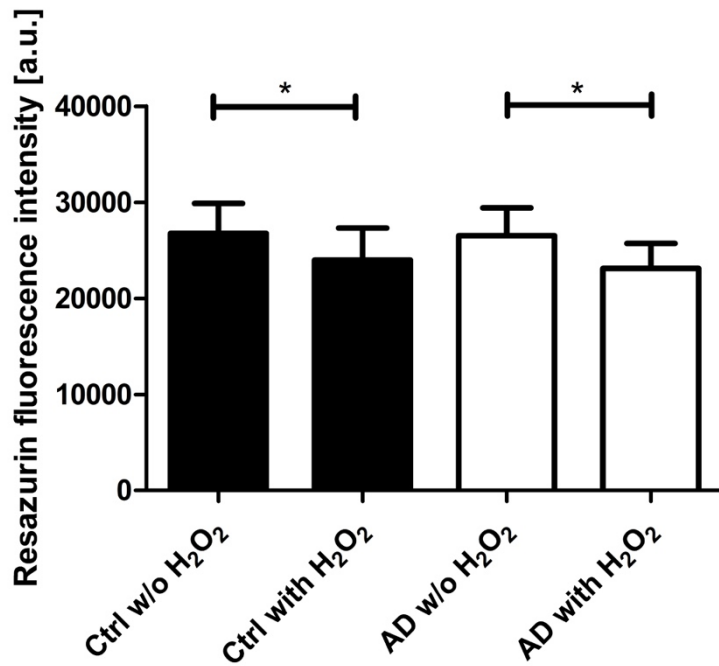


Figure 5: Viability of AD-model and control fibroblasts before and after the induction of oxidative stress. Atopic Dermatitis-model (AD) and control (Ctrl) fibroblasts were treated with 500 μ M H₂O₂. Cell viability was measured with the Resazurin assay. Cell viability was slightly reduced by H₂O₂ treatment. Results represent the means and standard error of the mean from 7 independent experiments. (a.u.=arbitrary units, w/o=without, * p <0.05)

To clarify whether the differences in oxidative capacity result in downstream biological processes, DNA damage was investigated. Anti-pH2AX is an antibody that detects phosphorylated H2AX, occurring after DNA damage. DNA strand breaks were made visible with immunofluorescence.

Figure 4 shows that H₂O₂ induced significantly more DNA damage in both AD-model and control fibroblasts than without treating them with H₂O₂. In AD-model fibroblasts DNA damage was 3.9 times higher and in control fibroblasts damage was 2.8 times higher after stimulation with H₂O₂ than without stimulation. There was no significant difference in the extent of damage between control and AD-model fibroblasts. However, before adding H₂O₂ the damage in control fibroblasts was 1.3 times higher than in AD-model fibroblasts. After adding H₂O₂ the extent of DNA damage was nearly the same in AD-model and control fibroblasts. The effect of inhibiting NADPH-oxidases by adding DPI could be seen as well. When DPI was added and therefore less ROS were produced there could be seen significantly less DNA damage after treatment with H₂O₂ than without the addition of DPI. In AD-model fibroblasts the amount of DNA damage was reduced by 54 % while in control fibroblasts the amount was

reduced by 60 % by adding DPI when treated with H_2O_2 in comparison to cells where no DPI was added. There was nearly no change in the amount of DNA damage when the cells were not treated with H_2O_2 , whether treated with DPI or not. Representative images of fluorescence staining are displayed in Figure 5.

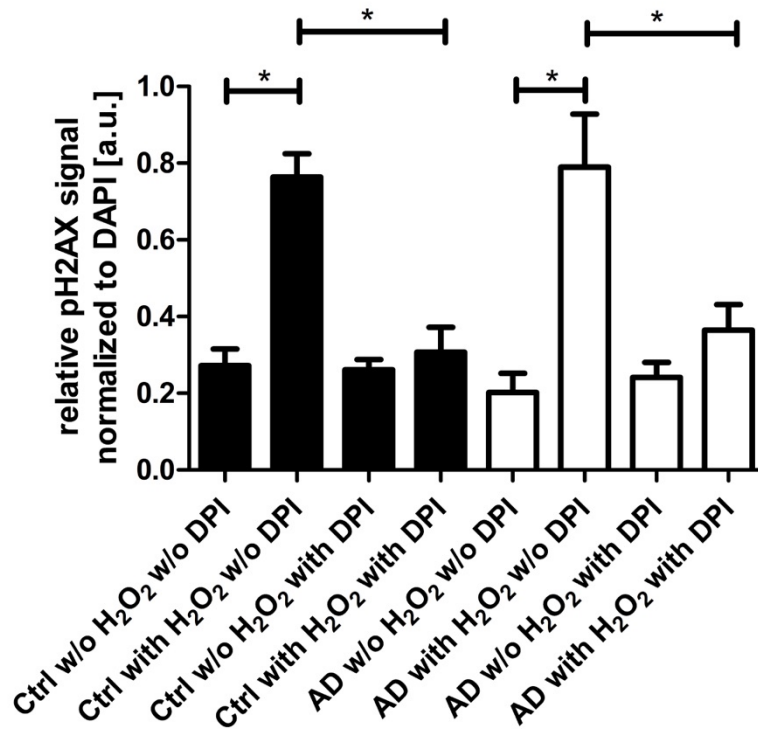


Figure 6: DNA-strand breaks in fibroblasts before and after oxidative stress induction and with or without inhibition of the reaction. Immunofluorescence in the Atopic Dermatitis-model (AD) and control (Ctrl) fibroblasts after treatment with diphenyleneiodonium chloride (DPI) and 500 μM H_2O_2 . Quantification of pH2AX fluorescence intensity normalized to DAPI. Treatment with H_2O_2 induced DNA-strand breaks which was abrogated by DPI. Results represent the means and standard error of the mean from 6 independent experiments. (a.u.=arbitrary units, w/o=without, * $p < 0.05$)

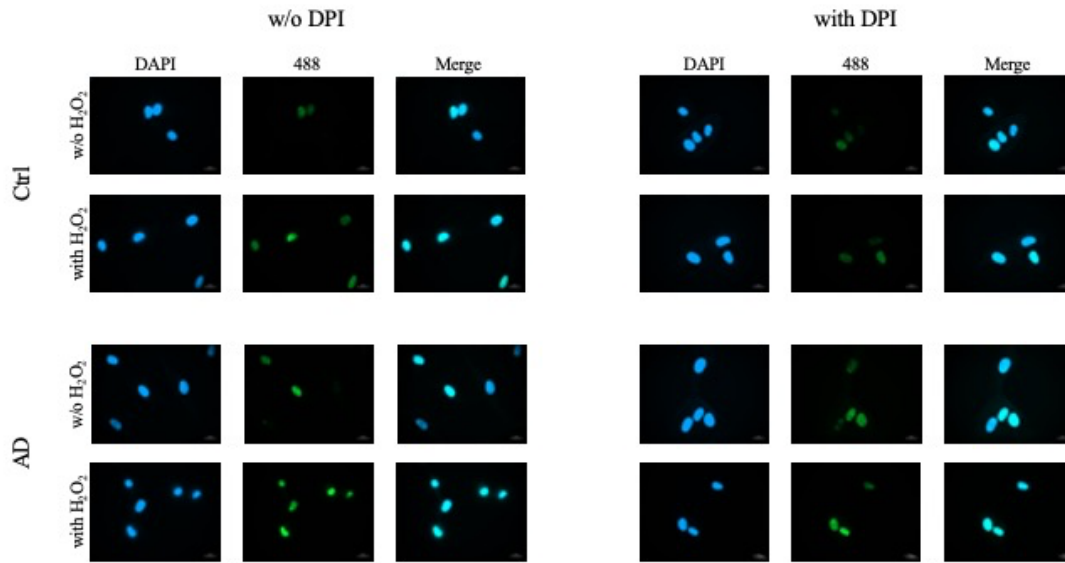


Figure 7: Representative images of immunofluorescence staining. Immunofluorescence in Atopic Dermatitis-model (AD) and control (Ctrl) fibroblasts after treatment with diphenyleneiodonium chloride (DPI) and 500 μ M H_2O_2 . Representative images of Alexa-Fluor 488 fluorescence staining and nuclear DAPI. (w/o=without)

For the identification of signaling cascades involved in redox balance stress related signaling cascades were analyzed via western blot.

Treatment with H_2O_2 induced phosphorylation of Erk both in control and AD-model fibroblasts as shown in Figure 6. In control fibroblasts the level of pErk was 1.2 times higher and in AD-model fibroblasts the level of pErk was 1.8 times higher with stimulation with H_2O_2 than without stimulation. Basal levels of pErk were 32 % lower in AD-model fibroblasts than in control fibroblasts. After treatment with H_2O_2 the levels of pErk in AD-model and control fibroblasts revealed no significant difference. The treatment of fibroblasts with DPI did not significantly change the amount of phosphorylated Erk.

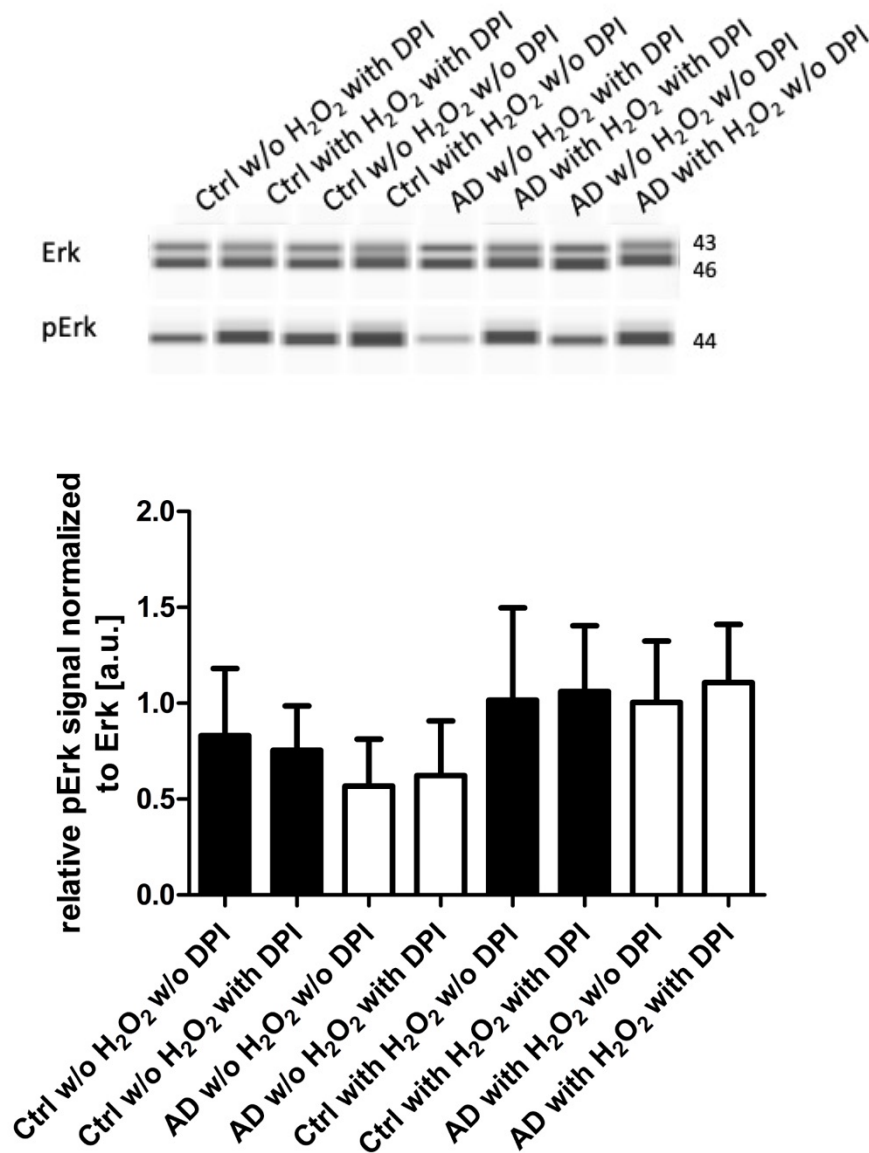


Figure 8: pErk expression in fibroblasts. Atopic Dermatitis model (AD) and control (Ctrl) fibroblasts were treated with 500 μ M H₂O₂ and diphenyleneiodonium chloride (DPI). Erk and pErk levels were determined by western blotting and protein levels of pErk normalized to Erk. Expression of pErk was higher after stressing with H₂O₂ for one hour. Basal pErk levels were slightly lower in Atopic Dermatitis fibroblasts compared to control fibroblasts. Results represent the means and standard error of the mean from 3 independent experiments. (a.u.=arbitrary units, w/o=without)

Figure 7 displays that the level of pP38 was significantly higher after stimulating control fibroblasts as well as AD-model fibroblasts with H₂O₂. In control fibroblasts the level of pP38 was 13.4 times higher after treatment with H₂O₂ than without treatment with H₂O₂. In AD-model fibroblasts the level of pP38 was 8.5 times higher after treatment with H₂O₂ than without treatment with H₂O₂. With as well as without treatment with H₂O₂ the amount of pP38 was

higher in AD-model fibroblasts than in control fibroblasts. Before treatment with H₂O₂ the level of pP38 was 1.9 times higher and after treatment with H₂O₂ the level of pP38 was 1.2 times higher in AD-model fibroblasts than in control fibroblasts. As well as in the level of pErk DPI did not seem to have a clear influence towards the amount of pP38.

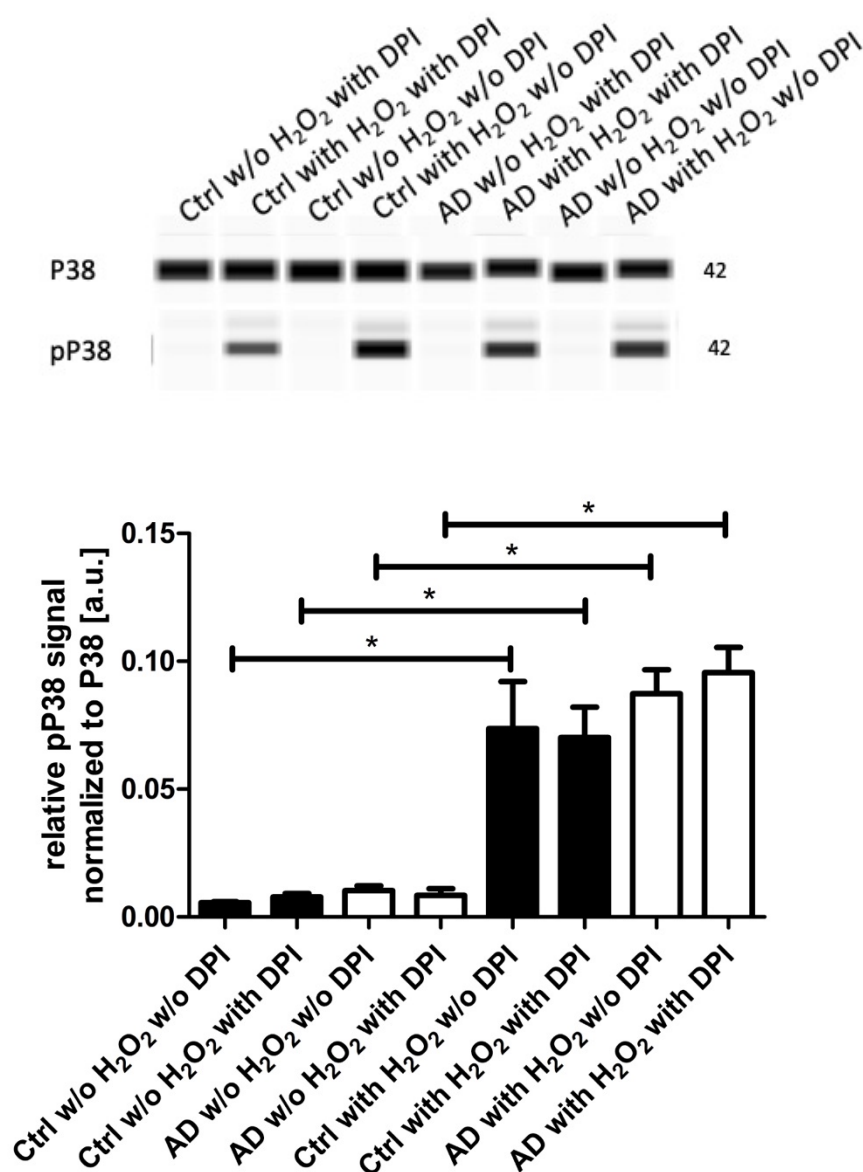


Figure 9: pP38 expression in fibroblasts. Atopic Dermatitis-model (AD) and control (Ctrl) fibroblasts were treated with 500 μ M H₂O₂ and diphenyleneiodonium chloride (DPI). P38 and pP38 levels were determined by western blotting and protein levels of pP38 normalized to p38. Phosphorylation of P38 was increased after stimulation with H₂O₂. In AD-model fibroblasts the level of pP38 was higher after H₂O₂ treatment than in control cells. Results represent the means and standard error of the mean from 3 independent experiments. (a.u.=arbitrary units, w/o=without, *p<0.05)

Results of western blotting of p Hsp27 are pictured in Figure 8. p Hsp27 was induced by treatment with H_2O_2 in both control and AD-model fibroblasts although the results were not significant. After treatment with H_2O_2 the level of p Hsp27 was 2.1 times higher in control fibroblasts than without H_2O_2 . In AD-model fibroblasts the level of p Hsp27 was nearly the same with or without treatment with H_2O_2 . Additionally, the basal levels of p Hsp27 were 2.2 times higher in AD-model fibroblasts in comparison to control fibroblasts while it was nearly similar after treatment with H_2O_2 . The addition of DPI increased the level of p Hsp27 1.3 times in AD-model fibroblasts and 1.4 times in control fibroblasts when treated with H_2O_2 in contrast to when no DPI was added. Addition of DPI to unstressed cells increased the level of p Hsp27 1.1 times in control fibroblasts and decreased the level 0.8 times in AD-model fibroblasts in comparison to when no DPI was added.

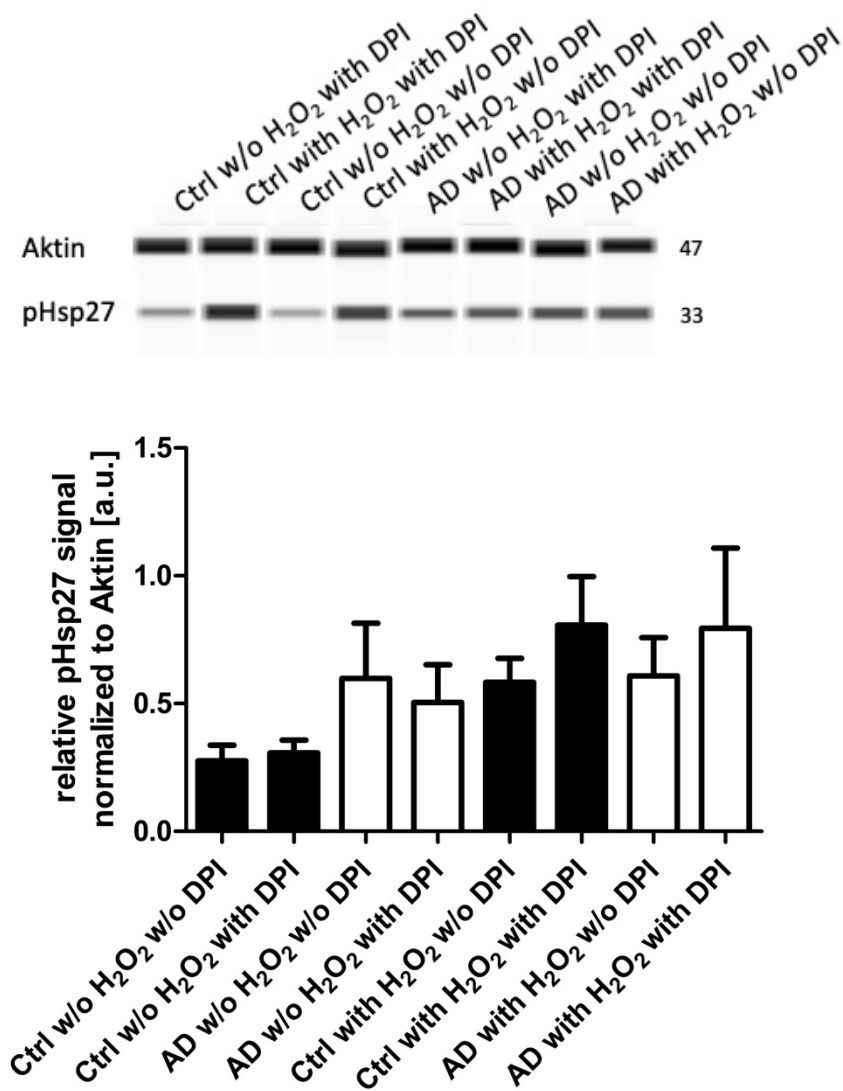


Figure 10: pHsp27 expression in fibroblasts. Atopic Dermatitis-model (AD) and control (Ctrl) fibroblasts were treated with 500 μ M H₂O₂ and diphenyleneiodonium chloride (DPI). Aktin and pHsp27 levels were determined by western blotting and protein levels of pHsp27 normalized to Aktin. Basal pHsp27 levels were higher in AD-model fibroblasts than in control fibroblasts. The levels were elevated after stressing the cells with H₂O₂. Results represent the means and standard error of the mean from 3 independent experiments. (a.u.=arbitrary units, w/o=without)

p38 signaling is considered to be a pro-apoptotic signal (Kralova et al. 2008), pErk is associated with cell proliferation and survival (Roberts and Der 2007) and pHsp27 is characterized as an anti-apoptotic protein (Gibert et al. 2011). Nonetheless, all three signaling pathways are activated after stress induction with H₂O₂, so there are both pro-apoptotic and pro-survival signaling pathways active.

Taken together, the results indicate that fibroblasts in an AD model react more strongly towards oxidative stress than control fibroblasts. Moreover, AD-model fibroblasts have a higher basal level of ROS. In response to mild oxidative stress induction by H₂O₂ fibroblasts produce ROS which results in a positive feedback cycle of ROS generation. This response can be prevented by inhibiting NADPH-oxidases. The consequence of oxidative stress is activation of different cell signaling pathways for example activation of Erk and p38 pathways as well as induction of pHsp27. AD fibroblasts react more likely with apoptosis than with survival programs towards oxidative stress than control fibroblasts. Another consequence of oxidative stress is DNA damage.

4.2. Fibroblasts prime keratinocytes to react more sensitive to oxidative stress

In order to understand the interaction, respectively to see if there is any interaction at all between keratinocytes and fibroblasts, crosstalk experiments have been conducted. For that the supernatant of fibroblasts, control as well as AD-model fibroblasts, was given onto keratinocytes. To determine how much time was needed for cells to produce mediators and secrete them into the medium different experimental designs based on previous observations were carried out. It did not make a difference whether the cells had only one hour or 24 hours to secrete mediators into the medium as well as there was no measurable difference whether the supernatant was poured onto the other cells for one hour or for 24 hours (data not shown). For practical reasons one hour time for secretion was chosen. 24 hours for the supernatant being on the other type of cells was chosen because this way the amount of time was the same as the AD-model cytokines being on the cells. After 24 hours keratinocytes were treated with H₂O₂ and the level of oxidative stress was measured with the DCFDA assay.

Results are pictured in Figure 10. The treatment with H₂O₂ induced a significantly higher level of oxidative stress in keratinocytes than in cells without treatment with H₂O₂. In control keratinocytes the level of ROS was 2.9 times higher with H₂O₂ treatment than without H₂O₂ treatment. In AD-model keratinocytes the level of ROS was 3.1 times higher with H₂O₂ treatment than without. In control-supernatant treated keratinocytes the level of ROS was 3.3 times higher with H₂O₂ treatment than without H₂O₂ treatment. In AD-supernatant treated keratinocytes the level of ROS was 3.4 times higher with H₂O₂ treatment than without treatment. Without treatment with H₂O₂, there were no big differences in the level of oxidative stress in control, AD-model and control- and AD-supernatant treated keratinocytes. The level

of oxidative stress was a little higher in AD-model keratinocytes than in control keratinocytes and the level was also a little higher in AD-supernatant treated keratinocytes than in control-supernatant treated keratinocytes. AD-model keratinocytes had a little higher level of oxidative stress compared to AD-supernatant treated keratinocytes. After treatment with H_2O_2 AD-model keratinocytes showed a 1.2 times higher level of ROS than control keratinocytes as well as control-supernatant treated keratinocytes showed a 1.1 times higher level than control keratinocytes. AD-supernatant treated keratinocytes showed a quite similar level of ROS as AD-model keratinocytes.

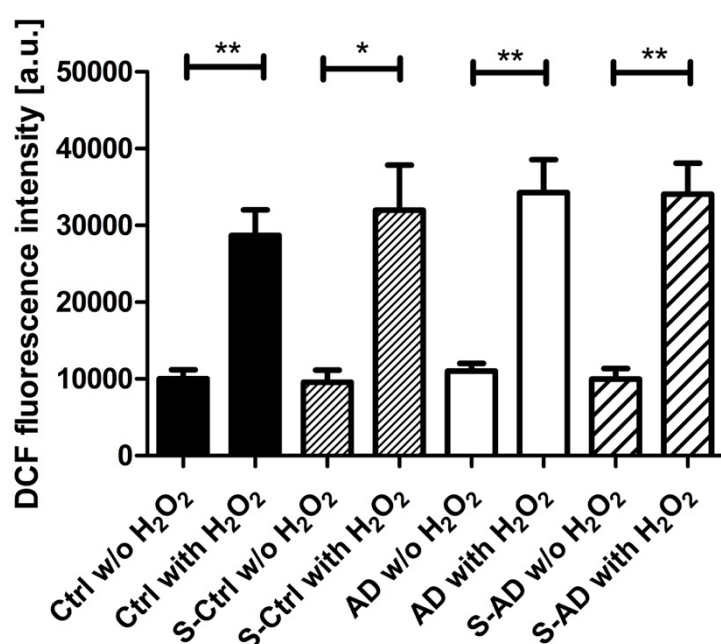


Figure 11: Reaction towards oxidative stress induction in keratinocytes after treatment with fibroblast supernatant. Control (Ctrl), Atopic Dermatitis-model (AD), AD-supernatant treated (S-AD) and control-supernatant-treated (S-Ctrl) keratinocytes were treated with 500 μM H_2O_2 . Reactive oxygen species (ROS) production was measured via DCFDA assay. Oxidative stress induction led to significantly elevated ROS levels while there was no significant difference between control keratinocytes, treatment with AD-cytokines or treatment with fibroblast supernatant on keratinocytes. Results represent the means and standard error of the mean from 3 independent experiments. (a.u.=arbitrary units, w/o=without, * $p<0.05$, ** $p<0.01$)

To determine whether the inhibition of ROS production has an effect if keratinocytes are not treated with the disease specific cytokines but with supernatant from control or AD-model fibroblasts, DPI was added to keratinocytes. Results are displayed in Figure 11.

As seen before, H_2O_2 increased the level of ROS. When DPI was added the effect of H_2O_2 was inhibited. In control-supernatant treated keratinocytes which were stimulated with H_2O_2 the

level of ROS was reduced by 88 % when treated with DPI, in comparison to control-supernatant treated keratinocytes which were stimulated with H₂O₂ but which were not treated with DPI. In AD-supernatant treated keratinocytes which were stimulated with H₂O₂ the treatment with DPI reduced the level of ROS by 83 % in comparison to AD-supernatant treated keratinocytes which were stimulated with H₂O₂ but without the treatment with DPI. DPI did not only reduce the reaction towards the induction of oxidative stress but also decreased the basal levels of ROS in keratinocytes. DPI reduced the basal level of oxidative stress by 67 % in control-supernatant treated keratinocytes and by 61 % in AD-supernatant treated keratinocytes.

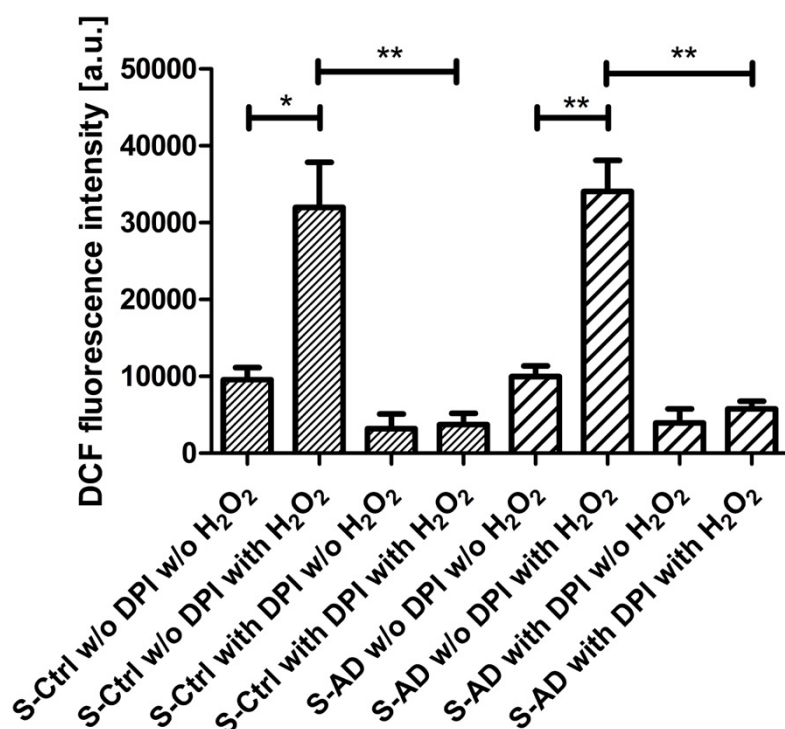


Figure 12: Inhibition of the reaction towards oxidative stress induction in supernatant treated keratinocytes. Control-supernatant (S-Ctrl) and AD-supernatant (S-AD) stimulated keratinocytes were treated with 500 μ M H₂O₂ and with diphenyleneiodonium chloride (DPI) which is a pan-NADPH-oxidase inhibitor. Reactive oxygen species (ROS) production was measured via DCFDA assay. DPI inhibited ROS production in all cells, whether treated with control or AD supernatant. Results represent the means and standard error of the mean from 3 independent experiments. (a.u.=arbitrary units, w/o=without, * p <0.05, ** p <0.01)

4.3. Keratinocytes strongly elevate the level of oxidative stress in fibroblasts

To analyze whether keratinocytes secrete factors that lead to a sensitization of fibroblasts to oxidative stress we next treated fibroblasts with keratinocyte supernatant.

Supernatant of keratinocytes, control as well as AD-model keratinocytes, was given onto fibroblasts and after 24 hours fibroblasts were treated with H₂O₂ and the level of oxidative stress was measured via DCFDA assay. Results are shown in Figure 13.

After stimulating fibroblasts with keratinocyte supernatant there was a higher level of oxidative stress than in control or AD-model fibroblasts. The basal level of ROS was 3.2 times higher in control-supernatant treated fibroblasts than in control fibroblasts. In AD-supernatant treated fibroblasts the basal level of ROS was 3.3 times higher than in AD-model fibroblasts. The level of ROS was significantly higher in all fibroblasts after stimulation with H₂O₂. In control fibroblasts the level of ROS was 4.5 times higher with H₂O₂ treatment than without treatment. In AD-model fibroblasts the level of ROS was 5.5 times higher with H₂O₂ treatment than without treatment. In control-supernatant treated fibroblasts the level of ROS was 6.3 times higher with H₂O₂ treatment than without treatment. In AD-supernatant treated fibroblasts the level of ROS was 5.5 times higher with H₂O₂ treatment than without treatment. The level of oxidative stress was significantly higher in supernatant treated cells after stimulation with H₂O₂ than in control or AD-model fibroblasts after stimulation with H₂O₂. The level of ROS was 4.4 times higher in control-supernatant treated keratinocytes than in control keratinocytes after stimulation with H₂O₂. In AD-supernatant treated keratinocytes the level of ROS was 3.3 times higher than in AD-model keratinocytes after stimulation with H₂O₂. The control-supernatant provoked a significantly stronger reaction in fibroblasts than the supernatant from AD-model keratinocytes.

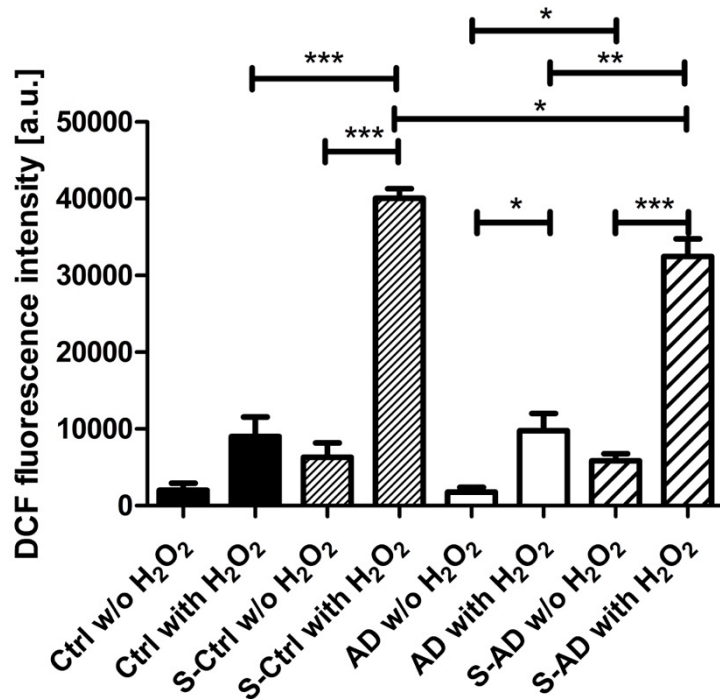


Figure 13: Reaction towards oxidative stress induction in fibroblasts after treatment with keratinocyte supernatant. Control (Ctrl), Atopic Dermatitis-model (AD), control-supernatant (S-Ctrl) and AD-supernatant (S-AD) treated fibroblasts were treated with 500 μ M H₂O₂. Reactive oxygen species (ROS) production was measured via DCFDA assay. Treatment with H₂O₂ led to ROS production in all fibroblasts, however, levels in fibroblasts stimulated with keratinocyte supernatant were much higher than in control fibroblasts or fibroblasts treated with AD-cytokines. Results represent the means and standard error of the mean from 3 independent experiments. (a.u.=arbitrary units, w/o=without, * p <0.05, ** p <0.01; *** p <0.001)

Figure 14 displays the effect of DPI on supernatant treated fibroblasts. As seen before H₂O₂ increased the level of ROS in all fibroblasts. DPI decreased this effect both in control- as well as in AD-supernatant treated fibroblasts and additionally decreased the level of ROS in those fibroblasts that were not treated with H₂O₂. In control-supernatant treated fibroblasts the basal level of oxidative stress was reduced by 72 % when treated with DPI. In AD-supernatant treated fibroblasts the basal level of oxidative stress was also reduced by 72 % when treated with DPI. In control-supernatant treated fibroblasts which were treated with H₂O₂ the treatment with DPI reduced the level of ROS by 68 % in comparison to control-supernatant treated fibroblasts which were treated with H₂O₂ but without treatment with DPI. In AD-supernatant treated fibroblasts which were treated with H₂O₂ the treatment with DPI reduced the level of oxidative stress by 65 % in comparison to AD-supernatant treated fibroblasts which were treated with H₂O₂ but without treatment with DPI.

DPI inhibited the level of oxidative stress in control- as well as in AD-supernatant treated fibroblasts both on a basal level as well as after treatment with H₂O₂.

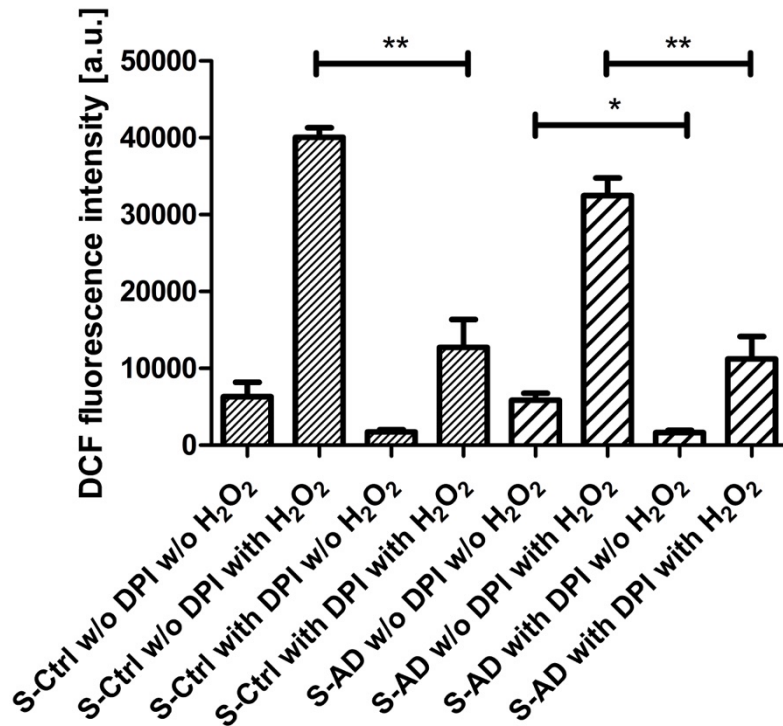


Figure 14: Inhibition of the reaction towards oxidative stress induction in supernatant treated fibroblasts. Control-supernatant (S-Ctrl) and AD-supernatant (S-AD) treated fibroblasts were treated with 500 μ M H₂O₂ and with diphenyleneiodonium chloride (DPI) which is a pan-NADPH-oxidase inhibitor. Reactive oxygen species (ROS) production was measured via DCFDA assay. The induction of oxidative stress in fibroblasts with H₂O₂ was inhibited by DPI. Results represent the means and standard error of the mean from 3 independent experiments. (a.u.=arbitrary units, w/o=without, * p <0.05, ** p <0.01)

In conclusion, both keratinocytes and fibroblasts produce ROS in response to mild oxidative stress, thus leading to a hyperproduction of ROS which can easily overcome antioxidative defenses. The crosstalk experiments revealed that additionally both fibroblasts and keratinocytes secrete factors into the supernatant that can prime the other type of cells to react more sensitive to subsequent oxidative stress. While keratinocytes react very sensitive to even small concentrations of H₂O₂, fibroblasts produce smaller amounts of ROS in response to treatment with H₂O₂. Treating fibroblasts with supernatant from conditioned keratinocytes however, ROS production after treatment with H₂O₂ is increased up to the same extent as seen in keratinocytes. The reaction of fibroblasts as well as of keratinocytes towards oxidative stress is mediated by NADPH-oxidases and can be inhibited by DPI.

5. Discussion

5.1. Fibroblasts are part of a cycle of ROS-generation leading to stronger inflammation in AD

AD is a chronic inflammatory skin disease and evidence spreads that AD is associated with elevated levels of ROS (Sivaranjani et al. 2013, Weidinger and Novak 2016). As it is known that keratinocytes in AD show increased levels of oxidative stress (Emmert et al. 2020), a question leading to this work was whether fibroblasts show a similar reaction. Outcome indicates that fibroblasts also have their stake in the development of AD.

Results show that AD-model fibroblasts exhibit increased basal levels of intracellular ROS compared to control fibroblasts. Treatment of AD-model fibroblasts with H₂O₂ further increases intracellular ROS and AD-model fibroblasts react more sensitive towards this treatment than control fibroblasts. This shows that in AD not only AD-model keratinocytes have a higher level of ROS and show a different reaction towards oxidative stress (Emmert et al. 2020) but fibroblasts do also have their own specific reaction in an AD model and seem to participate in the development of an AD environment.

Another conclusion drawn from these results is that AD is characterized by an increased oxidative state in the participating cells of the skin. As keratinocytes and fibroblasts show increased levels of ROS in an AD model, a further question would be whether other AD participating cells like immune cells also show an increased oxidative state. As immune cells do not stay in one place but are able to wander around, it could be assumed that they do not show an increased oxidative state. On the other hand, as immune abnormalities are part of the development of AD (Furie et al. 2017) it seems reasonable for immune cells to also participate in the misguided oxidative stress reaction.

Another question emerging from the results is whether the increased oxidative state is a driver of the development of AD or if it is a consequence of the disease. As antioxidant therapies have yet mostly failed to prove their benefit (Steinhubl 2008), it seems that oxidative stress might be a consequence of AD and the state of the disease can not be influenced by antioxidant therapies. Nonetheless, there are multiple reasons why these trials have not proven their effectiveness. One is that most of the antioxidant therapies have been chosen because of their easy availability and not because they were proved to be the best antioxidants. Another possibility is that the trials have not lasted long enough (Steinhubl 2008). This indicates that there is still a need for

clinical trials with effective antioxidants and a long enough duration until conclusions about the effect of antioxidants can be made. However, there are studies that name benefits for the use of antioxidative therapies (Bertino et al. 2020). For example, N-acetylcysteine shows antioxidant and anti-inflammatory activities and may be considered helpful as an adjuvant therapy in neurodegenerative diseases (Tardiolo et al. 2018). Also, animal studies have revealed promising in vivo effects of antioxidants for the prevention or the treatment of liver diseases, but in humans it does not appear to be effective yet, which could be explained by the route of administration, in animal studies antioxidants were given via oral or intraperitoneal injection (Li et al. 2015). Another point is that healthy food and a moderate consumption of wine have a link to cancer prevention and may also prevent various chronic diseases, biological mechanisms are associated with the consumption of antioxidants and polyphenols that are contained in vegetables and fruits and their products (Snopek et al. 2018). These last-mentioned studies indicate that an effective antioxidant therapy could play a big role in the prevention and therapy of various chronic diseases. Still, first there have to be found effective antioxidants which can be used on humans, but it remains possible that reducing oxidative stress in AD might decrease the severity of the disease.

As seen in the results of the DCF-assays, the pan-NADPH oxidase inhibitor DPI inhibits the increase of cellular oxidative stress and therefore it can be assumed that ROS are produced by fibroblasts themselves. Therefore, it can be suggested that fibroblasts react to oxidative stress with the production of more ROS, thus resulting in a positive feedback cycle of ROS generation, which leads to even stronger inflammation. This would be a pro-argument for oxidative stress as a driver of AD (Borgia et al. 2022), respectively oxidative stress could be the driver as well as the consequence of AD, the situation resulting even more in a vicious circle.

As it is known that in keratinocytes NOX1 is the NADPH oxidase in question (Emmert et al. 2020), it would be interesting to know if it is the same one in fibroblasts. Additionally, another point is to find out whether the already described more specific NADPH oxidases (Altenhöfer et al. 2015) could be a target in patients with AD and if inhibiting those NADPH oxidases could diminish the extent of the disease.

As it can be seen in the immunofluorescence, treatment with H₂O₂ induces DNA double-strand breaks in fibroblasts. That is something one could have assumed before, as it is known that oxidative stress causes DNA damage (Heinrich et al. 2014). Between AD-model and control fibroblasts only a minor difference was detected for the extent of damage in immunofluorescence assay. This indicates that in an AD-model the effect of oxidative stress towards DNA damage is the same as in a control, even if there is a higher level of oxidative

stress in AD-model fibroblasts than in control fibroblasts. That could mean that the ability of fibroblasts to cope with oxidative stress is unaffected by the disease, at least concerning the protection against DNA damage, while the production of ROS could be part of the fibroblasts' role in AD.

Cell viability is slightly reduced by H₂O₂ treatment both in AD-model and control fibroblasts compared to untreated controls. That shows the general toxic effect of H₂O₂ but indicates no difference between AD-model and control fibroblasts. The reduced viability is expected as a consequence to the damage caused by oxidative stress. Seeing no significant difference in the cell viability between AD-model and control cells could undermine the point that AD does not affect the ability of fibroblasts to survive oxidative stress.

Treatment with H₂O₂ induces phosphorylation of Erk and P38 and activation of Hsp27 both in control and AD-model fibroblasts. Thus, both pro-apoptotic and pro-survival pathways are activated. The activated signaling pathways lead to the promotion of inflammation by activating the production of pro-inflammatory cytokines (Zarubin and Han 2005). It therefore can be suggested that also fibroblasts play a role in the development of AD by promoting inflammatory events. AD is characterized by a pro-inflammatory cytokine milieu as well as it is clearly distinct from normal skin especially with respect towards terminal differentiation and some immune pathways (Suárez-Fariñas et al. 2011). Taken together, keratinocytes, immune cells and also fibroblasts play a role in the development of AD.

An unanswered question is if one of the signaling cascades is activated more strongly or if they are balanced. A pro argument for the second option would be that the viability of AD-model fibroblasts does not differ towards control fibroblasts and therefore pro- and anti-apoptotic signals seem to be balanced. The basal level of pErk is slightly lower in AD-model fibroblasts than in control fibroblasts, while after stress induction the level of pErk seems to be even between AD-model and control fibroblasts. As Erk is part of a cascade which is associated with cell proliferation and survival (Guo et al. 2020), it could be assumed that AD-model fibroblasts have a little decreased survival ability because of the decreased basal level of pErk in AD-model fibroblasts. However, after stress induction with H₂O₂ there is no difference in the level of pErk between AD-model and control fibroblasts. Thus, it can be concluded that their ability to survive oxidative stress is quite similar.

Stress induction with H₂O₂ increases the level of pP38 significantly in control as well as in AD-model fibroblasts compared to the untreated control. In AD-model fibroblasts the level of pP38 is higher than in control fibroblasts before as well as after treatment with H₂O₂. As pP38 is part of a signaling cascade that is considered to be pro-apoptotic, its induction by oxidative stress

seems to be a logic consequence (Dodeller and Schulze-Koops 2006). This is in line with the result that the cell viability is lowered by treatment with H_2O_2 . However, the Resazurin assay displayed no difference in the viability between control and AD-model fibroblasts while the western blots indicate that AD-model fibroblasts have a stronger activated pro-apoptotic pathway. This could mean that there have to be other activated pathways which act as a counterbalance towards this more strongly activated pP38 cascade in AD-model fibroblasts in comparison to control fibroblasts. Another conclusion is that the increased levels of pP38 in the AD-model just picture the fact of an increased oxidative state. However, there might be other, more strongly activated pathways that help the fibroblasts to cope better with elevated oxidative stress. It is unclear how important the influence of pP38 in this situation is.

pHsp27 is induced by treatment with H_2O_2 in both control and AD-model fibroblasts although not in a significant way. As pP38 is induced by oxidative stress and is able to activate pHsp27, the phosphorylation of Hsp27 could be a downstream part of activated pP38 as well as it might be caused by the increased stress itself (Anckar and Sistonen 2011). The basal level of pHsp27 is higher in AD-model fibroblasts than in control fibroblasts. After treatment with H_2O_2 the level of pHsp27 seems to be equal in AD-model and control fibroblasts. This could depict the result that in an AD-model, fibroblasts have a higher basal level of ROS and therefore more pHsp27 is activated as a protection against the negative effects of stress. The level of pHsp27 being similar in AD-model and control fibroblasts after stress induction could undermine the assumption that there have to be other pathways or protective mechanisms which help the fibroblasts to cope with oxidative stress. But even in this case, the level of pHsp27 being similar in AD-model and control fibroblasts should be further evaluated.

Addition of DPI does not significantly change the levels of pErk, pP38 or pHsp27 one way or another. This inhibitor of peroxidases changes the amount of ROS as seen in the DCF assays but does not change the activation of those signaling cascades including pErk, pP38 and pHsp27. This could mean that the signaling cascades are activated by signals unaffected of the level of ROS or at least unaffected by the amount of ROS produced by NADPH-oxidases. Therefore, another question yet unanswered is whether the inhibition of other, stronger activated pathways might help with the treatment of AD.

A weak point with all the western blots results is that they are not quite representative because of the small number of experiments. Additional experiments with kinetic enzyme activity assays could further underline the present experiments. Furthermore, many other signaling cascades could be involved and some more should be examined before making a definite conclusion.

5.2. AD-model fibroblasts induce an AD-like state in keratinocytes

Another point to examine in this work was whether fibroblasts and keratinocytes influence each other. Therefore, oxidative stress in keratinocytes was measured while some of the cells were treated with supernatant of fibroblasts before.

Treatment with H₂O₂ induces a significantly higher level of oxidative stress in keratinocytes in comparison to untreated cells, as to be expected (Galiniak et al. 2022). In the basal levels of oxidative stress there were no big differences in control, AD-model, control-supernatant treated and AD-supernatant treated keratinocytes. After stimulating cells with H₂O₂, the level of ROS was higher in AD-model keratinocytes than in control keratinocytes, as was shown before (Emmert et al. 2020). Equally, after stimulating with H₂O₂ the level of ROS was higher in AD-supernatant treated keratinocytes than in control-supernatant treated keratinocytes. This shows that AD-model fibroblasts produce mediators, which might be for example cytokines or interleukins, which induce a stronger reaction towards oxidative stress in keratinocytes. It would be interesting in future experiments to get to know which specific factors are involved. Comparing the level of ROS between AD-model keratinocytes and AD-supernatant treated keratinocytes, they seem quite similar. The results indicate that treatment of keratinocytes with supernatant from AD-model fibroblasts induces a similar reaction towards oxidative stress compared to treatment with the AD cytokine mix.

The experiment suggests that AD-model fibroblasts produce mediators of an unknown kind into their surroundings and that these can induce an AD-like state in keratinocytes. Adding DPI as a pan-NADPH oxidase inhibitor inhibits the stress induction by H₂O₂ and also decreases the basal level of ROS in keratinocytes. Therefore, the ROS production seems to be mediated by NADPH-oxidases as a reaction towards oxidative stress in fibroblasts and keratinocytes.

For this reason, the inhibition of NADPH-oxidases seems to be a promising possibility to reduce oxidative stress in fibroblasts as well as in keratinocytes.

5.3. Keratinocytes increase the level of oxidative stress in fibroblasts up to the same level as in keratinocytes

By performing the experiment the other way around, one could see that keratinocytes seem to influence fibroblasts in a much stronger way.

As expected because of the former DCFDA-assays, H_2O_2 induces an increased oxidative state in fibroblasts whether treated or not treated with supernatant from keratinocytes. Fibroblasts treated with keratinocyte supernatant show a higher basal level of ROS than control or AD-model fibroblasts. After the induction of oxidative stress, the level of ROS in control-supernatant treated fibroblasts as well as in AD-supernatant treated fibroblasts is significantly much higher than in control or AD-model fibroblasts.

Surprisingly, fibroblasts react much stronger in response towards supernatant from keratinocytes than towards an AD typical cytokine mix. Now, the pressing question is what exactly provokes a strong reaction like this.

To speculate on the mediators, as ROS are produced in response to signals from cytokines and growth factors (Baek and Lee 2016) it could be assumed that cytokines and growth factors might be contained in the supernatant. Cytokines and signaling molecules known for their pro-oxidative role are for example interleukin 3 and 6, angiotensin II, platelet-derived growth factor, fibroblast growth factor, transforming growth factor $\beta 1$ and TNF- α (Gabrielli et al. 2012). All of these factors could be part of the mediators promoting a reaction like the one in question.

The reason for a reaction that strong could on the one hand be found in the mediators that induce the reaction. On the other hand, the reason might be that the intracellular reaction to the same mediator could be different in keratinocytes compared to fibroblasts.

Extracellular signals of oxidative stress act via three main intracellular signaling pathways which include inhibition of protein tyrosine phosphatases (PTP), activation of MAPK cascades or other kinases and activation of specific transcription factors, and these pathways cause an intracellular increase in oxidative stress (Cannito et al. 2010). Signaling pathways implicated by an increased oxidative state in endothelial cells are for example nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B), nuclear factor erythroid 2-related factor (Nrf2) and antioxidant response element (ARE), resulting in expression of antioxidative enzymes, regulation of cellular proliferation and apoptosis in inflammatory states (Liang et al. 2017). Additionally, ROS induce various biological processes that include for example a transient elevation of intracellular Ca^{2+} concentration, phosphorylation of specific proteins, activation of specific transcription factors and other determinants of gene expression, while they also act as signaling molecules (Poljsak and Milisav 2012).

All of these mechanisms could be part of the underlying processes that lead to the strong reaction of fibroblasts towards keratinocyte supernatant.

DPI decreases significantly the level of oxidative stress in fibroblasts that have been treated with keratinocyte supernatant. Therefore, one could assume that NADPH oxidases play a major role in the strong reaction towards the supernatant.

The supernatant of control keratinocytes promotes a higher level of oxidative stress than supernatant of AD-model keratinocytes. This suggests that cellular responses in AD-model cells are different to control cells. Again, all of the mediators mentioned above could be involved but as DPI inhibits the reaction in such a strong way, NADPH oxidases seem to have a big role.

Another explanation for the differences in the reaction of keratinocytes and fibroblasts towards the opposing supernatant could be that the epidermis contains a higher concentration of antioxidants than the dermis (Baek and Lee 2016) which could be the reason for the keratinocytes not reacting that strong to fibroblast supernatant. The fibroblasts might have a poorer antioxidant capacity towards the mediators in the supernatant, thus leading to a hyperproduction of ROS which can easily overcome antioxidant defenses. Nonetheless, that doesn't exclude the possibility that fibroblasts and keratinocytes produce different mediators and that these are the reason for the different reaction.

Another possibility one has to consider is that the level of ROS being higher in control-supernatant treated fibroblasts than in AD-supernatant treated fibroblasts might be due to the level being in the higher end of the measurement accuracy and the precision can't be granted in these heights. Anyway, the fact that it is that high is enough to think about the reasons for being that high.

To conclude, the supernatant experiments revealed that both keratinocytes and fibroblasts secrete factors into the supernatant that can prime the other cells to react more sensitive to subsequent oxidative stress. The secreted factors could lead to a reinforcement of oxidative stress in neighboring tissues. While keratinocytes react very sensitive to even small concentrations of H_2O_2 , fibroblasts produce only small amounts of ROS in response to H_2O_2 . Treating fibroblasts with supernatant from conditioned keratinocytes however, ROS production is increased as a reaction towards H_2O_2 treatment to the same extent as seen in keratinocytes. The reaction towards oxidative stress in fibroblasts as well as in keratinocytes can be inhibited by DPI, therefore it can be assumed that it is mediated by NADPH-oxidases. A summarized overview of the level of oxidative stress in fibroblasts is displayed in Figure 15.

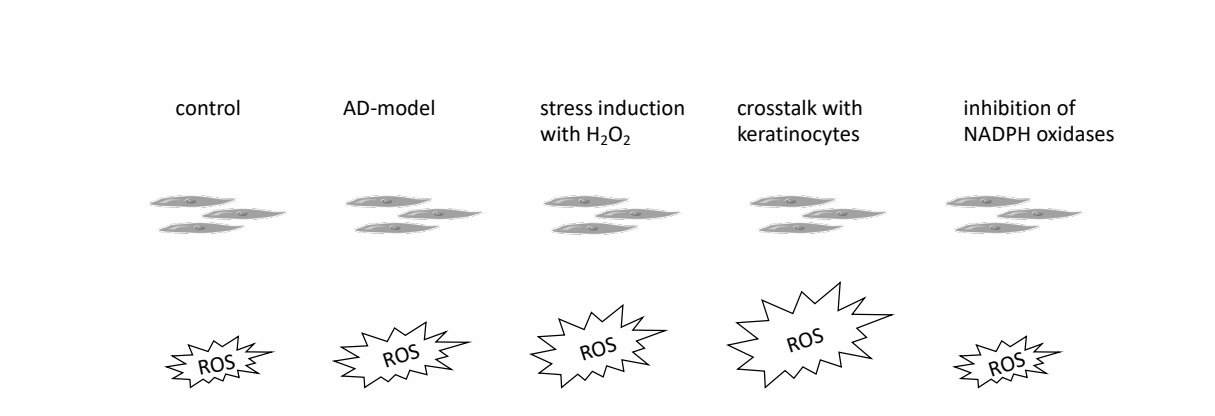


Figure 15: Model of the level of oxidative stress in fibroblasts. Control fibroblasts show a basal level of reactive oxygen species (ROS). AD-model fibroblasts show increased levels of ROS, which are even more increased after stress induction with H_2O_2 . After treatment of fibroblasts with supernatant from conditioned keratinocytes the level of ROS is the highest. By treating fibroblasts with an inhibitor of NADPH oxidases the level of ROS is decreased to the level of control fibroblasts.

6. Summary

The aim of this study was to explore the reaction of fibroblasts towards oxidative stress in an Atopic Dermatitis (AD) model as well as the consequences of oxidative stress. The interaction between keratinocytes and fibroblasts in relation with oxidative stress was another point of interest.

For the experiments, fibroblasts and keratinocytes were cultivated and stimulated with AD-specific cytokines. The sensitivity of fibroblasts towards oxidative stress was measured with a 2',7'-dichlorofluorescein diacetate (DCFDA)-assay, stress was induced by stimulation with hydrogen peroxide (H_2O_2). To inhibit intracellular reactive oxygen species (ROS) production the pan nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium (DPI) was used. Viability of the cells was measured with a Resazurin-assay, desoxyribonucleic acid (DNA) damage was investigated with immunofluorescence and participating signaling cascades were identified via western blot. In order to understand the interaction of fibroblasts and keratinocytes crosstalk experiments have been conducted.

It could be shown that AD-model fibroblasts exhibit elevated basal levels of intracellular oxidative stress compared to a control. Stress induction leads to even higher levels of ROS and AD-model fibroblasts react more sensitive to this treatment than control fibroblasts. By inhibiting NADPH-oxidases the level of oxidative stress is reduced, therefore it can be assumed that fibroblasts produce ROS in response to oxidative stress which leads to a positive feedback cycle of ROS generation and even stronger inflammation. This indicates that fibroblasts participate in the development of AD. The cell viability is not reduced in AD-model fibroblasts compared to a control, while the viability is reduced in both AD-model and control fibroblasts after the induction of oxidative stress. The induction of oxidative stress leads to DNA damage which can be abrogated by inhibiting NADPH oxidases, while there is no difference in the extent of damage between AD-model and control fibroblasts. The induction of oxidative stress leads to an activation of both pro-apoptotic and pro-survival pathways and promotes inflammation.

Crosstalk experiments showed that fibroblasts induce a stronger reaction towards oxidative stress in keratinocytes. Vice versa, keratinocytes also induce a stronger reaction towards oxidative stress in fibroblasts but in a much more powerful way, the level of oxidative stress in fibroblasts is increased up to the same level as in keratinocytes. In both cases the reaction could be suppressed by inhibiting NADPH oxidases, therefore it can be assumed that the reaction is mediated by NADPH oxidases. It can be assumed that both keratinocytes and fibroblasts secrete

factors that prime the other type of cells to react more sensitive to subsequent oxidative stress and lead to a reinforcement of oxidative stress in neighboring tissues.

It is important to learn more about the involved NADPH oxidases and to find ways to inhibit them in order to develop effective therapies to treat AD. The participation of fibroblasts should be considered in the treatment of the disease as well.

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8. Affidavit

Herewith I declare that this dissertation has been written completely independently and unaided apart from advices given by my doctoral supervisor and my other academic teachers, and that no other sources other than the ones given here have been used. Furthermore, I declare that this work has never been submitted at any other time and anywhere else as a dissertation to acquire a doctoral degree.

Date, Signature

9. Acknowledgements

At this point I want to thank everybody who helped me with my dissertation.

First, I want to express my great gratitude to my doctoral supervisor Prof. Dr. rer. nat. Hila Emmert for her amazing help and support. I cannot imagine that anyone could have done it any better.

Additionally, I want to thank Prof. Dr. med. Stephan Weidinger for his help and advice.

My sincere thanks go to Anke Rose, Steffen Bachmann and everybody else in the working group and at the Department of Dermatology, Venerology and Allergy, who helped me and was part of the friendly working atmosphere.

My family is largely responsible for helping me through my time in the lab and at the writing desk. I could not have done it without you.

Thank you.