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ORIGINAL ARTICLE

Dupilumab normalizes correlates of lysosomal function in atopic dermatitis

Christina Bürgler[1] | **Danielle Dutoit**[2] | **Susanne Radonjic-Hoesli**[1] | **Kathrin Thormann**[1] | **Mathias Lehmann**[1] | **Shida Yousefi**[2] | **Hans-Uwe Simon**[2,3] | **Dagmar Simon**[1]

[1]Department of Dermatology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland

[2]Institute of Pharmacology, University of Bern, Bern, Switzerland

[3]Institute of Biochemistry, Brandenburg Medical School, Neuruppin, Germany

Correspondence
Dagmar Simon, Department of Dermatology, Inselspital, Anna-Seiler-Allee 33, CH-3010 Bern, Switzerland.
Email: dagmar.simon@insel.ch

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Abstract

Background: Atopic dermatitis (AD) is characterized by skin barrier dysfunction and immune dysregulation. Autophagy, which is important for the epidermal differentiation, is impaired in AD. The treatment with dupilumab, an interleukin (IL)-4/IL-13 receptor blocker, has been shown to reduce skin inflammation and restore the skin barrier.

Objectives: This study aimed to investigate the effect of dupilumab on the expression of key proteins involved in autophagy and lysosomal degradation.

Methods: We performed immunofluorescence staining and microscopic analyses of skin specimens of AD patients, taken before and under (6–10 weeks) therapy with dupilumab, to investigate the expression of autophagy-related (ATG) 5 and ATG7 proteins, beclin-1, microtubule-associated protein light chain 3 (LC3B), sequestosome-1 (p62), lysosomal proteases (cathepsins B, D and L), serine protease inhibitors (SERPINB3, SERPINB4) as well as IL-33 and thymic stromal lymphopoietin (TSLP).

Results: The expression of LC3B and p62 as well as SERPINB3 and SERPINB4 was highly increased in untreated AD skin compared to non-lesional skin and normal skin and decreased upon dupilumab therapy. In contrast, the AD-associated increased expression of both ATG5 and ATG7 further increased under therapy. Before therapy, cathepsin D and L expression levels were significantly lower compared to normal skin, but increased following the initiation of dupilumab therapy. The increased expression of IL-33 and TSLP in the epidermis of AD patients correlated with that of LC3B and p62.

Conclusions: Our study provides further evidence that autophagy is inhibited in lesional AD skin owing to lysosomal dysfunction. Upon dupilumab therapy, a restoration of dysregulated key players of autophagy is observed.

Abbreviations: AD, atopic dermatitis; ATG, autophagy-related; IL, interleukin; LC, microtubule-associated protein light chain; NLS, nonlesional skin; NS, normal skin; SERPIN, serine protease inhibitor; TNF-α, tumor necrosis factor-alpha; TSLP, thymic stromal lymphopoietin.

KEYWORDS
atopic dermatitis, autophagy, dupilumab, skin barrier

INTRODUCTION

Atopic dermatitis (AD), a chronic inflammatory skin disease commonly observed in children and adults, is based on a genetic predisposition.[1,2] Skin barrier disruption, owing to both mutations, for example, filaggrin, and the exposome, as well as immune dysregulation are major factors in the pathogenesis of AD.[3,4] While an inflammatory response can be induced as a consequence of a disrupted skin barrier, the resulting chronic type 2-biased inflammation can further promote skin barrier dysfunction in AD.[5] Dupilumab, an antibody blocking the receptor for interleukin (IL)-4 and IL-13 t, is very effective in improving clinical signs and symptoms in patients with AD.[6,7] Moreover, dupilumab reduces cutaneous inflammation and seems to restore the skin barrier in AD as suggested by transcriptome and protein expression level studies.[8–10]

Autophagy is a noninflammatory highly conserved lysosomal degradation process to preserve cellular homeostasis. It involves the formation of autophagosomes, which are large vesicles formed by a double-membrane, engulfing cellular contents that need to be degraded. Subsequently, autophagosomes fuse with lysosomes, where lysosomal proteases degrade the infused cargo, carrying misfolded proteins and damaged organelles.[11] The autophagic process depends on the availability of autophagy-related (ATG) proteins, which are mainly involved in the formation of autophagosomes. Under physiological conditions, a basal level of autophagy is upheld. The process of autophagy needs to be tightly controlled, since dysregulated activity might contribute to cell death, inflammation and cancer.

As a consequence, numerous diseases, including neurodegenerative and autoimmune diseases, as well as metabolic disorders have been associated with dysregulated autophagy.[12] There are controversial reports on the effects of cytokines, for example, tumor necrosis factor (TNF)-α and IL-17, involved in the pathogenesis of inflammatory skin diseases on autophagy in keratinocytes.[13–16] Recently, we demonstrated that TNF-α plays a dual role as it facilitates the induction of autophagy in keratinocytes in an initial phase, but inhibits autophagy by reducing the levels and enzymatic activities of lysosomal cathepsins after long-term exposure.[17]

This study aimed at investigating the effects of dupilumab therapy on the epidermal barrier with special focus on proteins regulating autophagy and lysosomal degradation. Specifically, we were interested whether dupilumab might improve the previously reported dysregulated autophagic process and lysosomal dysfunction in AD skin.

METHODS

Tissue samples

Skin specimens taken for routine diagnostics of adult patients with AD were obtained from the archives of the Department of Dermatology, Inselspital. Basic demographic and clinical data of the patients are summarized in Supplementary Table S2. Specimens of lesional AD skin were taken before and after 6–10 weeks of dupilumab therapy (loading dose of 600 mg dupilumab followed by a maintenance dose of 300 mg every 2 weeks) from patients without concomitant systemic therapy ($n = 10$), and nonlesional skin (NLS) before treatment ($n = 5$). Skin from patients undergoing plastic surgery served as healthy controls (normal skin [NS]; $n = 3$). The study was approved by the Ethics Committee of the Canton of Bern. All patients gave informed consent for using data and biologic material for retrospective analyses.

Immunofluorescence staining

The specimens were analyzed using indirect immunofluorescence techniques as previously described.[17,18] Antibodies directed against autophagy markers (autophagy-related [ATG] 5 and ATG7 proteins, beclin-1, microtubule-associated protein light chain 3 [LC3B], sequestosome-1 [p62]), lysosomal proteases (cathepsins B, D and L), serine protease inhibitors (SERPINB3, SERPINB4) as well as epidermal alarmins IL-33 and thymic stromal lymphopoietin (TSLP) were used (Supplementary Table S1). Evaluation was performed using a confocal laser scanning microscope (LSM 510; Carl Zeiss Jena GmbH) at magnifications ×400 and ×630. For each staining and patient, 4–10 representative images were taken (Supplementary Table S3). To quantify the intensity of expression in the epidermis, Imaris software version 8.2.0 (Bitplane) and Image J (provided by the National Institutes of Health; https://imagej.nih.gov/ij/download.html) were applied.

Statistical analysis

Analyses were done using Graph Pad software Prism 9. For comparison of immunofluorescence data between groups and before-after therapy, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests and paired *t*-test, respectively, were applied. To correlate the expressions of alarmins and autophagy markers, linear regression analysis was done. *p*-Values < 0.05 were considered statistically significant.

RESULTS

Increased expression of autophagy markers ATG5 and ATG7 before and after treatment with dupilumab

The autophagic process is tightly regulated by the ATG protein family. ATG5 forms a complex with ATG12 and ATG16L, which functions as ubiquitin-like conjugating system, essential for the formation of autophagosomes and their fusion with lysosomes.[19] ATG5 and ATG7 were expressed in the cytoplasm of keratinocytes in all layers of the epidermis, though mainly in the basal and suprabasal layers (Figure 1). ATG5 expression was significantly higher in lesional AD skin compared to NLS and NS. Interestingly, upon dupilumab therapy, an additional increase of both ATG5 and ATG7 expression was noted in the skin of AD patients (Figure 1). These findings indicate a possible induction of autophagy in AD skin before as well as under therapy with dupilumab.

Decreased expression of LC3B and p62 upon treatment with dupilumab

The ATG12-ATG5-ATG16L1 complex together with ATG7 and ATG3 stimulates the conjugation of LC3 with phosphatidylethanolamine (PE) forming lipidated LC3-II which is important for membrane elongation and autophagosome closure.[20] The isoform LC3B is commonly used as an autophagy marker.[21,22] LC3B was present in all layers of the epidermis in AD skin before and after dupilumab therapy, NLS, and NS (Figure 1). A striking observation was the presence of intensively stained LCB3+ spots indicating autophagosomes particularly in the upper suprabasal and granular layers of AD skin. Upon dupilumab therapy, a significant decrease of LC3B+ spots was noted (Figure 1).

The autophagy receptor p62 links ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome.[23,24] p62 is selectively degraded during the autophagic process, but it accumulates in case autophagy is inhibited.[24] We detected p62 mainly in the granular layers, while its expression in the basal and suprabasal layers was minimal in AD skin and absent in NS (Figure 1). The quantification revealed a significant higher expression of p62 in lesional AD skin compared to AD skin under dupilumab therapy, NLS and NS. Moreover, we observed a weak expression of beclin-1 in the epidermis of AD skin, NLS and NS (data not shown).

Both observations the high number of LC3B-positive autophagosomes and the accumulation of p62 in lesional AD skin are suggestive of an impaired autophagic degradation.[17,25–27]

Dupilumab increases the expression of lysosomal proteases cathepsin D and cathepsin L

After fusion of autophagosomes with acidic lysosomes forming autolysosomes, the engulfed contents is degraded allowing a recycling of essential molecules.[21,28] The proteases cathepsin D, cathepsin L and cathepsin B are essential for proper lysosomal function.[29,30]

In line with our recent report, we found a significantly lower expression of cathepsin D and cathepsin L in lesional AD skin compared to NS (17). Upon dupilumab therapy, the expression of both cathepsins D and L increased toward levels seen in NS (Figure 2). In contrast, cathepsin B expression was similar in AD skin, NLS and NS (data not shown).

Dupilumab decreases the expression of the serine protease inhibitors B3 and B4 which are highly expressed in lesional AD skin

Based on RNA-seq analyses, a significant increased expression of the serine/cysteine protease inhibitors SERPINB3 and SERPINB4 in AD skin compared to NS has been reported.[31] Both serpins are located in the cytosol, but also in lysosomes, nuclei, and mitochondria.[32] While SERPINB3 was expressed in all layers of the epidermis, SERPINB4 was detected in the suprabasal layers. Both were predominantly found in the cytoplasm of keratinocytes, with a dotted pattern in the upper layers, suggesting its presence in lysosomes.[32] Our findings revealed a higher expression of SERPINB3 and B4 in lesional AD skin compared with AD skin upon dupilumab therapy, NLS and NS (Figure 2).

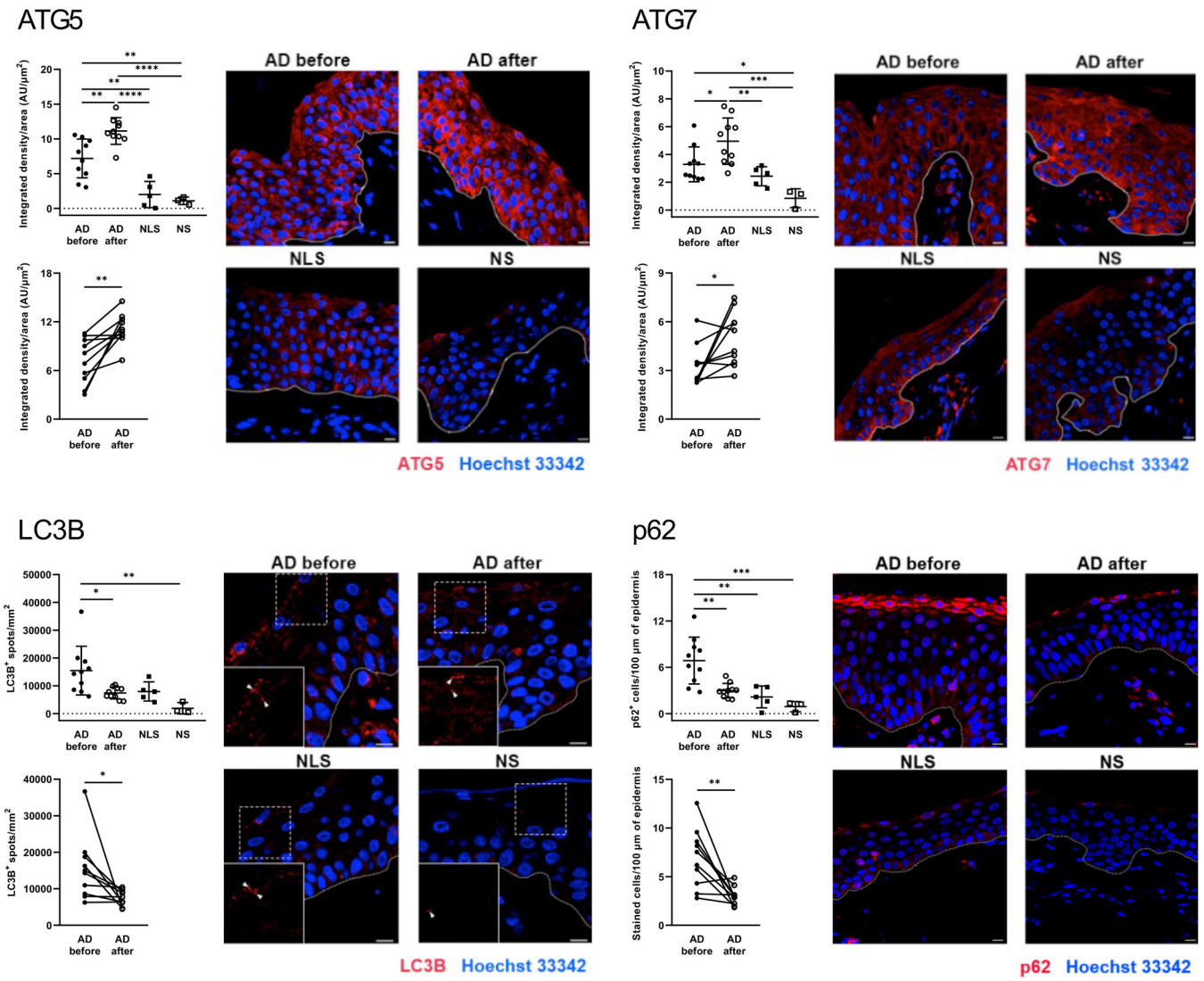

FIGURE 1 The elevated expression of autophagy-related (ATG) 5 and ATG7 in the epidermis of atopic dermatitis (AD) skin is further increased upon induction of dupilumab therapy. At the same time, protein light chain 3 (LC3B) and p62 expression levels decrease. Graphs in upper panels show a quantification of the expression (integrated intensity per area, LC3B positive spots per mm^2; mean ± SD) before ($n = 10$) and after ($n = 10$) therapy, nonlesional skin (NLS; $n = 5$), and normal skin (NS; $n = 3$). One-way analysis of variance was performed. For comparison before-and-after treatment expression, paired *t*-test was applied (lower panels). Representative images of immunofluorescence staining of ATG5, ATG7, LC3B, and p62 are presented (scale bar = 10 µm; the white dashed lines indicate the dermal-epidermal junction, arrows mark LC3B positive spots in inserts). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

The expression of TSLP and IL-33 correlates with LC3B and p62

In addition to their role as cytokines initiating Th2 inflammation in AD, TSLP has been demonstrated to increases the expression of IL-33, which has been implicated in the regulation of autophagy in keratinocytes.[33,34] We observed a significantly higher expression of TSLP and cytoplasmic IL-33 in the epidermis of lesional AD compared to NLS and NS that decreased upon dupilumab therapy (Figure 3). The expression of TSLP significantly correlated with that of p62 ($p = 0.004$) and LC3 ($p = 0.024$), respectively. For IL-33, we found a correlation with p62 expression ($p = 0.003$) (Figure 3).

DISCUSSION

This is the first report on the effects of dupilumab on proteins involved in the regulation of autophagy in keratinocytes in AD. Autophagy plays an important role in the development and differentiation of the epidermis.[35,36] AD has been associated with epidermal barrier dysfunction as well as impaired autophagy.[17]

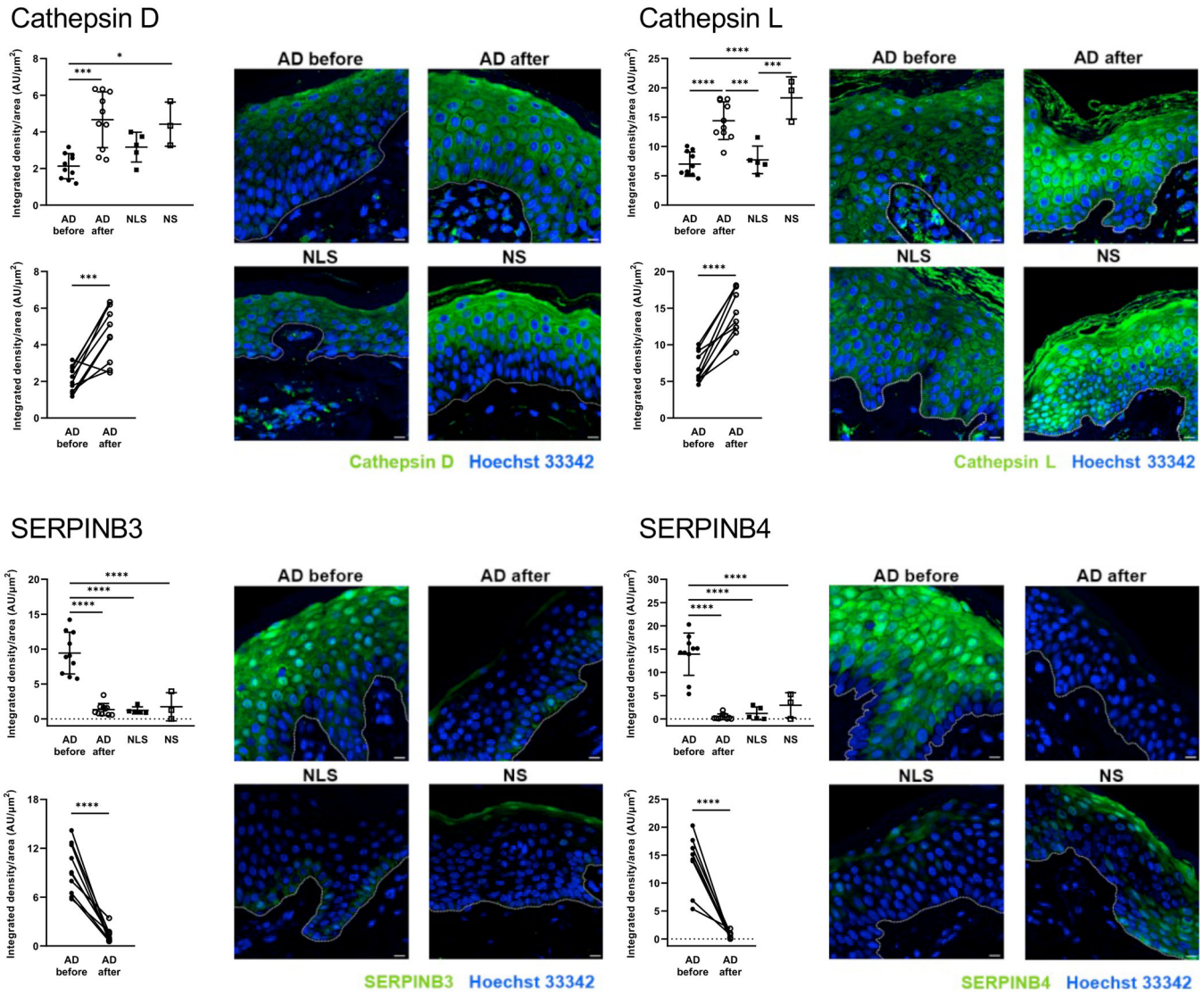

FIGURE 2 The expression levels of lysosomal proteases cathepsin D and cathepsin L are increased whereas those of the serine protease inhibitors SERPINB3/4 are decreased in atopic dermatitis (AD) skin upon dupilumab therapy. Graphs in upper panels show a quantification of the expression (integrated intensity per area; mean ± SD) in the epidermis of AD skin before ($n = 10$) and after ($n = 10$) therapy with dupilumab, nonlesional skin (NLS; $n = 5$), and normal skin (NS; $n = 3$). One-way analysis of variance was performed. For comparison before- and-after treatment expression, paired *t*-test was applied (lower panels). Representative images of the immunofluorescence staining of cathepsins D and L, and SERPINB3 and B4 are presented (scale bar = 10 µm; the white dashed lines indicate the dermal-epidermal junction). Isotype control staining is shown in Supplementary Figure S1. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

In agreement with our recent study, we observed an accumulation of LC3 and p62 in the uppermost layers of the epidermis in AD that is most likely a result of reduced degradation in autophagolysosomes.[17] The complex of p62 with polyubiquitinated cargos binds to LC3 associated with the inner membrane of the autophagosomes where it is selectively sequestered. As both p62 and LC3 are constantly degraded, their accumulation reflects dysfunctional autophagy.[26,27] Long-term exposure with the pro-inflammatory cytokine TNF-α promotes p62 expression in primary keratinocytes, which together with elevated LC3B-II levels indicates an impaired clearance in autophagolysosomes.[17] An increased p62 expression upon TLR stimulation of keratinocytes has been shown to be associated with increased proliferation and inflammatory cytokine production.[23] A recent study reported IL-4, IL-13, or a combination of both block the autophagic flux in keratinocytes resulting in significantly increased p62 levels.[37] Disturbed autophagy leads to an impairment of tight junctions and thus epidermal barrier dysfunction. Interestingly, human beta-defensin 3 via aryl hydrocarbon receptor (AhR) interacting with LC3 promotes the autophagy process.[37] Thus, it seems likely that blocking the IL-4/IL-13 receptor has a direct effect on improving autophagy in the epidermis of AD skin. The

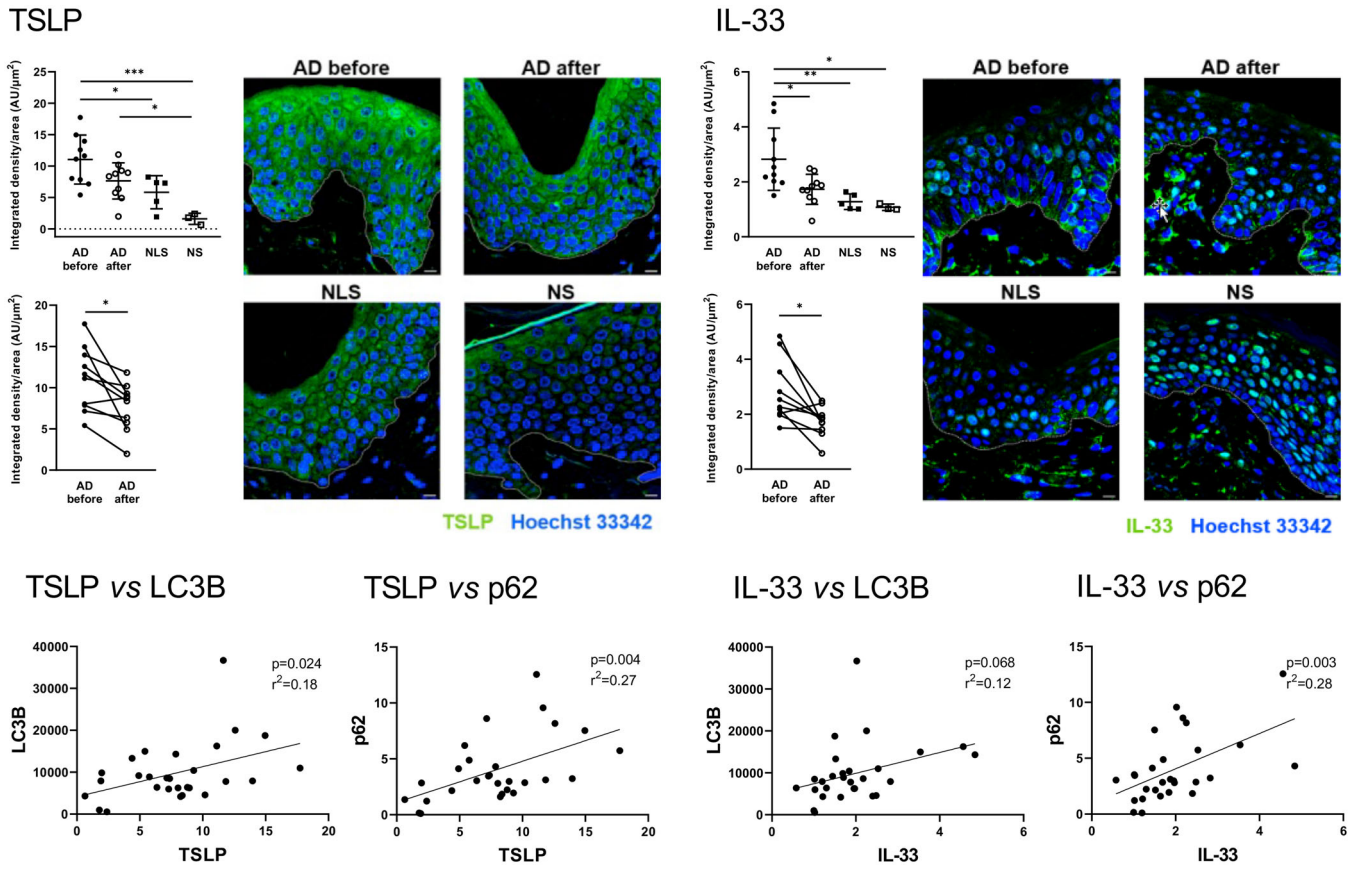

FIGURE 3 The expression levels of thymic-stromal lymphopoietin (TSLP) and interleukin (IL)-33 are increased in atopic dermatitis (AD) skin compared to nonlesional (NLS) and normal (NS) skin, and correlate with the expression of autophagy markers LC3B and p62. In the upper panel, the expression levels (integrated intensity per area; mean ± SD) in the epidermis of AD skin before ($n = 10$) and after ($n = 10$) therapy with dupilumab, NLS ($n = 5$), and NS ($n = 3$), comparison between groups (one-way analysis of variance; paired *t*-test), and representative images of immunofluorescence staining of TSLP and IL-33 are shown. Graphs in lower panel demonstrate the correlation between each TSLP and IL-33 with LC3B and p62, respectively (linear regression analysis, r^2 and p values are given). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

increased ATG5 and ATG7 levels might be interpreted as an adjustment to overcome the blocked autophagic flux in untreated AD epidermal cells, whereas they may reflect a true increase of autophagy and restoration of the epidermal barrier upon dupilumab therapy. The factors that trigger ATG5 and ATG7 expression under these conditions remain to be identified.

A proper lysosomal function is critical for early and late differentiation of keratinocytes.[35,36] Lysosomal proteases including cathepsin D and cathepsin B are required for proper lysosomal function, and any alteration of their levels or enzymatic activities may result in lysosomal dysfunction.[30] Inflammatory cytokines, for example, TNF-α, may reduce the levels and enzymatic activities of lysosomal cathepsins.[17] Indeed, in lesional AD skin, decreased expression of cathepsins D and L was found, whereas, upon dupilumab, an increase of cathepsins D and L was observed in parallel with decreased LC3B and p62 expression. The latter findings point to a possible improvement of the autophagic flux in keratinocytes of dupilumab-treated skin.

In agreement with the results of our study, the protease inhibitors SERPINB3/B4 (also called squamous cell carcinoma antigens [SCCA] 1 and 2) have been reported to be upregulated by IL-4 and IL-13, and highly expressed in AD skin.[31,38–40] By clustering of differentially expressed genes of AD skin, those encoding SERPIN and S100 as well as KRT6A/B and KRT16 proteins, known to be involved in epidermal differentiation and proliferation, were found in the same cluster.[41] Allergen exposure to the skin induced SERPINB3 expression promoting barrier dysfunction and early inflammatory responses in a mouse model.[42] In addition to their functions in inhibition of cell death and cell proliferation, SERPINB3 may also be involved in regulating autophagy by inhibiting cathepsin L activity.[43,44] In AD skin, dupilumab significantly downregulated mRNA expression of SERPINB4.[9,45] Our study

supports this finding, as we observed reduced protein expression of SERPINB3/B4 in lesional AD skin as a consequence of dupilumab therapy.

Interestingly, we noticed an association between the expression of alarmins TSLP and IL-33 and autophagy markers p62 and LC3. Upon inhibition of autophagy, an increased expression of IL-33 in co-localization with LC3 and p62 in keratinocytes, resulting in accelerated wound healing, has been observed in a mouse model.[33] In contrast, other authors have reported that TSLP and IL-33 treatment did not have any effect on LC3 levels and p62 accumulation in keratinocytes.[37] TSLP has been demonstrated to upregulate nuclear IL-33 followed by a reduced production of skin barrier proteins.[34] TSLP and IL-33 were found among the dysregulated genes in both lesional and non-lesional AD skin.[45] To note, an increase of IL-33 mRNA was noted upon dupilumab therapy.[45] By applying immunofluorescence staining, we observed a decrease of both cytosolic IL-33 and TSLP expression in AD skin upon dupilumab, whereas no effect on IL-33 expressed in the nuclei was noted in a previous study.[10]

Taken together, there is increasing evidence that autophagy is dysregulated in AD. Based on results of our and other studies, we hypothesize the following sequential events:

- inflammatory cytokines, including the alarmins TSLP and IL-33, TNF-α, IL-4 and IL-13, affect the expression of protease inhibitors,
- protease inhibitors, for example, SERPINs, inhibit cathepsin activity in autophagolysosomes,
- as a consequence, the autophagic flux is blocked in keratinocytes, resulting in an accumulation of LC3 and p62.

Whether the normalization of autophagy upon dupilumab therapy is due to direct effects on keratinocytes or indirect ones via suppression of inflammation, requires further investigation.

AUTHOR CONTRIBUTION

All authors contributed substantially to conception and design of the study. Danielle Dutoit, Shida Yousefi, Kathrin Thormann and Christina Bürgler performed data acquisition and data analysis. Danielle Dutoit, Christina Bürgler, Dagmar Simon and Hans-Uwe Simon interpreted the data. Christina Bürgler and Dagmar Simon drafted the manuscript. All authors revised the manuscript critically for important intellectual content.

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CONFLICTS OF INTEREST STATEMENT

Hans-Uwe Simon is a consultant for GlaxoSmithKline and Sanofi. Dagmar Simon has been an investigator, advisory board member, or consultant for AbbVie, AstraZeneca, Galderma, LEO, Lilly, Novartis, Pfizer, Sanofi Genzyme. Christina Bürgler has been an advisory board member for Sanofi, Novartis and Amryt. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Canton of Bern, Switzerland. All patients in this manuscript have given written informed consent for participation in the study and the use of their deidentified, anonymized, aggregated data and their case details (including photographs) for publication.

ORCID

Christina Bürgler https://orcid.org/0000-0003-1412-1869
Susanne Radonjic-Hoesli https://orcid.org/0000-0002-5742-0765
Kathrin Thormann https://orcid.org/0000-0002-4486-3591
Mathias Lehmann https://orcid.org/0000-0002-7894-8219
Shida Yousefi https://orcid.org/0000-0002-9855-4305
Hans-Uwe Simon https://orcid.org/0000-0002-9404-7736
Dagmar Simon https://orcid.org/0000-0001-8965-9407

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