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Original Article

LEKTI domain 6 displays anti-inflammatory action in vitro and in a murine atopic dermatitis model

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ABSTRACT

Background: Lympho-epithelial Kazal-type-related inhibitor (LEKTI) is a serine protease inhibitor consisting of multiple domains. A loss of function mutation is described in Netherton patients that show severe symptoms of atopic lesions and itch.
Objectives: LEKTI domain 6 (LD6) has shown strong serine protease-inhibitory action in *in vitro* assays and thus it was tested *in vitro* and *in vivo* for potential anti-inflammatory action in models of atopic skin disease.
Methods: Human skin equivalents were treated with LD6 and an inflammatory reaction was challenged by kallikrein-related endopeptidase 5 (KLK5). Furthermore, LD6 was tested on dorsal root ganglia cells stimulated with KLK5, SLIGRL and histamine by calcium imaging. The effect of topically administered LD6 (0.4–0.8%) in lipoderm was compared to a topical formulation of betamethasone-diproprionate (0.1%) in a therapeutic setting on atopic dermatitis-like lesions in NC/Nga mice sensitized to house dust mite antigen. Endpoints were clinical scoring of the mice as well as determination of scratching behaviour.
Results: KLK5 induced an upregulation of CXCL-8, CCL20 and IL-6 in skin equivalents. This upregulation was reduced by pre-incubation with LD6. KLK5 as well as histamine induced calcium influx in a population of neurons. LD6 significantly reduced the calcium response to both stimuli. When administered onto lesional skin of NC/Nga mice, both LD6 and betamethasone-dipropionate significantly reduced the inflammatory reaction. The effect on itch behaviour was less pronounced.
Conclusion: Topical administration of LD6 might be a new therapeutic option for treatment of lesional atopic skin.

1. Introduction

Atopic dermatitis (AD) is a chronic relapsing pruritic inflammatory skin condition. AD is commonly associated with elevated levels of immunoglobulin E (IgE) often directed against environmental allergens. The prevalence of AD symptoms in children increased over time in most geographical regions. Although treatment options increase, therapy gaps still exist [1]. Particularly there are still limited topical pharmaceutical treatment options for AD. Those frequently used, like topical calcineurin inhibitors or glucocorticoids, are prone to dose limiting side effects [2]. According to current understanding of the pathophysiology of AD, barrier disruption in the epidermis is a pivotal trigger factor of this disease. There is consensus that proteases play a central role in the initiation and maintenance of barrier disruption [3]. However, current symptomatic therapeutics for AD, such as glucocorticoids and calcineurin inhibitors or monoclonal antibodies like dupilumab, do not directly affect protease activity and often yield unsatisfactory effect on

Abbreviations: AD, atopic dermatitis; DRG, dorsal root ganglia; ELISA, Enzyme linked immunosorbent assay; FBS, fetal bovine serum; Fura-2AM, fura-2-acetoxymethyl ester; HBSS, hank's balanced salt solution; HDM, house dust mite allergen; IL, interleukin; IgE, immunoglobulin E; KCl, sodium chloride; LD6, LEKTI domain 6, LEKTI: Lympho-epithelial Kazal-type-related inhibitor; Ms, millisecond; PBS, phosphate buffered saline; TARC, thymus and activation regulated chemokine, FT-HSE: Full-thickness human skin equivalents, CCL20: C C-motif Chemokin ligand 20

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barrier disruption, although a recent study on dupilumab indicates some benefits on barrier function (dupilumab significantly improves skin barrier function in patients with moderate-to-severe atopic dermatitis). LEKTI (lympho-epithelial Kazal-type related inhibitor) is a 15-domain broad acting protease inhibitor, which is cleaved into active domains in the outermost skin layer [4]. There is a significant link between reduced transcriptional expression of *SPINK5* (the LEKTI encoding gene) and AD in humans [5,6], which is corroborated by functional data in keratinocytes from AD patients [7]. Patients with Netherton syndrome with a loss of function mutation of *SPINK5* frequently show inflammatory skin lesions, severe itch and atopic sensitizations [8]. It has been shown that another frequent mutation in LEKTI leads to enhanced protease activity in skin accompanied by enhanced thymic stromal lymphopoietin (TSLP) expression and disturbed barrier function [9]. Protease-activated-Receptors (PARs) are G protein-coupled receptors, which are activated by proteolytic cleavage by proteases including KLK5 [10]. Activation of PAR-2 in the skin effects many important processes concerning e.g., epidermal barrier defects and transduction of itch [11]Thus this study was performed to elucidate if topical administration of the LEKTI domain 6 (LD6) might have inhibitory action in cells critically involved in barrier disruption (keratinocytes) and perception of itch (dorsal root ganglia neurons). The dorsal root ganglia (DRG) contain the cell bodies of afferent sensory nerves, and it has been demonstrated that the same sensory receptor repertoire present at sensory terminals are also present on the cell bodies within the DRG [12]. In turn LD6 was tested in a chronic mouse model of AD, namely the NC/Nga mice sensitized to a relevant allergen (house dust mite allergen) [13,14]. LD6 was selected as the domain to study, as this is a very well characterized domain with several *in vitro* characterizations [15,16].

2. Material and methods

2.1. Expression of LEKTI domain 6 and purification

For *in vitro* studies, LEKTI domain 6 was provided by Azitra, Inc. and activity and purity was determined by protease inhibitory assays and mass spectrometry, respectively. For *in vivo* studies LD6 was recombinantly expressed as described previously [15] with slight modifications (e.g. expression vector was changed to pBAD/gIII). Purification was performed by means of HPLC. The LD6 containing peak was fractionated lyophilized and distributed by Pharis Biotec GmbH, Hannover, Germany. Characterization of LD6 domains is provided in supplementary methods file.

3. Human skin equivalents

Three-dimensional human skin cultures (full-thickness human skin equivalents = FT-HSE) were constructed as described, with some modifications [17]. Details are provided in the supplementary methods file.

4. Isolation of dorsal root ganglia neurons and calcium imaging on DRG cell culture

Preparation and cultivation of the dorsal root ganglia (DRG) neurons from female BALB/c mice was done as described previously [14]. Details can be found in supplementary methods file.

5. Mice

Fourteen female BALB/c mice (BALB/cAnNCrl, 7 ± 1 weeks old, body weight 18 ± 1.5 g) were obtained from Charles River (Sulzfeld, Germany). Forty female NC/Nga (NC/NgaTndCrlj, 6 ± 1 weeks old, body weight 17 ± 1.5 g) mice were purchased from Charles River Japan Laboratories (Tokyo, Japan). The animals were conventionally housed in standard type III macrolon cages at 22 ± 2 °C with 55 ± 10% humidity and a 12:12-hour light:dark cycle. The mice were fed with a standard pellet diet (ssniff Spezialdiäten GmbH, Soest, Germany) and received tap water *ad libitum*. After arrival the mice were randomly allocated into groups of four mice per cage and habituated to their new environment for two weeks before experimental procedures were conducted. Cages were changed weekly. One week prior to experiments, animal caretakers and experimenters habituated the mice to handling procedures. Animals were tail handled and enrichment (nesting materials with red plastic houses and alum tunnels) was offered. The study was conducted according to the German Welfare Act and National Animal Welfare Guidelines. The study protocol was ethically approved by local authorities (LaGeSo institute, Berlin, Germany; permit number G0234/17 and T0264/18). Potential 3 R applications were thoroughly reviewed and if identified will be implemented into future studies with this model. Humane endpoints were predefined. They were divided into clinical parameters (bodyweight reduction > 20%, motor dysfunctions, stereotypic behavior, bad general condition) and model-specific parameters (excessive ear swelling, skin ulceration, lasting intensive itch > 2 h after allergen application). Humane endpoints were not reached during the study.

6. Murine model of atopic dermatitis in NC/Nga mice

NC/Nga mice were clipped with electric clippers (Isis, Aesculap Schermaschinen GmbH, Suhl, Germany) and depilated with depilating cream (Veet®, Reckitt GmbH, Heidelberg, Germany) in the neck and back region one day before sensitization procedure started. Each cage with n = 4 mice was randomly allocated into one of the 4 experimental groups. For sensitization mice received 30 µl of 10 mg/mL house dust mite allergen (HDM, *Dermatophagoides farinae*, (GREER, Lenoir, NC, USA)) suspended in Paraffinum perliquidum (Caesar & Loretz GmbH, Hilden, Germany) onto the back and 10 µL onto the right ear twice weekly. To enhance the sensitization procedure, tape stripping with adhesive Tesa® tape (10 x on the depilated skin; tesa SE, Norderstedt, Germany) was performed once a week before the first of two HDM sensitizations that were performed per week (see Fig. S1). As soon as visible lesions had developed, tape stripping was terminated. Treatment with LD6 (0.4% in lipoderm lotion, Spirig Pharma, Egerkingen, Switzerland, n = 8), betamethasone dipropionate (0.1% in lipoderm, n = 8) or vehicle (0.4% BSA (Sigma-Aldrich) in lipoderm, n = 8) on back skin and right ear skin was started on day 17, where the mice showed a mean lesional score of 2. One group remained untreated, i.e. it did not receive any medical treatment nor HDM. The dose selection for betamethasone dipropionate (Sigma-Aldrich) was according to a former study in which 0.1% betamethasone dipropionate inhibited itch behaviour and inflammation in NC/Nga mice [11]. As loss in body weight was observed in the former study, it was decided to start betamethasone dipropionate treatment every other day from the very beginning. LD6 was produced by recombinant expression and the whole amount of 100 mg was calculated for a topical administration for 3 weeks maximum. After conservative calculation of total LD6 amount needed, the experiment was started with 0.4% and the dose was increased to 0.8% at day 23. As stability of LD6 at room temperature was decreased, we decided to prepare the cream freshly once a week and keep it refrigerated. This procedure was tested before the experiments with pig skin in Franz diffusion cells and led to high dermal penetration (data not shown).

During the experimental period, ear and back skin thickness, body weight, clinical scores were monitored twice a week and scratching behavior once every week. The clinical score was determined as according to the following system: no symptoms, 0; mild, 1; moderate, 2; severe, 3 and extreme, 4. The scores for erosions, edema, and erythema as well as skin dryness were added

resulting in a maximum score of 16. To monitor scratching behavior mice were video monitored for 60 min directly after sensitization with HDM once a week. Video monitoring was performed with mouse pairs (belonging to the same treatment group). Only repeated strokes with the hindlimb directed to the area of HDM sensitization were counted as scratching bout. All mice were sacrificed on day 30 (24 h after the last HDM challenge) and tissue (back and ear skin, *Lymphonodus auricularis dexter*) was collected from each group.

Samples were processed (or stored) for histology, western blot and cytokine determination and lymph node weights. Total IgE in serum was measured by means of an ELISA (IgE ELISA Ready-SET-Go!, Fisher, Scientific, Germany).

7. Real-time PCR

Lysis of the samples and mRNA isolation was performed with RNeasy Mini Kit (Quiagen, Hilden, Germany) as well as digestion of DNA was performed with RNase-Free DNase Kit (Quiagen), according to manufacturer's protocol. Details about protocol can be found in supplementary methods file.

8. Histology and immune histology

Skin equivalents were fixed in a mixture of 7.5% glutaraldehyde and 3% paraformaldehyde (both Carl Roth, Karlsruhe, Germany) and then incubated in 1% osmium tetroxide (Chempur, Karlsruhe, Germany). After dehydration in an ascending ethanol series the samples were embedded in a mixture of agar 100 resin, dodecenyl succinic anhydride, methyl-5-norbornene-2,3-dicarboxylic anhydride and 2,2-dimethoxypropane 30 (Agar Scientific, Stansted, UK). Semi-thin sections (0.5 µm) were stained with modified Richardson`s stain [18]. Sections were examined using an Axioimager M1 microscope (Carl Zeiss, 73447 Oberkochen, Germany). Images were taken with an AxioCam MRm version 3 FireWire monochrome camera and the AxioVision software, version 4.5 (Carl Zeiss, Germany).

Tissue samples from rostral neck skin or ear skin were excised, fixed in neutral buffered formalin (4% solution). After dehydration in an ascending ethanol series (70 – 100%) the samples were embedded in paraffin sectioned and stained with haematoxylin-eosin (Roth, Arlesheim, Switzerland). Edema and cell influx were evaluated semiquantitatively (no influx, no edema, 0; mild, 1; moderate, 2; and severe influx, severe edema, 3) in a blinded manner in skin sections (n = 8 for treatment groups, n = 7 for untreated control).

To assess the level of Filaggrin in the ear skin, we prepared 6 µm-thick paraffin sections and stained them with a Filaggrin-specific antibody (Poly 19058, BioLegend, USA) at a 1:1000 dilution. We used a donkey anti-rabbit IgG conjugated to Alexa Fluor 647 as the secondary antibody at a 1:800 dilution (Jackson ImmunoResearch, Baltimore, USA). A rabbit IgG control (Solid phase absorbed, Agilent Dako x0936, Santa Clara, CS, USA) served as negative control (see Fig. S4). Nuclei counterstaining (DAPI) was performed with ROTI Mount FluorCare DAPI (Carl Roth GmbH + Co. KG, Germany) according to manufacturer`s instruction. We visualized the fluorescence signal with a Leica Dmi8 microscope (Wetzlar, Germany) and a 20X objective. Fluorescence was graded visually and blinded by the author on a scale from 1 to 5 (1 as low, 3 as moderate and 5 as high – 0–5 scale).

9. Cytokine determination of murine skin tissues

One part of the ear skin tissue was snap-frozen in liquid nitrogen. Cytokine determination for skin tissues was performed as described previously [13]. Details about protocol can be found in supplementary methods file.

10. Statistical analysis

All data are displayed as mean (± SD). Statistical significance of the difference was estimated at the 5% and 1% levels of probability. Data were tested for normality distribution graphically and with D'Agostino & Pearson test. As the data were normally distributed, parametric tests were performed. For comparisons of more than 2 groups we used a one-way ANOVA, followed by Dunnett's or Sidaks multiple comparison test. Data that were obtained over multiple time points, as in the in vivo experiment, were analysed with two-way ANOVA, followed by Dunnett's or Sidak's multiple comparisons test. Comparisons of proportions (study with dorsal root ganglia) were made by means of the Fisher exact test. The data analysis was performed with Prism version 8 (GraphPad Software, San Diego, CA, USA).

11. Results

11.1. Stimulation of human skin constructs with KLK5 induced skin barrier defects and a cytokine expression that is reduced by LD6

As expected, histological evaluation revealed that skin constructs consisted of a dermal and an epidermal compartment. The epidermal equivalent was multilayered and showed characteristics of epidermal layers of *in vivo* skin with high variability between groups (vehicle treated control, KLK5, KLK5 + LD6). The control constructs showed the formation of a regular epidermal layer architecture, including a *stratum basale*, *stratum spinosum*, *stratum granulosum* and a multilayered *stratum corneum*. However, the constructs in the KLK5 group lacked epidermal keratinization and showed only occasionally cells that clearly corresponded to the *stratum granulosum*. The surface of the KLK5 constructs was irregular, appeared frayed and the superficial cells were detached, indicating a disruption of the intercellular connections. The epidermal layers of the KLK5 + LD6 group displayed a great variety. However, in the majority of the samples (n = 8), a stratum granulosum had formed, while the stratum corneum was discontinuous or in other samples continuous preserved. The surface of all constructs of the KLK5 + LD6 group showed no signs of excessive detachment of superficial cells, in line with the control constructs (Fig. 1b)Incubation of the FT-HSEs with KLK5 (0.4 µmol/L) led to a significant upregulation of IL-6, IL-33, CXCL8 and CCL20 gene expression (Fig. 1a). IL-25 was not amplificated in the constructs. A pre-incubation with 1 µmol/L LD6 and therapeutical treatment with 0.5 µmol/L reduced their up-regulation (significantly for CXCL8).

11.2. Impact of LD6 on SLIGRL, KLK5, histamine and IL-31 on calcium signals induced in DRGs

SLIGRL (100 µmol/L), a tethered ligand for PAR-2, induced distinct calcium signals in roughly 9% of neurons. This was significantly reduced by co-incubation with LD6 (2.5 µmol/l, Fig. 2a). The PAR-2-activating protease KLK5 (150 nmol/L) also induced a calcium signal to a similar extend as SLIGRL (10% of neurons). This was again reduced by LD6 (0.6 µmol/L; 3% of neurons), however, to observe a significant effect, a pre- and co-incubation with LD6 was necessary (Fig. 2a). Similarly, the histamine (500 nmol/L) induced signaling (13% of neurons) was also significantly reduced by pre- and co-incubation with LD6 (2.5 µmol/L; 8% of neurons; Fig. 2a/b), whereas the IL-31 (1 µg/L) induced activation (1% of neurons) was not affected by pre- and co-incubation with LD6 (2.5 µmol/L; Fig. 2a). LD6 itself at the selected concentrations does not lead to any activation of neurons (data not shown).

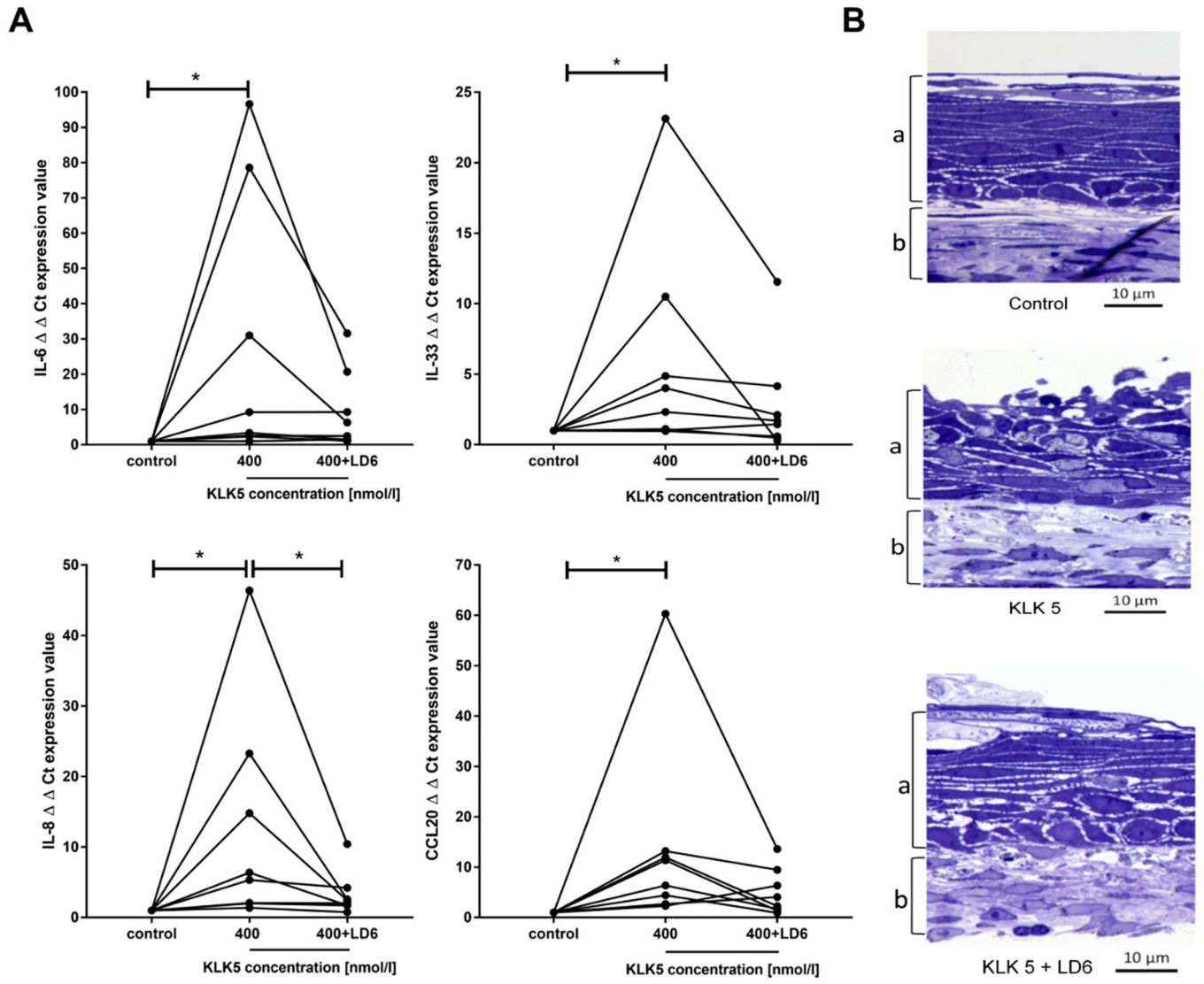

Fig. 1. Stimulation of Full-thickness human skin equivalents with KLK5 induced a cytokine expression that was reduced by LD6N/TERT-1 (keratinocytes) were co-cultivated with fibroblasts and endothelial cells to obtain a human 3D skin construct (A). Incubation with KLK5 (400 nmol/L) led to significant upregulation of IL-6, IL-33, CXCL-8 and CCL20. LD6 (1 µmol/L) was used for pre-incubation and follow on treatments were performed with 500 nmol/L. Samples were analyzed 6 hours after KLK5 treatment. Data are shown for mRNA regulation of IL-6, IL-33, CXCL-8 and CCL20 and compared to house keeping gene GAPDH. N = 8 constructs per group, performed in three independent settings. Statistical analysis was performed by Kruskal-Wallis test with multiple comparisons to the controls, *p < 0.05, followed by Wilcoxon test for KLK 400 nmol/L and recombinant human KLK 400 nmol/L+ LD6 *p < 0.05. *p < 0.05.Histologically prepared FT-HSE (B), thickness of sections were around 5 µm, a) epidermal equivalent (N/Tert-G1), b) dermal equivalent human fibroblasts and endothelial cells.

11.3. A topical formulation of LD6 and betamethasone reduces lesions in the murine NC/Nga model of AD

The disease severity of the murine atopic like lesions was monitored according to the four major clinical symptoms of atopic dermatitis, namely excoriations, edema, erythema and skin dryness. After starting HDM application, the lesional score gradually increased to a mean score of 2 at day 16. Treatment was started on day 17 and continued to day 29. Topical administration of 0.4% (0.8% from day 23 on) and 0.1% betamethasone (every other day) resulted in reduction in skin lesions compared to the vehicle-only (0.4% BSA) group. This difference became significant on day 23 for betamethasone diproprionate and day 26 (9 days after start of treatment) for LD6 (Fig. 3A). In parallel ear thickness was measured of the right HDM treated mouse ear. A slight increase in ear swelling (mean 20 µm) was observed, when topical treatment started at day 17. Again, topical treatment with LD6 and betamethasone could reduce ear swelling significantly starting from day 23 (7 days after treatment start). However, the reduction was more pronounced in the betamethasone group (Fig. 3B). Itch was determined on days 1, 11, 15, 22 and 29. A steady increase in itch behavior was observed over time. This was not observed in non-sensitized NC/Nga mice. Neither betamethasone, nor LD6 administration reduced the itch response significantly (Fig. S2). Body weight was not significantly affected by the treatment procedure, although a slight decrease of body weight was observed in the betamethasone dipropionate group (Fig. S3).

The histological evaluation confirmed the *in vivo* findings: At day 30 vehicle treated mice had markedly increased edema formation and inflammatory cell influx. This was significantly reduced by topical treatment with LD6 and betamethasone compared to vehicle treatment (Fig. 3 C and Table 1). Also epidermal hyperproliferation was reduced in ear and back skin treated with LD6 or betamethasone dipropionate (Fig. 3D and Table 1). Filaggrin, a fundamental structural protein for barrier function was significantly decreased in vehicle

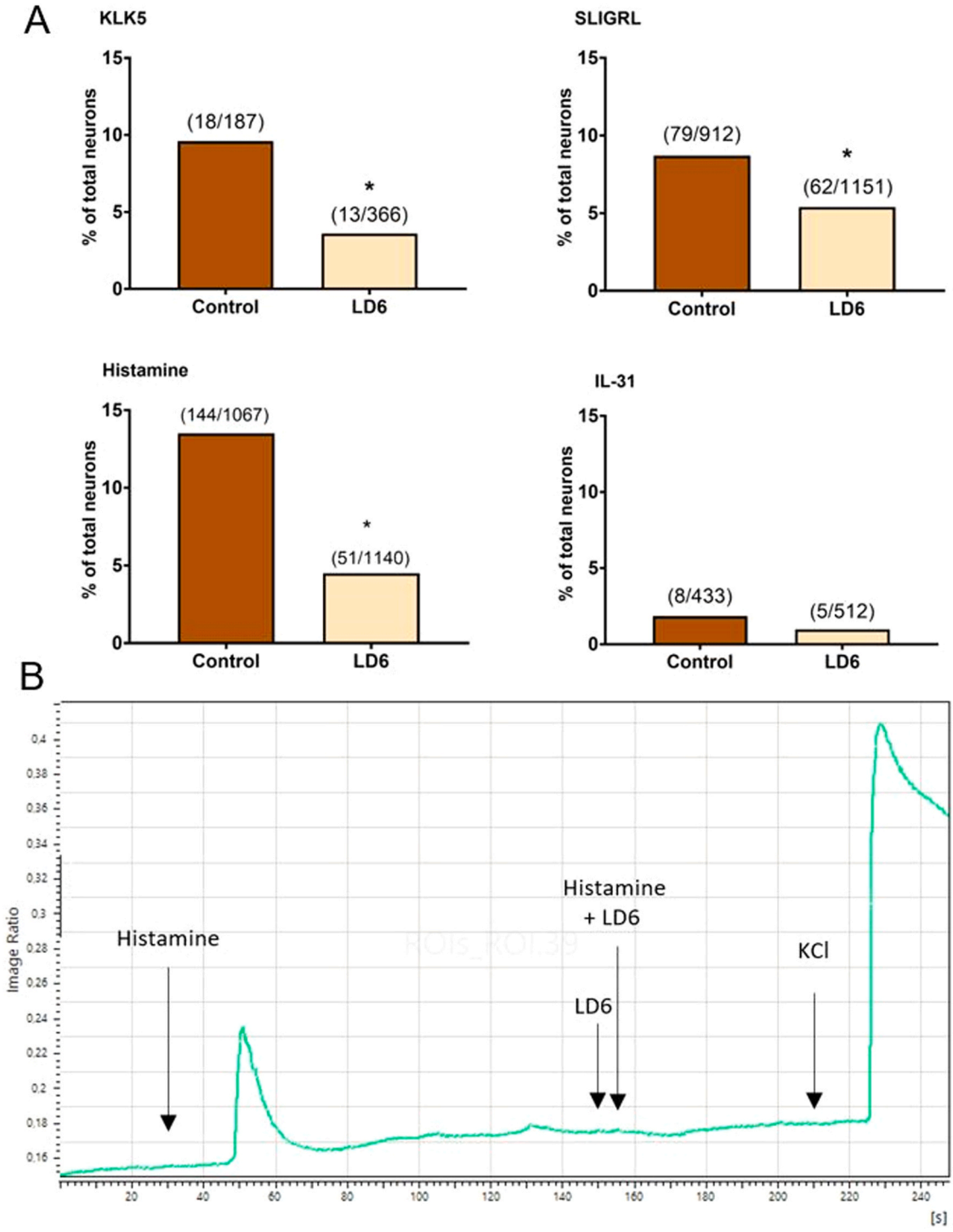

Fig. 2. Murine dorsal root ganglia were stimulated with KLK5 (150 nmol/L), SLIGRL (100 µmol/L), histamine (500 nmol/L) and IL-31 (1 µg/mL) (A). All stimuli induce calcium signals in subpopulations of DRG. For stimulation with KLK5 LD6 (600 nmol/L) was both pre- and co-incubated. LD6 (2.5 µmol/L) was only co-incubated with SLIGRL. The IL-31 and histamine stimulations were compared to a pre- and co-incubation with LD6 (2.5 µmol/L). For KLK5 n = 3, for SLIGRL n=4, for IL-31 n=3 and for histamine n=3 independent experiments per group, absolute numbers of neurons tested in brackets $^{*}p < 0.05$, $^{**}p < 0.01$ compared to stimulus alone. B) representative calcium response of a DRG neuron reacting to histamine (500 µmol/L). This reaction was abolished in the presence of LD6 (2.5 µmol/L), the response to KCl confirms the reaction of a sensory neuron.

treated and HDM challenged mouse skin, this disruption was almost completely prevented by LD6 and betamethasone dipropionate (Fig. 4).

The levels of pleiotropic pro-inflammatory cytokines like IL-1β, IL-6, KC and the Th2 cytokines IL-13 and TARC were determined in HDM challenged ear tissue (Table 1). All cytokines except for IL-13were strongly diminished by treatment with LD6 or betamethasone, compared to the vehicle (BSA in lipoderm) treatment. The local lymph node (*Ln. auricularis*), was significantly enlarged in vehicle treated mice by HDM treatment, again, this was significantly reduced in LD6 and betamethasone dipropionate (Table 1). Total IgE was measured in serum of all mice, values of vehicle treated and HDM challenged mice are vastly increased and reduced in mice treated with LD6 and betamethasone dipropionate (Table 1).

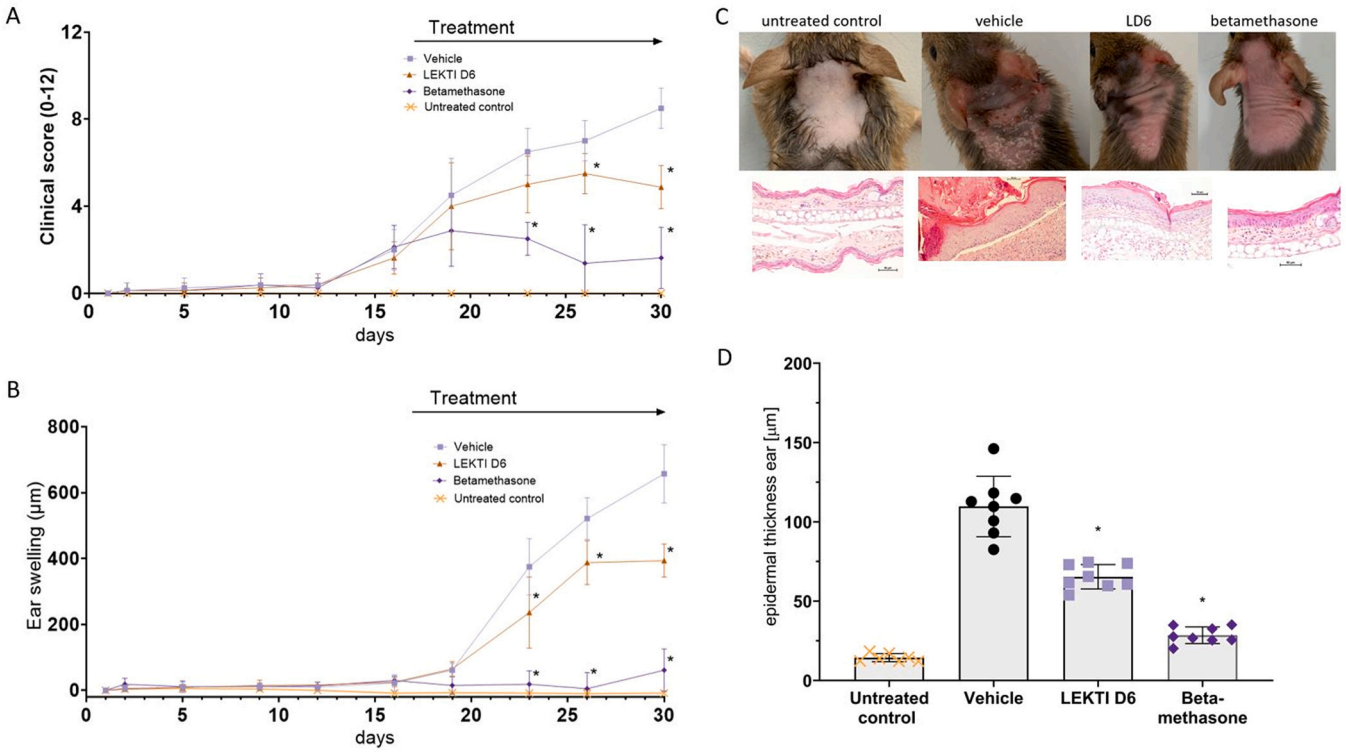

Fig. 3. Topical LD6 reduces the inflammatory response to topical house dust mite antigen in NC/Nga mice.(A) NC/Nga mice sensitized to house dust mite antigen (HDM) were treated topically with vehicle (0.4% BSA), LD6 (0.4–0.8%) or betamethasone dipropionate (0.1%) in lipoderm after first visible lesions occurred (Score = 2). Lesion score was evaluated by two persons blinded to the treatment allocation. (B) Ear swelling (right ear) of NC/Nga mice sensitized to HDM. Ear thickness was measured 24 h after topical HDM application and the respective treatment with vehicle (0.4% BSA), LD6 or betamethasone dipropionate in lipoderm (C) Macroscopic and histological evaluation of mouse skin (ear) at the end of the experiment. (D) Determination of epidermal thickness in histological specimens at the end of the experiment. N = 8/group, *p < 0.05 compared to vehicle (0.4% BSA) treated mice.

12. Discussion

Although new drugs like dupilumab or Janus kinase inhibitors recently entered the market for the treatment of atopic dermatitis (AD), particularly the topical treatment options are still limited. Additionally, the frequently used glucocorticoids and calcineurin inhibitors are prone to side effects, particularly with their long-term use [1].

Starting *in vitro* experiments in human skin constructs (FT-HSE model) and murine dorsal root ganglia neurons, the broad acting serine protease inhibitor LD6 displayed encouraging results indicating a benefit for both, the allergic inflammatory pathophysiology as well as sensory perception (itch). *In vivo*, LD6 reduced the inflammatory process as shown by blinded evaluated lesion scores, histology and cytokine content in inflamed tissue. Still, the anti-inflammatory potential of betamethasone dipropionate was stronger compared to LD6, but these results indicate that topical administration of LD6 might be of benefit particularly in those scenarios, where enhanced protease activity is part of inflammatory response or where mutations of LEKTI might hamper their inhibitory potential [19].

It has been described, that kallikreins are up-regulated in lesional skin of atopic dermatitis [6] [20]. Particularly KLK5 [21], KLK7 [22] and KLK14 [23] play a role under inflammatory skin conditions and all three are inhibited by LEKTI fragments [16,24], although LD6 has been reported to have no inhibitory activity on KLK14 in one study [24]. Recently, *Staphylococcus aureus* (*S. aureus*) has been described as a trigger factor to increased KLK activity in keratinocytes [25]. This is of interest as enhanced colonization of *S. aureus* is frequently observed in AD patients and might link barrier disruption due to enhanced KLK activity with a changed microbiome in AD lesions [26].

Surprisingly, the anti-itch effects that were indicated by the *in vitro* experiments were not confirmed *in vivo* in the NC/Nga mouse model. However, even betamethasone dipropionate did not significantly reduce itch behavior in contrast to a former study [13]. One reason might be that treatment with betamethasone

Table 1
Cytokine concentration in ear skin, IgE in serum, local lymph node weight and epidermal thickness as well as histological evaluation of back skin after topical application of LD6 and betamethasone diproprionate to house dust mite challenged NC/Nga mice in a chronic setting.

	Untreated control	Vehicle + HDM	LD6 + HDM	Betamethasone + HDM
IL-1β (pg/mg)	151 ± 44	638 ± 164	267 ± 243 *	158 ± 50 *
IL-6 (pg/mg)	76 ± 30	351 ± 238	166 ± 200	67 ± 37*
IL-13 (pg/mg)	446 ± 168	216 ± 71	164 ± 39	267 ± 93
CXCL1/KC (pg/mg)	133 ± 59	1712 ± 603	954 ± 746	532 ± 245*
TARC (pg/mg)	241 ± 174	257 ± 72	98 ± 75 *	146 ± 44*
IgE (ng/mL)	0.1 ± 0.01	41.59 ± 13.1	33.29 ± 3.3	13.4 ± 10.3 *
LN weight (mg)	5 ± 1	24 ± 7	15 ± 4 *	7 ± 3 *
Back skin epidermal thickness (µm)	8 ± 1	130 ± 20	73 ± 9 *	36 ± 11 *
Back skin histological evaluation (edema and cell influx)	0.2 ± 0.4	2.8 ± 0.4	1.6 ± 0.5 *	0.6 ± 0.5 *

Results are expressed as mean ± SD (n = 8 per group). *P < .05 (Dunnett's multiple comparison test) vs. HDM + vehicle group.

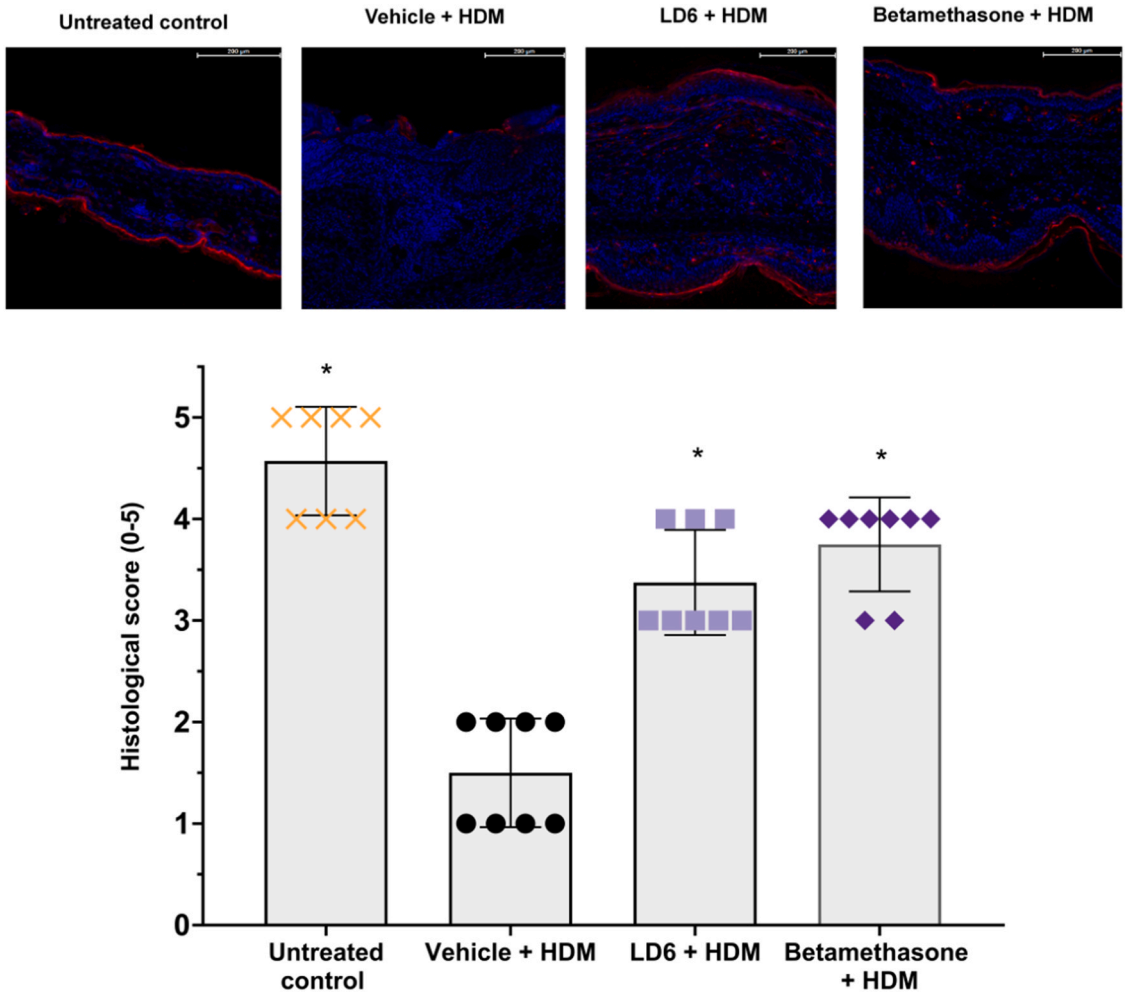

Fig. 4. Challenge with house dust mite antigen reduces filaggrin expression in the epidermis, topical administration of LD6 or betamethasone-dipropionate almost completely restored filaggrin expression (in red) in mouse epidermis (bar represents 200 µm). Six µm sections of paraffin embedded mouse ears were stained with a filaggrin-specific antibody and counterstained with DAPI. A blinded examination of sections according to a score system from 1 (low) to 5 (high filaggrin expression) revealed a drop to 1.5 in the vehicle group, that was restored to 3.4 by LD6 and 3.75 by betamethasone diproprionate. N = 8/group, $^{*}p < 0.05$ compared to vehicle (0.4% BSA) treated mice.

dipropionate was started on an every other day schedule, the former study started with daily treatment and was later reduced to every other day due to severe loss in body weight in treated mice. This occurred in the present study as well, but on a lower level (Fig. S3).

Although we enhanced LD6 from 0.4% to 0.8% within the study, we were limited in total amount of LD6 and thus it might be worthwhile to test higher concentrations in follow-on studies. It is noteworthy that non-protease related calcium signaling (by histamine) was also significantly reduced by LD6 *in vitro*. As SLIGRL is a tethered ligand for PAR-2 it was not expected that a protease inhibitor like LD6 reduced SLIGRL induced an intracellular calcium increase to the same extend as it reduced KLK5 signaling. The exact mechanism of the inhibitor potential *in vitro* warrants further investigation.

NC/Nga mice are frequently used for mechanistic studies as well as for testing new therapeutic options of AD [13,27,28]. The advantage of this model is that lesions are induced with a relevant allergen (house dust mite antigen) and the phenotype is quite translational to the human counterpart (constant itch, Th2 cytokines, lichenification and enhanced IgE levels) [29]. Although the inhibitory action of LD6 is moderate, there is the possibility that subpopulations of AD patients might benefit from LD6 substitution. Recent systematic analyses reveal, that several endotypes of AD patients exist, some with particular changes in barrier function [30]. In this context, the filaggrin results (Fig. 4) further support, that LD6 can restore barrier function in allergic inflammatory states. In addition, several polymorphisms in the LEKTI coding gene *SPINK5* have been described [31] and are partly related to disease severity [32]. Further, a down-regulation of *SPINK5* has been described in a microarray analysis of AD patients in Korea [5]. Thus, particularly patients with down-regulated *SPINK5* expression, dysfunctional LEKTI or enhanced KLK activity might benefit from topical LD6 substitution.

13. Conclusions

A repeated/chronic administration of LEKTI domain 6 reveals significant anti-inflammatory properties in a mouse model of atopic dermatitis. These results indicate that topical administration of a body-own serine-protease inhibitor might be of benefit in AD patients where an enhanced protease/kallikrein activity or decreased LEKTI activity is observed.

Ethics approval

The study was conducted according to the national guidelines for the Care and Use of Laboratory Animals. All protocols described herein have been approved by local authorities (LAGeSo; study protocol G0234/17 and T0264/18).

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CRediT authorship contribution statement

Pascal Canbolat: Investigation, Methodology, Formal analysis, Visualization. **Jenny Wilzopolski:** Investigation, Methodology, Formal analysis, Visualization. **Sabine Kaessmeyer:** formal analysis, visualization. **Marc Rüger:** Resources, Methodology. **Hans-Jürgen Mägert:** Conceptualization, Resources. **Wolf-Georg Forssmann:** Conceptualization, Resources. **Wolfgang Bäumer:** Supervision, Conceptualization, Formal analysis, Writing – original draft, Funding acquisition.

Declaration of Competing Interest

The authors have no conflict of interest to declare

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdermsci.2024.03.004.

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