ORIGINAL ARTICLE

Patients With Atopic Dermatitis Sensitized to Pet Dander Mount IgE and T-Cell Responses to Mammalian Cystatins, Including the Human Self-Protein

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Abstract

Background: Immediate and delayed-type hypersensitivity reactions to pet-borne allergens are common in atopic diseases. In atopic dermatitis (AD), controversy surrounds the contribution to the disease of cross-reactivity to self-proteins. Human cystatin A and the cat allergen Fel d 3 belong to the cystatins, an evolutionary conserved protein family. The objective of the present study was to assess cross-reactivity between mammalian cystatins and to analyze T-cell responses to cystatin in AD patients sensitized to pet dander.
Methods: cDNA coding for dog cystatin was cloned from dog skin. Sera from 245 patients with IgE-mediated sensitization to cat and dog dander were tested for IgE binding to recombinantly expressed feline, canine, and human cystatin. Of these, 141 were also diagnosed with AD.
Results: Cystatin-specific IgE was detected in 36 patients (14.7%), of whom 19 were considerably affected by AD. Within the AD patients, 9 had measurable IgE against all 3 cystatins. Cystatin-sensitized AD patients did not differ from non–cystatin-sensitized patients in terms of disease severity, age, or total IgE levels. T-cell cytokine measurements showed elevated IL-4 levels after stimulation with feline and human cystatin.
Conclusion: The humoral response suggests that in addition to Fel d 3, the homologous protein from dog might play a role in allergy. Furthermore, human cystatin appears to be capable of driving a type 2 immune response in sensitized AD patients and may therefore be considered a so-called autoallergen, as proposed for other evolutionary conserved proteins.

Key words: Allergy. Sensitization. Atopic dermatitis. Pet. IgE. T cell. Cross-reactivity. Autoallergy. Autoreactivity. Fel d 3. Can f 8. Cystatin. Cytokine.

Resumen

Antecedentes: Las reacciones de hipersensibilidad de tipo inmediato y retardado a los alérgenos que están en las mascotas son comunes en las enfermedades atópicas. En este estudio, en pacientes con dermatitis atopica (DA), se analiza la reactividad cruzada con las autoproteínas y su contribución a la enfermedad. Tanto la cistatina A humana como el alérgeno felino Fel d 3 pertenecen a la familia de las cistatinas, una familia de proteínas conservadas evolutivamente. El objetivo del presente estudio fue evaluar la reactividad cruzada entre las cistatinas de mamíferos y analizar la respuestas de las células T a la cistatina en pacientes con DA sensibilizados a la caspa de las mascotas.
Métodos: El ADNc que codifica la cistatina de perro se clonó a partir de piel de perro. Se analizaron sueros de 245 pacientes con sensibilización por IgE a la caspa de gato y perro para determinar la unión de IgE a cistatina felina, canina y humana expresada de forma recombinante, respectivamente. De estos 245 pacientes, 141 fueron diagnosticados de DA.
Resultados: Se detectó IgE específica frente a cistatina en el 14,7% (36) de los pacientes, de los cuales 19 padecían DA. Dentro de los pacientes con DA, 9 tenían IgE medible contra las tres cistatinas. Los pacientes con DA sensibilizados frente a cistatina no difirieron de los pacientes no sensibilizados con cistatina en términos de gravedad de la enfermedad, edad o niveles totales de IgE. El análisis de citocinas de células T reveló niveles elevados de IL-4 después de la estimulación con cistatina felina y humana.
Conclusión: La respuesta humoral sugiere que, además de Fel d 3, la proteína homóloga de perro también podría desempeñar un papel en la alergia. Además, la cistatina humana parece ser capaz de promover una respuesta inmune de tipo 2 en pacientes con DA sensibilizados y, por lo tanto, puede considerarse un autoalérgeno, como se ha propuesto para otras proteínas conservadas evolutivamente.

Palabras clave: Alergia. Sensibilización. Dermatitis atópica. Mascota. IgE. Célula T. Reactividad cruzada. Autoalergia. Autorreactividad. Fel d 3. Can f 8. Cistatina. Citocina.

Introduction

Hypersensitivity reactions to cat allergens are common in atopic individuals. In the general adult population, the frequency of sensitization to cat and dog epithelia ranges from 10% to 12% [1,2]. Atopic dermatitis (AD) often precedes other atopic diseases such as allergic asthma and rhinitis, and sensitization via the skin is therefore believed to be at the origin of the so-called atopic march. It has been consistently reported that 19%-25% of children affected with AD are sensitized to cat dander [3,4]. At present, 8 cat allergens are known, with Fel d 1 being the major one [5]. Recent epidemiological studies have shown that specific IgE to animal allergen components can be linked to symptoms. Specific IgE to Fel d 1 and Fel d 4 were significantly associated with asthma upon contact with cats [6]. A cross-sectional and longitudinal population-based study has shown that sensitization to Fel d 1 in early childhood is a predictor of cat allergy at age 16 years [7]. In cat-allergic children, elevated IgE-titers to Fel d 2 and Fel d 4 were linked to atopic dermatitis [8]. Fel d 3 is a minor allergen that is recognized by 10% of cat-allergic patient sera [9]. It has not yet been analyzed regarding a possible link with clinical symptoms. Fel d 3 is an 11-kDa dander protein that belongs to the cystatin family, more precisely a type I cystatin or cystatin A, which was cloned from a cat skin library [9]. Type I cystatins function as competitive inhibitors of papain-like cysteine proteases, are highly conserved in mammals, and are abundantly expressed in blood and epithelial cells including keratinocytes [10,11].

IgE autoreactivity is a common feature in AD [12,13], and cross-reactivity appears to be an underlying mechanism. Studies investigating the so-called autoallergens reported proinflammatory cytokine responses in sensitized patients, suggesting that autoallergy can indeed contribute to amplifying inflammation in AD [14-16]. Cross-reactivity between autoallergens and pet-borne allergens has not been evaluated in detail to date. In addition to serum albumins, proteins of the cystatin family have a strong potential to cross-react between man and pet. Human cystatin A (stefin A) is localized mainly intracellularly, although it is also detectable in sweat and in the medium of cultured keratinocytes. It has functions in skin integrity, since human cystatin A is one of the precursor proteins of the cornified cell envelope in keratinocytes and plays a role in epidermal development and maintenance [17,18]. Human cystatin A interferes with foreign enzymes [19] and virus-induced apoptosis and can disturb herpes simplex virus 1 production [20]. The potential of cystatin A to inhibit cysteine proteases leads to reduced immune responses to house dust mite allergens, since Der p 1 and Der f 1 are in fact cysteine proteases [21,22]. Nevertheless, its localization in skin and lung in combination with high enzymatic activity may predispose human cystatin A to be encountered by the immune system during an ongoing inflammation. Pre-existing sensitization to Fel d 3 could further boost T_H2 responses and autoreactivity to homologous human cystatin.

Therefore, this study aimed to investigate IgE and T-cell responses to Fel d 3 and potential auto- and cross-reactive cystatins from dog and humans in sensitized patients with and without AD.

Material and Methods

Patients and Sera

Adult patients with hypersensitivity reactions to dog or cat but no symptoms of AD were selected from the outpatient population of the National Unit of Immunology-Allergology of the Centre Hospitalier de Luxembourg based on a history of specific IgE to cat and/or dog dander (ImmunoCAP, Thermo Fisher Scientific). The mean age was 35 years (range, 13-73), and 46% were female. Of the patients selected, 26 had asthma, 16 had asthma associated with rhinitis and/or conjunctivitis, and 6 had rhinitis with or without conjunctivitis. Detailed information was not available for 56 patients. Patients with and without hypersensitivity reactions to dog or cat who also had AD according to the criteria of Hanifin and Rajka [23] were recruited at the Department of Dermatology and Allergy at Hannover Medical School based on a history of specific IgE to cat and/or dog dander. Their mean age was 35 years (range, 18-72), and 37% were female. The Scoring Atopic Dermatitis (SCORAD) tool revealed the median (SD) severity value to be 41 (17.5). AD sera were from the BioBank of the "Klinische Forschergruppe Autoimmunität 250", funded by the German Research Foundation (DFG). All patients gave their written informed consent. The study was conducted according to the Declaration of Helsinki and approved by the National Committee for Medical Research Ethics in Luxembourg and the Ethics Commitee of Hannover Medical School.

Cloning of Dog, Cat, and Human Cystatin

Shaved dog skin was obtained from a 14-year-old female bichon euthanized by a veterinarian. RNA was extracted from 1 g of tissue using the RNeasy Fibrous Tissue Kit according to the manufacturer's instructions (Qiagen). cDNA was generated using the SMART RACE cDNA amplification kit (Takara Bio). Forward and reverse primers were designed from a predicted dog cystatin A sequence, XM_545130.6 (3′RACE forward primer CCT GCC ACT CCA GAA GTC CAG; 5′RACE reverse primer AAA GCC TGT GAG CTC ATC ATC CTT GC). Primers were situated within the open reading frame and yielded fragments extending into the 5′ and 3′ untranslated region. In a second step, new primers with restriction enzyme recognition sites Nco I and Bgl II were designed to amplify the whole coding region for cloning into the pQE60 vector (Qiagen) (forward primer 5′ AGC CAC CAT GGT GCC TGG AGG CTT AAC TGA AGC C; reverse primer 5′ ACA AGA GAT CTA AAG CCT GTG AGC TCA TCA TCC TTG C).

Synthetic cDNAs based on the published sequences of Fel d 3 (AF238996; Q8WNR9) and human cystatin A (NM_005213; P01040) were ordered from Eurofins Genomics and cloned into pQE60. Codons were adapted for optimal expression in *Escherichia coli*.

Expression of Recombinant Cystatin

Recombinant cat, dog, and human cystatins carrying a C-terminal hexahistidine tag were expressed in *E coli* M15 (Qiagen). Protein production was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Roth), and recombinant proteins were obtained under nondenaturing conditions and

purified using immobilized metal ion affinity chromatography (HisTrap HP, GE-Healthcare) as described elsewhere [24]. Eluted recombinant proteins were further purified using ion exchange chromatography (Resource Q for Fel d 3, Resource S for human cystatin and Can f 8 [GE-Healthcare]). Fractions containing purified cystatin were pooled and dialyzed against phosphate-buffered saline (PBS). Purity of the recombinant protein was assessed using SDS-PAGE and silver staining, and identity was confirmed using N-terminal sequencing (PPSQ; Shimadzu, Kyoto, Japan). Lipopolysaccharide was removed from the protein preparations by applying the Endotoxin Removal Kit (Hyglos). Lipopolysaccharide was quantified in the final protein batches using the Limulus amebocyte lysate test (Associates of Cape Cod). In all the T-cell assays, respective amounts of lipopolysaccharide were applied as controls, as follows: Can f 8, 480 pg/mL; human cystatin, 33 pg/mL; and Fel d 3, 88 pg/mL.

Detection of Specific IgE by ELISA and ELISA Inhibition

Specific IgE to Fel d 3, Can f 8, and human cystatin were quantified by ELISA, as described elsewhere [25]. Briefly, each protein was coated overnight to microtiter plates (NUNC MaxiSorp) at a concentration of 5 µg/mL in PBS. The blocking buffer was 3% bovine serum albumin (Sigma-Aldrich) in PBS. Patient sera were diluted 1:3 or 1:5 in blocking buffer and incubated for 2 hours at room temperature on the microtiter plate. sIgE titers to cystatins were measured using a standard curve of serial dilutions of a patient serum with a known sIgE titer to cat serum albumin. Sera with sIgE to cystatin ≥0.35 kU/L were classed as positive. The sera of 7 healthy individuals were used as negative controls. For inhibition experiments, sera were preincubated for 2 hours with 100 µg/mL cat, dog, or human cystatin before adding to the microplate.

Extraction of Proteins From Animal Dander

Cat and dog dander were purchased from Allergon AB. Proteins were extracted in PBS at 4°C overnight from 2 g of dander, the suspension was centrifuged for 30 minutes at 5000*g*, and the supernatant was used in subsequent experiments.

SDS-PAGE and Immunoblot

Proteins were separated under reducing conditions using 15% SDS-PAGE and immunoblotted as described elsewhere [25]. Polyclonal anti–cystatin A antibody (Santa Cruz Biotechnology) raised against an N-terminal peptide of human cystatin A was used to detect cystatin in cat and dog dander. Incubation with an anti-goat IgG secondary antibody labeled with alkaline phosphatase (Sigma-Aldrich) followed by addition of nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Promega) made it possible to visualize cystatin-specific bands.

T-Cell Lines

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation, and T-cell lines were generated according to an established protocol [26]. More specifically, 2×10^6 PBMCs was cultured in the presence or absence of 2.5 µg/mL antigen at a density of 2×10^6/mL in Iscove medium (Biochrom KG) supplemented with 4% human heat-inactivated AB serum, 2 mM glutamine, 50 mg/mL gentamicin, 100 mg/mL penicillin and streptomycin, and nonessential amino acids. After 7 days, rhIL-2 (10 U/mL) was added to the culture, and after 14 days, cells were expanded with allogeneic, irradiated PBMCs (50 Gy) as feeder cells in the presence of phytohemagglutinin (10 µg/mL) and rhIL-2 (10 U/mL). After 4 weeks, T-cell lines were stimulated with concavalin A (10 µg/mL), and culture supernatants were harvested 18 hours after stimulation. ELISAs were performed according to the manufacturers' instructions and analyzed using a plate reader (FluoStar OPTIMA, BMG Labtech). ELISA kits for the detection of IL-4, IL-17, and IL-22 were purchased from R&D, and IFN-γ was obtained from ebioscience.

Screening for Genetic Variants Within the FLG Gene

The DNA of cystatin-sensitized AD patients was analyzed for variants in the FLG gene associated with AD (2282del4, S3247X, R501X, and R2447X) using Sanger sequencing of polymerase chain reaction (PCR) products. The PCR primers applied were as follows: R501X Forw CTGACTCTGCCCATGGACG Rev CCGGGTGTCCACGAA; 2282del4 Forw TCCCGCCACCAGCTCC Rev CCCTGAACGTCGAGACCT; R2447X Forw GTCAGCAGGAAGGTCTGGAC Rev GCCTTCCTCCACTGCTTGAC; S3247X Forw GATCCAGTGTGAGCCAGGAC Rev CTGAACGTCCAGACCTTCCC.

Statistical Analysis

The Mann-Whitney test was applied to calculate differences between 2 groups that do not follow a Gaussian distribution (GraphPad Prism, Version 5.02 and 7.04, GraphPad). Contingency tables and the Fisher exact test were used to analyze differences in the prevalence of IgE to a specific cystatin between groups.

Results

IgE-Mediated Sensitization to Pet Dander Is Associated With a Higher SCORAD Value

Patients sensitized to pet dander were identified by the presence of specific IgE (sIgE) to cat and/or dog dander. Phadia ImmunoCAP reagents e1 and e5 are part of the standard aeroallergen panel (Phadia ImmunoCAP sx1) used to assess sensitization to aeroallergens. In total, 245 patients were identified with significant amounts of IgE to cat or dog dander. Atopic patients with and without AD were grouped and analyzed separately. Patients without detectable respective IgE served as negative controls in subsequent experiments. Interestingly, in AD patients, IgE-mediated sensitization to dog and/or cat was associated with greater disease severity, as indicated by the mean SCORAD value (Figure 1A; *P*=.0455;

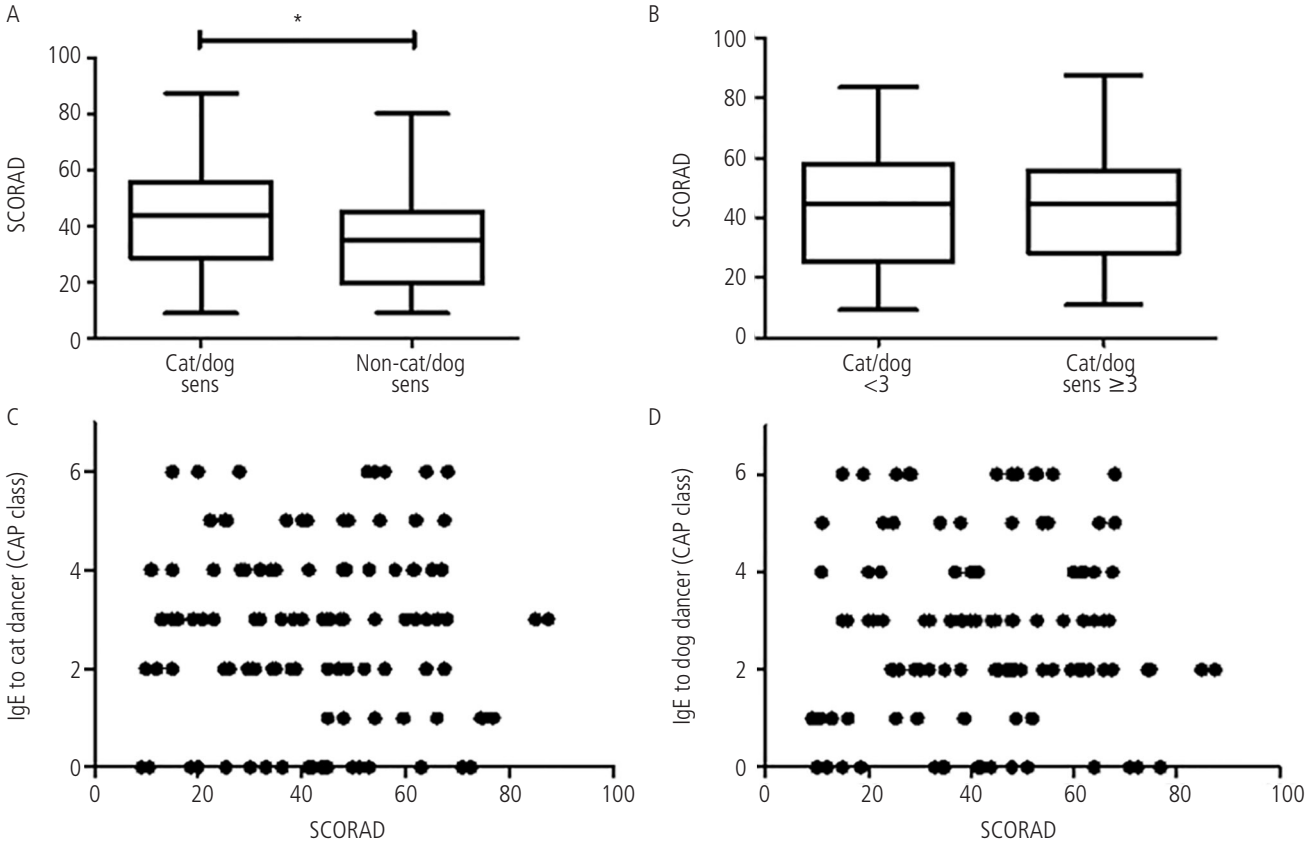

Figure 1. IgE-mediated sensitization to cat and/or dog dander in atopic dermatitis (AD) patients is associated with greater disease severity. A, IgE sensitization data from 40 AD patients without IgE-mediated sensitization to dog and cat dander (e1 and e5 Cap class 0) was used for statistical comparison with 141 AD patients with IgE-mediated sensitization to cat and/or dog dander, as detected by Phadia ImmunoCAP. B-D, Greater disease severity was not associated with the amount of IgE to cat or dog dander. In B, patients are stratified according to ImmunoCAP class as depicted. SCORAD indicates Scoring Atopic Dermatitis. *P<.05, Mann-Whitney test.

mean SCORAD cat/dog-sensitized 44.0 [18.0] vs non–cat/dog-sensitized 35.5 [19.5]). This association appears to be independent of the concentration of dog/cat dander IgE within the serum or within the total IgE (Figure 1B-D).

Dog Cystatin Is Expressed in Dog Dander

In order to investigate the presence of an allergen homologous to the cat allergen Fel d 3 in dog dander, cystatin was searched for using Western blot of cat and dog dander extracts and by applying a commercially available polyclonal antibody. In both cat and dog dander extracts, several bands were detectable within the predicted size range of 11 kDa and higher (Figure 2A). The presence of a dog cystatin gene had been predicted based on genetic similarities, which we could use as a starting point to clone the sequence by rapid amplification of cDNA ends PCR. The resulting cDNA sequence was submitted to the European Bioinformatics Institute (EMBL-EBI, Hinxton, UK) and is now accessible under LT994967 (cDNA) and F1PHB6 (protein). The allergen name Can f 8 was allocated by the WHO/IUIS Allergen Nomenclature Sub-Committee. To study cystatin-specific IgE binding and T-cell cytokine secretion, Fel d 3, Can f 8, and human cystatin were expressed in *E coli* (Figure 2B). Alignment of the amino acid sequences of dog, cat, and human cystatin A shows large stretches of highly conserved sequences (Figure 2C). The sequence identities were 83.7% between Fel d 3 and Can f 8, 75.5% between Fel d 3 and human cystatin, and 78.6% between dog and human cystatin.

Patients Sensitized to Animal Dander Carried IgE to 1 or More Mammalian Cystatins

Sera from 245 patients with significant amounts of IgE to cat or dog dander (see Supplemental Figure 1), of whom 141 also had AD, underwent IgE-ELISA by applying the recombinant cat, dog, and human cystatins, respectively. Cystatin-specific IgE was detected in 36 patients (14.7%), of whom 19 had AD (Figure 3A). Within the AD patients, most patients were sensitized to more than 1 cystatin, and 9 carried measurable sIgE against all 3 cystatin species (Figure 3B). Non-AD patients were more frequently sensitized to Fel d 3 (12.5%) and Can f 8 (14.4%) and less frequently to human cystatin (5.8%). However, the difference with AD patients was not significant (Figure 3). Of the AD patients, 7.8% were sensitized to Fel d 3, 10.6% to Can f 8, and 9.9% to human cystatin. Of interest, patients with exclusively IgE-mediated

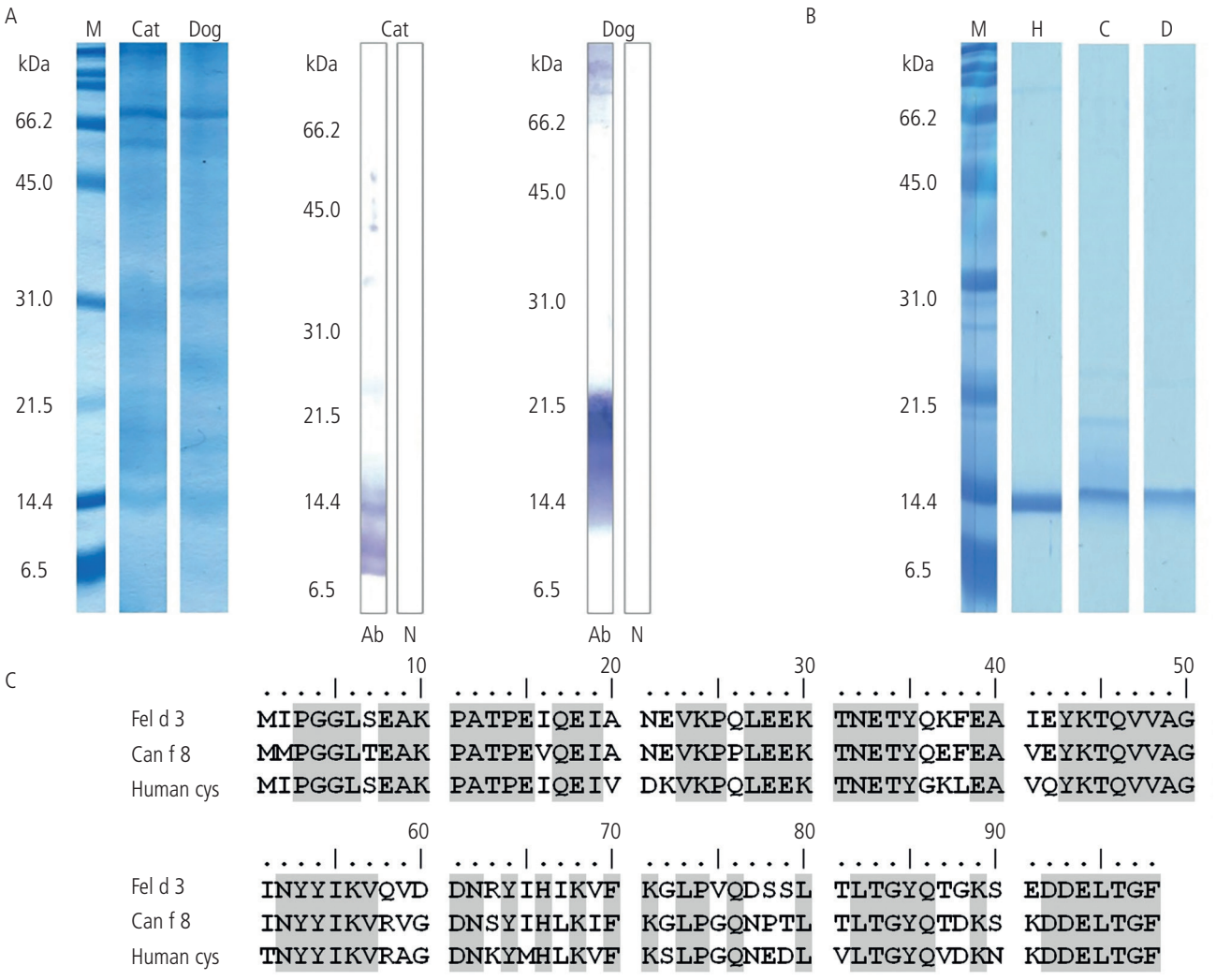

Figure 2. Dog cystatin is present in dog dander and is highly homologous to cat and human cystatin. A, Coomassie blue staining (left) of cat and dog dander extracts as well as immunoblotting (middle, right) after applying a commercially available polyclonal anticystatin antibody (Ab). B, Recombinantly expressed human (H), dog (D), and cat (C) cystatins are analyzed using Coomassie blue–stained SDS-PAGE under reducing conditions. C, Sequence alignment of cat (Q8WNR9), dog (F1PHB6), and human cystatin (P01040). Amino acid residues shared between all 3 cystatins are shaded in gray. N indicates negative control; M, size marker.

sensitization to human cystatin (no parallel sensitization to pet-borne cystatins) are found only in patients with AD, underlining that IgE autoreactivity is a hallmark of AD.

IgE-Mediated Sensitization to Cystatin Is Not Associated With Disease Severity or Levels of sIgE to Pet Dander

Regarding the clinical characteristics of pet dander–sensitized AD patients, IgE-mediated sensitization to cystatin was not associated with titers of sIgE to cat or dog dander (Supplemental Figure 3). In addition, total IgE levels, age, and severity of AD, as determined by SCORAD, were not associated with measurable sIgE to cystatins (Supplemental Figure 2). Regarding comorbidities, 6/19 cystatin-sensitized AD patients also had asthma, while 4/19 did not (no data were available for 9/19).

No Elevated Frequencies of FLG Variants Were Found in AD Patients With IgE-Mediated Sensitization to Cystatin

Four genetic variants of the *FLG* gene, which encodes the well-investigated skin barrier protein filaggrin, have been associated with the development of AD, namely, 2282del4, S3247X, R501X, and R2447X [27,28]. Epidemiological studies showed that having a cat at home enhances the effect of carrying the 2282del4 *FLG* variant on the development of IgE-mediated sensitization [29] and AD [30]. To investigate whether *FLG* variations occur more frequently in cystatin-sensitized AD patients, the 4 gene loci were analyzed using Sanger sequencing. Of 19 AD patients with detectable IgE to cystatin, 4 carried the heterozygous variant 2282del4, and no patient was a carrier of the *FLG* variants S3247X, R501X,

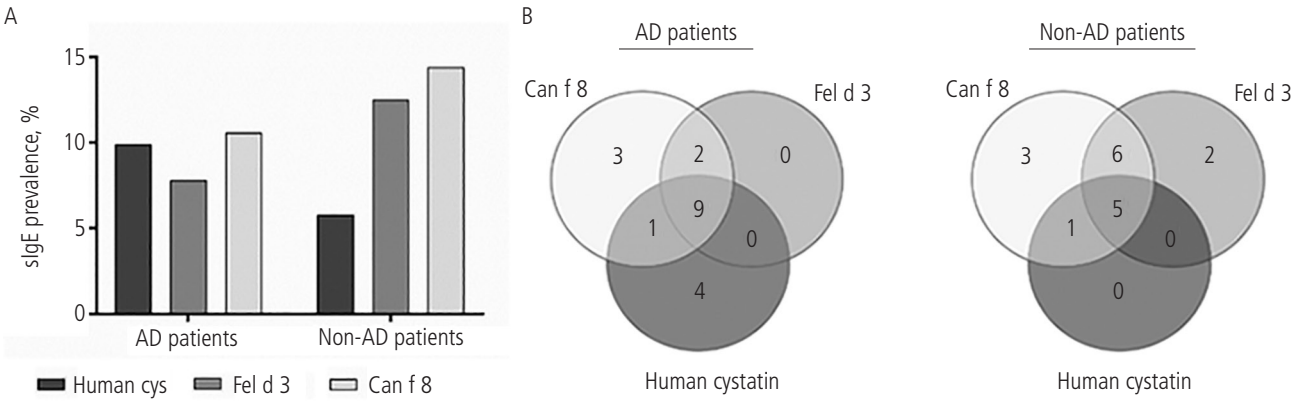

Figure 3. Detection of specific IgE to cat (Fel d 3), dog (Can f 8), and human cystatins by ELISA. A, Frequency of patients with detectable IgE-mediated sensitization to different cystatins within cat- and/or dog dander–sensitized individuals. Patients with (n=141) and without (n=104) atopic dermatitis (AD) are depicted separately. B, Schematic drawing to visualize the degree of polysensitization in AD and non-AD patients with IgE-mediated sensitization to cystatins, as indicated.

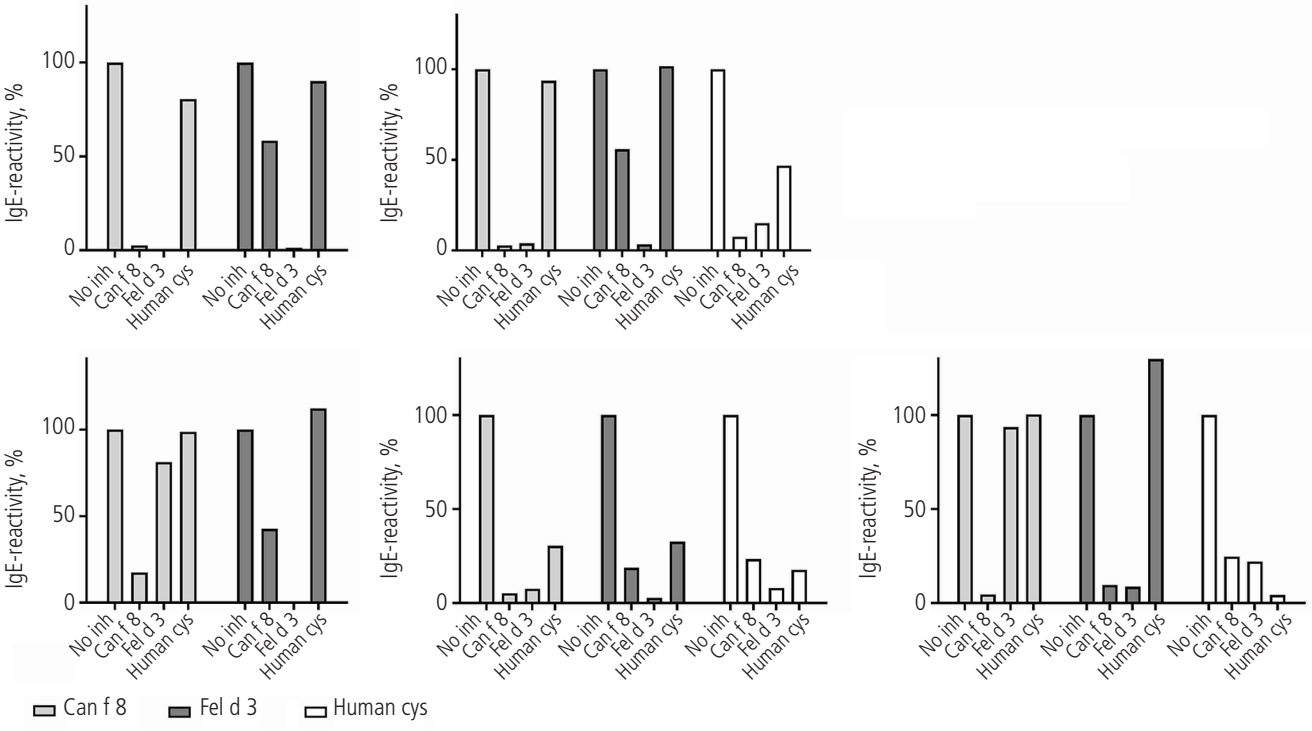

Figure 4. IgE-inhibition assays in sera of patients sensitized to cystatin. Inhibitions were performed with 100 μg/mL. The color code refers to coated allergens. Each graph represents a single patient.

or R2447X. Since the 2282del4 variant has been reported in 13.6% of AD patients in Germany [31] and in 16.1% in the Netherlands [29], our data do not indicate a specific association with sensitization to cystatin.

Human, Feline, and Canine Cystatin Share B-Cell Epitopes

In order to investigate IgE cross-reactivity, serum IgE binding to recombinant cystatins was assessed by ELISA with or without preincubation with different cystatins. In some patients, Fel d 3 was able to inhibit IgE binding to Can f 8 and human cystatin, pointing to primary sensitization to cat (Figure 4 upper panel and lower panel, middle), whereas in another patient, primary sensitization seemed to originate from Can f 8 (Figure 4, lower panel, right). IgE directed toward human cystatin mostly recognized cross-reactive epitopes, as human cystatin was able to substantially inhibit IgE binding to dog or cat cystatin in only 1 of the patient sera, (Figure 4, lower panel, middle). The fact that this patient displayed the highest amount of cystatin-specific IgE within

the group of AD patients leads us to question the impact of this reactivity on his disease.

T-Cell Reactivity to Cystatin in Sensitized Patients

To investigate the quality of the adaptive immune response to human cystatin, PBMCs were stimulated using recombinant cystatins over 4 weeks to propagate putative specific T cells. Since T-cell autoreactivity has been reported to occur frequently in AD, the T-cell response was investigated in atopic patients with AD and respective IgE-mediated sensitization. As a control, the cells were also stimulated in parallel in vitro with lipopolysaccharide in amounts resembling the detected endotoxin contamination of the respective recombinantly expressed cystatins. Subsequently, cells were mitogen-activated, and the cytokines IL-4, IL-17, IL-22, and IFN-γ were assessed using ELISA.

Cells from sensitized donors released a median (SD) of 106.0 (85.0) pg/mL IL-4 in response to human cystatin and 20.0 (184.6) pg/mL IL-4 in response to Fel d 3. These cytokine levels were significantly higher than control stimulation with lipopolysaccharide, which yielded in 7.9 (42.9) pg/mL IL-4. In healthy donors and AD patients without IgE-mediated sensitization to cystatin, no measurable cytokine secretion significantly exceeded the values of control lipopolysaccharide stimulation. Given the high interpatient variability, data are displayed as the stimulation ratio (index) to enhance visibility (Figure 5).

Discussion

Cystatins are evolutionary conserved proteins in mammals. Consequently, their presence in animal dander other than that of cat and their role in IgE-cross-reactivity is worthy of investigation. In the original characterization of Fel d 3, 10% of the sera reacted with the *E coli*–produced protein, whereas 60%-90% reacted in the plaque immunoassay, where the allergen was expressed as a bacterial fusion protein [9]. In the present work, we identified a homolog of Fel d 3 in dogs and successfully cloned dog cystatin, Can f 8, from dog skin.

Among the 245 patients analyzed in this study, we detected IgE-mediated sensitization to Can f 8 in about 12% of pet dander–sensitized patients, whilst 10% were sensitized to Fel d 3. The in vitro experiments performed here suggest a certain degree of IgE cross-reactivity, since most patients' Fel d 3–specific IgE could be trapped by Can f 8 and vice

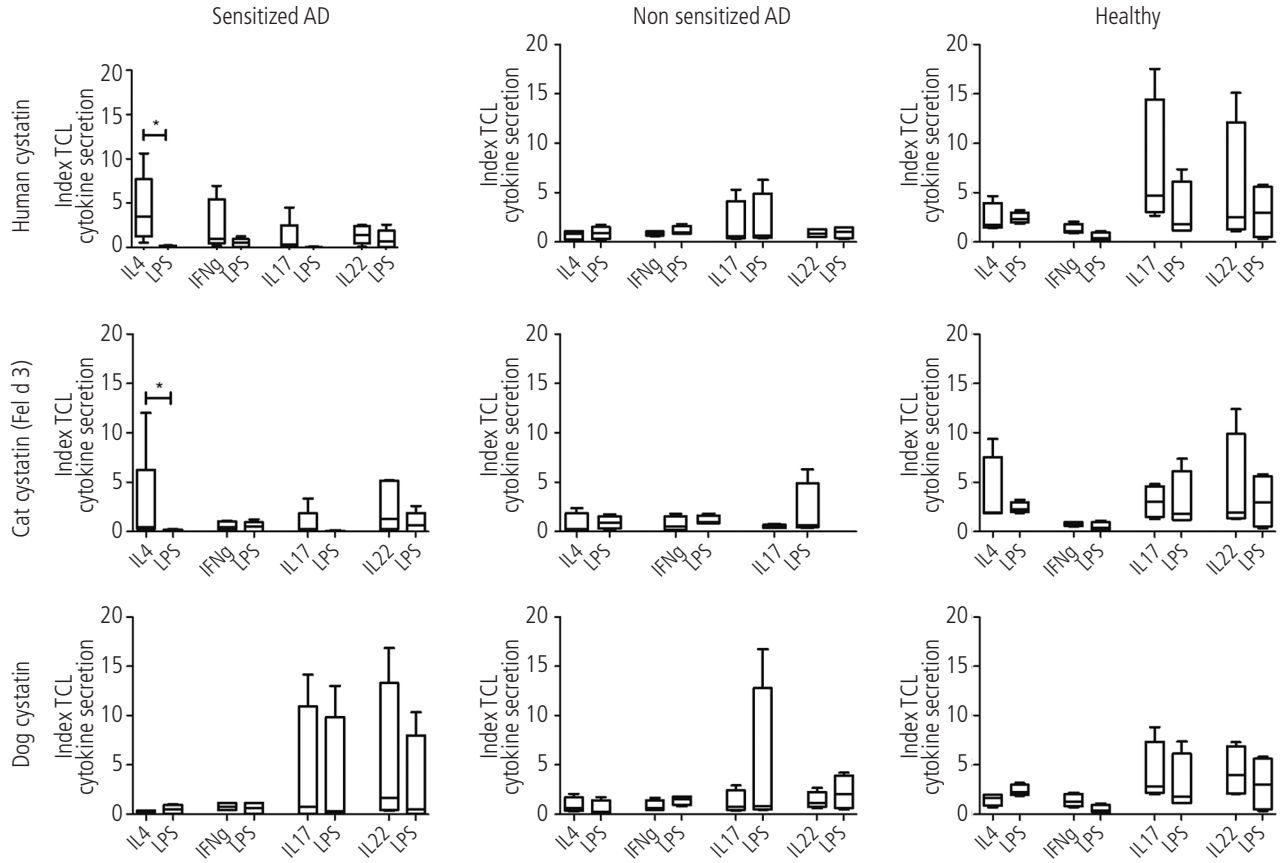

Figure 5. Cytokine profiling of 3-week cystatin-propagated T-cell lines (TCL) derived from sensitized AD patients, nonsensitized AD patients, and healthy donors by means of ELISA. For means of comparison, T cells were propagated for 3 weeks using liposaccharide (LPS) amounts resembling the endotoxin contamination of the respective recombinantly expressed cystatin. Data are displayed as stimulation ratio of T-cell line/nonstimulated T cells (index) to enhance visibility. n=5, *P<.05, Mann-Whitney test.

versa. Our data suggest that cystatins are—as for lipocalins or albumins—a family of pet-borne allergens.

Furthermore, since we detected sIgE against human cystatin A in about 8% of pet dander–sensitized individuals, we can conclude that patients are frequently sensitized to 1 or more cystatins. We observed IgE cross-reactivity between all 3 cystatins in vitro. However, a prerequisite for human cystatin to act as an antigen is its presentation via MHC complexes; therefore, the question remains whether cystatin is part of the human peptidome. By harnessing the power of high-throughput proteomics, T-cell epitopes presented by MHC molecules on the surface of antigen-presenting cells can now be identified using mass spectrometry. By this technique, cystatin was shown to be presented via MHC I and II in healthy donors [32-36]. We and others previously discussed that during an ongoing inflammatory episode of AD, cellular components can be released into the surrounding skin tissue as a consequence of cell destruction by scratching [37]. The surrounding type 2 inflammatory milieu is believed to facilitate sensitization subsequently, whilst inborn properties of the antigens may further foster the procedure [38,39]. This theory is underlined by the fact that exclusive IgE sensitization to the human cystatin without parallel sensitization to pet-borne cystatins was only detected in atopic patients with AD. Early exposure to cats was found to increase the likelihood of developing AD during childhood in individuals carrying a mutation in the filaggrin gene. However, in the patients we studied, no association with the 4 most common filaggrin mutations was detected.

T-cell responses to cat allergens have been investigated in several studies, with most emphasis on the major allergen Fel d 1. Use of MHC class II multimers for staining of Fel d 1–specific T cells revealed a central memory phenotype with low expression of skin-homing markers [40], but strong T_H2 polarization, as determined by expression of surface markers and secretion of IL-5 [41,42]. Specific allergen immunotherapy (SIT) is indeed considered a promising option for sensitized patients who cannot avoid the allergen. However, newer studies focus solely on Fel d 1 [43-45]. Interestingly, SIT with synthetic Fel d 1 peptide epitopes reduces expression of the T_H2 cell surface marker CRTh2 on Fel d 1–specific T cells [46]. Consistent with these observations on Fel d 1, Fel d 3, and human cystatin, we observed T_H2 T-cell responses in sensitized individuals, namely, induction of IL-4. The lack of IL-4 in Can f 8–stimulated samples remains speculative, although it may be a result of the relatively small numbers of individuals assessed in this assay.

The immune responses of pet-derived lipocalins have previously been compared to that of homologous human proteins. While human lipocalins appear to induce a T_H1 response in cells from healthy donors, pet allergens led to increased levels of IL-13 [47]. Therefore, the intrinsic properties of pet-derived lipocalins are thought to predispose them to induce a type 2 response. In individuals with IgE-mediated sensitization to pet lipocalins, the immune response has been reported to be dominated by both IL-4 and IL-5 in the presence of pet and human lipocalins [48].

Cross-reactivity to pet allergens, therefore, appears to be a plausible mechanism underlying the sensitization we observed to several homologous cystatins. Cross-reactivity is frequent in allergens that are grouped into allergen families according to their structure or function. It can also affect various atopic diseases, such as food, skin, and respiratory allergy. One of the best studied cross-reactions between pollen and plant food is birch pollen–related syndrome, which has a significant clinical impact [49]. The clinical impact of cross-reactivity to human autoallergens is difficult to estimate. Although in the present study we were able to show IgE binding to and T-cell proliferation in response to human cystatin, disease severity was not associated with sIgE to cystatin. One may presume a clinical relevance for cross-reactivity between fungal and human allergens, since the latter cause skin lesions in sensitized patients' skin. More precisely, recombinantly produced human manganese superoxide dismutase (MnSOD) has been applied to patients' skin in an atopy patch test [50], which is used for controlled assessment of the delayed-type (type 4–like) hypersensitivity skin reaction to protein allergens [51]. Although the responses to single autoallergens may appear rather modest, the high frequency of sensitization to self-antigens in 23%-91% AD patients [13] and the large number of autoallergens identified as playing a role in AD (>140) [12] may have a significant impact, leading to chronic inflammation. We therefore believe that focusing on Fel d 1 with regard to ongoing studies and treatment options may carry a certain risk, since it overlooks minor allergens such as Fel d 3.

In conclusion, we identified cystatins as a new family of cross-reactive animal allergens. Although most AD patients with sIgE to Fel d 3 and/or Can f 8 had sIgE to human cystatin, an association with disease severity could not be established.

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Conflicts of Interest

LMR declares grants to his institution and personal fees from Novartis outside the scope of this work. TW has received institutional research grants from LEO Pharma and Novartis and has provided consultancy services for AbbVie, Janssen, Galderma, LEO, Sanofi-Genzyme, and Novartis. He has also lectured at educational events sponsored by AbbVie, Janssen, Celgene, Galderma, LEO Pharma, Sanofi, and Novartis and is involved in performing clinical trials for various pharmaceutical industries that manufacture drugs used for the treatment of atopic dermatitis. The remaining authors declare that they have no conflicts of interest.

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