

Peptides from allergenic lipocalins bind to formyl peptide receptor 3 in human dendritic cells to mediate T_H2 immunity



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Background: How T_H2-mediated allergic immune responses are induced is still under investigation.

Objective: In an *in vitro* system we compared the effect of lipocalin allergens and nonallergenic homologues on human monocyte-derived dendritic cells (DCs) to investigate how they polarize naive CD4⁺ T_H cells. Microarray data gained with these DCs showed a significant difference in expression of formyl peptide receptors (FPRs). Activation of FPR3 in human monocyte-derived DCs leads to inhibition of IL-12 production. Low concentrations of IL-12 during T-cell priming biases immune responses toward T_H2. We hypothesize that binding of allergenic lipocalins to FPR3 might be a mechanism for induction of allergic immune responses.

Methods: We examined whether lipocalins and FPR3 colocalize within the cells by using confocal microscopy. With calcium mobilization assays of FPR3-transfected HEK 293 cells, we measured FPR3 signaling in response to allergenic and nonallergenic lipocalins. Silencing of FPR3 in DCs and pretreatment with an antagonistic peptide were used to assess the function of FPR3 in T_H2 induction.

Results: FPR3 and lipocalins colocalize in the same vesicles in DCs. Cathepsin S–digested allergenic lipocalins, but not digestion products of nonallergenic homologues, activate FPR3 signaling. FPR3 silencing in DCs or pretreatment with an antagonistic peptide restores IL-12 and induces IL-10 expression by DCs treated with lipocalin allergens, attenuating the T_H2 bias and inducing IL-10 production in cocultured T_H cells.

Conclusion: We describe a novel molecular mechanism for induction of T_H2-mediated allergic immune responses. (J Allergy Clin Immunol 2020;145:654–65.)

Key words: Lipocalin allergens, formyl peptide receptor 3, human monocyte-derived dendritic cells, T_H2 polarization, antagonistic peptide

Abbreviations used

DC:	Dendritic cell
EEA:	Early endosomal antigen
FACS:	Fluorescence-activated cell sorting
FPR:	Formyl peptide receptor
iDC:	Immature monocyte-derived dendritic cell
mDC:	Dendritic cell treated with maturation cocktail
MHCII:	MHC class II
MUP:	Major urinary protein
siRNA:	Small interfering RNA

Dendritic cells (DCs) build the interface of innate and adaptive immune responses and are major players in determining the type and magnitude of adaptive immune responses. Correct T_H cell polarization is necessary for establishment of an immune response best suited to combat the invading antigen. DCs provide T cell–polarizing cytokines on contact with microbes: IL-12 for T_H1 cell differentiation^{1,2} characterized by production of IFN- γ ³ and fitted to defeat intracellular pathogens; TGF- β for differentiation of T_H17 cells characterized by production of IL-17⁴ and equipped to fight extracellular pathogens and fungi; and IL-10, TGF- β , or both to induce peripheral regulatory T cells characterized by production of IL-10 and TGF- β and limiting excessive immune responses.⁵ Although there is ample evidence that DCs are also required to direct immune responses toward allergic T_H2 differentiation after interaction with allergens,^{6,7} the DC-derived stimulus for T_H2 induction is still under investigation.^{3,8} Discussed are, among others, a specialized DC subset,^{7,9} weak affinity of CD4⁺ T cells to the presented antigen,¹⁰ intracellular location of the antigen,¹¹ extracellular-signal regulated kinase activation in DCs leading to inhibition of IL-12 production,¹² and uptake through mannose receptor.^{13,14} To date, the absence or low concentrations of IL-12 during T-cell priming by DCs is still the best characterized prerequisite for T_H2 differentiation. Unraveling the regulatory chain for priming of T_H2 cells remains challenging.

The protein family of lipocalins contains the most important mammal-derived respiratory allergens and nonallergenic proteins. Lipocalins have a common tertiary structure but low amino acid homology, usually 30% to 40%. In an earlier study¹⁵ we used members of this protein family to equip DCs with the ability to induce either allergic or nonallergic immune responses according to the allergenicity of the lipocalins in an *in vitro* model system. Specifically, we used the major dog allergen Can f 1 (NP_001003190.1) and its human homologue, the tear-derived nonallergenic Lcn-1 (NC_000009.12, NP_001239546.1), as well as the cat allergen Fel d 4 (NM_001009233.1) and its nonallergenic homologue major urinary protein (MUP; NG_016729, hCG1795014), which share almost 60% of their

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amino acids (see Fig E1 in this article's Online Repository at www.jacionline.org), an unusually high homology within this family. When allergenic lipocalins were added to DCs, they expressed less maturation markers and IL-12. Cocultured naive CD4⁺CD45RA⁺ allogeneic T cells developed a T_H2 cytokine signature.¹⁵

To elucidate the molecular events of this interaction, we performed a transcriptome comparison of DCs treated with allergenic and nonallergenic lipocalins. We found formyl peptide receptors (FPRs) to be among the most prominently differentially regulated genes. FPRs are G protein-coupled receptors expressed mainly in leukocytes with the capacity to interact with a wide variety of peptide ligands. FPRs possess multiple functions in controlling inflammation, myeloid cell trafficking in host response to infection, tissue injury, and wound healing.

Detailed knowledge of FPR signaling is scarce, as described in a recent review.¹⁶ FPR1 and FPR2 are expressed on the cell surfaces of leukocytes and a variety of other cell types and are specifically involved in controlling inflammation-induced pathophysiologic conditions through interaction with N-formyl peptides.^{17,18} In DCs FPR1 and FPR2 expression is low or absent (see Fig E2 in this article's Online Repository at www.jacionline.org),¹⁹ whereas FPR3 is expressed mainly in small intracellular vesicles. FPR3 interacts with nonformylated peptides, and its overall function is still unclear.²⁰ One of the specific functions attributed to FPR3 is interference with LPS-induced IL-12 production on ligand binding shown with the synthetic agonistic peptide WKYMVM, which binds to all 3 human FPRs.¹⁹ On the other hand, the antagonistic synthetic peptide WRW4 is able to inhibit WKYMVM-induced Ca²⁺ mobilization in monocytes and DCs and acts as an antagonist for FPR3-induced DC migration.²¹

Here, for the first time, we describe a molecular mechanism leading to an allergic immune response. We demonstrate that in monocyte-derived DCs interaction of lipocalins with FPR3, which only occurs with allergenic members of the family, leads to downregulation of IL-12 production in DCs. This promotes the development of T_H2 cells from naive T_H cells in allogeneic mixed leukocyte reactions, a hallmark of allergic immune responses. Silencing of FPR3 expression or addition of an FPR3-antagonistic peptide prevent the allergen-induced decrease in IL-12 production. Interfering with FPR3 expression also induces IL-10 expression in DCs and leads to a suppressor phenotype in cocultured T_H cells. Thus manipulating FPR3 activation might serve for drug development.

METHODS

Recombinant lipocalins

Preparation of recombinant Can f 1, Fel d 4, Fel d 7, Lcn-1, and MUP was as described elsewhere.¹⁵ In short, genes encoding mature proteins were cloned into pQE-70 (Qiagen, Hilden, Germany) or pet 21d (Addgene, Watertown, Mass) vectors. Recombinant proteins were purified by means of a combination of Ni-affinity chromatography and size-exclusion chromatography. Contaminating LPS was removed by using Detoxi-Endotoxin Removing Gel (Thermo Fisher Scientific, Rockford, Ill). Selected preparations were determined to be nearly endotoxin free (<0.01 EU/μg) by using the Limulus assay. Recombinant Equ c 1 and Rat n 1 were purchased (Rekom Biotech, La Zubia, Spain, and Indoor Biotechnology, Cardiff, United Kingdom). Recombinant lipocalins (20 μg/mL) were used to stimulate DCs from days 6 to 8 of culture throughout the experiments.

Fluorescent dye labeling of lipocalins

Protein-labeling kits with Alexa Fluor 488 and Alexa Fluor 594 were purchased from Molecular Probes (Invitrogen, Eugene, Ore) and used according to the manufacturer's protocol.

Cell preparations

Generation of monocyte-derived DCs. Human monocyte-derived DCs were prepared from peripheral blood monocytes, essentially as described.²²⁻²⁴ We obtained anonymous human blood components from the local blood bank (Central Institute for Blood Transfusion and Immunology, Innsbruck Medical University, Innsbruck, Austria) according to the guidelines of the local blood bank and the tenets of the Helsinki Protocol and approved by the independent ethics committee of Innsbruck Medical University.

T-cell preparation. Bulk T cells were isolated from rosettes that had formed with neuraminidase-treated sheep red blood cells during the monocyte isolation procedure by lysing the sheep red blood cells with ammonium chloride, as previously described.²⁵ Naive CD4⁺ T cells were isolated from bulk T cells by using the naive CD4⁺ T-cell isolation kit purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), according to the manufacturer's protocol.

DC-T-cell cocultures. DCs (2.5×10^5) and allogeneic naive CD4⁺CD45RA⁺ T cells (2×10^6) were cocultured in 24-well plates for 5 days.

HEK 293 cell transfection with FPR3

For FPR3 cloning, FPR3 cDNA was amplified from reverse-transcribed mRNA isolated from monocyte-derived DCs by using specific primers: forward, 5' CAGTCGATATCCGAAACCAACTTCTCCATTCC; reverse, 5' ATCAGTGC GGCCGCCATTGCTTGTAATCCGTC. The PCR product was cleaned (Wizard; Promega, Madison, Wis) and subjected to A tailing for cloning into pGemT vector. The sequence was verified (Microsynth, Balgach, Switzerland), and the plasmid was used to produce the *EcoRV*/*NotI* insert for subsequent cloning into pcDNA3.1 His-B. This plasmid was linearized and used for transfection into HEK 293 cells (ATCC, Manassas, Va) by means of Ca₂PO₄ transfection in 24-well plates. Twenty-four hours later, cells were subjected to selection with G418 (Biochrom, Berlin, Germany). Clones were collected after 20 days and seeded into 6-well plates. At confluence, we controlled cells for FPR3 expression by using fluorescence-activated cell sorting (FACS) analysis (PE-anti-human FPR3; BioLegend, San Diego, Calif), transferred them to flasks, and expanded and froze them at 10⁷ cells/mL in their sixth passage for further experiments.

Relative quantification of FPR3 transcripts

Total RNA was extracted from DCs treated or not with the indicated lipocalins for 48 hours by using TRIzol (Gibco BRL, Thermo Fisher, Waltham, Mass). Random primed cDNA was prepared (Superscript II RNase H-reverse transcriptase; Life Technologies, Vienna, Austria) from total RNA. Quantitative PCR analysis was performed on a CFX96 RT C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif) by using SSoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). Sequences for primers (synthesized by Microsynth) specific for FPR3 cDNA were selected by using the Primer Express software (Applied Biosystems, Foster City, Calif): forward, 5' TGGTGTGGGAAGATGGAAACC; reverse, 5' CAGATGGTGTGTGACTGTGCG.

Immunocytochemical analysis and confocal microscopy

For FPR3 colocalization, DCs were allowed to adhere to poly-L-lysine (100 μg/mL for 1 hour at 37°C; Sigma, St Louis, Mo)-coated cover glass slides within a 24-well plate for 30 minutes. Fluorescent dye (Alexa Fluor 594)-labeled recombinant lipocalins (10 μg/mL) or LysoTracker Red (Invitrogen Life Technologies, Eugene, Ore) were added for 1 hour. Cells were fixed in

Fix&Perm Solution (An Der Grub Bio Research, Kaumberg, Austria) containing 1 µg/mL 4'-6-diamidino-2-phenylindole dihydrochloride for 10 minutes. Immunostaining was performed with polyclonal rabbit anti-human FPR3 IgG (LSBio, Seattle, Wash), followed by Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen Life Technologies, Eugene, Oreg).

FPR3 and cell organelle staining was performed with DCs fixed in Fix&Perm Solution (An Der Grub Bio Research) containing 1 µg/mL 4'-6-diamidino-2-phenylindole dihydrochloride for 10 minutes, followed by immunostaining performed with polyclonal goat anti-FPR3 antibody (Santa Cruz Biotechnology, Dallas, Tex) together with rabbit polyclonal anti-Early Endosomal Antigen (EEA)-1 (Abcam, Cambridge, United Kingdom), anti-Rab5, or anti-Rab7 antibody (Cell Signaling, Danvers, Mass), followed by the secondary antibodies Alexa Fluor 488- or Alexa Fluor 594-conjugated donkey anti-goat (Life Technology, Carlsbad, Calif) or Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-rabbit antibody (Invitrogen Life Technologies) or fluorescein isothiocyanate-labeled anti-human MHC class II (MHCII)-specific antibody (Becton Dickinson, Franklin Lakes, NJ).

Confocal microscopy was performed with a spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Waltham, Mass) connected to a Zeiss AxioObserver Z1 microscope (Zeiss, Oberkochen, Germany). Images were acquired with the Volocity software (PerkinElmer) by using a 63× oil immersion objective with a numeric aperture of 1.4.

Ca²⁺ mobilization assay

HEK 293 cells and HEK 293 cells overexpressing FPR3 were seeded in poly-L-Lysine (Sigma)-coated black-framed clear-bottom 96-well plates (PerkinElmer) at a density of 1.5×10^5 cells/well in a total volume of 50 µL/well culture medium (Dulbecco modified Eagle medium with 0.5% FCS, 1% HEPES, and 1 mmol/L glutamine) and incubated at 37°C for 5 to 6 hours. Ca²⁺ mobilization assays were conducted by using the Fluo-4 Direct Calcium Assay Kit (Invitrogen Life Technologies), according to the manufacturer's protocol. Briefly, 50 µL of 2× Fluo-4 Direct Calcium Reagent loading solution supplemented with 5 mmol/L probenecid was added to each well and incubated for 30 minutes at 37°C, followed by 30 minutes of incubation at room temperature in the dark. Assay plates were placed into the PHERAstar FS kinetic fluorescence plate reader (BMG Labtech, Ortenberg, Germany) to measure changes in intracellular calcium levels in response to receptor activation. One hundred intervals with an interval time of 1 second and 10 flashes per well per interval were recorded (excitation, 485 nm; emission, 520 nm). The basal fluorescence signal was recorded for 20 seconds, followed by an addition of 25 µL of compounds dissolved in Fluo-4 Direct Calcium Assay Buffer by means of direct injection and 80 seconds of continuous recording.

Cathepsin S processing of lipocalins

For processing of lipocalins with active cathepsin S, 200 µg of each lipocalin was incubated for 24 hours at 37°C with 15 µg of active human recombinant cathepsin S (BioVision, Milpitas, Calif) in 100 µL of sterile degradation buffer containing 100 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L sodium acetate (pH 5), and 2 mmol/L dithiothreitol.^{26,27} Samples were stored at -20°C and used for Ca²⁺ mobilization assays. We used high-concentration lipocalin protein solutions to omit buffer exchange of proteins with cathepsin S digestion buffer. In addition, digests of Lcn-1 and Can f 1 were withdrawn after 1, 5, 10, 24, and 48 hours and applied on a 12% Bis-Tris gel. As a control, the lipocalins were incubated for 48 hours at 37°C in digestion buffer without cathepsin S. The gels were cut into 2 parts between the 10- and 15-kDa markers. The upper parts with the larger proteins were stained with InstantBlue (Expedeon, Harston, United Kingdom), and the lower part was silver stained according to the manufacturer's protocol with the ProteoSilver Kit (Sigma-Aldrich, St Louis, Mo).

Identification of peptides in cathepsin S-digested lipocalins

Can f 1, Lcn-1, Fel d 4, and MUP were subjected to cathepsin S digestion as above and analyzed with the UltiMate 3000 nano-HPLC System (Dionex,

Germering, Germany) coupled through a nanospray ionization source to a Q Exactive HF Mass Spectrometer (Thermo Scientific, Vienna, Austria) using instrument settings according to the method of Hoernes et al.²⁸ A database search was performed with ProteomeDiscoverer (version 2.2; Thermo Scientific) with the search engine Sequest HT. Tandem mass spectrometric spectra were searched against a contaminants database (635 entries) to which 5 protein sequences (Can f 1, MUP, Lcn-1, Fel d 4, and cathepsin S) were added. The following settings were applied. Enzyme for protein cleavage was set to unspecific. Variable modifications were N-terminal protein acetylation and methionine oxidation. Precursor mass tolerance was set to 10 ppm; fragment mass tolerance was 20 mmu. The maximum false discovery rate for peptides was 1%.

ELISA

Culture supernatants of human monocyte-derived DCs were analyzed for IL-12 (p70), IL-10 (BD PharMingen, San Diego, Calif), and IL-6 (R&D Systems, Minneapolis, Minn). Cell supernatants of cocultures of human monocyte-derived DCs with allogeneic CD4⁺CD45RA⁺ naive T cells (5 days) were analyzed for IFN-γ, IL-13, IL-4, and IL-10 (BD PharMingen).

FPR3 silencing

DCs were cultured at 2.5×10^5 cells/well seeded in 24-well plates in 500 µL of culture medium (RPMI 1640 supplemented with 10% FCS, 2 mmol/L of L-glutamine, and 800 U/mL GM-CSF [specific activity, 1.1×10^6 U/mg]; Leukomax; Novartis, Basel, Switzerland) and 20 U/mL IL-4 (Strathmann, Hannover, Germany) without antibiotics. On days 1 and 3, cells were transfected with 120 nmol/L (1.6 ng/µL) small interfering RNA (siRNA; 5' UCA UUC UUG UCC UGA UUA A 3') with 3' deoxythymidine dinucleotide overhangs or control siRNA ready to use duplexes (Mycrosynth) with Lipofectamine 2000 (Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol. On day 6, DCs were harvested and used for further experiments. Reduction of FPR3 expression was determined by using intracellular FACS analysis for each experiment with polyclonal anti-FPR3 IgG (LS Bio, Seattle, Wash) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen Life Technologies). Cells were analyzed with a FACS-Canto (Becton Dickinson, Franklin Lakes, NJ), and data were handled with FlowJo software (TreeStar, Ashland, Ore).

Agonist, antagonist, and neutralizing antibody treatment

In specified experiments we used an agonist, WKYMVm (Tocris, Bristol, United Kingdom), or an antagonist, WRW4 (Tocris), to FPR3^{17,29} or a neutralizing anti-IL-12 antibody (Abcam). The agonist or antagonist was added to the cell culture at 1 µmol/L and the antibody was added at 4 µg/mL 30 minutes before the lipocalins.

Statistics

Data were handled with Prism software (GraphPad Software, La Jolla, Calif), and paired Student *t* tests were performed to evaluate significance.

RESULTS

Verification of differential expression of FPR3

To evaluate microarray data obtained by comparing expression data of DCs treated with either allergenic or nonallergenic lipocalins,¹⁵ we performed RT-quantitative PCR with cDNA obtained from immature DCs either treated for 48 hours with the nonallergenic lipocalins Lcn-1 and MUP or their allergenic homologues Can f 1 and Fel d 4 to assess expression of FPR3. Dendritic cells treated with maturation cocktail (mDCs) or immature monocyte-derived dendritic cells (iDCs) served as controls. In DCs treated with Lcn-1, MUP, or maturation cocktail, FPR3 is

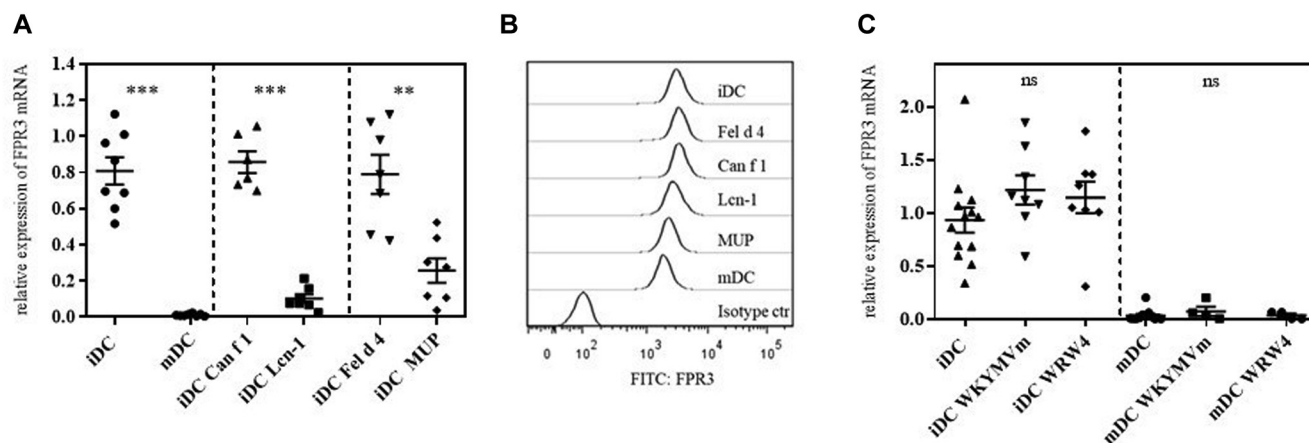


FIG 1. FPR3 expression in DCs. **A**, cDNA prepared from untreated iDCs or mDCs, Lcn-1, Can f 1, Fel d 4, or MUP as indicated for 48 hours was subjected to quantitative PCR by using the SYBR Green technique. **B**, DCs obtained with the same culture conditions were stained for FPR3 and analyzed. **C**, Expression levels (cDNA) in DCs treated with FPR3 ligands, as indicated. Expression levels are relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. *ns*, $P > .05$, $**P < .01$, and $***P < .001$.

hardly detectable, whereas levels remain similar in iDCs or iDCs treated with Can f 1 or Fel d 4 (Fig 1, A).

To evaluate FPR3 expression on the protein level, we performed intracellular staining and FACS analyses on cells obtained under the same conditions (Fig 1, B). We found a slight decrease in FPR3 expression in DCs treated with nonallergenic lipocalins or maturation cocktail.

To assess whether ligand binding influences FPR3 expression, we measured mRNA expression levels of FPR3 in monocyte-derived iDCs and mDCs treated with an agonist (WKYMVm) or an antagonist (WRW4). As shown in Fig 1, C, downregulation of FPR3 expression occurs when DCs encounter maturation stimuli independent of ligand binding.

Colocalization of lipocalins and FPR3 in endosomes

DCs adherent to PLL-coated cover glass slides were incubated with fluorescent dye-labeled lipocalins for 1 hour and stained with FPR3-specific antibodies to assess the feasibility of lipocalins taken up by DCs to interact with FPR3 located in small intracellular vesicles.³⁰ We could identify vesicles that stained for both lipocalins and FPR3, allowing for interaction of the receptor with putative lipocalin-derived ligands (Fig 2, A).

To identify the FPR3-containing vesicles, we used markers for cell vesicles and MHCII. We found colocalization of FPR3 with EEA-1 and Rab5, both markers for early endosomes, and Rab7, a marker for late endosomes³¹⁻³³ but not with LysoTracker or MHCII, markers for lysosomes and late endosomal MHCII compartments, respectively (Fig 2, B).

Allergenic, but not nonallergenic, lipocalin-derived peptides and cathepsin S digests interact with FPR3 expressed by HEK 293 cells

To investigate whether lipocalins interact with FPR3, we transfected HEK 293 cells with the FPR3-encoding expression vector pcDNA3.1 to induce FPR3 expression (see Fig E3 in this article's Online Repository at www.jacionline.org). We seeded

stably transfected cells into 96-well plates and prepared them for Ca^{2+} measurement. When we added the whole proteins, both Lcn-1 and Can f 1 induced Ca^{2+} mobilization in HEK 293 cells independently of FPR3 expression (Fig 3, A), indicating unspecific activation. Because FPR3 is a receptor for peptides, we decided to use the N-terminal 15-amino-acid peptides after the signal sequence of Can f 1 and Lcn-1 (see Fig E1 in this article's Online Repository at www.jacionline.org) to determine whether they are able to induce Ca^{2+} mobilization through FPR3. As shown in Fig 3, B, the Can f 1-derived peptide induces Ca^{2+} mobilization in FPR3-transfected HEK 293 cells in a concentration-dependent manner but not in control HEK 293 cells, whereas the Lcn-1-derived peptide does not. The FPR-specific agonistic synthetic peptide WKYMVm¹⁹ was used as a positive control for FPR3 activation.

The endocytic pathway produces peptides from protein antigens and thus protein allergens. Therefore we decided to generate peptides more closely resembling DC-processed lipocalins by incubating lipocalins with the major protease found along the endocytic pathway in DCs, cathepsin S.^{34,35} Breakdown products of the digested lipocalins Lcn-1 and Can f 1 were subjected to PAGE and stained with colloidal InstantBlue in the upper part and silver stained in the lower part containing the peptides. As shown in Fig 3, C, the generated peptides differ in size.

Additionally, we analyzed cathepsin S digests using HPLC coupled to a mass spectrometer (see Table E1 in this article's Online Repository at www.jacionline.org). We could not find overlapping peptides in the allergenic lipocalins, which do not appear in the nonallergenic lipocalins. Nevertheless, in the Ca^{2+} mobilization assay the digests prepared from Can f 1 and Fel d 4 induce Ca^{2+} mobilization in FPR3-expressing HEK 293 cells but not in control HEK 293 cells, whereas Lcn-1 and MUP digests do not (Fig 3, D).

Additionally, we induced Ca^{2+} mobilization in FPR3-expressing HEK 293 cells with cathepsin S digests of recombinant Fel d 7 and the commercially purchased recombinant allergens Equ c 1 and Rat n 1 (see Fig E4, A, in this article's Online Repository at www.jacionline.org). However, the

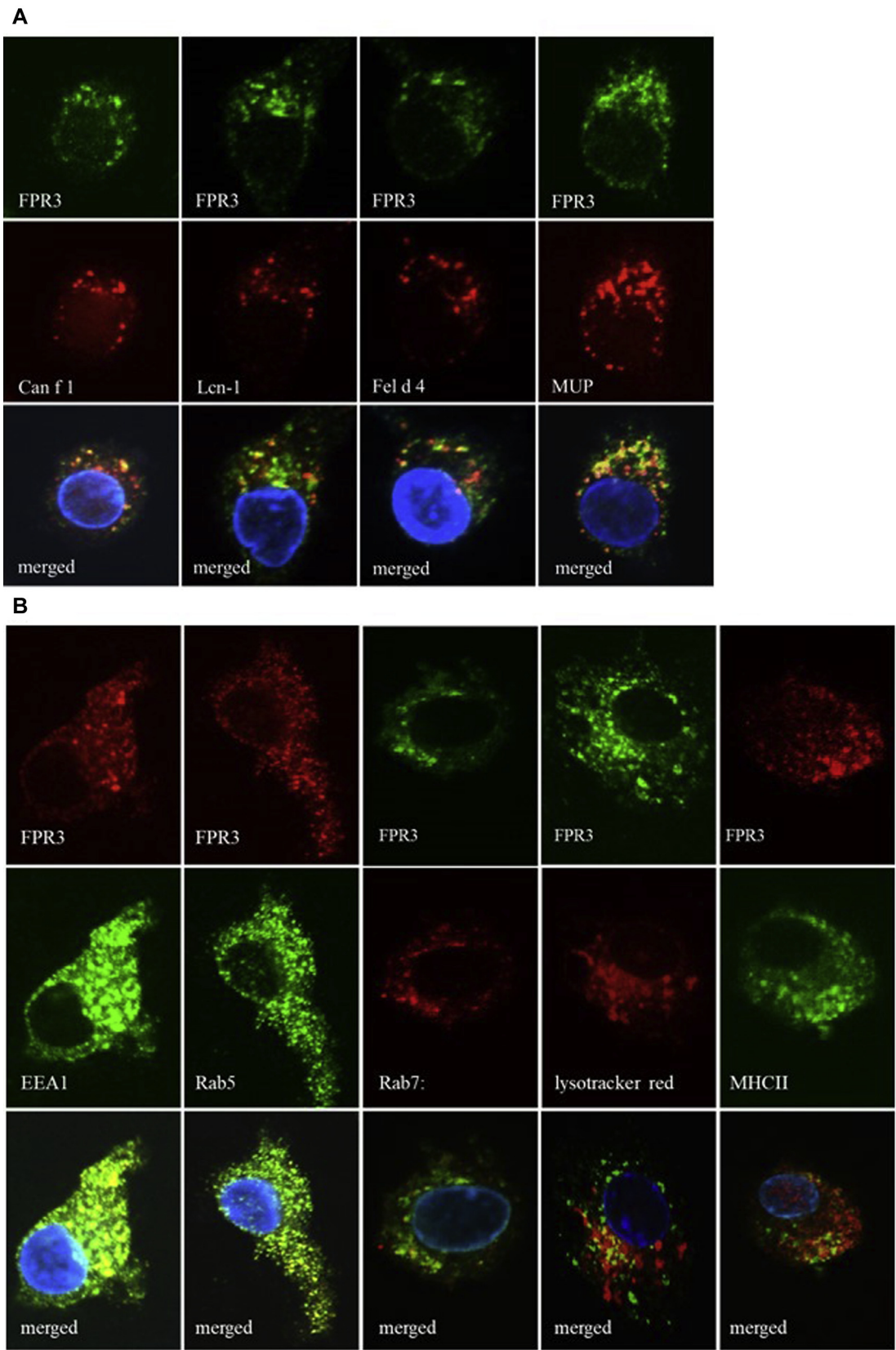


FIG 2. Intracellular localization of lipocalins and FPR3. **A**, DCs were incubated for 1 hour with Alexa Fluor 594–stained lipocalins (red) as indicated, fixed, permeabilized, and stained for FPR3 (green). **B**, DCs were treated with LysoTracker and stained for FPR3 (LysoTracker-treated DCs) or remained untreated and were stained for FPR3 and EEA-1, Rab5, Rab7, or MHCII, as indicated.

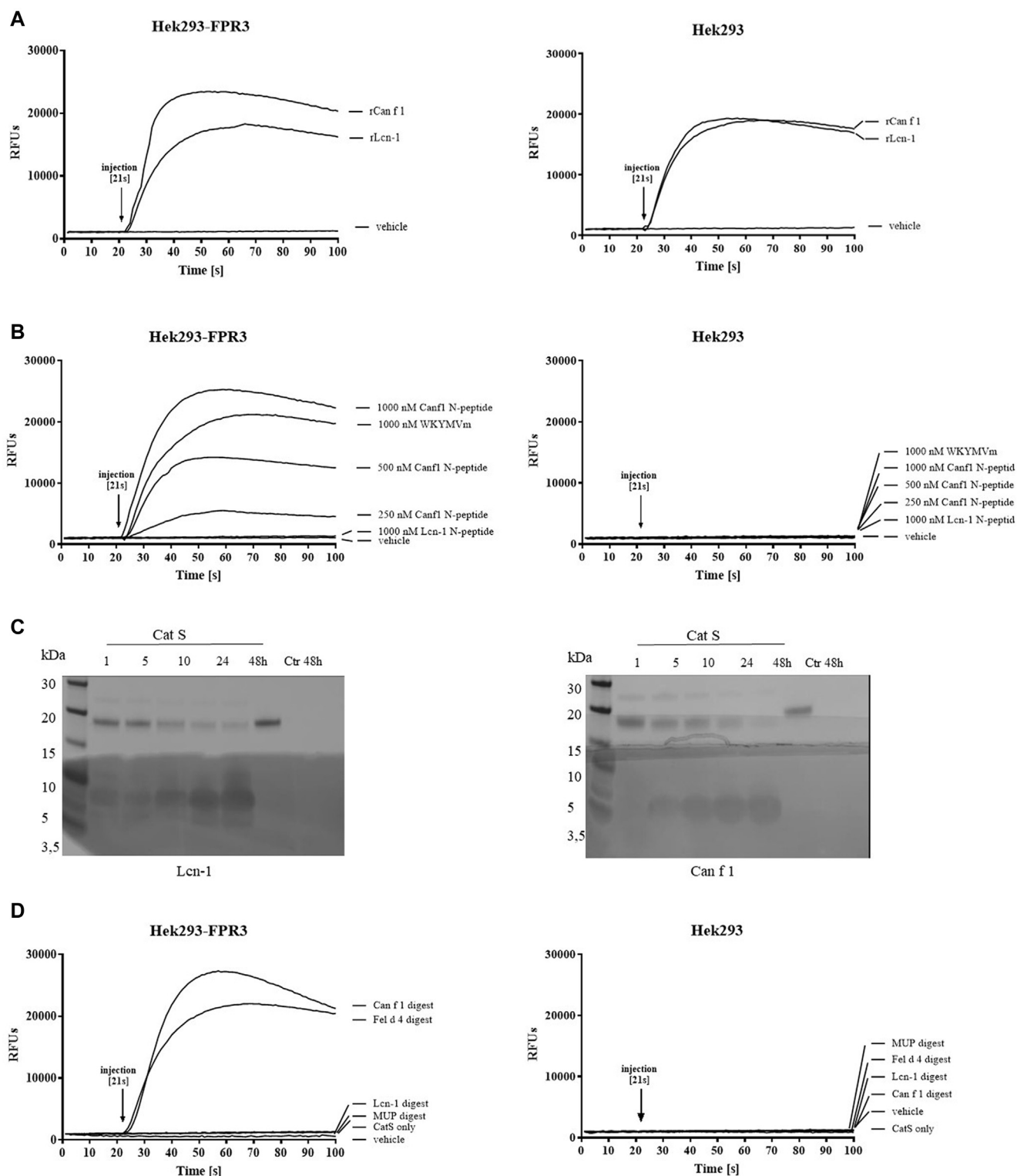


FIG 3. Ca^{2+} mobilization measurements with lipocalin proteins, peptides, and digests. **A**, Recombinant Lcn-1 (25 μL), recombinant Can f 1 (20 $\mu\text{g}/\text{mL}$), or vehicle. **B** and **D**, N-terminal 15-amino-acid peptides of Can f 1 and Lcn-1 (25 μL) at indicated doses and positive control WKYMVm (Fig 3, **B**) or cathepsin S (25 μL) digests of Can f 1, Fel d 4, MUP, and Lcn-1 (10 $\mu\text{mol}/\text{L}$; Fig 3, **D**) were added to FPR3-expressing HEK 293 cells or untransfected HEK 293 cells. **C**, Digests of Lcn-1 and Can f 1 were analyzed on polyacrylamide gels. Cat S, Cathepsin S; RFU, relative fluorescent units.

nonlipocalin respiratory allergen Fel d 1 did not induce Ca^{2+} mobilization through FPR3 (see Fig E4, B).

Silencing of FPR3 in DCs restores IL-12 production after activation with allergenic lipocalins and attenuates $\text{T}_\text{H}2$ induction

Low or absent IL-12 levels during T-cell activation favor $\text{T}_\text{H}2$ differentiation and suppress $\text{T}_\text{H}1$ differentiation (see Fig E5, A–D, in this article's Online Repository at www.jacionline.org for IL-12–neutralizing antibodies in DC cultures).^{1,36} Neutralized IL-12 in DC cultures treated with the nonallergenic lipocalins Lcn-1 and MUP (see Fig E5, A) induce naive T_H cells to differentiate toward a $\text{T}_\text{H}2$ phenotype characterized by lower IFN- γ (see Fig E5, B) and greater IL-13 (see Fig E5, C) and IL-4 (see Fig E5, D) production. FPR3 activation by the agonistic peptide WKYMVM leads to IL-12 downregulation (see Fig E5, E).¹⁹ DCs treated with this agonist and nonallergenic lipocalins activate naive T_H cells to gain a $\text{T}_\text{H}2$ phenotype (see Fig E5, F–H) similar to the cocultures with neutralizing anti-IL-12 antibodies. Therefore allergen-induced activation of FPR3 could be a mechanism to bias for $\text{T}_\text{H}2$ immune responses.

To address this, we silenced FPR3 expression in DCs by using siRNA, achieving a silencing efficiency of at least 65% (see Fig E6 in this article's Online Repository at www.jacionline.org); incubated these DCs with lipocalins for 24 hours; and measured IL-12 content in the supernatant. As shown in Fig 4, A, IL-12 production is restored by DCs silenced for FPR3 and treated with the allergens Can f 1 and Fel d 4, reaching levels similar to those in Lcn-1- and MUP-treated DCs. We detected no significant difference in IL-12 production by silencing of FPR3 in DCs treated with Lcn-1 and MUP (Fig 4, A). This is in concordance with the data shown above showing that only allergenic lipocalin-derived peptides interact with FPR3 and reduce IL-12 production.

We verified these data with the additional lipocalin allergens Fel d 7, Rat n 1, and Equ c 1 (see Fig E7, A, in this article's Online Repository at www.jacionline.org). Naive $\text{CD}4^+\text{CD}45\text{RA}^+$ T cells cocultured with allergen-treated DCs silenced for FPR3 produce less IL-13 (Fig 4, B) and IL-4 (Fig 4, C). IFN- γ production was not affected (Fig 4, D). We obtained similar results when DCs were treated with the additional lipocalin allergens Fel d 7, Equ c 1, and Rat n 1 (see Fig E7, B–D). $\text{T}_\text{H}2$ signature cytokine production by T cells cocultured with DCs treated with the nonallergenic lipocalins Lcn-1 and MUP was not altered (see Fig E7, E and F).

Thus interfering with FPR3 activation hinders polarization of naive $\text{CD}4^+$ T cells toward $\text{T}_\text{H}2$ by DCs treated with allergenic lipocalins.

The FPR3 antagonist WRW4 inhibits allergen-mediated FPR3 signaling, IL-12 decrease, and $\text{T}_\text{H}2$ differentiation

Peptides as therapeutics are of increasing importance, as reviewed by Dunn,³⁷ and advances in peptide immunotherapy for treatment of allergy are considered a promising approach.³⁸ The only available antagonistic peptide for FPR3, WRW4, was used by Shin et al²¹ to show the inhibition of FPR3 signaling induced by an endogenous ligand. Therefore we tested whether this peptide also inhibits the described consequences of allergen-induced FPR3 activation. We measured allergen-induced FPR3 signaling and IL-12 production of DCs treated

with allergenic lipocalins in the presence or absence of the antagonistic peptide and cytokine production of T_H cells activated by these DCs. Indeed, the FPR3 antagonist WRW4 inhibits Can f 1 digest-induced FPR3 signaling measured in FPR3-expressing HEK 293 cells (Fig 5, A). In DCs treated with the FPR3 antagonist, the decrease in IL-12 production induced by the allergenic lipocalins Can f 1, Fel d 4 (Fig 5, B), Fel d 7, Rat n 1, and Equ c 1 (see Fig E8, A, in this article's Online Repository at www.jacionline.org) and $\text{T}_\text{H}2$ cytokine production of naive $\text{CD}4^+$ T cells activated by these DCs (Fig 5, C and D, and see Fig E8, B and C) is reduced to similar levels as in FPR3-silenced DCs. However, we were not able to restore IL-12 production with the FPR3 antagonist WRW4 when IL-12 production of DCs was reduced by a respiratory allergen not belonging to the lipocalin family, Fel d 1 (see Fig E8, A, right row). Again, WRW4 had no effect on IFN- γ production of T cells cocultured with allergen-treated DCs (Fig 5, E) or cytokine production of T cells cocultured with DCs treated with the nonlipocalin allergen Fel d 1 (see Fig E8, B and C, right rows) or untreated DCs or DCs treated with nonallergenic lipocalins (see Fig E8, E and F).

Lipocalin allergens elicit IL-10 production in DCs silenced for FPR3 or exposed to the antagonistic peptide WRW4, and T cells activated by these DCs produce IL-10

Because interfering with FPR3 signaling in DCs leads to attenuated $\text{T}_\text{H}2$ differentiation but, despite restored IL-12 production, does not restore $\text{T}_\text{H}1$ differentiation in cocultured T cells (Figs 4, B–D, and 5, C–E), we further investigated the phenotype of DCs with impaired FPR3 activity. We found that the effects of FPR3 silencing or WRW4 treatment are not restricted to restored IL-12 production (Figs 4, A, and 5, B). IL-10 (Fig 6, A and B) also reached similar or greater levels as in DCs treated with nonallergenic lipocalins. IL-6 expression does not significantly change by interfering with FPR3 expression (see Fig E9, A and B, in this article's Online Repository at www.jacionline.org). We show similar results for DCs treated with the lipocalin allergens Fel d 7, Rat n 1, and Equ c 1 in Fig E9, C and D. This altered IL-10 expression indicates a broader relevance of FPR3 in controlling cytokine responses during DC-lipocalin-allergen interaction. Both methods to interfere with FPR3 signaling in DCs do not restore expression of accessory molecules (CD40, CD80, CD86) by allergen-treated DCs (Fig 6, C–F, and see Fig E9, E and F). These results suggest that interfering with FPR3 signaling in DCs treated with lipocalin allergens induces tolerogenic properties in DCs, which can induce peripheral tolerance by promoting naive T_H cells to differentiate into IL-10–producing T_H cells.^{37,39,40} Therefore we measured IL-10 levels in coculture supernatants of lipocalin allergen-treated DCs with impaired FPR3 signaling and naive T_H cells. Indeed, we found increased IL-10 production in cocultures with DCs either silenced for FPR3 (Fig 6, G, and see Fig E9, G) or treated with WRW4 (Fig 6, H, and see Fig E9, H) and treated with lipocalin allergens compared with nonallergenic lipocalins or DCs with intact FPR3 signaling.

DISCUSSION

The availability of 2 homologous pairs of lipocalins, each containing an allergenic and a nonallergenic member of the protein family, provided us with the opportunity to compare their

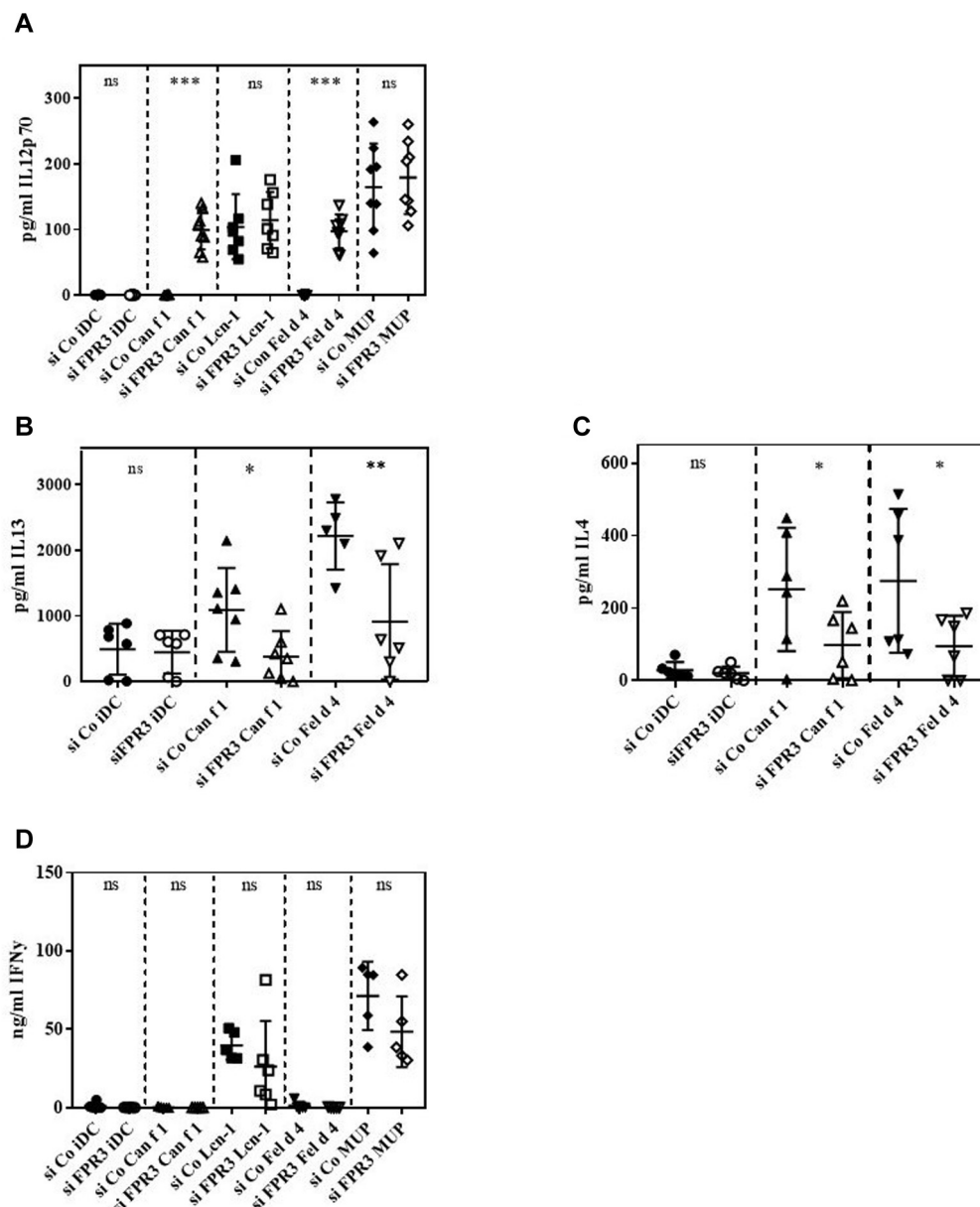


FIG 4. Cytokine production of allergen-treated DCs silenced for FPR3 expression and cocultured CD4⁺CD45RA⁺ T cells. **A**, Supernatants of DCs silenced for FPR3 expression or controls activated with lipocalins, as indicated, were analyzed by means of ELISA for IL-12 p70. **B-D**, Supernatants of day 5 cocultures of DCs silenced for FPR3 expression and treated with Can f 1 or Fel d 4, as indicated, and naive CD4⁺CD45RA⁺ T cells were analyzed for IL-13 (Fig 4, B), IL-4 (Fig 4, C), and IFN-γ (Fig 4, D) content. ns, $P > .05$; * $P < .05$; ** $P < .01$; and *** $P < .001$.

effect on human monocyte-derived DCs. In an allogeneic coculture of monocyte-derived DCs and CD4⁺CD45RA⁺ naive T_H cells, we previously showed that addition of the highly homologous lipocalins with and without allergenic potential induce differentiation to T_H2 or T_H1 cells, respectively. We started to evaluate the microarray data obtained from lipocalin-treated DCs¹⁵ and found the family of FPRs to be among the most prominently upregulated genes caused by addition of allergenic, but not nonallergenic, lipocalins. DCs do not express 2 members of this family: FPR1 and FPR2. For FPR3, however, expression by myeloid DCs (see Fig E2, A and B)⁴¹ and downregulation of IL-12 in response to receptor activation was reported.^{19,42}

Because low or absent IL-12 promotes T_H2 responses (see Fig E5),⁴³⁻⁴⁵ its restriction by activating FPR3 can lead to an enhanced T_H2 response.

After verifying the differential expression of FPR3 in DCs treated with Can f 1 or Fel d 4 and Lcn-1 or MUP (Fig 1, A and B) and showing that FPR3 downregulation is independent of ligand binding (Fig 1, C), we localized FPR3 to evaluate the possibility of interaction with lipocalins after uptake. We found FPR3 to be colocalized with both allergenic and nonallergenic lipocalins after 1 hour of incubation within iDCs in small vesicles (Fig 2, A), which we identified as early and late endosomes (Fig 2, B). This enables the interaction with and activation of FPR3 by lipocalins.

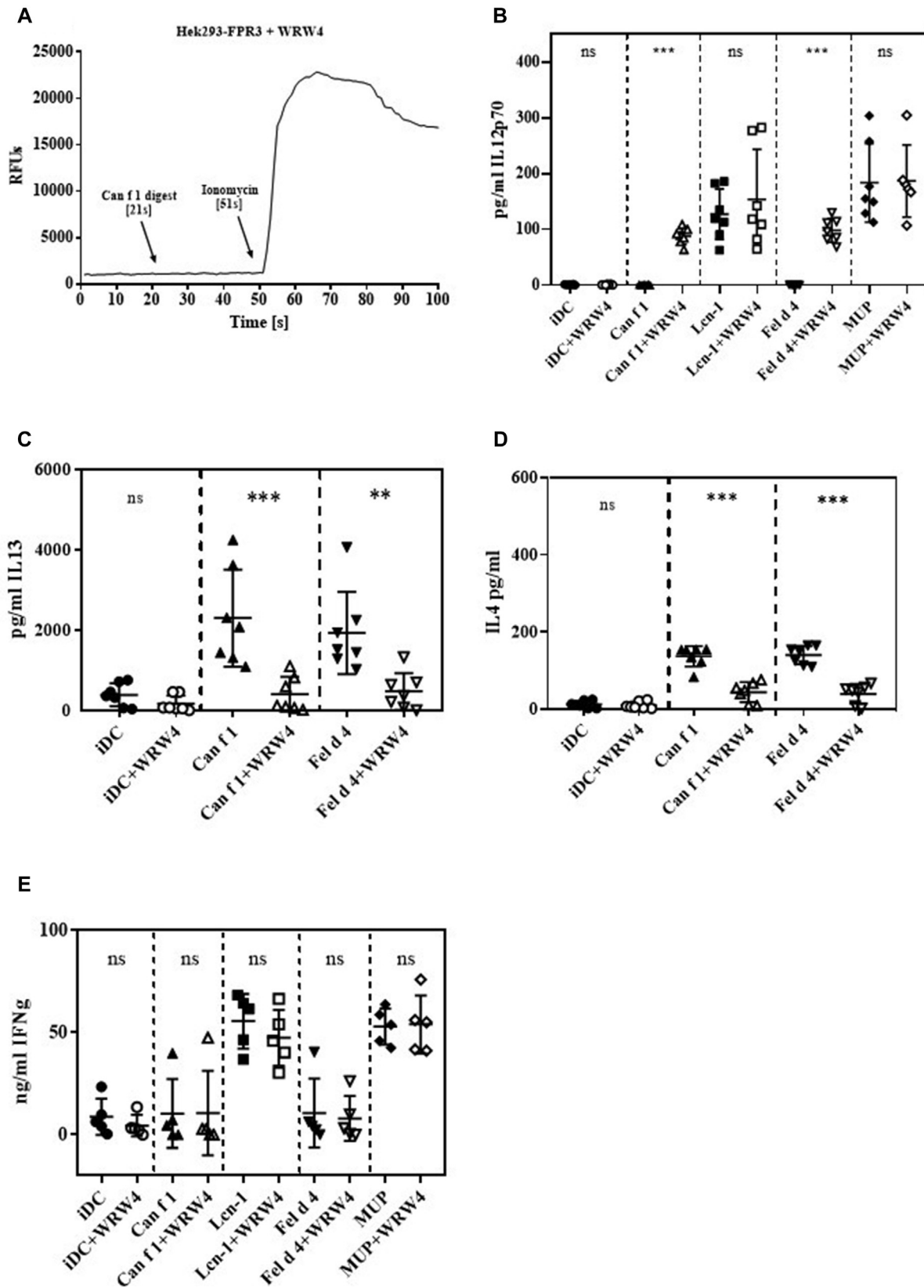


FIG 5. Effect of WRW4 on FPR3-induced T_H2 bias. **A**, WRW4 ($1 \mu\text{mol/L}$) was added before addition of Can f1 digest and ionomycin to HEK 293-FPR3 cells. **B**, IL-12 production by DCs treated with allergenic and nonallergenic lipocalins in the presence or absence of $1 \mu\text{mol/L}$ WRW4, as indicated. **C-E**, IL-13 (Fig 5, C), IL-4 (Fig 5, D), and IFN- γ (Fig 5, E) content in supernatants of cocultures of $CD4^+CD45RA^+$ naive T_H cells and DCs treated with allergenic and nonallergenic lipocalins in the presence or absence of WRW4, as indicated. ns, $P > .05$, ** $P < .01$, and *** $P < .001$.

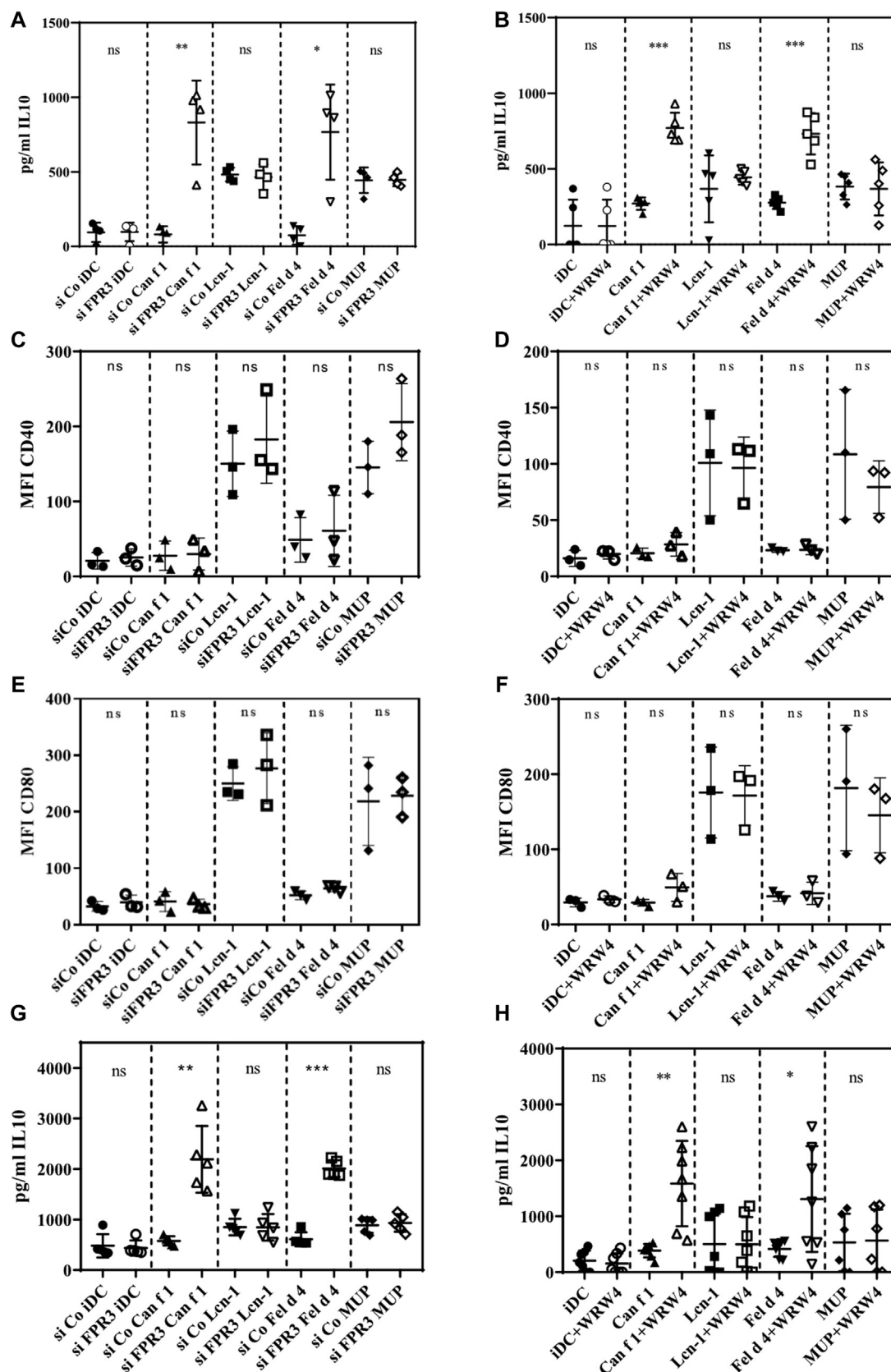


FIG 6. Characterization of DCs with impaired FPR3 activation. **A-F**, DCs silenced for FPR3 expression or treated with the FPR3 antagonist WRW4, as indicated, were analyzed for IL-10 concentrations in supernatants of DCs by means of ELISA (Fig 6, **A** and **B**) and expression of the accessory molecules CD40 (Fig 6, **C** and **D**) and CD80 (Fig 6, **E** and **F**). **G** and **H**, Supernatants cocultured with naive CD4⁺CD45⁺ T cells were analyzed for IL-10 by means of ELISA. ns, $P > .05$, * $P < .05$, ** $P < .01$, and *** $P < .001$.

To test whether such an interaction occurs, we transfected HEK 293 cells with an expression plasmid carrying FPR3 cDNA and produced several stably transformed HEK 293/FPR3 cell lines. Fig E3 shows FPR3 expression of the cell line used in the experiments. All FPR3-transfected cell lines express FPR3 also on their surfaces, which facilitates Ca^{2+} mobilization measurements in response to FPR3 activation used to assess the binding capacity of lipocalins to FPR3. As shown in Fig 3, A, intact lipocalins induce Ca^{2+} mobilization in HEK 293 cells, even when untransfected and thus not expressing FPR3. We interpret this as FPR3-independent signaling. Because FPR3 is a peptide receptor and in DCs is mainly located in early and late lysosomes (Fig 2, B), we hypothesized that instead of intact proteins, processed lipocalins might be binding partners of FPR3. We show that this is true for peptides derived from the allergenic lipocalins Can f 1 and Fel d 4 but not for peptides derived from the nonallergenic homologues Lcn-1 and MUP. Thus the FPR3-binding capacity of lipocalin-derived peptides is restricted to allergenic lipocalins. To date, this is the first and only property restricted to allergenic lipocalins capable to explain their different effects on DCs. A respiratory allergen not belonging to the lipocalin family, Fel d 1, does not bind to FPR3 (see Fig E4, B) or induce IL-12 production in DCs with impaired FPR3 activation (see Fig E8, A) or alter the outcome in cocultured naive T_H cells (see Fig E8, B-D). Therefore it remains to be further investigated whether FPR3 signaling can be induced by allergens other than lipocalins.

We further characterized the peptides obtained by means of cathepsin S digestion of lipocalins using HPLC coupled to a mass spectrometer (see Table E1). Of the peptides identified by using mass spectrometry, none appeared in the allergens only, eliminating an easy approach to search for FPR3-binding candidates. However, 2 of 139 Can f 1-derived peptides and 3 of 126 Lcn-1-derived peptides approximately span the regions we used for the peptides in our FPR3-binding experiments.

Based on published results on downregulation of IL-12 production in DCs by FPR3 activation^{19,42} and skewing toward a $\text{T}_\text{H}2$ response at low IL-12 concentrations (see Fig E5),^{43,44} the observed interaction of processing products of allergenic lipocalins points to a novel mechanism to explain their allergenicity. Data gained by silencing FPR3 in DCs, showing enhanced IL-12 production when treated with allergenic lipocalins and a constrained differentiation toward $\text{T}_\text{H}2$ when these DCs were used to activate naive CD4^+ T cells (Fig 4), underscore the importance of the interaction of FPR3 with the allergenic lipocalins for the development of allergic immune responses. The capacity of the only known FPR3 antagonist, WRW4, to inhibit the lipocalin allergen-induced activation of FPR3 and thus skewing of the induced immune response toward $\text{T}_\text{H}2$ (Fig 5 and see Fig E8) allows for the interpretation that the antagonist WRW4 interferes with allergen-induced downregulation of IL-12 in DCs to a similar level as FPR3 gene silencing. However, interfering with FPR3 activation in DCs does not lead to $\text{T}_\text{H}1$ differentiation by allergen-treated DCs, as indicated by low IFN- γ production (Figs 4, D, and 5, E), but rather restrains T cells from $\text{T}_\text{H}2$ cytokine production. The missing induction of $\text{T}_\text{H}1$ cells activated by DCs silenced for FPR3 or treated with WRW4 and activated with allergens instigated further characterization of these DCs.

We chose to measure the expression of accessory molecules and 2 additional cytokines known to be produced by DCs and with an effect on biasing of T_H differentiation: the proinflammatory cytokine IL-6⁴⁶ and the immunosuppressive cytokine IL-10.⁵

Although IL-6 production is not affected by interference with FPR3 activation (see Fig E9, A-D), IL-10 is significantly upregulated (Fig 6, A and B). Together with the impaired upregulation of costimulatory molecules, as shown in Fig 6, C-F, and Fig E9, E and F, this indicates that DCs with impaired FPR3 signaling resemble features of tolerogenic DCs.^{5,47,48}

Induction of IL-10 often occurs together with proinflammatory cytokines, and concurrent upregulation of the proinflammatory IL-12 and induced IL-10, as shown here, might actually negatively regulate immune responses. This opened the possibility that T cells differentiating in response to these DCs might acquire immunosuppressive properties. Thus we measured IL-10 production in T cells differentiated in response to DCs with impaired FPR3 activation and treated with lipocalin allergens and found that IL-10 was induced (Fig 6, G and H). IL-10-producing DCs have already been shown to hold great potential to improve the treatment of allergic diseases.^{5,9,49,50} Therefore interfering with FPR3 expression in DCs might open new opportunities for therapeutic intervention. Because FPR polymorphisms have been matched with a potential significance in pathology,⁵¹ we speculate that they might help explain different susceptibilities toward allergic responses to lipocalin allergens.

In summary, for the first time, we describe a molecular mechanism to induce allergic immune responses used by at least 5 allergenic members of the lipocalin family.

Clinical implications: FPR3-targeting antagonistic peptides can be adapted for therapeutic intervention.

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Peptides identified in sample Canf1, Lcn-1, Fel d 4, and MUP when analyzing 1µg and 100µg sample each

Protein	Peptide Description	Peptide Identification							Can f 1		Lcn-1		Fel d 4		MUP		
		Accession: O18873	Accession: P31025	Accession: Q5VFH6	Accession: P00001	Abundance: 1µg	Abundance: 100µg	Abundance: 1µg	Abundance: 100µg	Abundance: 1µg	Abundance: 100µg	Abundance: 1µg	Abundance: 100µg				
P31025	AAGARGLST				1	932.48	1.99	High	18.34			2.63E+06					
P31025	ADGGKHVYIR				1	1143.62	1.95	High	33.21			7.04E+06					
P31025	ADGGKHVYIRSHV				27	1299.72	3.53	High	25.12			3.62E+09	5.11E+08				
P31025	ADGGKHVYIRSHVK				14	1750.97	6.01	High	26.95			3.22E+08	3.89E+07				
435476	ADNGLR				1	758.42	1.96	High	23.94	3.87E+07	4.29E+06						
O18873	ADQVEKPD				3	1127.52	2.43	High	20.21	1.02E+08	1.20E+07						
O18873	ADQVEKPDV				18	1214.55	2.68	High	19.84	9.75E+09	1.77E+09						
O18873	ADQVEKPDVS				6	1313.62	2.65	High	25.62	2.41E+08	1.51E+07						
O18873	ADQVEKPDVPTPM				1	1642.76	2.73	High	31.67	1.71E+07	5.60E+05						
O18873	ADQVEKPDVPTPMILK				17	1755.85	3.45	High	40.4	3.06E+09	1.10E+08						
O18873	ADQVEKPDVPTPMI	1xOxidation [M1S]			2	1771.84	2.25	High	36.52	3.33E+07	9.04E+03						
O18873	ADQVEKPDVPTPMIL				6	1868.93	3.34	High	47.06	1.79E+08	1.24E+07						
O18873	ADQVEKPDVPTPMILK				33	1992.03	5.03	High	38.7	8.17E+09	4.03E+08						
O18873	ADQVEKPDVPTPMILK	1xOxidation [M1S]			16	2013.02	4.03	High	35.09	2.39E+08	9.06E+07						
O18873	AKGLNQELE				2	1114.61	2.59	High	34.02	5.13E+07	5.07E+06						
O18873	AKITML				3	676.41	2.05	High	35.09	8.74E+08	1.02E+08						
O18873	AKITMLT				3	777.45	2.01	High	32.54	2.08E+09	2.67E+08						
P31025	AKVTML				1	662.39	1.95	High	30.25			2.06E+09	2.61E+08				
P31025	ALDEFEKAAGARG				5	1334.67	3.16	High	32.89			9.50E+07	8.18E+06				
P31025	ALDEFEKAAGARGLS				10	1534.79	3.66	High	38.51			1.27E+07	4.52E+07				
P31025	ALDEFEKAAGARGLSLT				6	1635.83	4.25	High	39.11			1.16E+08	3.64E+06				
P31025	ALDEFEKAAGARGLSLTE				3	1764.88	5.02	High	39.1			1.91E+07					
P31025	ALDEFEKAAGARGLSSTESLPRQS				3	2659.40	3.67	High	48.12			1.65E+07	1.35E+06				
O18873	AMTADQVEKPDVPTPMILK				8	2300.15	4.73	High	40.92	1.74E+08	1.11E+07						
O18873	AMTADQVEKPDVPTPMILK	1xOxidation [M2]			2	2316.15	3.7	High	39.1	1.98E+07	2.01E+06						
P31025	AMTVDFPEPMNL				3	1681.76	3.81	High	47.45			4.55E+07					
P31025	AMTVDFPEPMNL	1xOxidation [M2]			1	1697.75	2.52	High	44.3			8.14E+06	2.27E+05				
P31025	AMTVDFPEPMNL				1	1768.79	2.05	High	46.53			2.84E+06					
O18873	AQGGNLEAKITM				1	1360.73	3.28	High	31.02	2.74E+07	1.44E+06						
O18873	AQGGNLEAKITMLT				3	1574.86	3.25	High	40.17	8.71E+07	4.10E+06						
O18873	AQGGNLEAKITMLTNGSQCNITVVL				1	3873.00	3.72	High	49.29	6.10E+06							
Q5VFH6	AREPVSQKLK				16	1270.71	3.73	High	19.02			2.07E+08	2.31E+07				
Q5VFH6	ARGSEVADSSVE				2	1334.62	2.91	High	19.25			1.26E+07	1.39E+06				
P25774	ARHPHF				44	961.44	2.37	High	33.14	7.45E+09	7.11E+08	6.53E+09	8.00E+08	6.21E+09	6.89E+08	7.65E+09	8.48E+08
P31025	ASDEEDQVS				13	1092.47	2.32	High	27.66			1.73E+10	3.87E+09				
P31025	ASDEEDQVSGTWYLYLK				6	1840.86	4.58	High	47.05			3.24E+07	5.81E+05				
P31025	ASDEEDQVSGTWYLYKAMTVDR				1	2143.99	2.11	High	53.57			3.11E+06	6.88E+05				
P31025	ASDEEDQVSGTWYLYKAMTVDR				1	2344.18	2.44	High	62.23			1.32E+06	9.05E+04				
Q5VFH6	ASDVKEEKGSMR				12	1692.82	5.13	High	22.99					5.60E+08	7.10E+07		
Q5VFH6	ASDVKEEKGSMRVF				2	1938.96	3.81	High	35.38					7.19E+06	4.65E+05		
P31025	AVLEKTEPKGYT				13	1450.74	4.01	High	24.9			3.51E+08	4.26E+07				
P31025	AVLEKTEPKGYTADGGKHVIA				1	2186.11	3.55	High	23.25			1.48E+07	1.96E+06				
P31025	AVLEKTEPKGYTADGGKHVYIR				7	2731.44	7.01	High	31.3			1.55E+08	4.90E+06				
P31025	AVLEKTEPKGYTADGGKHVYIRSHV				4	3182.70	4.97	High	34.51			9.27E+07					
O18873	AYEGGRVY				14	1068.55	2.54	High	36.9	1.79E+09	1.27E+08						
O18873	AYEGGRVYF				7	1181.63	2.92	High	44.6	2.42E+08	1.14E+07						
O18873	AYEGGRVYFQPSVPR				9	1846.00	5.37	High	38.34	1.04E+08	6.00E+06						
O18873	AYEGGRVYFQPSVDHLYVCEGLH				2	4542.36	3.1	High	57.77	1.15E+07							
O18873	CEGLHGRVDR				2	1297.64	2.51	High	22.76	8.46E+07	1.10E+07						
P25774	DASPYK				17	843.39	2.1	High	22.32	3.80E+08	4.03E+07	2.46E+08	3.12E+07	2.05E+08	2.66E+07	2.36E+08	3.13E+07
Q5VFH6	DEYLLH				6	902.46	2.07	High	41.41					6.61E+09	7.41E+08		
Q5VFH6	DEYLLHLLWKTDRFPQ				1	2262.19	2	High	63.2					1.93E+06			
P31025	DFEKAAGARG				3	1021.51	2.28	High	18.78			4.98E+07	2.33E+06				
P31025	DFEKAAGARGLS				6	1221.62	2.72	High	26.62			3.85E+09	5.66E+08				
P31025	DFEKAAGARGLSLT				8	1322.67	2.94	High	27.72			4.18E+08	4.65E+07				
P31025	DFEKAAGARGLSLTESLPRQS				3	1451.71	2.7	High	28.01			7.88E+07	6.12E+06				
P31025	DFEKAAGARGLSLTESLPRQSL				4	2346.24	3.63	High	41.49			4.61E+07	1.82E+06				
P31025	DFEKAAGARGLSLTESLPRQSET				2	2576.33	3.06	High	41.66			1.41E+07	5.97E+05				
O18873	DFREFSR				4	956.46	2.42	High	29.6	1.44E+08	1.12E+07						
O18873	DFREFSRK				1	1155.59	3.35	High									
O18873	DFREFSRAAG				1	1212.61	3.28	High	26.27	1.66E+07	1.40E+06						
O18873	DFREFSRAAGLN				4	1439.74	2.94	High	32.09	4.86E+08	2.98E+07						
O18873	DFREFSRAAGLNQLE				3	1557.80	3.05	High	31.88	5.50E+07	4.45E+06						
O18873	DFREFSRAAGLNQLELE				3	2052.05	4.89	High	42.6	7.24E+07	7.36E+06						
P31025	DGGKHVYIR				6	1228.68	3.5	High	26.08			6.31E+07	8.43E+06				
P50012	DGPNVSPFQNGDGYLSEGGSSGGGGS				2	3021.33	3.48	High	12.27			8.26E+07					
P00001	DGSMRVF	1xOxidation [M4]			4	827.37	2	High	26.95							1.32E+08	1.49E+07
P31025	DHYIFY				1	857.38	2	High	46.55			4.57E+08	9.57E+07				
P31025	DHYIFYCE				2	1089.43	1.97	High	45.56			2.15E+09	1.93E+08				
P31025	DHYIFYCEGLHGKPYR				1	2062.98	3.93	High	37.43			6.34E+06					
O18873	DHYLYL				1	823.40	1.93	High	42.81	1.77E+08	1.39E+07						
O18873	DHYLYLCE				1	1055.45	2.02	High	42.52	1.32E+07	9.09E+05						
P00001	DILETDYDHYFFYNKNGKETFLMFL				1	4509.18	2.78	High									
Q5VFH6	DKTDSGYTVVY				1	1387.71	3.49	High	34.52					1.21E+07	8.69E+05		
Q5VFH6	DKTRFPQ				4	891.47	2.79	High	27.08					2.04E+07	3.20E+06		
27894337	DLALLK				1	672.43	1.93	High	27.83							9.23E+06	1.12E+06
P25774	DUNGKEYW				9	1024.47	2.46	High	30.29	1.57E+08	1.23E+07	1.27E+08	9.94E+04	1.81E+08	1.07E+07	2.11E+08	2.11E+07
P25774	DUNGKEYWL				6	1137.56	2.68	High	48.2	2.29E+07	1.60E+06	2.37E+07	1.38E+06	1.83E+07	8.64E+05		
P25774	DUNGKEYVLK				17	13											

P31025	PFPMLE	1aOxidation (M4)	2	895.39	1.91	High	33.74			1.58E+08	1.10E+07				
P25774	PFSPYEI		10	852.41	2.05	High	48.72			5.14E+08	2.38E+07	2.21E+08	1.92E+07		
O18873	FSRAGLN		7	892.50	2.74	High	17.52	1.05E+08	5.09E+06						
O18873	FSRAGLNQ		3	1020.56	2.47	High	18.32	4.08E+07	7.52E+05						
QSVFHE	FVHTKEN		7	1021.51	2.69	High	25.26					4.28E+08	6.92E+07		
QSVFHE	FVHTKENG		2	1078.53	2.7	High	25.63					1.23E+08	1.74E+07		
QSVFHE	FVHTKENCK		2	1206.63	2.88	High	22.14					4.43E+07	5.64E+06		
QSVFHE	FVHTKENGACT		27	1410.68	3.58	High	23.87					4.26E+08	6.34E+08		
QSVFHE	FVHTKENGACTE		9	1539.73	3.51	High	24.48					2.33E+08	2.60E+07		
QSVFHE	FVAREPOVS		6	1083.51	2.5	High	28.1					1.41E+08	1.54E+07		
QSVFHE	FVAREPOVSQSLK		2	1580.84	4.07	High	27.7					3.31E+07	1.77E+06		
P00001	FYNKMK		3	1036.51	2.07	High	18.69							2.77E+08	9.86E+05
P00001	FYNKNKNG		3	1097.57	3.44	High	18.28							3.46E+08	1.62E+07
O18873	GEHQRDR		3	1065.59	2.37	High	15.05	1.79E+08							
QSVFHE	GEWYSL		1	1080.51	2.16	High	64.96					5.86E+07			
435476	GGGGGGGGGGSSRSS		1	1319.63	2.52	High	21.68					1.89E+06	3.79E+05		
435476	GGGGGGGGGGSSGR		1	1088.54	2.15	High	22.22					6.30E+05	1.91E+05		
P31025	GONLEAKVT		1	888.48	2.67	High	22.07			3.06E+07	4.22E+06				
P31025	GONLEAKVTML		2	1132.60	3.55	High	41.08			3.68E+07	4.27E+06				
P00001	GIDKENIF		7	935.48	2.5	High	37.26							3.99E+08	6.15E+07
P00001	GIDKENIFD		10	1050.51	2.16	High	38.5							2.66E+09	6.18E+08
P00001	GIDKENIFLT		6	1264.64	2.44	High	40.37							1.65E+08	1.74E+07
P25774	GIDSDASYPK		9	1215.55	2.64	High	28.94	2.74E+07	2.45E+06	2.68E+07	2.37E+06	3.21E+07	3.13E+06	3.58E+07	4.26E+06
P25774	GKEWVLVK		11	1022.57	2.64	High	33.65	2.12E+07	1.34E+06	1.50E+07	1.36E+06	3.60E+07	4.35E+06	4.65E+07	7.54E+06
O18873	GWYLYK		48	794.46	2.3	High	26.61	1.88E+11	1.12E+10						
O18873	GWYLYKAMT		1	1057.58	2.15	High	39.89	7.81E+06	4.48E+05						
O18873	GORVVF		2	705.40	1.96	High	30.56	6.99E+10	1.01E+10						
O18873	GORVYFI		6	818.49	2.15	High	40.23	1.58E+09	2.17E+08						
O18873	GORVYFQSPVPR		3	1482.85	2.97	High	34.57	2.16E+07	1.42E+06						
P31025	GRCOEYK		1	815.41	1.93	High	20.4			1.94E+06	1.91E+05				
O18873	GRDPEQSOEAL		2	1229.58	2.95	High	23.56	2.26E+07	2.35E+06						
O18873	GRDPEQSOEALF		10	1358.62	3.28	High	22.81	1.45E+09	1.64E+08						
O18873	GRDPEQSOEALFDR		2	1178.81	3.86	High	31.14	8.54E+06							
O18873	GRDPEQSOEALFDRFSRAGLN		4	2779.34	4.24	High	43.05	1.60E+07	9.75E+05						
O18873	GRDPEQSOEALFDRFSRAGLNDEL		4	3391.65	4.62	High	48.21	9.49E+07							
P31025	GRDPPNNLE		1	1042.53	2.19	High						1.51E+08	8.32E+06		
P31025	GTWYLYKAMT		6	1070.53	2.28	High	44.68			1.77E+08	1.79E+07				
P31025	GTWYLYKAMTVOREPFEMMLE		2	2430.15	3.03	High	55.92			6.74E+06	7.97E+05				
P31025	GTVLYVGRDPPNNLE		3	1538.86	3.72	High	25.02			4.45E+07	5.08E+06				
QSVFHE	GVTYFMQSGCRKVKLVGAIGTSAGI		1	4013.05	3.14	High									
QSVFHE	HEEENVV		1	855.38	2.09	High	18.93					1.65E+07			
P00001	HOIDKENIF		7	1072.54	2.93	High	31.94							7.08E+08	7.14E+07
P00001	HOIDKENIFD		17	1187.57	2.92	High	31.87							6.24E+09	7.41E+08
P00001	HOIDKENIFDOLT		8	1400.70	3.97	High	44.13							3.95E+08	2.05E+07
QSVFHE	HGYNIL		3	765.46	2.17	High	42.67					1.61E+08	1.75E+07		
QSVFHE	HGYNILD		3	880.49	2.08	High	41.37					9.92E+07	1.11E+07		
QSVFHE	HGYNILDLT		2	1094.62	2.48	High	54.07					6.36E+06	3.79E+05		
P00001	HIDYLGNSLT		1	1219.60	2.15	High								3.11E+08	5.30E+07
QSVFHE	HIKALDNNLS		13	1184.63	2.91	High	25.89					2.66E+07	1.45E+06		
QSVFHE	HLMDKTRFPK		3	1515.81	4.22	High	37.85								
P31025	HYATIR		3	873.51	2.34	High	26.85			3.02E+07	3.82E+06				
P00001	IDENKIF		5	878.46	2.42	High	35.64							6.40E+08	9.34E+07
P00001	IDENKIFD		11	993.49	2.35	High	34.31							7.14E+09	8.96E+08
P00001	IDENKIFDOLT		8	1207.62	2.12	High	45.17							5.06E+07	5.92E+06
P25774	IDNKGDSDA		4	1047.50	2.34	High	20.78	8.20E+06	1.04E+06	9.30E+06	1.03E+06	1.02E+07	8.61E+05	8.27E+06	
P25774	IDNKGDSDASYPK		1	1685.80	2.82	High									
P25774	IDSDASYPK		15	1158.53	2.89	High	26.41	3.87E+07	3.98E+06	2.98E+07	4.13E+06	2.76E+07	4.62E+06	5.15E+07	5.81E+06
P00001	IEENGSMR		23	836.41	2.46	High	18.49							2.58E+09	1.41E+08
QSVFHE	IEENGSMR		3	935.43	2.54	High	17.15					3.09E+07	2.63E+05		
P25774	IDNKGDSDASYPK		1	1798.89	2.62	High									
P31025	IRSHVK		1	852.54	2.02	High	18.71			2.20E+06	1.74E+05				
P31025	ILUPRO		1	739.48	1.92	High	29.34			6.06E+08	6.76E+07				
P31025	ILUPROSET		2	1056.60	2.14	High	29.54			1.39E+08	1.42E+07				
P25774	ILPDSQWR		65	1100.57	2.55	High	41.16	2.16E+09	1.11E+08	1.53E+09	1.16E+08	2.38E+09	9.30E+07	2.97E+09	2.11E+08
P25774	ILPDSQWRE		3	1232.62	2.1	High	41.27			3.07E+07	1.07E+06				
P25774	ILPDSQWREK		39	1357.71	3.51	High	35.38	2.09E+08	9.72E+06	1.42E+08	1.35E+07	2.11E+08	8.55E+06	3.06E+08	2.14E+07
P25774	ILPDSQWREKGCVT		3	1717.86	2.68	High	38.74					1.10E+07	1.14E+06	2.08E+07	1.83E+06
P31025	ILPDSQWYLYKAMT		1	1612.80	1.99	High	36.54								
O18873	IQSPVPR		21	796.47	2.14	High	15.54	3.59E+11	2.63E+10	7.73E+06	3.19E+05				
QSVFHE	ISGEWYSL		1	1067.54	1.91	High	61.37					4.20E+07	3.33E+06		
QSVFHE	ISGEWYSL		1	1180.62	2	High									
P08551	ISNDLS		5	889.50	2.68	High	27.29					1.07E+08	1.37E+07		
O18873	KDTVAVSGKWLYK		5	1494.83	5.06	High	33.17	4.87E+07	3.62E+06						
O18873	KDTVAVSGKWLYKAMT		3	1797.96	4.17	High	40.79	3.70E+07	2.69E+04						
O18873	KDTVAVSGKWLYKAMTADQVPEKPO		3	3175.96	6.15	High	46.24	2.38E+07							
P00001	KENIFD		2	765.38	1.95	High	28.89							3.67E+10	7.65E+09
P00001	KENIFD		2	878.46	2.15	High	45.93							3.37E+08	5.25E+07
P00001	KENIFDOLT		18	979.51	2.58	High	44.36							2.09E+10	2.79E+09
27894337	KELENTLE		1	1085.55	2.03	High	11.9								
P25774	KEYVLVK		12	965.55	3.34	High	33.47	2.66E+07	2.18E+06	2.26E+07	2.26E+06	1.94E+07	1.88E+06	3.16E+07	1.62E+06
P25774	KISDSDASYPK		2	1343.65	2.93	High	25.59			4.14E+06	5.49E+05			8.88E+06	1.09E+06
P08551	KISDSDASYPKALYGEQDRLRLAEDATNM		1	4012.06	3.1	High						1.25E+07	1.50E+06		
QSVFHE	KISGEWYSL		2	1195.64	2.24	High	54.55					4.11E+06	2.76E+05		
QSVFHE	KISGEWYSL		2	1308.72	1.92	High	61.07								
P00001	KISGEWYSL		2	1181.62	2.63	High	51.37							2.66E+07	1.97E+06
P00001	KNKNGETFL		1	1163.64	2.8	High									
P00001	KNKNGETFLM		2	1294.68	3.27	High	33.75							4.27E+07	
O18873	KPDSVTPM		1	874.43	2.13	High	23.16	4.80E+07	5.99E+06						
O18873	KPDSVTPMI		14	987.52	3.09	High	35.24	3.65E+09	3.37E+08						
O18873	KPDSVTPMIL		6	1003.51	3	High	34.95	7.93E+07	1.34E+07						
O18873	KPDSVTPMILK		4	1100.60	3.26	High	44.16	5.43E+07	3.40E+06						
O18873	KPDSVTPMILK		8	1228.70	4.59	High	33.61	1.12E+08	7.19E+06						
BSA-1351507	KOTALVELK		3	1142.71	2.15	High	40.06							8.36E+06	
P25774	KSNPRLP		4	1038.61	3.02	High	25.71	9.34E+06	6.81E+05	6.06E+06	6.81E+05				
P25774	KSNPRLPQSDVD		3	1454.76	3.29	High	30.16	1.18E+07	1.30E+06			1.34E+07	1.46E+06		
P25774	KSNPRLPQSDVWR		2	1796.94	2.66	High	36.58								
P31025	KTEDEPKY		5	1038.51	2.16	High									
P31025	KTEDEPKYATDGGKHVA		12	1773.88	5.08	High	16.74			1.86E+08	1.71E+07				
P31025	KTEDEPKYATDGGKHVAYIR		12	2319.21	6.27	High	27.35			2.77E+08	1.50E+07				
P31025	KTEDEPKYATDGGKHVAYIRSHVK		11	2770.46	7.6	High	31.18	1.16E+08	1.19E+06						
QSVFHE	KTRFPK		2	776.44	2.02	High	15.9								

P25774	NNPRLPSVD	27	1239.63	2.9	High	33.6	1.47E+08	1.13E+07	1.58E+08	1.66E+07	1.45E+08	1.30E+07	1.43E+08	2.40E+07
P25774	NNPRLPSVDVWR	17	1581.81	4.17	High	40.41	5.52E+07	2.69E+06	6.92E+07	2.69E+06	6.81E+07	3.75E+06	3.91E+07	6.00E+06
P25774	NNPRLPSVDVWRK	4	1838.95	4.33	High	35.57	3.61E+06							
P25774	NNPRLPSVDVWRKGCVT	3	1399.10	2.3	High	38.5			8.89E+06	9.71E+04			5.01E+06	1.78E+05
P25774	NRLPSVDWR	1	1370.72	2.9	High	39.22							1.29E+06	7.31E+04
P00001	NSLTFK	1	796.42	2.03	High	26.6							5.62E+07	6.30E+06
P00001	NSLTFKNGCNT	1	1285.58	1.96	High									
P25774	NNNGNNG	19	933.39	2.43	High	33.55	6.89E+08	6.69E+07	5.20E+08	5.39E+07	5.17E+08	9.03E+07	5.90E+08	8.49E+07
P25774	PSVDWR	3	874.41	2.8	High	41.23			3.68E+06	1.57E+05	1.83E+06	2.00E+04		
P25774	PSVDWRK	2	1331.54	2.18	High									
O18873	PEPQSVTPMILK	1	1213.61	3.5	High	39.66	2.96E+06							
O18873	PEPQSVTPMILK	8	1454.79	4.76	High	38.18	2.79E+07							
P31025	PKNLEALEDEFAKAGARLS	1	2230.15	2.79	High	48.48			1.98E+06	9.45E+04				
P25774	PGREDELV	2	1076.57	2.62	High	33.05	4.05E+06							
O18873	QCCNTV	1	904.46	2.01	High	35.15	6.32E+07	7.75E+06						
QSVFH6	QEGHVNIL	6	1022.56	2.91	High	43.55					4.60E+08	5.69E+07		
QSVFH6	QEGHVNILD	9	1137.59	2.59	High	42.36					3.63E+08	3.30E+07		
QSVFH6	QEGHVNILDLT	2	1353.72	2.41	High	54.96					1.80E+07	1.05E+06		
O18873	QEVPEKPS	1	944.46	1.92	High	18.74	4.01E+07	4.85E+06						
O18873	QEVPEKPS	15	1028.49	2.51	High	18.45	6.26E+09	8.62E+08						
O18873	QEVPEKPSDV	5	1127.56	2.65	High	24.45	1.01E+08	1.20E+07						
O18873	QEVPEKPSDVTPMI	5	1569.78	2.92	High	39.67	2.05E+08	7.05E+06						
O18873	QEVPEKPSDVTPMILK	10	1810.96	4.4	High	38.04	1.76E+08							
O18873	QNTVVLH	4	923.53	2.36	High	34.07	3.33E+08	3.49E+07						
O18873	QSPVR	8	963.88	2.12	High	19.57	3.43E+08	4.59E+07						
BS4-153307	QTAIVELK	1	914.62	1.51	High	46.86						1.45E+06		
P25774	QYDNGKD	7	1178.61	2.62	High	33.64	1.51E+07	5.20E+05	9.80E+06	1.13E+06	1.36E+07	1.18E+06	2.12E+07	2.53E+06
O18873	RDEQSOEAL	1	1172.55	2.9	High									
O18873	RDEQSOEAL	8	1301.60	3.18	High	23.25	8.05E+08	1.04E+08						
O18873	RDEQSOEALDEFRFSRAGKLN	1	2722.32	3.89	High	42.66	6.09E+06	4.18E+05						
O18873	RDEQSOEALDEFRFSRAGKLNQLE	3	3334.63	4.42	High	48.34	4.82E+07							
P31025	RDKNPNNLE	4	985.51	2.17	High	14.58			3.46E+07	5.12E+06				
P31025	RDKNPNNLE	4	1298.67	2.76	High	29.66			5.08E+07	3.39E+06				
P31025	RDKNPNNLEALEDEFAKAGARLS	2	2501.27	2.68	High	47.51			1.40E+07	8.13E+05				
P31025	REFEM	5	808.37	1.97	High	33.43			1.54E+09	1.83E+08				
P31025	REFEM	1xOxidation [M6]	8	824.36	1.18	High	22.31		2.11E+08	3.01E+07				
P31025	REFEMN	66	922.41	2.56	High	29.58			5.53E+10	7.84E+09				
P31025	REFEMN	1xOxidation [M6]	137	938.40	2.65	High	21.15		1.57E+09	3.19E+08				
P31025	REFEMNL	3	1035.49	2.52	High	42.91			3.32E+07	3.84E+06				
P31025	REFEMNL	1xOxidation [M6]	8	1051.49	2.68	High	33.23		1.85E+08	1.95E+07				
P31025	REFEMNLE	15	1164.54	2.92	High	40.74			2.66E+08	2.06E+07				
P31025	REFEMNLE	1xOxidation [M6]	28	1180.53	2.96	High	30.7		2.06E+09	2.01E+08				
QSVFH6	REPDSQALK	16	1399.67	3.31	High	18.03					4.34E+08	3.70E+07		
BS4-153307	RHPEVAVVYLK	22	1439.81	4.93	High	37.84	3.40E+07	8.54E+06	9.99E+06	8.88E+06	1.85E+07	2.15E+07		1.02E+07
P25774	RHPSF	2	790.40	2.08	High	30.02	7.70E+07						1.85E+08	
P25774	RILPSVDWR	10	1256.67	3.02	High	39.08	1.01E+07	3.75E+05	6.53E+06		1.04E+07	3.18E+05	4.23E+07	2.39E+06
P25774	RILPSVDWRK	1	1533.81	2.98	High	33.84							7.63E+06	
QSVFH6	RSNDIS	4	804.42	1.97	High	22.32					4.99E+08	2.84E+07		
QSVFH6	RSNDISK	1	932.52	2.47	High	17.87					1.07E+07	2.01E+06		
QSVFH6	RSNDISKS	6	1132.63	2.54	High	28.41					6.25E+08	1.23E+08		
QSVFH6	RSNDISKS	5	1189.65	2.9	High	29.86					6.35E+07	9.61E+06		
P25774	SDASYPK	42	930.42	2.57	High	21.88	1.74E+09	1.84E+08	1.17E+09	1.49E+08	1.29E+09	1.68E+08	1.87E+09	2.43E+08
P00001	SDCKEKEEGSDMR	3	1654.72	3.79	High	23.02							3.52E+07	1.18E+05
P31025	SDEEKQDV	16	1021.43	2.19	High	27			1.76E+10	4.18E+09				
P31025	SDEEKQDVSG	2	1078.45	2.38	High	27.32			1.04E+10	1.37E+07				
P31025	SDEEKQDVSGTWYK	4	1769.82	3.69	High	47.02			2.68E+07	7.64E+05				
P31025	SDEEKQDVSGTWYKAMTVDR	2	2443.14	2.7	High	52.15			1.49E+06	1.56E+05				
QSVFH6	SDCKEKEENG	1	1247.61	2.4	High	20.3					9.18E+06	1.08E+06		
QSVFH6	SDCKEKEENGSMR	10	1621.79	4.63	High	22.69					3.81E+08	4.50E+07		
QSVFH6	SDCKEKEENGSMRVF	1	1867.92	2.76	High	35.32					4.13E+06	1.38E+05		
P25774	SPFPHS	10	939.45	2.99	High	49.76	1.40E+09	1.25E+08	2.50E+09	1.01E+08	1.67E+09	1.52E+08	2.23E+09	1.79E+08
QSVFH6	SPVPHKENGKCT	1	1497.72	3.76	High									
QSVFH6	SGEWSYSL	1	1067.54	1.95	High	61.37								
O18873	SGKWYK	5	881.49	2.43	High	30.69	7.14E+08	7.59E+07						
O18873	SGKWYKAMT	5	1184.61	3.3	High	38.98	4.35E+07	2.98E+06						
P31025	SGTWYK	1	854.44	1.99	High	35.7			9.53E+07	2.54E+07				
P31025	SHVDCHYFP	2	1308.64	2.48	High	33.51			1.14E+08	1.23E+07				
P31025	SILPROSET	5	1143.64	2.48	High	32.07			2.50E+08	3.16E+07				
P00001	SNFDSK	7	810.40	2.11	High	26.19							1.13E+09	1.38E+08
P00001	SNFDSKIS	2	1010.52	2.24	High	38.15							5.96E+07	
P00001	SNFDSKISG	2	1067.54	2.65	High	37.71							1.77E+07	1.85E+06
QSVFH6	SNDSIK	52	1776.41	2.56	High	20.56					4.46E+10	1.04E+09		
QSVFH6	SNDSIKS	5	889.50	2.5	High	36.64					8.05E+07	1.01E+07		
QSVFH6	SNDSIKS	52	976.53	2.67	High	32.55					3.99E+10	6.70E+09		
QSVFH6	SNDSIKSG	13	1033.55	2.86	High	33.17					4.01E+08	5.26E+08		
QSVFH6	SNDSIKSGEWSYL	2	1182.60	2.71	High	34.65					1.06E+07	8.77E+05		
QSVFH6	SNDSIKSGEWSYLSDVAKKEIE	1	2937.55	2.79	High	63.61					2.43E+06	7.46E+05		
QSVFH6	SNDSIKSGEWSYLSDVAKKEIEENG	2	3611.83	3.49	High	61.58					1.98E+06			
P25774	SNPNRILP	42	930.31	2.51	High	30.26	1.78E+09	1.90E+08	1.56E+09	1.57E+08	1.68E+09	2.10E+08	1.43E+09	3.05E+08
P25774	SNPNRILPD	35	1025.54	2.26	High	29.32	4.82E+08	3.97E+07	4.30E+08	4.37E+07	4.09E+08	4.19E+07	6.60E+08	7.14E+07
P25774	SNPNRILPDS	45	1112.57	2.93	High	28.84	6.29E+08	1.02E+08	5.85E+08	6.13E+07	7.55E+08	1.19E+08	8.26E+08	8.90E+07
P25774	SNPNRILPDSVD	40	1326.86	3.24	High	33.78	7.72E+08	5.57E+07	5.93E+08	6.02E+07	5.98E+08	1.58E+07	5.75E+08	8.94E+07
P25774	SNPNRILPDSVWR	43	1568.85	3.85	High	34.38	4.37E+08	2.13E+07	3.71E+08	1.56E+07	4.03E+08	2.69E+07	3.05E+08	4.07E+07
P25774	SNPNRILPDSVWRK	8	1797.89	3.84	High	40.57	1.14E+07	7.35E+05	1.48E+07	4.49E+05	1.22E+07	9.94E+05	8.80E+06	1.30E+06
P25774	SNPNRILPDSVWRK	30	1925.98	4.93	High	35.85	1.30E+08	3.53E+06	9.90E+07		1.40E+08	8.62E+06	1.31E+08	8.89E+06
P25774	SNPNRILPDSVWRKGCVT	14	2286.13	3.99	High	36.63	4.49E+07	2.50E+06	7.93E+07	1.78E+06	4.88E+07	3.57E+06	7.99E+07	6.48E+06
O18873	SRAGLNQLE	5	1357.74	3.99	High	29.99	9.30E+07	7.10E+06						
435476	SRSGGGGGGGLSGSGSSR	2	1475.73	2.46	High	19.39			2.19E+06	1.70E+06	4.33E+06	9.74E+05		
435476	SRSGGGGGGGLSGSGSSRSS	3	1649.80	3.12	High	19.12			2.77E+06	2.99E+06	7.25E+06	1.67E+06		
435476	SRSGGGGGGGLSGSGSSRSSY	5	1812.86	4.06	High	20.03			3.60E+06	4.65E+06	6.06E+06	1.36E+07	1.79E+06	
P31025	STESILPQSG	2	1230.67	2.59	High									
P25774	SVWRK	2	919.46	2.03	High	21.79	3.54E+07	2.75E+05						
O18873	SVTNTLK	5	888.53	2.24	High	35.13			2.80E+08	2.97E+07				
P31025	TADEGKHWYAIR	1	1400.76	2.34	High	25.62					2.41E+06	2.31E+05		
O18873	TADEQVPEKPS	5	1315.60	2.78	High	20.78	1.56E+08	1.95E+07						
O18873	TADEQVPEKPSDVTPMI	3	1856.89	3.19	High	40.63	1.89E+07	1.84E+05						
O18873	TADEQVPEKPSDVTPMILK	7	2098.07	4.92	High	38.95	7.04E+07	2.90E+06						
P25774	TAQYHONGKD	1	1497.76	2.07	High	47.36							3.06E+06	2.16E+05
P25774	TAQYHONGKDSDASYPK	1	2409.16	2.57	High	46.63								

[illegible]

Figure E1: Sequence alignment. Lcn-1 was aligned with Can f 1 (A) and MUP with Fel d 4 (B) using LALIGN, (SIB Swiss Institute of Bioinformatics Lausanne Switzerland). The sequences share roughly 60% of their amino acids. Sequences of the N-terminal peptides of Lcn-1 and Can f 1 used in Ca^{2+} mobilization assays are included.

Figure E2: Expression of FPR1, FPR2 and FPR3 on DC. Day 6 human monocyte derived DC were collected for mRNA and cDNA preparation and subjected to qPCR for FPR1, 2 and 3 as indicated (A) or stained for FPR1, FPR2 or FPR3 as indicated and subjected to FACS analysis. ***: $P < .001$.

Figure E3: FPR3 expression analysis of Hek293 cells. A: Hek 293 cells stably transfected with an FPR3 encoding plasmid (solid line) or un-transfected (dashed line) were stained for FPR3 or with an isotype control (dotted line). B: stably FPR3 transfected Hek293 cells were stained with rabbit polyclonal anti human FPR3 and Alexafluor488-conjugated goat anti rabbit antibody and subjected to confocal microscopy. One plane of the confocal acquisition is shown. This cell line was used for Ca^{2+} - mobilization experiments.

Figure E4: Ca^{2+} mobilization measurements in H293-FPR3 cells with additional lipocalin allergens. 25 μl (10 μM) Cathepsin S digests of lipocalin allergens Fel d 7, Equ c 1, Rat n 1 and Lcn-1 (A) or Fel d 1 (B) were used to induce FPR3 signaling.

Figure E5: Response of naïve $\text{CD4}^+\text{CD45RA}^+$ naïve T cells to DC with impaired IL-12 production. DC were treated with anti IL-12 antibody (A-D) or WKYMMv (E-H) to reduce IL-12 production (A and E) in DC. Supernatants of co-cultures with naïve $\text{CD4}^+\text{CD45}^+$ T cells were analyzed for IFN γ (B and F), IL-13 (C and G) and IL-4 (D and H). ns: $P > .05$, *: $P < .05$, ***: $P < .001$.

Figure E6: Representative FACS analysis of DC silenced for FPR3. DC silenced for FPR3 (solid line) or treated with control siRNA (dashed line) were subjected to intracellular staining with FPR3 and FACS analysis. When $\geq 60\%$ of cells were silenced for FPR3 expression they were used for further experiments.

Figure E7: Cytokine production of allergen treated DC silenced for FPR3 expression and co-cultured CD4^+ T cells. A: Supernatants of DC silenced for FPR3 expression or controls, activated with lipocalins as indicated were analyzed for IL-12 p70 content by ELISA. B: IL-13, C: IL-4 and D: IFN γ content in supernatants of day 5 co-cultures of DC

silenced or not for FPR3 expression and treated with Fel d 7, Rat n 1 or Equ c 1 as indicated, and naïve CD4⁺CD45RA⁺ T cells. E: IL-13 and F: IL-4 content in supernatants of day 5 co-cultures of DC silenced or not for FPR3 expression and untreated (iDC) or treated with Lcn-1 or MUP as indicated, and naïve CD4⁺CD45RA⁺ T cells. ns: P > .05, *: P < .05, **: P < .01, ***: P < .001.

Figure E8: Cytokine production of allergen treated DC pretreated with WRW4 and co-cultured CD4⁺CD45RA⁺ T cells. A: Supernatants of DC pretreated with WRW4 or controls and activated with lipocalins as indicated were analyzed for IL12 p70 content by ELISA. B: IL-13, C: IL-4 and D: IFN γ content in supernatants of day 5 co-cultures of DC pretreated or not with WRW4 and treated with Fel d 7, Rat n 1 or Equ c 1 as indicated, and naïve CD4⁺CD45RA⁺ T cells. E: IL-13 and F: IL-4 content in supernatants of day 5 co-cultures of untreated DC (iDC) or DC treated with Lcn-1 or MUP and pretreated or not with WRW4 as indicated, and naïve CD4⁺CD45RA⁺ T cells. ns: P > .05, **: P < .01, ***: P < .001.

Figure E9: Characterization of DC with impaired FPR3 activation and activated with additional lipocalin allergens.

DC silenced for FPR3 expression or treated with the FPR3 antagonist WRW4 and treated with lipocalins as indicated were analyzed for IL6 (A-D) and IL10 (C and D, right part of graphs) concentrations in supernatants by ELISA, and expression of accessory molecule CD86 (E,F) by FACS. Supernatants of co-cultures of DC treated as indicated with naïve CD4⁺CD45RA⁺ T cells were analyzed for IL10 by ELISA (G and H). ns: P > .05, *: P < .05, **: P < .01, ***: P < .001.

Table E1: Identification of peptides derived from Cathepsin S digests of Can f 1, Lcn-1, Fel d 4 and MUP using HPLC coupled to a mass spectrometer.

Peptides are alphabetically ordered, yellow: Cathepsin S derived peptides, green: peptides approximately spanning the synthetic Lcn-1 peptide and orange: peptide approximately spanning the synthetic Can f 1 peptide used in the initial Ca²⁺ mobilization assay.