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Cleavage of Fibrinogen by Proteinases Elicits Allergic Responses Through Toll-Like Receptor 4

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Abstract

Proteinases and the innate immune receptor Toll-like receptor 4 (TLR4) are essential for expression of allergic inflammation and diseases such as asthma. A mechanism that links these inflammatory mediators is essential for explaining the fundamental basis of allergic disease but has been elusive. Here, we demonstrate that TLR4 is activated by airway proteinase activity to initiate both allergic airway disease and antifungal immunity. These outcomes were induced by proteinase cleavage of the clotting protein fibrinogen, yielding fibrinogen cleavage products that acted as TLR4 ligands on airway epithelial cells and macrophages. Thus, allergic airway inflammation represents an antifungal defensive strategy that is driven by fibrinogen cleavage and TLR4 activation. These findings clarify the molecular basis of allergic disease and suggest new therapeutic strategies.

Allergic asthma is a chronic inflammatory airway disease that is characterized by both airway obstruction and enhanced systemic and airway allergic inflammation marked by interleukin-4 (IL-4)–secreting T helper 2 (T_H2) cells, eosinophils, and serum immunoglobulin E (IgE). Proteinases able to elicit T_H2 cell–driven allergic responses are secreted by fungi (1) and can be found in natural sources linked to allergic disease such as pollens (2) and dust mite antigens like Der p 1 (3, 4). Nonetheless, nonproteinase allergens such as ovalbumin also possess allergenic activity. Prior studies have linked TLR4, a microbial pattern-recognition receptor, to both proteinase-dependent and -independent allergic responses in mice (5–7), but a mechanism that explains the importance of proteinases and TLR4 in diverse allergic contexts involving both proteinase-active and -inactive allergens remains unknown.

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To study the role of TLR4 in the context of proteinase-dependent allergic inflammation, we assessed wild-type (WT) and *Tlr4*^{-/-} mice after intranasal exposure to a fungal proteinase derived from *Aspergillus oryzae* (PAO) [endotoxin content: 1.7×10^{-3} endotoxin units (EU)/ μ g]. Consistent with our prior observations (2), WT mice challenged with PAO developed canonical features of asthma, including airway hyperresponsiveness, airway infiltration by eosinophils, enhanced production of the mucin gene transcript *Muc5ac*, goblet cell metaplasia of the airway, and enhanced production of transcripts for the proinflammatory cytokines IL-4, IL-5, and IL-13 (fig. S1, A to G). In contrast, all of these allergic parameters, with the exception of lung IL-4 transcripts, were either attenuated or abrogated in *Tlr4*^{-/-} mice. Thus, TLR4 is essential for the expression of proteinase-dependent asthma-like disease in mice.

Quantification of IL-4-secreting cells from whole lungs of proteinase-challenged mice (fig. S1H) confirmed the equivalent presence of IL-4-producing cells in the lung regardless of mouse genotype, suggesting that T_H2 cell development and recruitment occurred independently of TLR4. We then immunized mice against ovalbumin and confirmed that ovalbumin-specific T_H2 cell development was equivalent or enhanced in *Tlr4*^{-/-} mice compared with controls (fig. S1I). Induction of total IgE, an IL-4- and T_H2 cell-dependent process (8–11), was also identical between WT and *Tlr4*^{-/-} mice (fig. S1J).

Both the fungus *A. niger* and proteinase-free ovalbumin (1.8×10^{-3} EU/ μ g) also induced TLR4-dependent allergic lung disease in controls compared with proteinase-challenged WT mice (fig. S2). The consistent defect in disease expression seen in *Tlr4*^{-/-} mice was durable because identical reductions in allergic disease parameters were seen after 2 and 4 weeks of ovalbumin immunization (fig. S2). Thus, TLR4 was required for the development of allergic airway disease, regardless of allergen proteinase content, but was dispensable for T_H2 responses.

We confirmed the reduced IL-5 transcript production in *Tlr4*^{-/-} mice by assessing secreted IL-5 levels in bronchoalveolar lavage fluid (fig. S3A). Type 2 innate lymphoid cells (ILCs) secrete IL-5 and IL-13, but not IL-4, in the setting of airway proteinase challenge (12), suggesting that these cells might be influenced by TLR4. Indeed, relative to vehicle-challenged animals, ILCs failed to be recruited as robustly into bronchoalveolar lavage fluid in *Tlr4*^{-/-} mice relative to WT animals after proteinase challenge, potentially accounting in part for the reduced T_H2 cytokine production in *Tlr4*^{-/-} mice (fig. S3, B and C).

We next considered whether other TLRs played a role in proteinase-dependent allergic lung disease. Most TLRs signal through one of two major adapter proteins, MyD88 and TRIF, whereas TLR4 signals through both adapters (13). Mice deficient in either MyD88 or TRIF showed an enhanced or identical disease phenotype as genotype matched control mice when challenged with *A. niger* spores (fig. S4). In contrast, mice deficient in both MyD88 and TRIF showed complete disease abrogation. Thus, proteinase-dependent allergic lung disease is mediated through TLR4 and not other TLRs.

Because TLR4 does not determine T_H2 responses, we turned to macrophages to further explore how TLR4 controls allergic disease. Relative to naïve cells, bone marrow-derived macrophages (BMDMs) expressed distinct transcriptional programs when activated by lipopolysaccharide (LPS), interferon- γ (IFN- γ), and PAO (Fig. 1A). Specific genes induced by PAO included lysozyme (*Lyz*), macrophage receptor with a collagenous structure (*Marco*), and secretory leukoprotease inhibitor (*Slpi*), all of which have been linked to antifungal immunity (14–16), and their induction was dependent on TLR4 (Fig. 1B). PAO did not induce genes linked to previously characterized macrophage phenotypes, including IFN- γ -activated type 1 macrophages (M1) (nitric oxide synthase 2 (NOS2)) and IL-4-

activated M2 (arginase 1 and FIZZ1) (17) (Fig. 1, C and D). Alveolar macrophages from mice treated with PAO similarly showed up-regulation of lysozyme, MARCO, and SLPI (Fig. 1E). Thus, PAO induced a macrophage phenotype marked by expression of a distinct transcriptional profile that included genes with antifungal properties.

We next determined if PAO-activated macrophages were capable of restraining fungal growth in vitro. Relative to naïve BMDMs, as assessed by both microscopy and colorimetric quantification, only IFN- γ - and PAO-activated macrophages efficiently controlled fungal growth when the conidia of *A. niger* were added to cultures (Fig. 1, F and G, and fig. S5). Human monocyte-derived macrophages were similarly responsive to PAO treatment, although less so to IFN- γ compared with mouse BMDMs (Fig. 1H). However, control of fungal growth through PAO, but not IFN- γ , required the presence of TLR4 (Fig. 1, F to H). Again, MyD88 and TRIF were individually dispensable for control of fungal growth in macrophages activated by PAO, but deletion of both adapters abrogated the ability of mouse macrophages to control fungal growth (fig. S6). These in vitro findings correlated with a reduced ability of *Tlr4*^{-/-} mice to clear *A. niger* conidia from the airway after a single inhalational challenge (Fig. 1I).

Unlike other macrophage activators, PAO-dependent inhibition of fungal growth (fungistasis) required the presence of serum, suggesting that fungal proteinases acted through both a serum factor and TLR4 to induce macrophage antifungal immunity (Fig. 1J). Fibrinogen, a proposed TLR4 ligand (18), is the functional mammalian analog of the arthropod factors pro-Spätzle and coagulogen, which regulate anti-fungal immunity through Toll (19).

To determine if fibrinogen mediates proteinase-dependent fungistasis, we added it (1.8×10^{-5} EU/ μ g) to BMDM cultures, with and without fungal and endogenous proteinases, and the conidia of *A. niger*. Only when stimulated by PAO in the presence of serum did BMDMs exhibit robust fungistatic activity (Fig. 2A). Identical results were obtained in experiments in which PAO was substituted with the endogenous proteinase thrombin, which converts fibrinogen to fibrin as the terminal step in the clotting cascade while also creating additional cleavage products that do not participate in clot formation (Fig. 2B). These results suggested that rather than fibrinogen in per se, fibrinogen cleavage products (FCPs) were required to induce fungistasis. FCPs created by incubating fibrinogen with PAO or thrombin induced fungistasis to a comparable degree as whole serum and proteinase when added to BMDMs (Fig. 2, A and B). Moreover, the thrombin inhibitor hirudin (3.4×10^{-3} EU/ μ g) neutralized both PAO- and thrombin-dependent fungistasis that was induced in the presence of serum (Fig. 2C). Another abundant serum protein and putative TLR4 ligand, fibronectin (20), had no effect on macrophage fungistasis in either native or cleaved forms (fig. S7).

In addition to fungistasis (Fig. 2, D and E), FCPs also induced in BMDM the expression of mRNA for IL-13R α 1, a component of the IL-13 receptor that is required for expression of allergic airway disease (21), and the airway mucin gene *Muc5ac* through TLR4 (Fig. 3A). FCPs yielded similar findings and also induced fungistatic activity in human primary airway epithelial cells (Fig. 3, B and C).

Together, these findings support a model in which both endogenous and exogenous airway proteinase activities with allergenic potential produce alternate TLR4 ligands from fibrinogen that license innate immune cells to respond to T_H2 cells, as required for full expression of allergic airway disease. To test this model, we first administered intranasally to mice the maximum tolerated dose of FCPs, 0.6 mg per dose (Fig. 4A), which induced modest airway eosinophil recruitment and *Muc5ac* gene expression but failed to induce airway hyperresponsiveness and IL-4-secreting cells (Fig. 4, B to F). Thus, FCPs appeared

to influence only innate immune cells and specifically did not induce T_H2 responses that are required for robust allergic lung disease.

We conducted additional studies to confirm that airway proteinase activity was required for allergic lung disease using the proteinase inhibitor hirudin. Our in vitro studies indicated that hirudin, a known thrombin antagonist, could also inhibit PAO-mediated fungistasis (Fig. 2C), suggesting that hirudin may possess broad-spectrum antiproteinase activity. In a dose-dependent manner, hirudin progressively and significantly attenuated PAO-dependent allergic lung disease while leaving unaffected robust lung IL-4 responses (Fig. 4, G to K), a phenotype that resembles that of *Trl4*^{-/-} mice challenged with diverse allergens (figs. S1 and S2). Hirudin further inhibited ovalbumin-dependent allergic airway disease, suggesting that ovalbumin challenge activates an endogenous proteinase, possibly thrombin, to achieve the fibrinogenolysis that is necessary for disease expression (fig. S8). Together, these studies confirm the importance of airway fibrinogenolysis for the expression of allergic lung disease, regardless of the proteinase content of the inhaled allergen.

Although previous studies have shown that TLR4 contributes to T_H2 responses (5, 22, 23), we have shown here that TLR4 is not required for T_H2 cell development but rather is required for responsiveness of innate airway cells to T_H2 cells. Our findings do not exclude the possibility that bacterial endotoxin, a canonical TLR4 ligand, could mediate T_H2 responses under some conditions, as shown previously (5), but additional studies are needed to determine the contribution of fibrinogenolysis to this observation. Although independent of TLR4, T_H2 cells nonetheless develop through a proteinase-dependent pathway (24), suggesting that proteinases coordinate both innate and adaptive allergic pathways that together lead to allergic inflammation and disease (fig. S9).

Ultimately, mammalian TLR4 preserves the crucial role of arthropod Toll by linking proteinase-dependent fibrinogenolysis to antifungal immunity. However, in addition to fungi, mammals must also defend against other proteinase-associated pathogens such as helminth parasites (25) and, potentially, viruses (26). Although highly effective, the ancient proteinase-Toll-based defensive strategy is also susceptible to aberrant activation in response to innocuous proteinase sources such as pollens and many allergens, both with and without intrinsic proteinase activity. Clarification of the contribution of true infections to common allergic airway disorders such as allergic rhinitis, asthma, and chronic rhinosinusitis will determine the usefulness of interrupting FCP-TLR4 signaling as a therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The data presented in this paper are tabulated in the main paper and in the supplementary materials. Microarray data are available through the National Center for Biotechnology Information Gene Expression Omnibus, accession number GSE48609. We thank Y. Qian, T. Bird, and Y. Zhang for excellent technical assistance. Funding was provided by NIH grants HL75243, AI057696, and AI070973 (to D.B.C.); CA125123 (to C.J.C.); and T32GM088129 and R25GM56929 (to V.O.M.) and the C.N. and Mary V. Papadopoulos Charitable Fund from the Biology of Inflammation Center.

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Fig. 1. PAO induces a distinct fungistatic macrophage phenotype through TLR4

(A to D) BMDMs from WT or *Tlr4*^{-/-} mice were left unstimulated (N) or cultured in the presence of lipopolysaccharide (L), IFN- γ (I), PAO (P), or IL-4 (4) as indicated. (A) Heat map depicting the relative expression of 252 gene probes, as assessed by microarray ($P < 0.01$ and fold change > 1.5 , comparing PAO to each of the other groups) of WT BMDMs. (B) Polymerase chain reaction (PCR)-based analysis of lysozyme, MARCO, and SLPI. (C) NOS2 expression in IFN- γ - and PAO-activated WT macrophages. (D) Arg1 and Fizz1 expression in IL-4- and PAO-activated WT macrophages. (E) MARCO, SLPI, and lysozyme (LYZ) mRNA expression in alveolar macrophages derived from mice challenged with PBS (S) or PAO (P). (F) BMDMs from WT (top row) and *Tlr4*^{-/-} (bottom row) mice were treated with IFN- γ , LPS, IL-4, or PAO for 24 hours and then cultured with *A. niger* conidia. Photomicrographs depict filamentous fungal growth. (G) Quantification of fungal growth in the same experiment, as assessed by XTT assay ($n = 3$ replicates per group). (H) PAO-treated human peripheral blood monocyte-derived macrophages were cultured under the same conditions as in (F) and assessed by XTT assay for their ability to restrain fungal growth ($n = 3$ patients). (I) WT and *Tlr4*^{-/-} mice were intranasally challenged with 400,000 *A. niger* conidia, and lungs were harvested and fungal colony forming units (CFU) were determined over 7 days. (J) Fungistatic potential of BMDMs was determined as in (G), but in the presence (+) and absence (-) of fetal bovine serum (FBS). Data are presented as means $\pm$ SEM (error bars) from one of three comparable experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

0.01 ; $***P < 0.001$ by Mann-Whitney (two group comparisons) and Kruskal-Wallis (three or more group comparisons) tests.

Fig. 2. Fibrinogen mediates PAO-dependent fungistasis through TLR4

BMDMs were cultured for 24 hours in the presence of FBS, PAO, fibrinogen (FG), FCP, or thrombin (THR), as indicated, and then inoculated with the conidia of *A. niger* for 24 hours, testing the requirement of (A) PAO and (B) thrombin for induction of fungistasis and (C) the effect of the thrombin inhibitor hirudin on PAO- and thrombin-dependent fungistasis, as assessed by XTT assay. (D) PAO and (E) thrombin were further compared with FCPs alone for their ability to induce fungistasis in the presence or absence of the *Tlr4* gene ($n = 3$ replicates per group). Data are presented as means $\pm$ SEM (error bars) from one of four comparable experiments. * $P < 0.05$ by Mann-Whitney test.

Fig. 3. FCPs up-regulate IL-13ra1 and Muc5AC on airway epithelium

(A) WT and *Tlr4*^{-/-} mouse primary airway epithelial cells were cultured in the presence of IL-13 or FCPs for 24 hours, and *Il13ra1* and *Muc5ac* gene transcripts were analyzed by quantitative PCR ($n = 3$). (B) *Il13ra1* and *Muc5ac* transcripts were similarly analyzed from human primary airway epithelial cells 24 hours after treatment with FCPs or left unstimulated (Un) ($n = 4$). (C) Unstimulated and FCP-pretreated human airway epithelial cells were assessed for their ability to inhibit the growth of *A. niger* by XTT assay ($n = 3$ replicates per group.). Data are presented as means $\pm$ SEM (error bars) from one of three (murine) or two (human) comparable experiments. * $P < 0.05$; *** $P < 0.001$ by Mann-Whitney test.

Fig. 4. Fibrinolysis is necessary but insufficient for expression of robust allergic airway disease

(A) C57BL/6 mice were challenged intranasally with PAO or FCPs as indicated, after which (B) airway hyperresponsiveness, (C) total bronchoalveolar lavage fluid (BALF) inflammatory cells, (D) lung Muc5AC transcripts, and (E) total lung IL-4⁺ secreting cells were quantitated. (F to J) C57BL/6 mice were intranasally challenged with PAO without and with hirudin or hirudin alone on alternating days for 2 weeks, and the indicated parameters were assessed ($n = 3$ mice per group). Data are presented as means $\pm$ SEM (error bars) from one of three comparable experiments. Data are averages $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$ by Kruskal-Wallis test. R_{RS}, respiratory system resistance.