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Allergens as Immunomodulatory Proteins: The Cat Dander Protein Fel d 1 Enhances TLR Activation by Lipid Ligands

Jurgen Herre,^{*,†} Hans Grönlund,[‡] Heather Brooks,[†] Lee Hopkins,[†] Lisa Waggoner,[§] Ben Murton,[¶] Monique Gangloff,[¶] Olaniyi Opaleye,[¶] Edwin R. Chilvers,^{*} Kate Fitzgerald,[§] Nick Gay,[¶] Tom Monie,[¶] and Clare Bryant[†]

Allergic responses can be triggered by structurally diverse allergens. Most allergens are proteins, yet extensive research has not revealed how they initiate the allergic response and why the myriad of other inhaled proteins do not. Among these allergens, the cat secretoglobulin protein Fel d 1 is a major allergen and is responsible for severe allergic responses. In this study, we show that similar to the mite dust allergen Der p 2, Fel d 1 substantially enhances signaling through the innate receptors TLR4 and TLR2. In contrast to Der p 2, however, Fel d 1 does not act by mimicking the TLR4 coreceptor MD2 and is not able to bind stably to the TLR4/MD2 complex in vitro. Fel d 1 does, however, bind to the TLR4 agonist LPS, suggesting that a lipid transfer mechanism may be involved in the Fel d 1 enhancement of TLR signaling. We also show that the dog allergen Can f 6, a member of a distinct class of lipocalin allergens, has very similar properties to Fel d 1. We propose that Fel d 1 and Can f 6 belong to a group of allergen immunomodulatory proteins that enhance innate immune signaling and promote airway hypersensitivity reactions in diseases such as asthma. *The Journal of Immunology*, 2013, 191: 000–000.

The innate immune system is intrinsically linked with allergy. Pattern recognition receptors are involved in allergen sampling, nonspecific allergen elimination, and the maintenance of immune tolerance and homeostasis in response to allergens (1). An allergic response can be triggered by many different stimuli, for example, grass pollen, animal dander, foods, insect venoms, pharmaceutical products, chemicals, latex, and metals (2). The exact mechanisms by which major allergens are recognized by the host are largely unknown, but recent work suggests that TLRs play a crucial role in the response to two common allergens, house dust mite protein Der p 2 (3–5) and the metal nickel (6).

Der p 2 is a lipid-binding protein that sensitizes ligand-induced signaling through TLR4 and TLR2 (3, 4, 7). TLR4, in combination with MD2 and CD14, recognizes bacterial LPSs, and TLR2, in a heterodimer with either TLR1 or TLR6, recognizes di- and tri-

acylated lipoproteins (8) and lipoteichoic acid (LTA). TLR5 recognizes the bacterial protein flagellin (9, 10). Ligand recognition by TLRs then activates innate immune signaling pathways (11). Both MD2 and Der p 2 belong to a small family of lipid-binding proteins that have a β -sandwich or cup-type fold (12). These proteins recognize lipid by intercalating their acyl chains into the hydrophobic core of the β -sandwich. Thus, one potential mechanism by which Der p 2 enhances TLR4 signaling is to mimic MD2 by binding to TLR4. The Der p 2/TLR4 protein complex may then signal similarly to MD2/TLR4 to activate innate immune signaling (4). In mouse models of allergic asthma the effects of Der p 2 are markedly reduced in TLR4 knockout mice and can be prevented in wild-type mice by administration of a TLR4 antagonist (7). House dust mite extracts carrying flagellin can induce TLR5-dependent allergic responses in mice, although the molecular mechanism by which this occurs is unclear (5). Nickel sensitization in humans results from direct, lipid-independent activation of TLR4 by Ni^{2+} . Receptor activation is dependent on the presence of two histidine residues, H456 and H458, which coordinate the Ni^{2+} atom (or other metal ions such as Co^{2+}), promoting TLR4 dimerization and subsequent receptor activation. Murine TLR4 lacks these histidines and consequently is not activated by nickel (6, 13).

Another clinically important allergen is the cat dander protein Fel d 1, which is the most common cause of severe allergic responses to cats in humans (14). In contrast to Der p 2, this allergen has an entirely α -helical structure (15) and is thus unlikely to act as a mimetic of MD2. Fel d 1 can bind to the mannose receptor, but immune signaling is not initiated following engagement of this receptor (16). Thus, the mechanism by which this protein initiates an allergic response remains unclear.

In this study we propose a mechanism by which Fel d 1 is recognized by the host to activate immune signaling. Fel d 1 enhances LPS- and LTA- but not flagellin-induced TLR signaling. Unlike Der p 2, the mechanism for Fel d 1 enhancement of LPS-induced TLR4/MD2 activation does not involve the protein binding to the TLRs, but it does require the presence of CD14. The dog dander protein Can f 6 (17), a structurally distinct allergen from

^{*}Department of Medicine, University of Cambridge School of Medicine, Cambridge CB2 0QQ, United Kingdom; [†]Department of Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, United Kingdom; [‡]Department of Clinical Neuroscience, Karolinska Institute, SE-171 76 Stockholm, Sweden; [§]Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605; and [¶]Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom

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Address correspondence and reprint requests to Dr. Tom Monie or Dr. Clare Bryant, Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1QW, U.K. (T.M.) or Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, U.K. (C.B.). E-mail addresses: tpm22@cam.ac.uk (T.M.) or ceb27@cam.ac.uk (C.B.)

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Abbreviations used in this article: bFel d 1, baculovirus Fel d 1; BMDM, bone marrow-derived macrophage; IMP, immunomodulatory protein; LTA, lipoteichoic acid; rFel d 1, recombinant Fel d 1.

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Fel d 1 and a member of the lipocalin family of allergens, also enhances LPS-induced activation of TLR4 signaling, although, unlike Fel d 1, this protein has some MD2-independent effects. We propose, therefore, that animal allergen proteins form a novel class of IMPs that enhance TLR signaling and hence play a critical role in initiating allergic responses. The mechanism for TLR enhancement of signaling involves formation of a complex of bacterial lipids, such as LPS, with allergen and suggests that inhibitors of TLR2 and TLR4 may represent a new class of therapeutic compounds for the treatment of common allergic diseases.

Materials and Methods

Protein production

Fel d 1 was expressed and purified in *Escherichia coli*. Histidine-tagged and washed inclusion body Fel d 1 was solubilized in guanidine and loaded on a Ni²⁺-chelate affinity column. The isolated recombinant Fel d 1 (rFel d 1) preparation was further purified by size exclusion chromatography and equilibrated in PBS. rFel d 1 was subsequently purified from endotoxins on a Detoxi-Gel (Pierce, Rockford, IL) according to the manufacturer's instructions and stored at -80°C until required (15).

For expression in baculovirus, MultiSite Gateway cloning (Invitrogen) was used to produce an N-terminal receptor for advanced glycation end products secretion signal followed by Fel d 1 (chains 2 and 1) and C-terminal V5 epitope and 6-His tags in a pDEST48 vector (Invitrogen). Bacmids were generated in DH10Bac cells (Invitrogen) following the manufacturer's protocol, purified, and transfected into Sf9 cells to produce a P1 virus stock, which was subsequently amplified and the titer determined to give bFel d 1 virus.

Sf9 cells at a density of 1 million/ml were infected with bFel d 1 virus (multiplicity of infection of 1) for 3 d. Clarified supernatants were filtered following supplementation with ammonium sulfate to a final concentration of 300 mM. bFel d 1 was recovered using a HiTrap Butyl FF column (GE Healthcare) equilibrated in 300 mM ammonium sulfate and 25 mM Tris-HCl (pH 8). Protein was eluted in 25 mM Tris-HCl (pH 8). Fractions containing bFel d 1 were pooled and further purified using Ni-NTA resin before being eluted in 150 mM NaCl, 25 mM Tris-HCl (pH 8), and 300 mM imidazole. Eluted fractions were concentrated and further purified on an S75 column that had been washed in 1 M NaOH and equilibrated in tissue culture grade PBS to minimize LPS contamination. rFel d 1 was tested for endotoxin contamination using the Endosafe-PTS assay (Charles River Laboratories, Margate, U.K.). This assay system is based on the *Limulus* amoebocyte lysate assay utilizing Food and Drug Administration-licensed disposable cartridges with detection limits from 0.01 to 10 endotoxin units/ml.

Can f 6 was produced as previously described (17). Picia-derived Fel d 1 and Der p 2, as well as natural cat allergen preparations, were from Indoor Biotechnologies (Charlottesville, VA).

Biotinylated LPS pull-down

Biotinylated ultrapure *E. coli* 011:B4 LPS (1 mg/ml; InvivoGen) was immobilized on 20 µl Strep-Tactin Sepharose bead slurry (IBA). Additional proteins were added to the beads in 10-µl aliquots at 1 mg/ml concentration and incubated at room temperature with agitation for 20 min. Beads were recovered by centrifugation and washed three times in PBS plus 0.05% Tween 20. Beads were boiled in SDS-PAGE sample loading buffer with 5 mM DTT to release bound proteins and the samples were analyzed by SDS-PAGE.

TLR4/MD2 expression and purification

Human TLR4 ectodomain (E27-K631) and human MD2 (Q19-N160) fused to a thrombin-cleavable protein A tag were coexpressed in *Trichoplusia ni* cell culture. The complex was purified via IgG-Sepharose 6 (Amersham Pharmacia Biotech) affinity purification, followed by on-bead thrombin cleavage, cation exchange, and size exclusion through Sepharose 200. The protein was concentrated to 2 mg/ml.

Native PAGE gel

Purified samples of TLR4/MD2, Fel d 1, CD14, OVA, and LPS in PBS were used at a concentration of 1 mg/ml. A mixture of 1 µl of each component was made and incubated for 30 min at room temperature. Native loading buffer (1 µl) was added to the mixture and 2 µl final mixture was loaded onto 6% native-PAGE gel, run, and silver stained.

Transient transfection analysis

HEK293 cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. HEK293 cells were transfected as previously described (18). Briefly, cells were seeded at 3×10^4 /well in a 96-well plate and transiently transfected 2 d later. TLR2, TLR4, TLR5, and CD14 were cloned into pcDNA3 and MD2 was subcloned into pEFIREs. Expression vectors containing cDNA encoding TLR4, MD2, and CD14 (1 ng/well of each), a NF-κB transcription reporter vector encoding firefly luciferase (5 ng/well pNF-κB-luc; Clontech), and a constitutively active reporter vector encoding *Renilla* luciferase (5 ng/well pHRG-TK; Promega), together with empty vector to ensure an optimal amount of DNA, were mixed with jetPEI (Polyplus Transfection) according to the manufacturers' instructions. TLR2 was cotransfected with CD14 and reporter plasmids. TLR5 was cotransfected with reporter plasmids. After 48 h, cells were stimulated with KDO2-lipid A (a gift from Professor C. Raetz, Duke University, Durham, NC) diluted in DMEM supplemented with 0.1% FCS in the presence, or absence, of Fel d 1 protein. TNF-α stimulation (1 ng/ml) was used as a positive control. The cells were washed with PBS, lysed, and luciferase activity was quantified using the Dual-Luciferase kit (Promega) according to the manufacturer's instructions.

Bone marrow-derived macrophage stimulation

Mice were bred under specific pathogen-free conditions at Harlan UK or the Department of Veterinary Medicine, University of Cambridge (Cambridge, U.K.). Mice were housed in isolators or in filter-top cages and provided with sterile water and food ad libitum. TLR4^{-/-} mice on a C57BL6/J background were described previously (19). C57BL6/J mice were purchased from Harlan UK.

Bone marrow-derived macrophages (BMDMs) were isolated from femurs and tibiae of mice killed by cervical dislocation, then cultured in BMDM medium (RPMI 1640 medium supplemented with 10% [v/v] FCS, 2 mM glutamine, 5% [v/v] horse serum, 1 mM sodium pyruvate, and 10 µg/ml gentamicin) in petri dishes. For maintenance of BMDMs in culture, this medium was further supplemented with 20% (v/v) of supernatant taken from L929 cells (a murine M-CSF-producing cell line) (20, 21). For experiments, cells were plated onto 96-well plates at a plating density of 2×10^5 cells/well. Cells were stimulated with ligand in the presence or absence of Fel d 1. The small-molecule TLR4 inhibitor CRX-526 (22) was provided by GlaxoSmithKline Vaccines (Hamilton, MT) as a lyophilized powder. It was resuspended at a concentration of 1 mg/ml in a diluent of endotoxin-free sterile water containing 2% glycerol and 0.2% triethanolamine, at a pH of 7–7.4, using a Covaris sonicator and repeated cycles of heating and vortexing. Resuspension was performed at GlaxoSmithKline (Stevenage, U.K.). The final solution was stored at 4°C.

Generation of PBMCs

Human peripheral blood granulocytes were obtained from healthy normal human donors according to the method of Haslett et al. (23) under the study protocol (UK06/Q0108/281) titled "The Inflammatory Response of Human Leukocytes." Briefly, plasma was separated by centrifugation at room temperature and the erythrocyte/leukocyte layer was sedimented by the addition of 6% dextran and diluted with warmed PBS. The suspension was allowed to sediment before the upper leukocyte-rich layer was removed and pelleted by centrifugation. The pellet was resuspended in platelet-poor plasma and underlayered with freshly prepared Percoll gradient. Following centrifugation the monocyte layer was harvested and further purified with CD14 MACS beads (Miltenyi Biotec) as per the manufacturer's protocol. Resultant cells were plated in 96-well plates in RPMI 1640 enriched with L-glutamine, penicillin/streptomycin, 10% FBS, and GM-CSF (R&D Systems, Abingdon, U.K.; 100 ng/ml) and cultured for 7 d before use.

Measurement of cytokine production

To determine cumulative TNF-α production, supernatants were taken at 24 h posttreatment and stored at -80°C until analyzed with the DuoSet ELISA development system (R&D Systems, Abingdon, U.K.).

Statistics

Data for HEK-transfected cells are presented as representative experiments from an average of at least three repeats (18, 24). BMDM data are presented as mean data from at least three separate biological repeat experiments (25). Graphs were generated using GraphPad Prism and the data analyzed using one-way ANOVA and Tukey multiple comparison test for significant differences. Results are expressed as the means ± SEM of *n* separate experiments. A *p* value <0.05 was regarded as statistically significant.

Results

Fel d 1 enhances lipid-induced signaling through both TLR2 and TLR4

To determine whether Fel d 1 (similarly to Der p 2) is able to modulate innate immune signaling, we expressed and purified rFel d 1. This protein was made in *E. coli* and possessed <0.5 ng LPS/mg protein (data not shown). The effect of rFel d 1 on TLR4/MD2 signaling was tested in a reconstituted HEK293 cell-culture assay. LPS, as expected, induced a concentration-dependent increase in relative luciferase activity, but in the presence of Fel d 1 (10 ng/ml) the response to LPS was increased by ~15-fold (Fig. 1A). Next, we tested whether Fel d 1 also enhanced signaling through TLR2 in response to the ligand LTA. We found that LTA-induced TLR2 signaling was also enhanced in the presence of Fel d 1 (Fig. 1B). To rule out the possibility that Fel d 1 enhanced signaling from cell surface receptors in a nonspecific manner, we carried out equivalent assays with both transiently transfected and endogenous TLR5. Fel d 1 did not modify signaling induced by the TLR5 protein ligand flagellin in either instance (Fig. 1C, Supplemental Fig. 1). This suggests that the activity of Fel d 1 to enhance TLR signaling is restricted to those receptors that recognize lipids.

Fel d 1 potentiates the production of proinflammatory cytokines in primary immune cells

The rFel d 1 used in this study causes airway hyperresponsiveness in mice and children by unknown mechanisms (26, 27). To determine whether Fel d 1 enhances innate responses in cells other than transfected HEK293 cells, proinflammatory cytokine (TNF- α) production was measured from murine BMDMs stimulated with LPS, LTA, or the di- and triacylated lipopeptides Pam2CSK4 and Pam3CSK4. We required higher concentrations of Fel d 1 to stimulate the murine macrophages compared with the concentration required for activation of the HEK293 cells transfected with TLR4/MD2/CD14. These data are very similar to those from

Trompette et al. (4), where higher concentrations of Der p 2 were required to activate mouse macrophages than for HEK cells transfected with TLR4/MD2/CD14. Fel d 1 enhanced TNF- α production in response to all four bacterial lipid ligands (Fig. 2A–C). Fel d 1 enhancement of LPS-induced TNF- α production was inhibited by the TLR4 antagonist CRX-526, confirming that Fel d 1 sensitizes TLR4 signaling in monocyte/macrophage-like cells (Fig. 2D). In primary human PBMCs Fel d 1 also enhanced LPS-induced TNF- α production in six separate donors (Fig. 2E). Human cells, as expected, required 5- to 10-fold lower concentrations of LPS for TNF- α stimulation in comparison with mouse BMDMs.

In contrast to our *E. coli*-produced rFel d 1 protein used in these experiments, natural Fel d 1 is glycosylated. A recent study showed that sulfated galactose residues present in these glycans bind to mannose receptors and cause Fel d 1 to be internalized (16). To determine whether the glycosylation status of Fel d 1 influences the sensitization of TLR signaling, we compared the properties of a partially glycosylated Fel d 1 produced in the yeast *Pichia*, glycosylated natural Fel d 1 depleted of LPS, as well as our own *Baculovirus*-produced Fel d 1 in terms of their respective sensitizing effects on TLR4 signaling in BMDMs. These protein preparations all enhanced TLR4 signaling in BMDMs in a similar fashion to the *E. coli*-derived Fel d 1, showing that the TLR-sensitizing effects of this protein are independent of glycosylation (Fig. 2F) and thus mannose receptor activity. Fig. 2A, 2D, and 2F include TLR4-deficient cells as controls. In each case the signal enhancement seen in the presence of Fel d 1 was abolished in TLR4^{-/-} cells, demonstrating that the observed response depends entirely on this receptor.

The enhancement of TLR4 signaling mediated by Fel d 1 is dependent on both CD14 and MD2

We next determined whether, similar to Der p 2, Fel d 1 could sensitize TLR signaling in the absence of MD2 or CD14. Using

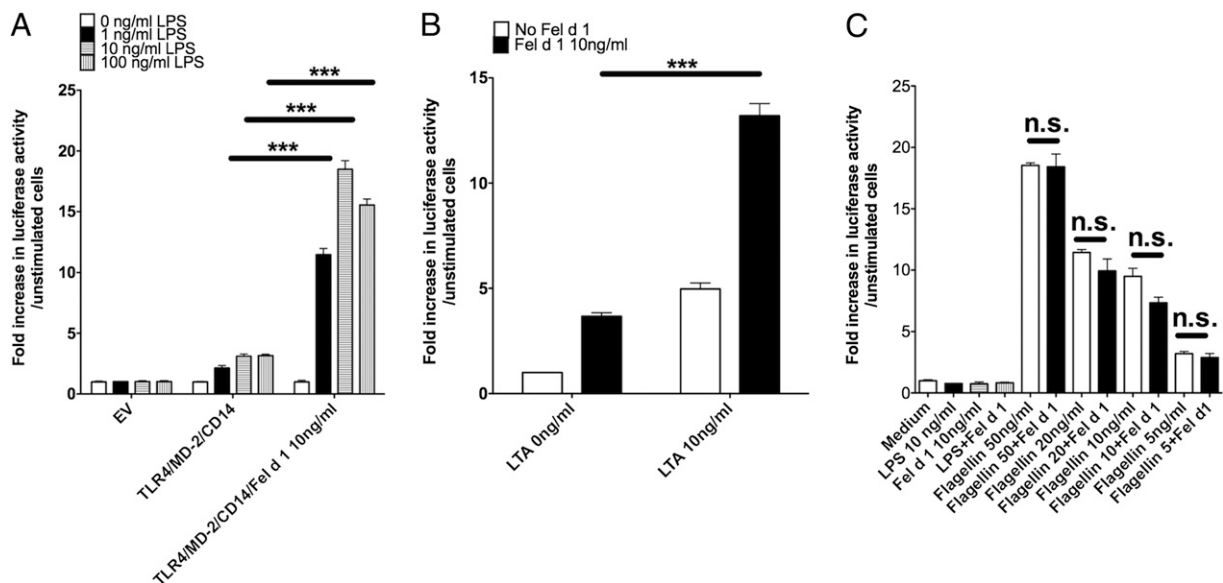


FIGURE 1. Fel d 1 enhances signaling by ligands of TLR2 and TLR4 but not TLR5. **(A)** HEK293 cells transiently transfected to express TLR4, CD14, MD2, an NF- κ B-luciferase reporter (pNF- κ B-luc) and a constitutively active luciferase reporter control (pRG-TK) were treated for 6 h with LPS (1–100 ng/ml) in the presence or absence of Fel d 1 (10 ng/ml). Cells were lysed and the NF- κ B-luciferase reporter was read on a FLUOstar Omega (BMG Labtech) luminometer. Data are normalized against the unstimulated control. The graph is one representative experiment from four separate experiments. **(B)** HEK293 cells transiently transfected with TLR2, CD14 pNF- κ B-luc, and pRG-TK were stimulated with LTA at 10 ng/ml in the presence and absence of Fel d 1 (10 ng/ml). Data are normalized against the unstimulated control. Data are shown from one representative experiment from three separate experiments. **(C)** HEK293 cells were transiently transfected with TLR5, NF- κ B-luc, and pRG-TK, then stimulated with flagellin (5–50 ng/ml) in the presence or absence of Fel d 1 (10 ng/ml). Data normalized against unstimulated control are shown as one representative experiment from five separate experiments. Data represent means $\pm$ SEM of a representative experiment from four to five repeat experiments. *** p < 0.001.

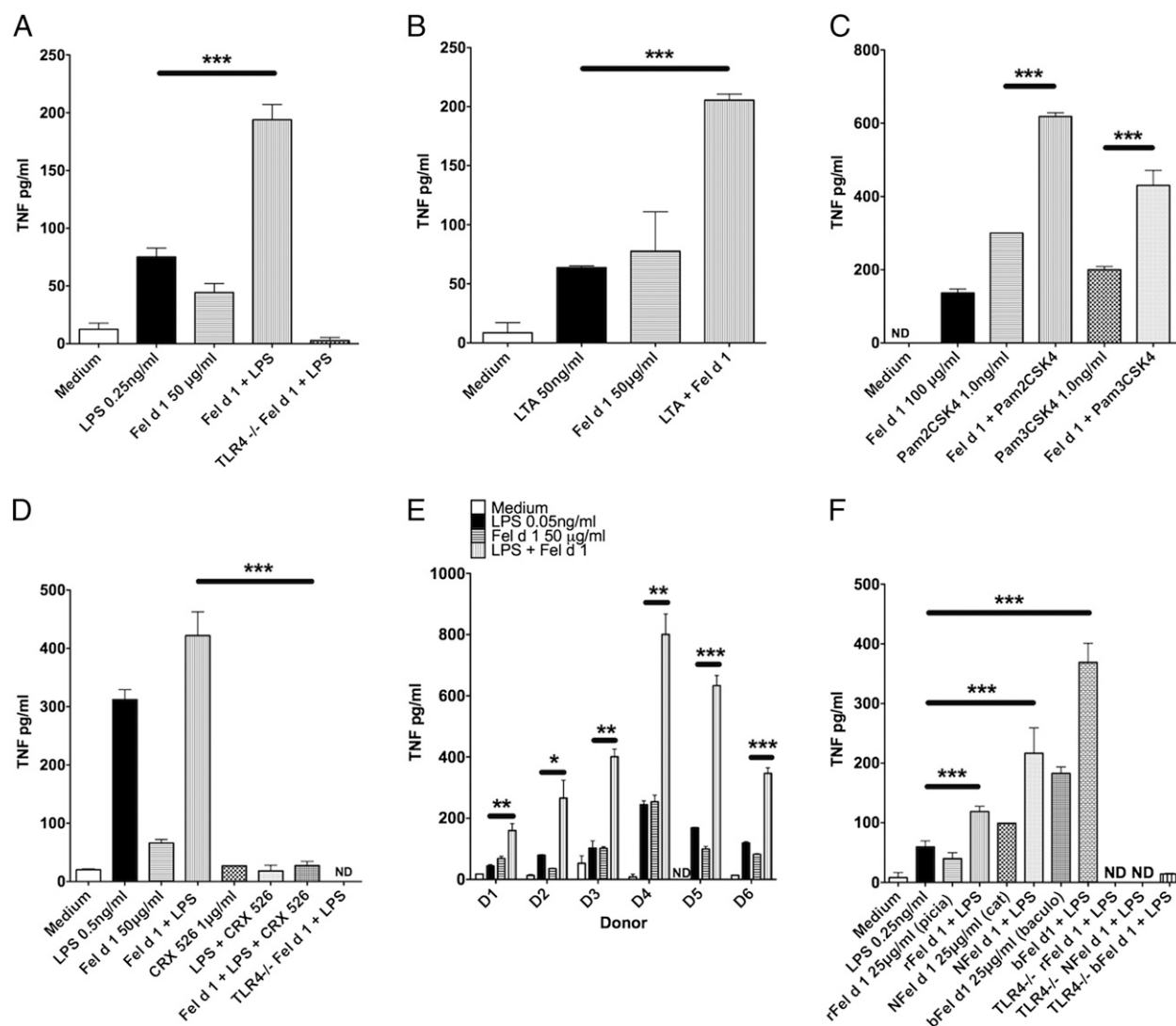


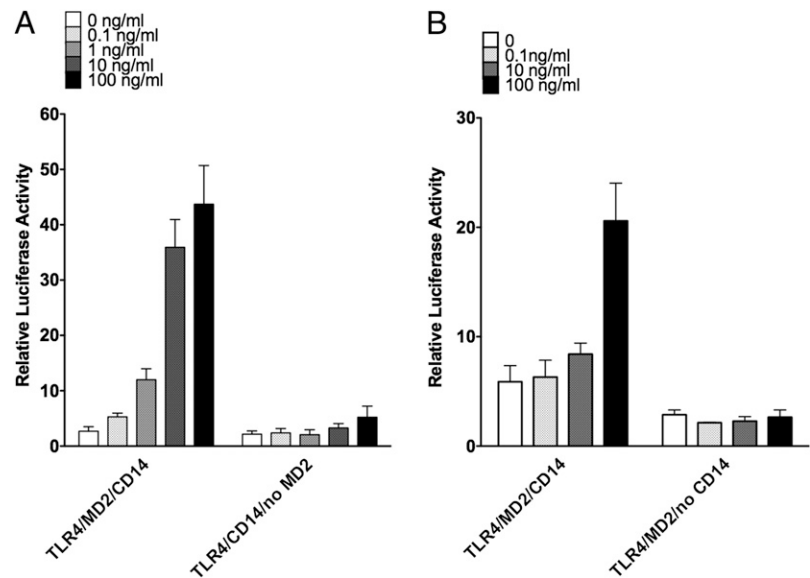
FIGURE 2. Fel d 1 enhances expression of the proinflammatory cytokine TNF- α in primary cells of the innate immune system. **(A)** BMDMs from wild-type and TLR4^{-/-} C57BL/6J mice were treated for 24 h with LPS at 0.5 ng/ml, Fel d 1 at 50 μ g/ml, or both, as indicated. TNF- α levels were measured by an ELISA. Data from five separate experiments were pooled and expressed as means $\pm$ SEM. **(B)** Wild-type BMDMs were treated with LTA at 50 ng/ml with or without Fel d 1 at 50 μ g/ml for 24 h. TNF- α levels were measured by an ELISA. Data from three separate experiments were pooled and expressed as means $\pm$ SEM. **(C)** Wild-type BMDMs treated for 24 h with di- and triacylated lipid activators of TLR1/2 and TLR2/6 (Pam3CSK4 at 1.0 ng/ml, Pam2CSK4 at 1.0 ng/ml) with or without Fel d 1 at 100 μ g/ml. TNF- α levels were measured by an ELISA. Data from two separate experiments were pooled and expressed as means $\pm$ SEM. **(D)** Wild-type and TLR4^{-/-} BMDMs were pretreated with the TLR4 small molecule inhibitor CRX-526 for 60 min and then treated for 24 h with LPS at 0.5 ng/ml with or without Fel d 1 at 50 μ g/ml. CRX-526 was maintained throughout the stimulation period. TNF- α levels were measured by an ELISA. Data from two separate experiments were pooled and expressed as means $\pm$ SEM. **(E)** PBMCs derived from healthy donors as part of an ethically approved research program were treated for 24 h with LPS at 0.05 ng/ml with or without Fel d 1 at 50 μ g/ml. Donors are shown separately to demonstrate interindividual variation. TNF- α levels were measured by an ELISA. Data are shown from six healthy donors. **(F)** BMDMs from wild-type and TLR4^{-/-} mice treated with LPS 0.25 ng/ml and three alternative sources of Fel d 1 (recombinant protein expressed in the yeast *Pichia pastoris* [rFel d 1], natural protein from the cat depleted of LPS [NFel d 1], and bFel d 1) each at 25 μ g/ml. TNF- α levels were measured by an ELISA. Data from three separate experiments were pooled and expressed as means $\pm$ SEM. * p < 0.05, ** p < 0.005, *** p < 0.001.

HEK293 cells transfected with TLR4 and CD14 in the absence of MD2, we observed that Fel d 1 induced only a small increase in signaling (1.9-fold) even at the highest concentration tested (100 ng/ml), compared with a 16-fold increase when MD2 was present (Fig. 3A). A similar result was seen when CD14, an extrinsic membrane protein required to deliver LPS to TLR4/MD2, was absent (Fig. 3B). These results show that the bioactivity of Fel d 1 in upregulating LPS signaling is dependent on the presence of both MD2 and CD14. These data also show that Fel d 1, unlike Der p2, cannot substitute for MD2 (4) or for CD14.

Fel d 1 does not form a stable complex with TLR4/MD2 but does bind to LPS

Our data suggest that, unlike Der p 2, Fel d 1 does not mimic MD2 and act as a coreceptor for TLR4, but rather it enhances signaling by a different mechanism. One possibility is that this allergen facilitates transfer of LPS to CD14 and MD2. To test this hypothesis we asked first whether either recombinant or natural Fel d 1 is able to form a complex in vitro with TLR4/MD2 or TLR4 alone. To do this we used native PAGE and visualized the proteins by silver staining. Fel d 1 preparations were highly pure and showed no contaminating

FIGURE 3. Signal enhancement by Fel d 1 requires the TLR4 coreceptor MD2 and CD14. **(A)** HEK293 cells were transfected with components of the TLR4 signaling complex—either TLR4, MD2, and CD14, or TLR4 and CD14, an NF- κ B-luciferase reporter (pNF- κ B-luc) and a constitutively active luciferase reporter control (phRG-TK)—and then stimulated with Fel d 1 (0.1–100 ng/ml). The graph is one representative experiment from three separate experiments. **(B)** HEK293 cells were transfected with components of the TLR4 signaling complex—either TLR4, MD2, and CD14, or TLR4 and MD2, an NF- κ B-luciferase reporter (pNF- κ B-luc) and a constitutively active luciferase reporter control (phRG-TK)—and then stimulated with Fel d 1 (0.1–100 ng/ml). The graph is one representative experiment from three separate experiments.



bands (Fig. 4A, lane 1, 4B, lane 2). Addition of LPS alone to TLR4/MD2 (Fig. 4A) or to TLR4 alone (Fig. 4B) induced receptor dimerization and oligomerization as shown by changes in the migration of the TLR4-containing species. However, we were unable to observe formation of a complex between rFel d 1 and TLR4/MD2 (Fig. 4A) or natural Fel d 1 and TLR4 (Fig. 4B) in either the presence or absence of LPS. Fel d 1 can, however, interact directly with LPS, as streptavidin-coated beads were able to precipitate significant amounts of Fel d 1, but not the control GST, when coincubated with biotinylated LPS (Fig. 4C). Fel d 1 showed no nonspecific binding to the streptavidin-coated beads.

Lipid presentation may be a common mechanism for the action of animal allergens

Given that both Der p 2 and Fel d 1 enhance TLR signaling, we wondered whether lipid presentation by different allergen proteins could provide a more generic mechanism for animal allergen recognition in the host. To test this hypothesis, we generated a structurally unrelated recombinant dog dander allergen, Can f 6 (17), to determine whether this protein could also enhance ligand-induced TLR signaling. Can f 6, similar to Fel d 1, sensitized TLR4/MD2/CD14 responses and enhanced LPS-induced signaling in BMDMs (Fig. 5A). In contrast, the model allergen OVA (that is

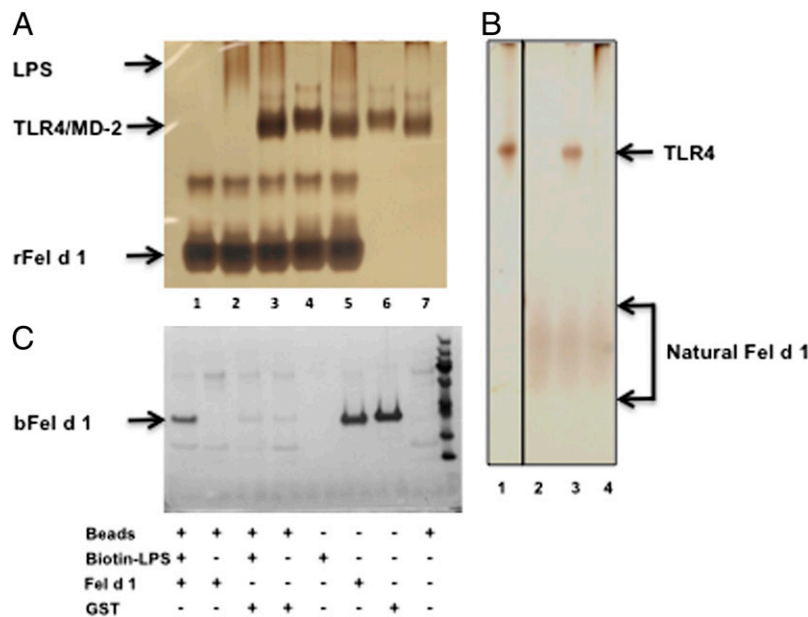
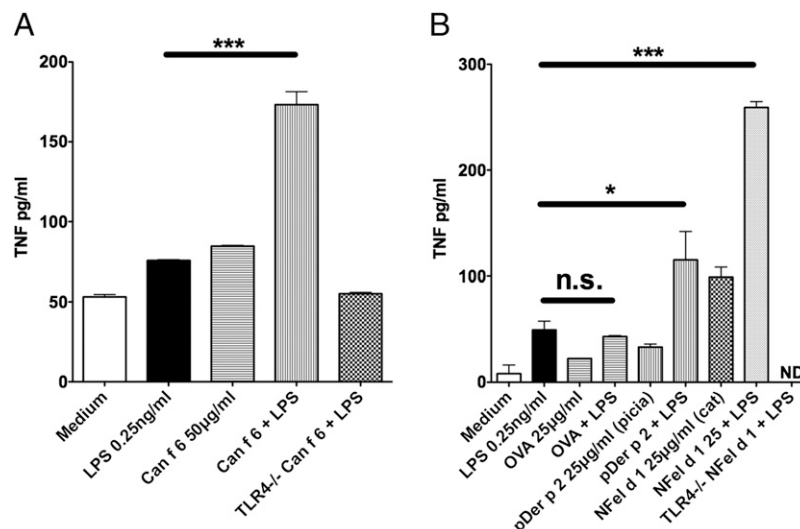


FIGURE 4. Fel d 1 interacts with LPS, but not with the TLR4/MD2 complex. **(A)** rFel d 1 does not interact with TLR4/MD2. Silver-stained gel of rFel d 1 that has been incubated with LPS and components of a copurified TLR4/MD2 complex. Each component (1 μ g) was added in the order listed and incubated at room temperature for 30 min: 1, rFel d 1 alone; 2, rFel d 1 plus LPS; 3, rFel d 1 plus LPS plus TLR4/MD2; 4, rFel d 1 plus TLR4/MD2; 5, rFel d 1 plus TLR4/MD2 plus LPS; 6, TLR4/MD2; 7, TLR4/MD2 plus LPS. The position of migration for each component is shown. rFel d 1 migrates as two bands, consistent with the formation of a dimer and tetramer. **(B)** Natural Fel d 1 does not form a complex with TLR4 either in the presence or absence of LPS. Each of the listed components (1 μ g) was incubated together at room temperature for 30 min: 1, TLR4 alone; 2, natural Fel d 1 alone; 3, natural Fel d 1 plus TLR4; 4, natural Fel d 1 plus LPS plus TLR4. No shift in the band for natural Fel d 1 was observed. **(C)** Fel d 1 interacts with LPS. rFel d 1 from baculovirus (bFel d 1) was incubated with biotin-LPS and the complex was pulled down using streptavidin-conjugated beads (lane 1). The position of bFel d 1 is marked. Lanes 2–4 control for nonspecific binding of bFel d 1 to the streptavidin-coated beads, nonspecific binding of proteins to biotin-LPS, and nonspecific binding of GST to the streptavidin-coated beads, respectively. Lanes 5–8 indicate assay inputs.

FIGURE 5. Allergens from three different structural classes sensitize signaling by TLR4 and TLR2. **(A)** Wild-type and TLR4^{-/-} BMDMs treated with LPS at 0.25 ng/ml in the presence or absence of the dog lipocalin allergen Can f 6 at 50 µg/ml for 24 h. TNF-α levels were measured by an ELISA. Data from three separate experiments were pooled and expressed as means ± SEM. **(B)** Wild-type and TLR4^{-/-} BMDMs treated with LPS (0.25 ng/ml), the house dust mite allergen Der p 2 (25 µg/ml), the model allergen protein OVA (20 µg/ml), or cat-derived Fel d 1 (25 µg/ml) either singly or in combination for 24 h. TNF-α levels were measured by an ELISA. Data from three separate experiments were pooled and expressed as means ± SEM. **p* < 0.05, ****p* < 0.001.



not a recognized allergen in humans) had no enhancing effect on TLR4 signaling. Der p 2, as expected, enhanced LPS-induced TLR4 responses, albeit to a lesser extent than did natural Fel d 1 (Fig. 5B). Together these results suggest that animal dander proteins employ a shared mechanism for enhancement of TLR signaling (Fig. 6).

Discussion

Despite Fel d 1 being responsible for ~80% of all human allergic responses to cats, little is known about how it is recognized by the host (2). In this study, to our knowledge, we show for the first time that the major cat and dog allergens, Fel d 1 and Can f 6, cause a substantial amplification of LPS/TLR signaling in both a transfected cell model and in primary, macrophage-like cells. Importantly, the model allergen OVA, which is not a recognized airways allergen in humans, has no effect on TLR signaling. Unlike the house dust mite allergen Der p 2, these molecules do not act by mimicking the TLR4 coreceptor MD2. Instead, they appear to bind microbial lipid PAMPs directly and transfer them to the receptors at the cell surface in a mechanism that depends on CD14. Our work and that of others (4) also shows that, at least in part, Der p 2 also enhances LPS-induced TLR4/MD2 signaling. We propose, therefore, that lipid binding and transfer is a common property of allergen IMPs.

In the absence of MD2, a high concentration of Fel d 1 induces a very low level of TLR4/CD14 activation, but even this signal is dependent on CD14. This CD14 and MD2 dependence indicates

that Fel d 1 does not work mechanistically by substituting for their functions. This, along with the fact that Fel d 1 also enhances LTA-induced activation of TLR2, suggests that the IMPs may be increasing the availability of lipids to CD14 and the TLR signaling complex. Alternatively, Fel d 1 may facilitate the assembly of TLR signaling complexes in membrane microdomains, thus lowering the activation threshold (28) (Fig. 6).

Although the IMPs appear to have a similar mechanism for enhancing innate immune signaling, they all have very diverse three-dimensional structures. Der p 2 is a member of a small family of lipid-binding proteins and has a similar β-cup structure to MD2. In the Der p 2 crystal structure electron density can be seen that most likely corresponds to at least one fatty acyl chain, and by comparison with MD2 it is likely that this molecule can accommodate a hexa-acyl glycolipid such as LPS (29, 30). Fel d 1, alternatively, is a heterodimer of two related chains that forms a structure with eight α-helices stabilized by intramolecular disulfide bonds. The subunit interface forms a hydrophobic cavity that may represent the binding site for microbial lipid ligands of the TLRs. The third IMP we have studied is the newly described Can f 6, which causes sensitization in 35% of patients allergic to dogs. It is a lipocalin allergen, a family that also includes dog Can f 1, Can f 2, Can f 4, cat Fel d 4, and Equ c 1 from the horse (17). Lipocalins form an eight-stranded β-barrel structure with a hydrophobic cavity to which small lipophilic molecules, such as pheromones, can bind (31). It is probable that, similar to Der p 2 and Fel d 1, these allergens will bind to the lipid ligands of the TLRs.

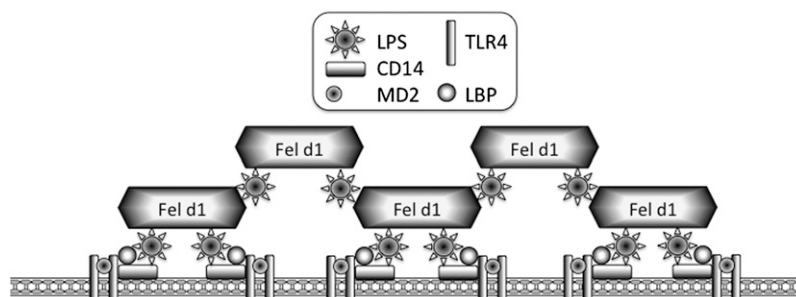


FIGURE 6. Model of LPS sensitization by dander proteins: Animal dander proteins loaded with environmentally derived lipid pathogen-associated molecular patterns associate with cell membrane, facilitating lipid presentation or transfer to receptor complexes. Dander proteins, in the presence of low LPS concentrations, could cluster together with LPS to form larger complexes that then promote greater clustering of TLR4-bearing lipid rafts, leading to increased receptor activation. At higher LPS exposure levels (such as would not be seen from environmental sources), this effect is not seen because maximal receptor activation has already been achieved.

Previous studies showed that TLR4 in particular is required to develop allergic responses to Der p 2, at least in a mouse model of asthma. These studies also showed that TLR4 function is likely to be required not only in innate immune cells but also in the airway epithelia (7). Sensitization to inhaled allergens is caused by the generation of allergen-specific IgE Ab response, and several epitopes have been defined in Fel d 1 and other IMPs (15, 17). To generate an Ab response the allergen must be taken up and presented by dendritic cells in a Th2 polarizing cytokine environment. In the case of Fel d 1, uptake by dendritic cells may be mediated by cell surface mannose receptors (16), but this process appears to be independent of TLR2/4 activation (Fig. 3). A possible hypothesis for allergen action is that IMPs stimulate TLR signaling in the airway epithelium leading to the production of Th2 cytokines, such as IL-4 and IL-13 (5, 32). TLR signaling might also undermine the barrier function of the epithelium, allowing allergens to access innate cells within the lamina propria (33, 34). In this regard it is known that the protein kinase C δ isoform and myosin L chain kinase are activated by TLR2 and TLR4 (35). These kinases can promote the disassembly of tight junctions by phosphorylating regulatory molecules. In hypersensitivity responses it is possible that activation of TLRs by allergens also increases the permeability of the respiratory epithelia, allowing access to allergen-specific IgE. Aggregates of IgE and IMPs would then ligate Fc ϵ receptors, leading to activation of mast cells and rapid release of inflammatory mediators.

In conclusion, we have shown that a number of mammalian IMPs enhance TLR signaling in response to lipid ligands. Agonists and antagonists to TLRs, therefore, may provide new therapeutic targets to modulate and treat allergic responses to animal-derived allergens.

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Disclosures

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Supplementary Figure Legends:

Supplementary Figure 1:

HEK293 cells were transiently transfected with either TLR5, NFkB-luc and pHRG-TK (TLR5+) or just NFkB-luc and pHRG-TK (TLR5-) then stimulated with flagellin (5-50 ng/ml) in the presence or absence of Fel d 1 (10 ng/ml). Stimulation in the absence of transfected TLR5 enables the contribution of endogenous TLR5 to be determined. Data normalized against unstimulated control are shown as the mean and s.e.m of one representative experiment from duplicate experiments. No statistically significant difference was observed between stimulation in the absence or presence of Fel d 1.

Supplementary Figure 1

