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# Exonic mutations associated with atopic dermatitis disrupt LEKTI action and enhance its degradation

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## **Short title**

Exonic mutations of LEKTI associated with AD

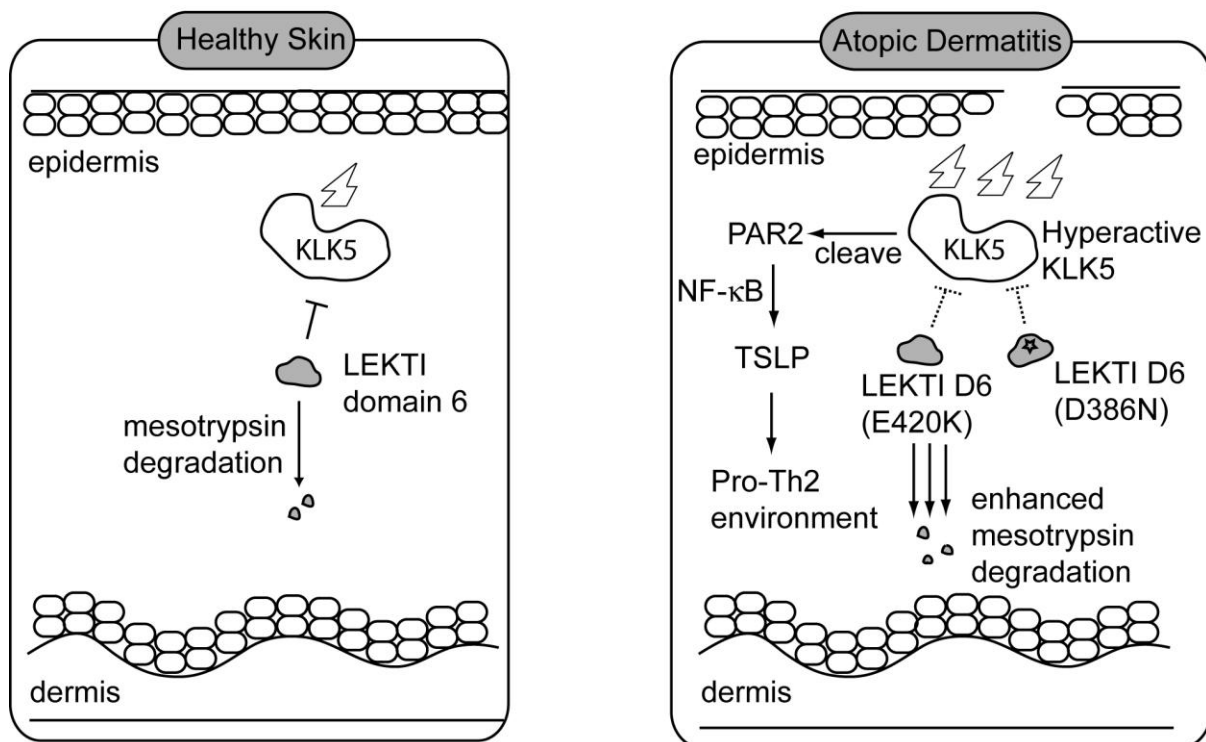
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## Keywords

Atopic dermatitis, exonic mutation, KLK5 protease, LEKTI activity, mesotrypsin degradation

### Exonic mutations of *SPINK5* in atopic dermatitis



## GRAPHICAL ABSTRACT

KLK5 protease is responsible for skin homeostasis and is under the tight inhibitory control of the multidomain proteinase inhibitor, LEKTI, encoded by *SPINK5*. D386N SNP at the active site of LEKTI disrupts its inhibitory activity, whereas E420K SNP enhances its degradation by mesotrypsin. Both SNPs contribute to the enhanced protease activity of KLK5 and thus disrupt the skin barrier, leading to AD. In addition, hyperactive KLK5 cleave PAR2 and activate TSLP leading to Pro-Th2 innate immune response independent of skin barrier function and environmental factors.

## Abstract

**Background:** Skin desquamation is facilitated by serine proteases KLK5 and KLK7, which are tightly regulated by lymphoepithelial Kazal-type related inhibitor (LEKTI). LEKTI itself is controlled through degraded by mesotrypsin. Here, we sought to determine whether LEKTI exonic mutations associated with atopic dermatitis (AD) affect the protease inhibitory activity of LEKTI or its susceptibility to mesotrypsin degradation.

**Methods:** The inhibitory activities of the LEKTI domain 4 (D4) and D6 WT and AD associated mutants on the enzyme activities of KLK5 and KLK7 were compared using fluorogenic substrates. A keratinocyte cell culture system using HaCat cells was established to assess the role of D6 WT and D386N on triggering inflammation via the induction of thymic stromal lymphopoietin (TSLP). A degradation assay was used to assess the susceptibility of D4 and D6 mutants to mesotrypsin degradation.

**Results:** Enzymatic assays revealed that the D6 D386N mutation affected the inhibitory activity of LEKTI on KLK5 but not KLK7. Other exonic mutations on D6 (N368S, V395M and E420K) and D4 (R267Q) did not alter LEKTI inhibition. The D386N mutation disrupted the role of D6 in suppressing TSLP induction by KLK5 in HaCat cells. Although WT D4 is more susceptible to mesotrypsin degradation than WT D6, the D4 R267Q mutant was more resistant to mesotrypsin degradation, whereas the D6 E420K mutant showed enhanced mesotrypsin-mediated degradation.

**Conclusion:** Exonic mutations in D6, which previously have been associated with AD, may cause a disruption of inhibitory activity on KLK5 or enhance the degradation by mesotrypsin.

## INTRODUCTION

The stratum corneum (SC) layer of skin epidermis comprises anucleated corneocytes that are surrounded by a protein envelope filled with water and keratins attached through corneodesmosomes. During skin homeostasis, an important event in the process of epidermal desquamation involves digestion of corneodesmosomes by proteolytic enzymes.<sup>1,2</sup> Kallikrein (KLK)-related peptidases comprise a class of serine peptidases with trypsin-like substrate specificity. Of the fifteen KLK peptidases identified to date, KLK5 and KLK7 are both expressed in the outermost SC layer and are involved in the epidermal desquamation process.<sup>3</sup> Desquamation by these serine proteases is tightly regulated by various serine protease inhibitors present in the skin.<sup>4</sup> One of these inhibitors is lympho-epithelial Kazal-type related inhibitor (LEKTI), a 15-domain Kazal serine protease inhibitor encoded by the *SPINK5* gene. Various fragments of the LEKTI domains have been shown to inhibit KLK5 and KLK7.<sup>5</sup> Keratinocyte-specific mesotrypsin is responsible for regulating the turnover of LEKTI in the skin by degrading the protein over time.<sup>6</sup>

Skin barrier dysfunction is the main cause of many inflammatory skin diseases. Netherton Syndrome (NS) is caused by nonsense mutations in *SPINK5*<sup>7,8</sup> or null mutation of *FLG*, the gene that codes for filaggrin.<sup>9,10</sup> Unregulated proteolytic activities of both KLK5 and KLK7 are responsible for NS development,<sup>11</sup> while the filament-aggregating protein filaggrin plays an important role in the formation of the skin barrier function.<sup>12</sup> Cork et al. in 2006 proposed that premature desquamation can result in skin barrier dysfunction,<sup>13</sup> with the premature breakdown of the corneodesmosome occurring in response to uncontrolled KLK peptidase activity in the skin.<sup>13</sup> Dysfunctional skin barrier leads to frequent bacterial and viral infections, enhanced trans-epidermal water loss, and other inflammatory-type reactions. Defective skin barrier function is also one of the causes in the development of AD. AD is caused by mutation of *FLG*<sup>14</sup> and polymorphism in *SPINK5*<sup>8</sup>, leading to insufficient LEKTI

inhibitory activity and thus excessive activation of KLK5.<sup>15</sup> However, hyperactive KLK5 can also cleave protease-activated receptor 2 (PAR2) and activate NF- $\kappa$ B to produce thymic stromal lymphopoietin (TSLP), which lead to chemokines that attract Th2 cells and expression of pro-Th2 cytokines.<sup>16,17</sup> This is an innate immune response and independent of skin barrier function and adaptive immune system, but still leading to an cascade of allergic inflammation and AD.<sup>18</sup>

Several polymorphisms in the *SPINK5* gene are significantly associated with Netherton syndrome<sup>19</sup> and, in Northeast China<sup>20</sup> and Japan,<sup>21</sup> AD has been associated with single nucleotide polymorphism (SNPs) in the *SPINK5* gene. Gene function is differentially affected by SNP location (promoter, deep intronic, close to the 3' end of the gene, and exonic), with certain SNPs linked to changes in protein expression levels, function, and mRNA transcript levels, among other changes. Exonic mutations are crucial, as they can directly affect how the protein is expressed and its function. For instance, exonic mutations in *SPINK5* cause defective protein function at three different levels: it disrupts furin-mediated proteolytic activation; it causes a loss of inhibition against the target serine protease; and it alters LEKTI degradation by mesotrypsin, which in turn further exacerbates the loss of inhibition on the serine protease.

SNP rs2303067, which causes an exonic mutation (E420K) in the LEKTI domain 6 (D6), was shown to be significantly associated with the risk of AD in two studies in a British population.<sup>22</sup> The D6 domain is known to strongly inhibit KLK5 and KLK7.<sup>23</sup> This E420K mutant alters the proteolytic activation of LEKTI, thus resulting in enhanced epidermal proteolytic activity.<sup>24</sup> The two non-synonymous SNPs in D6, rs2303064 and rs2306067, have been linked with AD among Japanese patients.<sup>25</sup> Variants, including N368S (rs2303063), D386N (rs2303064) and V395M (rs17775319) are documented in the dbSNP database.<sup>26</sup> In a Korean population, a haplotype containing SNPs rs2303064 (D386N) and rs6892205

(R267Q) showed significantly different allelic or genotypic distributions between AD cases and controls.<sup>27</sup> The SNP rs6892205 (R267Q) is associated with non-atopic hand dermatitis in a Taiwanese nursing population.<sup>16</sup> This R267Q mutation is found in the LEKTI domain 4 (D4), for which the inhibitory activity has never been studied.

Despite the well-established association between *SPINK5* polymorphisms and AD, no study has elucidated the effects of these mutations on the inhibitory function and the degradation of LEKTI domains. Using chromogenic enzymatic and mesotrypsin degradation assays, we found that certain exonic SNPs associated with AD disrupted the inhibitory activity of LEKTI on KLK5, whereas other SNPs enhanced the degradation of LEKTI by mesotrypsin. These findings will help to shed light on the manifestation of AD and aid in the design of novel therapeutic strategies to manage the disease.

## METHODS

### Determination of LEKTI level in skin and *SPINK5* haplotypes

Volunteer subjects were recruited through multiple recruitment programs in a 10 yr period. The subjects were asked to fill in a survey questionnaire. Based on the answers to the questionnaire and the results of the skin prick tests conducted, the participants were classified as AD cases, intermediates, controls or others. The AD cases could further be classified based on severity as mild, moderate or severe. On the basis of chronicity, they were classified as chronic and non-chronic. Dead skin cells from stratum corneum of these subjects were collected from the antecubital fossae (Ac) of both the arms of subjects using an adhesive tape disc (D-squame tape, CuDerm corporation) via tape stripping method (repeatedly sticking and peeling for a total of 50 times). The tapes are placed in a 1.5 ml microcentrifuge tube and 0.1X PBS buffer is added to the tubes. The samples undergo 3 cycles of 15 min freeze and 3 min thaw. This is followed by sonication. The tape is then removed from the tube and

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the samples undergo lyophilization to concentrate skin proteins. These skin proteins were transferred to nitrocellulose membranes and probed with antibodies specific to LEKTI. Chemiluminiscence is used to view the membranes. The results were viewed and analyzed using GeneSys software. Comparison of LEKTI levels were performed using GraphPad prism. DNA samples of the subjects were obtained via buccal cells. Genotyping was used to determine the haplotypes of the individuals. LEKTI levels of subjects are categorized based on the haplotypes and studied.

### **Expression and purification of recombinant LEKTI domains**

Genes coding for D4 and D6 (codon optimised for bacterial expression vector system) were purchased from Genscript (NJ, USA) and subcloned into a pet-M plasmid using the restriction sites for *Bam*HI and *Eco*RI (Figure S3). The sequence was confirmed using Big-Dye sequencing, and plasmids containing the respective genes were transformed into SHuffle Competent *E. coli* (New England Biolabs; MA, USA) and plated on LB agar plates. Individual colonies were picked and cultured overnight at 30°C in 4 ml LB with 100 µg/ml ampicillin. Overnight culture (1 ml) was used to inoculate 1 L of LB medium containing ampicillin. The bacteria were cultured until the absorbance at 600 nm reached 0.6, following which protein expression was induced using 0.3 mM IPTG overnight at 20°C. The cells were then harvested, resuspended in nickel binding buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 5 mM imidazole), and sonicated. The cell lysate was centrifuged and then further purified using nickel affinity chromatography (Roche; BSL, Switzerland) followed by gel filtration using a Superdex 75 column (GE Healthcare; MA, USA).

## Site-directed mutagenesis

LEKTI gene constructs with single amino acid mutations were generated using a modified QuikChange site-directed mutagenesis kit (Thermo Fisher Scientific; Waltham, MA, USA). First, the forward primer and the complementary reverse primer with the desired mutation were designed using the software SnapGene Viewer and analyzed using the IDT oligoanalyzer tool. The recombinant plasmid containing the desired gene was amplified using the KOD hot-start polymerase (Novagen; Madison, WI, USA) with an extended extension time of 3 min. After amplification, the product was digested using Dpn1 (Thermo Fisher Scientific; Waltham, MA, USA) for 1 h at 37°C to digest the methylated wild-type plasmid and then transformed into competent DH5 $\alpha$  cells. The plasmids were then isolated and sequenced to confirm the mutation. These plasmids were then transformed into SHuffle Competent *E. coli* (New England Biolabs; MA, USA). The expression and purification of these mutants were carried out as mentioned in the previous section.

## Circular dichroism (CD) spectroscopy

The overall secondary structures of the expressed proteins and their mutants were verified by acquiring Far-UV CD spectra using the Jasco J-18 spectropolarimeter (Jasco, Japan) with a Hellma quartz cuvette of 1.0 mm path length. For all CD experiments, 20 mM Tris-HCl pH-8.0 was used with 200 mM NaCl buffer. On average, 5 spectra scans were taken using a cuvette with a 0.1 cm path length. All scans were measured from 260 nm to 190 nm with a scan speed of 50 nm/min, spectral resolution of 0.1 nm, bandwidth of 1.0 nm, and response time of 8 sec.

## **Protease inhibition assay**

We used active recombinant human (rh) KLK5 and KLK7 (R&D Systems; Minneapolis, MN, USA) to measure serine protease inhibition by the expressed LEKTI domains and their mutants. The fluorogenic substrate for KLK5 was Boc-VPR-AMC (R&D Systems; Minneapolis, MN, USA), with an excitation wavelength of 380 nm and an emission wavelength of 460 nm, whereas the substrate for KLK7 was MCA-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys(Dnp)-NH<sub>2</sub> (R&D Systems; Minneapolis, MN, USA), with wavelengths of 320 nm and 405 nm, respectively. The assay conditions were prepared as per the manufacturer's recommended protocol. Final assay concentrations were 23.3 nM KLK5 with 66.6  $\mu$ M substrate and 51.2 nM KLK7 with 6.6  $\mu$ M substrate. The protease activity is measured as a function of fluorescence intensity produced by the cleavage of fluorophore tagged substrates. The emission was measured using the Tecan infinite series multi-plate reader in kinetic mode. To screen the LEKTI domains and their mutants for inhibitory activity, the protease and inhibitor were initially incubated together for 10 min before adding the substrate to initiate the assay reaction. Each of the recombinant D4, D6, and mutants were screened using this method. For the IC<sub>50</sub> calculation, a dose response curve (log [inhibitor] vs percentage inhibition) was plotted using the non-linear curve fitting function in the graph pad prism software. All the experiments were carried out in triplicates.

## **Induction of TSLP mRNA expression in HaCat cells**

We established a keratinocyte cell culture system, based on a previously reported method<sup>16</sup>, to demonstrate the effect of the D386N mutant on triggering an inflammatory response in the skin. HaCat human keratinocyte cells were seeded into 96-well plates in DMEM supplemented with 10% FBS and 0.06 mM CaCl<sub>2</sub>. After overnight culture, the cells were incubated with 400 nM rhKLK5 (R&D Systems) with or without WT D6 or the D6 D386N

mutant (4  $\mu$ M final concentration) for 3 h. After incubation, the media was removed, and the cells were used for direct cDNA synthesis using the SuperScript III CellsDirect cDNA Synthesis Kit (Thermo Fischer Scientific). cDNA quality was evaluated using Nanodrop spectrophotometry. cDNA was then used for qPCR to assess the relative expression of *TSLP* mRNA using the Thermo Fisher Scientific Maxima SYBR Green qPCR Master Mix. Supplemental Figure S4 shows the primers used for the qPCR reaction. The results were normalized against *HPRT* and analyzed using ABI Prism 7000 SDS software (Applied Biosystems). Supplemental Figure S5 shows a scheme of the cell culture system protocol. The cell culture experiments were carried out in triplicate wells followed by RT-PCR, which was again carried out with 3 experimental repeats. Unpaired t-test from GraphPad prism was used to calculate the p-value. A p-value of  $< 0.05$  is considered statistically significant.

### **Degradation assay by mesotrypsin**

To investigate the effect of point mutations on the mesotrypsin-mediated degradation of LEKTI<sup>6</sup>, active mesotrypsin (rh-trypsin 3/PRSS3, R&D Systems; Minneapolis, MN, USA) was incubated with wild-type and mutated forms of LEKTI in a 1:2 ratio (mesotrypsin:LEKTI) in a buffer containing 50 mM Tris pH 7.4, 100 mM NaCl, 5 mM CaCl<sub>2</sub> at room temperature. Samples were collected at various intervals (0, 0.5, 1, 2, 3, 12, 24, and 48 h). Collected samples were run on SDS-PAGE, and band intensities were quantified using ImageJ software. The intensity at 0 hr was considered as 100% and all values were calculated relative to 0 hr.

## RESULTS

### **The GAG haplotype of SPINK5 affects the level of LEKTI on skin upon induction**

The GAG haplotype occurs if there is a combination of SNPs (G, A and G) in minor alleles present at R267 (rs6892205), N368 (rs2303063) and E420 (rs2303067), respectively, of *SPINK5*. This haplotype occurs in 23.4% of the Singapore Chinese population but does not associate with the development, severity or chronicity of AD in this population (unpublished result). This haplotype, however, is found to affect the level of SPINK5 on skin in two groups of subjects, namely low (constitutive levels) and high (induced levels). The level of SPINK5 is lower in individuals with the GAG haplotype as compared to the other haplotypes when SPINK5 is induced (Figure 1). This phenomenon prompts us to investigate the effects of these exonic SNPs on the activities and stabilities of individual LEKTI domains.

### **Recombinant D4 and D6 and their mutants are stably expressed in the *E. coli* soluble fraction**

LEKTI genes were successfully subcloned into petM plasmids, as confirmed by sequencing. Plasmids were then transformed into SHuffle Competent *E. coli* T7-express cells for expression. All wild-type LEKTI domains and their mutants were found in the soluble fraction. The protein was initially purified using Ni-NTA affinity chromatography and the elute was subjected to subsequent purification through gel filtration (Figure S1A). The CD spectra of LEKTI WT and mutants were measured in the far UV range (260 nm to 190 nm). The D6 domain showed more helical content than the D4 domain. There were no significant differences in the secondary structures of WT D4 and D6 as compared with their mutants (Figure S1B).

## **The D386N mutation decreases LEKTI D6 protease inhibition against KLK5 but not KLK7**

A fluorescence spectroscopy-based experiment using serine proteases, rhKLK5 and Boc-VPR-AMC fluorogenic substrate was used to evaluate the protease inhibition by the LEKTI WT D6 and D4 compared with their mutant counterparts. We found that both WT D4 and WT D6 could inhibit KLK5 and KLK7. Interestingly, most of the mutants had no significant effect on the inhibitory activities of both domains. However, the D386N mutation in D6 significantly reduced the inhibitory activity as compared with WT D6 (10-times lower activity; Table 1). Figure 2 shows the IC<sub>50</sub> plots for KLK5 and KLK7 inhibition by D4, D6, and their respective mutants.

## **D386N mutant of D6 fails to inhibit TSLP activation in keratinocytes**

The D386N loss-of-function mutation in D6 is proposed to permit not only uncontrolled desquamation but also activate pro-inflammatory pathways in the skin.<sup>28</sup> Here, we used an in vitro cell culture system to study KLK5-mediated activation of TSLP expression, an epithelial cell-derived cytokine expressed in the skin. These assays were performed in the presence or absence of D6 WT or the D386N loss-of-function mutant. We found that HaCat cells incubated with KLK5 for 3 h showed significantly higher (~9-fold) TSLP mRNA levels as compared with the untreated control (Figure 3). However, in the presence of WT D6, TSLP mRNA levels maintained closer to that of the untreated control, presumably due to the inhibition of KLK5 activity. In contrast, in the presence of the D386N mutant, TSLP mRNA levels were increased 8-fold, presumably because the mutated D6 failed to inhibit KLK5.

## **SNP mutants of D4 and D6 show differential susceptibility to degradation by mesotrypsin**

Finally, a mesotrypsin degradation assay was performed to study the effect of SNP mutations on LEKTI turnover. We incubated both the D4 and D6 domains and their respective mutants with mesotrypsin and assessed the rate of degradation of the LEKTI domains using densitometry of bands separated by SDS-PAGE (Figure 4). We found that the D4 domain was generally more susceptible to mesotrypsin degradation than the D6 domain. Compared with the WT protein, D4 bearing the R267Q mutation had significantly enhanced resistance to mesotrypsin, suggesting that this mutation might offer a protective effect against the development of AD. Compared with WT D6 and the other mutants, the D6-E420K mutant degraded the fastest in the presence of mesotrypsin. Thus, the E420K mutation may hinder the proper regulation of the skin proteases by LEKTI, leading to skin barrier dysfunctions and the development of AD.

## **DISCUSSION**

Uncontrolled serine protease activity in the skin can lead to premature epidermal desquamation in the epidermis and result in various skin diseases.<sup>29</sup> LEKTI encoded by the *SPINK5* gene is a Kazal-type protease inhibitor responsible for controlling serine protease activity. *SPINK5* (LEKTI) polymorphisms have long been associated with skin disorders, such as AD and Netherton syndrome.<sup>30</sup> These mutations might affect the proper functioning of LEKTI at multiple stages. Various studies have sought to investigate the effects of these mutations on LEKTI expression and post-translational modification.<sup>31</sup> However, the effect of exonic mutations on the inhibitory function and degradation of LEKTI have not been studied to date. In the current study, D4 and D6 domains bearing AD-associated exonic mutants were generated to evaluate the structural and functional effects of these mutations on skin homeostasis. All of the LEKTI domains (WT and mutants) were able to be expressed in *E.*

*coli* cells and purified successfully as soluble proteins. Far-UV CD spectra showed that none of the point mutations significantly affected the secondary structure of the domains.

D6 is known to inhibit KLK5 and KLK7, the two predominant serine proteases in skin.<sup>32</sup> We performed a chromogenic enzyme assay using KLK5 and KLK7 and their respective substrates to evaluate the function of WT and mutant LEKTI D4 and D6 domains. We show that both WT D4 and D6 inhibited KLK5 and KLK7 with similar IC<sub>50</sub> values (Table 1), and most of the mutants tested had no significant bearing on this inhibitory function, except D6 D386N. The D386N mutation caused a more than 10-fold decrease in the inhibitory activity of D6 against KLK5 (Table 1) but had no effect on KLK7. D386 is located at the P3' position of the active site loop and is thought to bind to the S3' site on the protease (Figure S2); the other tested D6 mutation sites are all located away from the active site loop. A closer assessment of the chemical nature of the S3' sites of KLK5 and KLK7 shows that the S3' site of KLK5 has a much higher positive surface charge as compared with the S3' site of KLK7 (Figure 5). Hence, KLK5 would preferentially interact with inhibitors with negative residues at the P3' position, as in the case of residue D386 in D6. Mutation of residue D386 to N386 at the P3' position would affect the charge-charge interaction between the inhibitor and KLK5, but not in KLK7. These results suggest that the effect of the exonic SNP on the inhibitory activity of LEKTI will depend on the target serine protease with which it interacts.

In addition to the premature desquamation, increased serine protease activity (KLK5 but not KLK7) in the skin can activate pro-inflammatory pathways. This occurs through protease-activated receptor-2 (PAR-2)-mediated overexpression of a pro-Th2 cytokine, TSLP, which results in the infiltration of eosinophils and mast cells into the epidermis.<sup>33</sup> The epidermis will also communicate with the neighboring sensory neurons through TSLP, which causes the sensation of itching on this skin.<sup>34</sup> Hence, uncontrolled activity of KLK5 could result in AD-like lesions in the skin via the activation of pro-inflammatory pathways. LEKTI

plays a major role in controlling this serine protease activity, and any loss of function in LEKTI could result in the activation of atopic lesions. In the current study, the D386N mutation, which is associated with AD, led to a loss in the inhibitory activity of D6. Using an in vitro cell culture system, we demonstrated that the D386N mutant of D6 failed to control the catalytic activity of KLK5, resulting in the increased production of TSLP (Figure 3).

Although LEKTI tightly binds to KLKs to regulate their activities, these complexes must dissociate for KLK to perform its proteolytic reaction. This is achieved through the degradation of bound LEKTI by epidermal mesotrypsin.<sup>6,35</sup> Epidermal mesotrypsin expressed in the upper layers of the epidermis has unique properties: it cannot digest high molecular weight protein, and it demonstrates resistance to most naturally occurring serine protease inhibitors. This inherent resistance to inhibitors is due to the presence of an Arg residue close to the catalytic triad at position 198, instead of a Gly residue, as noted in most other serine proteases.<sup>33</sup> The resistance of mesotrypsin towards LEKTI inhibition allows it to degrade the LEKTI domains. A degradation assay was performed to study the effect of the SNP mutations on the degradation of LEKTI domains mediated by mesotrypsin. The D4 R267Q mutant showed a slower rate of degradation as compared with its WT counterpart, whereas the D6 E420K mutant showed a faster degradation rate. Serine proteases prefer basic residues (Arg or Lys) in the P1 site of their substrate. Hence, the presence of Arg in WT D4 or a Lys residue in the case of the mutant D6 would facilitate the faster degradation of LEKTI by mesotrypsin.

In addition to the presence of favorable amino acids at the P1 site, substrate flexibility may also influence the rate of degradation. D4 domains have a higher backbone dynamic, which may also result in its faster degradation.<sup>36</sup> Exonic SNPs that cause a faster degradation of the LEKTI domain, independent of its inhibitory activity, could also lead to uncontrolled proteolytic activity and the development of AD.

Following our observation of reduced LEKTI levels in induced conditions, the SNPs rs2303063, rs2303064, rs17775319, rs2303067 and rs6892205 were chosen for analysis in the present study. Different significant associations for these mutations have been reported in North Chinese, Korean, Japanese, Taiwanese, and British populations.<sup>20-23,26</sup> None of the associations, however, are significant in the Singapore Chinese population. Association studies performed in other populations, including those from North Germany and the Netherlands, did not find a significant association between *SPINK5* and atopy or AD.<sup>34,35</sup> There could be a number of reasons for this difference in association. AD occurs due to genetic as well as lifestyle factors; it is difficult to determine why associations differ in different populations. The current study is limited to *in vitro* effect of functional defect on LEKTI inhibition of KLK5 and KLK7. Identification of novel physiological targets of LEKTI in the skin and *in vivo* skin model will be needed to better understand the physiological and pathophysiological pathways in the skin.

## **ACKNOWLEDGMENTS**

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## **CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest.

## **AUTHOR CONTRIBUTIONS**

K.R. designed and performed the experiments with assistance from A.S.M. on the TLP experiments. K.R. and Y.K.M. wrote the manuscript. F.T.C. provided information on SNPs

associated with AD. Y.K.M. coordinated and supervised all experiments, and secured funding.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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**Table 1.** IC<sub>50</sub> values of LEKTI domain 4 and 6 against KLK5 and KLK7

|                               | LEKTI D6 |       |              |       |       | LEKTI D4 |       |
|-------------------------------|----------|-------|--------------|-------|-------|----------|-------|
|                               | WT       | N368S | D386N        | V395M | E420K | WT       | R267Q |
| KLK5<br>IC <sub>50</sub> (nM) | 9.5      | 3.76  | <b>87.92</b> | 3.31  | 13.86 | 10.52    | 9.83  |
| KLK7<br>IC <sub>50</sub> (nM) | 0.13     | 0.2   | <b>0.04</b>  | 0.07  | 0.1   | 0.03     | 0.02  |

## Figure Legends

**Figure 1.** Comparison of the induced levels of LEKTI on the stratum corneum of individuals. Individuals with high levels of LEKTI (above 150 AU) were categorized based on the presence of the GAG haplotype. Subjects with the GAG haplotype have significantly lower induced levels of LEKTI when compared to those with other haplotypes. A Mann-Whitney test was performed in order to compare the two groups and generate the P value.

**Figure 2.** Dose-response curve for the inhibition of Kallikrein protease-5 and 7 (KLK5 and KLK7). Inhibition of (A) KLK5 and (B) KLK7 by WT LEKTI domain 6 (black), D386N (blue), N368S (magenta), V395M (red), and E420K (green) mutants. Inhibition of (C) KLK5 and (D) KLK7 by the WT LEKTI domain 4 (black) vs. the R267Q (blue) mutant. All experiments were carried out in triplicates and  $IC_{50}$  was calculated using GraphPad prism software.

**Figure 3.** Relative TSLP gene expression (level of mRNA) after incubating HaCat cells with KLK5 in the presence of WT LEKTI domain 6 or the D386N mutant. Empty bar = treated sample, striped bar = untreated media control. The insert table shows the fold-change in TSLP mRNA expression in HaCat cells as compared with the untreated control. All experiments were carried out in triplicates. P-value was calculated using unpaired t-test and a p-value of  $< 0.05$  is considered statistically significant.

**Figure 4.** Mesotrypsin degradation of LEKTI domains. The WT LEKTI domains and their respective mutants were incubated with mesotrypsin, and the samples were collected at different time points. The collected samples were analyzed using SDS-PAGE. Band intensity was quantified using ImageJ. The % of the intact protein was plotted vs time of incubation.

Left panels: WT LEKTI domain 4 (open circle) and R267Q mutant (closed circle). Right panels: WT LEKTI domain 6 (open circle), D386N (closed circle), N368S (open square), V395M (closed square) and E420K (open triangle) mutants.

**Figure 5.** Comparison of the surface charges of KLK5 and KLK7. (A) Surface charge diagram of KLK5 and KLK7 (red, negative; blue, positive), with the S1 site indicated by the black arrow and the S3' site marked with a green arrow and circled in yellow. The S3' pocket on KLK5 is positively charged, whereas the one in KLK7 is neutral. (B) Sequence alignment of the N-terminal regions of KLK5 and KLK7 with selected S3' site residues marked with green arrows. These residues contribute partly to the positive charge at the S3' pocket of KLK5.

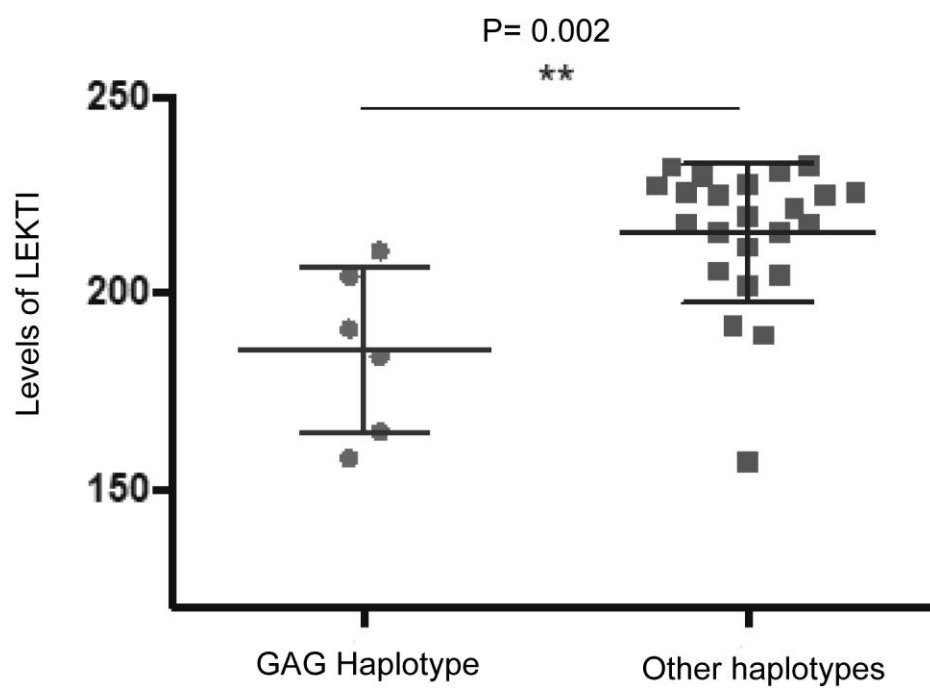


**Figure 1**

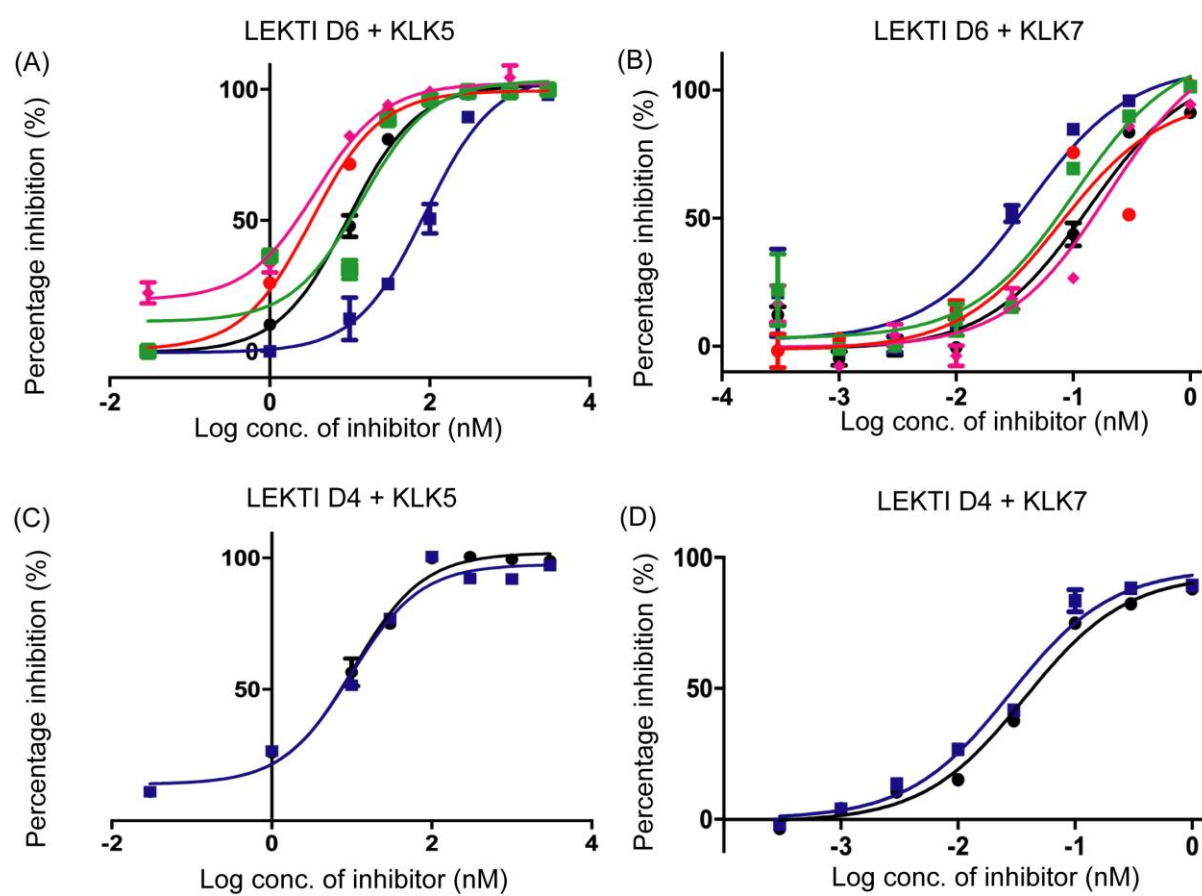
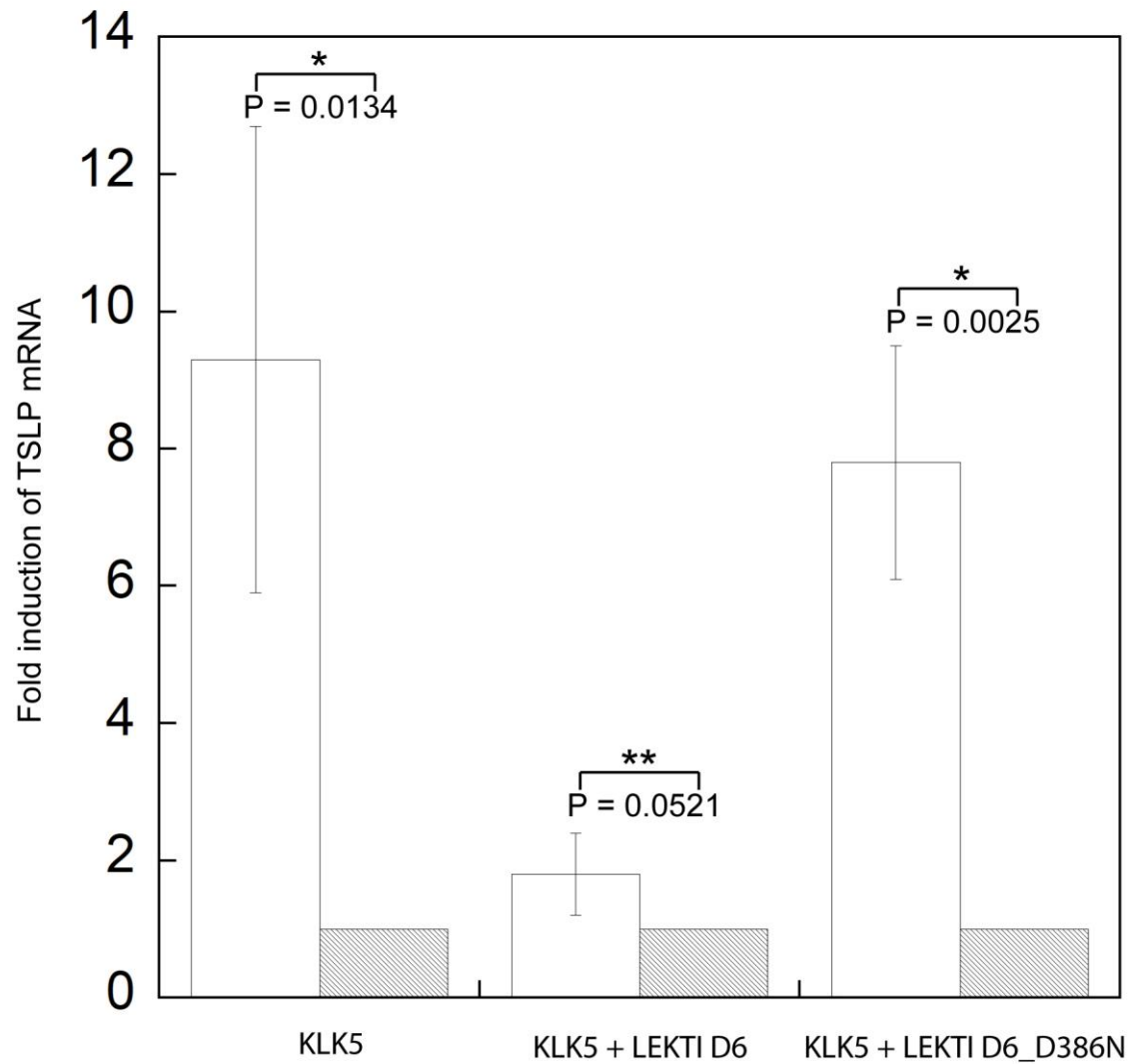


**Figure 2**



|                       | Fold induction of TSLP mRNA |
|-----------------------|-----------------------------|
| KLK5                  | 9.3 ± 3.4                   |
| KLK5 + LEKTI D6       | 1.8 ± 0.6                   |
| KLK5 + LEKTI D6_D386N | 7.8 ± 1.7                   |

**Figure 3**

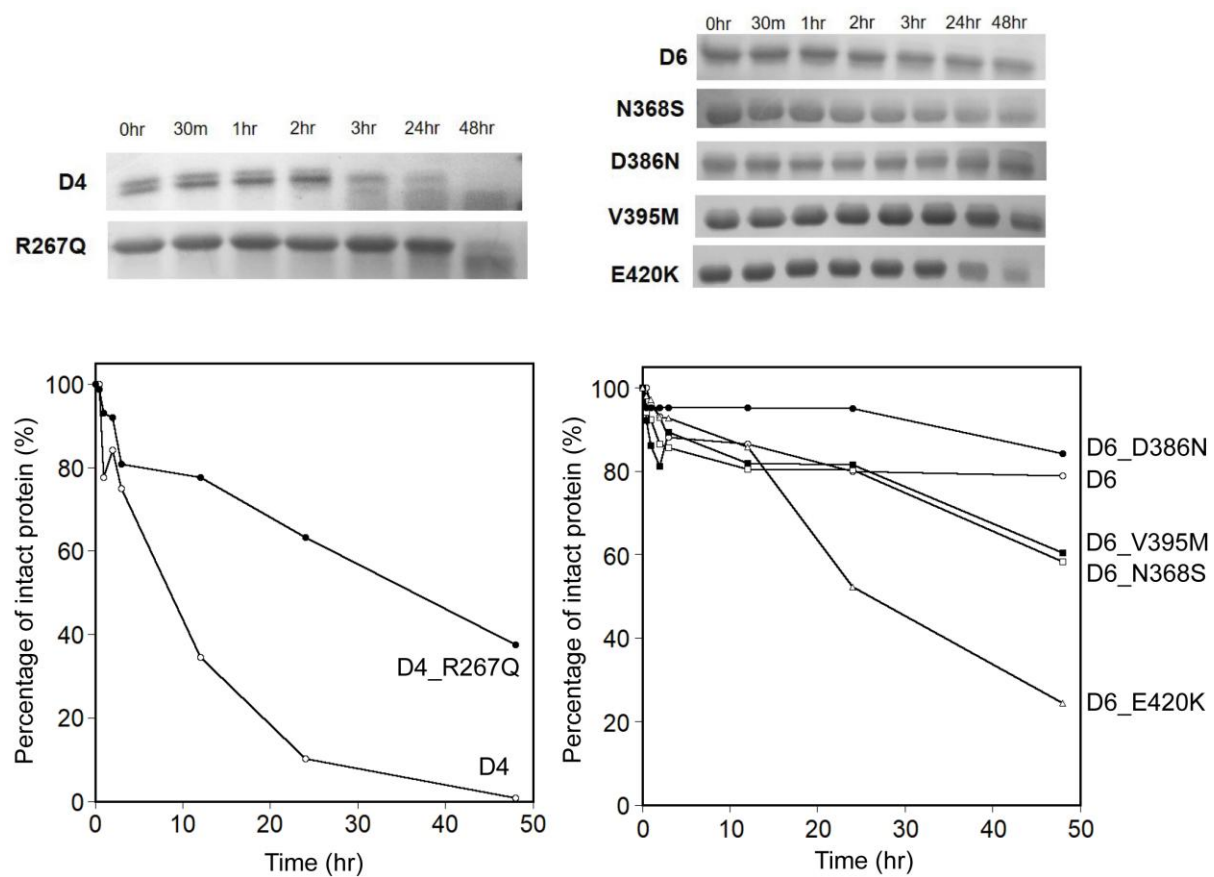


**Figure 4**

Figure 1 displays the surface representation of the active site of Human KLK5 (left) and Human KLK7 (right). The surfaces are colored blue, red, and white, representing different chemical environments. The S3' pocket is indicated by a green arrow, and the S1 pocket is indicated by a black arrow. The S3' pocket is located on the left side of the active site, and the S1 pocket is located on the right side.

k1k5 IINGSDCDMHTQPWQAALLRPNQLYCGAVLVHPQWLLTAAHCRKKVFRVRLGHYSLSPV  
k1k7 IIDGAPCARGSHPWQVALLSG-NQLHCGGVLVNERWVLTAACHKMNEYTVHLGSDTLG--  
\*: \*: \* : \*\*: \*\* : \*: \*\* : : : \* : \*

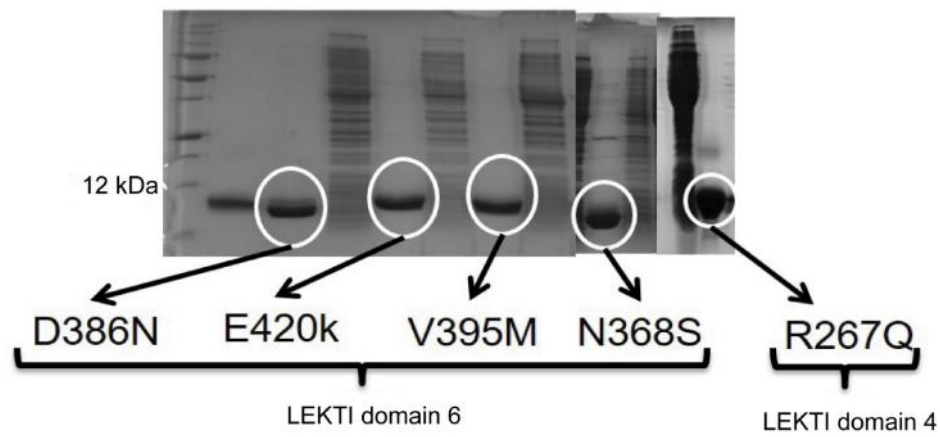
### Figure 5

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(A)



(B)

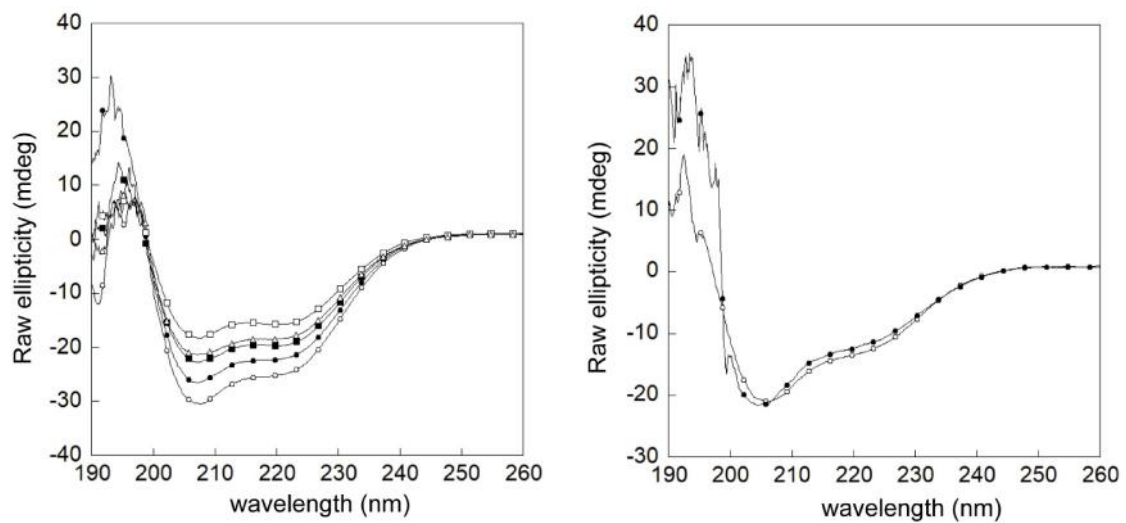


**Figure S1.** SDS-PAGE gel of purified LEKTI domains and their respective mutants. (A) The circled band is the eluate after nickel affinity purification and the adjacent lanes are the flow-through from the Ni-NTA column. (B) Overlays of FarUV-CD spectra of WT LEKTI domains and their respective mutants. Left panel: WT (closed circle), N368S (open circle), D386N (closed square), V395M (open square), and E420K (open triangle) mutants of the LEKTI domain 6 (D6). Right panel: WT (open circle) and R267Q (closed circle) mutant of LEKTI D4.

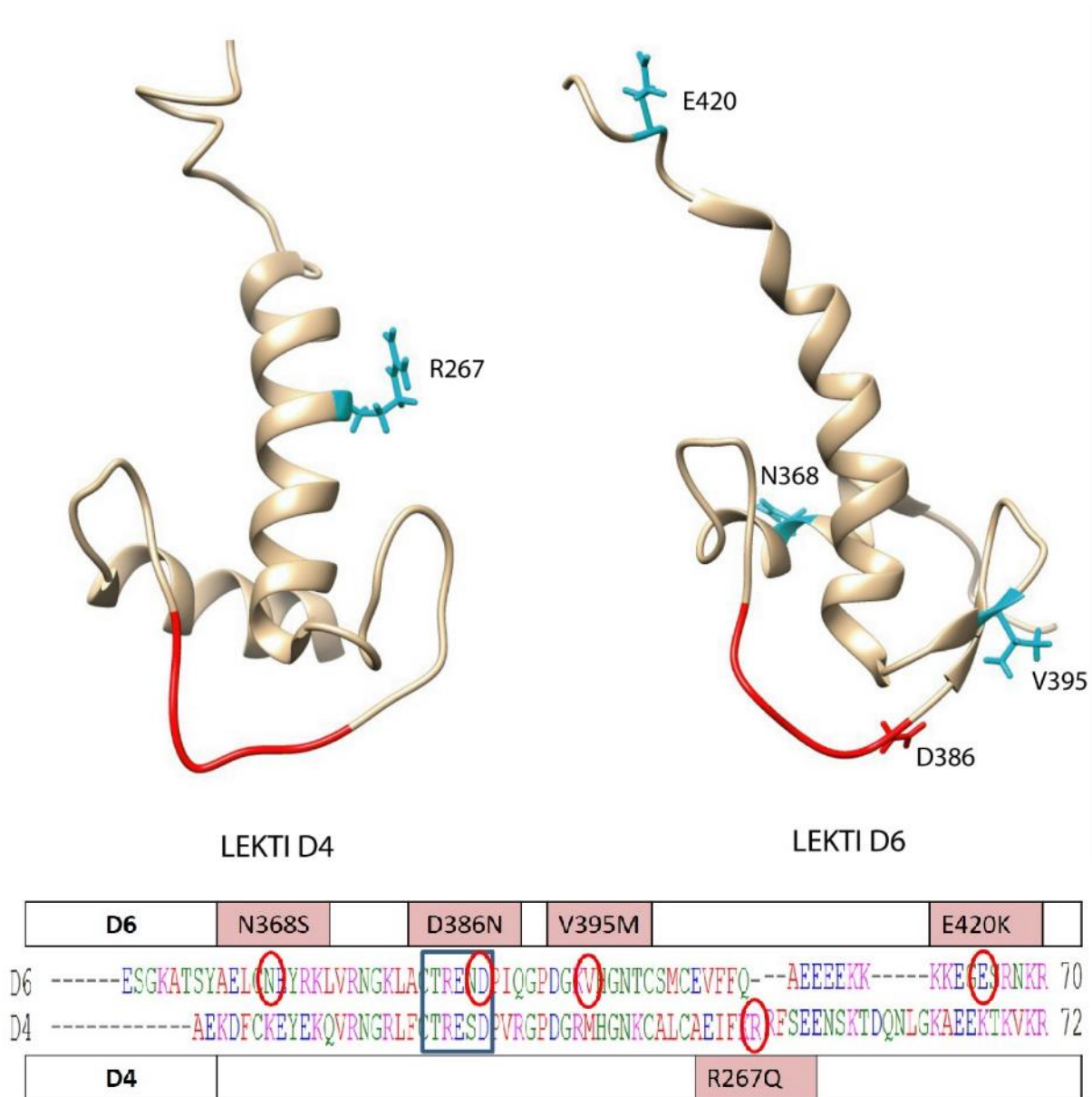


**Figure S2.** Structures and sequence alignment of the LEKTI domain 4 (D4) and domain 6 (D6). In the structure, residues P3 to P3' ("CTRES" in D4 and "CTREND" in D6) at the reactive site loop are colored red. Residues that are mutated in this study are shown as stick models in cyan and are labelled. In the sequence alignment of D4 and D6, positions of the selected mutations are circled in red. The reactive site loops are boxed in blue.

**(A) LEKTI Domain 4**

GCTGAAAAAGACTTCTGTAAAGAATATGAAAAACAAGTTCGCAATGGCCGCCTGTTCTGTACCCGTGAATCGGA  
CCCGGTTCTGTGGTCCGGATGGCCGTATGCATGGTAACAAATGCGCGCTGTGTGCCGAAATTTTAAACGTCGCT  
TCAGCGAAGAAAACAGTAAACCGACCAAAACCTGGGTAAAGCCGAAGAAAAAACGAAAGTCAAACGCTGA

AEKDFCKEYEKQVRNGRLFCTRESDPVRGPDGRMHGNKCALCAEIFKRRFSEENSKTDQNLGKAEKTKVKR

**(B) LEKTI Domain 6**

GAAAGCGGCAAGGCAACGTCCTATGCTGAACTGTGTAAACGAATACCGTAAACTGGTCCGTAATGGCAAACGG  
CGTGATACCCGTGAAAACGATCCGATTCAGGGCCCGGACGGTAAAGTGCATGGCAATACCTGCAGCATGTGTGA  
AGTGTTTTTCCAAGCGGAAGAAGAAGAAAAGAAGAAAAAGAAGGCGAATCTCGTAACAAGCGC

ESGKATSYAELCNEYRKLVRNGKLACTRENDPIQGPDGKVHGNTCSMCVFFQAEKKKKEGESRNKR

**Figure S3.** Bacterial codon optimized DNA and protein sequences (underlined) of (A) LEKTI domain 4 and (B) LEKTI domain 6.

|               | Forward Primer (5'-3') | Reverse primer (5'-3') | Amplicon<br>Size |
|---------------|------------------------|------------------------|------------------|
| Human<br>HPRT | GCCCTGGCGTCGTGATTAGT   | AGCAAGACGTTTCAGTCCTGTC | 136              |
| Human<br>TSLP | GAGTGGGACCAAAAGTACCG   | GAGTTTCCGAATAGCCTGGG   | 185              |

**Figure S4.** Primers used for qPCR in the study.

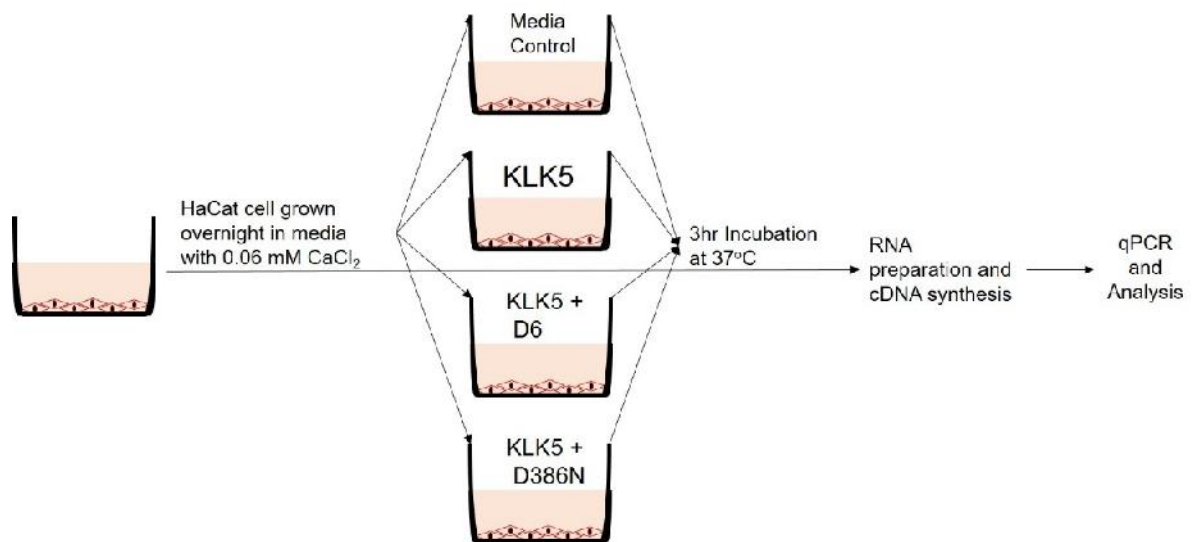


**Figure S5.** Schematic diagram of the HaCat cell culture system used to assess thymic stromal lymphopoietin (TSLP) expression.