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**Dermatophagoides pteronyssinus** Major Allergen 1 Activates the Innate Immune Response of the Fruit Fly *Drosophila melanogaster*

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Some allergens with relevant protease activity have the potential to directly interact with host structures. It remains to be elucidated whether this activity is relevant for developing their allergic properties. The major goal of this study was to elucidate whether allergens with a strong protease activity directly interact with modules of the innate immune system, thereby inducing an immune response. We chose *Drosophila melanogaster* for our experiments to prevent the results from being influenced by the adaptive immune system and used the armamentarium of methods available for the fly to study the underlying mechanisms. We show that *Dermatophagoides pteronyssinus* major allergen 1 (Der p 1), the major allergen of the house dust mite, efficiently activates various facets of the *Drosophila* innate-immune system, including both epithelial and systemic responses. These responses depend on the immune deficiency (IMD) pathway via activation of the NF-κB transcription factor Relish. In addition, the major pathogen associated molecular pattern recognizing receptor of the IMD pathway, peptidoglycan recognition protein–LC, was necessary for this response. We showed that Der p 1, which has cysteine protease activity, cleaves the ectodomain of peptidoglycan recognition protein–LC and, thus, activates the IMD pathway to induce a profound immune response. We conclude that the innate immune response to this allergen-mediated proteolytic cleavage represents an ancient type of danger signaling that may be highly relevant for the primary allergenicity of compounds such as Der p 1. *The Journal of Immunology,* 2013, 190: 366–371.

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Asthma bronchiale is one of the most common chronic inflammatory diseases of the lung. It is characterized by marked inflammation, airway obstruction, bronchial hyperreactivity, and atopy (1). Inflammatory responses, resulting from the interaction between IgE Abs and allergens, are of central importance for disease development (2). Although this hallmark of allergic reactions appears to involve only components of the adaptive immune system, an essential role for innate immune responses in disease development has recently become apparent (3–5). Airway epithelial cells appear to be of central importance and are involved in orchestrating the entire allergenic response (6). Within these epithelial cells, modules of the innate immune system, namely NF-κB factors, are believed to function as central integrators, receiving input from diverse sources and orchestrating inflammatory responses in the lung (5, 7–9).

Contact with allergens is often the trigger that induces an asthmatic episode. Mechanistically, the interaction between allergens and the epithelium may differ substantially depending on the class of allergen involved. Some allergens, especially those found in the house dust mite or in fungi, are characterized by a very potent proteolytic activity (10). Thus, their interaction with the epithelium involves a proteolytic attack that provokes a series of specific and nonspecific responses. *Dermatophagoides pteronyssinus* major allergen 1 (Der p 1), the major allergen of the house dust mite, increases the permeability of epithelial cells in vivo, presumably via degradation of tight junction proteins. In addition, a more specific response may be induced by these proteolytically active allergens. Their interaction with epithelial cells can induce the release of cytokines, such as IL-6 and IL-8, or induce fluid secretion from submucosal glands (11). The mechanism underlying this response depends upon protease-activated receptors (PARs). PARs are activated by proteolytic cleavage, which can be mediated by Der p 1 (12, 13). Thus, Der p 1 may serve as the prototype of proteolytically active allergens. Elucidating the way in which these allergens interact with epithelial cells and manipulate their activity may improve our understanding of why certain proteins are highly allergenic and others are not.

To investigate whether allergens are able to induce an innate immune response, adaptive-immune responses must be excluded. Therefore, we used the fruit fly *Drosophila melanogaster*, because this invertebrate completely relies on its innate immunity (14). Two major signaling pathways operate within the immune system of *Drosophila*, both mediated via NF-κB transcription factors: the Toll and immune deficiency (IMD) pathways. These pathways share surprising architectural homology with the vertebrate TLR and the TNF-α receptor pathways, respectively (15). As in ver-

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tebrates, the major role of these signaling pathways is to sense pathogen-associated molecular patterns (PAMPs) derived from microbes or fungi, either directly (IMD) or via soluble recognition proteins (Toll). Recently, an alternative mechanism of immune activation was characterized in Drosophila, in which triggering of the IMD pathway is possible without any direct pathogen contact. Proteases are able to cleave the PAMP-recognizing receptors within this pathway, ultimately resulting in antimicrobial peptide gene (AMP) expression (16). This may be a simple response that also detects classical danger signals, such as pathogen-borne proteases (17).

In this study, we wanted to test whether the potent allergen of the house dust mite, Der p 1, was able to induce a pure innate-immune response. Therefore, we challenged the different immune-competent organs of Drosophila with highly purified Der p 1 preparations, quantified the outcome, and identified the signaling pathways involved.

**Materials and Methods**

**Recombinant Der p 1**

Unglycosylated Der p 1 was expressed in *Pichia pastoris* and purified chromatographically to homogeneity. Quality control included determination of endotoxin, detection of proteolytic activity, and assessment of purity by SDS-PAGE, reverse phase–HPLC, and size exclusion–HPLC (18). The endotoxin content was found to be far <0.02 EU/µg protein, and the purity was clearly >95%. We also tested whether the preparation contained PAMPs, which may induce an immune response. HEK293 cells (10,000 cells/well) were transiently transfected with plasmids (200 ng/ml) containing the coding sequences for the receptors TLR2, TLR4, NOD1, and NOD2, because containing within the allergen preparation (such as lipopeptides [TLR2], LPS [TLR4] or components of proteoglycans, such as γ-D-glutamyl-meso-diaminopimelic acid [NOD1] or muramyldipeptide [NOD2]) activate these receptors. The transfected cells were then incubated with PAMPs, which may induce an immune response. HEK293 cells (10,000 cells/well) were transiently transfected with plasmids (200 ng/ml) containing the coding sequences for the receptors TLR2, TLR4, NOD1, and NOD2, because containing within the allergen preparation (such as lipopeptides [TLR2], LPS [TLR4] or components of proteoglycans, such as γ-D-glutamyl-meso-diaminopimelic acid [NOD1] or muramyldipeptide [NOD2]) activate these receptors. The transfected cells were then incubated with PAMPs, which may induce an immune response.

**Luciferase assay**

S2 cells were suspended in Schneider’s Drosophila medium (Genaxxon, Ulm, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany) and 2% penicillin/streptomycin (Life Technologies-BRL, Life Technologies, Darmstadt, Germany) and plated at a density of 1.5 × 10⁶ cells/ml in six-well plates. After 24 h, cells were transfected (in the presence of Cellfectin (Invitrogen, Life Technologies)) with plasmids containing a firefly luciferase gene under the transcriptional control of a dipterincin promoter or a drosomycin promoter (16). Luciferase activities were measured using the Dual-Glo luciferase assay system (Promega, Mannheim, Germany) and a luminometer (Becton Dickinson, Heidelberg, Germany) to allow for normalization. Transiently transfected S2 cells were stimulated overnight with recombinant Der p 1 preparation (Supplemental Fig. 1).

**Preparation and stimulation of fat bodies and activation of the epithelial immune response**

Fat bodies from early third–instar larvae were prepared manually in Schneider’s Drosophila medium (Genaxxon) and stimulated with Der p 1 (500 and 1000 nM) or *A. lwoffii* (10⁵ CFU/ml) for 2 to 6 h. After incubation, the medium was removed, and the fat bodies were lysed in TRIzol reagent (Invitrogen, Life Technologies). A Relish-yfp fusion protein was introduced into the fat bodies using the Gal4/universal activating sequence (UAS) system (19, 20). Thus, the corresponding UAS line (UAS-rel-yfp) was crossed with the fat body–specific driver line (UAS-Csp-rlsh). Fat bodies from the resulting larvae were prepared as described above. Experimental samples were treated with Der p 1 (100 nM) for 2 h. Controls were incubated in *Drosophila* Ringer’s solution alone. In addition, mutant strains defective for the Relish gene (*w¹¹¹B, Rel¹⁵⁸*) or the PGRP-LC gene (ind7) were used. The fat bodies from third-instar larvae were isolated and subsequently treated with Der p 1.

To determine whether Der p 1 is able to activate the epithelial immune response, we challenged late second–instar larvae of the strain *yw*D¹⁰¹, which contains a *drosomycin-promotor:gfp* insertion, essentially as described previously (21). Der p 1–containing solution (1 µM) was mixed with an equal amount of crushed banana. Larvae were incubated on this mixture for 30 min. After this, they were transferred to normal medium at 18°C and scored for *gfp* expression 24 h later. Control animals were treated identically, except that buffer, rather than the Der p 1–containing solution, was mixed with banana.

**Real-time PCR**

RNA isolation was performed using TRIzol reagent (Invitrogen, Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed using matching primer pairs for each gene of interest. Nonstimulated fat bodies or wild-type fat bodies from *w¹¹¹B* flies were used as controls. qRT-PCR was performed in a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using Mesa Green (Eurogentec, Köln, Germany). Probe sets were normalized against the housekeeping gene actin 42A.

**Western blotting of FLAG-tagged peptidoglycan recognition protein–LC**

Stably transfected Drosophila S2 cells were cultured and passaged every 3–4 d in Insectom SF express medium (Biochrom), supplemented with 10% heat-inactivated FCS, 2 µg/ml penicillin, and 2 U/ml streptomycin (PAA, Pasching, Austria). Additionally, stably transfected cells were selected using Geneticin sulphat (G418) solution (Roth, Karlshue, Germany) at 1 mg/ml final concentration. Peptidoglycan recognition protein–LC (PGRP-LC) expression was induced by adding CuSO₄ (500 µM; Merck, Darmstadt, Germany) for 24 h. Der p 1 stimulation was performed in a 24-well cell culture cluster. Before Der p 1 treatment, cells were washed and incubated in PBS. A total of 1 × 10⁶ cells/well was incubated with Der p 1 (50 µM final concentration) for 3 h at 25°C. Cells were collected in 100 µl Laemmli buffer (60 mM Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-ME, 0.01% bromophenol blue).

**Results**

**Activation of the epithelial immune response by Der p 1**

To elucidate whether Der p 1 is able to induce aspects of the fruit fly’s immune response, we first challenged late second–instar larvae of the genotype *yw*D¹⁰¹ [containing a *drosomycin-promotor:gfp* construct (23)] with Der p 1 (500 nM) and checked for the development of GFP fluorescence as a marker of *drosomycin* expression. For this, we challenged the larvae briefly (30 min), returned them to normal medium, and analyzed them 24 h later. This expression is seen consistently in most parts of the airway epithelium of *Drosophila* larvae (Fig. 1A), whereas control animals treated otherwise identically (but without Der p 1) did not show this reaction (Fig. 1B). Fluorescence is primarily seen in those parts of the tracheal system that are close to the spiracles and in the major branches (i.e., those parts of the airway system that are closest to the outside world).

**Influence of Der p 1 on the expression of AMP genes in S2 cells and isolated fat bodies**

In a second set of experiments, S2 cells were transiently transfected with reporter constructs (the promoters of the AMP genes *drosomycin* and *dipterincin* fused to luciferase), enabling quantitation of their activation (16, 17). We incubated the cells with different

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concentrations of Der p 1. Gram-negative A. lwoffii and Gram-positive B. subtilis were used as positive controls. We did not observe any differences in the response induced by 10 and 100 nM Der p 1 in either of the pathways analyzed. The allergen preparation was two to three times more potent in activating the dipthericin promoter (Fig. 2A) compared with the drosomycin promoter (Fig. 2B). Although expression of these AMP-coding genes cannot exclusively be attributed to activation of the IMD pathway or the Toll pathway, respectively, these results imply that the IMD pathway is more relevant for the more pronounced induction of diptericin expression.

To exclude artifacts associated with the long-term culture of the S2 cells, we also performed the experiments using fresh, ex vivo organ preparations. We incubated isolated larval fat bodies (20) with either Der p 1 or A. lwoffii and quantified the expression of drosomycin and diptericin using qRT-PCR (Fig. 3). Under these conditions, we observed almost no induced drosomycin expression (preferentially reflecting Toll pathway activation). A very moderate level of activation was observed only after 6 h of incubation (Fig. 3A). In contrast, diptericin expression was enhanced significantly after exposure to Der p 1 at two concentrations and the Gram-negative bacteria, with maximal responses observed after 6 h (Fig. 3B).

We attempted to elucidate the mechanism underlying innate immune activation by Der p 1 using a cysteine protease inhibitor (E64). Preincubation of the Der p 1 preparation with this compound significantly reduced its activity upon challenge with isolated fat bodies, especially at later time points, which might reflect kinetic effects of the inhibition (Fig. 4A). To learn more about the mechanisms involved, we preincubated S2 cells either with bacteria (E. coli) or Der p 1 (Fig. 4B), challenged the cells for a second time with Der p 1 24 h later, and measured the activation of the immune response. Preincubation with bacteria primed the immune response in S2 cells, resulting in a doubling of the response to the second stimulus (Der p 1 24 h later; Fig. 4B). Preincubation with Der p1 instead of the bacteria reversed this effect (Fig. 4B), indicating a long-lasting inhibition or impairment of the underlying signaling pathway.

Signal-transduction pathways

To elucidate the Der p 1–induced signaling events activated by Der p 1 that lead to the expression of AMP genes, we used fluorescence microscopy to visualize the activation of NF-κB transcription factors through their induced translocation to the nuclei of fat body cells isolated from transgenic flies. Therefore, we crossed the lsp2-Gal4 driver line (specific for fat body expression) with the FIGURE 1. Der p 1 induced an epithelial immune response in the airways. (A) Incubation of Drosophila larvae carrying a drosomycinP-gfp construct with Der p 1 (100 nM) induced expression of the reporter in the trachea only (arrow). (B) Control larvae of the same genotype and age were treated exactly as those above, but Der p 1 was omitted. Median larval length is ~2 mm.

FIGURE 2. The immune system of Drosophila S2 cells was activated by Der p 1. S2 cells were transiently transfected with constructs for use in a luciferase assay. The promoters of the genes encoding two AMP genes, drosomycin (A) and diptericin (B), were each fused to luciferase, enabling the measurement of bioluminescence upon promoter activation. The cells were incubated with either Der p 1 or bacteria (B. subtilis and A. lwoffii; 10^7 CFU/ml) for 16 h. Mean values ± SD are given. *p < 0.05 versus control. RLU, Relative light unit.

FIGURE 3. Induction of AMP gene expression in the fat body of Drosophila. Der p 1 induces drosomycin (A) and diptericin (B) expression in the fat body of larval Drosophila. Isolated fat bodies were stimulated with either Der p 1 (10 or 100 nM) or A. lwoffii (10^7 CFU/ml) for 2, 4, or 6 h. The fold expression relative to untreated control tissues is given. Mean values ± SD are given. *p < 0.05 versus control.
flies defective in this gene (\textit{E. coli} / Der p 1), resulted in a stronger immune response than that seen in cells stimulated with bacteria alone (\textit{E. coli}–). Cells for which the first challenge was performed with the allergen (Der p 1/Der p 1 compared with Der p 1/−) showed an almost 2-fold reduction in the immune response. Asterisks denote statistically significant differences from matching controls.

UAS-Relish-eYFP, UAS-dorsal-eGFP, or UAS-dif-eCfp effector line (20). Freshly isolated fat bodies from the corresponding larvae were challenged with Der p 1. Dorsal and Dif showed no evidence of nuclear translocation upon incubation with Der p 1, indicating that signaling pathways converging onto them (e.g., the Toll pathway) are not activated by Der p 1. However, a marked translocation of Relish-associated fluorescence from the cytoplasm (Fig. 5A) to the nuclei of the fat body cells (Fig. 5B, 5C) was observed. Based on these results, we concluded that only the IMD pathway, with its terminal element, the NF-κB transcription factor Relish, is essential for the fly’s Der p 1–induced immune response (24). To further elucidate the underlying signaling pathways, we used mutants defective in the transcription factor Relish (\textit{rel}–/−) and subsequently challenged their fat bodies with Der p 1. Even at very high Der p 1 concentrations, these flies showed no Der p 1–induced increase in diptericin expression (Fig. 6A), indicating that Relish is essential for mediating Der p 1–induced effects. The proximal part of the IMD pathway comprises the pattern recognition receptor (PRR), PGRP-LC (25). There was also no increase in \textit{dipertcin} expression upon Der p 1 challenge in flies defective in this gene (\textit{pgrp-lc}−/−/−) (Fig. 6A), suggesting that Der p 1 interacts directly with this most proximal molecule in the IMD pathway.

\textbf{Physical interaction of Der p 1 with PGRP-LC}

To further test the hypothesis that Der p 1 physically interacts with PGRP-LC, we used S2 cells stably transfected with a FLAG-tagged version of PGRP-LC. The FLAG-peptide is inserted at the C-terminal end of PGRP-LC, thus tagging the ectodomain of the receptor molecule. Because the construct is under transcriptional control of a metallothionein promoter, incubation with CuSO$_4$ can induce protein production substantially. After induction, the FLAG-tagged full-length protein is visible at a band of ∼60 kDa in the Western blot (Fig. 6B, lanes 1 and 3, respectively). Equal amounts of cells were incubated or not with Der p 1, and cell homogenates were run on a SDS gel electrophoresis, blotted, and probed with an anti-FLAG Ab. Incubation with Der p 1 (Fig. 6B, lane 2) led to a complete disappearance of this band representing the full-length protein. Instead, another Flag-tagged band of ∼15–17 kDa appeared in this preparation, which almost exactly represents the molecular mass of the complete ectodomain of PGRP-LC including the FLAG-tag (Fig. 6B, lane 2). This implies that Der p 1 cleaves the nearly complete ectodomain via a single recognition site in this domain that is close to the membrane. The noninduced (ni) cells are loaded in the third lane, expressing only very low levels of FLAG-tagged PGRP-LC. These results led us to propose a model regarding the mechanism of Der p 1–induced immune activation. Under control conditions, the IMD pathway is not activated (Fig. 6C). Upon contact with bacteria (recognized by PGRP-LC), the pathway is activated, leading to translocation of the NF-κB transcription factor Relish into the nucleus (Fig. 6D). Der p 1 directly interacts with PGRP-LC, presumably by proteolytic cleavage, thus activating the IMD pathway via this alternative route (Fig. 6E).

\textbf{Discussion}

It is not well understood why certain proteins are highly allergenic but others are not. One peculiar and highly potent group of
allergens, comprising the major allergen of the house dust mite (Der p 1) and numerous allergens derived from fungi, is characterized by a pronounced intrinsic protease activity. In addition to its primarily physical effect (i.e., cleavage of epithelial cell junctions), which culminates in weakening of the epithelial barrier, interactions with target cells that induce very specific responses have been characterized. These proteases can activate PRRs, thus inducing a specific response in the corresponding cells. Sensing the activity of proteases may be a very straightforward strategy, because protease release is often associated with the presence of pathogenic microbes. Their presence can be associated with tissue damage, invasion, and cell migration. In addition, vertebrate host proteases are essential for an effective immune response. Thus, unexpected proteolytic activity within the body points to a current infection, either because the pathogens release proteases of their own or they damage host tissues, which, in turn, may lead to the liberation of endogenous proteases. The sensing of proteolytic activity developed early in evolution and appears to be an integral part of immune systems; it has been demonstrated for the Toll pathway in insects (35). If the immune system does not respond properly to the occurrence of proteolytic activity, the presence of a pathogen could be missed and, more importantly, the cleavage of PRRs would render the immune system “blind.”

Allergens possessing proteolytic activity may act in the same manner, meaning that they interact with the innate-immune system through their proteolytic activity, causing nonspecific activation. Thus, early activation of the innate-immune system by specific allergens may markedly increase their allergenic potential. In contrast, fruit flies can come into contact with peptidases from other sources that may have very similar properties. Other compounds with allergenic potential from fruits, such as papain from papaya, may be most relevant in this context. Flies feeding on these fruits...
may ingest the corresponding peptidases, where they immediately come into contact with the intestinal epithelium. Presumably to counteract the unwanted activation of this arm of the immune system by ingested peptidases, the major PRR PGRP-LC is not present or is present at only very low levels in the midgut of the fly (36). Our own observations support this finding.

The results of this study have a broader relevance in that they demonstrate an interaction between allergens and the innate-immune system that might be of great relevance to a subgroup of allergens (i.e., those with a definite protease activity). Cleavage activation of PRRs may not be restricted to PARs; it may also be a mechanism that accounts for many molecular hallmarks of the innate-immune system. This type of activation, without any direct contact with PAMPs, may represent a classical danger signal. Thus, proteases that manage to invade the body are interpreted as a danger signal, in the same way as those produced by invading microorganisms or released by endogenous cells.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplementary Figure 1: Der p 1 preparations are devoid of detectable amounts of contaminants. HEK293-cells (10 000 cells/well) were transiently transfected with plasmids (200 ng/ml) containing the coding sequences for the receptors TLR2 (top left), TLR4 (top right), NOD1 (bottom left), or NOD2 (bottom right). The transfected cells than were then stimulated with positive control compounds Pam3C-SK4 (1-100 nM; TLR2), LPS (1-100nM; TLR4) iDAP (NOD1), MDP (NOD2, both at 10–500 ng/ml), or TNF-α (1 ng/ml). In addition, three different Der p 1 (5-100 nM) concentrations were tested. After incubation for 18 h, the amount of the cytokine, IL-8, in the cell culture supernatant was measured using an ELISA-Kit (Invitrogen, life technologies, Germany). The values are mean values (± S.D.) from three independent experiments.