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

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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 secretoglobin 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: 1529–1535.

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-fold type (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 Ni₂⁺. Receptor activation is dependent on the presence of two histidine residues, H456 and H458, which coordinate the Ni²⁺ atom (or other metal ions such as Co²⁺), 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/MDF2 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...
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 a 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 titers determined to give bFel d 1 virus.

S99 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 and the medium was centrifuged to remove debris. Proteins were concentrated to 2 mg/ml using a Centricon-10 device (Millipore, Bedford, MA).

Transcript reporter vector encoding firefly luciferase (5 ng/well pNF-κB-luc; Promega) together with the appropriate reporter plasmids. The NF-κB reporter vector encoding Renilla luciferase (5 ng/well pGL3-TK; Promega) was cotransfected with CD14 and reporter plasmids. Transfection was performed 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, 10 mM sodium pyruvate, and 10 μg/ml gentamicin) in petri dishes. For experiments, cells were plated onto 96-well plates at a plating density of 2 × 10⁵ 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 50% deionized 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/Q1018/281) 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. Resistant cells were plated in 96-well plates in RPMI 1640 enriched with 10% FBS, 1% L-glutamine, penicillin/streptomycin, 100 U/ml penicillin, and 10 mg/ml streptomycin.

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.). The final solution was stored at 4°C.

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 values ± 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 (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 (similarly to *Der p 2*) is able to sensitize TLR signaling in the absence of MD2 or CD14, 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 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.

*Fig. 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-κB–luciferase reporter (pNF-κB-luc) and a constitutively active luciferase reporter control (phRG-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-κB–luciferase reporter was read on a FLUOstar Omega (BMG Labtech) luminometer. Data are normalized against the unstimulated control. LPS-induced 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<sup>−/−</sup> 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
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 p 2, 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 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−/− C57BL6/J mice were treated for 24 h with LPS at 0.5 ng/ml, Fel d 1 at 50 μg/ml, or both, as indicated. TNF-α levels were measured by an ELISA. Data from five separate experiments were pooled and expressed as means ± SEM. (B) Wild-type BMDMs were treated with LTA at 50 ng/ml with or without Fel d 1 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. (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 μg/ml. TNF-α levels were measured by an ELISA. Data from two separate experiments were pooled and expressed as means ± 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 μg/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 ± 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 μg/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 μg/ml. TNF-α levels were measured by an ELISA. Data from three separate experiments were pooled and expressed as means ± SEM. *p < 0.05, **p < 0.005, ***p < 0.001.
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

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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 LTA-induced activation of TLR2, suggesting 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 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.

**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 mannosse 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 ε 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 epithelium, allowing access to allergen-specific IgE. Aggregates of IgE and IMPs would then ligate Fce 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.

Acknowledgments
We thank Prof. S. Akira for provision of the TLR4−/− mice.

Disclosures
The authors have no financial conflicts of interest.

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