Development and preliminary clinical evaluation of a peptide immunotherapy vaccine for cat allergy

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Background: Allergic sensitization to cat allergens is common and represents a major risk factor for asthma. Specific immunotherapy (SIT) is effective but cumbersome and associated with IgE-dependent adverse events. Immunotherapy targeting allergen-specific T cells, with synthetic peptides representing T-cell epitopes, might improve safety and reduce the duration of treatment.

Objective: We sought to define major T-cell epitopes of Fel d 1 for peptide immunotherapy, generate a peptide vaccine, and evaluate its safety and tolerability in subjects with cat allergy.

Methods: We determined the binding affinities of Fel d 1 peptides for 10 commonly expressed HLA-DR molecules. Functionally immunodominant peptides were identified by means of proliferation and cytokine secretion. Histamine-releasing activity was assessed, and a peptide vaccine was formulated. Safety and tolerability were evaluated in a dose-ranging phase IIa clinical trial.

Results: MHC-binding sequences were identified throughout Fel d 1. Some regions contained multiple overlapping T-cell epitopes that bound multiple MHC molecules. Immunodominant sequences were identified on the basis of proliferative and cytokine (IFN-γ, IL-10, and IL-13) responses. Cat allergen extract, but not peptides, induced histamine release in blood basophils. A single administration of peptide vaccine was safe and well tolerated. The dose of vaccine resulting in the greatest inhibition of the late-phase skin response to intradermal whole allergen challenge was 3 nmol.

Conclusions: Fel d 1 contains multiple overlapping MHC-binding motifs. A peptide vaccine comprising the immunodominant regions of the allergen was safe and well tolerated when given to subjects with cat allergy as a single dose. The dose of vaccine resulting in the greatest reduction in late-phase skin response was defined for future clinical development. (J Allergy Clin Immunol 2011;127:89-97.)

Key words: *Immunotherapy, allergy, T cell, epitope, peptide, MHC, immune tolerance, vaccine*

Cat allergy is one of the most common allergic sensitizations and is strongly associated with asthma.[1,2] Children sensitized to cats are more likely to have severe asthma than those sensitized to other allergens.[3] Current treatment options for allergy to cats are largely symptomatic. However, allergen-specific immunotherapy (SIT) is available for desensitization to cats and has been demonstrated to be clinically effective in the treatment of both allergic rhinitis and asthma.[4-8] SIT has a duration of action that exceeds the treatment period.[9] Furthermore, SIT has the potential to prevent new allergic sensitizations in children,[10] and 3 years of SIT prevented the development of asthma (odds ratio for no asthma, 4.6; 95% CI, 1.5-13.7) over a period of at least 7 years after withdrawal of therapy.[11-13] However, SIT with native proteins is associated with a high frequency of treatment-related reactions that can be severe and occasionally life-threatening. Thus reducing the allergenicity of immunotherapy approaches represents an important unmet need in the treatment of allergy.

In an attempt to reduce allergenicity, improve safety, and reduce treatment times, T-cell epitopes from the major cat allergen Fel d 1[14] with a reduced capacity (vs whole allergen) to cross-link IgE on effector cells were identified and used to treat subjects with cat allergy. Improvements in clinical and surrogate outcomes were observed in most,[15-17] but not all,[18] studies. More recently, the prototype of the current vaccine consisted of a mixture of 12 peptides.[19-22] This mixture was not designed on the basis of MHC-binding characteristics. Indeed, our subsequent MHC-binding analysis, as reported in this study, demonstrated that not all of the original 12 peptides displayed binding to the panel of common HLA-DR molecules evaluated. Thus it was possible to rationally reduce the number of peptides in the vaccine without substantially compromising population coverage. This enabled the product to comply with the requirements of the regulatory authorities for a well-characterized product (eg, the ability to resolve each peptide and associated impurities and degradants from the mixture). A further advantage introduced in the current formulation of the vaccine is the addition of an agent (thioglycerol) to prevent dimerization of peptides containing cysteine residues (which could potentially increase the chance of IgE cross-linking). Formulation studies on the vaccine demonstrated

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Supported by Circassia Ltd.

Disclosure of potential conflict of interest: R. P. Hafner and P. Laidler are employees of Circassia Ltd. A. B. Kay is a founder, stockholder, and consultant for Circassia Ltd. M. Larché is founder, stockholder, and consultant of Circassia Ltd and a founding scientist of Adiga Life Sciences and has received research support from both companies. The rest of the authors have declared that they have no conflict of interest.

Received for publication July 28, 2010; revised November 3, 2010; accepted for publication November 23, 2010.

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0091-6749/$36.00

doi:10.1016/j.jaci.2010.11.029

Abbreviations used
AD: Atopic dermatitis
EPSR: Early-phase skin response
HEP: Histamine equivalent in prick testing
LPSR: Late-phase skin response
PPD: Purified protein derivative of *Mycobacterium tuberculosis*
SAE: Severe adverse event
TEAE: Treatment-emergent adverse event

that homodimers and heterodimers were formed among peptides in the mixture in the absence of thioglycerol.

Conceptually, the rationale behind the development of the current vaccine was as follows. First, highly soluble peptide sequences of minimal length and displaying promiscuous MHC binding would form the basis of a vaccine containing major T-cell epitopes of the allergen with extensive population coverage. Second, the vaccine, delivered without adjuvant, would lead to recognition of epitopes in a noninflammatory milieu, resulting in the induction of tolerant T-cell responses similar to encounter of the immune system with ubiquitous self-antigen epitopes.

Safety and tolerability were evaluated after a single administration in an incremental dose cohort study. Additionally, the clinical arm of this study aimed to define the dose of vaccine providing the greatest reduction in the magnitude of the late-phase skin response (LPSR) to whole cat allergen extract because this surrogate marker has been associated with clinical efficacy.[23]

METHODS

Peptides

For *in vitro* studies, Fel d 1 peptides were synthesized by using standard Fmoc chemistry, purified (>90%) by means of HPLC, and presented as a lyophilized solid (Advanced Biotechnology Centre, Imperial College London, United Kingdom, or Bachem, Wirral, United Kingdom; see Table E1 in this article's Online Repository at www.jacionline.org). The peptide vaccine for the clinical study (ToleroMune Cat; also known as Cat-PAD) was an equimolar mixture of 7 peptides from Fel d 1 supplied as a frozen concentrate containing peptides (200 nmol/mL) and was diluted with placebo to prepare individual doses. The individual peptide sequences were as follows: CPAVKRDVDLFLT, EQVAQYKALPVVLENA, KALPVVLENARILNCV, RILKNCVDAKMTEEDKE, KENALSLLDKIYTSPL, TAMKKIQDCYVENGLI, and SRVLDGLVMTTIS SSK. The peptides were synthesized by Bachem according to current Good Manufacturing Practice; formulated, filled, and finished by Nova Laboratories (Leicester, United Kingdom), also according to current Good Manufacturing Practice; tested at Gen-Probe (Livingston, United Kingdom) and released in accordance with the Clinical Trials Q3 Directive.

Subjects

Vaccine design study. The study received prior approval from the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust. Subjects (18-65 years of age) provided informed consent and had a documented history of allergy on exposure to cats in addition to a positive skin prick test response to cat allergen extract (3 mm greater than that elicited by the negative control), a specific cat allergen IgE level of greater than 0.35 IU/mL, or both.

Clinical study. The study received prior approval from the Landesamt fűr Gesundheit und Soziales Ethik-Kommission des Landes Berlin. Subjects were male or female (18-65 years of age) with a history of rhinoconjunctivitis with or without controlled asthma (Global Initiative for Asthma 2006 classification 1; http://www.ginasthma.com/) on exposure to cats for at least 1 year, were willing to provide written informed consent, and were able to comply with the study requirements. Further details are provided in the Methods section of this article's Online Repository at www.jacionline.org.

Purification of HLA-DR molecules and HLA-DR peptide-binding assays

HLA-DR molecules were purified from HLA-homozygous EBV cell lines by means of affinity chromatography with the monoclonal anti-DR antibody L-243 coupled to protein A–Sepharose CL 4B gel (GE Healthcare, Saclay, France), as previously described.[24]

Identification of predicted core epitope-binding motifs

Each peptide-MHC interaction identified by using physical binding assays was further analyzed *in silico* with the Immune Epitope Database (www.immuneepitope.org/).[25-27] Core MHC-binding motifs predicted according to the method of Sturniolo et al[28] were recorded.

Proliferation assays, measurement of cytokines, and histamine release assays

Immunologic reactivity of peptides was evaluated using PBMCs. Methods for measurements of proliferation, cytokine release, and histamine release in PBMCs are described in detail in the Methods section of this article's Online Repository.

Clinical study design

This was a randomized, double-blind, placebo-controlled, escalating, single-dose evaluation of the safety and tolerability of intradermal and subcutaneous injections of ToleroMune Cat run at the Allergie-Centrum-Charité in Berlin. The study design is summarized in Fig E1 (in this article's Online Repository at www.jacionline.org), and the procedures performed at each visit are summarized in Table E2 (in this article's Online Repository at www.jacionline.org). Further details of the study design are described in the Methods section of this article's Online Repository.

Primary and secondary efficacy measurements

The primary efficacy measurement was the change (vs baseline) in mean diameter of the LPSR 8 hours after intradermal challenge with whole cat allergen on day 21 after vaccine or placebo injection. LPSR was induced by means of intradermal injection of 0.010 histamine equivalent in prick testing (HEP) units of cat allergen (Laboratorios Leti, Madrid, Spain). Two injections were administered, separated by 30 minutes, into the volar surface of the left and right forearms, respectively. A ballpoint pen outline of each response was obtained (by using adhesive tape) 8 hours later. The longest and orthogonal diameters were measured and recorded. The secondary efficacy measurement was the mean diameter of the early-phase skin response (EPSR) 15 minutes after the same intradermal injections.

Safety measurements

Safety parameters were as follows: adverse events, physical examination, vital signs, clinical laboratory tests (hematology, blood biochemistry, and urinalysis), spirometric FEV_1, visual analog score of breathlessness and nasal symptoms, and local reactions at the injection site.

Adverse events

At each visit, the investigator determined whether any adverse events had occurred by asking nonleading questions. Adverse event reporting began from the point of informed consent and ended at treatment phase visit 2.

TABLE I. Summary of the results obtained with the Fel d1 peptides and HLA-DR molecules

Peptides	DR1	DR3	DR4	DR7	DR11	DR13	DR15	B3	B5	B4
Fel d 1 chain 1										
1-17*	15385	1	50	2363	1250	280	17	553	1581	10000
12-28	5000	75	200	18	>6250	380	111	922	1215	1000
23-38*	4	>250	2	3	1	15	0.1	1843	18	350
29-45*	3	4	78	208	30	2	1	138	1	20
39-55*	179	6	14	99	125	100	77	2765	75	133
48-63	4100	150	2500	>4167	1875	400	8046	1382	4301	>50000
54-69*	4362	200	300	29	1	8	12	1843	3226	2835
Fel d 1 chain 2										
1-16	641	>250	333	90	1394	>400	1322	691	>10573	220
7-23	1669	?	300	15	1113	?	2103	184	108	?
20-35	192	138	11	1	22	2	73	1382	0.4	475
29-44	38462	225	>3333	177	>6250	380	>11494	>4608	1860	45000
40-55*	23	200	50	31	2081	>400	2	922	2151	0.2
48-63	1669	138	1333	156	406	>400	210	>4608	610	1665
56-71*	462	113	3	188	16	>400	184	>4608	215	92
67-82	231	63	20	271	500	220	73	246	2151	750
77-92	3077	2	13	904	391	>400	210	2304	1613	2500

Results are expressed as relative binding ratio obtained by dividing the IC50 of the peptide by the reference peptide.
Lower numbers correspond to higher binding affinity. Reference peptide nanomolar binding constants are provided.
Numbers in red (ratio of 20 or less) = high affinity binding, those in green (ratio of 20-100) = moderate affinity binding.
"?" indicates that it was not possible to calculate affinity due to solubility problems.
Each peptide-MHC combination was evaluated in 3-6 independent experiments.
Peptides in blue were evaluated in proliferation/cytokine assays. Peptides in orange were poorly soluble at neutral pH.
*Peptides included in the vaccine. Sequence 1-17 evaluated in functional studies modified to residues 3-15 in the vaccine.

Statistical analysis

Proliferation data were analyzed by comparing 12 replicate wells of cells and medium with 12 replicate wells of cells and the test peptide/antigen by using the Mann-Whitney test.

For the clinical study, a comparison of each ToleroMune Cat dose with pooled placebo from the same route of administration was made by using an analysis of covariance model with treatment as a factor and baseline measurements as covariates.

RESULTS

Multiple, overlapping MHC-binding sequences within Fel d 1

The binding affinity of 16 Fel d 1 peptides for 10 commonly expressed HLA-DR molecules was determined and transformed into ratios of affinity between the test peptide and positive control peptide to account for the differences in sensitivity of the binding assays (Table I). Arbitrary thresholds were established to define high- and moderate-affinity binding. All of the MHC class II molecules except HLA-DRB3 bound at least 1 Fel d 1 sequence. Several peptides contained multiple overlapping HLA-DR–binding sequences capable of binding to different HLA-DR molecules with different affinities. Three of the 16 peptides screened (chain $1^{48\text{-}63}$, chain $2^{29\text{-}44}$, and chain $2^{48\text{-}63}$) failed to bind to the HLA-DR molecules analyzed and were not evaluated further. Fig 1 presents an overview of HLA-DR–binding regions with the Fel d 1 molecule, including *in silico* predicted minimal core binding motifs and sequences used in published tetramer analyses.[29-31]

PBMC proliferation assays

Of the 13 peptides with demonstrable affinity for HLA-DR, 2 (chain $1^{12\text{-}28}$ and chain $2^{20\text{-}35}$) were insoluble at neutral pH and were not assayed further. As a result of their limited binding (HLA-DR7 only), 2 further peptides (chain $2^{1\text{-}16}$ and chain $2^{7\text{-}23}$) were excluded from proliferation and cytokine assays. The remaining 9 peptides were assayed in PBMC cultures from 100 subjects with cat allergy. There appeared to be a dose-dependent increase in proliferation for each peptide, although in some cases this was not statistically different from that seen with medium alone. Responses to individual peptides were weaker than to whole allergen extract, as expected. Because of MHC restriction of T-cell responses to peptides, it was expected that only a proportion of subjects would mount a proliferative response to any given peptide, and thus at the population level, the proliferative responses observed were low. Of note, the proliferative response to the 7-peptide vaccine was equivalent to whole allergen extract (Fig 2). Strong responses were observed to the positive control antigen purified protein derivative of *Mycobacterium tuberculosis* (PPD) because the study population was immunized with BCG.

PBMC cytokine responses

Separate PBMC cultures were established at higher cell density to evaluate the secretion of IFN-γ, IL-13, and IL-10 in response to individual peptides. Fig 3 summarizes the IFN-γ (Fig 3, *A*), IL-13 (Fig 3, *B*), and IL-10 (Fig 3, *C*) responses to the 9 peptides, the 7-peptide vaccine, cat dander extract, and the positive control antigen PPD (BCG-vaccinated population). All peptides induced a cytokine response in greater than 20% of the population. Generally, responses were weak to moderate, as expected. Cytokine responses to whole cat dander extract were observed in the majority of subjects with moderate-to-high levels of cytokines. Of note, the response to the 7-peptide vaccine was equivalent to that to the whole cat dander extract.

Peripheral blood basophil histamine release

Robust histamine release was observed at all concentrations of whole cat dander allergen extract (as low as 10 ng/mL). In contrast, histamine release elicited by any individual peptide (or the 7-peptide vaccine, data not shown) at any concentration was

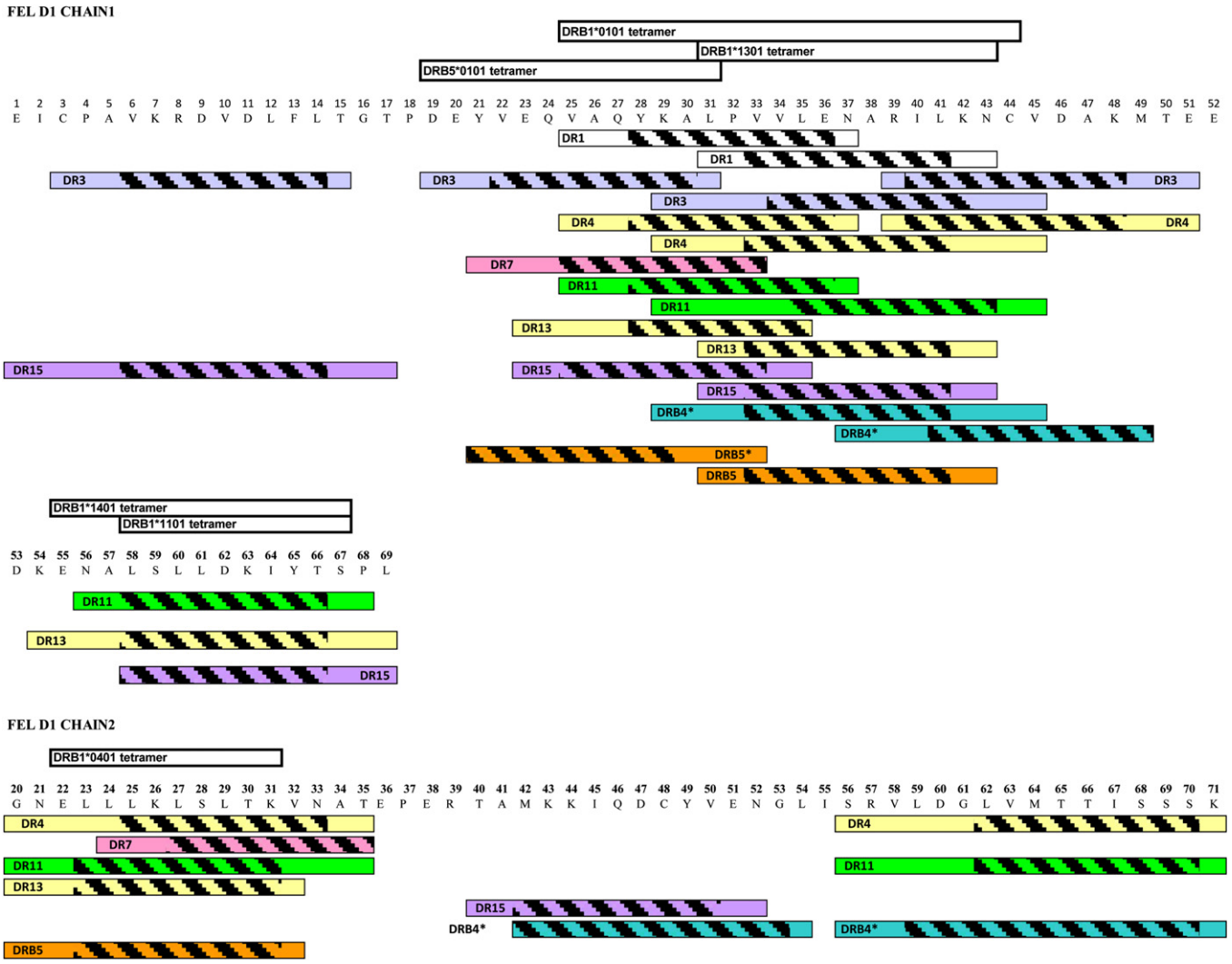

FIG 1. Epitope map of Fel d 1 chains 1 and 2. The figure shows the primary sequence of Fel d 1 chains 1 and 2 (partial). The location of MHC-binding sequences is shown in *colored bars* (see Table I). The restricting MHC element is indicated at the side of each bar. The predicted core binding motif *(hatched region of the bar)* is shown. Published MHC class II tetramer sequences are provided.

less than 5% of total release (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus individual peptides and the vaccine were at least 1,000-fold less able to induce basophil histamine release than native allergen.

Clinical study

Eighty-eight of 148 subjects screened met the inclusion/exclusion criteria. Forty were randomized to receive ToleroMune Cat or placebo by means of intradermal injection, and 48 were randomized to receive ToleroMune Cat or placebo by means of subcutaneous injection (see Fig E1). Ten of 40 subjects assigned to intradermal administration and 12 of 48 subjects assigned to subcutaneous administration received placebo. All subjects completed the study. At screening, cat-specific IgE levels and EPSR and LPSR measurements were captured, and the results are plotted in Fig E3 (available in this article's Online Repository at www.jacionline.org). There was a weak but statistically significant correlation ($P = .02$) between the cat-specific IgE level and the magnitude of the LPSR. It is interesting to note that some subjects had substantial EPSRs and LPSRs with low levels of cat-specific IgE, whereas conversely, a number of subjects had high levels of cat-specific IgE but did not show significant EPSRs and LPSRs to intradermal cat allergen.

Safety

There were no serious adverse events (SAEs) during the study, and no subject withdrew because of an adverse event. The most common treatment-emergent adverse events (TEAEs) in the intradermal ToleroMune Cat cohorts were nasopharyngitis, cough, and headache. In the subcutaneous cohort the most commonly reported TEAEs were nasal congestion and respiratory symptoms. In the intradermal cohort no subjects receiving the active preparation had a reduction in FEV_1 of greater than 20% from baseline or reported asthma-like symptoms during

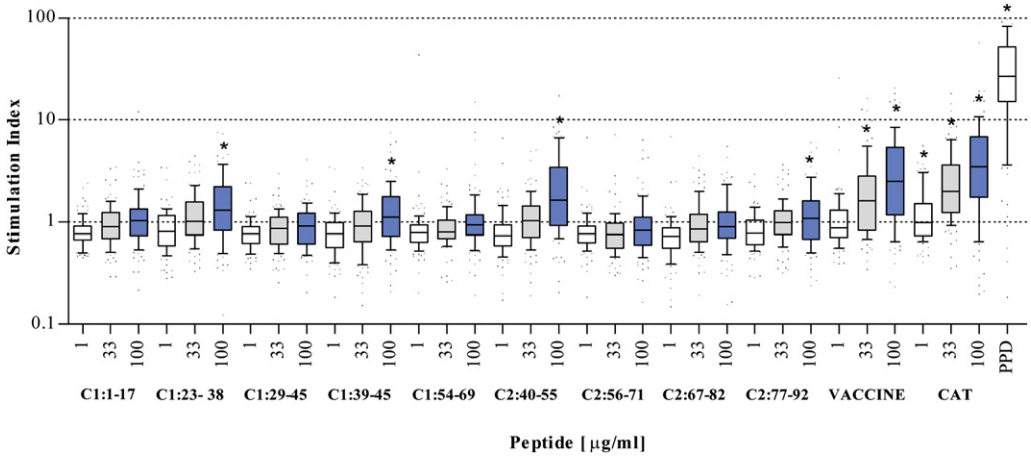

FIG 2. Proliferative responses of 100 subjects with cat allergy to T-cell epitopes of Fel d 1. Data are presented as stimulation indices for comparison between subjects. Twelve replicates were established for each culture condition. PPD (BCG vaccinated population) acted as a positive control. *Bars* indicate medians with interquartile ranges, and *whiskers* show 10th to 90th percentiles. **P* < .05 versus medium alone (Mann-Whitney test).

the 8-hour postdosing period. In the subcutaneous group 1 patient had a 29% decrease in FEV_1 4 hours after 0.3 nmol of ToleroMune Cat. After leaving the clinic 8 hours after dosing, the subject had moderate asthma symptoms, which were treated with salbutamol and which the investigator believed were possibly related to the study drug. A second subject in the subcutaneous group had a 25% decrease in FEV_1 8 hours after 3 nmol of ToleroMune Cat. After leaving the clinic 8 hours after dosing, this subject had mild asthma symptoms, which were treated with salbutamol and which the investigator believed were possibly related to the study drug. One subject receiving placebo had a 19% decrease in FEV_1 at 7 hours after dosing. After leaving the clinic 8 hours after dosing, this subject had mild asthma symptoms, which were treated with salbutamol and which the investigator believed were possibly related to the study drug.

In both the intradermal and subcutaneous cohorts, the visual analog score scores of breathlessness and nasal symptoms were low throughout the 8-hour postdosing period. There were no clinically significant changes in any laboratory parameter or electrocardiographic findings after treatment, and no adverse findings on physical examination were observed at follow-up.

All subjects treated with ToleroMune Cat or placebo experienced transient and self-limiting local reactions at the injection site 15 minutes after intradermal injection. These reactions comprised erythema in all and wheals in the majority of subjects. Most subjects who received subcutaneous ToleroMune Cat or placebo experienced no local reactions. A detailed description of all adverse events is provided in the Methods section of this article's Online Repository.

Effect of ToleroMune Cat on skin responses to allergen

Fig 4, *A*, shows the relationship between the dose of ToleroMune Cat administered intradermally and the change in mean diameter of the LPSR compared with that elicited by placebo. Fig 4, *B*, shows the change in mean area of the LPSR. Three nanomoles administered intradermally resulted in a reduction in the LPSR of approximately 40% (placebo, 10%). None of the changes in reaction size achieved statistical significance (vs placebo), but a trend was observed at the 3-nmol dose. ToleroMune Cat administered subcutaneously at doses of 0.03, 0.3, 3, and 12 nmol resulted in smaller changes in the mean diameter of the LPSR than were achieved with placebo (data not shown). More substantial changes were seen with the 1- and 20-nmol dose administered subcutaneously, although this result must be interpreted with caution because there were also substantial changes in the EPSR, which suggests the allergen used for the challenge had lost potency. There were no consistent changes in the EPSR with intradermal or subcutaneous administration.

DISCUSSION

The purpose of this study was (1) to identify the immunodominant T-cell epitopes best suited for peptide immunotherapy from the major cat allergen Fel d 1, (2) to develop a peptide-based therapeutic vaccine for the treatment of cat allergy, (3) to evaluate the safety and tolerability of the vaccine in subjects with allergic rhinoconjunctivitis (with or without mild asthma) triggered by cats, and (4) to identify a vaccine dose for future efficacy studies. Vaccines composed of short synthetic peptide sequences offer the potential advantage of decreased IgE-mediated adverse events during therapy. Indeed, our data demonstrate that peptides of 13 to 17 amino acids and the 7-peptide vaccine itself were at least 1,000-fold less allergenic *in vitro* than native allergen.

To define immunodominant epitopes, we used biochemical techniques to determine the MHC class II–binding affinities of peptides spanning the majority of Fel d 1, together with proliferative and cytokine responses induced in PBMCs of subjects with cat allergy. A number of previous studies have identified T-cell epitopes in Fel d 1,[32-37] but little information is available regarding MHC restriction. Many of these studies demonstrated that the majority of proliferative responses were stimulated by peptides from Fel d 1 chain 1, particularly the region from amino acids 9 to 55. Based on these data, 2 large (27mer) overlapping peptides (designated IPC-1 and IPC-2, representing amino acids 7-55 of Fel d 1 chain 1) were developed for clinical application.[15-18] Modest clinical efficacy was reported in some of these

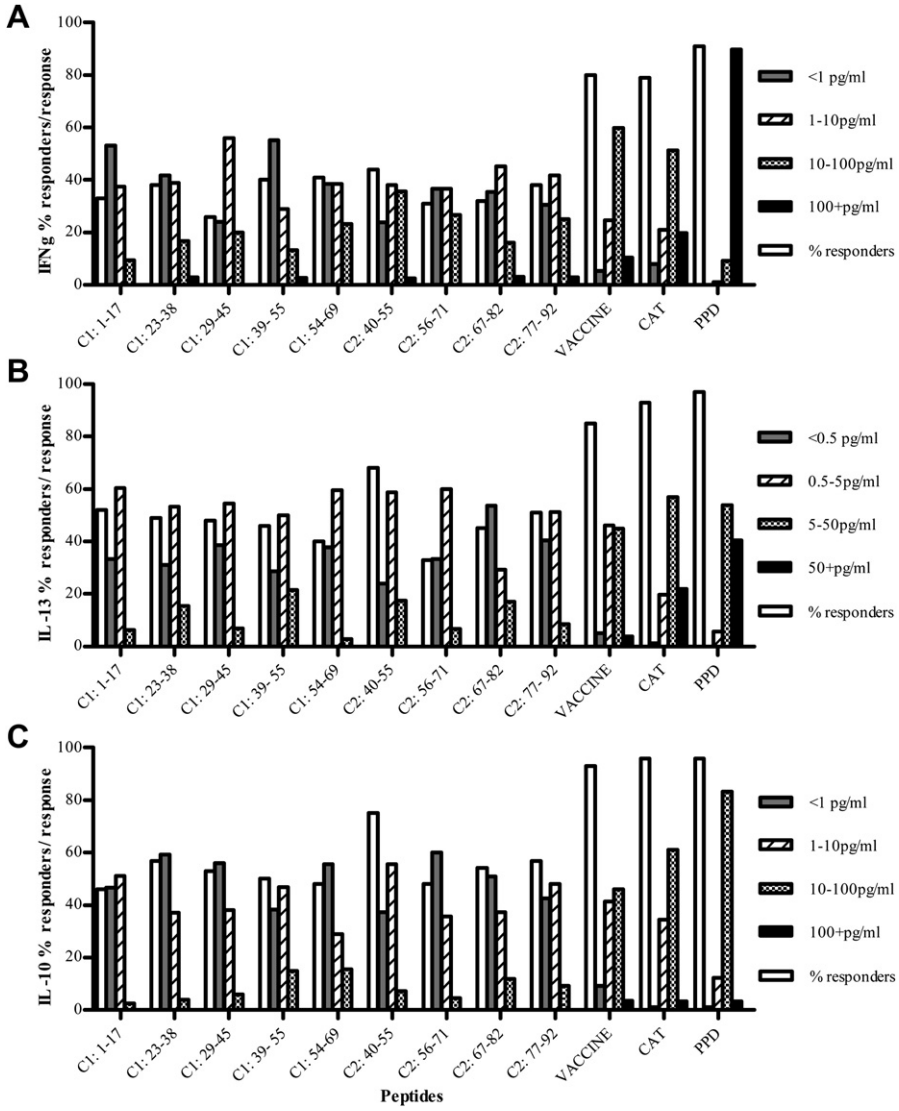

FIG 3. Distribution and strength of cytokine responses to peptide/antigen. Strength of response was arbitrarily defined based on the detection range of each assay. *Bars* show percentages of subjects making a detectable response *(% responders)* followed by a breakdown of the percentage of these subjects making responses of each strength. **A**, IFN-γ (n = 87). **B**, IL-13 (n = 89). **C**, IL-10 (n = 91).

studies, but our data indicate that the selection of peptides (IPC-1/IPC-2; chain 1 only) might have been too limited because we have identified important epitopes in other regions of the molecule, particularly in chain 2. Furthermore, the limited overlap (spanning the sequence KALPV) of the 2 peptides in questions results in the loss of several MHC-binding motifs (Fig 1).

Currently, few data are available concerning the MHC-binding characteristics of Fel d 1 sequences. We determined the binding constants of Fel d 1 peptides for 10 common HLA-DR molecules. Our data provide the first MHC restriction map of an allergen and define a number of functional T-cell epitopes. We previously defined the DR1- and DR4-binding characteristics of 2 of the peptides (chain $1^{29\text{-}45}$: DRB1*0101; Chain $1^{23\text{-}38}$: DRB4*0405, 0408) evaluated in the current study.[38] Recently, the use of HLA-DRB1*0101 tetramers in 3 independent studies has confirmed the functional binding of the chain $1^{29\text{-}45}$ peptide to DRB1*0101. Campbell et al[29] used a tetramer containing the Fel d 1 chain 1 sequence KALPVVLENARILKNCV (chain $1^{29\text{-}45}$) to monitor epitope-specific T cells in a model of allergic airway inflammation in HLA-DRB1*0101 transgenic mice. Bateman et al[30] generated a tetramer containing the sequence LPVVLENARILKNCVDAK (chain $1^{31\text{-}48}$) and examined the frequency and phenotype of Fel d 1–specific T cells in subjects with cat allergy with atopic dermatitis (AD) and healthy control subjects. A DRB1*0101 tetramer reported by Kwok et al[31] used the peptide VAQYKALPVVLENARILKNC, which, based on our analysis (Fig 1), contains 2 overlapping epitopes. All 3 of these tetramer reagents recognize T cells specific for the same core epitope (VVLENARIL), whereas in addition, the tetramer described by Kwok et al should be capable of detecting T cells specific for a second core epitope YKALPVVLE. Kwok et al also described 5 more tetramers containing peptides from Fel d 1. Their results are in close agreement with our own using different but complementary techniques. With the exception of a DRB1*1401 (not included in our binding analysis)–restricted epitope, all tetramers (peptide-MHC combinations) align precisely

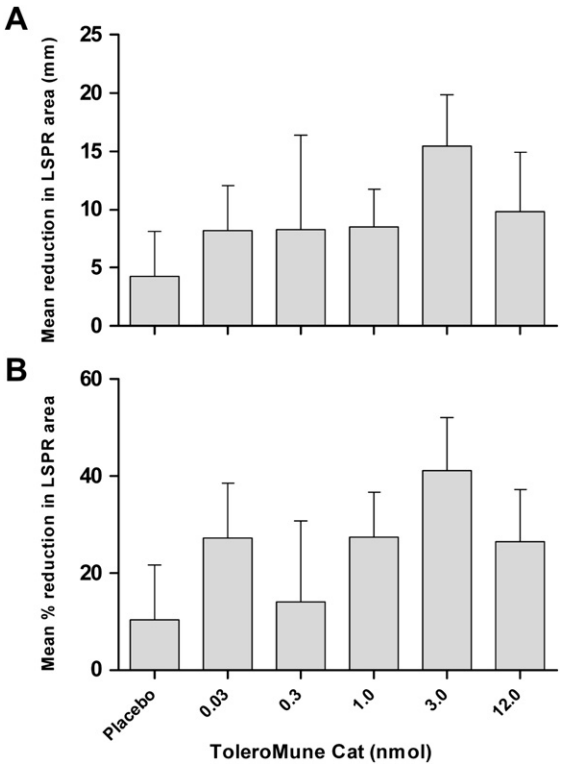

FIG 4. The effect of ToleroMune Cat on the magnitude of the LPSR. Mean reduction (in millimeters; **A**) and mean percentage reduction **(B)** in 8-hour LPSR area between baseline and posttreatment (intradermal) challenge with cat allergen extract are shown. Five cohorts received a single intradermal injection of vaccine or placebo (n = 8 per group: 6 active and 2 placebo). No statistically significant changes were seen (analysis of covariance). A trend ($P = .1$ LPSR in millimeters; $P = .09$ in LPSR area) was observed at 3 nmol. *Bars* are shown as means with standard errors.

with our binding data and the core binding motifs predicted by the Immune Epitope Database.[26,28] Three of the tetramers identified contain sequences from a region that our analysis has identified as epitope rich and thus desirable for inclusion in a peptide vaccine. Our analysis of physical binding of peptides to common HLA-DR molecules suggests that the derived 7-peptide vaccine will have broad population coverage. Southwood et al[39] calculated that a similar panel of MHC alleles would be representative of 83.9% to 98.8% of subjects in ethnically diverse populations. Furthermore, analysis of predicted HLA-DP and DQ binding of our peptides by using the Immune Epitope Database suggests that multiple common DP and DQ motifs are present within the vaccine peptides, making it likely that the vaccine will interact with MHC class II molecules in all subjects.

Cellular assays were used to confirm the functionality of epitopes identified by means of MHC-binding assays. In addition to proliferative responses, cytokine production (IL-10, IL-13, and IFN-γ) was evaluated and proved to be more sensitive. Interestingly, there was no correlation between the degree of promiscuity in MHC binding and the magnitude, frequency, or both of cellular responses. This might have been due to the ability of some or all of the peptides to bind to MHC molecules not evaluated in this study (eg, HLA-DP and HLA-DQ). In contrast to a previous report, no individual peptide appeared to elicit a response skewed to any single cytokine. Reefer et al[37] screened 20 peptides from Fel d 1, measuring IL-5, IL-10, IL-13, and IFN-γ. Responses from subjects with cat allergy and healthy control subjects (no measureable serum IgG or IgE to Fel d 1) were compared with those from "modified T_H2" subjects with a negative skin test response to cat extract and Fel d 1 but with high-titer IgG and no IgE to Fel d 1. Interestingly, peptides derived from the N-terminus of Fel d 1 chain 2 preferentially elicited either IFN-γ or IL-10 responses. Furthermore, a high frequency (50%) of HLA-DR7 was observed in the "modified T_H2" group, and IL-10 responses were higher in these $DR7^+$ subjects. *In silico* analysis predicted multiple (7) HLA-DR binding motifs within the first 24 residues of chain 2. In our own binding assays, we were only able to confirm high-affinity binding for one of these, HLA-DR7, which bound the sequences chain $2^{1\text{-}16}$ and chain $2^{7\text{-}23}$. As a result of the limited HLA-DR–binding characteristics of the N-terminal region of chain 2, we did not evaluate proliferative and cytokine responses and are thus unable to confirm the selective IL-10– and IFN-γ–inducing activities of these sequences. More recently, Bateman et al[40] identified overlapping T-cell epitopes restricted by HLA-DQB1*06 (Fel d 1 chain 2 residues 5-18) and HLA-DPB1*0401 (Fel d 1 chain 2 residues 6-18) in subjects with AD exacerbated by exposure to cats. In general, subjects with AD had higher IL-4 responses and lower IFN-γ responses (determined by using ELIspot) than healthy control subjects. Interestingly, higher IL-10 responses were observed in control subjects, supporting the findings of Reefer et al[37] in relation to this region.

As part of the preclinical evaluation of each peptide, whole-blood basophil histamine release assays were performed in subjects with cat allergy. Our data, in agreement with previous studies, suggest that short synthetic peptide sequences retain little IgE binding and only rarely activate basophils in comparison with frequent responses to the whole allergen.

The primary outcome of this study was safety and tolerability. Our data indicate that ToleroMune Cat was safe and well tolerated when administered by means of intradermal injection at doses up to 12 nmol and when administered by means of subcutaneous injection at doses up to 20 nmol. Three nanomoles of ToleroMune Cat contain approximately 35 μg of Fel d 1 peptides. Effective maintenance doses for cat dander immunotherapy are around 15 μg of Fel d 1[5] and are normally reached after prolonged dose escalation. The finding that doses of ToleroMune Cat as high as 12 nmol when administered intradermally and 20 nmol when administered subcutaneously were safe and well tolerated creates the possibility to administer doses without lengthy dose escalation.

The greatest reduction in the LPSR was observed after intradermal administration of 3 nmol of ToleroMune Cat. Interestingly, intradermal administration of 3 nmol of ToleroMune Cat appeared to be more effective than either 1 or 12 nmol at reducing the size of the LPSR, suggesting that dose-escalation regimens using peptide immunotherapy might be counterproductive, as we had previously speculated.[41] The change in area of the LPSR of 40% after a single injection of 3 nmol of ToleroMune Cat is comparable with that achieved after 12 weeks of dosing with grass pollen extract[42] or 1 year of treatment with birch immunotherapy[43] and greater than the effect reported after 12 to 18 months of treatment with sublingual immunotherapy.[44] The observed change also correlates closely with data from studies using a prototype Fel d 1 peptide vaccine.[19,20] The current vaccine represents an improvement on the prototype because it is pharmacologically defined and compliant with regulatory requirements. This suggests that a short course of peptide immunotherapy with a fixed dose of peptides might be able to achieve therapeutic effects

comparable with those of longer courses with conventional allergen. Further studies involving repeat administration of intradermal ToleroMune Cat evaluating the effect on rhinoconjunctivitis symptom scores are required.

In conclusion, we have identified numerous MHC class II–binding motifs in the major cat allergen Fel d 1. Using cellular assays, we have defined "immunodominant" sequences with low histamine-releasing potential from which we derived a therapeutic peptide vaccine for the treatment of allergy to cats. The results of our initial safety and tolerability study indicate that the vaccine is safe and well tolerated. Furthermore, we have defined the dose of vaccine displaying the greatest efficacy in a surrogate clinical outcome marker, which can be used in future clinical studies.

We thank Nisha Faruk for technical assistance, Maxine Aizen for subject recruitment in the United Kingdom, and Andrew Bysice for assistance with figures. We also thank Dr Enriquez Fernandes-Caldas for the supply of cat dander allergen extract and Drs Alessandro Sette and Bjoern Peters of the La Jolla Institute for Allergy and Immunology for their assistance in identifying predicted core HLA-DR binding regions and predicted HLA-DP and DQ sequences.

Clinical implications: Peptide epitopes of the major cat allergen have been identified and formulated into a peptide vaccine. A single dose was safe and well tolerated when administered to subjects with cat allergy.

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METHODS

Clinical characteristics of subjects in the clinical study

Subjects were required to have a positive skin prick test response to cat allergen with a wheal diameter at least 3 mm larger than that produced by the negative control and to have an LPSR to 0.010 HEP units of cat allergen 8 hours after intradermal injection of greater than 25 mm in diameter. Female subjects of childbearing potential were required to practice an acceptable form of contraception. Subjects were excluded from the study if they had asthma falling under Global Initiative for Asthma (2006) classification 2 (partly controlled) and 3 (uncontrolled) and if they had an FEV_1 of less than 80% of normal value, a history of anaphylaxis to cat allergen, an acute-phase skin response to cat allergen with a wheal diameter of greater than 30 mm, or a cat-specific IgE level of greater than 100 kU/L. Subjects with hay fever who could not complete the clinical study outside the pollen season and subjects who had received allergen immunotherapy during the last 5 years or cat dander immunotherapy ever were also excluded. Furthermore, subjects being treated with anti-IgE antibody, corticosteroids, cromones, antihistamines other than loratadine, leukotriene inhibitors, anticholinergics, α-adrenergic agonists, and tricyclic antidepressants were excluded from the study. Additional exclusion criteria included subjects for whom administration of adrenaline was contraindicated (eg, subjects with acute or chronic symptomatic coronary heart disease or severe hypertension), subjects being treated with β-blockers, and subjects who had completed or were undergoing ongoing treatment with tranquillizers or psychoactive drugs. In addition, subjects with symptoms of a clinically relevant illness, including urticaria factitia, within 6 weeks before the screening visit and subjects with clinically relevant abnormalities detected on physical examination or 12-lead electrocardiography or vital signs outside normal limits and laboratory values that were outside the normal ranges were excluded. Female subjects who were pregnant, lactating, or planning a pregnancy during the study were also excluded. Finally, subjects were also excluded if they had a significant history of alcohol or drug abuse; had a history of hepatitis B, hepatitis C, or HIV; had previously been randomized into this study or had received a prototype of Cat-PAD previously; had a history of severe drug allergy or anaphylactic reaction to food; had planned travel outside the study area for a substantial portion of the study period; had received treatment with an investigational drug within 6 months before study screening; had participated in a study with a new formulation of a marketed drug 1 month before study screening, were deemed by the investigator to have questionable reliability in their ability to comply with the protocol and provide accurate information, were unable to communicate or to understand the requirements of the study, or had any psychiatric disorder that would impair communication between the subject and investigator; had any significant disease or disorder that in the opinion of the investigator might either put the subject at risk because of participation in the study or influence the results of the study or the subject's ability to participate in the study; had a known allergy to ToleroMune Cat or thioglycerol; or had a dependent relationship with either the sponsor or investigator.

Clinical study design

The overall study design is shown in Fig E1. The Schedule of assessments for the study is shown in Table E2. Subjects attended for an initial screening visit and then a separate baseline challenge 6 to 8 days before study medication administration (treatment phase visit 1, day 1). At baseline challenge, subjects were injected with 0.010 HEP units of cat allergen (Laboratorios Leti), and the EPSR at 15 minutes and the LPSR at 8 hours were documented. At treatment phase visit 1, day 1, subjects received either intradermal or subcutaneous injections of Cat-PAD. The intradermal cohort received a single intradermal injection of either Cat-PAD (0.03, 0.3, 3, and 12 nmol) or placebo, whereas the subcutaneous cohort received a single subcutaneous injection of either Cat-PAD (0.03, 0.3, 3, 12, and 20 nmol) or placebo. Groups of 8 subjects received treatment at each dose level (6 received Cat-PAD and 2 received placebo). The first group received 0.03 nmol of Cat-PAD, and each subsequent group received the next dose level, providing the previous dose was well tolerated on a safety review blind to treatment. The protocol permitted selection of one further dose below a dose that had been well tolerated to better define the dose-response relationship. A dose of 1 nmol was selected for both the intradermal and subcutaneous cohorts. At treatment phase visit 2 on day 21 (±3 days) after treatment, EPSRs and LPSRs to 0.010 HEP units of cat allergen were measured, and safety evaluations were repeated.

Proliferation assays

PBMCs were freshly isolated from peripheral blood by means of density gradient centrifugation on Histopaque (Sigma, St Louis, Mo). The cells were washed in heparinized (1 U/mL) HEPES-buffered medium (Sigma) and resuspended in RPMI 1640 medium supplemented with 10% pooled human AB serum (Sigma), 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin (Complete Medium; components from Sigma). Proliferation cultures were established in 96-well flat-bottomed plates at 2×10^5 cells per well in a total volume of 200 μL of medium. Twelve replicates at each of 3 concentrations (1 μg, 33 μg, and 100 μg/mL) were established. Twelve replicates of medium and cells alone acted as the negative control, 12 wells of cat dander allergen extract (100 μg/ml; Laboratorios Leti S.A., Madrid, Spain) acted as the positive whole allergen control, and PPD (10 μg/mL) acted as the positive recall antigen (the population assayed were vaccinated with BCG) control. Cells were incubated at 37°C in a humidified incubator gassed with 5% CO_2 in air for 7 days. Cultures were pulsed for the last 8 to 16 hours of culture with 37 kBq of tritiated thymidine and harvested onto glass-fiber filters. Proliferation, as correlated with tritiated thymidine incorporation, was quantified by means of liquid scintillation spectroscopy (Top Count; PerkinElmer, Waltham, Mass).

Cytokine assays

PBMCs were isolated by means of density gradient centrifugation, and their concentration was adjusted to 5×10^6 cells/mL in complete medium at room temperature. Two hundred fifty microliters of cell solution was distributed into appropriate wells of the 48-well plate containing 250 μL of the appropriate control, antigen, or peptide concentration. Cultures were incubated for 5 days, whereupon 300 μL of supernatant was removed from the top of the well and stored at −20°C before measurement of cytokines by means of ELISA (IL-10, IL-13, and IFN-γ; Pelikine, Sanquin, Amsterdam, The Netherlands), according to the manufacturer's instructions.

Histamine release assays

Histamine release assays were performed on whole blood according to the manufacturer's instructions (Histamine Assay 2015; Beckman Coulter, Fullerton, Calif). Diluted blood was incubated for 45 minutes with each peptide/control allergen preparation in a 5-point log dilution series (10 μg/mL to 1 ng/mL for peptides and 100 μg/mL to 10 ng/mL for cat allergen extract). The dose range of whole cat dander allergen extract was 10-fold higher than for peptide on the basis that each peptide represented approximately 10% of the length of the primary sequence of Fel d 1. After centrifugation, supernatants were acylated, and acylated histamine concentrations measured by means of ELISA. Results were expressed as the percentage of total release, the latter obtained from supernatants of blood samples processed through 2 freeze-thaw cycles.

Safety evaluation: Extent of exposure

In total, 66 subjects received either intradermal or subcutaneous doses of Cat-PAD, and 22 subjects received placebo. Table E3 summarizes the extent of exposure. Thirty subjects received intradermal administrations of Cat-PAD: 5 groups of 6 subjects who received 0.03 nmol, 0.3 nmol, 1 nmol, 3 nmol, and 12 nmol administered as a single dose. Thirty-six subjects received subcutaneous administrations of Cat-PAD: 6 groups of 6 subjects who received 0.03 nmol, 0.3 nmol, 1 nmol, 3 nmol, 12 nmol, or 20 nmol administered as a single dose.

Adverse events

Brief summary of adverse events. Table E4 summarizes the incidence of SAEs and TEAEs in both the intradermal and subcutaneous injection cohorts. There were no deaths or other SAEs in any cohort, and no subjects withdrew as a result of adverse events.

In the intradermal cohort the greatest number of TEAEs occurred in those subjects who received the 12-nmol Cat-PAD dose, but these included a number of TEAEs (nasopharyngitis, tonsillitis, dysmenorrhea, cough, and hypertension) that were not considered to be related to the treatment. In the

subcutaneous cohort the greatest number of TEAEs occurred in those subjects who received placebo.

Analysis of adverse events. Tables E5 and E6 present TEAEs by body system for the intradermal and subcutaneous cohorts, respectively. In the intradermal cohort the most commonly reported TEAEs in subjects who received Cat-PAD were nasopharyngitis, cough, and headache. Nasopharyngitis occurred in 8 subjects (26.7% of the 30 subjects who received Cat-PAD), whereas headache and cough occurred in 2 (6.7%) subjects each. No cases of asthma were reported after intradermal Cat-PAD treatment. One case of asthma did, however, occur in the pooled placebo group. One case of allergic respiratory symptoms occurred in a subject receiving 1 nmol of Cat-PAD. Vertigo, nausea, chest discomfort, headache, balance disorder, tremor, allergic respiratory symptoms, throat irritation, and skin irritation were thought to be possibly related to treatment. No TEAE was thought to be probably or definitely related to treatment. No TEAEs were regarded as severe, 10 subjects had moderate TEAEs, and 9 subjects mild TEAEs. The TEAEs of moderate severity included fatigue, nausea, nasopharyngitis and headache (0.03 nmol), bronchitis (0.3 nmol), allergic respiratory symptoms and balance disorder (1 nmol), headache and nasopharyngitis (3 nmol), vertigo and dysmenorrhea (12 nmol), and cardiovascular disorder and dyspnea (placebo). No dose-related increases in the number of TEAEs were observed up to and including the 3-nmol dose. At the 12-nmol dose, an increase in the number of TEAEs and subjects with TEAEs was observed, but as noted previously, these included a number of TEAEs (nasopharyngitis, tonsillitis, dysmenorrhea, cough, and hypertension) that were not considered to be related to the treatment.

In the subcutaneous cohort the most commonly reported TEAEs in subjects who received Cat-PAD were in the respiratory, thoracic, and mediastinal body system, with 9 subjects (25% of the 36 subjects who received Cat-PAD) having an adverse event in this body system. Asthma occurred in 1 subject after 0.3 nmol of Cat-PAD (moderate) and 1 subject after 3 nmol of Cat-PAD (mild). No TEAE was thought to be probably or definitely related to treatment. The majority of adverse events were mild, 4 were moderate, and none were severe. The adverse events of moderate severity were asthma (0.3 nmol), headache (1 nmol), AD (20 nmol), and creatine phosphokinase increase (placebo). No dose-related increases in the number of TEAEs were observed.

In each cohort approximately half the TEAEs were mild, with the remainder were of moderate severity. No severe TEAEs were reported. For the intradermal cohort, the majority of TEAEs (approximately two thirds) were assessed as not related to treatment. For the subcutaneous cohort, the majority of TEAEs (approximately three quarters) were assessed as related in some way to treatment. Tables E7 and E8 present treatment-related TEAEs by body system for the intradermal and subcutaneous cohorts, respectively.

Deaths, other SAEs, and other significant adverse events. There were no deaths or other SAEs during the study. There were no other significant adverse events.

Local reactions at the injection site

All subjects treated with Cat-PAD or placebo experienced local reactions at the injection site 15 minutes after intradermal injection. These comprised erythema in all subjects and wheals in the majority of subjects in the 1-, 3-, and 12-nmol groups (including those receiving placebo) that were transient and self-limiting. No local reactions were recorded at other time points. In contrast, the majority of subjects had no local reactions after subcutaneous injection of Cat-PAD or placebo. At 15 minutes, 2 subjects who received 3 nmol of Cat-PAD had a local reaction consisting of erythema only that was transient and self-limiting. A single subject in the 0.03-nmol dose group had a local reaction at 8 hours, but this was not erythema or a wheal. Table E9 summarizes the frequency of erythema and wheals in the intradermal and subcutaneous cohorts at the 15-minute assessment point.

Safety conclusions

- There were no deaths, SAEs, or other significant adverse events in the study.
- No subjects withdrew from the study because of adverse events.
- The proportion of subjects experiencing TEAEs was comparable in the intradermal and subcutaneous groups, and the overall incidence of TEAEs was low.
- FEV_1 expressed as a mean percentage of predose values was not reduced by Cat-PAD during the 8 hour after the dose period in either the intradermal or subcutaneous cohorts. No subjects experienced a reduction in FEV_1 to less than 80% and less than 70% of predose values in the intradermal cohort. One subject in each of the 0.3-, 1-, and 3-nmol dose groups experienced a reduction in FEV_1 to less than 80% of predose values in the subcutaneous cohort, but no subjects experienced a reduction to less than 70% of predose values.
- Visual analog score scores of breathlessness and nasal symptoms were low and remained low throughout the 8-hour postdose period at all doses for both the intradermal and subcutaneous cohorts.
- All subjects treated with Cat-PAD or placebo experienced transient and self-limiting local reactions at the injection site 15 minutes after intradermal injection. The majority of subjects who received subcutaneous Cat-PAD or placebo experienced no local reactions.
- There were no clinically significant changes in any laboratory parameters after treatment.
- No adverse findings on physical examination were observed at follow-up.
- There were no clinically significant changes in vital signs or electrocardiographic results at follow-up.
- Cat-PAD was safe and well tolerated when administered by means of intradermal injection at doses of up to 12 nmol and when administered by means of subcutaneous injection at doses of up to 20 nmol.

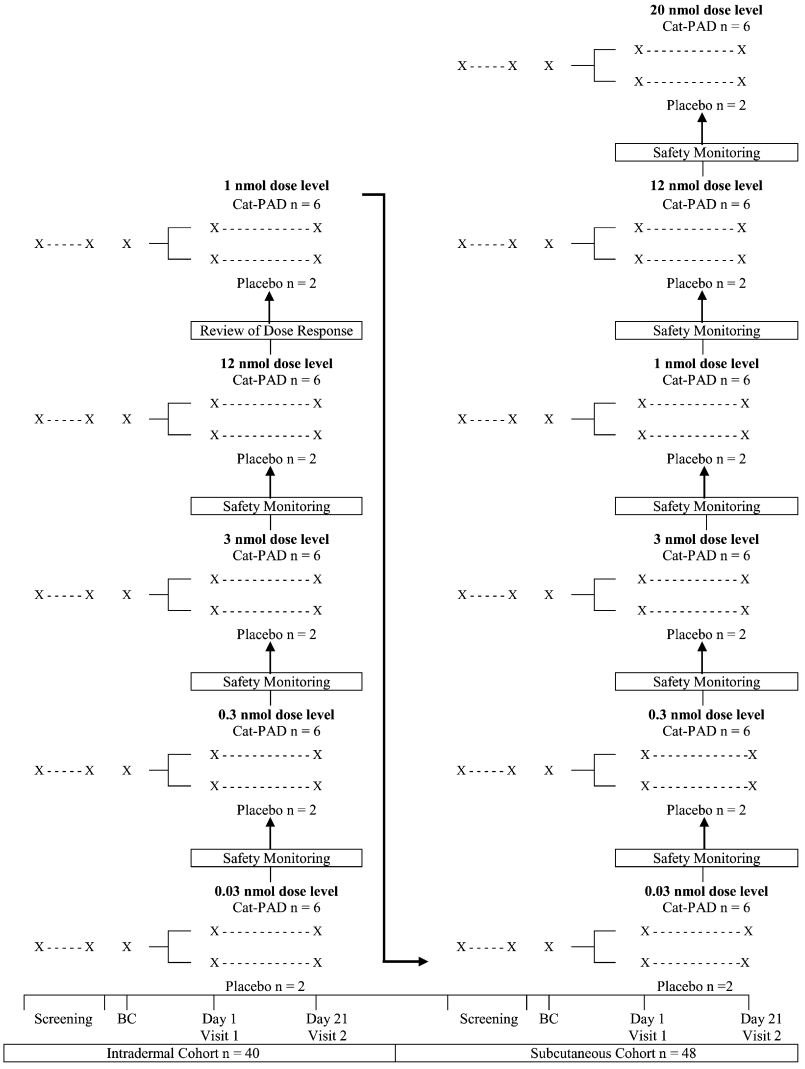

FIG E1. Overall study design. The intradermal cohort was studied first, followed by the subcutaneous cohort. Groups of 8 subjects participated at each dose level (6 active and 2 placebo), starting with the lowest dose level. The dose was only increased after a blind review of safety data to confirm that it was safe to do so. The protocol permitted an additional dose less than a dose that had been well tolerated to further delineate the dose-response curve. A dose of 1 nmol was administered both intradermally and subcutaneously. *BC*, Baseline challenge.

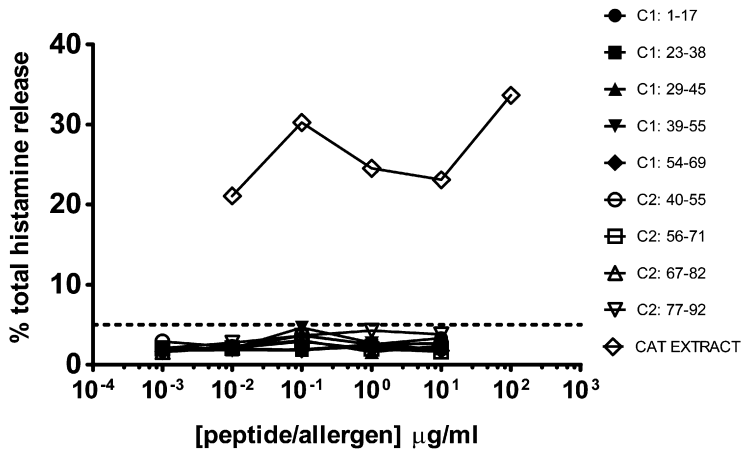

FIG E2. Cat allergen, but not peptides, induces histamine release *in vitro*. The ability of peptides (10^{-3} to 10 μg/mL) and whole cat dander allergen extract (10^{-2} to 100 μg/mL) to activate peripheral blood basophils was evaluated. Data represent the mean percentage of total histamine release (n = 46 subjects with cat allergy).

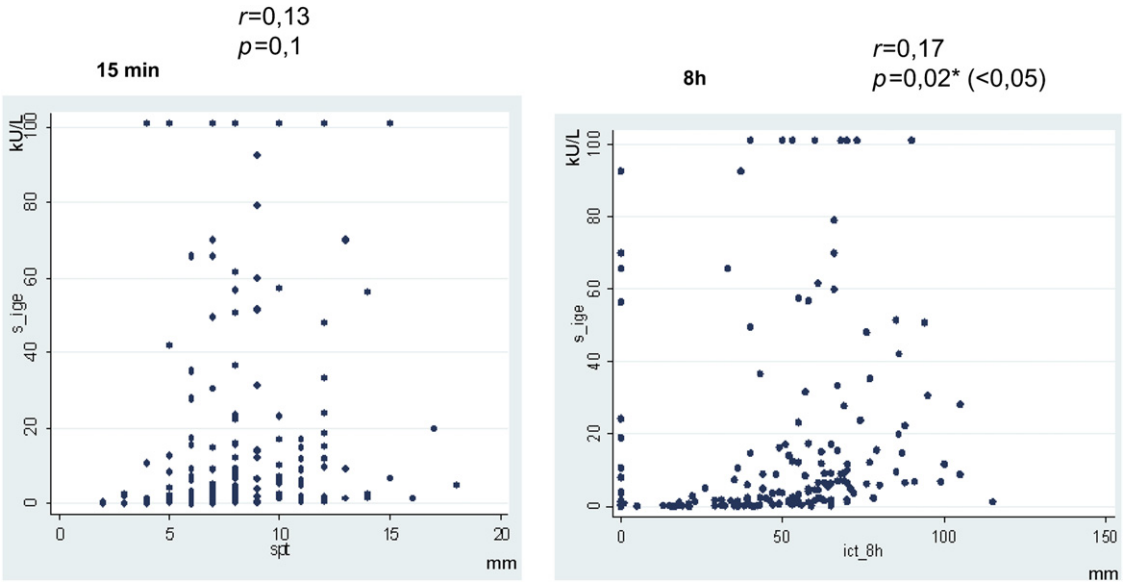

FIG E3. Relationship between cat-specific IgE levels and sizes of EPSRs and LPSRs. EPSRs were measured at 15 minutes and LPSRs at 8 hours after an intradermal dose of 0.010 HEP units of cat allergen administered on the volar aspect of each forearm.

TABLE E1. Fel d 1 peptide sequences

Fel d 1 Chain 1																
1-17	E	I	C	P	A	V	K	R	D	V	D	L	F	L	T	G	T
12-28	L	F	L	T	G	T	P	D	E	Y	V	E	Q	V	A	Q	Y
23-38	E	Q	V	A	Q	Y	K	A	L	P	V	V	L	E	N	A	
29-45	K	A	L	P	V	V	L	E	N	A	R	I	L	K	N	C	V
39-55	R	I	L	K	N	C	V	D	A	K	M	T	E	E	D	K	E
48-63	K	M	T	E	E	D	K	E	N	A	L	S	L	L	D	K	
54-69	K	E	N	A	L	S	L	L	D	K	I	Y	T	S	P	L	
Fel d 1 Chain 2																
1-16	V	K	M	A	E	T	C	P	I	F	Y	D	V	F	F	A	
7-23	C	P	I	F	Y	D	V	F	F	A	V	A	N	G	N	E	L
20-35	G	N	E	L	L	L	K	L	S	L	T	K	V	N	A	T	
29-44	L	T	K	V	N	A	T	E	P	E	R	T	A	M	K	K	
40-55	T	A	M	K	K	I	Q	D	C	Y	V	E	N	G	L	I	
48-63	C	Y	V	E	N	G	L	I	S	R	V	L	D	G	L	V	
56-71	S	R	V	L	D	G	L	V	M	T	T	I	S	S	S	K	
67-82	I	S	F	S	K	D	C	M	G	E	A	V	Q	N	T	V	
77-92	A	V	Q	N	T	V	E	D	L	K	L	N	T	L	G	R	

TABLE E2. Schedule of Assessments

Study Phase	**Screening**	**Baseline Challenge**	**Treatment**		
Visit	**Screening**	**Baseline**	**Visit 1**	**Telephone Check**	**Visit 2**
Time	**Day -28 to Day -14**	**Day -7±1**	**Day 1**	**Day 7 (±2 days)**	**Day 21 (±3 days)**
Informed consent	X				
Demography and medical history	X				
Physical examination	X				X
Vital signs	X	X[a]	X[b]		X[a]
Automated blood pressure recording	X				
Skin prick testing to cat dander	X				
Blood sample for total IgE	X				
Blood sample for cat specific IgE	X				
12-lead ECG	X		X		X
Spirometry (FEV1)	X	X[a]	X[c]		X[a]
VAS breathlessness	X	X[a]	X[d]		X[a]
VAS nasal symptoms	X	X[a]	X[d]		X[a]
Blood sample for haematology/biochemistry	X		X		X
Urine dipstick	X		X		X
Blood sample for tryptase	X		X[e]		
Pregnancy test	X		X		X
Concomitant medication	X	X	X		X
Cutaneous response to cat allergen[f]	X	X			X
Dosing			X		
Examination of the injection site[f]			X[d]		X
Recording of AEs	X	X	X[d]	X	X

[a]Before and 8 hours after intradermal allergen testing.
[b]Pre dose and hourly after dosing.
[c]Measured immediately prior to dosing and then at 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours after dosing.
[d]Measured immediately prior to dosing, at 15, 30 and 45 minutes and then at 1, 2, 3, 4, 5, 6, 7 and 8 hours after dosing.
[e]Repeated after 3 hours only in the event of suspected anaphylactic response.
[f]Including digital photography.

TABLE E3. Extent of Exposure

	Cat-PAD 0.03 nmol	Cat-PAD 0.3 nmol	Cat-PAD 1 nmol	Cat-PAD 3 nmol	Cat-PAD 12 nmol	Cat-PAD 20 nmol	Cat-PAD Total	Pooled Placebo
Intradermal	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**N/A**	**n = 30**	**n = 10**
Number of Subjects Dosed	6	6	6	6	6	N/A	30	10
Number of Doses	6	6	6	6	6	N/A	30	10
Subcutaneous	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n= 36**	**n = 12**
Number of Subjects Dosed	6	6	6	6	6	6	36	12
Number of Doses	6	6	6	6	6	6	36	12

N/A = Not applicable, top intradermal dose was 12 nmol.

TABLE E4. Summary of Treatment-Emergent Adverse Events and Serious Adverse Events

	Cat-PAD 0.03 nmol n (%)	Cat-PAD 0.3 nmol n (%)	Cat-PAD 1 nmol n (%)	Cat-PAD 3 nmol n (%)	Cat-PAD 12 nmol n (%)	Cat-PAD 20 nmol n (%)	Pooled Placebo n (%)
Intradermal	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**N/A**	**n = 10**
Number of TEAEs	6	2	6	2	11	N/A	6
Subjects with TEAEs	2 (33.3)	2 (33.3)	3 (50.0)	2 (33.3)	6 (100.0)	N/A	4 (40.0)
Subjects with SAEs	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	N/A	0 (0.0)
Subcutaneous	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 12**
Number of TEAEs	7	6	2	6	3	2	16
Subjects with TEAEs	3 (50.0)	2 (33.3)	2 (33.3)	3 (50.0)	3 (50.0)	2 (33.3)	9 (75.0)
Subjects with SAEs	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

N/A = Not applicable, top intradermal dose was 12 nmol.

TABLE E5. Summary of Proportion of Subjects in the Intradermal Cohort with Treatment-Emergent Adverse Events by Body System

Adverse Events	Cat-PAD 0.03 nmol (n = 6) n (%)	Cat-PAD 0.3 nmol (n = 6) n (%)	Cat-PAD 1 nmol (n = 6) n (%)	Cat-PAD 3 nmol (n = 6) n (%)	Cat-PAD 12 nmol (n = 6) n (%)	Pooled Placebo (n = 10) n (%)
CARDIAC DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
Cardiovascular disorder	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
EAR AND LABYRINTH DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Vertigo	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
GASTROINTESTINAL DISORDERS	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Nausea	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	1 (16.7)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Chest discomfort	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Fatigue	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
INFECTIONS AND INFESTATIONS	2 (33.3)	1 (16.7)	2 (33.3)	1 (16.7)	4 (66.7)	1 (10.0)
Bronchitis	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cystitis	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Nasopharyngitis	2 (33.3)	0 (0.0)	2 (33.3)	1 (16.7)	3 (50.0)	1 (10.0)
Tonsillitis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
NERVOUS SYSTEM DISORDERS	1 (16.7)	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)	2 (20.0)
Balance disorder	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Headache	1 (16.7)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	1 (10.0)
Migraine	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
Tremor	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
REPRODUCTIVE SYSTEM AND BREAST DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Dysmenorrhoea	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	2 (33.3)	2 (20.0)
Allergic respiratory symptom	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Asthma	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
Cough	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)
Dyspnoea	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
Throat irritation	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
SKIN AND SUBCUTANEOUS TISSUE DISORDERS	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Skin irritation	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
VASCULAR DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Hypertension	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)

Subjects who had more than one event within a body system were counted once.
Subjects who had more than one event assigned to the same preferred term were counted once.

TABLE E6. Summary of Proportion of Subjects in the Subcutaneous Cohort with Treatment-Emergent Adverse Events by Body System

Adverse Events	Cat-PAD 0.03 nmol (n = 6) n (%)	Cat-PAD 0.3 nmol (n = 6) n (%)	Cat-PAD 1 nmol (n = 6) n (%)	Cat-PAD 3 nmol (n = 6) n (%)	Cat-PAD 12 nmol (n = 6) n (%)	Cat-PAD 20 nmol (n = 6) n (%)	Pooled Placebo (n = 12) n (%)
EYE DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Eye pruritis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Lacrimation increased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
GASTROINTESTINAL DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Gastritis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Chest discomfort	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
IMMUNE SYSTEM DISORDERS	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (16.7)
Hypersensitivity	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Seasonal allergy	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
INFECTIONS AND INFESTATIONS	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)	0 (0.0)
Nasopharyngitis	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Oral herpes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)
INVESTIGATIONS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (33.3)
Alanine aminotransferase increased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Blood creatinine phosphokinase increased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (25.0)
Platelet count decreased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Musculoskeletal pain	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
NERVOUS SYSTEM DISORDERS	1 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Dizziness	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Headache	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	1 (16.7)	2 (33.3)	1 (16.7)	3 (50.0)	2 (33.3)	0 (0.0)	2 (16.7)
Allergic respiratory symptom	0 (0.0)	1 (16.7)	1 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)
Asthma	0 (0.0)	1 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Dyspnoea	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Nasal congestion	1 (16.7)	1 (16.7)	0 (0.0)	2 (33.3)	1 (16.7)	0 (0.0)	1 (8.3)
Pharyngolaryngeal pain	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Throat irritation	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Wheezing	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
SKIN AND SUBCUTANEOUS TISSUE DISORDERS	1 (16.7)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (8.3)
Dermatitis atopic	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Seborrhoeic dermatitis	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Urticaria	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)

Subjects who had more than one event within a body system were counted once.
Subjects who had more than one event assigned to the same preferred term were counted once.

TABLE E7. Summary of Proportion of Subjects in the Intradermal Cohort with Treatment-Related Treatment-Emergent Adverse Events by Body System

Adverse Events	Cat-PAD 0.03 nmol (n = 6) n (%)	Cat-PAD 0.3 nmol (n = 6) n (%)	Cat-PAD 1 nmol (n = 6) n (%)	Cat-PAD 3 nmol (n = 6) n (%)	Cat-PAD 12 nmol (n = 6) n (%)	Pooled Placebo (n = 10) n (%)
EAR AND LABYRINTH DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Vertigo	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
GASTROINTESTINAL DISORDERS	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Nausea	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Chest discomfort	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
INFECTIONS AND INFESTATIONS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
Nasopharyngitis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
NERVOUS SYSTEM DISORDERS	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Balance disorder	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Headache	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Tremor	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	1 (10.0)
Allergic respiratory symptom	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Asthma	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)
Throat irritation	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
SKIN AND SUBCUTANEOUS TISSUE DISORDERS	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Skin irritation	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)

Treatment-related refers to TEAEs where the causality was assessed as unlikely, possible, probable or highly probable.
Subjects who had more than one event within a body system were counted once.
Subjects who had more than one event assigned to the same preferred term were counted once.

TABLE E8. Summary of Proportion of Subjects in the Subcutaneous Cohort with Treatment-Related Treatment-Emergent Adverse Events by Body System

Adverse Events	Cat-PAD 0.03 nmol (n = 6) n (%)	Cat-PAD 0.3 nmol (n = 6) n (%)	Cat-PAD 1 nmol (n = 6) n (%)	Cat-PAD 3 nmol (n = 6) n (%)	Cat-PAD 12 nmol (n = 6) n (%)	Cat-PAD 20 nmol (n = 6) n (%)	Pooled Placebo (n = 12) n (%)
EYE DISORDERS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Eye pruritis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Lacrimation increased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Chest discomfort	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
IMMUNE SYSTEM DISORDERS	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Hypersensitivity	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
INFECTIONS AND INFESTATIONS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Nasopharyngitis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
INVESTIGATIONS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (33.3)
Alanine aminotransferase increased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Blood creatinine phosphokinase increased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (16.7)
Platelet count decreased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
NERVOUS SYSTEM DISORDERS	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Headache	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	1 (16.7)	2 (33.3)	0 (0.0)	3 (50.0)	2 (33.3)	0 (0.0)	2 (16.7)
Asthma	0 (0.0)	1 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Dyspnoea	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Nasal congestion	1 (16.7)	1 (16.7)	0 (0.0)	2 (33.3)	1 (16.7)	0 (0.0)	1 (8.3)
Pharyngolaryngeal pain	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Throat irritation	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Wheezing	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
SKIN AND SUBCUTANEOUS TISSUE DISORDERS	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (8.3)
Dermatitis atopic	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
Seborrhoeic dermatitis	1 (16.7)[a]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Urticaria	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)

Treatment-related refers to TEAEs where the causality was assessed as unlikely, possible, probable or highly probable.
Subjects who had more than one event within a body system were counted once.
Subjects who had more than one event assigned to the same preferred term were counted once.
[a]Causality was missing, so seborrhoeic dermatitis assumed to be treatment-related.

TABLE E9. Frequency of Erythema and Wheals in the Intradermal and Subcutaneous Cohorts 15 minutes After Injection

	Cat-PAD 0.03 nmol n (%)	Cat-PAD 0.3 nmol n (%)	Cat-PAD 1 nmol n (%)	Cat-PAD 3 nmol n (%)	Cat-PAD 12 nmol n (%)	Cat-PAD 20 nmol n (%)	Pooled Placebo n (%)
Intradermal	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**N/A**	**n = 10**
Erythema	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	N/A	10 (100.0)
Wheal (swelling)	1 (16.7)	0	6 (100.0)	5 (83.3)	6 (100.0)	N/A	5 (50.0)
Subcutaneous	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 6**	**n = 12**
Erythema	0	0	0	2 (33.3)	0	0	0
Wheal (swelling)	0	0	0	0	0	0	0

N/A = Not applicable, top intradermal dose was 12 nmol.